Handbook of
Hydrocarbon and Lipid Microbiology

SPRINGER
REFERENCE

Series Editors: Kenneth N. Timmis (Editor-in-Chief)
Matthias Boll · Otto Geiger · Howard Goldfine · Tino Krell
Sang Yup Lee · Terry J. McGenity · Fernando Rojo
Diana Z. Sousa · Alfons J. M. Stams · Robert Steffan · Heinz Wilkes

Otto Geiger *Editor*

Biogenesis of Fatty Acids, Lipids and Membranes

Springer

Handbook of Hydrocarbon and Lipid Microbiology

This handbook is the unique and definitive resource of current knowledge on the diverse and multifaceted aspects of microbial interactions with hydrocarbons and lipids, the microbial players, the physiological mechanisms and adaptive strategies underlying microbial life and activities at hydrophobic material:aqueous liquid interfaces, and the multitude of health, environmental and biotechnological consequences of these activities.

More information about this series at http://www.springer.com/series/13884

Otto Geiger
Editor

Biogenesis of Fatty Acids, Lipids and Membranes

With 161 Figures and 19 Tables

Springer

Editor
Otto Geiger
Centro de Ciencias Genómicas
Universidad Nacional Autónoma de México
Cuernavaca, Morelos, Mexico

ISBN 978-3-319-50429-2 ISBN 978-3-319-50430-8 (eBook)
ISBN 978-3-319-50431-5 (print and electronic bundle)
https://doi.org/10.1007/978-3-319-50430-8

Library of Congress Control Number: 2018961436

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This work is dedicated to my extended German-Mexican family:
Gertrud, Hanna, Jörg, Johannes, and Sofia Geiger
Isabel M. López Lara, Otto Antonio, and Carmen Geiger López

Preface

Nucleic acids carry the information and proteins do most of the work, but there is no life without membranes. Biological membranes are composed of proteins and amphiphilic lipids, i.e., a group of molecules consisting of hydrophobic hydrocarbon chains which are connected to a hydrophilic head group. In an aqueous environment, amphiphilic lipids tend to aggregate, and, if their molecular shape is more or less cylindrical, they assemble into lipid bilayers as encountered in most membranes. In this volume of the Handbook, the biochemistry of the biogenesis of membranes and of their building blocks, fatty acids, and membrane lipids will be covered as well as their genetics and functional genomics. Examples of membrane homeostasis emphasize the flexibility and adjustability of biological membranes. For a more complete picture, we also tried to include the biogenesis of other lipid-derived biomolecules encountered in microbes.

Biological membranes show remarkable features, such as regions of distinct fluidity, giving rise to what is known as lipid rafts or microdomains. The bilayer of most biological membranes seems to be asymmetric, as the inner leaflet usually shows a different lipid composition than the outer leaflet. Lipid composition of biological membranes depends on physiological conditions experienced by an organism; composition is not a constant and maybe changed upon adjusting to other physiological conditions, employing lipid remodeling and de novo lipid synthesis. There is lipid transfer between different membranes inside a cell, and finally, gradients of ions and charges across biological membranes are frequent features. In recent years, lipidomic and structural studies revealed the existence of many more membrane lipids, sometimes limited to very specialized microbes or organelles. Confronted with such complexity, it is obvious that experimental approaches to study biological membranes are demanding, not always satisfactory and still need to be improved in many cases. We are only beginning to understand the functioning and importance of biological membranes. Besides lipids, proteins are the other important components of biological membranes, and different functional associations, integrations, penetrations, and movements of proteins in connection with membranes are described, often endowing these membranes with special functional properties.

Humans, animals, and plants are the most obvious and visible organisms for us on our planet, and we are usually focused on them when thinking on our immediate

needs and pleasures. However, it is "the microbes" which guarantee ongoing life on earth, and we need to understand all aspects of their biology in order to help our planet.

I hope that besides the experts, newcomers to the field will find the chapters of this book interesting and understandable in the hope that "hot topics research" on the biogenesis of fatty acids, lipids, and membranes will become accessible to a large readership and hopefully to the interested general public.

Finally, I would like to thank the people who made this work possible:

- The editor in chief, Kenneth Timmis, who did a wonderful job in getting me on the way with this volume and who always had constructive inputs during the evolution of the volume
- The people at Springer, especially Simone Giesler, Sharmila Thirumaniselvan, and Sylvia Blago, who were at the production interface and did all the manuscript processing
- All the contributors of a chapter to this volume
- Finally, my wife, Isabel, our son, Otto Antonio, and our daughter, Carmen, who came to understand that family efforts were necessary to ready this volume for the Handbook

Thank you all very much!
Otto Geiger
Centro de Ciencias Genómicas
Universidad Nacional Autónoma de México
Cuernavaca, Morelos, Mexico
December 2018

Acknowledgments

I am glad that during my life I could dedicate most of my time to do what I most like to do: research in biological sciences. I like to thank my teachers and friends for the training and lessons they had in wait for me: Helmut Görisch, Harald Sobek, Rudi Müller, Karl-Heinz van Pée, and Franz Lingens at the University of Hohenheim, Eugene P. Kennedy at Harvard Medical School, as well as Ben Lugtenberg and Herman Spaink at Leiden University. I am grateful to the funding agencies that made research possible: Alexander von Humboldt Foundation, Deutsche Forschungsgemeinschaft, Howard Hughes Medical Institute, UC-Mexus Conacyt, MIT-Mexico CONACYT Seed Fund, Consejo Nacional de Ciencia y Tecnología México (Ciencia Básica 253549, Fronteras de la Ciencia 118), Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica, and Universidad Nacional Autónoma de México. I am most grateful to all my collaborators and especially those who passed through the laboratory and with whom I was allowed to share the exciting moments of discovering some novelty. Special thanks to Lourdes Martínez-Aguilar and to Isabel López-Lara, and "Gracias México!"

Contents

Part I Biochemistry of Biogenesis of Fatty Acids and Lipids 1

1 Fatty Acids: Introduction 3
Eric R. Moellering, Victoria L. Prince, and Roger C. Prince

2 Synthesis of Acetyl-CoA from Carbon Dioxide in Acetogenic Bacteria 25
A. Wiechmann and V. Müller

3 Formation of Fatty Acids 43
Isabel M. López-Lara and Otto Geiger

4 Formation of Isoprenoids 57
Jordi Pérez-Gil, Manuel Rodríguez-Concepción, and Claudia E. Vickers

5 Formation of Bacterial Glycerol-Based Membrane Lipids: Pathways, Enzymes, and Reactions 87
Otto Geiger, Christian Sohlenkamp, and Isabel M. López-Lara

6 Ornithine Lipids and Other Amino Acid-Containing Acyloxyacyl Lipids 109
Christian Sohlenkamp

7 Bacterial Sphingolipids and Sulfonolipids 123
Otto Geiger, Jonathan Padilla-Gómez, and Isabel M. López-Lara

8 Phenolic Lipids Synthesized by Type III Polyketide Synthases 139
Akimasa Miyanaga and Yasuo Ohnishi

9 Lipid A 151
Russell E. Bishop

10 Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria 163
Olaf Schneewind and Dominique Missiakas

11 Mycolic Acids: From Chemistry to Biology 181
Mamadou Daffé, Annaïk Quémard, and Hedia Marrakchi

12 Lipid Intermediates in Bacterial Peptidoglycan Biosynthesis 217
Hélène Barreteau, Didier Blanot, Dominique Mengin-Lecreulx, and Thierry Touzé

13 *Marinobacter* as a Model Organism for Wax Ester Accumulation in Bacteria 237
Carolann M. Knutson, Eric M. Lenneman, and Brett M. Barney

14 Vitamin Formation from Fatty Acid Precursors 259
Michael F. Dunn

15 Functional Roles of Non-membrane Lipids in Bacterial Signaling 273
María J. Soto, N. Calatrava-Morales, and Isabel M. López-Lara

16 Formation of Lipochitin Oligosaccharide Signaling Molecules . . . 291
V. Poinsot and F. Couderc

17 Metabolism and Regulation of Glycerolipids in Yeast 315
Vanina Zaremberg, Suriakarthiga Ganesan, and Brittney N. Shabits

18 Metabolism and Roles of Sphingolipids in Yeast *Saccharomyces cerevisiae* 341
Jihui Ren and Yusuf A. Hannun

19 Nonpolar Lipids in Yeast: Synthesis, Storage, and Degradation 363
Karin Athenstaedt

20 Modeling Lipid Metabolism in Yeast 375
Eduard J. Kerkhoven

Part II Genetics and Functional Genomics of Biogenesis of Fatty Acids and Lipids **389**

21 Fatty Acid Synthesis and Regulation 391
Isabel M. López-Lara and María J. Soto

22 Components and Key Regulatory Steps of Lipid Biosynthesis in Actinomycetes 409
Gabriela Gago, Ana Arabolaza, Lautaro Diacovich, and Hugo Gramajo

23 Type III Polyketide Synthases Responsible for Phenolic Lipid Synthesis 435
Akimasa Miyanaga and Yasuo Ohnishi

24 Wax Ester and Triacylglycerol Biosynthesis in Bacteria 445
H. M. Alvarez, M. A. Hernández, O. M. Herrero, M. P. Lanfranconi, R. A. Silva, and M. S. Villalba

25 Biogenesis of Medium-Chain-Length Polyhydroxyalkanoates 457
Ryan Kniewel, Olga Revelles Lopez, and M. Auxiliadora Prieto

26 Storage of Hydrophobic Polymers in Bacteria 483
Luísa S. Serafim, Ana M. R. B. Xavier, and Paulo C. Lemos

27 Players in the Nonpolar Lipid Game: Proteins Involved in Nonpolar Lipid Metabolism in Yeast 509
Karin Athenstaedt

Part III Biochemistry of Biogenesis of Membranes 523

28 Membrane Lipid Biogenesis 525
Howard Goldfine

29 Biosynthesis and Evolution of Archaeal Membranes and Ether Phospholipids 539
Yosuke Koga

30 Functional Roles of Individual Membrane Phospholipids in *Escherichia coli* and *Saccharomyces cerevisiae* 553
William Dowhan, Mikhail Bogdanov, Eugenia Mileykovskaya, and Heidi Vitrac

31 Bacterial Lipid Domains and Their Role in Cell Processes 575
Adrián F. Alvarez and Dimitris Georgellis

32 Outer Membrane Vesicles of Bacteria: Structure, Biogenesis, and Function 593
Armaity Nasarabadi, James E. Berleman, and Manfred Auer

33 Flip-Flopping Membrane Proteins: How the Charge Balance Rule Governs Dynamic Membrane Protein Topology 609
Mikhail Bogdanov, Heidi Vitrac, and William Dowhan

34 Lactose Permease: From Membrane to Molecule to Mechanism 637
Lan Guan and H. Ronald Kaback

35 Role of the BAM Complex in Outer Membrane Assembly 651
Fernando Navarro-Garcia

36 Structure: Function of Transmembrane Appendages in Gram-Negative Bacteria 671
Miguel Ángel Díaz-Guerrero, Meztlli O. Gaytán, and Bertha González-Pedrajo

37 Fatty Acid-Binding Proteins, a Family of Lipid Chaperones 691
Masato Furuhashi

38 Protein Lipidation, Elucidation by Chemical Proteomics, and Its Functional Roles 707
Gemma Triola

39 Membrane-Disrupting Proteins 729
Jeremy H. Lakey and Gregor Anderluh

40 Modeling Lipid Membranes 741
Pouyan Khakbaz, Viviana Monje-Galvan, Xiaohong Zhuang, and Jeffery B. Klauda

Part IV Membrane Homeostasis **761**

41 Membrane Formation and Regulation 763
Megan E. Ericson and Charles O. Rock

42 Regulation of Membrane Lipid Homeostasis in Bacteria upon Temperature Change 775
M. C. Mansilla and D. de Mendoza

43 Membrane Homeostasis in Bacteria upon pH Challenge 787
Christian Sohlenkamp

44 Contributions of Membrane Lipids to Bacterial Cell Homeostasis upon Osmotic Challenge 801
T. Romantsov and J. M. Wood

45 Membrane Homeostasis upon Nutrient (C, N, P) Limitation 823
F. Schubotz

46 Autophagy in Stationary Phase of Growth 849
José L. Aguilar-López and Soledad Funes

Index 867

About the Series Editor-in-Chief

Kenneth N. Timmis
Emeritus Professor
Institute of Microbiology
Technical University Braunschweig
Braunschweig, Germany

Kenneth Timmis studied microbiology and obtained his Ph.D. at Bristol University. He undertook postdoctoral training at the Ruhr-University Bochum, Yale and Stanford, at the latter two as a Fellow of the Helen Hay Whitney Foundation. He was then appointed Head of an independent research group at the Max Planck Institute for Molecular Genetics in Berlin and subsequently Professor of Biochemistry in the University of Geneva, Faculty of Medicine. Thereafter, for almost 20 years, he was Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI), and concomitantly Professor of Microbiology in the Institute of Microbiology of the Technical University Braunschweig. He is currently Emeritus Professor in this institute.

The Editor-in-Chief has worked for more than 30 years in the area of environmental microbiology and biotechnology, has published over 400 papers in international journals, and is an ISI Highly Cited Microbiology-100 researcher. His group has worked for many years, *inter alia*, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, and on the ecology of hydrocarbon-degrading microbial communities, discovered the new group of marine oil-degrading hydrocarbonoclastic bacteria, initiated genome sequencing

projects on bacteria that are paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*), and pioneered the topic of experimental evolution of novel catabolic activities.

He is Fellow of the Royal Society, Member of the European Molecular Biology Organisation, Fellow of the American Academy of Microbiology, Member of the European Academy of Microbiology, and Recipient of the Erwin Schrödinger Prize. He is the founder and Editor-in-Chief of the journals *Environmental Microbiology*, *Environmental Microbiology Reports*, and *Microbial Biotechnology*.

About the Editor

Otto Geiger
Centro de Ciencias Genómicas
Universidad Nacional Autónoma de México
Cuernavaca, Morelos, Mexico

Major Research Line: Formation and Function of Bacterial Cell Surfaces

Otto Geiger studied biology at the University of Hohenheim in Stuttgart, Germany, and after investigating quinoproteins and their prosthetic group pyrroloquinoline quinone at the Institute of Microbiology, he received his doctoral title from the same institution in 1987. As a Feodor-Lynen Fellow of the Alexander von Humboldt Foundation, he did postdoctoral work at Harvard Medical School in the United States with Eugene P. Kennedy on the biosynthesis and function of periplasmic glucans in Gram-negative bacteria. Subsequently, together with Ben Lugtenberg and Herman Spaink, he investigated the biosynthesis of lipochitin oligosaccharide signals that cause nodule formation on legume host plants at the Institute of Molecular Plant Sciences, Leiden University, Netherlands. From 1993 to 1999, he was a Research Group Leader in the Department of Biotechnology at the Technical University of Berlin, where he habilitated for Biochemistry in 1997. Otto Geiger was an International Research Scholar (2002–2006) of the Howard Hughes Medical Institute. He currently holds the position of Professor (Investigador Titular C) at the Center for Genomic Sciences (formerly the Research Center for Nitrogen Fixation) at the National Autonomous University of Mexico (Cuernavaca) which he joined in 1999. Geiger's group discovered the phosphatidylcholine

synthase pathway, which constitutes a major route for phosphatidylcholine (lecithin) formation in bacteria as well as the biosynthesis pathway for ornithine-containing lipids, which are widespread bioactive lipids encountered in bacterial membranes. More recently, he discovered molecular mechanisms for lipid remodeling of bacterial membranes in response to different types of stress. Presently, he studies the molecular functions of phosphatidylcholine in bacteria as well as the biosynthesis, transport, and function of sphingolipids in bacteria. He functions as an Editor for the journal *BMC Microbiology* and for the *Handbook of Hydrocarbon and Lipid Microbiology*.

Contributors

José L. Aguilar-López Departamento de Genética Molecular, División de Investigación Básica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico, Ciudad de Mexico, Mexico

H. M. Alvarez Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina

Adrián F. Alvarez Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad de Mexico, Mexico

Gregor Anderluh Laboratory for Molecular Biology and Nanobiotechnology, National Institute of Chemistry, Ljubljana, Slovenia

Ana Arabolaza Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Karin Athenstaedt Institute of Molecular Biosciences, University of Graz, Graz, Austria

Manfred Auer Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Brett M. Barney Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN, USA

Hélène Barreteau Group Bacterial Cell Envelopes and Antibiotics, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris Sud, Université Paris-Saclay, Gif-sur-Yvette, France

James E. Berleman Lawrence Berkeley National Laboratory, Berkeley, CA, USA
Saint Mary's College of California, Moraga, CA, USA

Russell E. Bishop Department of Biochemistry and Biomedical Sciences, Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada

Didier Blanot Group Bacterial Cell Envelopes and Antibiotics, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris Sud, Université Paris-Saclay, Gif-sur-Yvette, France

Mikhail Bogdanov Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, McGovern Medical School, Houston, TX, USA

N. Calatrava-Morales Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain

F. Couderc Laboratoire IMRCP UMR5623 UPS/CNRS, Université Paul Sabatier, Toulouse, France

Mamadou Daffé Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, France

Lautaro Diacovich Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Miguel Ángel Díaz-Guerrero Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico

D. de Mendoza Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Universidad Nacional de Rosario, Rosario, Argentina

William Dowhan Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, McGovern Medical School, Houston, TX, USA

Michael F. Dunn Programa de Genómica Funcional de Procariontes, Centro de Ciencias Genómicas, Universidad Nacional Autonoma de México, Av. Universidad s/n, Col. Chamilpa, Cuernavaca, Morelos, Mexico

Megan E. Ericson Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA

Soledad Funes Departamento de Genética Molecular, División de Investigación Básica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico, Ciudad de Mexico, Mexico

Masato Furuhashi Department of Cardiovascular, Renal and Metabolic Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan

Gabriela Gago Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Suriakarthiga Ganesan Department of Biological Sciences, University of Calgary, Calgary, AB, Canada

Meztlli O. Gaytán Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico

Otto Geiger Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Dimitris Georgellis Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad de Mexico, Mexico

Howard Goldfine Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

Bertha González-Pedrajo Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico

Hugo Gramajo Department of Microbiology, Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Lan Guan Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX, USA

Yusuf A. Hannun Stony Brook Cancer Center, Stony Brook University, Health Science Center, Stony Brook, NY, USA

M. A. Hernández Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina

O. M. Herrero Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina

Oil m&s, Comodoro Rivadavia, Chubut, Argentina

H. Ronald Kaback Departments of Physiology, Microbiology, Immunology and Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, CA, USA

Eduard J. Kerkhoven Systems and Synthetic Biology, Department of Biology and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden

Pouyan Khakbaz Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD, USA

Jeffery B. Klauda Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD, USA

Biophysics Program, University of Maryland, College Park, MD, USA

Ryan Kniewel Department of Environmental Biology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

Carolann M. Knutson Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN, USA

Yosuke Koga University of Occupational and Environmental Health, Kitakyushu City, Japan

Munakata City, Fukuoka, Japan

Jeremy H. Lakey Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK

M. P. Lanfranconi Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina

Paulo C. Lemos LAQV-REQUIMTE, Departmento de Química, Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, Lisboa, Portugal

Eric M. Lenneman Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN, USA

Isabel M. López-Lara Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, Mexico

M. C. Mansilla Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Universidad Nacional de Rosario, Rosario, Argentina

Hedia Marrakchi Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, France

Dominique Mengin-Lecreulx Group Bacterial Cell Envelopes and Antibiotics, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris Sud, Université Paris-Saclay, Gif-sur-Yvette, France

Eugenia Mileykovskaya Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, McGovern Medical School, Houston, TX, USA

Dominique Missiakas Department of Microbiology, University of Chicago, Chicago, IL, USA

Akimasa Miyanaga Department of Chemistry, Tokyo Institute of Technology, Tokyo, Japan

Eric R. Moellering Synthetic Genomics, Inc., La Jolla, CA, USA

Viviana Monje-Galvan Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD, USA

V. Müller Department of Molecular Microbiology and Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt/Main, Frankfurt, Germany

Armaity Nasarabadi Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Fernando Navarro-Garcia Department of Cell Biology, Center for Research and Advanced Studies (Cinvestav), Mexico City, Mexico

Yasuo Ohnishi Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

Jonathan Padilla-Gómez Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Jordi Pérez-Gil Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Barcelona, Spain

Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD, Australia

V. Poinsot Laboratoire IMRCP UMR5623 UPS/CNRS, Université Paul Sabatier, Toulouse, France

M. Auxiliadora Prieto Department of Environmental Biology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

Victoria L. Prince Department of Family and Preventive Medicine, University of Utah School of Medicine, Salt Lake City, UT, USA

Roger C. Prince Stonybrook Apiary, Pittstown, NJ, USA

Annaïk Quémard Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, France

Jihui Ren Stony Brook Cancer Center, Stony Brook University, Health Science Center, Stony Brook, NY, USA

Olga Revelles Lopez Bioprocess Laboratory D-BSSE, ETH Zürich, Basel, Switzerland

Charles O. Rock Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA

Manuel Rodríguez-Concepción Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Barcelona, Spain

T. Romantsov Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Olaf Schneewind Department of Microbiology, University of Chicago, Chicago, IL, USA

F. Schubotz MARUM and Department of Geosciences, University of Bremen, Bremen, Germany

Luísa S. Serafim CICECO – Aveiro Institute of Materials, Departamento de Química, Universidade de Aveiro, Aveiro, Portugal

Brittney N. Shabits Department of Biological Sciences, University of Calgary, Calgary, AB, Canada

R. A. Silva Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina

Christian Sohlenkamp Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

María J. Soto Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain

Thierry Touzé Group Bacterial Cell Envelopes and Antibiotics, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris Sud, Université Paris-Saclay, Gif-sur-Yvette, France

Gemma Triola Department of Biological Chemistry and Molecular Modelling, Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

Claudia E. Vickers Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD, Australia

Commonwealth Science and Industry Research Organization (CSIRO), Land and Water, EcoSciences Precinct, Brisbane, QLD, Australia

M. S. Villalba Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina

Oil m&s, Comodoro Rivadavia, Chubut, Argentina

Heidi Vitrac Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, McGovern Medical School, Houston, TX, USA

A. Wiechmann Department of Molecular Microbiology and Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt/Main, Frankfurt, Germany

J. M. Wood Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Ana M. R. B. Xavier CICECO – Aveiro Institute of Materials, Departamento de Química, Universidade de Aveiro, Aveiro, Portugal

Vanina Zaremberg Department of Biological Sciences, University of Calgary, Calgary, AB, Canada

Xiaohong Zhuang Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD, USA

Part I

Biochemistry of Biogenesis of Fatty Acids and Lipids

Fatty Acids: Introduction

1

Eric R. Moellering, Victoria L. Prince, and Roger C. Prince

Contents

1 Introduction 4
2 Fatty Acid Metabolism 4
2.1 Fatty Acid Synthesis 4
2.2 Fatty Acid Elongation, Desaturation, and Assembly into Glycerolipids 9
2.3 Fatty Acid Degradation 10
3 Types of Fatty Acids 12
3.1 Saturated Fatty Acids 12
3.2 Monoenoic Fatty Acids 12
3.3 Polyenoic Fatty Acids 15
4 Research Needs 17
References 17

Abstract

Fatty acids have been part of the biosphere from its beginning, providing a high calorie foodstuff in metabolism, the structural basis of membranes, an important class of intra-and extracellular signaling molecules, and many other functions. They are a diverse group of molecules, although most are linear aliphatic molecules with a terminal carboxylic acid. Here, we provide an overview of their

E. R. Moellering
Synthetic Genomics, Inc., La Jolla, CA, USA
e-mail: emoellering@hotmail.com

V. L. Prince
Department of Family and Preventive Medicine, University of Utah School of Medicine, Salt Lake City, UT, USA
e-mail: vlprince@gmail.com

R. C. Prince (✉)
Stonybrook Apiary, Pittstown, NJ, USA
e-mail: rogercprince@gmail.com

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_2

nomenclature, their synthesis, and their biodegradation and an outline of some of the major classes of fatty acids.

1 Introduction

Fatty acids have been part of the biosphere from its beginning (Nagy and Bitz 1963), providing a high calorie foodstuff in metabolism (Freedman and Bagby 1989), the structural basis of membranes (Segré et al. 2001), and an important class of intra- and extracellular signaling molecules (Jimenez et al. 2012; Jump et al. 2013). They are also important chemical feedstocks (Metzger 2009). Fatty acids are a diverse group of molecules, although most are linear aliphatic molecules with a terminal carboxylic acid. The most abundant possess 16 or 18 carbon atoms with up to two unsaturated bonds, but they range from the shortest, propionic acid (from the Greek *protos* ("first") and *pion* ("fat"); the smallest carboxylic acid with fatty acid properties) to simple species with at least 34 carbons (e.g., geddic acid (Vrkoslav et al. 2009), isolated from the beeswax of *Apis dorsata*) and more complicated types such as the mycolic acids of the mycobacteria, which can have 80 carbon atoms or more (Takayama et al. 2005). The degree of unsaturation progresses up to at least six unsaturated bonds (e.g., docosahexaenoic acid, initially isolated from hog brains (Hammond and Lundberg 1953), and a plethora of other variants are known (see Table 1)). It is not yet clear what roles all these components play – for example, Carballeira et al. (1997) identified 37 different fatty acids in *Thermotoga maritima* and 18 in *Pyrococcus furiosus*, while Rontani et al. (2005) identified up to 25 in different marine aerobic anoxygenic phototrophic bacteria. To put these numbers into perspective, a recent study of a single butter sample reported 430 different fatty acids (albeit only 15 were major components), of which about half had more than two double bonds (Schröder and Vetter 2013).

2 Fatty Acid Metabolism

2.1 Fatty Acid Synthesis

Biosynthesis of fatty acids is carried out by a set of highly conserved enzymatic reactions first elucidated over a half century ago (Toomey and Wakil 1966; Vagelos et al. 1969; Lynen 1969). The canonical pathway utilizes one acetyl-CoA and 6–8 malonyl-CoA equivalents in the NAD(P)H-dependent production of a C14–C18 saturated acyl chain (Fig. 1), where iterative condensation of acetyl moieties on the growing acyl chain involves two rounds of reduction separated by a dehydration step. Variation in primer and chain extension substrates, as well as the termination reaction specificity in some microbes, allows for the synthesis of longer chain fatty acids or, for example, branched chain fatty acids (Kaneda 1991). There is also a

Table 1 Some examples of complex fatty acids

Class	Example	Etymology	Notes
α,ω-Dicarboxylic acids			
Linear	Azelaic acid (nonanedioic acid)	Prepared by nitric acid hydrolysis of oleic acid (Fr. *azote*, nitro, Gr. *elaic* olive)	Important commercial chemical, used as an antibacterial in cosmetic creams, and primes systemic defense responses in *Arabidopsis* (Shah 2009)
	Equisetolic acid (triacontanedioic acid)	L. *Equisetum,* horsetail	From spores of *Equisetum* (horsetail) (Brune and Haas 2011)
	Heptacosane-1,27-dioic acid		One of several virulence factors of *Legionella* (Geiger 2010)
Branched	Diabolic acid (3,16-dimethyl octacosanedioic acid)	Gr. *diabollo*, to mislead	First isolated from *Butyrivibrio* (Klein et al. 1979)
Monounsaturated	Traumatic acid (Dodec-2-enedioic acid)	Gr. *trauma,* wound	Potent wound healing signaling agent in plants (English et al. 1939)
Diunsaturated	Muconic acid (hexa-2,4-dienoic acid)	Related to mucic acid (2,3,4,5-tetrahydroxyhexanedioic acid), itself made by nitric acid oxidation of galactose	Three isomers – E,E, E,Z, and Z,Z. A potentially important intermediate for bioplastics (Curran et al. 2013)
Allenic	Mycomycin ((−)-3,5,7,8-*n*-tridecatetraene-10,12-diynoic acid) (Celmer and Solomons 1953)	Killed molds on a Sabouraud's agar plate	Originally thought to be produced by the actinomycete
			Nocardia acidophilus, (Johnson and Burdon 1947) but now known to be produced by Basidiomycetes such as *Odontia bicolor* (Bu'Lock 1964)
	Labellanic acid ((−)-octadeca-5,6-dienoic acid)	Initially isolated from a labiate (*Leonotis nepetaefolia*) (Bagby et al. 1965)	

(*continued*)

Table 1 (continued)

Class	Example	Etymology	Notes
2,3-dicarboxylic acids	Ceriporic acid (1-heptadecene-2,3R–dicarboxylic acid)	Isolated from *Ceriporiopsis subvermispora*, a lignin-degrading basidiomycete (Amirta et al. 2003)	
Hydroxylated	Ricinoleic acid (12-hydroxy-9-*cis*-octadecenoic acid)	From castor oil, itself from *Ricinus communis*. Castor oil got its name as a replacement for beaver oil (*Castor fiber*, European beaver). Also produced by *Claviceps purpurea*, the ergot fungus (Meesapyodsuk and Qiu 2008)	About 90% of the triglycerides of the seed oil. Formed the original basis for the Castrol brand of lubricants (Corley 1988)
Epoxylated	Vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid)	From *Vernonia anthelmintica* (an ironweed) seed oil	About 70% of the triglycerides of the seed oil (Barford et al. 1963)
Furan fatty acids	12,15-epoxy-13-methyleicosa-12,14-dienoic acid	First identified in Northern Pike (*Esox lucius*) (Glass et al. 1975)	Subsequently identified widely, including in bacteria (Shirasaka et al. 1995). These molecules have potent anti-inflammatory activity (Wakimoto et al. 2011)
Cyclopropanated	Mycolic acids	From *Mycobacterium tuberculosis*	*M. tuberculosis* mycolic acid has two cyclopropane rings in fatty acids with 77–82 carbon atoms (Qureshi et al. 1978). Cyclopropanation apparently essential for viability (Barkan et al. 2009)
	Lactobacillic acid (*cis*-11,12-methylene octadecanoic acid)	From *Lactobacillus arabinosus* (Hofmann and Lucas 1950)	Similar compounds subsequently found in a range of bacteria (Grogan and Cronan 1997)

ω-Cyclohexyl fatty acids	ω-Cyclohexyl-undecanoic and tridecanoic acids	First identified in acidophilic thermophilic *Bacillus* species (Oshima and Ariga 1975), later reclassified as *Alicyclobacillus* (Wisotzkey et al. 1992)	Can make up 74–93% of the fatty acids in the organisms (Wisotzkey et al. 1992)
Halogenated	18-fluoro-*cis*-octadecenoic acid	Identified as the toxic principle of ratsbane, the powdered fruit of *Dichapetalum toxicarium* (Dear and Pattison 1963). The *trans* isomer is said to be twice as toxic	More than 300 naturally occurring halogenated fatty acids had been identified by 2002 (Dembitsky and Srebnik 2002)
Arseno-fatty acids	1-dimethylarsinoylpentadecanoic acid and 1-dimethylarsinoyl all-*cis*-4,7,10,13,16,19-docosahexanoic acid	Fish oils	Found along with arsenohydrocarbons and arsenobetaines (Taleshi et al. 2010)

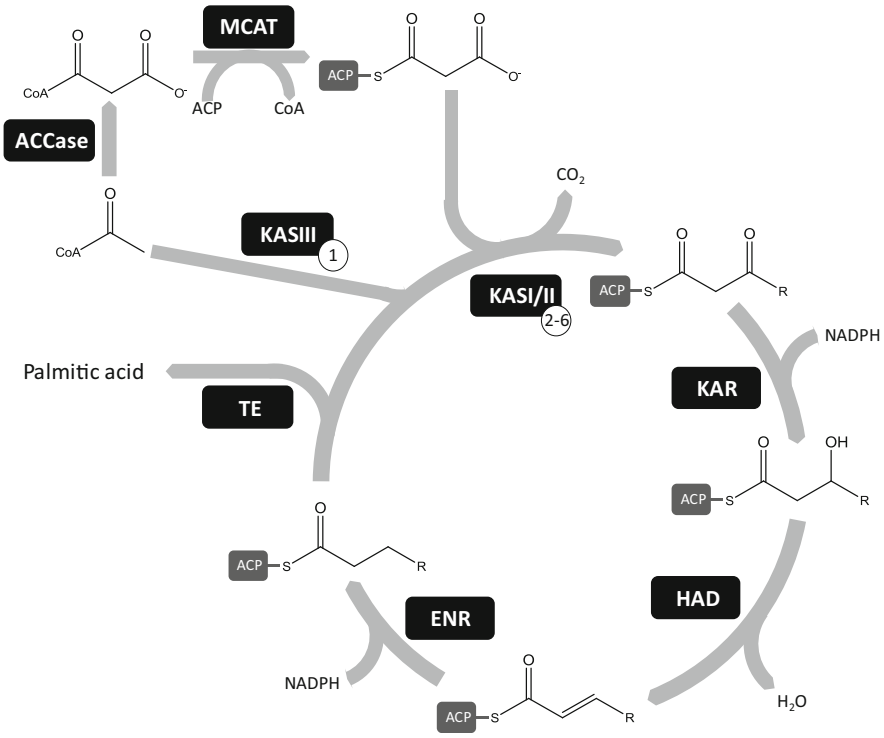


Fig. 1 General scheme of fatty acid synthase reactions leading to the synthesis of palmitic acid (C16:0). Enzymes (in *black bubbles*) for the provision of substrates for FAS are shown (acetyl-CoA carboxylase, *ACCase*; malonyl-CoA/acyl-carrier protein transacylase, *MCAT*). Ketoacyl synthase III (*KASIII*) initiates the first cycle of FAS with malonyl-ACP and acetyl CoA. The ketoacyl-ACP is then further reacted upon by ketoacyl-ACP reductase (*KAR*), hydroxyacyl-ACP dehydratase (*HAD*), and enoyl-acyl-ACP reductase (*ENR*) to complete the reduction of the β-carbon to a fully saturated state. KASI and II enzymes initiate elongation cycles 2–6 of FAS to produce C16 saturated fatty acyl-ACP, which is cleaved by thioesterase (*TE*) to produce palmitic acid

remarkable diversity in the structural organization of fatty acid synthase (FAS) enzyme complexes, which fall into two categories: the type I FAS comprised of large multifunctional complexes of one or two subunits and the "dissociated" type II FAS in which distinct/individual enzymes catalyze each step. In both cases the growing acyl chain is linked to a 4-phosphopantetheine cofactor bound to acyl carrier protein (ACP, existing as a domain in type I FAS) through a thioester bond (Schweizer and Hofmann 2004; Janssen and Steinbuchel 2014). It is common for eukaryotic microbes with a cytosolic localized FAS to harbor the type I form, although it is also present in the "CMN" group of bacteria (*Corynebacterium, Mycobacterium,* and *Nocardia*)(Schweizer and Hofmann 2004). It is tempting to speculate that the type I FAS are advantaged over type II in terms of simplifying regulation of expression to one or two genes and perhaps being more efficient by circumventing the need for free dissociation of acyl-ACP intermediates. Yet, type II

FAS are present in the majority of prokaryotes studied to date and appear to be ubiquitous in the plastid organelles of phototrophic eukaryotes. In vitro reconstitution and kinetic analyses of type II FAS systems from *Escherichia coli* and *Synechococcus* sp. PCC7002 have revealed significant differences in terms of which step limits overall fatty acid synthesis (Yu et al. 2011; Kuo and Khosla 2014). While the rationale for this evolved difference in flux control remains unclear, it was posited that it may be related to distinct means of fatty acid desaturation occurring in these two species (during fatty acid synthesis in *E. coli* and during glycerolipid assembly in *Synechococcus* sp.). In the polyphyletic group of microbes we refer to as microalgae, many of the green algae accumulate fatty acids with C16 and C18 chain lengths, whereas in heterokonts (e.g., eustigmatophytes and diatoms), C18 fatty acids are minor components that are largely replaced by C20 (or C22) fatty acids (Lang et al. 2011; Vieler et al. 2012). In most type II FAS systems, the presence of three ketoacyl synthases (KAS) results in the co-synthesis of C16 and C18 fatty acids: KASIII for FAS initiation, KASI for elongation to C16, and KASII to elongate from C16 to C18. Interestingly, the annotated genome sequencing of the eustigmatophyte *Nannochloropsis gaditana CCMP1779* revealed no KASII ortholog – suggesting the initial production of only palmitic acid C16:0 by FAS and its subsequent elongation to the C20 fatty acids that constitute ~30% of total fatty acids by some other enzyme (Vieler et al. 2012). From these examples, one can glean the potential advantage of maintaining the FAS as discrete dissociated parts that can be modified independently in terms of expression regulation and/or kinetic parameters to optimally suit the metabolic requirements downstream of FAS.

2.2 Fatty Acid Elongation, Desaturation, and Assembly into Glycerolipids

Synthesis is only the beginning for most fatty acids – as the overwhelming majority does not accumulate in the free acid form but rather is desaturated, elongated (or further chemically modified as described below), and assembled into glycerolipids. Through a myriad of genera- and species-specific variations in substrate specificity of these activities, their localization and regulation, as well as the trafficking of intermediates across membranes and organelles, there are 100s of distinct fatty acids and 1,000s of lipid species into which they are bound. In the simplest case, monounsaturation of stearoyl-ACP (or stearoyl-CoA, if the glycerolipid assembly pathway is eukaryotic and non-plastidial) at the delta 9 position produces oleyl-ACP/oleyl-CoA, which is then used to assemble oleic acid moieties into glycerolipids. A significantly more complex case is found in the synthesis of long-chain polyunsaturated fatty acids (PUFAs) such as 20:5 and 22:6, where several desaturases and elongases constitute two parallel pathways for synthesis. In some species this requires swapping the acyl-chain from CoA to glycerolipid intermediate multiple times (Vieler et al. 2012; Bellou et al. 2016). Here it must be noted that fatty acid desaturation can also occur during fatty acid synthesis. Many prokaryotes, including *E. coli*, are capable of incorporating a *cis*-

double bond initiated by FabA-dependent isomerization of the *trans*-2-decenoyl to the *cis*-3-decenoyl intermediate which results in the production of 16:1ω7 and 18:1ω7 (Kass and Bloch 1967). O_2-independent synthesis of PUFAs by FAS-like polyketide synthases (PKSs) is observed in a small number of microbes, including the marine bacterium *Shewanella* and some protists in the thraustochytrid family (Metz et al. 2001; Ye et al. 2015; Yoshida et al. 2016). While desaturases predominately catalyze the formation of *cis*-double bonds, *trans*-Δ-3-hexadecenoic acid (trans 16:1,ω13) is found in some green algae (and most higher plants) (Ohnishi and Thompson 1991; Dubertret et al. 1994). This *trans*-fatty acid is formed by a distinct desaturase (Gao et al. 2009) and specifically esterified to chloroplast-localized phosphatidylglycerol – although at levels below those that would require our vegetables or algae-based supplements to be labeled as "trans-fat" foods in the grocery store!

Along the way, elongases can act to increase the chain length of fatty acids beyond C18. These multifunctional enzymes catalyze a reaction sequence homologous to FAS, but use long-chain acyl-CoAs as primers, and malonyl CoA for chain length extension, and different isoforms are capable of producing fatty acids up to at least C26 in many eukaryotic microbes (Schweizer and Hofmann 2004). While representing only a minor fraction of total fatty acids, these very-long-chain fatty acids (VLCFAs) are often essential for cell viability as components of both sphingolipids and GPI anchors, as demonstrated in yeast (Tehlivets et al. 2007).

Fatty acids are assembled into glycerolipids by stepwise acylation of the *sn*-1 and *sn*-2 hydroxyls of glycerol-3-phosphate using either acyl-CoAs or acyl-ACPs (Riekhof et al. 2005; Gibellini and Smith 2010). The phosphatidic acid thus produced is used as substrate in the synthesis of a variety of phospholipids, glycolipids, sulfolipids, and betaine lipids that, in varying ratios depending on the species, are the major constituents of most microbial membranes. In addition, fatty acids can be converted to storage lipids such as triacylglycerols commonly found in eukaryotic microbes, as well as wax esters in some bacteria (Ishige et al. 2003). Though often depicted as linear biosynthetic pathways, a growing body of work has revealed the occurrence of Lands' cycle glycerolipid acyl-editing (Lands 1960) in some microbes, which adds to the diversity of membrane lipids through the remodeling activity of specific acyltransferases (Das et al. 2001). Several chapters in this volume are dedicated to various aspects of microbial glycerolipid metabolism.

2.3 Fatty Acid Degradation

The ability of microbes to catabolize fatty acids through β-oxidation is nearly as ubiquitous as the capacity for de novo fatty acid synthesis. This is not surprising when considering that energy dense fatty acids are an attractive source of energy to drive metabolism and provide carbon skeletons for the production of macromolecules in growing cells. Microbial β-oxidation is not only important for microbes which we consider decomposers but is just as important for microbes (e.g., phototrophic algae) that accumulate storage lipids under environmental stresses, only to

metabolize them to support growth when permissive conditions return (Poirer et al. 2006). The metabolic "undoing" of a fatty acid shares many mechanistic similarities with its synthesis, and in the early days of lipid biochemistry, the two pathways were believed to be the same reversible pathway operating in opposite directions (Lynen 1964). Since then the biochemistry of β-oxidation has been studied in detail, and several variations are known in different microbes. One example in eukaryotic microbes is the presence of β-oxidation in both the peroxisomes and mitochondria. While yeasts appear to only harbor the peroxisomal pathway, the fungus *Aspergillus nidulans* harbors and utilizes both depending on the acyl chain length being degraded (Maggio-Hall and Keller 2004). β-oxidation utilizes acyl-CoAs as substrate regardless of locale – but a significant difference exists in the first enzymatic step. In peroxisomes an acyl-CoA oxidase dependent on electron transfer to O_2 catalyzes the first step, whereas in mitochondria an acyl-CoA dehydrogenase is linked to the electron transport chain (Kim and Miura 2004).

Fatty acids enter mitochondrial β-oxidation differently depending on their length. Short- (<6 carbons) and medium-chain (6–12 carbons) fatty acids freely cross mitochondrial membranes without utilizing transporters and are activated to their CoA derivatives within the mitochondria (Schonfeld and Wojtczak 2016). Contrarily, long-chain fatty acids (LCFA, >12 carbons) do not freely diffuse into mitochondria and must utilize a carnitine((*R*)-(−)-3-hydroxy-4-(trimethylammonio)butyrate) shuttle to enter (Longo et al. 2016). Unlike short- and medium-chain fatty acids that are activated to their CoA form in the mitochondria, LCFAs are activated to their CoA derivative in the cytoplasm. Once activated, LCFAs are transferred to carnitine by carnitine palmitoyltransferase 1 (CPT-1), which is associated with the outer membrane of mitochondria, forming an acyl-carnitine and a free coenzyme A (Nakamura et al. 2014). The acyl-carnitine is transported into the mitochondrion by carnitine-acylcarnitine translocase in exchange for a free carnitine. Finally, carnitine palmitoyltransferase 2 (CPT-2), on the inner mitochondrial membrane, transfers the acyl group to a coenzyme A in the mitochondrial matrix, liberating carnitine to be transported back out to the cytosol (Montgomery et al. 2013). Regeneration of carnitine by CPT-1 appears to be the key rate-limiting and regulatory step of β-oxidation of LCFA (Nada et al. 1995). The difference in the uptake of fatty acids of various lengths may explain, at least in part, the metabolic advantages seen with medium-chain fatty acids in comparison to LCFA, specifically an increase in mitochondrial oxidative capacity, increased mitochondrial respiration, and a decrease in reactive oxygen species production (Montgomery et al. 2013).

The *trans*-2-enoyl-CoA produced within the mitochondria is further metabolized by three highly conserved reactions (enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and ketothiolase) to yield an acetyl CoA and a shortened acyl-CoA that reenters the next β-oxidation cycle (Hiltunen et al. 2003; Fujita et al. 2007). Prokaryotic microbes appear unanimous in their use of acyl-CoA dehydrogenases to initiate β-oxidation, which can allow for anaerobic growth on fatty acids as a sole carbon source as long as an alternative terminal electron acceptor is present (Campbell et al. 2003). Of course, an exception to this has been reported in

Arthrobacter nicotianae, from which a short-chain acyl-CoA oxidase enzyme has been purified and characterized; its biological role remains to be elucidated (Sztajer et al. 1993). Noticeably absent from the microbial "β-oxidizers" are the cyanobacteria studied to date (von Berlepsch et al. 2012), but given that they represent only a fraction of a percent of the estimated number of cyanobacterial species, an exception to this observation seems plausible.

Given their central role in metabolism, one might expect that spills of vegetable oils would disappear quickly from the environment. Alas, this is not always true, and polymerization can lead to long-lasting contamination (Mudge 1997) unless spill response is prompt and thorough (Bucas and Saliot 2002).

3 Types of Fatty Acids

3.1 Saturated Fatty Acids

Fully saturated fatty acids (Table 2) are important components of most phospholipids and storage lipids. In light of the synthesis by the sequential addition of two-carbon units, it is no surprise that the vast majority of fatty acids with more than ten carbons have an even number of carbon atoms, and in general it is these that have common names (Table 2). Short-chain fatty acids are moderately toxic to microbes (Royce et al. 2013), and the larvae of some insects, such as the corn earworm (*Heliothis zea*) and fall armyworm (*Spodoptera frugiperda*), excrete caprylic acid to inhibit the germination of conidia of the ascomycete *Beauveria bassiana* (Smith and Grula 1982). Pelargonic acid is sold as a broad-spectrum herbicide (Dayan et al. 2009), and the alkyl esters of C8 to C12 fatty acids, along with C8 to C10 fatty alcohols, are used as selective pruning agents for fruit trees (Cathey et al. 1966). Long-chain fatty acids, such as palmitate (C16), inhibit methanogenesis (Silva et al. 2016).

Hydrocarbon-degrading bacteria readily assimilate longer alkanes to fatty acids and use them for both membrane and storage lipids (Davis 1964; Makula and Finnerty 1968). In general, these fatty acids have the same carbon length as the alkanes, but additional minor compounds can be detected in consortia degrading crude oil (Aries et al. 2001).

Eukaryotic cells, including yeasts, use palmitate and myristate in directing proteins to and from membranes and organelles by attaching (and removing) these fatty acids to cysteine or glycine residues (Linder and Deschenes 2007). Myristoyltransferase may be an important chemotherapeutic target in trypanosomes (Herrera et al. 2016).

3.2 Monoenoic Fatty Acids

There are two isomeric forms for each unsaturated bond in a fatty acid, the *cis* (Z) and *trans* (E) form. Most biological unsaturated fatty acids are the *cis* form, but

Table 2 Etymologies of saturated fatty acids

Carbon number	Common name	Etymology
C1	Formic	L. *formicum*, ants
C2	Acetic	L. *acetum*, vinegar
C3	Propionic	Gr. *pro pion*, initial fat
C4	Butyric	L. *butyrum*, (rancid) butter
C5	Valeric	L. *Valeriana*, valerian
C6	Caproic	L. *caper*, goat
C7	Enanthic	Gr. *oenos*, wine
C8	Caprylic	L. *caper*, goat
C9	Pelargonic	L. *Pelargonium*, geranium
C10	Capric	L. *caper*, goat
C12	Lauric	L. *Laurus*, laurel
C14	Myristic	L. *Myristica*, nutmeg
C16	Palmitic	Fr. *palmitique*, palm
C18	Stearic	Gr. *stear*, tallow
C20	Arachidic	L. *arachis*, peanut
C22	Behenic	Ar. Ben-oil tree, *Moringa oleifera*
C24	Carnaubic, lignoceric	P. *carnauba,* wax
		L. *ligno*, wood, *ceram* wax
C26	Cerotic	Gr. *keros*, wax
C28	Montanic	L. *montanus*, mountain, extracted from lignite
C30	Melissic	Gr. *melissa*, bee
C32	Lacceric	Hi. Lakh, resin from scale insects
C33	Psyllic	L. *Psylla*, wax-secreting plant louse
C34	Geddic	T. *Gedda*, beeswax (*Apis dorsata*)

Purists would insist that propionic acid is the smallest molecule with true fatty acid properties
Ar. Arabic, *Fr.* French, *Gr.* Greek, *Hi.* Hindi, *L.* Latin, *P.* Portuguese, *T.* Telugu

trans-fatty acids are well known. For example, milk contains vaccenic acid (*trans* 18:1, ω7) and a range of conjugated linoleic (C18:2) acids (a few percent of total fatty acids (Nudda et al. 2005), and these seem to have beneficial effects on human health (Banni et al. 2001; Da Silva et al. 2015). Bacteria have long been known to contain *trans*-fatty acids in their membrane phospholipids (Keweloh and Heipieper 1996; Heipieper et al. 2003), increasing the amount of the *trans* form in response to solvents (Junker and Ramos 1999).

The commonest *cis*-monoenoic fatty acids are the ω9 series, exemplified by oleic acid, and fatty acids with similar unsaturation often have oleic appended as a modifier to the saturated molecule's name (e.g., caproleic, palmitoleic, gadoleic, etc.; see Table 3). Here, it is worth noting that there are two alternative and potentially confusing nomenclatures for describing the position of the unsaturation: either n or ∇, counting from the acid moiety, or ω, counting from the other end, although some sources use n in place of ω. Table 3 uses the ω notation.

Table 3 Etymologies of some monoenoic fatty acids

Carbon number	Position of unsaturation	Common name	Etymology
C10	*cis* 10:1, ω1	Caproleic	L. *caper*, goat
	cis 10:1, ω6	Obtusilic	L. *Lindera obtusiloba* (Japanese spice bush)
C12	*cis* 12:1, ω3	Lauroleic	L. *Laurus*, laurel
	cis 12:1, ω7	Denticetic	L. *denti*, teeth, *cetus*, whale – toothed whale
	cis 12:1, ω8	Linderic	L. *Lindera obtusiloba* (Japanese spice bush)
C14	*cis* 14:1, ω5	Myristoleic	L. *Myristica*, nutmeg
	cis 14:1, ω9	Physeteric	L. *Physeter*. Sperm whale
C16	*cis* 16:1, ω7	Palmitoleic	Fr. *palmitique*, palm
	*trans*16:1, ω7	Palmitelaidic	Fr. *palmitique*, palm
C18	*cis* 18:1, ω7	Asclepic	L. *Asclepia*, milkweed
	trans 18:1, ω7	Vaccenic	L. *vacca*, cow
	cis 18:1, ω9	Oleic	L. *olea*, olive
	trans 18:1, ω9	Elaidic	Gr. *elaia*, olive
	cis 18:1, ω12	Petroselinic	L. *Petroselinum*, parsley
	trans 18:1, ω12	Petroselaidic	L. *Petroselinum*, parsley
C20	*cis* 20:1, ω7	Paullinic	L. *Paullinia*, guarana
	cis 20:1, ω9	Gondoic	J. *gondou,* pilot whale
	cis 20:1, ω11	Gadoleic	L. *Gadus*, cod
C22	*cis* 22:1, ω9	Erucic	L. *Eruca*, kale
C24	*cis* 24:1, ω9	Nervonic aka selacholeic	From cerebroside "nervone," aka from Gr. *selachos*, shark
C26	C26:1, ω9	Ximenic	L. *Ximenia americana*, tallow wood

Fr. French, *Gr.* Greek, *J.* Japanese, *L.* Latin

Unsaturated fatty acids have significantly lower melting points than their saturated cousins, for example, the saturated C18 stearic acid has a melting point of 70 °C, while C18:1 oleic acid has a melting point of 14 °C (Knothe and Dunn 2009) – this was the motivation for converting liquid vegetable oils to margarines by partial hydrogenation (Chrysan 2005), albeit in the process producing significant amounts of *trans*-fatty acids (Alonso et al. 2000). There has been significant concern over the health effects of these "unnatural" *trans* isomers, although perhaps overstated (de Souza et al. 2015; Kleber et al. 2015). The stereochemistry of the unsaturation also affects the melting point – oleic acid (*cis* 18:1, ω9) has a melting point of 14 °C, while elaidic acid (*trans* C18:1, ω9) has a melting point of 45 °C (Knothe and Dunn 2009).

Microbes seem to take advantage of these phenomena to tailor their fatty acid composition to their growth temperature; *Clostridia* (Chan et al. 1971), *Candida* (McMurrough and Rose 1973), *Navicula* (Teoh et al. 2004), and a variety of facultative psychrophiles (Rossi et al. 2009) have been shown to increase the amount

of unsaturation of their fatty acids at lower temperatures. Mink (*Mustela vison*) seem to increase the levels of unsaturated fatty acids in their extremities to live at cold temperatures (Mustonen et al. 2007), but apocryphal stories that polar bear (*Ursa maritimus*) paws exhibit the same phenomenon may not be correct (Pond et al. 1992). On the other hand, *Vibrio* (Morita et al. 1993) and *Colwellia* (Hashimoto et al. 2015) seem to isomerize *cis*- to *trans*-fatty acids as the growth temperature increases.

3.3 Polyenoic Fatty Acids

Polyunsaturated fatty acids played a significant role in the fine arts of the fifteenth century when it was discovered that linseed oil (*Linum usitatissimum*) was a "drying oil" (Lazzari and Chiantore 1999) that polymerized to a dry varnish on exposure to light and air. Boiling the oil initiates polymerization, and "thickens" it, so that drying after application is speeded, and metal catalysts can serve the same function. Mixing pigments into the boiled oil allowed the development of oil painting. The storage triglycerides of some cultivars of linseed (flax) contain more than 65% linolenic acid in their seed oil (all *cis*-C18:3; Green 1986), and it is these three unsaturated bonds that make this molecule such an effective substrate for oxidative polymerization, including the protection of cricket and baseball bats. Tung oil (*Vernicia fordii*) is another drying oil, in this case containing triglycerides of eleostearic acids (*cis,trans, trans* (80%) and all-*trans* C18:3; Dyer et al. 2002).

Of course there are many potential isomers for the polyenoic acids; Table 4 lists some of the more common C18 isomers, and many others surely exist. Two are classified as essential for the human diet (linoleic (ω6) and α-linolenic acids (ω3); Burr et al. 1932), although it is becoming evident that several of their desaturation/elongation products are more influential in human health (e.g., all-*cis*-5,8,11,14-eicosatetraenoic (arachidonic acid), all-*cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), and all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA); Ruxton et al. 2004). These lipids are incorporated into cellular structures and also form the foundation for a variety of signaling cascades in mammalian cells (Ryan et al. 2014) and as such are involved in many aspects of health and disease. Supplementing humans with ω3 fatty acids decreases inflammatory gene expression from neutrophils (Weaver et al. 2009), whereas increased ω6 consumption is associated with increased inflammation (de Batlle et al. 2012). Furthermore, low levels of serum ω3 lipids (especially DHA and EPA) are associated with more severe strokes and poorer outcomes (Song et al. 2015), while an increased total ω3:ω6 ratio was associated with decreased squamous cell carcinoma (Wallingford et al. 2013). This information is interesting in light of nutritional recommendations and food availability. Over the course of the twentieth century the amount of linoleic acid available in the diet increased significantly, from ~3% of available energy to >7%, largely due to the introduction of linoleic-rich vegetable oils such as soybean and corn oil (Blasbalg et al. 2011). The low-fat trend that swept the field of nutrition in the 1980s also encouraged increased polyunsaturated consumption at the expense of saturated fat (Berge 2008), but while this led to an increased availability of ω6, there has not been a concordant rise in ω3 availability

Table 4 Some octadecenoic fatty acids

Stereochemistry	Trivial name	Etymology
cis,cis-5,11-octadecadienoic ω7	Ephedric	L. *Ephedra,* a gymnosperm
cis,cis-6,11-octadecadienoic ω7	Cilienic	L. *cilium*, eyelash, from *Tetrahymena* a ciliate
cis,cis-9,12-octadecadienoic ω6	Linoleic	Gr. *linon* – flax
trans,trans,*cis*-5,9,12-octadecatrienoic ω6	Columbinic	En. Columbine, *Aquilegia*
cis,cis,trans-8,10,12-octadecatrienoic ω6	α-Calendic	L. *Calendula,* marigold
All-*cis*-8,10,12-octadecatrienoic ω6	β-Calendic	L. *Calendula,* marigold
trans,cis,trans-8,10,12-octadecatrienoic ω6	Jacaric	L. *Jacaranda*
trans,cis,trans-9,11,13-octadecatrienoic ω5	Punicic	L. *Punica*, pomegranate
cis,trans,trans-9,11,13-octadecatrienoic ω5	α-eleostearic	Gr. *eleo*, oily, Gr. *stear*, tallow
All-*trans*-9,11,13-octadecatrienoic ω5	β-eleostearic	Gr. *eleo*, oily, Gr. *stear*, tallow
trans,trans,cis-9,11,13-octadecatrienoic ω5	Catalpic	L. *Catalpa*
All-*cis*-5,9,12-octadecatrienoic ω6	Pinolenic	L. *Pinus*
All-*cis*-6,9,12-octadecatrienoic ω6	γ-Linolenic	Gr. *linon* – flax
All-*cis*-9,12,15-octadecatrienoic ω3	α-Linolenic	Gr. *linon* – flax
All-*trans*-9,12,15-octadecatrienoic ω3	α-Linolenelaidic	Gr. *linon* – flax
cis,cis,cis,trans-8,10,12,14-octadecatetraenoic ω4	Ixoric	L. *Ixora* a shrub

En. English, *Gr*. Greek, *L*. Latin

(Blasbalg et al. 2011). While vegetable oils have been major contributors to increased dietary ω6 fats, changes in agriculture and aquaculture have also altered dietary availability of polyunsaturated fats. Animals raised on grass have more ω3-fats and a higher ω3:ω6 ratio than feed-lot raised animals (Daley et al. 2010; McDaniel et al. 2013), and farm-raised Atlantic salmon have a ω3:ω6 ratio of 3.6 ± 1.8, while wild salmon's ratio is 11 ± 2.7 (Blanchet et al. 2005). Interestingly, the ratio in farmed rainbow trout and wild rainbow were not significantly different at around 4.6 (Blanchet et al. 2005). Dairy products from cows fed grass have substantially more ω3 fats than those from their grain-fed compatriots (Benoit et al. 2014). There are many ways in which dietary lipids might affect health, and historically research has focused on changes in inflammation, cholesterol, and lipid membranes (Simopoulos 2008), but there is growing interest in the influence of dietary lipids on the microbiome (Conlon and Bird 2015). Mice fed dairy products from pasture-fed cows not only had a decrease in inflammatory markers in adipocyte cells, they also had an increase in paneth and goblet cells in the gut, which are known to be protective and to participate in microbiota homeostasis (Benoit et al. 2014). Another study showed that feeding mice a diet rich in ω6 fats caused intestinal

dysbiosis (Ghosh et al. 2013). In a mouse model of alcoholic liver disease, animals fed a diet rich in saturated fat with alcohol were protected against endotoxemia and liver steatosis in comparison to animals fed ω6-rich corn oil and alcohol. In these animals there were also significant alterations in gut microbiota with a reduction in Bacteroidetes and increased Proteobacteria and Actinobacteria (Kirpich et al. 2016). This is obviously a complex picture, made more complex by the feedback from the microbiome, where metabolites produced by the microbiome, especially short-chain fatty acids, likely contribute to the health of the host (Kirpich et al. 2016).

As noted above, polyunsaturated fatty acids form the foundation for a variety of signaling cascades in mammalian cells (Ryan et al. 2014), and linolenic acid is the start of the jasmonate pathway in plants (Pérez and Goossens 2013) and algae (Jusoh et al. 2015).

Polyunsaturation also affects physical properties. A second unsaturation lowers the melting point of fatty acids below that of the monoenes, such that C18:2 linoleic acid has a melting point of −7 °C (Knothe and Dunn 2009) and polyenoic acids usually have even lower melting points.

4 Research Needs

This volume contains an up-to-date overview of many aspects of the biogenesis of fatty acids, lipids, and membranes. This is an exciting time for such studies because new analytical and genomic techniques are revolutionizing the field.

References

Alonso L, Fraga MJ, Juárez M (2000) Determination of *trans* fatty acids and fatty acid profiles in margarines marketed in Spain. J Am Oil Chem Soc 77:131–136

Amirta R, Fujimori K, Shirai N, Honda Y, Watanabe T (2003) Ceriporic acid C, a hexadecenylitaconate produced by a lignin-degrading fungus, *Ceriporiopsis subvermispora*. Chem Phys Lipid 126:121–131

Aries E, Doumenq P, Artaud J, Acquaviva M, Bertrand JC (2001) Effects of petroleum hydrocarbons on the phospholipid fatty acid composition of a consortium composed of marine hydrocarbon-degrading bacteria. Org Geochem 32:891–903

Bagby MO, Smith CR Jr, Wolff IA (1965) Laballenic acid. A new allenic acid from *Leonotis nepetaefolia* seed oil 1. J Org Chem 30:4227–4229

Banni S, Angioni E, Murru E, Carta G, Paola Melis M, Bauman D, Dong Y, Ip C (2001) Vaccenic acid feeding increases tissue levels of conjugated linoleic acid and suppresses development of premalignant lesions in rat mammary gland. Nutr Cancer 41:91–97

Barford RA, Herb SF, Luddy FE, Magidman P, Riemenschneider RW (1963) Alcoholysis of *Vernonia anthelmintica* seed oil and isolation of methyl epoxyoleate. J Am Oil Chem Soc 40:136–138

Barkan D, Liu Z, Sacchettini JC, Glickman MS (2009) Mycolic acid cyclopropanation is essential for viability, drug resistance, and cell wall integrity of *Mycobacterium tuberculosis*. Chem Biol 16:499–509

Bellou S, Triantaphyllidou IE, Aggeli D, Elazzazy AM, Baeshen MN, Aggelis G (2016) Microbial oils as food additives: recent approaches for improving microbial oil production and its polyunsaturated fatty acid content. Curr Opin Biotechnol 37:24–35

Benoit B, Plaisancie P, Geloen A, Estienne M, Debard C, Meugnier E, Loizon E, Daira P, Bodennec J, Cousin O, Vidal H, Laugerette F, Michalski MC (2014) Pasture v. standard dairy cream in high-fat diet-fed mice: improved metabolic outcomes and stronger intestinal barrier. Br J Nutr 112:520–535

Berge AFL (2008) How the ideology of low fat conquered America. J Hist Med Allied Sci 63:139–177

Blanchet C, Lucas M, Julien P, Morin R, Gingras S, Dewailly E (2005) Fatty acid composition of wild and farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Lipids 40:529–531

Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR (2011) Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. Am J Clin Nutr 93:950–962

Brune T, Haas K (2011) *Equisetum* species show uniform epicuticular wax structures but diverse composition patterns. AoB Plants 2011:plr009

Bucas G, Saliot A (2002) Sea transport of animal and vegetable oils and its environmental consequences. Mar Pollut Bull 44:1388–1396

Bu'Lock JD (1964) Polyacetylenes and related compounds in nature. Prog Org Chem 6:86–134

Burr GO, Burr MM, Miller ES (1932) On the fatty acids essential in nutrition. 3. J Biol Chem 97:1–9

Campbell JW, Morgan-Kiss RM, Cronan JE Jr (2003) A new *Escherichia coli* metabolic competency: growth on fatty acids by a novel anaerobic beta-oxidation pathway. Mol Microbiol 47:793–805

Carballeira NM, Reyes M, Sostre A, Huang H, Verhagen MF, Adams MW (1997) Unusual fatty acid compositions of the hyperthermophilic archaeon *Pyrococcus furiosus* and the bacterium *Thermotoga maritima*. J Bacteriol 179:2766–2768

Cathey HM, Steffens GL, Stuart NW, Zimmerman RH (1966) Chemical pruning of plants. Science 153:1382–1383

Celmer WD, Solomons IA (1953) Mycomycin. III. The structure of mycomycin, an antibiotic containing allene, diacetylene and cis, trans-diene groupings. J Am Oil Chem Soc 75:1372–1376

Chan M, Himes RH, Akagi JM (1971) Fatty acid composition of thermophilic, mesophilic, and psychrophilic *Clostridia*. J Bacteriol 106:876–881

Chrysan MM (2005) Margarines and spreads. Food emulsifiers and their applications. In: Shahidi F (ed) Bailey's industrial oil and fat products, 6th edn. Wiley, New York, pp 307–326

Conlon MA, Bird AR (2015) The impact of diet and lifestyle on gut microbiota and human health. Nutrients 7(1):17–44

Corley TA (1988) A history of the Burmah Oil Company: 1924–66. Heinemann, London

Curran KA, Leavitt JM, Karim AS, Alper HS (2013) Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*. Metabol Eng 15:55–66

Da Silva MS, Julien P, Pérusse L, Vohl MC, Rudkowska I (2015) Natural rumen-derived trans fatty acids are associated with metabolic markers of cardiac health. Lipids 50:873–882

Daley CA, Abbott A, Doyle PS, Nader GA, Larson S (2010) A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. Nutr J 10:1

Das S, Castillo C, Stevens T (2001) Phospholipid remodeling/generation in Giardia: the role of the lands cycle. Trends Parasitol 17:316–319

Davis JB (1964) Microbial incorporation of fatty acids derived from n-alkanes into glycerides and waxes. Appl Microbiol 12:210–214

Dayan FE, Cantrell CL, Duke SO (2009) Natural products in crop protection. Bioorg Med Chem 17:4022–4034

de Batlle J, Sauleda J, Balcells E, Gomez FP, Mendez M, Rodriguez E, Barreiro E, Ferrer JJ, Romieu I, Gea J, Anto JM, Garcia-Aymerich J (2012) Association between omega3 and omega6 fatty acid intakes and serum inflammatory markers in COPD. J Nutr Biochem 23:817–821

de Souza RJ, Mente A, Maroleanu A, Cozma AI, Ha V, Kishibe T, Uleryk E, Budylowski P, Schünemann H, Beyene J, Anand SS (2015) Intake of saturated and trans unsaturated fatty acids

and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies. Brit Med J 351:h3978

Dear RE, Pattison FL (1963) Toxic fluorine compounds. XVIII. 1 the synthesis of the toxic principle of *Dichapetalum toxicarium* (18-Fluoro-*cis*-9-octadecenoic acid) and related ω-fluoro unsaturated acids. J Am Chem Soc 85:622–626

Dembitsky VM, Srebnik M (2002) Natural halogenated fatty acids: their analogues and derivatives. Prog Lipid Res 41:315–367

Dubertret G, Mirshahi A, Mirshahi M, Gerard-Hirne C, Tremolieres A (1994) Evidence from in vivo manipulations of lipid composition in mutants that the delta 3-trans-hexadecenoic acid-containing phosphatidylglycerol is involved in the biogenesis of the light-harvesting chlorophyll a/b-protein complex of *Chlamydomonas reinhardtii*. Eur J Biochem 226:473–482

Dyer JM, Chapital DC, Kuan JC, Mullen RT, Turner C, McKeon TA, Pepperman AB (2002) Molecular analysis of a bifunctional fatty acid conjugase/desaturase from tung. Implications for the evolution of plant fatty acid diversity. Plant Physiol 130:2027–2038

English J, Bonner J, Haagen-Smit AJ (1939) Structure and synthesis of a plant wound hormone. Science 90:329

Freedman B, Bagby MO (1989) Heats of combustion of fatty esters and triglycerides. J Am Oil Chem Soc 66:1601–1605

Fujita Y, Matsuoka H, Hirooka K (2007) Regulation of fatty acid metabolism in bacteria. Mol Microbiol 66:829–839

Gao J, Ajjawi I, Manoli A, Sawin A, Xu C, Froehlich JE, Last RL, Benning C (2009) FATTY ACID DESATURASE4 of *Arabidopsis* encodes a protein distinct from characterized fatty acid desaturases. Plant J 60:832–839

Geiger O (2010) Lipids and *Legionella* virulence. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology, 1st edn. Springer, Berlin, pp 3195–3202

Ghosh S, Molcan E, DeCoffe D, Dai C, Gibson DL (2013) Diets rich in n-6 PUFA induce intestinal microbial dysbiosis in aged mice. Br J Nutr 110:515–523

Gibellini F, Smith TK (2010) The Kennedy pathway – de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. IUBMB Life 62:414–428

Glass RL, Krick TP, Sand DM, Rahn CH, Schlenk H (1975) Furanoid fatty acids from fish lipids. Lipids 10:695–702

Green AG (1986) A mutant genotype of flax (*Linum usitatissimum* L.) containing very low levels of linolenic acid in its seed oil. Can J Plant Sci 66:499–503

Grogan DW, Cronan JE (1997) Cyclopropane ring formation in membrane lipids of bacteria. Microbiol Mol Biol Rev 61:429–441

Hammond EG, Lundberg WO (1953) A methyl docosahexaenoate: its isolation and characterization. J Am Oil Chem Soc 30:438–441

Hashimoto M, Orikasa Y, Hayashi H, Watanabe K, Yoshida K, Okuyama H (2015) Occurrence of trans monounsaturated and polyunsaturated fatty acids in *Colwellia psychrerythraea* strain 34H. J Basic Microbiol 55:838–845

Heipieper HJ, Meinhardt F, Segura A (2003) The *cis–trans* isomerase of unsaturated fatty acids in *Pseudomonas* and *Vibrio*: biochemistry, molecular biology and physiological function of a unique stress adaptive mechanism. FEMS Microbiol Letts 229:1–7

Herrera LJ, Brand S, Santos A, Nohara LL, Harrison J, Norcross NR, Thompson S, Smith V, Lema C, Varela-Ramirez A, Gilbert IH (2016) Validation of N-myristoyltransferase as potential chemotherapeutic target in mammal-dwelling stages of *Trypanosoma cruzi*. PLoS Negl Trop Dis 10:e0004540

Hiltunen JK, Mursula AM, Rottensteiner H, Wierenga RK, Kastaniotis AJ, Gurvitz A (2003) The biochemistry of peroxisomal beta-oxidation in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 27:35–64

Hofmann K, Lucas RA (1950) The chemical nature of a unique fatty acid. J Am Chem Soc 72:4328–4329

Ishige T, Tani A, Sakai Y, Kato N (2003) Wax ester production by bacteria. Curr Opin Microbiol 6:244–250

Janssen HJ, Steinbuchel A (2014) Fatty acid synthesis in *Escherichia coli* and its applications towards the production of fatty acid based biofuels. Biotechnol Biofuels 7:7

Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ (2012) The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev 76:46–65

Johnson EA, Burdon KL (1947) Mycomycin-a new antibiotic produced by a moldlike actinomycete active against the bacilli of human tuberculosis. J Bacteriol 54:281

Jump DB, Tripathy S, Depner CM (2013) Fatty acid–regulated transcription factors in the liver. Ann Rev Nutr 33:249–269

Junker F, Ramos JL (1999) Involvement of the *cis/trans* isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. J Bacteriol 181:5693–5700

Jusoh M, Loh SH, Chuah TS, Aziz A, San Cha T (2015) Elucidating the role of jasmonic acid in oil accumulation, fatty acid composition and gene expression in *Chlorella vulgaris* (Trebouxiophyceae) during early stationary growth phase. Algal Res 9:14–20

Kaneda T (1991) *Iso*- and *anteiso*-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288–302

Kass LR, Bloch K (1967) On the enzymatic synthesis of unsaturated fatty acids in *Escherichia coli*. Proc Natl Acad Sci U S A 58:1168–1173

Keweloh H, Heipieper HJ (1996) Trans unsaturated fatty acids in bacteria. Lipids 31:129–137

Kim JJ, Miura R (2004) Acyl-CoA dehydrogenases and acyl-CoA oxidases. Structural basis for mechanistic similarities and differences. Eur J Biochem 271:483–493

Kirpich IA, Petrosino J, Ajami N, Feng W, Wang Y, Liu Y, Beier JI, Barve SS, Yin X, Wei X, Zhang X, McClain CJ (2016) Saturated and unsaturated dietary fats differentially modulate ethanol-induced changes in gut microbiome and metabolome in a mouse model of alcoholic liver disease. Am J Pathol 186:765–776

Kleber ME, Delgado GE, Lorkowski S, März W, von Schacky C (2015) Trans fatty acids and mortality in patients referred for coronary angiography: the Ludwigshafen risk and cardiovascular health study. Eur Heart J 2015:ehv446

Klein RA, Hazlewood GP, Kemp P, Dawson RM (1979) A new series of long-chain dicarboxylic acids with vicinal dimethyl branching found as major components of the lipids of *Butyrivibrio* spp. Biochem J 183:691–700

Knothe G, Dunn RO (2009) A comprehensive evaluation of the melting points of fatty acids and esters determined by differential scanning calorimetry. J Am Oil Chem Soc 86:843–856

Kuo J, Khosla C (2014) The initiation ketosynthase (FabH) is the sole rate-limiting enzyme of the fatty acid synthase of *Synechococcus* sp. PCC 7002. Metab Eng 22:53–59

Lands WEM (1960) Metabolism of glycerolipids. II. The enzymatic acylation of lysolecithin. J Biol Chem 235:2233–2237

Lang I, Hodac L, Friedl T, Feussner I (2011) Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biol 11:124

Lazzari M, Chiantore O (1999) Drying and oxidative degradation of linseed oil. Polymer Deg Stabil 65:303–313

Linder ME, Deschenes RJ (2007) Palmitoylation: policing protein stability and traffic. Nat Rev Mol Cell Biol 8:74–84

Longo N, Frigeni M, Pasquali M (2016) Carnitine transport and fatty acid oxidation. Biochim Biophys Acta 1863:2422–2435

Lynen F (1964) The pathway from "activated acetic acid" to the terpenes and fatty acids. Proc Roy Karol Inst 1964:103–138

Lynen F (1969) Yeast fatty acid synthase. Meth Enzymol 14:17–33

Maggio-Hall LA, Keller NP (2004) Mitochondrial beta-oxidation in *Aspergillus nidulans*. Mol Microbiol 54:1173–1185

Makula R, Finnerty WR (1968) Microbial assimilation of hydrocarbons I. Fatty acids derived from normal alkanes. J Bacteriol 95:2102–2107

McDaniel J, Askew W, Bennett D, Mihalopoulos J, Anantharaman S, Fjeldstad AS, Rule DC, Nanjee NM, Harris RA, Richardson RS (2013) Bison meat has a lower atherogenic risk than beef in healthy men. Nutr Res 33:293–302

McMurrough I, Rose AH (1973) Effects of temperature variation on the fatty acid composition of a psychrophilic *Candida* species. J Bacteriol 114:451–452

Meesapyodsuk D, Qiu X (2008) An oleate hydroxylase from the fungus *Claviceps purpurea*: cloning, functional analysis, and expression in *Arabidopsis*. Plant Physiol 147:1325–1333

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293:290–293

Metzger JO (2009) Fats and oils as renewable feedstock for chemistry. Eur J Lipid Sci Technol 111:865–876

Montgomery MK, Osborne B, Brown SH, Small L, Mitchell TW, Cooney GJ, Turner N (2013) Contrasting metabolic effects of medium- versus long-chain fatty acids in skeletal muscle. J Lipid Res 54:3322–3333

Morita N, Shibahara A, Yamamoto K, Shinkai K, Kajimoto G, Okuyama H (1993) Evidence for *cis-trans* isomerization of a double bond in the fatty acids of the psychrophilic bacterium *Vibrio* sp. strain ABE-1. J Bacteriol 175:916–918

Mudge S (1997) Can vegetable oils outlast mineral oils in the marine environment? Mar Pollut Bull 34:213

Mustonen AM, Käkelä R, Nieminen P (2007) Different fatty acid composition in central and peripheral adipose tissues of the American mink (*Mustela vison*). Comp Biochem Physiol A 147:903–910

Nada MA, Abdel-Aleem S, Schulz H (1995) On the rate-limiting step in the beta-oxidation of polyunsaturated fatty acids in the heart. Biochim Biophys Acta 1255:244–250

Nagy B, Bitz SM (1963) Long-chain fatty acids from the Orgueil meteorite. Arch of Biochem Biophys 101:240–248

Nakamura MT, Yudell BE, Loor JJ (2014) Regulation of energy metabolism by long-chain fatty acids. Prog Lipid Res 53:124–144

Nudda A, McGuire MA, Battacone G, Pulina G (2005) Seasonal variation in conjugated linoleic acid and vaccenic acid in milk fat of sheep and its transfer to cheese and ricotta. J Dairy Sci 88:1311–1319

Ohnishi M, Thompson GA Jr (1991) Biosynthesis of the unique trans-delta 3-hexadecenoic acid component of chloroplast phosphatidylglycerol: evidence concerning its site and mechanism of formation. Arch Biochem Biophys 288:591–599

Oshima MI, Ariga T (1975) Omega-cyclohexyl fatty acids in acidophilic thermophilic bacteria. Studies on their presence, structure, and biosynthesis using precursors labeled with stable isotopes and radioisotopes. J Biol Chem 250:6963–6968

Pérez AC, Goossens A (2013) Jasmonate signaling: a copycat of auxin signaling? Plant Cell Environ 36:2071–2084

Poirier Y, Antonenkov VD, Glumoff T, Hiltunen JK (2006) Peroxisomal beta-oxidation-a metabolic pathway with multiple functions. Biochim Biophys Acta 1763:1413–1426

Pond CM, Mattacks CA, Colby RH, Ramsay MA (1992) The anatomy, chemical composition, and metabolism of adipose tissue in wild polar bears (*Ursus maritimus*). Can J Zoo 70:326–341

Qureshi N, Takayama K, Jordi HC, Schnoes HK (1978) Characterization of the purified components of a new homologous series of alpha-mycolic acids from *Mycobacterium tuberculosis* H37Ra. J Biol Chem 253:5411–5417

Riekhof WR, Sears BB, Benning C (2005) Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: discovery of the betaine lipid synthase BTA1Cr. Eukaryot Cell 4:242–252

Rontani JF, Christodoulou S, Koblizek M (2005) GC-MS structural characterization of fatty acids from marine aerobic anoxygenic phototrophic bacteria. Lipids 40:97–108

Rossi M, Buzzini P, Cordisco L, Amaretti A, Sala M, Raimondi S, Ponzoni C, Pagnoni UM, Matteuzzi D (2009) Growth, lipid accumulation, and fatty acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts. FEMS Microbiol Ecol 69:363–372
Royce LA, Liu P, Stebbins MJ, Hanson BC, Jarboe LR (2013) The damaging effects of short chain fatty acids on *Escherichia coli* membranes. Appl Microbiol Biotechnol 97:8317–8327
Ruxton CH, Reed SC, Simpson MJ, Millington KJ (2004) The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J Hum Nutr Diet 17:449–459
Ryan VH, Primiani CT, Rao JS, Ahn K, Rapoport SI (2014) Coordination of gene expression of arachidonic and docosahexaenoic acid cascade. PLOS One 9:e100858
Schonfeld P, Wojtczak L (2016) Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. J Lipid Res 57:943–954
Schröder M, Vetter W (2013) Detection of 430 fatty acid methyl esters from a transesterified butter sample. J Am Oil Chem Soc 90:771–790
Schweizer E, Hofmann J (2004) Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. Microbiol Mol Biol Rev 68:501–517
Segré D, Ben-Eli D, Deamer DW, Lancet D (2001) The lipid world. Orig Life Evol B 31:119–145
Shah J (2009) Plants under attack: systemic signals in defence. Curr Opin Plant Biol 12:459–464
Shirasaka N, Nishi K, Shimizu S (1995) Occurrence of a furan fatty acid in marine bacteria. Biochim Biophys Acta 1258:225–227
Silva SA, Salvador AF, Cavaleiro AJ, Pereira MA, Stams AJ, Alves MM, Sousa DZ (2016) Toxicity of long chain fatty acids towards acetate conversion by *Methanosaeta concilii* and *Methanosarcina mazei*. Microb Biotechnol 9:514–518
Simopoulos AP (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med 233:674–688
Smith RJ, Grula EA (1982) Toxic components on the larval surface of the corn earworm (*Heliothis zea*) and their effects on germination and growth of *Beauveria bassiana*. J Invert Pathol 39:15–22
Song TJ, Cho HJ, Chang Y, Choi K, Jung AR, Youn M, Shin MJ, Kim YJ (2015) Low plasma proportion of omega 3-polyunsaturated fatty acids predicts poor outcome in acute non-cardiogenic ischemic stroke patients. J Stroke 17:168–176
Sztajer H, Wagner G, Schmnid RD (1993) Bacterial short-chain acyl-CoA oxidase: production, purification and characterization. Appl Microbiol Biotechnol 39:708–713
Takayama K, Wang C, Besra GS (2005) Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. Clin Microbiol Rev 18:81–101
Taleshi MS, Edmonds JS, Goessler W, Ruiz-Chancho MJ, Raber G, Jensen KB, Francesconi KA (2010) Arsenic-containing lipids are natural constituents of sashimi tuna. Environ Sci Technol 44:1478–1483
Tehlivets O, Scheuringer K, Kohlwein SD (2007) Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta 1771:255–270
Teoh ML, Chu WL, Marchant H, Phang SM (2004) Influence of culture temperature on the growth, biochemical composition and fatty acid profiles of six Antarctic microalgae. J Appl Phycol 16:421–430
Toomey RE, Wakil SJ (1966) Studies on the mechanism of fatty acid synthesis. XVI. Preparation and general properties of acyl-malonyl acyl carrier protein-condensing enzyme from *Escherichia coli*. J Biol Chem 241:1159–1165
Vagelos PR, Alberts AW, Majerus PW (1969) Mechanism of saturated fatty acid biosynthesis in *Escherichia coli*. Meth Enzymol 14:39–43
Vieler A, Wu G, Tsai CH, Bullard B, Cornish AJ, Harvey C, Reca IB, Thornburg C, Achawanantakun R, Buehl CJ, Campbell MS, Cavalier D, Childs KL, Clark TJ, Deshpande R, Erickson E, Armenia Ferguson A, Handee W, Kong Q, Li X, Liu B, Lundback S, Peng C, Roston RL, Sanjaya SJP, Terbush A, Warakanont J, Zauner S, Farre EM, Hegg EL, Jiang N, Kuo MH, Lu Y, Niyogi KK, Ohlrogge J, Osteryoung KW, Shachar-Hill Y, Sears BB, Sun Y, Takahashi H, Yandell M, Shiu SH, Benning C (2012) Genome,

functional gene annotation, and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. PLoS Genet 8:e1003064

von Berlepsch S, Kunz HH, Brodesser S, Fink P, Marin K, Flugge UI, Gierth M (2012) The acyl-acyl carrier protein synthetase from *Synechocystis* sp. PCC 6803 mediates fatty acid import. Plant Physiol 159:606–617

Vrkoslav V, Míková R, Cvačka J (2009) Characterization of natural wax esters by MALDI-TOF mass spectrometry. J Mass Spectrom 44:101–110

Wakimoto T, Kondo H, Nii H, Kimura K, Egami Y, Oka Y, Yoshida M, Kida E, Ye Y, Akahoshi S, Asakawa T (2011) Furan fatty acid as an anti-inflammatory component from the green-lipped mussel *Perna canaliculus*. Proc Natl Acad Sci U S A 108:17533–17537

Wallingford SC, Hughes MC, Green AC, van der Pols JC (2013) Plasma omega-3 and omega-6 concentrations and risk of cutaneous basal and squamous cell carcinomas in Australian adults. Cancer Epidemiol Biomark Prev 22:1900–1905

Weaver KL, Ivester P, Seeds M, Case LD, Arm JP, Chilton FH (2009) Effect of dietary fatty acids on inflammatory gene expression in healthy humans. J Biol Chem 284:15400–15407

Wisotzkey JD, Jurtshuk P Jr, Fox GE, Deinhard G, Poralla K (1992) Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius, Bacillus acidoterrestris,* and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus gen. nov.* Int J Syst Bacteriol 42:263–269

Ye C, Qiao W, Yu X, Ji X, Huang H, Collier JL, Liu L (2015) Reconstruction and analysis of the genome-scale metabolic model of *Schizochytrium limacinum* SR21 for docosahexaenoic acid production. BMC Genomics 16:799

Yoshida K, Hashimoto M, Hori R, Adachi T, Okuyama H, Orikasa Y, Nagamine T, Shimizu S, Ueno A, Morita N (2016) Bacterial long-chain polyunsaturated fatty acids: their biosynthetic genes, functions, and practical use. Mar Drugs 14:94

Yu X, Liu T, Zhu F, Khosla C (2011) In vitro reconstitution and steady-state analysis of the fatty acid synthase from *Escherichia coli*. Proc Natl Acad Sci U S A 108:18643–18648

Synthesis of Acetyl-CoA from Carbon Dioxide in Acetogenic Bacteria

2

A. Wiechmann and V. Müller

Contents

1 Introduction 26
2 The Wood-Ljungdahl Pathway of CO_2 Fixation 27
3 Energy Conservation in Acetogenic Bacteria 30
3.1 Electron Transfer Within the WLP 30
3.2 Energy Conservation in Form of ATP 32
4 CO Fixation for Production of Acetyl-CoA and Acetate 34
5 CO_2 Fixation of Acetogens During Heterotrophic Growth 36
6 Research Needs 38
References 39

Abstract

Bacterial species, which are able to fix CO_2 + H_2 as only carbon and energy source to acetyl-CoA and further to acetate, are called acetogens. The pathway acetogenic bacteria possess is the linear, two-branched reductive acetyl-CoA pathway (Wood-Ljungdahl pathway), which they not only use to fix CO_2 + H_2 and/or CO to acetyl-CoA and further to acetate but also for redox balancing when growing on other carbon substrates. Reduction of CO_2 to acetate does not leave acetogens with any additional energy in form of ATP for their anabolism. In order to overcome these energetic constraints, acetogens possess additional membrane complexes which couple the electron transfer from reduced ferredoxin to H^+ or NAD^+ to a proton or sodium ion gradient across the membrane, which in turn can be used by a proton- or sodium ion-dependent ATP synthase for energy conservation. Since acetogens are able to live autotrophically by using H_2 + CO_2, they are considered to be valuable tools for the fixation of greenhouse gases. Genetic

A. Wiechmann · V. Müller (✉)
Department of Molecular Microbiology and Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt/Main, Frankfurt, Germany
e-mail: vmueller@bio.uni-frankfurt.de

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_4

modifications together with fermentative studies have converted these living artists to strong work horses for production of biofuels and synthetic compounds that help to prevent further global warming and the exploitation of our planet's fossil fuels.

1 Introduction

When life on earth started to evolve about 4.1 billion years ago in the Hadean period (Bell et al. 2015), the conversion of CO_2 to more complex organic carbon happened for the first time in an ancestral living cell. At this early time of our planet's history, the most abundant carbon source was CO_2 within in an atmospheric temperature of around 55°C up to 80°C (Sojo et al. 2016; Basen and Müller 2016). Today hydrothermal alkaline deep sea vents are a habitat for microorganisms with similar conditions as there were when the first life forms on our planet developed, which means a strong alkaline environment (pH 9–11), venting fluids with temperature gradients between 40°C and 90°C and high concentrations of dissolved H_2 and CH_4 but not much dissolved CO_2 (Martin et al. 2008). Authors of a recent review paper suggested that the last universal common ancestor (LUCA) was likely able to fix CO_2 to a methyl group by a simple pathway (Sojo et al. 2016). Confirming this, an experimental study compared phylogenies of 6.1 million protein-coding genes from sequenced prokaryotic genomes and showed that LUCA was likely to be a thermophilic, anaerobic organism, which fixed CO_2 and N_2 for biomass production. Interestingly, the reductive acetyl-CoA pathway, also named Wood-Ljungdahl pathway (WLP) for CO_2 fixation, seems to be the first pathway used for production of biomass (Poehlein et al. 2012). In the case of LUCA, the WLP used H_2, released by geological serpentinization, as an electron donor to firstly reduce ferredoxin (Fd), which is used to reduce CO_2 to CO and to a methyl group, respectively. Together with CO, a methyl group and CoA are combined to acetyl-CoA which is used as a precursor for carbon metabolism and biomass production. Concomitant to CO_2 reduction, experimental data predict a proton or sodium-ion motive force across a porous membrane or H^+/Na^+ antiporters within a primitive cell wall, supplying the cell with energy for ATP synthesis important for energy-requiring processes (Sojo et al. 2016; Weiss et al. 2016). The LUCA is proposed to be the common ancestor of the taxonomic domains of *Archaea* and *Bacteria* out of which methanogenic archaea and acetogenic clostridia are likely the first descendants (Weiss et al. 2016). After splitting off from LUCA, archaea modified the ancestral WLP to produce acetyl-CoA and methane and acetogenic bacteria modified the CO_2 fixation pathway to synthesize acetyl-CoA and from there acetate and biomass (Martin and Sousa 2016).

Bacteria must exhibit some strict characteristics for them to be categorized as acetogens: Bacteria, which are able to produce biomass from fixing two molecules of CO_2 via the WLP to acetyl-CoA and further to acetate and additionally couple this pathway to energy conservation, are called acetogens (Drake et al. 2008; Schuchmann and Müller 2016). Acetyl-CoA is also used as a precursor for anabolism, and depending on the bacterial species, also additional by-products can be

synthesized from acetyl-CoA or acetate (Poehlein et al. 2012; Schuchmann and Müller 2014). Acetogenic bacteria can also feed on other carbon sources, such as sugars and alcohols, and therefore use the WLP to reoxidize or oxidize electron carriers to maintain a stable redox balance (Schuchmann and Müller 2016).

It is important to clarify that not all bacteria which produce acetate are acetogenic bacteria. For example, belonging to the genus *Clostridia*, there are species, which harbor the WLP for energy conservation and hence produce acetate from two molecules of CO_2, such as *Clostridium autoethanogenum* and *Clostridium ljungdahlii* (Abrini et al. 1994; Tanner et al. 1993). However, the same genus also harbors species such as *Clostridium acetobutylicum* which produces acetate together with ethanol and butanol from fermentation of glucose (Jones and Woods 1986) and is not considered to be an acetogen. This example of acetogenic and non-acetogenic species within a genus gives a hint toward the difficulty of classifying acetogens using simple methods such as 16S rRNA analysis. Other genera, for instance, *Eubacterium* and *Thermoanaerobacter*, also harbor species that are either acetogenic or non-acetogenic. Until now, solely bacteria belonging to the genera *Sporumosa, Moorella*, and *Acetobacterium* have been found to only be acetogens (Drake et al. 2008; Groher and Weuster-Botz 2016). In some cases, bacteria were not classified directly as acetogens, while they were isolated but found to exhibit all characteristics to be an acetogen in later studies. For example, *Clostridium coccoides,* isolated from the mouse gut flora in 1976 (Kaneuchi et al. 1976), was identified as acetogenic bacterium about 20 years later when the strain was isolated from a human intestine (Kamlage et al. 1997). In total, acetogens have been found in 23 genera so far (Drake et al. 2008).

Until recently, only bacteria were known to use the WLP for the production of acetate from acetyl-CoA. Recent findings gave evidence for the existence of archaea harboring genes for acetate production. Genes of Bathyarchaeota collected from marine sediments were found to encode for enzymes such as a phosphotransacetylase and an acetate kinase and confirmed the latter as a functional enzyme. Together with those genes, genes for the WLP of methanogenic archaea were sequenced such as the methenyltetrahydromethanopterin cyclohydrolase and the 5,10-methylenetetrahydromethanopterin reductase. These findings reveal that evolution of archaea and bacteria is slightly more complex than developing from LUCA either into a methanogen or an acetogen (He et al. 2016).

This chapter summarizes the current knowledge about the pathway of CO_2 fixation in acetogenic bacteria and energy conservation. Examples of different species will further illustrate enzymatic modifications to highlight the complexity of the WLP and energy-conserving mechanisms and how those bacteria are advantageous for the production of chemicals and biofuel commodities.

2 The Wood-Ljungdahl Pathway of CO_2 Fixation

The WLP (reductive acetyl-CoA pathway) is used for the autotrophic metabolism in some sulfate-reducing bacteria, in methanogenic archaea and in acetogenic bacteria (Fuchs 1986). The first acetogenic bacterium discovered was *Clostridium aceticum*,

which has been isolated in 1936 by Klaas T. Wieringa (Wieringa 1936, 1939). However, the culture had been lost until 1979, when a spore stock of *C. aceticum* was found at UC Berkeley by Gerhardt Gottschalk during his sabbatical at the laboratory of Horace A. Barker (Braun et al. 1981). *Moorella thermoacetica* (formerly known as *Clostridium thermoaceticum*) was firstly described by Francis E. Fontaine in 1942, who isolated this obligate thermophilic bacterium from horse manure and showed that it produces acetate from various types of sugars, such as glucose, fructose, and xylose (Fontaine et al. 1942). Starting from there, Lars G. Ljungdahl and Harland G. Wood carried out investigations in *M. thermoacetica* toward the identification of the pathway in which CO_2 is fixed into acetate (Wood 1952). Much of the work L. Ljungdahl performed was to elucidate the methyl branch of the WLP, and H. Wood successfully identified the enzymatic complexity of the carbonyl branch. Details of the stepwise identification of the WLP can be found in various journal and review articles (Li et al. 1966; Ljungdahl and Wood 1969; Wood et al. 1986; Ragsdale 2008; Drake et al. 2008). In honor of the achievement of those two outstanding researchers, the reductive acetyl-CoA pathway is also referred to as Wood-Ljungdahl pathway. Other well-studied acetogens are, for example, *Acetobacterium woodii* and *Clostridium autoethanogenum* and will be, together with *M. thermoacetica*, the ones this book chapter is mainly referring to.

In contrast to other metabolic pathways, the WLP is a reductive and linear process, in which two molecules of CO_2 are converted to two molecules of H_2O and to one molecule of acetyl-CoA, which is further converted to acetate (Fig. 1). In this pathway, eight reducing equivalents [H] are needed for the reduction of two molecules of CO_2 and further intermediates to acetate (Reaction 1).

$$2\,CO_2 + 8\,[H] \rightarrow \text{acetate} + 2\,H_2O \tag{1}$$

The enzymes of the WLP are encoded in three gene clusters. For example, in the acetogenic bacterium *A. woodii*, genes for the formate hydrogenase are arranged in cluster I (Awo_c08190–Awo_c08260); genes for the formyl-THF synthetase, methenyl-THF cyclohydrolase, methylene-THF dehydrogenase, and methylene-THF reductase are found in gene cluster II (Awo_c09260–Awo_c09310); and genes encoding for a functional CODH/ACS are arranged in cluster III (Awo_c10670–Awo_c10760) (Poehlein et al. 2012).

For production of acetyl-CoA, each molecule of CO_2 is reduced with different enzymes which "divides" the WLP into two "branches": One molecule is reduced to CO by a carbon monoxide dehydrogenase (CODH) at the expense of two reducing equivalents in the carbonyl branch, and in the other, the methyl branch, the other CO_2 molecule is reduced to formate by a formate dehydrogenase or hydrogen-dependent CO_2 reductase (HDCR). This reaction also needs two reducing equivalents. At the expense of one adenosine triphosphate (ATP), formate is coupled to tetrahydrofolate (THF), which is an important cofactor for transfer of single carbon groups. This generates formyl-THF, which is then cyclized into methenyl-THF by a formyl-THF cyclohydrolase. Two other reducing equivalents are needed to generate methylene-

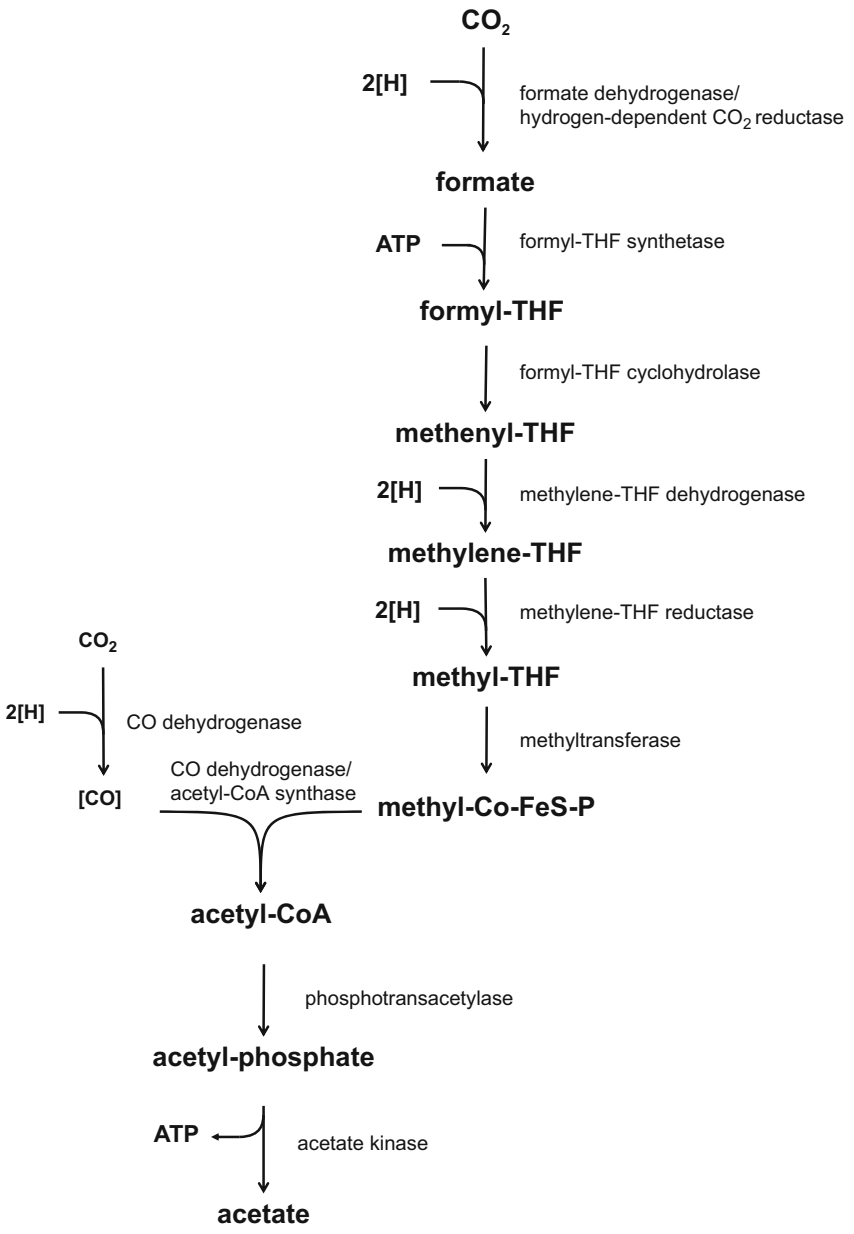


Fig. 1 General steps of the Wood-Ljungdahl pathway in acetogenic bacteria. One molecule of CO_2 is reduced via formate to a THF-bound methyl group. The methyl group is further transferred via a corrinoid iron-sulfur protein (*Co-FeS-P*) to the CO dehydrogenase/acetyl-CoA synthase. This enzyme complex oxidizes CO_2 to CO and then assembles acetyl-CoA by joining the methyl group together with CO and CoA. In the further enzymatic steps acetyl-CoA will be converted to acetate. *[H]* reducing equivalent, *THF* tetrahydrofolate

THF, and additional two electrons and protons are used for the production of methyl-THF using a methylene-THF-dehydrogenase in the first and a methylene-THF-reductase in the second step. With the help of a methyltransferase, the methyl group of methyl-THF is transferred from THF to a corrinoid iron-sulfur protein (Co-FeS-P), which in turn transfers the methyl group to the CODH-bound CO. Finally, the acetyl-CoA synthase (ACS) connects these two molecules and CoA for synthesis of acetyl-CoA. It is important to note that the CODH and the ACS form a complex for the simultaneous conversion of CO_2 via CO to acetyl-CoA. In total, acetogenic bacteria will have used one ATP during synthesis of formyl-THF but will also gain one ATP during synthesis of acetate from acetyl-CoA by substrate level phosphorylation (SLP). For this, acetyl-CoA is converted to acetyl-phosphate by a phosphotransacetylase, which recycles CoA for synthesis of the next molecule of acetyl-CoA in the WLP. After that, an acetate kinase transfers the phosphate to adenosine diphosphate (ADP) to synthesize ATP via SLP and to simultaneously produce acetate. In summary, one ATP is used during synthesis of formyl-THF, and one ATP is gained by the acetate kinase resulting in a net use/gain equation of zero molecules of ATP (Müller 2003; Schuchmann and Müller 2014).

For synthesis of biomass, it is assumed that acetogenic bacteria possess a pyruvate:ferredoxin oxidoreductase. This enzyme links the WLP to anabolism by the carboxylation of acetyl-CoA to pyruvate. For *M. thermoacetica*, it was shown that the PFOR can convert acetyl-CoA together with CO_2 to pyruvate and CoA at the expense of reducing equivalents, which probably are provided by Fd^{2-} (Furdui and Ragsdale 2000). It is important to note that the reverse reaction from pyruvate to acetyl-CoA and from there to the WLP can be used by acetogens to recycle their reducing equivalents which have been generated by heterotrophic growth (Ragsdale 2003).

3 Energy Conservation in Acetogenic Bacteria

3.1 Electron Transfer Within the WLP

As mentioned above, reducing equivalents are necessary for the electron and proton transfer during several redox steps of the WLP. Where do these reduced molecules come from? Atmospheric H_2 can either be used directly or by using electron carriers that have acquired their electrons by transfer from H_2 via a hydrogenase. In some acetogens such as *A. woodii*, this hydrogenase was found to be electron bifurcating (Schuchmann and Müller 2012). That means this enzyme can couple an exergonic transfer of electrons to a simultaneous endergonic electron transfer, usually by using flavins as cofactors. In case of the hydrogenase of *A. woodii* and *M. thermoacetica*, the exergonic electron transfer from H_2 ($[H_2/H^+]:E_0' = -414$ mV) to NAD^+ ($[NAD^+/NADH]:E_0' = -320$ mV) is driving the endergonic transfer of electrons from H_2 to ferredoxin (Fd; ($[Fd/Fd^{2-}]:E_0' = \sim -450$ mV) (Schuchmann and Müller 2014). Another important bifurcating enzyme found in some acetogens is the NADH-dependent reduced ferredoxin:NADPH oxidoreductase (Nfn), which links

the bifurcating electron transfer from two molecules of NADPH to the simultaneous reduction of NAD^+ and Fd in, for example, *C. autoethanogenum*. This enzyme is also found in *M. thermoacetica*, but here the reverse reaction is needed, which concomitantly transfers electrons from Fd^{2-} and NADH to reduce two molecules of $NADP^+$ (Huang et al. 2012; Mock et al. 2015). However, *A. woodii* probably lacks Nfn, since a BLASTx search using *nfnAB* of *C. autoethanogenum* and *M. thermoacetica* could not identify a homologue of the Nfn protein (https://blast.ncbi.nlm.nih.gov/Blast.cgi, unpublished data). It is likely that *A. woodii* does not harbor an Nfn homologue since this bacterium only uses NAD and not NADP as reducing equivalent in the WLP.

For reduction of CO_2 to CO, a strong reducing equivalent is required since the standard redox potential E_0' of CO_2/CO is as low as −520 mV. Therefore, the CODH was found to use Fd^{2-} which is the strongest available reducing equivalent for the WLP (Schuchmann and Müller 2014).

A. woodii harbors a hydrogen-dependent carbon dioxide reductase (HDCR) that can directly use electrons and protons from H_2 to reduce CO_2 to formate (Schuchmann and Müller 2013), whereas *C. autoethanogenum* possesses a formate hydrogen lyase, a complex of formate dehydrogenase and a bifurcating hydrogenase to couple the reduction of Fd and $NADP^+$([$NADP^+$/NADPH]:$E_0' = -320$ mV) and the conversion of CO_2 to formic acid (Mock et al. 2015). In case of *M. thermoacetica*, electrons needed for the reduction of CO_2 to formate are provided by NADPH (Huang et al. 2012).

Other enzymes of the WLP, such as the methylene-THF dehydrogenase and the methylene-THF reductase, also require reducing equivalents for the reduction of methenyl-THF to methylene-THF and further to methyl-THF. *A. woodii* uses NADH for both reactions (Bertsch et al. 2015; Schuchmann and Müller 2014), whereas *C. autoethanogenum* and *M. thermoacetica* were both shown to use electrons provided by NADPH for the reduction of methenyl-THF and NADH only for the reduction of methylene-THF (Mock et al. 2015; O' Brien et al. 1973). The methylene-THF reductase consists of several subunits which vary between the described species. In *A. woodii*, the methylene-THF reductase was shown to consist of three subunits: MetF, which contains a flavin and is the potential electron donor for reduction of methylene-THF; MetV, a smaller subunit which is potentially involved in electron transfer; and RnfC2 which contains a flavin and iron-sulfur clusters and might be the entry site of electrons provided by NADH. However, this enzyme is apparently not bifurcating as reduction of ferredoxin or stimulation of the reaction by ferredoxin was not observed (Bertsch et al. 2015). In contrast, the methylene-THF reductase of *M. thermoacetica* has been described as an hexa-heteromeric complex since MetF and MetV were found to be attached to HdrABC and MvhD. Homologues of HdrABC and MvhD were shown to be bifurcating enzymes for the reduction of heterodisulfides and Fd with H_2 or formate in methanogenic archaea (Mock et al. 2014). The genome of *C. autoethanogenum* predicts no flavoprotein or other NADH-oxidizing protein neighboring the *metF* and *metV* genes and, like in *M. thermoacetica*, may build a complex with other enzymes that contain a flavoprotein domain as shown for *M. thermoacetica* (Mock et al.

2015). Taken together, it may well be that the methylene-THF reductase in each *C. autoethanogenum* and *M. thermoacetica* is a bifurcating enzyme that couples the oxidation of 2 moles of NADH with the simultaneous reduction of methylene-THF and another still unknown electron acceptor (Mock et al. 2014, 2015).

3.2 Energy Conservation in Form of ATP

In the WLP, there is no net ATP synthesis by SLP. Thus, to overcome these energetic constrains, acetogens developed different strategies for the improvement of their ATP gain.

In addition to SLP, ATP can be also synthesized via ion-gradient driven phosphorylation. For acetogens, F_1F_o-ATP synthases have been shown to use H^+ or Na^+ gradients as driving force for ADP phosphorylation (Das and Ljungdahl 1997; Müller et al. 2001). So far two complexes are known that couple the energy released by exergonic electron transfer to build up a chemiosmotic ion gradient. Those complexes are either the Ech complex (energy-conserving hydrogenase) for export of H^+ and most likely also Na^+ which is driven by the transfer of electrons to Fd^{2-} onto either H^+ or Na^+ depending on the species (Hess et al. 2014) or the Rnf complex (*Rhodobacter* nitrogen fixation), which couples the electron transfer from Fd^{2-} onto NAD^+ to the export of H^+ or Na^+ (Biegel and Müller 2010; Schlegel et al. 2012; Hess et al. 2016).

M. thermoacetica harbors an Ech complex which consists of several subunits encoded in the gene cluster Moth_2184–2192 out of which Moth_2185 likely being the Fd^{2-}-oxidizing unit (EchF) and Moth_2186 the H^+-reducing unit (EchE) since they are homologous to EchE and EchF of the Ech complex described in *Methanosarcina barkeri* (Hedderich and Forzi 2005). In *M. thermoacetica*, the Ech complex may be connected to the formate dehydrogenase which would affect energy conservation (Moth_2193) (Huang et al. 2012; Mock et al. 2014). Taken together, the proposed model of energy conservation for *M. thermoacetica* is as follows: Assuming that the methylene-THF reductase of *M. thermoacetica* is electron-bifurcating and uses Fd as electron acceptor, which is not experimentally proven, the conversion of four molecules of H_2 plus two molecules of CO_2 to acetate by the WLP leads to an excess of two molecules of Fd^{2-}. The Ech complex uses these two molecules of Fd^{2-} for the reduction of four molecules of H^+ and simultaneous export of H^+. The exact amount of H^+ released per two molecules of Fd^{2-} is still unknown. However, assuming that two molecules of H^+ are exported and that around four molecules of H^+ are needed for the production of one molecule of ATP by the ATP synthase, the total energy conserved would be 0.5 molecules of ATP (Basen and Müller 2016; Schuchmann and Müller 2014).

Instead of an Ech complex, *A. woodii* and *C. autoethanogenum* each have been shown to possess an Rnf complex linked to energy conservation. The Rnf in acetogens was firstly described by Biegel et al. in 2009 who confirmed the existence of an *rnf* operon in *A. woodii* (Awo_c22060–Awo_c22010) encoding for the subunits RnfCDGAB (Biegel et al. 2009). Experiments using inverted membrane vesicles of *A. woodii* and inhibitor studies verified the presence of a membrane-bound

enzyme conducting ferredoxin:NAD oxidoreductase (Fno) activity together with simultaneous electrogenic export of Na^+ (Biegel and Müller 2010). The Fno activity was shown to be strictly dependent on Na^+, and the rate of Na^+ transfer was found to depend on the NAD^+ concentration (Hess et al. 2013). The chemiosmotic gradient of Na^+ then drives the Na^+-dependent F_1F_o ATP synthase, which needs 3.3 Na^+ for synthesis of one molecule of ATP (Matthies et al. 2014; Brandt and Müller 2015). Taken together, when reducing CO_2 to one molecule of acetate, 0.5 Fd^{2-} are left over to be used by the Rnf complex for export of one molecule of Na^+ (Fig. 2). Since the ATP synthase of *A. woodii* was found to use 3.3 Na^+ for the production of one molecule of ATP, overall, 0.3 molecules of ATP are synthesized (Schuchmann and Müller 2014).

The Rnf complex was shown to work in either direction for oxidization and reduction of Fd and NAD (Hess et al. 2013), which is depending on the current metabolic needs of the acetogens. The potential electron entry or exit units of this complex harboring 4Fe-4S clusters are RnfB for the oxidation or reduction of Fd^{2-}/Fd and RnfC, which contains an NADH-binding motif for reduction or oxidation of NAD^+/NADH (Biegel et al. 2011). The subunits RnfD/A and E together are possibly involved in translocation of Na^+, as comparative modelling studies of the Rnf

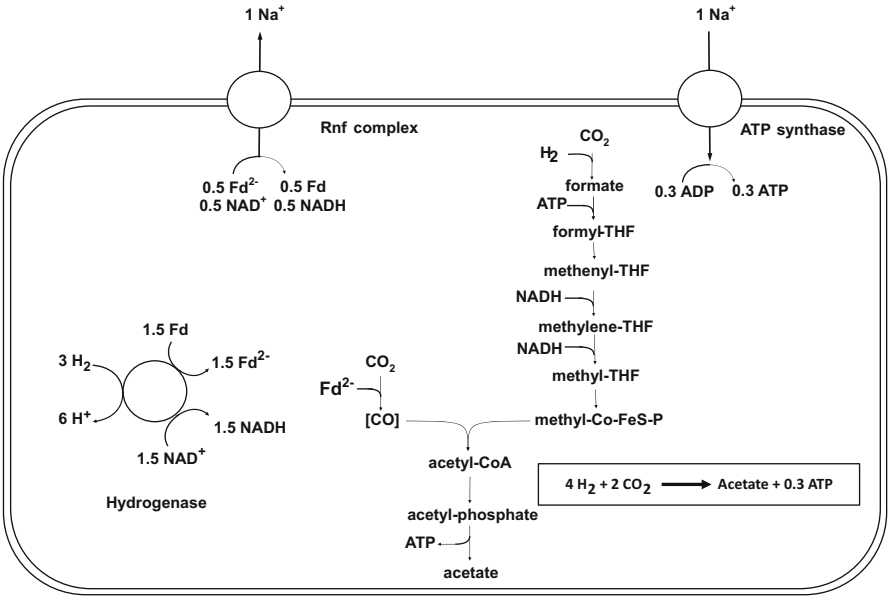


Fig. 2 Model of acetogenesis from H_2 + CO_2 and ATP synthesis in *Acetobacterium woodii*. A bifurcating hydrogenase drives the oxidization of three molecules of H_2 and links it with the endergonic reaction of reducing 1.5 molecules of Fd by coupling it with the exergonic reaction of reducing 1.5 molecules NAD^+. One molecule of Fd^{2-} is used for reduction of CO_2 to CO via the carbon monoxide dehydrogenase. The other 0.5 molecules of Fd^{2-} are used by the Rnf complex for the reduction of NAD^+, leading to the export of one molecule of Na^+ out of the cell. In the next step, the ATP synthase uses the electrochemical Na^+ gradient to generate 0.3 molecules of ATP. *THF* tetrahydrofolate, *Co-FeS-P* corrinoid iron-sulfur protein

complex with the well-known NADH/quinone oxidoreductase proposed Na^+-binding sites in these subunits (Biegel et al. 2011; Barquera 2014; Hreha et al. 2015).

In contrast to the Na^+-transferring Rnf complex of *A. woodii*, the Rnf complex of *C. autoethanogenum*, which is encoded in the *rnf*-operon (CAETHG_3227-CAETHG_3232), was found to be independent of Na^+, so that the translocation of H^+ is assumed to build up a chemiosmotic gradient for synthesis of ATP by the H^+-dependent ATP synthase (Mock et al. 2015). Evidence for H^+-dependent Rnf complex and ATP synthases have been described for its close relative *C. ljungdahlii* (Tremblay et al. 2013; Köpke et al. 2010).

Although both, *A. woodii* and *C. autoethanogenum*, use an Rnf complex for electron transfer and transmembranous Na^+ or H^+ import or export, the amount of ATP gained varies for these two organisms. This is due to the fact that *C. autoethanogenum* possesses an Nfn complex and that its methylene-THF reductase is potentially electron bifurcating, which enhances the availability of Fd^{2-} for use by the Rnf complex. Furthermore, the ATP synthase needs about four H^+ molecules to produce one molecule of ATP (Schuchmann and Müller 2014). Taking this in account, by converting four molecules of H_2 and two molecules of CO_2 to acetate, *C. autoethanogenum* should have 1.75 molecules of Fd^{2-} leftover which can be used by the Rnf complex to transfer electrons from Fd^{2-} to NAD, leading to an export of 3.5 molecules of H^+. These H^+ molecules are then used by the ATP synthase to produce ~0.9 molecules of ATP (Fig. 3) (Mock et al. 2015).

4 CO Fixation for Production of Acetyl-CoA and Acetate

Acetogens can also use CO as substrate for autotrophic growth, according to Reaction 2:

$$4\ CO + 2\ H_2O \rightarrow \text{acetate} + 2\ CO_2 \tag{2}$$

Using CO as carbon donor gives acetogens two advantages. First, acetogens do not need to use the CODH to reduce CO_2 to the intermediate CO, which leaves them with one remaining Fd^{2-}. Second, the bifunctional CODH can oxidize the additional three molecules of CO to CO_2, which is needed for the methyl branch of the WLP, resulting in the gain of three additional molecules of Fd^{2-}. Taking *A. woodii* as an example, growing on CO would lead to an excess of 2.5 moles of Fd^{2-} (and 0.5 Fd^{2-} would be used by the hydrogenase) which can be used as electron donor for energy conservation by the Rnf complex. Thus, electrons will be transferred to 2.5 molecules of NAD^+, concomitantly exporting 5 Na^+ which will then be taken up by the ATP synthase to enhance the ATP yield to a total amount of 1.5 molecules of ATP (Fig. 4). The higher ATP gain means that not only acetate but also other by-products could be synthesized from acetyl-CoA or acetate depending on the enzymes available in the genome of the acetogens and their functionality.

Growth experiments with *M. thermoacetica* on CO showed that this acetogen can grow up to the same cell density and produces up to 40% of the same level of acetate

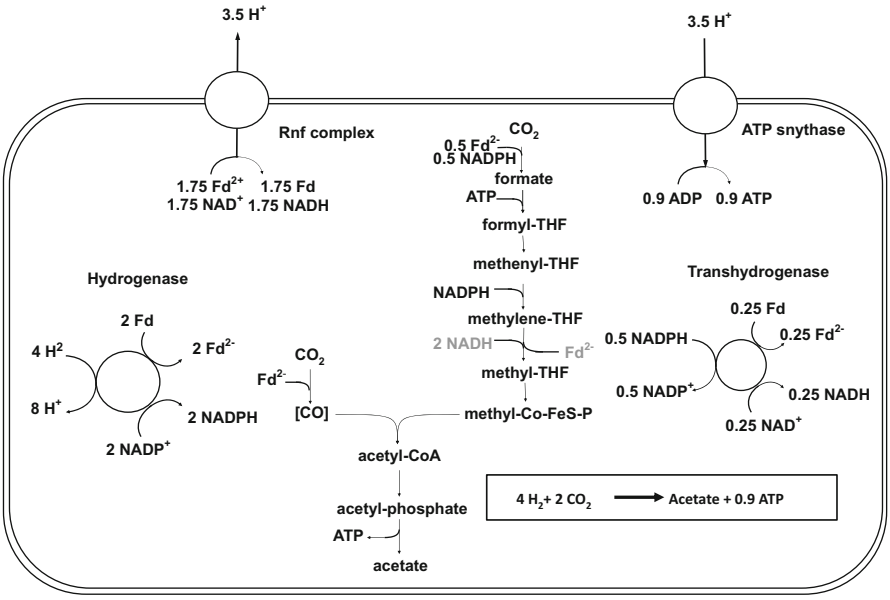


Fig. 3 Model of acetogenesis from H_2 + CO_2 and ATP synthesis in *Clostridium autoethanogenum*. A bifurcating hydrogenase drives the oxidization of four molecules of H_2 and links it with the endergonic reaction of reducing two molecules of Fd by coupling this with the exergonic reaction of reducing two molecules $NADP^+$. One molecule of Fd^{2-} is then used for reduction of CO_2 to CO via the carbon monoxide dehydrogenase and 0.5 molecules of Fd^{2-} together with 0.5 molecules of NADPH are used for the reduction of CO_2 to formate. 0.5 molecules of $NADP^+$ are used by the Nfn complex for the generation of 0.25 Fd^{2-} and 0.25. By using a bifurcating methylene-THF reductase, the oxidization of two molecules of a reducing equivalent (potentially NADH) drives the simultaneous reduction of methylene-THF with the reduction of an electron acceptor. Assuming that one molecule of Fd is the electron acceptor, in total 1.75 molecules of Fd^{2-} are generated which are used by the Rnf complex to reduce 1.75 NAD^+, leading to the export of 3.5 molecules of H^+ out of the cell. In the next step, the ATP synthase can use the electrochemical H^+ gradient to generate around 0.9 molecules of ATP. *THF* tetrahydrofolate, *Co-FeS-P* corrinoid iron-sulfur protein

when compared to its growth on glucose (Daniel et al. 1990). *C. autoethanogenum* and *C. ljungdahlii* are not only producing acetate but also ethanol when grown on only CO or when fed with a $CO/H_2/CO_2$ mixture (synthesis gas) (Abrini et al. 1994; Cotter et al. 2009). Furthermore, these two organisms were shown to be likely using an aldehyde: ferredoxin oxidoreductase (AOR) which converts acetate to acetaldehyde that can be further reduced to ethanol (Basen et al. 2014; Köpke et al. 2010; Mock et al. 2015). By using this enzyme, organisms can employ the advantage of gaining an ATP from synthesis of acetate compared to other organisms, which directly use acetyl-CoA as substrate for synthesis of acetaldehyde (Bertsch and Müller 2015a).

However, using CO is not that simple in case of *A. woodii*. This is because the HDCR was shown to be highly inhibited by CO. Hence, this acetogen is not able to grow on CO as the only carbon source or on a $H_2/CO_2/CO$ mixture. A ratio of H_2/

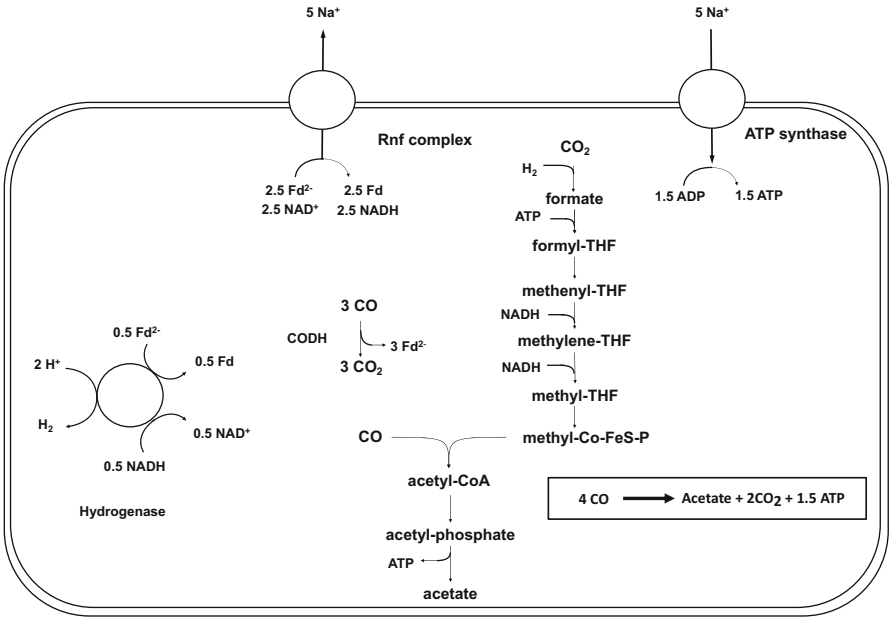


Fig. 4 Model of acetogenesis from CO and ATP synthesis in *Acetobacterium woodii*. Three molecules of CO_2 are generated by oxidizing three molecules of CO via the carbon monoxide dehydrogenase (*CODH*), which simultaneously generates three molecules of Fd^{2-}. The other molecule of CO can be directly used by the acetyl-CoA synthase for production of acetyl-CoA. 2.5 molecules of Fd^{2-} are used by the Rnf complex for reduction of 2.5 molecules of NAD^+, leading to an export of five molecules of Na^+ out of the cell, which are used by the ATP synthase to produce 1.5 molecules of ATP. The other 0.5 molecules of Fd^{2-} together with 0.5 molecules of NADH are used to confurcate electrons onto two molecules of H^+ for the generation of H_2 which is needed for reduction of CO_2 to formate in the WLP. *THF* thetrahydrofolate, *Co-FeS-P* corrinoid iron-sulfur protein

CO_2/CO (64%/16%/>5%) impaired the consumption of H_2 causing a strong reduction in growth and acetate production, which could be antagonized with the use of formate as second carbon donor instead of CO_2 (Bertsch and Müller 2015b). Therefore, it is very important to know the enzymatics of an organism very well before employing it as production platform for biofuels or other chemical compounds as discussed in Sect. 6.

5 CO_2 Fixation of Acetogens During Heterotrophic Growth

In addition to their ability to live autotrophically by fixing H_2 + CO_2 or CO, acetogens can also grow heterotrophically, which gives them the advantage to live from nourishing of various substrates. If acetogens would only grow on H_2 + CO_2, they would face a major energetic disadvantage since autotrophic growth is very energy limiting. Therefore, it is important for these bacteria to be able to use various

substrates in order to be competitive. Their main competitors are methanogens who occupy the same environmental niche and also grow on H_2 + CO_2. However, their end product of the WLP is methane which enables methanogens to conserve more energy and thus to grow faster (Amend and Shock 2001; Basen and Müller 2016), challenging acetogens to adapt or to die.

M. thermoacetica was shown to grow in addition to H_2 + CO_2 or CO on, for example, formate, methanol, hexoses, pentoses, methoxylated benzoic acids, and several two-carbon compounds. *M. thermoacetica* can also use thiosulphate, nitrate, and nitrite as alternative electron acceptors instead of CO_2. *A. woodii* was shown to grow, among others, on formate, methanol, ethanol, 1,2-propanediol, 2,3-butanediol, ethylene glycol, acetoin, lactate, glycerate, sugars, betaine, and several methoxylated aromatic acids. Less is known for *C. autoethanogenum*, but it was found to grow on substrates including xylose, pyruvate, rhamnose, and glutamate (Abrini et al. 1994).

The ability to ferment sugars such as glucose stoichiometrically only to acetate as shown for *M. thermoacetica* and *A. woodii* is called homoacetogenesis and results in the highest ATP yield known for glucose-fermenting bacteria, which potentially is 4.5 moles of ATP for *M. thermoacetica* and 4.3 moles of ATP for *A. woodii* (Reaction 3) (Schuchmann and Müller 2016).

$$\text{glucose} \rightarrow 3\ \text{acetate} \tag{3}$$

The conversion of glucose to acetate can be split into different steps. Taking *A. woodii* as an example, one molecule of glucose is first processed via the Embden-Meyerhof-Parnas pathway (EMP pathway, glycolysis) to two molecules of pyruvate. This pathway results in a yield of four molecules of ATP and two molecules of NADH (Thauer et al. 1977). After glycolysis, each molecule of pyruvate is converted to one molecule of acetyl-CoA and one molecule of CO_2 via the PFOR. This reaction concomitantly reduces one molecule of Fd, which needs to be reoxidized in the WLP together with the two molecules NADH left over from the EMP. Additionally, by using the WLP the two molecules of CO_2 will be further converted to acetate. Taking the 0.5 molecules of Fd^{2-} and 0.5 molecules of NADH for the hydrogenase to reduce two molecules of H^+ to one molecule of H_2 for the methyl branch and one molecule of Fd^{2-} for the reduction of CO_2 to CO in the carbonyl branch of the WLP leaves 0.5 molecules of Fd^{2-} for the electron transfer onto NAD^+ by the Rnf complex. This leads to the release of one molecule of Na^+ used by the ATP synthase to phosphorylate ADP to ATP, altogether resulting in a net energy gain of 4.3 molecules of ATP by fermentation of one molecule of glucose (Schuchmann and Müller 2016).

However, when using other carbon sources, energy in form of ATP may be used to reduce Fd by reverting the known pathways for energy conservation (ATP synthase and Rnf complex) so that Fd^{2-} can serve as electron donor for reduction of CO_2 to acetate within the WLP. An example of this reversion of energy transfer is the lactate metabolism in *A. woodii* (Reaction 4).

$$2\ \text{lactate} \rightarrow 3\ \text{acetate} \tag{4}$$

During conversion of two molecules of lactate to two molecules of pyruvate by the electron-bifurcating lactate dehydrogenase (LDH), four molecules of NADH will be reduced by electrons provided by lactate and two molecules of Fd^2. Those NADH molecules could then be used in the WLP to reduce the CO_2 which has been produced by conversion of pyruvate to acetyl-CoA by the PFOR that leads to the generation of two molecules of acetate and two molecules of ATP via SLP. However, since Fd^{2-} has been used by the LDH, no freely available Fd^{2-} will be left over to serve as a reducing equivalent. Hence, at least 0.9 molecules out of the 2.0 molecules of ATP synthesized are needed for the ATP synthase to export three molecules of Na^+. Those will then be used by the Rnf complex to further reduce 1.5 molecules of NADH to 1.5 molecules of Fd^{2-}. These 1.5 molecules of Fd^{2-} are needed to feed the hydrogenase (together with NADH) with electrons for the reduction iof H^+ to H_2 and for the reduction of CO_2 to CO in the carbonyl branch of the WLP. Taken together, released from conversion of two molecules of lactate to three molecules of acetate, 1.1 molecules of ATP are conserved (Weghoff et al. 2015).

Knowing the exact enzymatics for the WLP and of the energy-conserving complexes as shown here for the example of *A. woodii* helps to understand the conversion of substrates to certain products and product yields. By taking this as investigative background together with the genomic data available will facilitate the application of forward genetic studies to utilize acetogens in biotechnological approaches.

6 Research Needs

Global warming has started a long time ago. Since the beginning of industrialization, the temoerature of our planet has already risen, and since 1995, United Nations Climate Change Conferences are held to discuss plans to prevent the continuous rise of our planet's temperature. The aim is to avoid a total temperature rise of 2°C above the atmospheric temperature during preindustrial times (Liew et al. 2016). Greenhouse gases such as CO_2 are playing a big role in global warming, which are released mainly by fossil fuel combustion in our transport vehicles and by industry (Friedlingstein et al. 2014). However, CO_2 emission of transport vehicles together with industrial syngas emission is hard to tackle. We need innovative ways to lower the use of fossil fuels and also to recapture CO_2 or syngas from the industrial exhaust.

$CO + H_2$ can also be fixed by chemical processes such as the Fischer-Tropsch process (FTP), which converts syngas to low-carbon liquid hydrocarbons, but may only achieve a low efficiency depending on the syngas composition. In contrast, using acetogenic bacteria for syngas fermentation might be more efficient and has the additional advantage of directly converting syngas to biofuels such as bioethanol which can be used as fuel for vehicles or even, when chemically converted, also serve as jet fuel. Currently the company LanzaTech is installing the world's largest bioethanol production plant at the world's leading steel producer ArcelorMittal in

Ghent, Belgium, for the production of up to 104 million gallon of biofuels per year (Liew et al. 2016).

Another company, INEOS Bio, built a smaller (8 million gallon per year semi-commercial) facility together with the New Planet Energy Holdings, LLC, Florida, to use burned lignocellulose and municipal solid waste materials as substrates for gas fermentation and has already succeeded in production of bioethanol together with electrical power, showing that plant and municipal waste can be turned into fuels and energy (Liew et al. 2016).

These pioneering companies have shown that it is possible to use acetogens for the production of biofuels from syngas. Research is currently undertaken to enhance the range of products that can be synthesized from syngas, such as 2,3-butanediol and lactate by *C. ljungdahlii* or even higher alcohols (butanol, hexanol) and medium-chain fatty acids (butyrate and caproate) by co-cultures of the acetogen *C. autoethanogenum* and the non-acetogen *Clostridium kluyveri* (Köpke et al. 2011; Diender et al. 2016). In addition, using recently developed genetic approaches for gene deletions and insertions in *Clostridia* species, such as allelic exchange, triple cross, or CRISPR/Cas9 (Hoffmeister et al. 2016; Minton et al. 2016; Huang et al. 2016; Liew et al. 2016), will help to further modify acetogens to create pathways for production of various biofuels or synthetic materials. However, as mentioned above, it is important to know the enzymatics and energetics of each organism very well before starting genetic modifications (Bertsch and Müller 2015a), since an organism is only able to produce the desired product, if the engineered pathways still allow a positive ATP yield and reassure redox homeostasis.

Acknowledgments The work of the authors' laboratory is supported by grants from the Deutsche Forschungsgemeinschaft and the Federal Ministry of Education and Research.

References

Abrini J, Naveau H, Nyns E-J (1994) *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch Microbiol 161:345–351

Amend JP, Shock EL (2001) Energetics of overall metabolic reactions of thermophilic and hyperthermophilic archaea and bacteria. FEMS Microbiol Rev 25:175–243

Barquera B (2014) The sodium pumping NADH:quinone oxidoreductase (Na^+-Nqr), a unique redox-driven ion pump. J Bioenerg Biomembr 46:289–298

Basen M, Müller V (2016) "Hot" acetogenesis. Extremophiles 21:15–26

Basen M, Schut GJ, Nguyen DM, Lipscomb GL, Benn RA, Prybol CJ, Vaccaro BJ, Poole FL, Kelly RM, Adams MWW (2014) Single gene insertion drives bioalcohol production by a thermophilic archaeon. Proc Natl Acad Sci USA 111:17618–17623

Bell EA, Boehnke P, Harrison TM, Mao WL (2015) Potentially biogenic carbon preserved in a 4.1 billion-year-old zircon. Proc Natl Acad Sci USA 112:14518–14521

Bertsch J, Müller V (2015a) Bioenergetic constraints for conversion of syngas to biofuels in acetogenic bacteria. Biotechnol Biofuels 8:210

Bertsch J, Müller V (2015b) CO metabolism in the acetogen *Acetobacterium woodii*. Appl Environ Microbiol 81:5949–5956

Bertsch J, Öppinger C, Hess V, Langer JD, Müller V (2015) Heterotrimeric NADH-oxidizing methylenetetrahydrofolate reductase from the acetogenic bacterium *Acetobacterium woodii*. J Bacteriol 197:1681–1689

Biegel E, Müller V (2010) Bacterial Na^+-translocating ferredoxin:NAD^+ oxidoreductase. Proc Natl Acad Sci USA 107:18138–18142

Biegel E, Schmidt S, Müller V (2009) Genetic, immunological and biochemical evidence for a Rnf complex in the acetogen *Acetobacterium woodii*. Environ Microbiol 11:1438–1443

Biegel E, Schmidt S, Gonzalez JM, Müller V (2011) Biochemistry, evolution and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes. Cell Mol Life Sci 68:613–634

Brandt K, Müller V (2015) Hybrid rotors in F_1F_o ATP synthases: subunit composition, distribution, and physiological significance. Biol Chem 396:1031–1042

Braun M, Mayer F, Gottschalk G (1981) *Clostridium aceticum* (Wieringa), a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. Arch Microbiol 128:288–293

Cotter JL, Chinn MS, Grunden AM (2009) Influence of process parameters on growth of *Clostridium ljungdahlii* and *Clostridium autoethanogenum* on synthesis gas. Enzym Microb Technol 44:281–288

Daniel SL, Hsu T, Dean SI, Drake HL (1990) Characterization of the H_2- and CO-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. J Bacteriol 172:4464–4471

Das A, Ljungdahl LG (1997) Composition and primary structure of the F_1F_0 ATP synthase from the obligately anaerobic bacterium *Clostridium thermoaceticum*. J Bacteriol 179:3746–3755

Diender M, Stams AJ, Sousa DZ (2016) Production of medium-chain fatty acids and higher alcohols by a synthetic co-culture grown on carbon monoxide or syngas. Biotechnol Biofuels 9:82

Drake HL, Gossner AS, Daniel SL (2008) Old acetogens, new light. Ann N Y Acad Sci 1125: 100–128

Fontaine FE, Peterson WH, Mccoy E, Johnson MJ, Ritter GJ (1942) A new type of glucose fermentation by *Clostridium thermoaceticum* nov. sp. J Bacteriol 43:701–715

Friedlingstein P, Andrew RM, Rogelj J, Peters GP, Canadell JG, Knutti R, Luderer G, Raupach MR, Schaeffer M, Van Vuuren DP, Le Quere C (2014) Persistent growth of CO_2 emissions and implications for reaching climate targets. Nature Geosci 7:709–715

Fuchs G (1986) CO_2 fixation in acetogenic bacteria: variations on a theme. FEMS Microbiol Lett 39:181–213

Furdui C, Ragsdale SW (2000) The role of pyruvate ferredoxin oxidoreductase in pyruvate synthesis during autotrophic growth by the Wood-Ljungdahl pathway. J Biol Chem 275: 28494–28499

Groher A, Weuster-Botz D (2016) Comparative reaction engineering analysis of different acetogenic bacteria for gas fermentation. J Biotechnol 228:82–94

He Y, Li M, Perumal V, Feng X, Fang J, Xie J, Sievert SM, Wang F (2016) Genomic and enzymatic evidence for acetogenesis among multiple lineages of the archaeal phylum bathyarchaeota widespread in marine sediments. Nat Microbiol 1:16035

Hedderich R, Forzi L (2005) Energy-converting [NiFe] hydrogenases: more than just H_2 activation. J Mol Microbiol Biotechnol 10:92–104

Hess V, Schuchmann K, Müller V (2013) The ferredoxin:NAD^+ oxidoreductase (Rnf) from the acetogen *Acetobacterium woodii* requires Na^+ and is reversibly coupled to the membrane potential. J Biol Chem 288:31496–32502

Hess V, Poehlein A, Weghoff MC, Daniel R, Müller V (2014) A genome-guided analysis of energy conservation in the thermophilic, cytochrome-free acetogenic bacterium *Thermoanaerobacter kivui*. BMC Genomics 15:1139

Hess V, Gallegos R, Jones JA, Barquera B, Malamy MH, Müller V (2016) Occurrence of ferredoxin:$NAD^{(+)}$ oxidoreductase activity and its ion specificity in several Gram-positive and Gram-negative bacteria. PeerJ 4:e1515

Hoffmeister S, Gerdom M, Bengelsdorf FR, Linder S, Fluchter S, Ozturk H, Blumke W, May A, Fischer RJ, Bahl H, Dürre P (2016) Acetone production with metabolically engineered strains of *Acetobacterium woodii*. Metab Eng 36:37–47

Hreha TN, Mezic KG, Herce HD, Duffy EB, Bourges A, Pryshchep S, Juarez O, Barquera B (2015) Complete topology of the Rnf complex from *Vibrio cholerae*. Biochemistry 54:2443–2455

Huang H, Wang S, Moll J, Thauer RK (2012) Electron bifurcation involved in the energy metabolism of the acetogenic bacterium *Moorella thermoacetica* growing on glucose or H_2 plus CO_2. J Bacteriol 194:3689–3699

Huang H, Chai C, Li N, Rowe P, Minton NP, Yang S, Jiang W, Gu Y (2016) CRISPR/Cas9-based efficient genome editing in *Clostridium ljungdahlii*, an autotrophic gas-fermenting bacterium. ACS Synth Biol. (in press). https://doi.org/10.1021/acssynbio.6b00044

Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50:484–524

Kamlage B, Gruhl B, Blaut M (1997) Isolation and characterization of two new homoacetogenic hydrogen-utilizing bacteria from the human intestinal tract that are closely related to *Clostridium coccoides*. Appl Environ Microbiol 63:1732–1738

Kaneuchi C, Benno Y, Mitsuoka T (1976) *Clostridium coccoides*, a new species from the feces of mice. Int J Syst Evol Microbiol 26:482–486

Köpke M, Held C, Hujer S, Liesegang H, Wiezer A, Wollherr A, Ehrenreich A, Liebl W, Gottschalk G, Dürre P (2010) *Clostridium ljungdahlii* represents a microbial production platform based on syngas. Proc Natl Acad Sci USA 107:13087–13092

Köpke M, Mihalcea C, Liew F, Tizard JH, Ali MS, Conolly JJ, Al-Sinawi B, Simpson SD (2011) 2,3-butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. Appl Environ Microbiol 77:5467–5475

Li LF, Ljungdahl L, Wood HG (1966) Properties of nicotinamide adenine dinucleotide phosphate-dependent formate dehydrogenase from *Clostridium thermoaceticum*. J Bacteriol 92:405–412

Liew F, Martin ME, Tappel RC, Heijstra BD, Mihalcea C, Köpke M (2016) Gas fermentation-a flexible platform for commercial scale production of low-carbon-fuels and chemicals from waste and renewable feedstocks. Front Microbiol 7:694

Ljungdahl L, Wood H (1969) Total synthesis of acetate from co_2 by heterotrophic bacteria. Annu Rev Microbiol 23:515–538

Martin WF, Sousa FL (2016) Early microbial evolution: the age of anaerobes. Cold Spring Harb Perspect Biol 8:a018127

Martin W, Baross J, Kelley D, Russell MJ (2008) Hydrothermal vents and the origin of life. Nat Rev Micro 6:805–814

Matthies D, Zhou W, Klyszejko AL, Anselmi C, Yildiz Ö, Brandt K, Müller V, Faraldo-Gómez JD, Meier T (2014) High-resolution structure and mechanism of an F/V-hybrid rotor ring in a Na^+-coupled ATP synthase. Nat Commun 5:5286

Minton NP, Ehsaan M, Humphreys CM, Little GT, Baker J, Henstra AM, Liew F, Kelly ML, Sheng L, Schwarz K, Zhang Y (2016) A roadmap for gene system development in *Clostridium*. Anaerobe 41:104–112

Mock J, Wang S, Huang H, Kahnt J, Thauer RK (2014) Evidence for a hexaheteromeric methylene-tetrahydrofolate reductase in *Moorella thermoacetica*. J Bacteriol 196:3303–3314

Mock J, Zheng Y, Müller AP, Ly S, Tran L, Segovia S, Nagaraju S, Köpke M, Dürre P, Thauer RK (2015) Energy conservation associated with ethanol formation from H_2 and CO_2 in *Clostridium autoethanogenum* involving electron bifurcation. J Bacteriol 197:2965–2980

Müller V (2003) Energy conservation in acetogenic bacteria. Appl Environ Microbiol 69: 6345–6353

Müller V, Aufurth S, Rahlfs S (2001) The Na^+ cycle in *Acetobacterium woodii:* Identification and characterization of a Na^+ translocating F_1F_0-ATPase with a mixed oligomer of 8 and 16 kDa proteolipids. Biochim Biophys Acta 1505:108–120

O'Brien WE, Brewer JM, Ljungdahl LG (1973) Purification and characterization of thermostable 5,10-methylenetetrahydrofolate dehydrogenase from *Clostridium thermoaceticum*. J Biol Chem 248:403–408

Poehlein A, Schmidt S, Kaster A-K, Goenrich M, Vollmers J, Thürmer A, Bertsch J, Schuchmann K, Voigt B, Hecker M, Daniel R, Thauer RK, Gottschalk G, Müller V (2012) An ancient pathway combining carbon dioxide fixation with the generation and utilization of a sodium ion gradient for ATP synthesis. PLoS ONE 7:e33439

Ragsdale SW (2003) Pyruvate:ferredoxin oxidoreductase and its radical intermediate. Chem Rev 103:2333–2346

Ragsdale SW (2008) Enzymology of the Wood-Ljungdahl pathway of acetogenesis. Ann N Y Acad Sci 1125:129–136

Schlegel K, Welte C, Deppenmeier U, Müller V (2012) Electron transport during aceticlastic methanogenesis by *Methanosarcina acetivorans* involves a sodium-translocating Rnf complex. Febs J 279:4444–4452

Schuchmann K, Müller V (2012) A bacterial electron-bifurcating hydrogenase. J Biol Chem 287:31165–31171

Schuchmann K, Müller V (2013) Direct and reversible hydrogenation of CO_2 to formate by a bacterial carbon dioxide reductase. Science 342:1382–1385

Schuchmann K, Müller V (2014) Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. Nat Rev Microbiol 12:809–821

Schuchmann K, Müller V (2016) Energetics and application of heterotrophy in acetogenic bacteria. Appl Environ Microbiol 82:4056–4069

Sojo V, Herschy B, Whicher A, Camprubi E, Lane N (2016) The origin of life in alkaline hydrothermal vents. Astrobiology 16:181–197

Tanner RS, Miller LM, Yang D (1993) *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial rRNA homology group I. Int J Syst Evol Microbiol 43:232–236

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180

Tremblay P-L, Zhang T, Dar SA, Leang C, Lovley DR (2013) The Rnf complex of *Clostridium ljungdahlii* is a proton-translocating ferredoxin:NAD^+ oxidoreductase essential for autotrophic growth. mBio 4:e00406

Weghoff MC, Bertsch J, Müller V (2015) A novel mode of lactate metabolism in strictly anaerobic bacteria. Environ Microbiol 17:670–677

Weiss MC, Sousa FL, Mrnjavac N, Neukirchen S, Roettger M, Nelson-Sathi S, Martin WF (2016) The physiology and habitat of the last universal common ancestor. Nat Microbiol 1:16116. https://doi.org/10.1038/nmicrobiol.2016.116

Wieringa KT (1936) Over het verdwijnen van waterstof en koolzuur onder anaerobe voorwaarden. Antonie van Leeuwenhoek 3:263–273

Wieringa KT (1939) The formation of acetic acid from carbon dioxide and hydrogen by anaerobic spore-forming bacteria. Antonie Leeuwenhoek J Microbiol Serol 6:251–262

Wood HG (1952) A study of carbon dioxide fixation by mass determination of the types of C13-acetate. J Biol Chem 194:905–931

Wood HG, Ragsdale SW, Pezacka E (1986) The acetyl-CoA pathway of autotrophic growth. FEMS Microbiol Lett 39:345–362

Formation of Fatty Acids

3

Isabel M. López-Lara and Otto Geiger

Contents

1 Introduction 44
2 Biosynthesis 45
2.1 The Basic Type II Fatty Acid Biosynthesis Pathway 46
2.2 Modifications of Fatty Acids 47
3 Specificity of Acyltransferases 51
4 Fatty Acid Biosynthesis as Target for Antibiotic Development 51
5 Modification of the Fatty Acid Pathway for Biodiesel Production 52
6 Research Needs 53
References 53

Abstract

Fatty acids are the building blocks of diverse membrane lipids and therefore are essential for the viability of all cells, except the Archaea where side chains of membrane lipids are isoprenoids. Fatty acid biosynthesis is catalyzed in most bacteria by a group of highly conserved proteins known as the type II fatty acid synthase (FAS II) system. A key protein in this system is the acyl carrier protein (ACP) which acts as carrier of growing acyl chains during biosynthesis and as donor of acyl chains during transfer to target molecules. The *Escherichia coli* FAS II system is the model pathway of fatty acid biosynthesis in bacteria, and mainly the same set of enzymes is present in different bacteria. Variations of the basic set give rise to the diversity of fatty acid

I. M. López-Lara (✉)
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, Mexico
e-mail: isabel@ccg.unam.mx

O. Geiger
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico
e-mail: otto@ccg.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_5

composition of each organism. The intrinsic specificity of the acyltransferases is largely responsible for the fatty acid composition of complex lipids and signal molecules. Biosynthesis of fatty acids is a target for development of new antibiotics, especially against drug resistance microbes. Metabolic engineering of the fatty acid biosynthetic pathway is a promising tool to enhance production of biofuels.

1 Introduction

Fatty acids (FAs) are organic molecules consisting of an aliphatic carbon chain with a methyl group at one end of the chain and a carboxylic acid group (carbon number 1) at the other end. FAs are key components of the cell, and their synthesis is essential for all organisms except for Archaea, although the presence of FAs as part of lipoproteins has been described in Archaea (Pugh and Kates 1994). FAs occur in their free form only in small amounts; in most cells they are built up to form complex lipids with structural, energy storage, and signaling functions. In glycerophospholipids, which are usually the major components of cell membranes, FAs are ester-linked to a glycerol backbone, although there are many other membrane-forming lipids which are based on FAs (López-Lara and Geiger 2017). FAs are also constituents of the lipid A part of lipopolysaccharides which are constituents of the outer membrane of Gram-negative bacteria (reviewed by Whitfield and Trent 2014) and of lipoteichoic acids that contribute to the cell envelope of Gram-positive bacteria (Percy and Gründling 2014). They are also used for posttranslational protein modifications that are functionally important (Nakayama et al. 2012). Triacylglycerols are FA triesters of glycerol widely used as reserve compounds among Eukarya including yeast and fungi; however, the occurrence of triacylglycerols in bacteria seems to be restricted to the actinomycetes group (Alvarez and Steinbüchel 2002). Instead, most bacteria synthesize polymeric lipids such as poly (3-hydroxybutyrate) (PHB) or other polyhydroxyalkanoates (PHAs) as reserve material (Steinbüchel 2001). In many cases FAs are part of signals used in the microbial world for cell to cell communication (see Soto et al., ▶ Chap. 15, "Functional Roles of Non-membrane Lipids in Bacterial Signaling", this volume).

The FAs present in microbial lipids are mainly of four types: straight-chain saturated, straight-chain monounsaturated, branched-chain (predominantly iso and anteiso), and cyclopropane FAs. Besides these, polyunsaturated and hydroxylated FAs are abundant in some organisms, and ω-alicyclic FAs, either cyclohexyl or cycloheptyl, are major FAs in some thermoacidophilic bacilli (see Fig. 1 for examples of different FAs). In most of the naturally occurring unsaturated fatty acids (UFAs), the orientation of the double bonds is *cis* rather than *trans*. The *cis* orientation has an important effect on molecular structure because each double bond inserts a bend into the hydrocarbon chain (Fig. 1b). The nomenclature of FAs is complicated by the fact that there are at least five different systems in use. Details of FA nomenclature are explained in Gunstone and Herslöf (1992). The various groups of prokaryotes differ remarkably in the structure and synthesis of FA-derived lipids, which serve as reliable systematic marker molecules in chemotaxonomy (Osterhout et al. 1991). Unsaturated and branched-chain fatty acids increase the fluidity of the membrane and fulfill the function of thermal adaptation. The

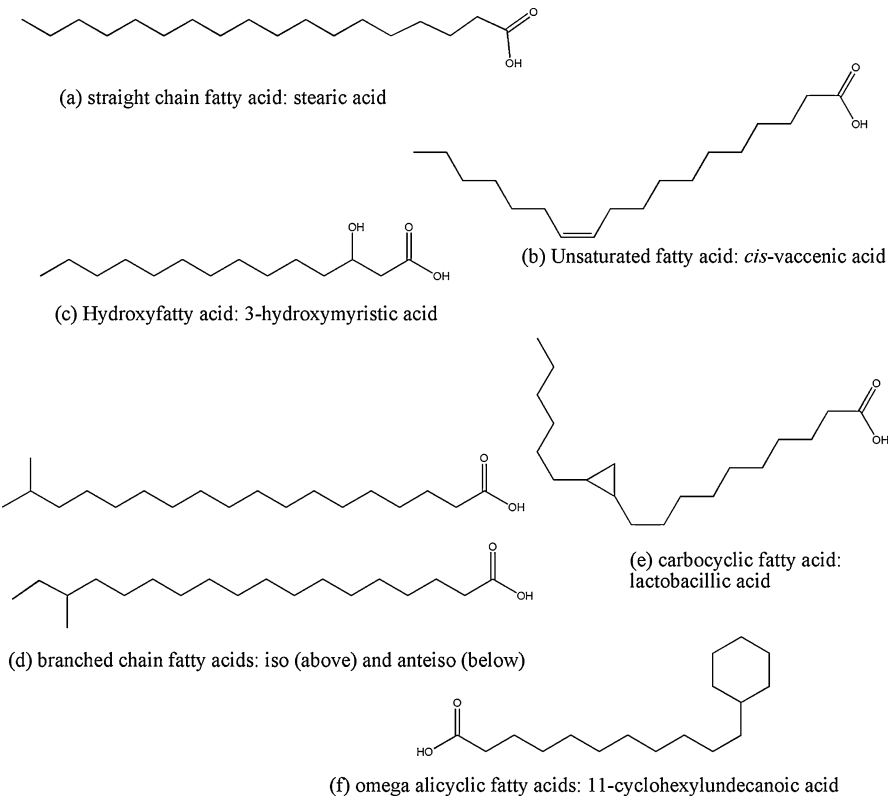


Fig. 1 Representative structures of fatty acids

higher the content of these FAs, the lower is the solid-to-liquid phase transition temperature of these lipids.

2 Biosynthesis

Although the reaction mechanism of de novo FA biosynthesis is essentially the same in all biological systems, there are different structures of fatty acid synthase (FAS) systems. The first class is represented by the dissociated type II FAS system which occurs in most bacteria as well as in organelles (chloroplasts, mitochondria, and apicoplast). In this case, the components are independent proteins which are encoded by a series of separated genes. In contrast, the highly integrated type I FAS multienzymes contain the various catalytic activities of the reaction sequence as discrete functional domains, either in a single polypeptide chain or, in some cases, on two different multifunctional proteins of comparable size. Type I FAS multienzymes are found characteristically in the eukaryotic cytoplasm, but also in some bacteria, i.e., mycobacteria (Cabruja et al. 2017). Microbial type I FAS are hexamers forming either α6β6 (fungi) or α6 (bacteria) oligomers, while

animal FAS are α2 dimers (Schweizer and Hofmann 2004). In this review we will describe mainly the type II FAS (Fig. 2). The *Escherichia coli* FAS is the paradigm for the study of this system, and X-ray structures and/or NMR structures of representative members of every enzyme of the type II pathway are now available (White et al. 2005).

2.1 The Basic Type II Fatty Acid Biosynthesis Pathway

A key feature of this system is that all fatty acyl intermediates are covalently connected to a small acidic protein, the acyl carrier protein (ACP), which shuttles them from one enzyme to another. The linkage between fatty acyl groups and ACP is achieved by a thioester bond in which the sulfhydryl of the ACP's 4′-phosphopantetheine prosthetic group is condensed with the carboxy group of a FA. Apo-ACP is converted to the active protein by holo-ACP synthase (AcpS), which transfer the 4-′-phosphopantetheine from coenzyme A (CoA) to apo-ACP (Fig. 2). In *E. coli* K12, there is a unique and essential ACP named AcpP, but other organisms have, besides the orthologous AcpP for general FA biosynthesis, other ACPs dedicated to the synthesis

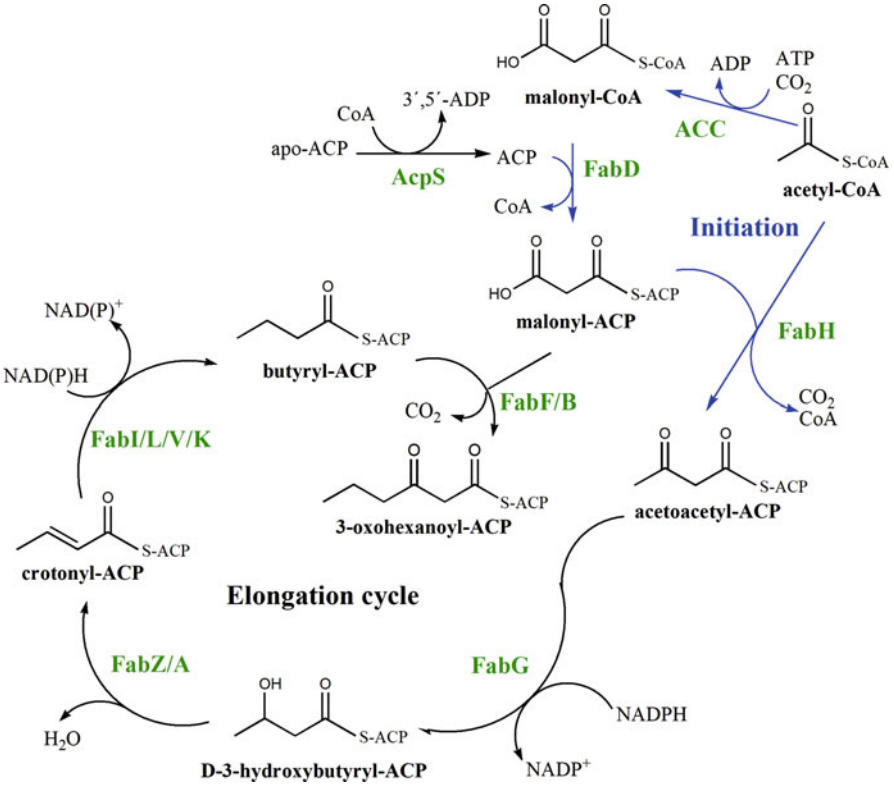


Fig. 2 The FAS II biosynthesis pathway. See the text for details

of specific FAs. The presence of multiple ACPs in one organism has been related to a more complex metabolism (Geiger and López-Lara 2002). In the FAS I system, the ACP is one of the domains of the polypeptide. Fatty acid biosynthesis proceeds in two stages, initiation and cyclic elongation as outlined in Fig. 2. Detailed reviews of the pathway and the enzymes involved have been published recently (Rock 2008; Parsons and Rock 2013; Beld et al. 2015). Acetyl-CoA carboxylase (ACC) catalyzes the first committed step of FA biosynthesis, the ATP-consuming carboxylation of acetyl-CoA. ACC is formed by four separate proteins and requires biotin as cofactor. The product of the reaction is malonyl-CoA, and the malonyl group is transferred to ACP by malonyl-CoA:ACP transacylase (FabD) to form malonyl-ACP. The condensation of malonyl-ACP with acetyl-CoA by 3-oxoacyl-ACP synthase III (FabH) with the formation of acetoacetyl-ACP and CO_2 is the initiation step in FAS. Elongation of fatty acids by C2 units goes through a cycle of reactions, each cycle involving a condensation, a first reduction, a dehydration, and a second reduction step. Each new round is initiated by an elongation condensing enzyme: 3-oxoacyl-ACP synthase II (FabF) or 3-oxoacyl-ACP synthase I (FabB). Both catalyze a Claisen condensation using malonyl-ACP to elongate the growing acyl chain by two carbons (Fig. 2). FabF is the elongation condensing enzyme present in most bacteria, while FabB is only present in bacteria that utilize the FabA system for unsaturated FA biosynthesis (Fig. 3). FabG is the 3-oxoacyl-ACP reductase, and only a single isoform of this enzyme has been identified so far in bacteria. The next step is the dehydration of the 3-oxoacyl-ACP to *trans*-2-enoyl-ACP by 3-hydroxyacyl-ACP dehydratases. Two isoforms, FabA and FabZ, catalyze the dehydration step although with different substrate specificities (Rock 2008). Each cycle is led to completion by an enoyl-ACP reductase which reduces the double bond in *trans*-2-enoyl-ACP using the cofactor NAD(P)H (Fig. 2). There are four isoforms of enoyl-ACP reductase. FabI of *E. coli* was the first described and is a member of the short-chain dehydrogenase/reductase (SDR) superfamily. The isoforms FabL and FabV are also members of the SDR superfamily, while FabK belongs to the NAD(P)H-dependent flavin oxidoreductase family. Several bacteria contain two enoyl-ACP reductases, *Bacillus* species contain FabI and FabL isoforms, and *Pseudomonas aeruginosa* contain FabI and FabV (Parsons and Rock 2013).

2.2 Modifications of Fatty Acids

The 3-hydroxyacyl-ACP dehydratase FabA not only can remove water during fatty acid elongation but is also able to catalyze the isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP (Fig. 3). For the elongation of *cis*-3-decenoyl-ACP to palmitoleyl-ACP, FabB is essential. Bioinformatic analyses have shown that the FabA/FabB pathway for unsaturated FA might be restricted to α- and γ-proteobacteria (Rock 2008). Variations of this pathway have been described in other bacteria. In *Streptococcus*, after the formation of *trans*-2-decenoyl-ACP by FabZ, the specific isomerase FabM isomerizes it to *cis*-3-decenoyl-ACP (Marrakchi et al. 2002). Surprisingly, in *Enterococcus* FabZ has isomerase activity, and FabZ/

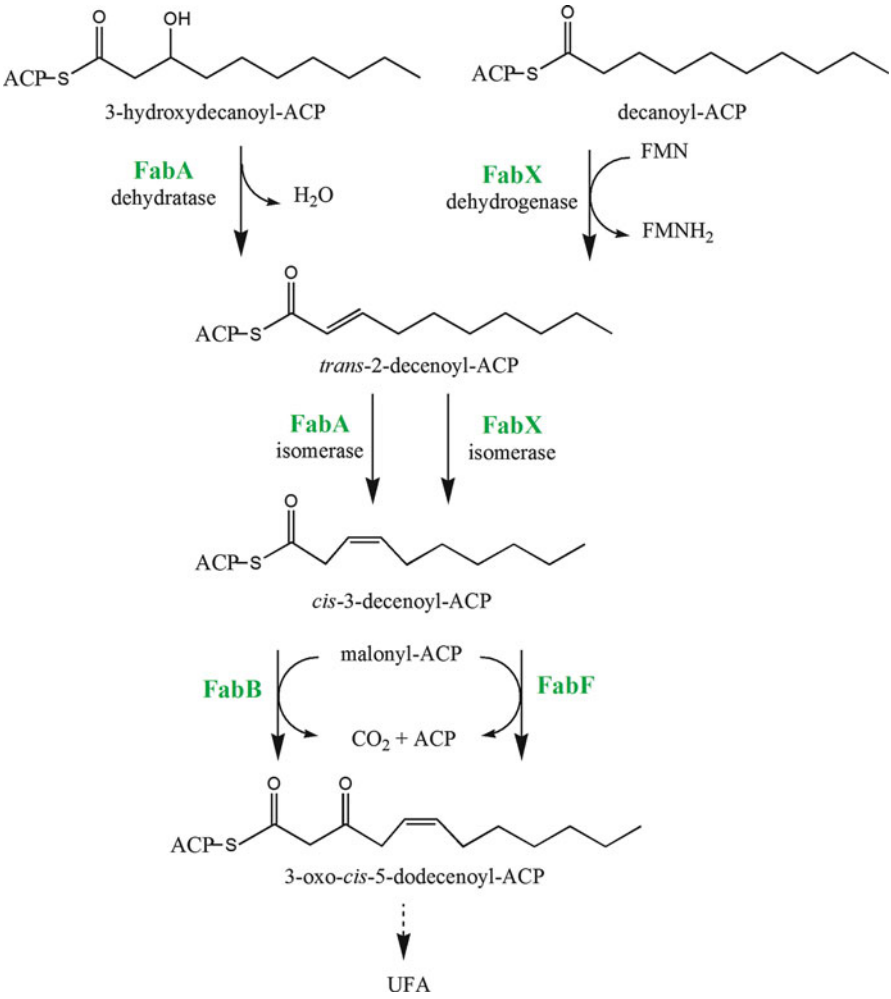


Fig. 3 Two different mechanisms for anaerobic biosynthesis of unsaturated FAs (UFAs) in bacteria. FabA is a dehydratase/isomerase that uses as substrate 3-hydroxy-decanoyl-ACP, while FabX is a dehydrogenase/isomerase that uses the fully saturated decanoyl-ACP. Organisms with the FabA system utilize the FabB condensing enzyme for elongation of UFAs, while organisms with FabX use FabF

FabF of *Enterococcus* can functionally replace the FabA/FabB system of *E. coli* (Wang and Cronan 2004). Recently, a new mechanism of anaerobic unsaturated FA biosynthesis has been described. FabX of *Helicobacter pylori* is a flavin enzyme that has both dehydrogenation and isomerase activities and proceeds via a backtracking mechanism to introduce a double bond into the fully saturated decanoyl-ACP (Fig. 3) (Bi et al. 2016). FabX homologues (previously designated as UfaA) are widespread in bacteria mainly in anaerobe organisms that lack a FabA homologue (Isabella and Clark 2011).

Another pathway for unsaturated FA synthesis in bacteria occurs by aerobic desaturation. In this case, the fatty acid is modified post-biosynthetically when fatty acyl residues are already attached to membrane phospholipids. This system is found in bacilli and cyanobacteria and represents a mechanism conserved in Bacteria and Eukarya (Aguilar and de Mendoza 2006).

Not all bacteria regulate membrane fluidity through the production of monounsaturated straight-chain FAs. Most Gram-positive bacteria use branched-chain fatty acids to modulate membrane fluidity. The branch is a methyl group in the iso- or anteiso-position in the chain (i.e., on the second or third carbon from the distal end of the chain, Fig. 1d). The branched chains are introduced into the pathway in the initiation phase by FabH enzymes with different specificities to that of *E. coli*. FabH of *E. coli* is selective for acetyl-CoA (Fig. 2), while FabH enzymes of bacteria that produce branched-chain FAs prefer the branched-chain substrates isovaleryl-CoA, isobutyryl-CoA, and 2-methylbutyryl-CoA. These biosynthetic primers are generated by degradation of the branched-chain amino acids leucine, valine, and isoleucine. Branched-chain amino acids are transaminated by a separate enzyme, and the resulting 2-oxoacids are oxidatively decarboxylated by the branched-chain 2-oxoacid dehydrogenase complex (also known as BDK) (Fig. 4) (Kaneda 1991).

The presence of 3-hydroxylated fatty acids is common in some bacteria, and these fatty acids are the primary FAs in lipid A (Whitfield and Trent 2014) as well as in ornithine-containing lipids (OL) (López-Lara and Geiger 2017). 3-Hydroxy-fatty acyl-ACPs are normal intermediates in the FAS II elongation cycle (Fig. 2). The acyltransferases involved in the addition of the primary FAs in lipid A biosynthesis (LpxA and LpxD) or in OL biosynthesis (OlsB) must have a higher affinity for the specific 3-hydroxy-fatty acyl-ACP than the FAS II enzymes and may thus efficiently remove the intermediate from the biosynthesis pathway. In contrast, *S*-2-hydroxylation usually is introduced after the fatty acyl group has been attached to the lipid A molecule and is catalyzed by the LpxO dioxygenase (Gibbons et al. 2008). Similar dioxygenases might be responsible for the introduction of 2-hydroxy substitutions on the fatty acyl group of OL, bacterial sphingolipids, or phosphatidylethanolamine (López-Lara and Geiger 2017).

In numerous representatives of the α-2 subgroup of the *Proteobacteria*, the lipid A carries a long (ω-1)-hydroxy fatty acid of 28 carbons (27-hydroxyoctacosanoic acid, 27OH-C28). Interestingly, a specialized ACP, AcpXL, is required for the transfer of 27OH-C28 to lipid A by the acyltransferase LpxXL (Basu et al. 2002). Between the genes for *acpXL* and *lpxXL,* four genes are located that encode homologues of fatty acid biosynthetic enzymes (Basu et al. 2002), and therefore presumably there is a complete set of FAS II enzymes dedicated to the biosynthesis of 27OH-C28 in which the acyl intermediates will be bound to the specialized AcpXL instead than to the housekeeping AcpP.

The conversion of pre-existing *cis*-unsaturated FAs to cyclopropane fatty acids (i.e., lactobacillic acid shown in Fig. 1e) is widespread in bacteria. The required methylation is carried out by cyclopropane fatty acid synthase (Cfa), which uses *S*-adenosylmethionine as the methyl donor to create the cyclopropane ring (Rock 2008).

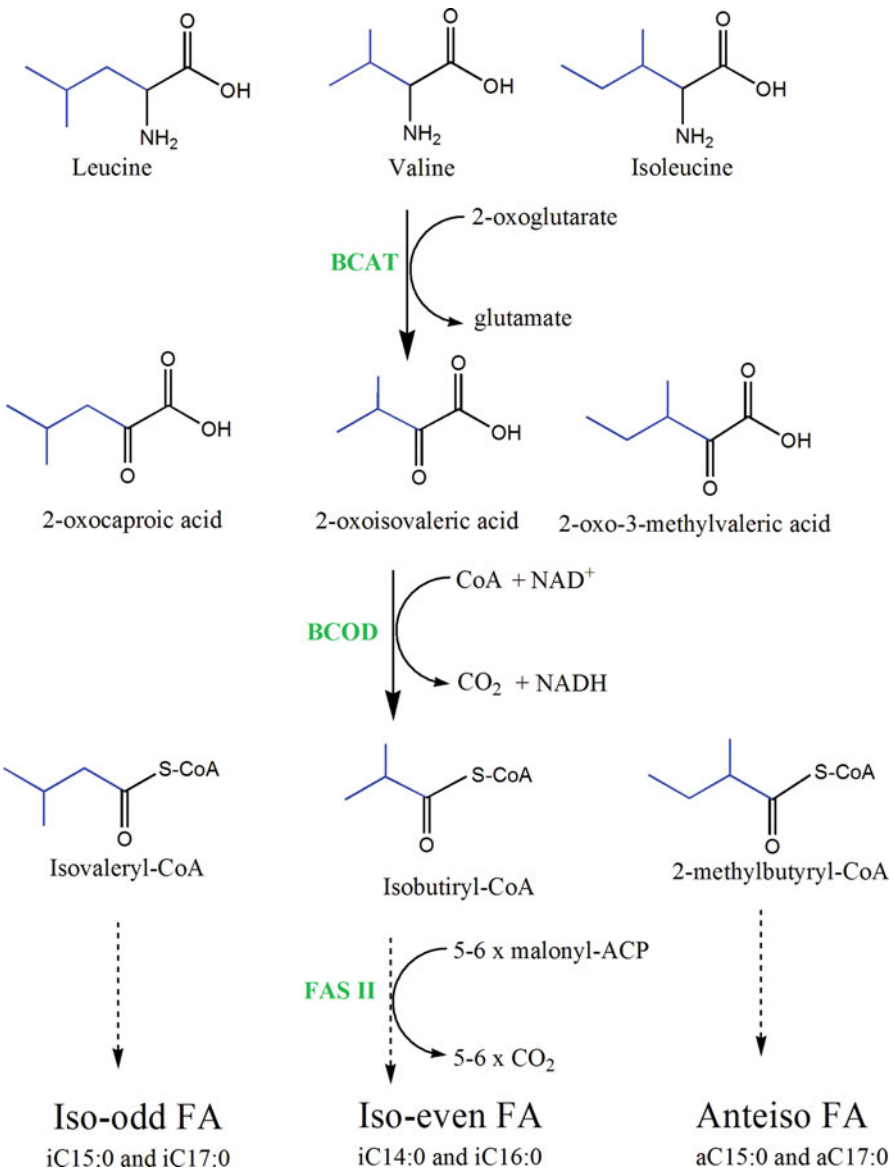


Fig. 4 Schematic representation of the biosynthesis of branched-chain FAs. Branched-chain amino acids are transaminated by a branched-chain amino acid transaminase (BCAT), and the branched-chain 2-oxoacid dehydrogenase complex (BCOD) synthesizes the acyl-CoA intermediates. The FAS II system uses the substrates isovaleryl-CoA, isobutyryl-CoA, and 2-methylbutyryl-CoA, and after five or six cycles of elongation using malonyl-ACP as building block, the respective branched-chain fatty acids are formed

A few bacteria have evolved a mechanism to convert existing *cis*-unsaturated FAs in phospholipids into *trans*-unsaturated fatty acids to adapt to environmental challenges. The isomerization is carried out by the periplasmic *cis-trans* isomerase (Cti) (Holtwick et al. 1997).

For the synthesis of the unusual α-β-unsaturated fatty acids, which are specific substituents of nodulation (Nod) factors, it seems that a specialized ACP, NodF, and a different elongation condensing enzyme, NodE, work in combination with enzymes of the basic FAS II system (Geiger and López-Lara 2002).

3 Specificity of Acyltransferases

In the biosynthesis of phospholipids, the bacterial glycerol-phosphate acyltransferases utilize the completed fatty acyl chains to form membrane phospholipids. Initially, the bacterial acyltransferases PlsB and PlsC of *E. coli* were studied, and it was shown that these two acyltransferases use acyl-thioesters of either CoA or ACP as the acyl donors (Rock 2008). However, most organisms use the PlsX/PlsY system, instead of the PlsB enzyme. PlsX converts acyl-ACP into acyl-phosphate and PlsY uses the acyl-phosphate to acylate glycerol-3-phosphate. In all bacteria, PlsC transfers a second acyl group to 1-acyl-glycerol-3-phosphate leading to phosphatidic acid formation (see Geiger et al., ▶ Chap. 5, "Formation of Bacterial Glycerol-Based Membrane Lipids: Pathways, Enzymes, and Reactions," this volume). Although PlsC is widely distributed, the PlsC homologue in *Bacillus subtilis* can use only acyl-ACP (Paoletti et al. 2007).

LpxA is the acyltransferase that catalyzes the first step of lipid A biosynthesis, the reversible transfer of the *R*-3-hydroxyacyl chain from *R*-3-hydroxyacyl-ACP to the 3-OH group of UDP-*N*-acetylglucosamine. *E. coli* LpxA is highly selective for *R*-3-hydroxymyristate. The structural basis of LpxA specificity for the fatty acid was clarified (Williams and Raetz 2007). Acyl homoserine lactones (AHLs) vary greatly in acyl-chain length, from C4-AHL up to C18-AHLs, and each AHL synthase produces specifically a certain type of AHL. Sequence analysis of the AHL synthase family fails to reveal a robust correlation between sequence composition and acyl-chain length. Therefore, to accommodate and create a preference for acyl-ACPs of different acyl-chain lengths, a number of sequence and tertiary structure differences must have evolved in different AHL synthases (Gould et al. 2004). In the AHL EsaI that produces 3-oxo-C6-AHL, a restrictive hydrophobic pocket was found (Watson et al. 2002) in contrast to the presence of a more relaxed hydrophobic tunnel present in the AHL LasI that produces 3-oxo-C12-AHL (Gould et al. 2004).

4 Fatty Acid Biosynthesis as Target for Antibiotic Development

There is urgent need for the development of new antibiotics because of the increase in multidrug-resistant bacteria. FAS II is emerging as a major target for the development of novel antibacterial agents (Zhang et al. 2006). FA synthesis is essential for

many bacterial pathogens, and the focus is to develop inhibitors targeting bacterial FAS II and not the structurally different mammalian type I FAS. The use of the antituberculosis drug isoniazid and the biocide triclosan validates this pathway as a target for antibacterial development. Triclosan is the best known FabI inhibitor (McMurry et al. 1998), and isoniazid is an antituberculosis drug that inhibits InhA, the homologue to FabI from *Mycobacterium tuberculosis* involved in mycolic acid biosynthesis (Banerjee et al. 1994). There are continuous efforts to produce new inhibitors of enoyl-ACP reductase. For example, the small molecule AFN-1252 is an inhibitor optimized against *Staphylococcus aureus* FabI that shows efficacy and safety in controlling acute bacterial infections (Hafkin et al. 2016). Drug discovery has also been focused in the enzymes acetyl-CoA carboxylase and the condensing enzymes FabH, FabF, and FabB (Yao and Rock 2015). Platensimycin and platencin are natural products discovered via a whole-cell screening using FabF of *Staphylococcus aureus* as a target. Platensimycin inhibits the elongation enzymes FabF and FabB, while platencin is a dual inhibitor of FabF/FabB and of FabH (Shang et al. 2015). There is evidence that FAS inhibitors may be useful against most emerging pathogens, and only *Streptococcus* has the ability to bypass FAS II inhibition through the use of exogenous FAs (Yao and Rock 2015).

5 Modification of the Fatty Acid Pathway for Biodiesel Production

Free FAs are only one catalytic step away from petroleum-derived diesel molecules such as FA methyl esters, FA ethyl esters, fatty alcohols, alkanes, and alkenes. Therefore, manipulation of the microbial FA biosynthesis pathway has been intensely studied with the aim to produce FAs or derivatives for substitution of diesel (reviewed by Janßen and Steinbüchel 2014). Several approaches that increase FA production have been identified. In bacteria, long-chain acyl-ACPs are the end products of FA synthesis, and acyl groups are directly transferred from acyl-ACP to lipids by acyltransferases. In order to obtain free FAs, an endogenous or heterologous thioesterase is overexpressed. Furthermore, biosynthesis of FAs in *E. coli* is feedback inhibited by acyl-ACP, but this inhibition is released by the overexpression of a thioesterase resulting in the production of free FAs. Usually, the FA β-oxidation pathway is blocked by deleting *fadD* (coding for acyl-CoA synthetase) and avoiding consumption of the FAs overproduced (Pech-Canul et al. 2011). Overexpression of some of the FA biosynthetic proteins is another strategy used in *E. coli*, but, in general, the modification resulting in higher FA production is the overexpression of thioesterases (Janßen and Steinbüchel 2014). Finally, in such a strain overproducing FAs, heterologous overexpression of specific enzymes leads to the production of the desired product. For example, alkanes have been produced from free FAs in *E. coli* by expressing FA reductase from the genes *luxCDE* of *Photorhabdus luminescens* that forms fatty aldehydes plus the expression of a decarbonylase from *Nostoc puntiforme* which converts the fatty aldehydes to alkanes (Howard et al. 2013).

6 Research Needs

FAS II has been extensively studied in the *E. coli* and *B. subtilis* model systems, and with the availability of hundreds of genome sequences, the pathway has been investigated in other organisms, mostly in important human pathogens. Here we have summarized the FA biosynthesis pathway prototypical of *E. coli*, but as more organisms are studied in detail, many variations are found. For example, it was thought that FabH was the universal initiator in the FAS II system, but in *Pseudomonas aeruginosa*, a new type of condensing enzyme, FabY, was described (Yuan et al. 2012). Functional screening of metagenomes for activities involved in FA biosynthesis will uncover even more diversity (Khan et al. 2016).

The structures of the different components of the FAS II systems of *E. coli* are known, and these data are being used to identify the mechanisms of interaction of ACP with the different enzymes (Finzel et al. 2015). Rather than an ACP-binding motif defined by primary structure, the enzymes of the type II system share 3-D surface features that account for their specific recognition of ACP and its thioesters (Zhang et al. 2003). Also, a putative binding site for ACP on the surface of the AHL synthase LasI includes a basic patch of residues (Gould et al. 2004). Cocrystallization of the different enzymes with their respective acyl-ACP will help to confirm the structure of the ACP binding site as well as to determine in more examples which structural features determine the specificity for the acyl chain (Finzel et al. 2015). Finally, the structural basis of the interaction of specialized ACPs with their corresponding enzymes and acyl chains should be investigated in order to understand the contribution of these specialized ACPs in channeling biosynthetic building blocks into certain pathways.

ACP is one of the most interactive proteins in bacteria (Butland et al. 2005), and the study of the physiological significance of many of these interactions is still subject of research.

Acknowledgments Research in our lab was supported by grants from Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México (DGAPA-UNAM; PAPIIT IN202616) and from Consejo Nacional de Ciencia y Tecnología-México (CONACyT-Mexico) (178359 and 253549 in Investigación Científica Básica as well as 118 in Investigación en Fronteras de la Ciencia). We thank José Espíritu Salazar, Ángeles Moreno-Ocampo, and Lourdes Martínez-Aguilar for their skillful technical assistance.

References

Aguilar PS, de Mendoza D (2006) Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. Mol Microbiol 62:1507–1514

Alvarez H, Steinbüchel MA (2002) Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol 60:367–376

Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, de Lisle G, Jacobs WR Jr (1994) *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 263:227–230

Basu SS, Karbarz MJ, Raetz CR (2002) Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in *Rhizobium leguminosarum*. J Biol Chem 277:28959–28971

Beld J, Lee DJ, Burkart MD (2015) Fatty acid biosynthesis revisited: structure elucidation and metabolic engineering. Mol BioSyst 11:38–59

Bi H, Zhu L, Jia J, Zeng L, Cronan JE (2016) Unsaturated fatty acid synthesis in the gastric pathogen *Helicobacter pylori* proceeds via a backtracking mechanism. Cell Chem Biol 23:1480–1489

Butland G, Peregrín-Alvarez JM, Li J, Yang W, Yang X, Canadien V, Starostine A, Richards D, Beattie B, Krogan N, Davey M, Parkinson J, Greenblatt J, Emili A (2005) Interaction network containing conserved and essential protein complexes in *Escherichia coli*. Nature 433:531–537

Cabruja M, Mondino S, Tsai YT, Lara J, Gramajo H, Gago G (2017) A conditional mutant of the fatty acid synthase unveils unexpected cross talks in mycobacterial lipid metabolism. Open Biol 7:160277

Finzel K, Lee DJ, Burkart MD (2015) Using modern tools to probe the structure-function relationship of fatty acid synthases. Chembiochem 16:528–547

Geiger O, López-Lara IM (2002) Rhizobial acyl carrier proteins and their roles in the formation of bacterial cell-surface components that are required for the development of nitrogen-fixing root nodules on legume hosts. FEMS Microbiol Lett 208:153–162

Gibbons HS, Reynolds CM, Guan Z, Raetz CR (2008) An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. Biochemistry 47:2814–2825

Gould TA, Schweizer HP, Churchill ME (2004) Structure of the *Pseudomonas aeruginosa* acyl-homoserinelactone synthase LasI. Mol Microbiol 53:1135–1146

Gunstone FD, Herslöf BG (1992) A lipid glossary. The Oily Press, Dundee

Hafkin B, Kaplan N, Murphy B (2016) Efficacy and safety of AFN-1252, the first *Staphylococcus*-specific antibacterial agent, in the treatment of acute bacterial skin and skin structure infections, including those in patients with significant comorbidities. Antimicrob Agents Chemother 60:1695–1701

Holtwick R, Meinhardt F, Keweloh H (1997) *Cis-trans* isomerization of unsaturated fatty acids: cloning and sequencing of the *cti* gene from *Pseudomonas putida* P8. Appl Environ Microbiol 63:4292–4297

Howard TP, Middelhaufe S, Moore K, Edner C, Kolak DM, Taylor GN, Parker DA, Lee R, Smirnoff N, Aves SJ, Love J (2013) Synthesis of customized petroleum-replica fuel molecules by targeted modification of free fatty acid pools in *Escherichia coli*. Proc Natl Acad Sci U S A 110:7636–7641

Isabella VM, Clark VL (2011) Identification of a conserved protein involved in anaerobic unsaturated fatty acid synthesis in *Neisseria gonorrhoeae*: implications for facultative and obligate anaerobes that lack FabA. Mol Microbiol 82:489–501

Janßen HJ, Steinbüchel A (2014) Fatty acid synthesis in *Escherichia coli* and its applications towards the production of fatty acid based biofuels. Biotechnol Biofuels 7:7

Kaneda T (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288–302

Khan R, Kong HG, Jung YH, Choi J, Baek KY, Hwang EC, Lee SW (2016) Triclosan resistome from metagenome reveals diverse enoyl acyl carrier protein reductases and selective enrichment of triclosan resistance genes. Sci Rep 6:32322

López-Lara IM, Geiger O (2017) Bacterial lipid diversity. Biochim Biophys Acta 1862:1287–1299

Marrakchi H, Choi KH, Rock CO (2002) A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*. J Biol Chem 277:44809–44816

McMurry LM, Oethinger M, Levy SB (1998) Triclosan targets lipid synthesis. Nature 394:531–532

Nakayama H, Kurokawa K, Lee BL (2012) Lipoproteins in bacteria: structures and biosynthetic pathways. FEBS J 279:4247–4268

Osterhout GJ, Shull VH, Dick JD (1991) Identification of clinical isolates of gram-negative nonfermentative bacteria by an automated cellular fatty acid identification system. J Clin Microbiol 29:1822–1830

Paoletti L, Lu YJ, Schujman GE, de Mendoza D, Rock CO (2007) Coupling of fatty acid and phospholipid synthesis in *Bacillus subtilis*. J Bacteriol 189:5816–5824

Parsons JB, Rock CO (2013) Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res 52:249–276

Pech-Canul A, Nogales J, Miranda-Molina A, Álvarez L, Geiger O, Soto MJ, López-Lara IM (2011) FadD is required for utilization of endogenous fatty acids released from membrane lipids. J Bacteriol 193:6295–6304

Percy MG, Gründling A (2014) Lipoteichoic acid synthesis and function in gram-positive bacteria. Annu Rev Microbiol 68:81–100

Pugh EL, Kates M (1994) Acylation of proteins of the archaebacteria *Halobacterium cutirubrum* and *Methanobacterium thermoautotrophicum*. Biochim Biophys Acta 1196:38–44

Rock CO (2008) Fatty acids and phospholipids metabolism in prokaryotes. In: Vance DE, Vance JE (eds) Biochemistry of lipids, lipoproteins and membranes, 5th edn. Elsevier, Amsterdam, pp 59–96

Schweizer E, Hofmann J (2004) Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. Microbiol Mol Biol Rev 68:501–517

Shang R, Liang J, Yi Y, Liu Y, Wang J (2015) Review of platensimycin and platencin: inhibitors of β-ketoacyl-acyl carrier protein (ACP) synthase III (FabH). Molecules 20:16127–16141

Steinbüchel A (2001) Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. Macromol Biosci 1:1–24

Wang H, Cronan JE (2004) Functional replacement of the FabA and FabB proteins of *Escherichia coli* fatty acid synthesis by *Enterococcus faecalis* FabZ and FabF homologues. J Biol Chem 279:34489–34495

Watson WT, Minogue TD, Val DL, Beck von Bodman S, Churchill MEA (2002) Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol Cell 9:685–694

White SW, Zheng J, Zhang YM, Rock CO (2005) The structural biology of type II fatty acid biosynthesis. Annu Rev Biochem 74:791–831

Whitfield C, Trent MS (2014) Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem 83:99–128

Williams AH, Raetz CR (2007) Structural basis for the acyl chain selectivity and mechanism of UDP-*N*-acetylglucosamine acyltransferase. Proc Natl Acad Sci U S A 104:13543–13550

Yao J, Rock CO (2015) How bacterial pathogens eat host lipids: implications for the development of fatty acid synthesis therapeutics. J Biol Chem 290:5940–5946

Yuan Y, Sachdeva M, Leeds JA, Meredith TC (2012) Fatty acid biosynthesis in *Pseudomonas aeruginosa* is initiated by the FabY class of β-ketoacyl acyl carrier protein synthases. J Bacteriol 194:5171–5184

Zhang YM, Wu B, Zheng J, Rock CO (2003) Key residues responsible for acyl carrier protein and beta-ketoacyl-acyl carrier protein reductase (FabG) interaction. J Biol Chem 278:52935–52943

Zhang YM, White SW, Rock CO (2006) Inhibiting bacterial fatty acid synthesis. J Biol Chem 281:17541–17544

Formation of Isoprenoids

4

Jordi Pérez-Gil, Manuel Rodríguez-Concepción, and Claudia E. Vickers

Contents

1 Introduction 58
2 Biosynthesis of Isoprenoids 59
2.1 Two Nonhomologous Pathways Produce the Universal Isoprenoid Precursors IPP and DMAPP 61
2.2 Prenyl Transferases 68
2.3 Terpene Synthases 70
3 Functional Classes of Microbial Isoprenoids 71
3.1 Biosynthesis of the Cell Wall 71
3.2 Membrane Architecture and Fluidity 72
3.3 Electron Transport 74
3.4 Conversion of Sunlight into Chemical Energy 75
3.5 Protein Translation 77

J. Pérez-Gil (✉)
Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Barcelona, Spain

Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD, Australia
e-mail: j.perezgil@uq.edu.au; jordi.perez@cragenomica.es

M. Rodríguez-Concepción
Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Barcelona, Spain
e-mail: manuel.rodriguez@cragenomica.es

C. E. Vickers
Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD, Australia

Commonwealth Science and Industry Research Organization (CSIRO), Land and Water, EcoSciences Precinct, Brisbane, QLD, Australia
e-mail: c.vickers@uq.edu.au; Claudia.Vickers@csiro.au

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_6

3.6 Protein Prenylation 77
3.7 Secondary Metabolites 78
4 Research Needs 79
References 81

Abstract

Isoprenoids, also known as terpenoids, are a diverse group of metabolites produced in all free-living organisms. They play an indispensable role in a wide variety of essential processes but also contribute to a better adaptation to the environment in the form of specialized secondary metabolites. In spite of their notable structural and functional diversity, all isoprenoids are synthesized from the same metabolic precursors, which are then converted into prenyl diphosphates of increasing length. Such basic prenyl diphosphate intermediates represent the starting point of downstream pathways leading to the formation of the vast diversity of end products. Here we present an overview of isoprenoid biosynthesis in microbes from the three kingdoms of life, namely, bacteria, archaea, and eukaryotic microorganisms (mainly microalgae and yeast), with a special emphasis on the research conducted during the last decade. We also discuss the main functional classes of isoprenoids occurring in these microorganisms by focusing in the representative model organisms of each kingdom. Finally, we examine key research needs in this field. This includes expanding our understanding of secondary isoprenoid metabolism in microbes, examining the evolutionary relationships between the two core biosynthetic pathways and improving our ability to engineer production of industrially useful isoprenoids in microbes.

1 Introduction

Isoprenoids are evolutionarily one of the oldest known classes of biomolecules. They were originally identified as key components of cell membranes and were widely used as taxonomic markers, even in fossil history (Chappe et al. 1979; Langworthy and Pond 1986). Today they represent the largest family of natural products, with more than 70,000 different compounds described (Dictionary of Natural Compounds: http://dnp.chemnetbase.com). Isoprenoids, also known as terpenoids, are produced in all free-living organisms. As a group, they are extraordinarily diverse structurally and functionally. They play essential primary roles in fundamental processes, including respiration (quinones), photochemical conversion (chlorophylls, bacteriochlorophylls, bacteriorhodopsins, carotenoids, plastoquinones), electron transport (cytochromes), protein translation and modification (prenylation), membrane integrity (sterols, bactoprenols, hopanoids, ether-type lipids in archaea), and antioxidant protection (carotenoids, quinones, isoprene) (Gershenzon and Dudareva 2007). These groups of compounds are highly

conserved in the evolutionary scale. Isoprenoids also have myriad secondary metabolic functions, and many of them are also industrially relevant compounds, having uses as biofuels, fragrances, drugs, pigments, or nutraceuticals (Vickers et al. 2015).

Plants are the organisms that provide most of the structural and functional diversity found in the isoprenoid family, primarily due to their very complex secondary metabolism for the interaction of the plant with the environment. Prokaryotes and eukaryotic microorganisms tend to have a less complex secondary isoprenoid metabolism. In model microbes such as *E. coli* and yeast, isoprenoid diversity is relatively limited, with isoprenoid products being mostly primary metabolites. In other classes of microbes, a more extensive secondary isoprenoid metabolism is present. For example, some non-photosynthetic bacteria and fungi produce nonessential carotenoids (which in photosynthetic organisms are usually considered as essential auxiliary pigments of the photosynthetic light-harvesting apparatus) to protect from photodynamic reactions and act as scavengers of oxygen radicals (Frank 1999); and some actinomycete and streptomyces strains produce isoprenoid-based antibiotics (Dairi 2005). However, sometimes the line between primary and secondary isoprenoids becomes rather blurry. This is the case, for instance, for isopentenyl tRNA. Addition of an isopentenyl molecule to some tRNA increases the efficiency of translation and reduces the sensitivity to the codon context both in eukaryotes and prokaryotes reducing the mutation rate (Persson et al. 1994). This provides the cell with a competitive advantage that is not environment-specific; is this a primary or secondary function?

2 Biosynthesis of Isoprenoids

Isoprenoid biosynthesis can be divided into a three-stage process that includes two relatively conserved core sets of reactions (1 and 2 below), and a third stage encompassing a set of variable reactions leading to the enormous variety of isoprenoid end products (Fig. 1):

1) Biosynthesis of the C5 prenyl phosphate universal isoprenoid precursors
2) Assembly of polyprenyl diphosphate backbones
3) Addition of functional groups and modification/decoration of the carbon backbone

These three stages take places in all organisms, but the third is especially diverse and extended in plants and support a tremendous variety of reactions including cyclization, dimerization, oxidation, or isomerization among others. This stage is more limited among prokaryotes, which mainly, but not only, produce functional isoprenoids through removal of the diphosphate and addition of the prenyl backbone to a target moiety (e.g., condensation to quinone head groups).

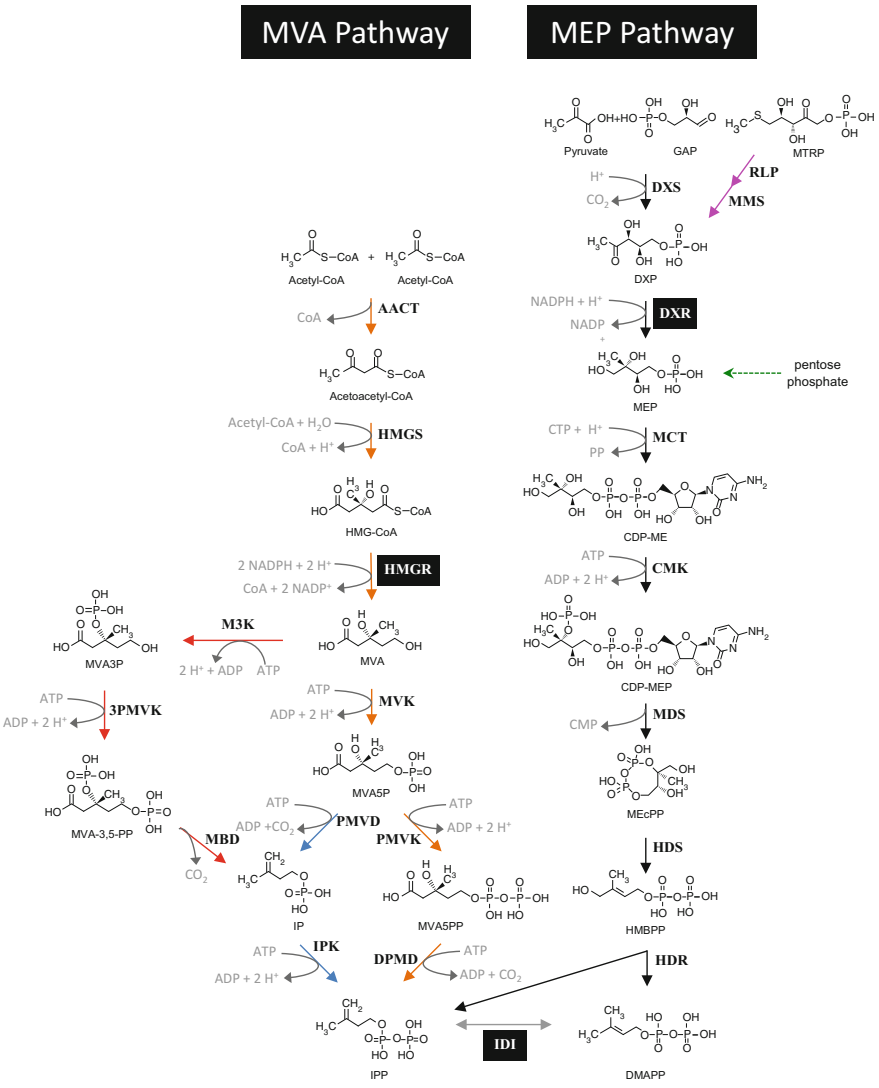


Fig. 1 Pathways for the biosynthesis of isoprenoid precursors in microbes. The canonical MEP pathway steps are shown in *black arrows* (*GAP* D-glyceraldehyde 3-phosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2-C-methyl-D-erythritol 4-phosphate, *CDP-ME* 4-diphosphocytidyl-2-C-methyl-D-erythritol, *CDP-MEP* 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate, *MEcPP* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, *HMBPP* 4-hydroxy-3-methylbut-2-enyl diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate). The alternative MEP pathway initiation discovered in *Rhodospirillum* is shown in *purple arrows* (MTRP, 5-methylthio-D-ribulose 1-phosphate), and the alternative pathway initiation proposed for *Synechocystis* is shown in *green dashed arrow*. The canonical or eukaryotic MVA pathway is presented in *orange arrows* (*HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA, *MVA* mevalonic acid, *MVA5P* 5-phosphomevalonate, *MVA5PP* 5-diphosphomevalonate). The alternative steps of the archaeal MVA pathway are shown in *blue arrows* (*IP* isopentenyl phosphate), and the alternative

2.1 Two Nonhomologous Pathways Produce the Universal Isoprenoid Precursors IPP and DMAPP

Despite their astonishing diversity both at the structural and functional level, all isoprenoids are derived from the same C5 universal building blocks, isopentenyl diphosphate (IPP), and its isomer dimethylallyl diphosphate (DMAPP). In nature, two chemically unrelated pathways are responsible for the synthesis of IPP and DMAPP. The mevalonate (MVA) pathway is present mainly in eukaryotes and archaea, whereas the 2-methyl 3-erythritol-4-phosphate (MEP) pathway is found in most bacteria (including many important pathogens). In plants both pathways coexist, but in different subcellular compartments: the MVA pathway is located in the cytosol, and the MEP pathway is located in the plastids. IPP is the final product in the MVA pathway, whereas the last step of the MEP pathway yields both IPP and DMAPP simultaneously (Fig. 1).

2.1.1 MVA Pathway

The MVA pathway was discovered during the 1960s and was the first pathway identified for the biosynthesis of isoprenoids (Lynen 1967). For many decades, it was considered to be the only pathway producing IPP/DMAPP and was therefore thought to be ubiquitous in all living organisms. The "canonical" MVA pathway is considered to be the pathway as it was elucidated in eukaryotic organisms, though plasticity is present in other organisms (see below for details; Fig. 1). In the first two steps of the canonical or eukaryotic MVA pathway, the sequential condensation of three molecules of acetyl-CoA produces acetoacetyl-CoA and then 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), catalyzed by the enzymes acetoacetyl-CoA thiolase (AACT) and HMG-CoA synthase (HMGS), respectively. HMG-CoA is then converted to MVA in an irreversible reaction that represents the first committed step of the pathway and is catalyzed by the enzyme HMG-CoA reductase (HMGR). MVA is sequentially phosphorylated to 5-phosphomevalonate by mevalonate kinase (MVK) and then to 5-diphosphomevalonate by 5-phosphomevalonate kinase (PMVK). Finally, decarboxylation of 5-diphosphomevalonate catalyzed by 5-diphosphomevalonate decarboxylase (DPMD) generates IPP. DPMD catalyzes a two-step reaction involving an ATP-dependent phosphorylation at a 3-OH position of the mevalonate moiety prior to the decarboxylation. An isomerase (IDI) forms DMAPP from IPP in a reaction that is required to complete a fully functional MVA pathway (Fig. 1). The enzymes involved in the "canonical" MVA pathway have been recently reviewed (Miziorko 2011).

This canonical pathway is also found in a few eubacteria, including three gram-positive cocci, *Staphylococcus aureus*, *Streptococcus pneumonia*, and *Enterococcus*

Fig. 1 (continued) thermoplasma-type MVA pathway is shown in *red arrows* (*MVA3P* mevalonate 3-phosphate, *MVA-3,5-PP* mevalonate 3,5-bisphosphate). Boxed enzymes correspond to those with more than one type described. Enzyme acronyms (*in bold*) are described in the text

faecalis and the spirochete *Borrelia burgdorferi* (Lombard and Moreira 2011). In addition to the canonical MVA pathway, some eubacterial species (e.g., *Listeria monocytogenes* and some *Streptomyces* strains) also contain the MEP pathway. In *Listeria monocytogenes* despite both pathways contribute to the synthesis of IPP/DMAPP, it has been described that HMGR is an essential gene, and the MEP pathway cannot support cell growth by itself (Heuston et al. 2012). However, MEP pathway mutants are impaired in infection of mice. Interestingly, the closely related nonpathogenic *Listeria innocua* contains the MVA pathway and functional genes for the four initial steps of the MEP pathway but lacks the last two, which have been lost during evolution possibly during adaptation to a nonpathogenic life cycle (Begley et al. 2008). In the case of *Streptomyces*, the MEP pathway is used mainly for the synthesis of essential isoprenoids, whereas the MVA pathway is used to produce specialized secondary metabolites such as antibiotics due to its transcriptional regulation activated during the late phase of growth (Hamano et al. 2002).

Archaea also harbor an MVA pathway but with variation from the canonical pathway. The increasing availability of fully sequenced archaeal genomes has allowed to conclude that this kingdom of life only uses the MVA pathway, as no single representative has been found to date that also contains the MEP pathway. However, gene analyses for the full set of enzymes of the MVA pathway systematically failed to identify the genes for the last two steps of the canonical pathway, PMVK and DPMD. These two genes are absent in most archaea with only a few exceptions including halophilic archaea and *Thermoplasma* relatives, which contain a DPMD-like gene and some thermoacidophilic archaea showing ortologues for both PMVK and DMPD (Lombard and Moreira 2011).

Recent experiments have demonstrated that IPP in most archaea is produced by switching the order of last phosphorylation and decarboxylation steps. In this alternative route, 5-phosphomevalonate is converted to isopentenyl phosphate (IP) by 5-phosphomevalonate decarboxylase (PMD) and subsequently phosphorylated by IP kinase (IPK) to yield IPP (Fig. 1). IPK was first identified in *Methanocaldococcus jannaschii* (Grochowski et al. 2006). More recently, the alternative MVA route was completed with the identification of a PMVD-encoding sequence in green non-sulfur bacteria (Dellas et al. 2013). Even more recently, a phosphomevalonate decarboxylase together with an isopentenyl phosphate kinase were identified and characterized in *Haloferax volcanii*, showing how the archaeon decarboxylation step occurs prior to phosphorylation (Vannice et al. 2014).

Furthermore, in 2014 a second alternative MVA pathway was suggested in *Archaea* (Azami et al. 2014). Very recent experiments have demonstrated the existence of a thermoplasma-type MVA pathway suggested to be unique among the archaeal order thermoplasmatales (Vinokur et al. 2016). In this new alternative route, MVA is sequentially phosphorylated to mevalonate 3-phosphate by mevalonate kinase (M3K) and then to mevalonate 3,5-bisphosphate by mevalonate 3-phosphate 5-kinase (3PMVK). Finally, an ATP-independent decarboxylation of mevalonate 3,5-bisphosphate catalyzed by mevalonate 3,5-bisphosphate decarboxylase (MBD) generates IP, which in turn is phosphorylated by the previously mentioned IPK to produce IPP (Fig. 1). M3K was first identified and characterized

in *Thermoplasma acidophilum* (Azami et al. 2014) and immediately after in another extreme acidophile, *Picrophilus torridus*. 3PMVK and MBD (Vinokur et al. 2016) have also been identified in *Thermoplasma acidophilum* to complete a fully functional alternative MVA pathway. The identification of this new pathway in the most acid-tolerant organisms has been associated to an evolutionary adaptation to extremely acidic environments.

2.1.2 2-C-Methyl-D-Erythritol 4-Phosphate Pathway (MEP)

Over the years following discovery of the MVA pathway, inconsistencies in labeling experiments led to significant confusion, as the labeling patterns could not be explained by the known biochemistry of the MVA pathway. In the early 1990s, new labeling experiments revealed the existence of an alternative pathway for IPP and DMAPP production from pyruvate and glyceraldehyde 3-phosphate (Rohmer et al. 1993). The MEP pathway, also known as the non-mevalonate, Rohmer or DXP pathway, comprises seven enzymatic steps (Fig. 1; (Frank and Groll 2017)) that are best characterized in the gram-negative model organism *Escherichia coli*. The first step involves the condensation of pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate to produce 1-deoxy-D-xylulose 5-phosphate (DXP) and eliminate one molecule of CO_2 in a reaction catalyzed by DXP synthase (DXS). In *E. coli*, DXP represents a branch point of the pathway as DXP is also used for the production of pyridoxal phosphate (vitamin B6) and thiamine diphosphate (vitamin B1). In the next step DXP is converted to MEP by the enzyme DXP reductoisomerase (DXR) in a reaction that involves an intramolecular rearrangement and posterior reduction and represents the first committed step of the pathway. In the next three successive steps, cytidylation, phosphorylation, and cyclization, reactions are catalyzed by the enzymes MEP cytidylyltransferase (MCT), 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK), and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) synthase (MDS), leading to the formation of MEcPP, a cyclic diphosphate molecule with an unusual structure cyclized through the phosphate groups (Fig. 1). In the sixth step, 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) synthase (HDS) catalyzes the opening reduction of the ring to form HMBPP. In the last step of the pathway, HMBPP is transformed simultaneously into a mixture of IPP and DMAPP at a 5:1 ratio by the enzyme HMBPP reductase (HDR). The simultaneous production of IPP and DMAPP by HDR makes the isomerase (IDI) a nonessential step of the MEP pathway. Despite this, it is well established that overexpression of IDI results in an increase in isoprenoid end products. This suggests that the enzyme can play an important role in balancing the availability of the universal building blocks for the production of downstream compounds. The MEP pathway has been reviewed elsewhere (Rodriguez-Concepcion and Boronat 2002). During the elucidation and characterization of the pathway, different nomenclatures were adopted to designate orthologous bacterial and plant genes leading to some confusion. A unified nomenclature for the MEP pathway genes, enzymes, and intermediates was proposed to mitigate this problem (Phillips et al. 2008).

2.1.3 Plasticity and Diversity in Isoprenoid Pathways

Since isoprenoids are essential, selection forces act to maintain isoprenoid production but not always necessarily at the individual enzyme level. Microorganisms show an astonishing biochemical and metabolic diversity (mainly in prokaryotes) as a consequence of their ability to adapt to a wide range of environmental conditions. This plasticity is also reflected on isoprenoid biosynthesis at many levels. In the last decade, extensive work has revealed the existence of a variety of alternative steps in both pathways as well as lateral gene transfer (LGT) events which transferred whole pathways and specific alternative steps between kingdoms (Perez-Gil and Rodriguez-Concepcion 2013).

The existence of two nonhomologous biochemically unrelated pathways to produce the same universal precursors represents the first and most obvious layer of diversity and plasticity. Also, as noted, the final two steps of the MVA pathway are variable in Archaea (Dellas et al. 2013), and some bacteria harbor an MVA pathway instead of a MEP pathway (Dairi 2005). However, plasticity can also be found at the enzyme level in both pathways at different enzymatic steps. These alternative steps are distributed in various phyla, but unlike the alternative archaeal MVA pathway, they are not necessarily widely distributed in one phylum.

In the MVA pathway, two different classes of homologous HMGR have been identified based on sequence alignments and phylogeny. Class 1 (HMGR-I) enzymes are found mainly in eukaryotes and archaea, whereas class 2 (HMGR-II) are predominantly found in bacteria harboring the MVA pathway. However, HMGR-II is present in some archaeal orders, including Thermoplasmatales and Archeoglobales, in what has unambiguously identified as a LGT event (Boucher et al. 2001). The two classes of HMGR enzymes, which have been proposed to arise by divergent evolution from a common ancestor, share less than 20% amino acid identity and show major differences around the active site. As a consequence of these differences, the two classes of enzymes show a different level of sensitivity to statins, a family of blockbuster drugs first isolated from fungi that inhibit HMGR activity. Statins are widely used to lower cholesterol levels in humans through strong inhibition of the activity of HMGR-I, but they show a very poor effect on HMGR-II enzymes. This might explain the substitution of HMGR-I by HMGR-II enzymes in bacteria as well as the ability of some archaea to incorporate and maintain this type of enzyme even in the presence of an active HMGR-I (Friesen and Rodwell 2004).

Different enzymes catalyzing the same reaction are also described in the MEP pathway. Two different enzymes can catalyze the first committed step of the pathway to produce MEP from DXP. Most bacteria have a canonical DXR enzyme (DXR-I) to catalyze this reaction, but recently a new type of DXR-like enzyme (DRL or DXR-II) was discovered in a heterogeneous group of MEP pathway-containing bacteria having no DXR homolog (Sangari et al. 2010). DXR-II belongs to a family of previously uncharacterized proteins with oxidoreductase features but with similarity to DXR-I only in the NADPH-binding domain and a different arrangement of the active sites (Perez-Gil et al. 2012a). Moreover, not all DXR-II proteins participate in the MEP pathway. Some organisms have a DXR-II homolog, but do not have a MEP pathway; in this case, the homolog does not in fact have DXR activity.

Phylogenetic analyses support a single origin of DXR-II and these DXR-II homologs, followed by functional divergence in different phyla. This represents a quite unique model of convergent evolution in the case of the nonhomologous DXR-I and DXR-II enzymes with DXR activity and divergent evolution in the case of DXR-II enzymes with different functions. The patchy distribution of DXR-II in specific eubacteria is best explained by gene loss (Carretero-Paulet et al. 2013). This mechanism would explain the unique presence of DXR-I in the majority of bacteria, the presence of only DXR-II in some, and the presence of both DXR-I and DXR-II in a few strains.

The last step in both MVA and MEP pathways is catalyzed by IDI. Two different types of enzymes have also been identified, IDI-I and IDI-II. Even though they catalyze the same reaction, the two classes show no sequence similarity, have unrelated structures, and have different reactions mechanisms involving different cofactors (Berthelot et al. 2012). Type I (IDI-I) enzymes, which were the first identified and studied extensively in the 1950s, are found in eukaryotes and in most bacteria. Type II (IDI-II) were first identified in *Streptomyces* 15 years ago (Kaneda et al. 2001) and then shown to be also present in archaea and some other bacteria. Type I enzymes are zinc metalloproteins utilizing a divalent metal in a well-established protonation−deprotonation reaction. In contrast, type II enzymes are flavoproteins that require a reduced form of flavin mononucleotide (FMN) produced by NADPH and Mg^{2+}. However, the mechanism of reaction is still under investigation. A radical mechanism was first proposed, but recently a protonation−deprotonation mechanism similar to the one observed for IDI-I was suggested (Berthelot et al. 2012). Interestingly, some bacteria possess either IDI-I or IDI-II, some possess both of them, but a large proportion do not contain homologs for any IDI protein. As IDI is not essential for the MEP pathway, it is feasible that they do not have an IDI activity.

In addition to enzymatic plasticity at specific nodes of the pathway, there is also some evidence that the MEP pathway can initiate from alternative central carbon precursors. The cyanobacterium *Synechocystis* PCC6803 has homologs for all the MEP pathway genes. However, no effect was observed on isoprenoid biosynthesis in photoautotrophically grown cells either when supplementing the pathway with pathway substrates/intermediates or when pharmacologically blocking the pathway (Ershov et al. 2002). This is surprising, as both approaches result in significant modification of isoprenoid production in other organisms. Conversely, feeding-labeled phosphorylated sugars of the pentose phosphate pathway resulted in incorporation of labeled IPP into isoprenoids (Perez-Gil and Rodriguez-Concepcion 2013). This suggested alternative pathway is not completely understood, but together these results suggest that photosynthesis-derived products of the pentose phosphate pathway could be used as alternative substrates for IPP biosynthesis.

A more recent but better understood alternative MEP pathway initiation route has been identified in another photosynthetic bacterium, *Rhodospirillum rubrum* (Warlick et al. 2012). In this alternative pathway, DXP is produced in two catalytic steps from 5-methylthio-D-ribulose 1-phosphate (MTRP) instead of pyruvate and G3P. MTRP is an intermediate of the methionine salvage pathway, and is isomerized

to 1-methylthio-D-xylulose 5-phosphate (MTXP) by a RuBisCO-like protein (RLP). MTXP is finally converted into DXP by MTXP methylsulfurylase (MMS). Genes encoding for RLP and MMS are present in several bacterial groups producing MTRP, making feasible a potential wider distribution of this alternative pathway initiation route. Interestingly, all these bacteria also have genes encoding DXS (the enzyme producing DXP from pyruvate and G3P), which still appears to be the main contributor to IPP/DMAPP production (Perez-Gil and Rodriguez-Concepcion 2013).

The contribution of these alternative routes linking the adenosylmethionine (SAM)-dependent polyamine metabolism or the pentose phosphate pathway with isoprenoid biosynthesis remains to be investigated. However, it is possible that bacteria harboring the canonical pathway as well as alternative routes could potentially use both pathways to synthesize their isoprenoids in response to specific environmental conditions.

The differential distribution of the two main pathways producing the universal precursors for the biosynthesis of isoprenoids, the MVA present in mammals (including humans), and the MEP present mostly in bacteria (including several pathogens as well as the protozoa parasite Plasmodium falciparum) along with the essential role of isoprenoids provides a new promising target for the development of much needed antibiotics (Rodriguez-Concepcion 2004). Potentially, drugs inhibiting any of the seven enzymatic steps of the MEP pathway could lead to the development of antibiotic drugs.

Additionally, the existence of different types of enzymes in some steps of both pathways provides a good opportunity for the development of highly specific drugs. Drugs targeting only HMGR-II (present in bacteria) but with no effect on HMGR-I (present in humans) could be used against eubacterial pathogens harboring this specific isoform (Friesen and Rodwell 2004). To date no good inhibitors showing specificity for HMGR-II have been identified yet. DXR-I and DXR-II represent another node species-specific. The active site of these enzymes shows a different arrangement what could be used to design highly specific antibiotics against only one of the enzyme types (Perez-Gil et al. 2012a). Most relevant would be the identification of drugs selectively targeting pathogens harboring DXR-II to develop a new generation of antibiotics without affecting beneficial bacteria in the human gut carrying DXR-I enzymes.

However, even the MEP pathway represents a promising target for the development of new antibiotics against pathogens the above-described remarkable diversity and plasticity observed in bacteria for isoprenoid biosynthesis requires special attention since represents mechanisms to overcome pharmacological blockage that apply to specific organisms. Several pieces of evidence showing the ability of bacteria to bypass inhibition of single enzymes have been already reported highlighting the requirement of accurate designs and rational development. Reduced uptake (Brown and Parish 2008), shunt pathways (Ershov et al. 2002), alternative enzymes (Perez-Gil and Rodriguez-Concepcion 2013), or spontaneous mutations (Perez-Gil et al. 2012b) are some of the mechanisms already described when inhibiting isoprenoid biosynthesis that requires higher level of attention. Currently, great effort

is focused on the identification and development of antibiotics directed against almost all of the enzymes of the MEP pathway. Despite all this effort to date, only one drug targeting the second enzyme of the MEP pathway (DXR), fosmidomycin (FSM), has reached clinical trials to fight malaria, and results show that further work is required. Phase II trials using FSM alone do not retrieve the expected cure rates, and phase III trials in combination with clindamycin also failed due to a parasite recrudescence after an initial promising high cure rate seven days posttreatment (Fernandes et al. 2015).

2.1.4 Evolution of the Pathways Producing Universal Precursors of Isoprenoids

It is often generalized that the MVA pathway is present in eukaryotes and (in slightly modified form) in archaea, whereas the MEP pathway is present in bacteria. However, a detailed exploration of the genomic data available reveals a much more patchy distribution of these pathways – in particular, among eubacteria and microalgae. In some cases, bacteria from the same phylogenetic group use different pathways; and in others, unrelated bacteria use the same pathway. Obviously, this distribution cannot be described by simple vertical inheritance of genetic information. Phylogenomic analyses have confirmed that the MEP pathway is restricted to bacteria and to the plastids of eukaryotes (Lombard and Moreira 2011). However, the canonical MVA pathway has been identified in bacterial and archaeal clades as well as the eukarya. Presence in prokaryotes has traditionally been attributed to lateral gene transfer (LGT) events among the three kingdoms of life. While LGT is not a rare phenomenon – for instance, LGT is thought to be responsible for almost 18% of *E. coli* genes (Lawrence and Ochman 1998) – LGT cannot exclusively explain the patchy distribution of the MVA pathway.

The absence of homologs for the last three steps of the classical MVA pathway in archaea was first explained by the recruitment of nonhomologous enzymes to catalyze the same reaction. This was supported by the discovery of the nonhomologous IDI-II in *Streptomyces* (Kaneda et al. 2001) that subsequently was found in most archaeal genomes. However, this is not the case for the remaining unidentified steps, which were recently identified to complete what now is generally accepted as the alternative MVA pathway in Archaea (Vannice et al. 2014). Despite sporadic cases of LGT, the alternative MVA pathway seems to be ancestral in archaea suggesting that actually three ways to produce IPP would exist – one of each for the three kingdoms of life (Lombard and Moreira 2011).

The uneven taxonomic distribution of the two major pathways has led to the proposal of two different hypotheses about their evolutionary origin. Some authors propose that the two pathways emerged in completely independent evolutionary events, one in the bacterial lineage and one in the archaeal lineage (Lange et al. 2000). However, recent phylogenetic studies suggest that not all the bacteria bearing the MVA pathway acquired the genes by LGT from archaea or eukaryotes (Lombard and Moreira 2011). This data provides the framework for a completely different evolutionary hypothesis: that the classical MVA pathway is in fact the common ancestral pathway, which was inherited in all three kingdoms and modified (in the

case of the alternate MVA pathway) or replaced (under appropriate selective conditions) by the MEP pathway following its evolution.

These two hypotheses have relevant implications with respect to the nature of the cenancestor (the last common ancestor of all organisms). An independent emergence of the two pathways implies the absence of lipid membranes in the cenancestor. In this case, the cenancestor would either be noncellular or surrounded by a mineral structure. In contrast, if the MVA pathway is in all three kingdoms, then it follows that this pathway was present in the cenancestor. This in turn implies the possibility that the common ancestor had membranes containing isoprenoids (Lombard and Moreira 2011). After being a matter of debate for many years, the presence of lipid membranes in the cenancestor is now becoming generally accepted (Lombard et al. 2012; Sojo et al. 2014).

The distribution and evolution of the two pathways in algae is even more complex. In all higher plants, the MEP pathway is always found in chloroplast, and the MVA is found in the cytosol; however, microalgae show a much more diverse distribution of the pathways. The chrysophyte *Ochromonas danica* and the red alga *Cyanidium caldarium* use both pathways, but the green algae *Scenedesmus obliquus*, *Chlorella fusca*, and *Chlamydomonas reinhardtii* produce all their isoprenoids through their chloroplastic MEP pathway. The euglenophyte *Euglena gracilis* was originally thought to produce all its isoprenoids (including plastidial isoprenoids) using the MVA pathway due to the lack of label incorporation in phytol from feeding labeled glucose or pyruvate. Later work supported a lack of label incorporation in phytol but also demonstrated label incorporation in carotenoids – demonstrating that, despite the lack of labeled phytol, the MEP pathway clearly contributes to plastidial isoprenoid biosynthesis (Lohr et al. 2012).

Evolution of eukaryotic phototrophs involved different symbiotic events that lead to the coexistence of both pathways. In the primary endosymbiosis, a heterotrophic eukaryote (harboring the MVA pathway) engulfed a cyanobacterium that ultimately became the plastid in some of the algae phyla (mostly green algae). Subsequently, another heterotrophic eukaryote (also harboring the MVA pathway) engulfed a green alga (already harboring both the MVA and MEP pathways) to become eukaryotic endosymbionts that were finally reduced to plastids (secondary endosymbiosis). Other secondary symbiotic events involving red algae have been suggested as well. In this scenario, different coexistence of the pathways has produced a variety of pathway distributions, including coexistence of the two pathways in algae. However, the overall story suggests that the MEP pathway is essential for all plastid-bearing organisms (Lohr et al. 2012).

2.2 Prenyl Transferases

Prenyl transferases (also known as prenyl diphosphate synthases) catalyze the condensation of an acceptor (that may or may not be isoprenoid-derived) to an allylic diphosphate. Three different types of prenyltransferase reactions are described. The first one catalyzes head-to-tail reactions (mainly producing *trans*-

prenyl diphosphates from DMAPP); the second catalyzes head-to-head reactions (using prenyl diphosphates to produce tri- and tetra-terpenes) or head-to-middle (leading to irregular isoprenoids); and the third involves the insertion of prenyl chains on a non-isoprenoid acceptor (alkylation), for example, in ubiquinone biosynthesis (Bouvier et al. 2005; Oldfield and Lin 2012).

The three core prenyl transferase enzymes involved in the biosynthesis of the main backbones for the production of isoprenoid end products are the enzymes geranyl diphosphate synthase (GDS), farnesyl diphosphate synthase (FDS), and geranylgeranyl diphosphate synthase (GGDS) (Fig. 2). The reaction is initiated at the primary allylic diphosphate, DMAPP; sequential addition of IPP results in the linear all-*trans*-prenyl phosphates of increasing length, starting with the C10 geranyl diphosphate (GPP), then the C15 farnesyl diphosphate (FPP), and then the C20 geranylgeranyl diphosphate (GGPP). A new C-C bond is generated between two

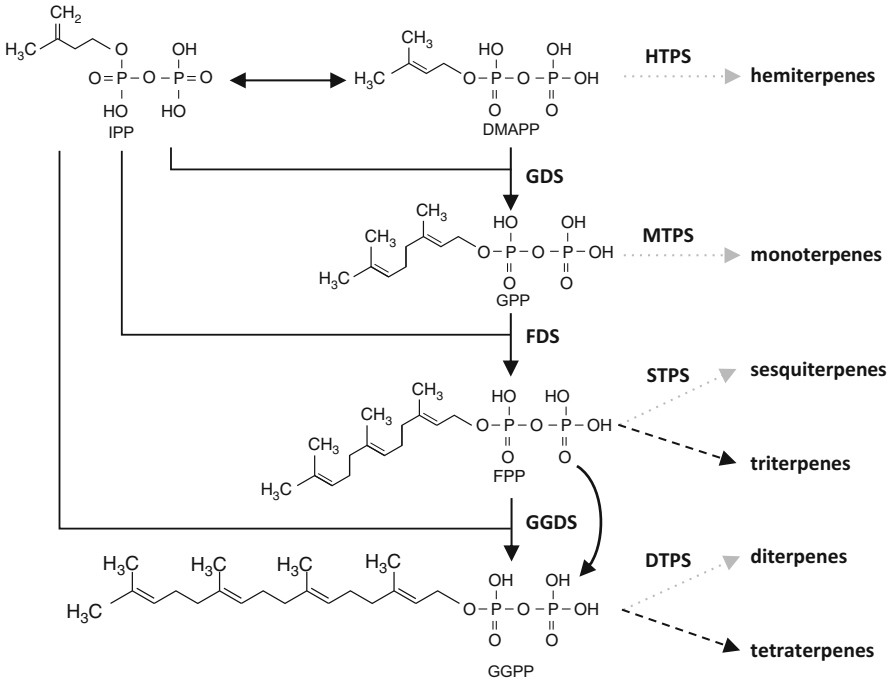


Fig. 2 Schematic representation of the biosynthesis of the isoprenoid end products' families from the universal building blocks, IPP (isopentenyl diphosphate), and DMAPP(dimethylallyl diphosphate). *Solid arrows* indicate head-to-tail condensation reactions leading to the formation of geranyl diphosphate, GPP; farnesyl diphosphate, FPP; and geranylgeranyl diphosphate, GGPP. Reactions are catalyzed by GDS, geranyl diphosphate synthase; FDS, farnesyl diphosphate synthase; and GGDS, geranylgeranyl diphosphate synthase, respectively. *Dashed arrows* indicate head-to-head condensations for the formation of triterpenes and tetra-terpenes. Hemiterpene synthases, HTPS; monoterpene synthases, MTPS; sesquiterpene synthases, STPS; and diterpene synthases, DTPS catalyze the conversion of basic backbones into isoprenoid end products, *dotted arrows*

prenyl units through a nucleophilic substitution releasing a pyrophosphate molecule (Sacchettini and Poulter 1997). In bacteria (as well as in plants), GGDS sequentially adds IPP to the allylic co-substrates DMAPP, GPP, and FPP to produce GGPP, whereas in yeast (as well as in mammals, and presumably all higher eukarya), GGDS use only FPP as a substrate. This suggests two different ways to produce GGPP involving one (GGDS) or two (FPPS/GGDS) enzymes. Prenyl diphosphates such as IPP, GPP, FPP, and GGPP are the polyprenyl backbones leading to isoprenoid end products' families of hemiterpenes, monoterpenes, sesquiterpenes, and diterpenes, respectively (Fig. 2).

2.3 Terpene Synthases

Terpene synthases (TPSs) catalyze a range of reactions (primarily dephosphorylation and cyclization) to convert the polyprenyl backbones into members of the different subfamilies of isoprenoids. These transformations may then be followed by a variety of modifications of the parental skeletal types to produce the vast family of isoprenoid metabolites described to date. In plants, the TPS family is particularly diverse and has been extensively studied (Bohlmann et al. 1998). There are various classification systems. TPS genes are generally divided into seven clades based on sequence similarity (Chen et al. 2011), whereas the proteins can be classified based on their mechanism of reaction or substrate specificity.

The two reaction mechanism groups, class I and class II, produce carbocations that ultimately lead to the formation of new carbon-carbon bonds; however, they differ in the way reactions are initiated. Class I enzymes catalyze heterolytic cleavage of an allylic diphosphate ester bond, whereas class II enzymes utilize protonation to initiate the reaction (Gao et al. 2012). Class I TPS enzymes are the most abundant and often produce multiple products, using either a single prenyl diphosphate substrate or more than one. This is due to the stochastic nature of bond rearrangements that can follow the formation of the carbocation intermediate. Alternatively, TPS enzymes can be classified based on substrate specificity for the length of the prenyl phosphate substrate. Hemiterpene synthases, monoterpene synthases, sesquiterpene synthases, and diterpene synthases use C5, C10, C15, and C20 prenyl phosphates as substrates, respectively. Most of the C5–C15, and some C20, terpenoids are volatile under normal environmental conditions.

Following the initial dephosphorylation (+/− cyclization), further alterations and decorations by oxidation, peroxidation, methylation, acylation, or cleavage may occur through the action of other enzymes, particularly cytochrome P450s. These decorations have an impact on the specific physical properties of the final compound and may be responsible for new biological activities.

Less is known about TPSs in prokaryotes, where relatively few TPSs have been identified. It has been known that bacteria produce odorous volatile compounds, presumably in the terpenoid family, since the nineteenth century; however, the first studies on these compounds only began in the 1960s, when Gerber and Lechevalier (Gerber and Lechevalier 1965) described the petrichor ("rain-wet-earth" smell)

isoprenoid, geosmin, produced by actinomycetes. It appears that it took over 50 years for the next research reporting volatile isoprenoids (isoprenoid hydrocarbons and alcohols) from bacteria to be published (Wilkins and Schöller 2009). While generally far fewer than in plants, bacteria also contain genes coding for terpene synthases. The encoded enzymes are class I TPSs (so far, no class II TPSs have been found in bacteria). However, they do not show sequence similarity with the plant or fungal genes and usually have low levels of similarity with other bacterial TPSs, making their identification through local sequence alignment challenging. Despite these differences at the primary sequence level, terpene synthases all typically display two highly conserved metal-binding motifs: an acidic amino acid-rich motif and a triad of residues located downstream that is fully conserved in all three classes of bacterial terpene synthases (mono-, sesqui-, and diterpene synthases) (Komatsu et al. 2008). Hemiterpene synthases have not been described in bacteria; instead, in the case of isoprene production in *Bacillus*, the hemiterpene isoprene is produced by HDR (also known as IspH), the terminal enzyme in the MEP pathway (Ge et al. 2016).

Based on the two conserved motifs, a search method for new TPSs was developed using hidden Markov (HMM) models and previously identified plant, fungal, and bacterial TPSs. This approach retrieved 41 new predicted synthases from the protein family (Pfam) database. A second-generation method using the new set of 41 bacterial sequences to train the HMM model leads to the identification of 140 presumptive terpene synthases. More recently, a third-generation model, trained with the previous 140 bacterial TPS, has expanded the family up to 262 genes, the biochemical function of some of which has also been determined. Results show that sesquiterpene synthases are by far the most prevalent TPS among prokaryotes (Yamada et al. 2015).

3 Functional Classes of Microbial Isoprenoids

It is currently considered that isoprenoids in microbes mainly fulfill essential biological roles in growth and survival. These processes include cell wall biosynthesis, membrane function or electron transport among others. However, some microbes, especially microalgae, also produce highly specialized isoprenoid-based secondary metabolites. Moreover, as more microbial TPS genes are discovered and the functions of their products determined, this view may change. In this section we briefly describe the main functional classes of isoprenoids identified to date focusing primarily on isoprenoid roles in the model microbial organisms for each kingdom but also examining some other interesting organisms/roles.

3.1 Biosynthesis of the Cell Wall

The bacterial cell wall is a rigid semipermeable structure surrounding the cytoplasmic membrane that maintains the cell shape and prevents deleterious effects of

internal osmotic pressure in the cell. The biosynthesis of the cell wall is a complex process that involves the transport of highly hydrophilic monomeric units across the cytoplasmic membrane that are then assembled to produce the main component of the cell wall, peptidoglycan (Scheffers and Pinho 2005). The key compound allowing this transport is an isoprenoid lipid carrier, undecaprenyl phosphate (UP), commonly referred to as bactoprenol (Fig. 3). UP is a C55 compound derived from a prenyl diphosphate produced by the sequential addition of 8 molecules of IPP onto FPP followed by dephosphorylation. This step is essential since the oligosaccharide transfer between the UDP-sugar and the lipid requires the monophosphate form of the lipid (Touz and Mengin-Lecreulx 2008).

3.2 Membrane Architecture and Fluidity

As already mentioned, cell membrane isoprenoids have a long history, both evolutionarily and academically as taxonomic markers. The cell membrane is a selectively permeable layer, basically containing lipids and proteins, which surrounds and encloses the contents of the cell. Glycerolipids are one of the main lipid components in the membrane; they are composed of a polar head and two alkane chains attached to a glycerol moiety. In archaea, the architecture of the membrane shows some unique features. Unlike any other form of life, archaeal membranes contain ether-type lipids with hydrocarbon chains bound to the glycerol moiety exclusively by ether linkages. In eukaryotes and eubacteria, fatty acyl hydrocarbon chains are linked to an *sn*-glycerol-3-phosphate backbone via an ester bond, whereas in archaea ether bonds link isoprenoid hydrocarbon chains to *sn*-glycerol-1-phosphate (Jain et al. 2014). Usually, the isoprenoid side chains are methyl-branched C20 and C25 prenyl phosphates derived from GGPP (Fig. 3).

Other isoprenoids in other microbes are also involved in the regulation of the fluidity of the membrane. In yeast, as a representative of eukaryotic microorganisms, ergosterol plays a similar role to that described for cholesterol (the most common sterol in vertebrates) or phytosterol (in plants) in the regulation of the membrane rigidity, fluidity, and permeability (Abe and Hiraki 2009). The reason for this specificity of sterols in each eukaryotic clade is not clear but must have some evolutionary rationale. In particular, the reason why fungi produce ergosterol is not clear since its biosynthesis is more energy-demanding compared to cholesterol, and structure-function studies have not been able to identify any advantage (Shrivastava and Chattopadhyay 2007). One recent explanation is that ergosterol may provide a better adaptation to the typical climatic instabilities of fungal ecological niches (Dupont et al. 2012). Interestingly, it was recently shown that some less evolutionary advanced species of fungi produce cholesterol instead of ergosterol (Weete et al. 2010). The biosynthetic pathway for ergosterol involves several catalytic steps, but the basic backbone derives from the head-to-head condensation of two molecules of FPP to produce the C30 squalene (Fig. 3).

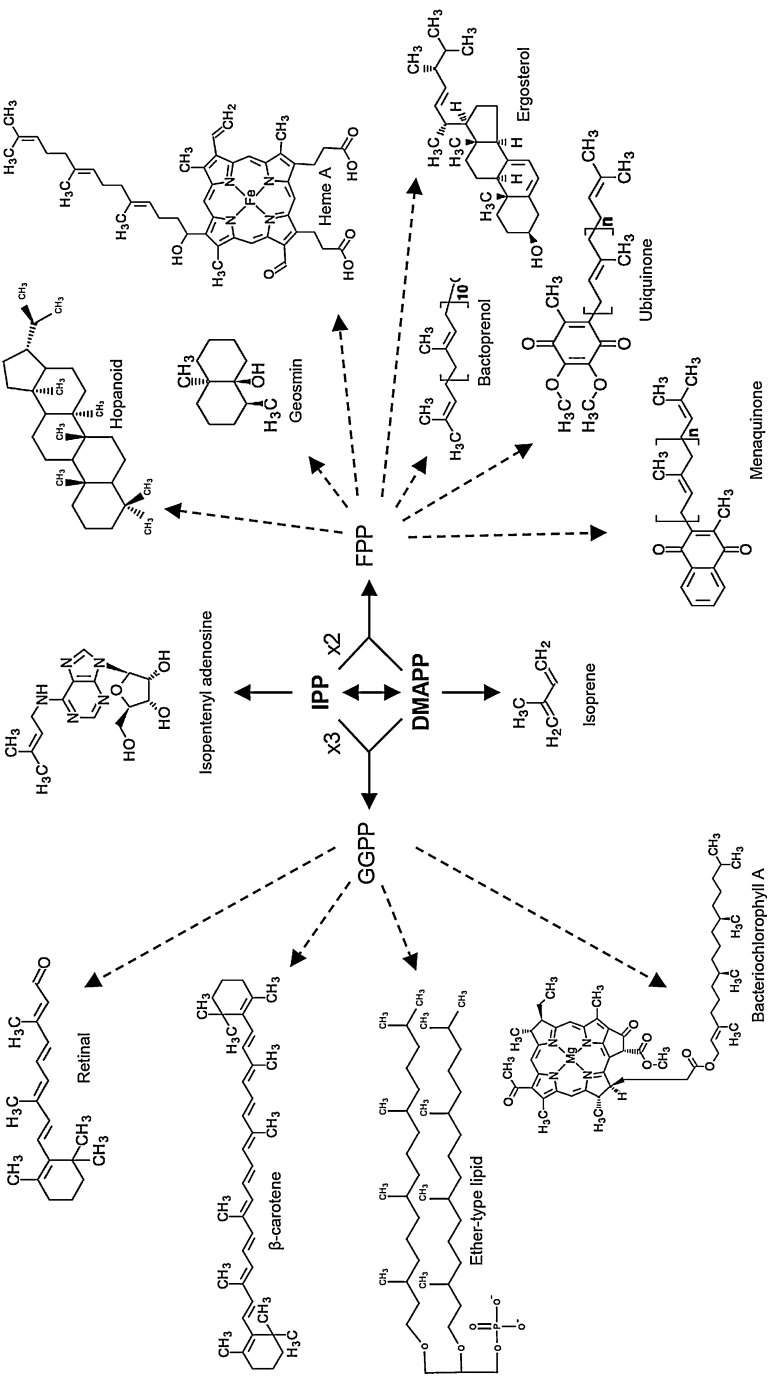


Fig. 3 Representative isoprenoids found in microorganisms encompassing the three kingdoms of life. Examples of isoprenoids derived from IPP, DMAPP, FPP, and GGPP are shown. *Solid arrows* indicate single-step reactions; *dashed lines* indicate multiple steps

In contrast, the distribution among microalgae is quite diverse with some producing mainly cholesterol, some others producing ergosterol and some of them producing a highly complex profile of sterols (Volkman 2003).

Most prokaryotes do not produce any kind of sterols. Membrane fluidity in these organisms is regulated through another set of isoprenoid-based molecules, hopanoids, which is found in about 50% of the species investigated. Hopanoids are pentacyclic triterpenoids produced from a basic skeleton of squalene showing a structure that resembles the one described for sterols (Saenz et al. 2015). Poulter and co-workers recently described an alternative path to squalene in bacteria (Pan et al. 2015).

Isoprenoids are also involved in the membrane organization by taking part on the formation of membrane microdomains as a result of the segregation of different lipids due to their physicochemical properties. Lipid rafts are better known in eukaryotes as functional membrane microdomains (FMM) enriched in particular lipids such as cholesterol (or ergosterol in yeast), which harbor and compartmentalize proteins involved in several cellular functions like signal transduction. They also contain specific proteins like flotillins, membrane-bound chaperones suggested to be responsible of the recruitment of functional proteins into the rafts. The existence of lipid rafts has been associated to the higher cellular complexity of eukaryotes. However, the existence of FMM in bacteria was recently reported. Most bacteria do not have two of the major components described for lipid rafts in eukaryotes, cholesterol, and sphingomyelin, but flotillin-like proteins have been identified both in bacteria and archaea. The existence of lipid rafts in bacteria still remains controversial, and the molecular structure of the lipids involved has not been elucidated. However, hopanoids and carotenoids have been suggested as the most plausible candidates for the assembly of FMM, whereas cardiolipin, a diphosphatidylglycerol lipid could play the role described for sphingomyelin in eukaryotes (Bramkamp and Lopez 2015).

3.3 Electron Transport

Isoprenoid quinones are membrane-bound compounds found in all living organisms with the only known exceptions of some obligatory fermentative bacteria and some methanogenic archaea. They are composed of a polar head, which interacts with proteins in the membrane, and an isoprenoid hydrophobic side chain that confers a lipid-soluble character and allows anchoring in the lipid layer. Isoprenoid quinones function mainly as electron and proton carriers in electron transfer chains (photosynthesis and respiration) but have also an important function as antioxidants. The reduced forms of isoprenoid quinones have antioxidant properties that protect membranes from lipid peroxidation and the effects of reactive oxygen species on membrane components. The great majority of naturally occurring isoprenoid quinones belong to the naphthoquinone family (polar head is a naphthoquinone ring) or the evolutionary younger benzoquinones (polar head is a benzoquinone group)

(Nitschke et al. 1995). In both families of compounds, the isoprenoid side chains are of a different length depending on the organism; they are usually 6–10 isoprene (C5) units although chains ranging from 1 to 14 units have been identified in different species (Fig. 3). Most often the side chain is fully unsaturated, but in some organisms it is partially or fully saturated. The reasons for the length and degree of saturation of the side chain are not fully understood, but these characteristics are often dependent on the growth temperature of specific species. It has been shown that quinones with different side-chain length can complement the absence of the endogenous ubiquinone in *E. coli* (Choi et al. 2009), suggesting that length is not critical for basic biological function.

Menaquinones (members of the naphthoquinone family) function in respiratory and photosynthetic electron transport chains of bacteria. Among benzoquinones, the most widespread and important are ubiquinones and plastoquinones. Ubiquinones participate in respiratory chains of eukaryotic mitochondria and some bacteria, whereas plastoquinones are components of photosynthetic electron transport chains of cyanobacteria. Menaquinones have low midpoint redox potential, and their appearance in early phase of evolution has been correlated with the reducing character of the ancient atmosphere. Reduced menaquinones become rapidly oxidized in the presence of oxygen; therefore these compounds cannot efficiently operate in an atmosphere containing oxygen. The evolutionary transition from menaquinones to ubiquinones (showing higher midpoint redox potential) occurred independently in some groups of prokaryotes as an adaptation to aerobic metabolism (Nitschke et al. 1995). Facultative anaerobes like *E. coli* produce ubiquinone when growing under aerobic condition but mainly produce menaquinone under anaerobic conditions.

An isoprenoid tail (hydroxyethyl farnesyl) derived form a FPP molecule is found both in HemeA and HemeO (Mogi et al. 1994), which are key components of cytochromes. Cytochromes are membrane-bound proteins containing a heme group that facilitate the movement of electrons. Heme groups are heterocyclic porphyrin rings made up of four pyrrolic groups joined together by methine bridges with a metal ion (usually iron) in the central position, bound to the four pyrrole nitrogen atoms (Fig. 3). There are different heme groups (A, B, C, D, O) showing different substitutions along the porphyrin ring. Cytochrome-based respiratory chains are present in eukaryotes, archaea and bacteria (O'Brian and Thony-Meyer 2002).

3.4 Conversion of Sunlight into Chemical Energy

Some microorganisms as well as higher plants are able to capture solar photons and convert the light energy into storage chemical energy. Two unrelated mechanisms have evolved to sense and harvest light for phototrophic growth, and both of them require isoprenoid-derived compounds. The first one is commonly known as photosynthesis and is dependent on photochemical reaction centers, and the second one is

based on membrane proteins binding retinal pigments and forming a light-driven proton pump.

In microbes there are two different types of photosynthesis, both of which use isoprenoid phytyl side chains for the light-harvesting pigments. Oxygenic photosynthesis uses chlorophylls for light harvesting and is found in algae, cyanobacteria, and some bacteria. Anoxygenic photosynthesis uses bacteriochlorophylls and is found in purple bacteria, green sulfur, and non-sulfur bacteria as well as in heliobacteria (Bryant and Frigaard 2006). The structure of these two pigments is similar, with a distinctive tetrapyrrole ring and a C20 phytyl tail derived from a molecule of GGPP that anchors the molecule to the photosynthetic membrane (Fig. 3). Major differences between these two families occur in the substitutions around the ring and substitutions on the phytyl tail, which modulates the specific wavelength of light that each pigment absorbs. Although phytyl is the most common tail in bacterial chlorophylls, other isoprenoids (farnesyl, geranylgeranyl, or 2,6 phytadienyl) have also been identified (Chew and Bryant 2007). These pigments play a central role in light harvesting and photochemistry. All chlorophototrophs also produce essential carotenoid pigments, which act primarily as photoprotective agents (protecting against reactive oxygen species that are generated during photosynthesis) but are also involved as light-harvesting helpers (Frank 1999). Carotenoids are a diverse subfamily of isoprenoids derived from the C20 backbone GGPP. Some non-photosynthetic microorganisms also synthesize carotenoids; in this case, they can be considered secondary metabolites since they are not essential but protect the organism from oxidative damage.

The second mechanism for light harvesting was originally identified in marine haloarchaea (Oesterhelt and Stoeckenius 1971). This mechanism employs membrane proteins called proteorhodopsins and bacteriorhodopsins, which bind isoprenoid-derived retinal; the retinal acts as a chromophore to harvest light, and the proteins function as proton pumps ultimately producing ATP. In addition to their originally described role, members of the diverse family of retinal-binding proteins have roles as transmembrane chloride pumps and photosensors (Fuhrman et al. 2008). Unlike chlorophylls, retinal – a relatively simple pigment – captures light without accessory pigments. During the last decades, this mechanism has also been found to be widespread among marine bacteria showing different absorption spectra (allowing them to live at different water depths where the wavelengths of light available are filtered) (Beja et al. 2000).

Retinal is best known as the vitamin A compound that forms the basis of animal vision. It is an isoprenoid-derived C20 compound (Fig. 3) produced in archaea and most bacteria by oxidative cleavage of the C40 carotenoid β-carotene (Fig. 3) at the 15,15′ bond to produce two identical molecules of retinal. In cyanobacteria retinal is also produced by the cleavage of β-carotene, but at least two carotenoid oxigenases are known to also produce retinal using apo-carotenoids with all-*trans* linear end groups as a substrate (Ruch et al. 2005; Scherzinger et al. 2006). The origin of these apo-carotenoids remains unclear, but it is likely that they are derived by asymmetrical oxidative cleavage at one end of a C40 carotenoid.

3.5 Protein Translation

Most of the bacterial and eukaryotic tRNAs that read codons starting with uracil are modified at the adenosine at position 37 (corresponding to the 3′ adjacent of the anticodon) by isopentenylation. The isopentenyl group of an IPP molecule is transferred to the amino group on position C_6 of the nucleoside (Fig. 3) to produce N^6-Δ^2-isopentenyl adenosine (i^6A). Isopentenyl adenosine (as well as other non-isoprenoid modifications at different positions) increases the efficiency of translation of the modified tRNA and reduces the sensitivity to the codon context. These chemical modifications in the anticodon loop of tRNAs alter the conformation and reduce flexibility, providing proper geometry that acts as a proofreading mechanism and allows optimal rate of protein synthesis (Persson et al. 1994).

A recent study has described the identification of geranylated RNA in some bacteria including *E. coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Salmonella enterica* (Dumelin et al. 2012); these observations represent the first example of oligoisoprenylated nucleic acids. The C10 isoprenoid geranyl binds to the sulfur atom at the 2-thiouridine position in around 7% of the first position of anticodons for tRNAs UUC, UUU, and UUG. RNA geranylation has been suggested as an alternative to selenation at low selenium levels. Geranylation of tRNA affects codon bias and frame shifting of the open reading frame during translation.

3.6 Protein Prenylation

Prenylation of proteins is a posttranslational modification that is mainly involved in facilitating protein association to the membrane and directing subcellular localization. It has been best studied in eukaryotes (especially mammalian and plant cells) but was first identified in the basidiomycete yeast *Rhodosporidium toruloides* and, a few years later, in the ascomycete yeast *Saccharomyces cerevisiae* in mating factor peptides. Prenylated peptides contain a C-terminal cysteine thioether linked to (most commonly) a molecule of farnesyl or hydroxyfarnesyl (derived from FPP) that is essential for its biological function (Omer and Gibbs 1994). This addition is catalyzed in yeast by the enzyme farnesyl protein transferase (FTP) (Zhang and Casey 1996). Less commonly, GGPP is involved, and two types of GGPP protein transferases have been described (Zhang and Casey 1996). These activities have also been identified in other eukaryotic microorganisms such as *Plasmodium falciparum*. Protein prenylation has so far been demonstrated only in eukaryotes, but recent work has also predicted that pathogenic bacteria can access eukaryotic machinery to prenylate their own proteins. These bacteria have evolved mechanisms to direct their effector proteins to the desired subcellular localization in the eukaryotic host cell. One of these mechanisms involves secretion of proteins containing prenylation motifs that are recognized by the prenylation machinery of the host. In-host prenylation of bacterial proteins has been identified using both farnesyl (by addition of FPP) and geranylgeranyl (by addition to GGPP) (Ivanov et al. 2010). In silico analyses show that most bacterial species contain effectors with the conserved

prenylation motif. However, to date no in-cell protein prenylation has been described in prokaryotes.

3.7 Secondary Metabolites

Secondary metabolites represent a diverse group of compounds playing different biological roles on the interaction with the environment and providing better fitness to the specific ecological niche. In some cases, the role of specific compounds is still not clear or still under debate. Higher plants and macroscopic fungi, rather than microbes, produce most of the terpenoid secondary metabolites known to date. The amount of these secondary metabolites found in microorganisms including bacteria, archaea, unicellular fungi (yeast), and microalgae is more restricted or, at least, less studied. Here, we will outline a few well-known examples, but these are by no means exhaustive; secondary metabolism is very complex.

Actinomycetes and specially *Streptomyces* are well known for their relatively large genome size (8–10 Mb), which correlates with a complex secondary metabolism, usually occurring at the late growth phase. Most of the secondary metabolites produced are antibiotics, and some of them are isoprenoid-derived molecules (Dairi 2005). Most *Streptomyces* produce their isoprenoids through the MEP pathway, but interestingly, some of them also possess the MVA pathway as a gene cluster on their genome. In this case, the MVA pathway genes are expressed only at very low levels during the growth phase, suggesting that the MEP pathway supports biosynthesis of essential isoprenoids. During the late phase of growth, the MVA genes are upregulated, and around 60% of cellular IPP is produced via the MVA pathway for biosynthesis of secondary metabolites such as antibiotics. Additionally, most of the genes involved in further modifications of the isoprenoid backbone are located in the flanking regions of the MVA cluster genes (Dairi 2005). However, *Streptomyces* species carrying only the MEP pathway also produce antibiotics. Actinomycetes are also well known for the production of other isoprenoid-derived secondary metabolites including some volatile terpenes, the aforementioned FPP-derived geosmin (Jiang et al. 2007) being perhaps the original and best-known example. Geosmin is also produced by other microorganisms such cyanobacteria, myxobacteria, and fungi.

All photosynthetic microorganisms produce carotenoids, which as noted play an essential role in light harvesting and photoprotection of the photosynthetic machinery (Takaichi 2011). Some produce sufficient amounts that they can provide a natural source of industrially relevant carotenoids, e.g., the chlorophytes *Haematococcus* for astaxanthin and *Dunaliella* for β-carotene (Fig. 3). However, a variety of non-photosynthetic microbes, including some yeast, a variety of bacteria, and a few archaea (mainly Halobacteriaceae (Calegari-Santos et al. 2016)), also produce carotenoids as secondary metabolites. Their main physiological function is thought to be as antioxidants, but a role has also been described as virulence factors in some eubacteria (Liu et al. 2005). Most of the prokaryotic carotenoids known to date are found in land bacteria, but marine bacteria represent a partially unexplored source of new carotenoid

structures still to be identified. Recently, a compilation of carotenoid structures and biosynthetic pathways from prokaryotes with more than 300 unique structures has been publicly released (http://bioinfo.imtech.res.in/servers/procardb/) (Nupur et al. 2016). Carotenoids are mainly C40 compounds (Fig. 3) derived from the head-to-head condensation of two molecules of GGPP (Fig. 2) that is then further modified by desaturation, cyclization, or oxidation to produce the vast number of structures identified. However, C30 structures derived from FPP (Furubayashi et al. 2014), and C50 structures produced by the addition of two molecules of IPP onto the C40 lycopene, have also been reported (Yang et al. 2015).

Another intriguing metabolite produced by many organisms including animals, plants, and bacteria is isoprene. Isoprene (2-methyl-1,3-butadiene) is the smallest C5 representative of the isoprenoid family but in terms of total production is the most important isoprenoid with a total annual production estimated at 600 Tg carbon equivalents, mostly from plants and microalgae (Guenther et al. 2006). This massive amount of isoprene affects atmospheric chemistry and contributes to local air pollution, especially in the presence of anthropogenic nitrous oxides. The physiological role of isoprene is still under debate but has been suggested to act as a protectant in response to abiotic stress in plants (Vickers et al. 2009). Isoprene is produced from DMAPP in a reaction catalyzed by the enzyme isoprene synthase (IPS) (Fig. 3). In addition to microalgae, many other microorganisms produce isoprene. Both gram-negative and gram-positive bacteria have been identified as isoprene emitters, with *Bacillus* showing the highest emission rates. However, for a long time attempts to identify an isoprene synthase responsible for the conversion of DMAPP in prokaryotes failed. Surprisingly, recent *in vitro* studies have demonstrated the ability of HDR/IspH, the last step of the MEP pathway catalyzing the simultaneous production of IPP and DMAPP from HMBPP, to produce isoprene directly from HMBPP in *Bacillus* (Ge et al. 2016).

4 Research Needs

Biosynthesis of isoprenoids is highly complex, not only in the decoration phase but also in the core IPP/DMAPP production pathways (MVA and MEP). A decade ago, we had a fairly clear idea of how isoprenoids were synthesized through the canonical MVA and MEP isoprenoid pathways – even though the MEP pathway was only fully elucidated 15 years ago (Rodriguez-Concepcion and Boronat 2002). In recent years, we have discovered a number of variations on these canonical pathways; indeed, there is an astonishing genetic and metabolic plasticity among microbes. In the future, we can probably expect that further biochemical complexity will be discovered. Moreover, decoration and modification biochemistry downstream of prenyl phosphate metabolism is still poorly understood for some classes of compounds, for example, the recently discovered strigolactone group, which was identified as a group of plant hormones only in 2008 (Umehara et al. 2008). We still have much to learn about this group and many other isoprenoids. In particular, while plant secondary isoprenoid metabolism is relatively well characterized, relatively little is

known about prokaryotic secondary isoprenoid metabolism. Recent analyses have revealed the existence of a widespread variety of putative terpene synthases in bacteria (Yamada et al. 2015) that could soon lead to the identification of new catalytic activities and new isoprenoid structures. A systematic approach to validate all these candidates is required.

The high plasticity in the pathways producing IPP/DMAPP and the patchy distribution of the pathways across the three kingdoms of life have relevant evolutionary repercussions regarding both the origin of the pathways and the relationship between the two. The case of the coexistence of both pathways in microalgae is a particularly interesting topic relevant to these issues. Although some retain the two pathways (MEP in the chloroplast and MVA in the cytosol, as described in higher plants), some others, like the model green alga organism *Chlamydomonas reinhardtii,* produce their isoprenoids solely from the chloroplast-localized MEP pathway (Lohr et al. 2012). This raises the question of whether the ancestor of green algae ever used the MVA pathway or if it was lost during evolution. Regardless, the absence of the MVA pathway indicates that mechanisms exist to transport MEP pathway-derived isoprenoids from the chloroplast for the biosynthesis of cytosolic isoprenoids. To date, very limited information about the export/import of MEP pathway prenyl phosphate intermediates is available. A better understanding of these processes could help us to understand isoprenoid biosynthesis in the cytosol of green algae.

Many isoprenoids are of industrial interest, with applications including biofuels, chemical feedstocks, industrial solvents, agricultural chemicals, essential oils, fragrances, nutraceuticals, pharmaceuticals, etc. However, availability from natural sources is often limited and insufficient for industrial applications. The structural complexity of many of these compounds makes chemical synthesis from petrochemical precursors complex, expensive, and often impossible. In this context biological platforms represent a very attractive environmentally friendly approach. However, while we understand the basic metabolic pathways allowing us to engineer production of many of these interesting targets, much less is known about the regulatory constraints that currently limit isoprenoid production titers/rates/yields at the industrial level (Vickers et al. 2012). For example, in silico simulations predict a 10% greater theoretical maximum yield for the MEP pathway compared with the MVA pathway (Gruchattka et al. 2013). However, the regulation of the MEP pathway has revealed to be highly complex (Ajikumar et al. 2010), and yields from current efforts to boost the pathway flux to optimize isoprenoid production are still far from the predictions. Most successful approaches for industrial isoprenoid production have been achieved using the MVA pathway (Paddon and Keasling 2014) or generating new synthetic circuits (Meadows et al. 2016). A better understanding of the regulatory controls on the MEP pathway might allow us to achieve the predicted increase in yields through the MEP pathway.

Acknowledgments Research at the authors' laboratories was supported by a Marie Curie International outgoing Fellowship within the 7th European Community Framework Programme to JPG, an ERA-IB-2 project funded by the Spanish MINECO (PCIN-2015-103) to MRC, and a Queensland Government Accelerate Fellowship to CEV.

References

Abe F, Hiraki T (2009) Mechanistic role of ergosterol in membrane rigidity and cycloheximide resistance in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1788:743–752

Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 330:70–74

Azami Y, Hattori A, Nishimura H, Kawaide H, Yoshimura T, Hemmi H (2014) (R)-mevalonate 3-phosphate is an intermediate of the mevalonate pathway in *Thermoplasma acidophilum*. J Biol Chem 289:15957–15967

Begley M, Bron PA, Heuston S, Casey PG, Englert N, Wiesner J, Jomaa H, Gahan CG, Hill C (2008) Analysis of the isoprenoid biosynthesis pathways in *Listeria monocytogenes* reveals a role for the alternative 2-C-methyl-D-erythritol 4-phosphate pathway in murine infection. Infect Immun 76:5392–5401

Beja O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN, DeLong EF (2000) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902–1906

Berthelot K, Estevez Y, Deffieux A, Peruch F (2012) Isopentenyl diphosphate isomerase: a checkpoint to isoprenoid biosynthesis. Biochimie 94:1621–1634

Bohlmann J, Meyer-Gauen G, Croteau R (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proc Natl Acad Sci U S A 95:4126–4133

Boucher Y, Huber H, L'Haridon S, Stetter KO, Doolittle WF (2001) Bacterial origin for the isoprenoid biosynthesis enzyme HMG-CoA reductase of the archaeal orders Thermoplasmatales and Archaeoglobales. Mol Biol Evol 18:1378–1388

Bouvier F, Rahier A, Camara B (2005) Biogenesis, molecular regulation and function of plant isoprenoids. Prog Lipid Res 44:357–429

Bramkamp M, Lopez D (2015) Exploring the existence of lipid rafts in bacteria. Microbiol Mol Biol Rev 79:81–100

Brown AC, Parish T (2008) Dxr is essential in *Mycobacterium tuberculosis* and fosmidomycin resistance is due to a lack of uptake. BMC Microbiol 8:78

Bryant DA, Frigaard NU (2006) Prokaryotic photosynthesis and phototrophy illuminated. Trends Microbiol 14:488–496

Calegari-Santos R, Diogo RA, Fontana JD, Bonfim TM (2016) Carotenoid production by halophilic archaea under different culture conditions. Curr Microbiol 72:641–651

Carretero-Paulet L, Lipska A, Perez-Gil J, Sangari FJ, Albert VA, Rodriguez-Concepcion M (2013) Evolutionary diversification and characterization of the eubacterial gene family encoding DXR type II, an alternative isoprenoid biosynthetic enzyme. BMC Evol Biol 13:180

Chappe B, Michaelis W, Albrecht P, Ourisson G (1979) Fossil evidence for a novel series of archaebacterial lipids. Naturwissenschaften 66:522–523

Chen F, Tholl D, Bohlmann J, Pichersky E (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant J 66:212–229

Chew AG, Bryant DA (2007) Chlorophyll biosynthesis in bacteria: the origins of structural and functional diversity. Annu Rev Microbiol 61:113–129

Choi JH, Ryu YW, Park YC, Seo JH (2009) Synergistic effects of chromosomal ispB deletion and dxs overexpression on coenzyme Q(10) production in recombinant *Escherichia coli* expressing *Agrobacterium tumefaciens* dps gene. J Biotechnol 144:64–69

Dairi T (2005) Studies on biosynthetic genes and enzymes of isoprenoids produced by actinomycetes. J Antibiot (Tokyo) 58:227–243

Dellas N, Thomas ST, Manning G, Noel JP (2013) Discovery of a metabolic alternative to the classical mevalonate pathway. Elife 2:e00672

Dumelin CE, Chen Y, Leconte AM, Chen YG, Liu DR (2012) Discovery and biological characterization of geranylated RNA in bacteria. Nat Chem Biol 8:913–919

Dupont S, Lemetais G, Ferreira T, Cayot P, Gervais P, Beney L (2012) Ergosterol biosynthesis: a fungal pathway for life on land? Evolution 66:2961–2968

Ershov YV, Gantt RR, Cunningham FX Jr, Gantt E (2002) Isoprenoid biosynthesis in *Synechocystis* sp. strain PCC6803 is stimulated by compounds of the pentose phosphate cycle but not by pyruvate or deoxyxylulose-5-phosphate. J Bacteriol 184:5045–5051

Fernandes JF, Lell B, Agnandji ST, Obiang RM, Bassat Q, Kremsner PG, Mordmuller B, Grobusch MP (2015) Fosmidomycin as an antimalarial drug: a meta-analysis of clinical trials. Future Microbiol 10:1375–1390

Frank HA (1999) Incorporation of carotenoids into reaction Center and light-harvesting pigment-protein complexes. In: Frank HA, Young AJ, Britton G, Cogdell RJ (eds) The photochemistry of carotenoids. Springer, Dordrecht, pp 235–244

Frank A, Groll M (2017) The methylerythritol phosphate pathway to isoprenoids. Chem Rev 117:5675–5703

Friesen JA, Rodwell VW (2004) The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biol 5:248

Fuhrman JA, Schwalbach MS, Stingl U (2008) Proteorhodopsins: an array of physiological roles? Nat Rev Microbiol 6:488–494

Furubayashi M, Saito K, Umeno D (2014) Evolutionary analysis of the functional plasticity of *Staphylococcus aureus* C30 carotenoid synthase. J Biosci Bioeng 117:431–436

Gao Y, Honzatko RB, Peters RJ (2012) Terpenoid synthase structures: a so far incomplete view of complex catalysis. Nat Prod Rep 29:1153–1175

Ge D, Xue Y, Ma Y (2016) Two unexpected promiscuous activities of the iron-sulfur protein IspH in production of isoprene and isoamylene. Microb Cell Factories 15:79

Gerber NN, Lechevalier HA (1965) Geosmin, an earthly-smelling substance isolated from actinomycetes. Appl Microbiol 13:935–938

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nat Chem Biol 3:408–414

Grochowski LL, Xu H, White RH (2006) *Methanocaldococcus jannaschii* uses a modified mevalonate pathway for biosynthesis of isopentenyl diphosphate. J Bacteriol 188:3192–3198

Gruchattka E, Hadicke O, Klamt S, Schutz V, Kayser O (2013) In silico profiling of *Escherichia coli* and *Saccharomyces cerevisiae* as terpenoid factories. Microb Cell Factories 12:84

Guenther A, Karl T, Harley P, Wiedinmyer C, Palmer PI, Geron C (2006) Estimates of global terrestrial isoprene emissions using MEGAN (Model of Emissions of Gases and Aerosols from Nature). Atmos Chem Phys 6:3181–3210

Hamano Y, Dairi T, Yamamoto M, Kuzuyama T, Itoh N, Seto H (2002) Growth-phase dependent expression of the mevalonate pathway in a terpenoid antibiotic-producing *Streptomyces* strain. Biosci Biotechnol Biochem 66:808–819

Heuston S, Begley M, Davey MS, Eberl M, Casey PG, Hill C, Gahan CG (2012) HmgR, a key enzyme in the mevalonate pathway for isoprenoid biosynthesis, is essential for growth of *Listeria monocytogenes* EGDe. Microbiology 158:1684–1693

Ivanov SS, Charron G, Hang HC, Roy CR (2010) Lipidation by the host prenyltransferase machinery facilitates membrane localization of *Legionella pneumophila* effector proteins. J Biol Chem 285:34686–34698

Jain S, Caforio A, Driessen AJ (2014) Biosynthesis of archaeal membrane ether lipids. Front Microbiol 5:641

Jiang J, He X, Cane DE (2007) Biosynthesis of the earthy odorant geosmin by a bifunctional *Streptomyces coelicolor* enzyme. Nat Chem Biol 3:711–715

Kaneda K, Kuzuyama T, Takagi M, Hayakawa Y, Seto H (2001) An unusual isopentenyl diphosphate isomerase found in the mevalonate pathway gene cluster from *Streptomyces* sp. strain CL190. Proc Natl Acad Sci U S A 98:932–937

Komatsu M, Tsuda M, Omura S, Oikawa H, Ikeda H (2008) Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol. Proc Natl Acad Sci U S A 105:7422–7427

Lange BM, Rujan T, Martin W, Croteau R (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci U S A 97:13172–13177

Langworthy TA, Pond JL (1986) Archaebacterial ether lipids and chemotaxonomy. Syst Appl Microbiol 7:253–257

Lawrence JG, Ochman H (1998) Molecular archaeology of the *Escherichia coli* genome. Proc Natl Acad Sci U S A 95:9413–9417

Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, Fierer J, Nizet V (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J Exp Med 202:209–215

Lohr M, Schwender J, Polle JE (2012) Isoprenoid biosynthesis in eukaryotic phototrophs: a spotlight on algae. Plant Sci 185-186:9–22

Lombard J, Moreira D (2011) Origins and early evolution of the mevalonate pathway of isoprenoid biosynthesis in the three domains of life. Mol Biol Evol 28:87–99

Lombard J, Lopez-Garcia P, Moreira D (2012) The early evolution of lipid membranes and the three domains of life. Nat Rev Microbiol 10:507–515

Lynen F (1967) Biosynthetic pathways from acetate to natural products. Pure Appl Chem 14:137–167

Meadows AL, Hawkins KM, Tsegaye Y, Antipov E, Kim Y, Raetz L, Dahl RH, Tai A, Mahatdejkul-Meadows T, Xu L, Zhao L, Dasika MS, Murarka A, Lenihan J, Eng D, Leng JS, Liu CL, Wenger JW, Jiang H, Chao L, Westfall P, Lai J, Ganesan S, Jackson P, Mans R, Platt D, Reeves CD, Saija PR, Wichmann G, Holmes VF, Benjamin K, Hill PW, Gardner TS, Tsong AE (2016) Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature 537:694–697

Miziorko HM (2011) Enzymes of the mevalonate pathway of isoprenoid biosynthesis. Arch Biochem Biophys 505:131–143

Mogi T, Saiki K, Anraku Y (1994) Biosynthesis and functional role of haem O and haem A. Mol Microbiol 14:391–398

Nitschke W, Kramer D, Riedel A, Liebl U (1995) From naphtho-to benzoquinones-(r) evolutionary reorganizations of electron transfer chains. Photosynthesis: From Light to Biosphere 1:945–950

Nupur LN, Vats A, Dhanda SK, Raghava GP, Pinnaka AK, Kumar A (2016) ProCarDB: a database of bacterial carotenoids. BMC Microbiol 16:96

O'Brian MR, Thony-Meyer L (2002) Biochemistry, regulation and genomics of haem biosynthesis in prokaryotes. Adv Microb Physiol 46:257–318

Oesterhelt D, Stoeckenius W (1971) Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. Nat New Biol 233:149–152

Oldfield E, Lin FY (2012) Terpene biosynthesis: modularity rules. Angew Chem Int Ed Engl 51:1124–1137

Omer CA, Gibbs JB (1994) Protein prenylation in eukaryotic microorganisms: genetics, biology and biochemistry. Mol Microbiol 11:219–225

Paddon CJ, Keasling JD (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nat Rev Microbiol 12:355–367

Pan JJ, Solbiati JO, Ramamoorthy G, Hillerich BS, Seidel RD, Cronan JE, Almo SC, Poulter CD (2015) Biosynthesis of Squalene from Farnesyl Diphosphate in bacteria: three steps catalyzed by three enzymes. ACS Cent Sci 1:77–82

Perez-Gil J, Rodriguez-Concepcion M (2013) Metabolic plasticity for isoprenoid biosynthesis in bacteria. Biochem J 452:19–25

Perez-Gil J, Calisto BM, Behrendt C, Kurz T, Fita I, Rodriguez-Concepcion M (2012a) Crystal structure of *Brucella abortus* deoxyxylulose-5-phosphate reductoisomerase-like (DRL) enzyme involved in isoprenoid biosynthesis. J Biol Chem 287:15803–15809

Perez-Gil J, Uros EM, Sauret-Gueto S, Lois LM, Kirby J, Nishimoto M, Baidoo EE, Keasling JD, Boronat A, Rodriguez-Concepcion M (2012b) Mutations in *Escherichia coli aceE* and *ribB* genes allow survival of strains defective in the first step of the isoprenoid biosynthesis pathway. PLoS One 7:e43775

Persson BC, Esberg B, Olafsson O, Bjork GR (1994) Synthesis and function of isopentenyl adenosine derivatives in tRNA. Biochimie 76:1152–1160

Phillips MA, Leon P, Boronat A, Rodriguez-Concepcion M (2008) The plastidial MEP pathway: unified nomenclature and resources. Trends Plant Sci 13:619–623

Rodriguez-Concepcion M (2004) The MEP pathway: a new target for the development of herbicides, antibiotics and antimalarial drugs. Curr Pharm Des 10:2391–2400

Rodriguez-Concepcion M, Boronat A (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol 130:1079–1089

Rohmer M, Knani M, Simonin P, Sutter B, Sahm H (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem J 295 (Pt 2):517–524

Ruch S, Beyer P, Ernst H, Al-Babili S (2005) Retinal biosynthesis in Eubacteria: in vitro characterization of a novel carotenoid oxygenase from *Synechocystis* sp. PCC 6803. Mol Microbiol 55:1015–1024

Sacchettini JC, Poulter CD (1997) Creating isoprenoid diversity. Science 277:1788–1789

Saenz JP, Grosser D, Bradley AS, Lagny TJ, Lavrynenko O, Broda M, Simons K (2015) Hopanoids as functional analogues of cholesterol in bacterial membranes. Proc Natl Acad Sci U S A 112:11971–11976

Sangari FJ, Perez-Gil J, Carretero-Paulet L, Garcia-Lobo JM, Rodriguez-Concepcion M (2010) A new family of enzymes catalyzing the first committed step of the methylerythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis in bacteria. Proc Natl Acad Sci U S A 107:14081–14086

Scheffers DJ, Pinho MG (2005) Bacterial cell wall synthesis: new insights from localization studies. Microbiol Mol Biol Rev 69:585–607

Scherzinger D, Ruch S, Kloer DP, Wilde A, Al-Babili S (2006) Retinal is formed from apo-carotenoids in *Nostoc* sp. PCC7120: in vitro characterization of an apo-carotenoid oxygenase. Biochem J 398:361–369

Shrivastava S, Chattopadhyay A (2007) Influence of cholesterol and ergosterol on membrane dynamics using different fluorescent reporter probes. Biochem Biophys Res Commun 356:705–710

Sojo V, Pomiankowski A, Lane N (2014) A bioenergetic basis for membrane divergence in archaea and bacteria. PLoS Biol 12:e1001926

Takaichi S (2011) Carotenoids in algae: distributions, biosyntheses and functions. Mar Drugs 9:1101–1118

Touz ET, Mengin-Lecreulx D (2008) Undecaprenyl phosphate synthesis. EcoSal Plus 2013; https://doi.org/10.1128/ecosalplus.4.7.1.7

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, Kyozuka J, Yamaguchi S (2008) Inhibition of shoot branching by new terpenoid plant hormones. Nature 455:195–200

Vannice JC, Skaff DA, Keightley A, Addo JK, Wyckoff GJ, Miziorko HM (2014) Identification in *Haloferax volcanii* of phosphomevalonate decarboxylase and isopentenyl phosphate kinase as catalysts of the terminal enzyme reactions in an archaeal alternate mevalonate pathway. J Bacteriol 196:1055–1063

Vickers CE, Gershenzon J, Lerdau MT, Loreto F (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. Nat Chem Biol 5:283–291

Vickers CE, Klein-Marcuschamer D, Kromer JO (2012) Examining the feasibility of bulk commodity production in *Escherichia coli*. Biotechnol Lett 34:585–596

Vickers CE, Behrendorff JBYH, Bongers M, Brennan TCR, Bruschi M, Nielsen LK (2015) Production of industrially relevant Isoprenoid compounds in engineered microbes. In: Kamm B (ed) Microorganisms in biorefineries. Springer, Berlin/Heidelberg, pp 303–334

Vinokur JM, Cummins MC, Korman TP, Bowie JU (2016) An adaptation to life in acid through a novel mevalonate pathway. Sci Rep 6:39737

Volkman JK (2003) Sterols in microorganisms. Appl Microbiol Biotechnol 60:495–506
Warlick BP, Evans BS, Erb TJ, Ramagopal UA, Sriram J, Imker HJ, Sauder JM, Bonanno JB, Burley SK, Tabita FR, Almo SC, Sweedler JS, Gerlt JA (2012) 1-methylthio-D-xylulose 5-phosphate methylsulfurylase: a novel route to 1-deoxy-D-xylulose 5-phosphate in *Rhodospirillum rubrum*. Biochemistry 51:8324–8326
Weete JD, Abril M, Blackwell M (2010) Phylogenetic distribution of fungal sterols. PLoS One 5: e10899
Wilkins K, Schöller C (2009) Volatile organic metabolites from selected *Streptomyces* strains. Actinomycetologica 23:27–33
Yamada Y, Kuzuyama T, Komatsu M, Shin-Ya K, Omura S, Cane DE, Ikeda H (2015) Terpene synthases are widely distributed in bacteria. Proc Natl Acad Sci U S A 112:857–862
Yang Y, Yatsunami R, Ando A, Miyoko N, Fukui T, Takaichi S, Nakamura S (2015) Complete biosynthetic pathway of the C50 carotenoid bacterioruberin from lycopene in the extremely halophilic archaeon *Haloarcula japonica*. J Bacteriol 197:1614–1623
Zhang FL, Casey PJ (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem 65:241–269

Formation of Bacterial Glycerol-Based Membrane Lipids: Pathways, Enzymes, and Reactions

5

Otto Geiger, Christian Sohlenkamp, and Isabel M. López-Lara

Contents

1 Introduction 88
2 Membrane-Forming Lipids in *Escherichia coli* 89
3 Formation of Glycerophospholipids in Bacteria 90
3.1 Formation of the Central Activated Intermediate CDP-Diacylglycerol 90
3.2 Diversification of Polar Head Groups in Bacterial Glycerophospholipids 91
4 Formation of Phosphorus-Free Glycerol-Based Membrane Lipids in Bacteria 97
4.1 Diacylglycerol 98
4.2 Glycolipids 99
4.3 Sulfolipids 99
4.4 Betaine Lipids 100
5 Modifications of Acyl Residues in Pre-existing Glycerol-Based Membrane Lipids of Bacteria 100
5.1 Modifications of Pre-Existing Glycerol-Based Lipids in the Alkyl Region of Fatty Acyl Residues 101
5.2 Biosynthesis of Plasmalogens 101
6 Conclusions and Research Needs 103
References 104

Abstract

The model bacterium *Escherichia coli* contains the phospholipids phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine as major membrane lipids,

O. Geiger (✉) · C. Sohlenkamp
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México
e-mail: otto@ccg.unam.mx; chsohlen@ccg.unam.mx

I. M. López-Lara
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, México
e-mail: isabel@ccg.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_8

and biosyntheses and functionalities of individual membrane lipids have mainly been studied in this organism. However, in other bacteria, additional and alternative glycerol-based membrane lipids are found, and in many cases neither their biosyntheses nor their functionalities are understood. Some Gram-negative bacteria have phosphatidylcholine in their standard repertoire, whereas many Gram-positive bacteria have glycosylated diacylglycerols and lysyl-phosphatidylglycerol in their membranes. Notably, phosphatidylinositol seems to be an essential lipid for *Mycobacterium tuberculosis* and Actinomycetes, and it might be formed in some proteobacteria. Under certain stress conditions, specific membrane lipids can be formed in order to minimize the stress exerted. For example, under phosphorus-limiting conditions of growth, some bacteria form glycerol-based membrane lipids lacking phosphorus such as glycolipids, sulfolipids, or betaine lipids. Challenge of proteobacteria with acid causes modifications of membrane lipids, such as formation of lysyl-phosphatidylglycerol. Modifications of the acyl residues of pre-existing glycerol-based membrane lipids include desaturation, cyclopropanation, *cis-trans* isomerization reactions, as well as bacterial plasmalogen biosynthesis.

1 Introduction

A primary role of lipids in cellular function is to form the lipid bilayer permeability barrier of cells. Also, there are at least two ways by which lipids can affect protein structure and function and thereby the functioning of a cell. In one way, protein function is influenced by specific protein-lipid interactions that depend on the chemical and structural anatomy of lipids (head group, backbone, alkyl chain length, degree of unsaturation, chirality, ionization, and chelating properties). In another way, protein function is also influenced by the unique self-association properties of lipids that result from the collective properties (fluidity, bilayer thickness, shape, and packing properties) of the lipids organized into membrane structures.

Glycerophospholipids are the primary building blocks of membranes, but other lipids are important components. Eukaryotic membranes contain phospholipids (glycerophospholipids and phosphosphingolipids), phosphorus-free glycerolipids and sphingosine-based lipids as well as steroids (Nelson and Cox 2017). Although highly decorated sphingolipid derivatives or the broad variety of steroids are absent in prokaryotes, *Bacteria* possess all the basic structures of membrane lipids found in *Eukarya*, albeit not in one single species. In *Eukarya*, sphingolipids and cholesterol cluster together in membrane lipid rafts, and numerous specific functions are associated with lipid rafts (Nelson and Cox 2017). Defined lipid domains exist also in *Bacteria* (Matsumoto et al. 2006), and it has been shown that the cell division-inhibiting protein MinD is associated with phosphatidylglycerol-containing lipid spirals in *Bacillus* (Barák et al. 2008). Apparently, bacteria organize many

signaling transduction processes in functional membrane microdomains (FMM), consisting of polyisoprenoid lipids, the scaffold protein flotillin, and FMM-associated proteins that oligomerize into functional complexes and signal transduction cascades (Lopez 2015).

Gram-negative bacteria possess functionally distinct inner (cytoplasmic) and outer membranes (Dowhan et al. 2008). However, there is evidence that in addition to their cytoplasmic membrane, some Gram-positive bacteria might have an outer membrane bilayer structure formed by long-chain mycolic acids and other membrane lipids (Hoffmann et al. 2008).

The membrane lipids of *Archaea* differ from those of *Bacteria* and *Eukarya* (see Koga, ▶ Chap. 29, "Biosynthesis and Evolution of Archaeal Membranes and Ether Phospholipids," this volume). In contrast to the lipids of *Bacteria* and *Eukarya* in which ester linkages bond the fatty acids to glycerol, the lipids of *Archaea* contain ether bonds between glycerol and their hydrophobic side chains. In addition, archaeal lipids lack fatty acids. Instead, the side chains are composed of repeating units of isoprene (Dowhan et al. 2008). However, the stereochemistry of the glycerol-phosphate backbone represents the most striking difference, as *Archaea* assemble their membrane lipids on a glycerol-1-phosphate backbone, whereas *Bacteria* and *Eukarya* employ a glycerol-3-phosphate backbone for their glycerol-based membrane lipids.

As other membrane lipids (lipopolysaccharides (see Bishop, ▶ Chap. 9, "Lipid A," this volume)), sphingolipids (see Geiger et al., ▶ Chap. 7, "Bacterial Sphingolipids and Sulfonolipids," this volume), ornithine-containing lipids (see Sohlenkamp, ▶ Chap. 6, "Ornithine Lipids and Other Amino Acid-Containing Acyloxyacyl Lipids," this volume), phenolic lipids (see Miyanaga and Onishi, ▶ Chap. 8, "Phenolic Lipids Synthesized by Type III Polyketide Synthases," this volume), hopanoids, and other isoprenoids (see Perez-Gil et al., ▶ Chap. 4, "Formation of Isoprenoids," this volume) are covered in separate chapters of this handbook, we will focus on glycerol-based membrane-forming lipids of *Bacteria* in this chapter.

2 Membrane-Forming Lipids in *Escherichia coli*

In the bacterial model organism, *Escherichia coli*, only three major membrane lipids phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin occur. In addition, Gram-negative bacteria usually have the lipid A-containing lipopolysaccharide in the outer monolayer of their outer membrane (see Bishop, ▶ Chap. 9, "Lipid A," this volume), and lipid A modification systems have been reviewed (Raetz et al. 2007; Henderson et al. 2016). However, even in *E. coli* many minor membrane phospholipids exist (Raetz 1986). For many of these minor phospholipids, neither their structures nor the genes/enzymes required for their biosyntheses are known. Presently, considerable efforts are ongoing to characterize also these minor bacterial lipids (Garrett 2016).

3 Formation of Glycerophospholipids in Bacteria

3.1 Formation of the Central Activated Intermediate CDP-Diacylglycerol

In *Bacteria*, glycerol-3-phosphate (Fig. 1) forms the backbone of all glycerophospholipid molecules. It can be synthesized by two different pathways, from glycerol directly or from the glycolytic intermediate dihydroxyacetone phosphate (DHAP). During growth on carbon sources other than glycerol, the biosynthetic glycerol-3-phosphate dehydrogenase GpsA reduces DHAP with NADH and forms glycerol-3-phosphate (Rock 2008). In contrast, during growth with glycerol as sole carbon source, the enzymes of the glycerol catabolic (*glp*) operon are induced. Bacteria can obtain glycerol-3-phosphate directly from the environment by employing the organophosphate/phosphate antiporter GlpT, or they can take up glycerol with the

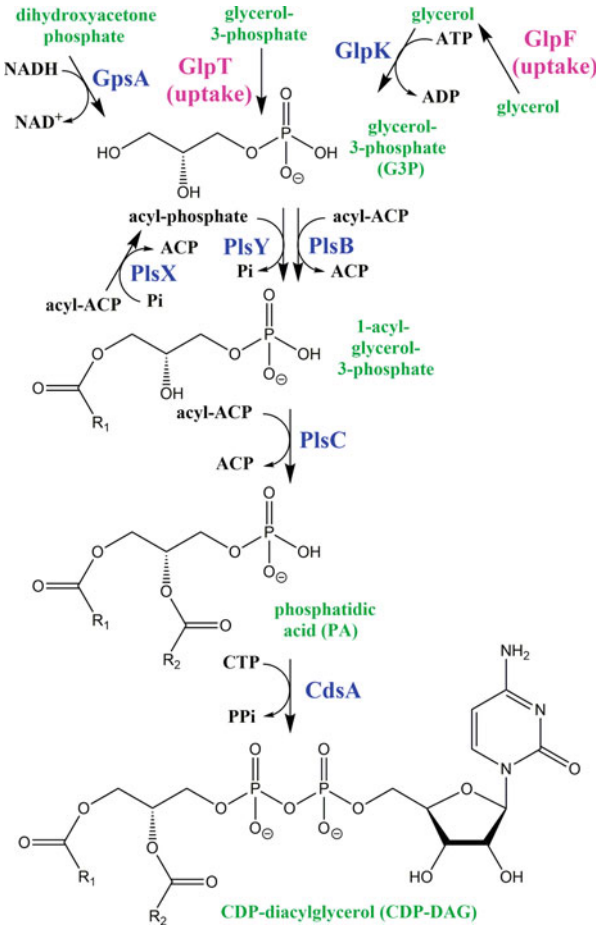


Fig. 1 Synthesis of phosphatidic acid and CDP-diacylglycerol in bacteria (for details see text). Names of enzymes are indicated in *blue* and of transporters in *pink* (Reproduced from López-Lara and Geiger (2017) with permission from Elsevier)

aquaglyceroporin GlpF and convert it to glycerol-3-phosphate employing glycerol kinase GlpK (Yao and Rock 2013).

In *E. coli*, the glycerol-3-phosphate acyltransferase PlsB can use acyl-CoA or acylated derivatives of acyl carrier protein (ACP) as acyl donors and is the major activity in this bacterium for catalyzing the first acylation at position 1 of glycerol-3-phosphate thereby forming 1-acyl-glycerol-3-phosphate (Yao and Rock 2013). However, the more widespread pathway for the initial acylation of glycerol-3-phosphate among bacteria involves PlsX and PlsY (Lu et al. 2006). In this pathway, PlsX catalyzes the conversion of acyl-ACP and inorganic phosphate (Pi) to acyl-phosphate and ACP. In a second step, PlsY transfers the acyl group from acyl-phosphate to glycerol-3-phosphate forming Pi and 1-acyl-glycerol-3-phosphate (Lu et al. 2006).

The second fatty acyl residue is added by another enzyme, the 1-acyl-glycerol-3-phosphate acyltransferase PlsC, to form phosphatidic acid (PA). Although PlsC from *E. coli* can use acyl-ACP or acyl-CoA as acyl donors, PlsC from many other bacteria uses exclusively acyl-ACP (Lu et al. 2006; Yao and Rock 2013). There is a tendency that most fatty acids at the 1-position are saturated, whereas most at the 2-position are unsaturated. The acylation specificity, however, is not absolute and can be altered by the supply of acyl donors.

The conversion of PA to CDP-diacylglycerol (CDP-diglyceride; CDP-DAG) requires CTP, is catalyzed by CDP-diglyceride synthase CdsA, and forms inorganic pyrophosphate (PPi) as the second product (Rock 2008) (Fig. 1).

3.2 Diversification of Polar Head Groups in Bacterial Glycerophospholipids

Enzymes that use CDP-diacylglycerol for the introduction of different head groups usually belong to the CDP-alcohol phosphotransferase family or to the phospholipase D (PLD) superfamily each displaying their characteristic protein sequence motifs as reviewed earlier (Sohlenkamp and Geiger 2016).

3.2.1 Biosynthesis of Phosphatidylglycerol

Phosphatidylglycerol phosphate synthase (PgsA) transfers glycerol-3-phosphate to CDP-diacylglycerol under the release of CMP thereby producing phosphatidylglycerol phosphate (PGP) (Kanfer and Kennedy 1964; Gopalakrishnan et al. 1986). There are three enzymes with PGP phosphatase activity (PgpA, PgpB, and PgpC) in *E. coli*, releasing Pi from PGP to form phosphatidylglycerol (PG) (Lu et al. 2011) (Fig. 2). PG or other negatively charged membrane lipids are required for initiation of DNA replication, control of cell division, and protein translocation across membranes (Dowhan et al. 2008) (see Dowhan et al., ▶ Chap. 30, "Functional Roles of Individual Membrane Phospholipids in *Escherichia coli* and *Saccharomyces cerevisiae*," this volume).

3.2.2 Biosynthesis of Lysyl-Phosphatidylglycerol and Other Aminoacylated PG Derivatives

Lysyl-phosphatidylglycerol (lysyl-PG) is a well-known membrane lipid in many Gram-positive bacteria (*Staphylococcus*, *Lactococcus*, *Bacillus*), and its formation increases the resistance of bacteria against cationic antimicrobial peptides (CAMPs) of the innate immune response. The staphylococcal MprF transfers lysine from charged lysyl-tRNA to PG forming lysyl-PG (Fig. 2) (Slavetinsky et al. 2016). The C-terminal domain of MprF is sufficient for full-level lysyl-PG production at the inner leaflet of the cytoplasmic membrane, whereas the N-terminal MprF domain translocates lysyl-PG from the inner to the outer leaflet and therefore acts as a flippase (Ernst et al. 2009). Paralogs of MprF are responsible for alanyl-PG formation in *Clostridium perfringens* and *Pseudomonas aeruginosa* and for arginyl-PG formation in *Enterococcus faecium*. Remarkably, *Bacillus subtilis* can form both stereoisomers, L- and D-alanyl-PG (Slavetinsky et al. 2016). Among the homologs of the C-terminal aminoacyl-PG synthase (aaPGS) domain, up to seven phylogenetically different types of proteins can be distinguished (Smith et al. 2015). Multiple aaPGS paralogs can occur in one organism, especially in actinobacterial species. In some cases, structural genes for aaPGS seem to be associated with genes encoding for putative hydrolytic enzymes as reported for *Rhizobium tropici* (Vinuesa et al. 2003), *P. aeruginosa* (Arendt et al. 2013), or *E. faecium* (Smith et al. 2015), and the predicted hydrolases seem to release the respective amino acid from the PG lipid molecule to the periplasmic space. In some Gram-negative bacteria, the MprF homolog LpiA is induced under acidic conditions forming lysyl-PG which seems to confer acid tolerance, nodulation competitiveness and increase the resistance to CAMPs (Vinuesa et al. 2003; Sohlenkamp et al. 2007).

3.2.3 Biosynthesis of Cardiolipin and Aminoacylated Cardiolipin Derivatives

For many years, only two different pathways for cardiolipin (CL) synthesis were known; one was the so-called bacteria-like CL synthase (Cls-I) pathway in which two molecules of PG react to form CL and glycerol (Fig. 2) and the eukaryote-like CL synthase (Cls-II) pathway in which one molecule PG reacts with CDP-DAG leading to the formation of CL and CMP. As implied by the names, one route was thought to be specific for prokaryotes, whereas the other was specific for eukaryotes. In *E. coli* CL is synthesized by the enzyme ClsA from two molecules of PG (Nishijima et al. 1988). ClsA from *E. coli* is a typical prokaryotic Cls, belonging to the PLD superfamily. However, *Streptomyces coelicolor* and other actinomycetes use the eukaryote-like pathway to synthesize CL (Sandoval-Calderon et al. 2009) (Fig. 2). The Cls from actinomycetes belongs to the CDP-alcohol phosphotransferase superfamily. *E. coli* has three structural genes for Cls (*clsA*, *clsB*, *clsC*). Whereas ClsA and ClsB catalyze the condensation of two PG molecules to obtain CL, ClsC synthesizes CL from PG and PE (Tan et al. 2012) (Fig. 2). Although multiple structural genes for Cls might be encoded in several bacterial genomes, it is presently not clear what their distinct functions might be. Particularly intriguing in CL biosynthesis is that distinct Cls enzymes might use different

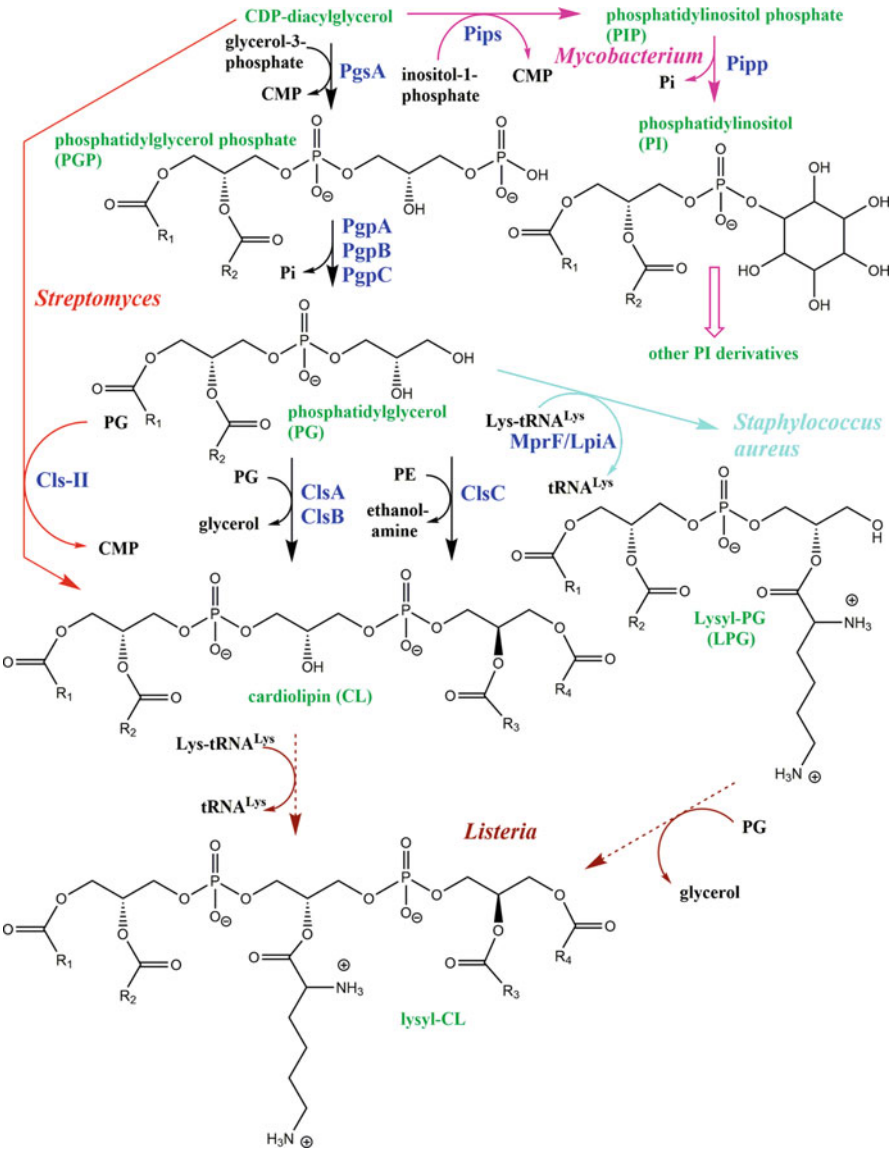


Fig. 2 Synthesis of anionic phospholipids (PG, CL, PI) and the aminoacylated derivatives of PG and CL in bacteria (for details see text). Names of enzymes are indicated in *blue*. Reactions occurring in *E. coli* are represented with *black arrows*. Reactions additionally occurring in *Streptomyces*, *Mycobacterium*, *Staphylococcus aureus*, or *Listeria* are indicated in *red, pink, light blue*, or *brown arrows*, respectively. Reactions with interrupted *arrows* are hypothetical and have not been shown experimentally to date (Reproduced from López-Lara and Geiger (2017) with permission from Elsevier)

energetics. Typical "bacterial" Cls catalyzes transesterification reactions, and the standard free energy change ($\Delta G'^0$) in such reactions is close to zero. In contrast, enzymes consuming CDP-DAG break a phosphoanhydride bond ($\Delta G'^0 = -30$ kJ/mol) and upon CL formation generate an ester linkage ($\Delta G'^0 = +14$ kJ/mol) resulting in a net standard free energy change of about −16 kJ/mol for the reaction. Therefore, this latter reaction might be required when an organism needs to synthesize high relative amounts of CL. In *Xanthomonas campestris*, a bifunctional CL/PE synthase which synthesizes CL from PG and CDP-DAG (Fig. 2) can also catalyze ethanolamine-dependent PE formation (Moser et al. 2014b). However, the gene encoding this bifunctional CL/PE synthase seems to be restricted to the orders *Xanthomonadales* and *Pseudomonadales*.

The aminoacylated derivatives of CL, alanyl-CL, and lysyl-CL (Fig. 2) have been described in different *Listeria* species. An aminoacyl-PG synthase is required for their formation, and if lysyl-PG formation is impaired in *Listeria*, also lysyl-CL formation is impaired, implying that there is no dedicated second enzyme required for the aminoacyl transfer to CL (Dare et al. 2014). Presently it is not clear whether the respective amino acid is transferred to CL or if a CL synthase can use the aminoacylated PG as substrate and convert it into lysyl-CL or alanyl-CL (Fig. 2).

Evidence suggests that cardiolipin controls the osmotic stress response and the subcellular location of the transporter ProP in *E. coli* (Romantsov et al. 2017) (see Romanstov and Wood, ▶ Chap. 44, "Contributions of Membrane Lipids to Bacterial Cell Homeostasis upon Osmotic Challenge," this volume).

3.2.4 Biosynthesis of Phosphatidylinositol (PI) and Mannosylated PI Derivatives

PI has been considered to be a typical eukaryotic lipid, and it is thought that only few bacteria can form PI. However, PI formation is widespread within the order Actinomycetales and has been reported for a few other bacteria, such as *Myxococcus*. Whereas eukaryotes form PI in a one-step reaction using CDP-DAG and inositol as substrates, bacteria possess phosphatidylinositol phosphate synthase (Pips) which catalyzes the reaction between inositol-1-phosphate and CDP-DAG, leading to the formation of phosphatidylinositol phosphate (PIP) (Fig. 2). In a second step, PIP is dephosphorylated by PIP phosphatase (Pipp) to PI (Morii et al. 2010) (Fig. 2). PI can be further modified with mannose residues and acyl groups forming the PI mannosides (reviewed by Sohlenkamp and Geiger 2016). PI has been reported to be essential in *Mycobacterium* (Jackson et al. 2000).

Although PI seems to be widespread among actinobacteria, its possible presence in Gram-negative bacteria has been subject to debate. As no obvious homologs of Pips seem to be encoded by genomes of Gram-negative bacteria, PI might be formed by a distinct pathway. A recent study on the thermophilic Bacteroidete *Rhodothermus marinus* reports on the presence of dialkylether glycerophosphoinositides (Jorge et al. 2015). In *R. marinus*, the bacterial dialkylether glycerophosphoinositol synthase (BEPIS) catalyzes the condensation of CDP-inositol and dialkylether glycerols to produce phosphoinositol ether lipids. Remarkably, BEPIS

homologs are found in other members of the *Cytophaga*, *Flavobacterium*, *Bacteroidetes* (CFB) group, and in some α-proteobacteria.

3.2.5 Biosynthesis of Phosphatidylethanolamine

The first step in the synthesis of phosphatidylethanolamine (PE) is the condensation of CDP-diacylglycerol with serine to form phosphatidylserine (PS) catalyzed by phosphatidylserine synthase (Pss) (Fig. 3). Pss enzymes belong to two different families: type I Pss is a PLD-like protein, whereas type II proteins belong to the CDP-alcohol phosphotransferase family (Sohlenkamp et al. 2004). For example, Pss from *E. coli* is a type I Pss, whereas Pss from *B. subtilis* and *S. meliloti* is a type II Pss. Though both types of Pss enzymes are unrelated on the sequence level, they both employ CDP-DAG and L-serine as substrates and form PS and CMP as products. Whereas type I Pss is restricted to a few γ-proteobacteria, type II Pss occurs in all three domains of life (Sohlenkamp et al. 2004).

In a second step, the decarboxylation of PS is catalyzed by PS decarboxylase (Psd) to yield PE (Fig. 3). Mutants deficient in *pss* or *psd* have been obtained in several bacteria (reviewed in Sohlenkamp and Geiger 2016). In general, these mutants are viable under specific growth conditions, and many require bivalent cations in the growth medium. Recently, a bifunctional cardiolipin/phosphatidylethanolamine synthase (CL/PE synthase) has been described in the plant pathogen *Xanthomonas campestris* which catalyzes the synthesis of PE from CDP-DAG and ethanolamine (Moser et al. 2014b) (Fig. 3). This same enzyme also functions as a Cls and, as many bacterial Clss, belongs to the PLD superfamily. In eukaryotes, PE synthesis occurs via the CDP-ethanolamine pathway, but this reaction is unknown to date in prokaryotes.

PE is required in *E. coli* for the proper folding and topological organization of certain membrane proteins within the lipid bilayer. For example, permeases, such as the lactose permease LacY, require the presence of PE for active transport of their substrates (Dowhan et al. 2008) (see Dowhan et al., ▶ Chap. 30, "Functional Roles of Individual Membrane Phospholipids in *Escherichia coli* and *Saccharomyces cerevisiae*," this volume).

3.2.6 Biosynthesis of Phosphatidylcholine

Although phosphatidylcholine (PC) is the major membrane-forming phospholipid in *Eukarya*, it is thought to be present in only about 15% of the bacteria (Sohlenkamp et al. 2003; Geiger et al. 2013). A well-known pathway for PC formation occurs by threefold methylation of PE using *S*-adenosylmethionine (SAM) as methyl donor and catalyzed by phospholipid *N*-methyltransferase (Pmt). In many bacteria, all three subsequent methylations are achieved by a single enzyme (Fig. 3). Some complex bacteria with large genomes, however, such as *Bradyrhizobium japonicum*, possess up to four distinct phospholipid *N*-methyltransferases with distinct substrate specificities. *B. japonicum* has phospholipid *N*-methyltransferases that predominantly perform the first, the second, or the third methylation (Hacker et al. 2008). Although Pmts are usually soluble enzymes, they have to be recruited to the membrane in order to methylate PE. Apparently, the Pmt from *A. tumefaciens* attaches to anionic

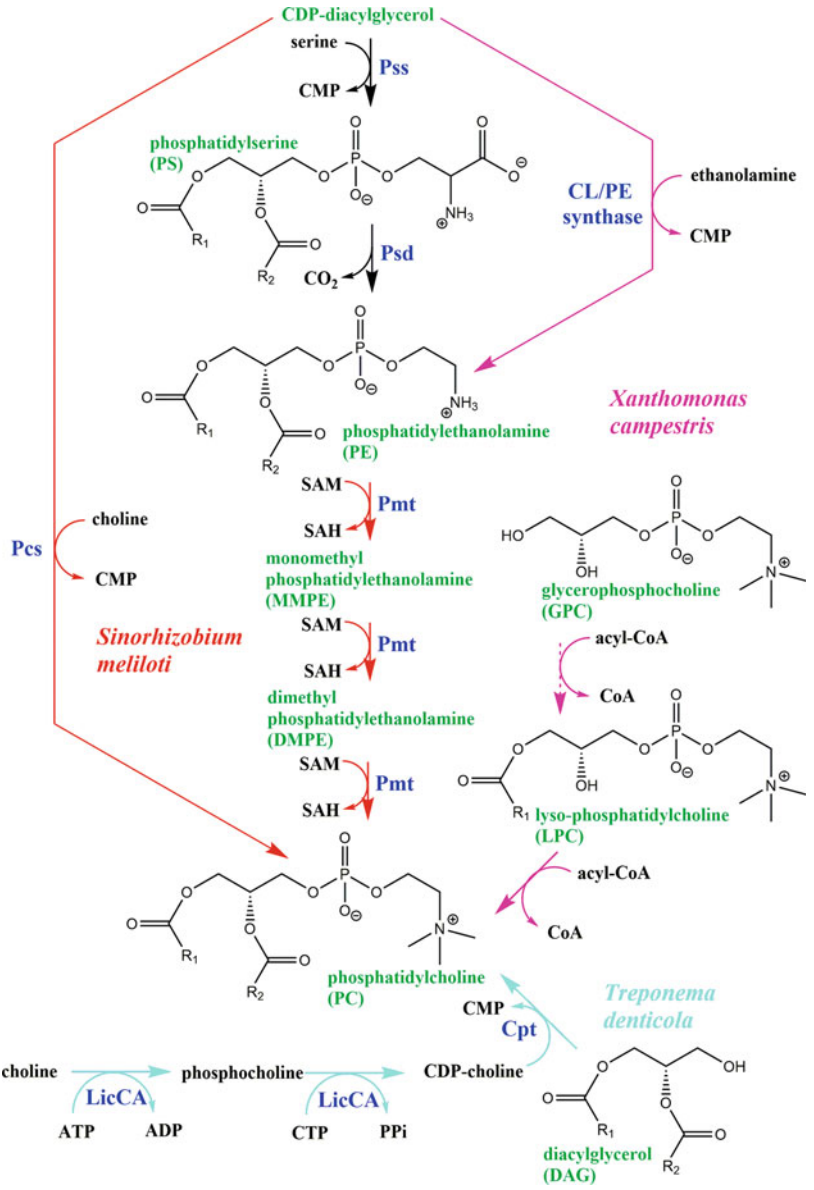


Fig. 3 Synthesis of zwitterionic phospholipids PE and PC in bacteria (for details see text). Names of enzymes are indicated in *blue*. Reactions occurring in *E. coli* are represented with *black arrows*. Reactions additionally occurring in *S. meliloti*, *X. campestris*, or *T. denticola* are indicated in *red*, *pink*, or *light blue arrows*, respectively. Reactions with interrupted *arrows* are hypothetical and have not been shown experimentally to date (Reproduced from López-Lara and Geiger (2017) with permission from Elsevier)

phospholipids (PG and CL) at the inner surface of the cytoplasmic membrane, undergoing a conformational change that keeps the enzyme associated to the membrane thus facilitating the methylation reaction (Danne et al. 2015). Increasing PC concentrations trigger membrane dissociation, suggesting that the membrane binding of Pmt is revoked by its end product PC (Danne et al. 2015).

Many PC-containing bacteria have a second pathway for PC formation, catalyzed by phosphatidylcholine synthase (Pcs), in which choline is condensed directly to CDP-diacylglycerol forming PC and CMP (Sohlenkamp et al. 2003) (Fig. 3). Pcs belongs to the CDP-alcohol phosphotransferase superfamily, and it displays a slightly modified CDP-alcohol phosphotransferase motif that makes the bioinformatic prediction of Pcs-encoding genes straightforward (Geiger et al. 2013). Although Pcs is widespread in PC-forming bacteria, to date, Pcs has not been found in eukaryotes (Sohlenkamp and Geiger 2016). *Eukarya* usually have another pathway, the CDP-choline pathway, to recycle free choline to PC. In the CDP-choline pathway, choline is phosphorylated to phosphocholine by choline kinase and further activated by CTP/phosphocholine cytidylyltransferase to CDP-choline. In a third step, catalyzed by CDP-choline/1,2-diacylglycerol choline phosphotransferase, the CDP-choline is condensed to the nonactivated lipid part 1,2-diacylglycerol forming PC and CMP. This latter eukaryote-like pathway exists in some bacteria such as *Treponema* (Kent et al. 2004; Vences-Guzman et al. 2017). Remarkably, in *Treponema* the prokaryotic *licCA*-encoded choline kinase and CTP/phosphocholine cytidylyltransferase activities are combined with the *cpt*-encoded CDP-choline/1,2-diacylglycerol choline phosphotransferase. Probably this latter gene has been acquired by *Treponema* by horizontal gene transfer from a eukaryotic host (Vences-Guzman et al. 2017).

The plant pathogen *X. campestris* uses glycerophosphocholine (GPC) as a substrate for PC formation (Moser et al. 2014a) (Fig. 3). A similar GPC-dependent pathway for PC formation exists in *Saccharomyces cerevisiae*. Two acyltransferase reactions are required to form PC from GPC. In both yeast and *X. campestris*, only enzymes for the second acyltransferase step catalyzing the conversion of lyso-PC to PC were demonstrated. To date, enzymes catalyzing the first acylation step have not been identified.

In *Legionella pneumophila*, bacterial PC seems to be required for multiple functions, such as attachment to the macrophage via the platelet-activating factor receptor, timely formation of a functioning type IV secretion system, as well as transition of the bacterium to a state of higher virulence (Conover et al. 2008).

4 Formation of Phosphorus-Free Glycerol-Based Membrane Lipids in Bacteria

The composition of lipids in the cell membrane can vary depending on the physiological situation of an organism (López-Lara et al. 2003). Under phosphorus-limiting conditions, membrane phospholipids of some bacteria are at least partially replaced by lipids containing no phosphorus as demonstrated in *B. subtilis*,

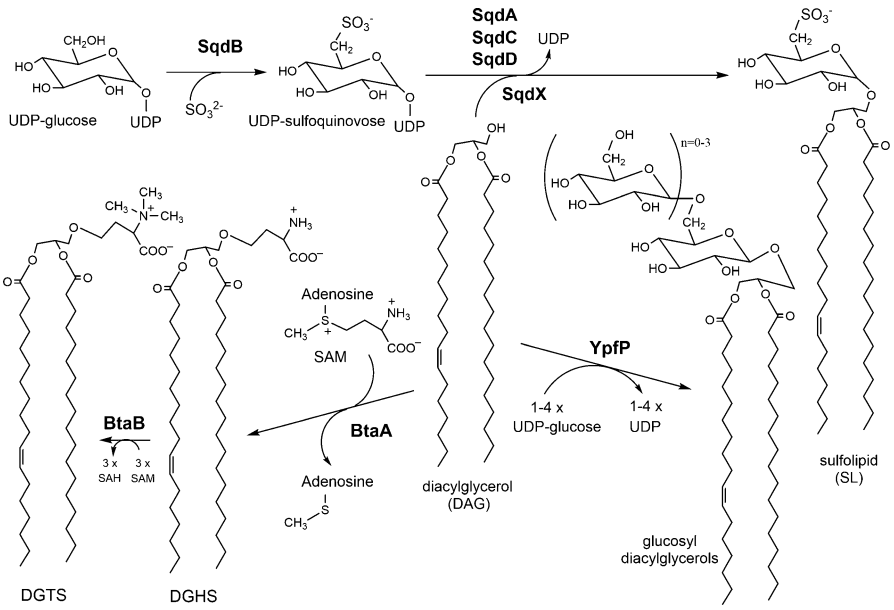


Fig. 4 Biosynthesis of diacylglycerol-derived phosphorus-free membrane lipids in bacteria (for details see text)

Pseudomonas diminuta, *Pseudomonas fluorescens*, *Sinorhizobium meliloti*, and *Rhodobacter sphaeroides* (reviewed in López-Lara et al. 2003). In *S. meliloti* these phosphorus-free lipids are the sulfolipid sulfoquinovosyl diacylglycerol (SL), ornithine-containing lipid (OL), and the betaine lipid diacylglyceryl trimethylhomoserine (DGTS), and in some other bacteria these phosphorus-free lipids include glycolipids. Notably, glycolipids, sulfolipids, and betaine lipids are all formed from the same precursor diacylglycerol (Fig. 4).

4.1 Diacylglycerol

Diacylglycerol (DAG) is the most simple glycerol-based membrane lipid as it has only two fatty acyl residues attached to the glycerol backbone, one to the 1- and one to the 2-position. Although DAG has important functions as second messenger in eukaryotic cells (Nelson and Cox 2017), in bacteria it is usually a minor lipid that might be toxic to bacteria when present in higher amounts in the membrane (Raetz and Newman 1978). There are a number of metabolic pathways that might lead to the formation of DAG, for example, the biosynthesis of lipoteichoic acid in Gram-positive bacteria, the formation of charged periplasmic glucans in Gram-negative bacteria at conditions of low osmolarity (reviewed in Sahonero-Canavesi et al. 2017), or the hydrolysis of membrane phospholipids by a phospholipase C when cultivated at growth-limiting concentrations of phosphate (Zavaleta-Pastor et al.

2010). DAG can either be phosphorylated by DAG kinase to PA and reenter de novo PL biosynthesis (Geiger et al. 2013) or it can be decorated with distinct head groups giving rise to glycolipids, sulfolipids, or betaine lipids (Fig. 4).

4.2 Glycolipids

Glycosyl diacylglycerols (GLs) are widespread membrane glycolipids in plants, animals, and Gram-positive bacteria. The *ypfP*-encoded UDP-glucosyltransferase from *B. subtilis* successively transfers up to four glucose residues from UDP-glucose to DAG (Jorasch et al. 1998) (Fig. 4), though in other bacteria, different activated sugar precursors, such as UDP-galactose or GDP-mannose, may be employed instead in glycosyltransferase reactions. Recently, reports on GLs in Gram-negative bacteria have been increasing as well. For example, under phosphorus-limiting conditions of growth, *Mesorhizobium loti* makes the processive glycosyltransferase Pgt causing GL formation (Devers et al. 2011).

An essential glycosyltransferase (MPN483) from the human pathogen *Mycoplasma pneumoniae* is also processive and relatively flexible with regard to its substrates. MPN483 can use UDP-galactose or UDP-glucose as sugar donors and DAG or ceramide derivatives as acceptors thereby producing a wide spectrum of compounds, some of them important antigens during infection by *Mycoplasma* (Klement et al. 2007). A more detailed compendium on glycolipids is provided in the review by Hölzl and Dörmann (2007).

4.3 Sulfolipids

The sulfolipid 6-sulfo-α-D-quinovosyl diacylglycerol (SQDG) occurs widely in photosynthetic organisms ranging from bacteria to seed plants (reviewed by Benning 2007) where it is associated with photosynthetic membranes. Although SQDG is not generally essential for photosynthesis, it is required for growth under phosphate-limiting growth conditions in *R. sphaeroides* and cyanobacteria. In *R. sphaeroides*, phosphate limitation of the wild type caused a significant reduction in the amount of all phospholipids and an increased amount of sulfolipid. SQDG has been suggested to function as a surrogate for phospholipids, particularly phosphatidylglycerol, under phosphate-limiting conditions (Benning 1998). At least four structural genes (*sqdA* and the operon *sqdBDC*) are involved in sulfolipid biosynthesis in *R. sphaeroides* (Benning 1998) (Fig. 4). SqdB (named as SQD1 in plants) is responsible for the formation of UDP-sulfoquinovose from UDP-glucose and sulfite (Benning 2007) (Fig. 4). *sqdB* is the only gene required for SQDG synthesis that has orthologs in all SQDG-producing organisms (Benning 2007). In the cyanobacterium *Synechococcus* sp. PCC 7942, the gene *sqdB* forms an operon with the gene encoding the lipid glycosyltransferase SqdX which is also known as the cyanobacterial SQDG synthase (Benning 2007) (Fig. 4). Plants use an SqdX homolog called SQD2 for SQDG synthesis (Shimojima 2011). SqdB and SqdX orthologs also seem to be the enzymes

responsible for SQDG formation in *Bacillus* species that had been isolated from phosphorus-poor environments (Gómez-Lunar et al. 2016). In contrast, a specific function of the genes *sqdDC* and *sqdA*, which are present in *R. sphaeroides* and other proteobacteria, has not been defined, but they seem to provide an alternative pathway for connecting sulfoquinovose to the DAG lipid anchor. Mutants deficient in SQDG formation have been obtained in *S. meliloti*, *Synechococcus* sp. PCC 7942, and *R. sphaeroides* (reviewed by Sohlenkamp and Geiger 2016), and they show only slightly altered phenotypes.

4.4 Betaine Lipids

Although PC is known to be the major membrane lipid in eukaryotes, some lower eukaryotic organisms possess the betaine lipid diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS) instead. DGTS occurs in a wide variety of lower green plants (green algae, bryophytes, and pteridophytes), chromophytes, fungi, and amoebae (reviewed in Sohlenkamp et al. 2003). In some α-proteobacteria, DGTS has been identified as a phosphorus-free membrane lipid that substitutes for PC under conditions of phosphate limitation. There is an apparent reciprocity between the content of PC and the content of DGTS, i.e., when PC is a major membrane lipid, often no DGTS is detected in the same organism, whereas in organisms where DGTS is a major lipid, PC is only found in trace levels. This suggests that DGTS and PC, both zwitterionic at physiological pH, are interchangeable at least with regard to essential functions for the respective organism.

Two structural genes from *R. sphaeroides*, *btaAB*, coding for two enzymes BtaA and BtaB involved in DGTS biosynthesis, have been characterized (Riekhof et al. 2005). The BtaA *S*-adenosylmethionine/diacylglycerol 3-amino-3-carboxypropyl transferase converts diacylglycerol (DAG) into diacylglyceryl-homoserine (DGHS), and during the formation of the ether bond, *S*-adenosylmethionine functions as donor of the homoseryl group. Subsequently, the *S*-adenosylmethionine/diacylglycerylhomoserine-*N*-methyltransferase BtaB catalyzes threefold methylation of DGHS in order to yield DGTS (Fig. 4). Expression of BtaA and BtaB usually requires induction by the transcriptional PhoB regulator (Krol and Becker 2004).

5 Modifications of Acyl Residues in Pre-existing Glycerol-Based Membrane Lipids of Bacteria

Although initially the substitution of glycerol-based membrane lipids with specific fatty acyl residues at the 1- or 2-position is defined during the formation of PA (see Sect. 3.1), acyl residues of existing glycerol-based membrane lipids can be modified in a number of ways. For example, fatty acyl residues can be removed from an existing glycerol-based membrane lipid, and the lysolipid formed can be reacylated with another fatty acyl residue as observed in some lipid cycles (Sahonero-Canavesi et al. 2017). Also, modifications might occur either in the alkyl region (i.e.,

desaturation, cyclopropanation, *cis-trans* isomerization) or in the ester-linked region (plasmalogen biosynthesis) of a fatty acyl residue in existing glycerol-based lipids.

5.1 Modifications of Pre-Existing Glycerol-Based Lipids in the Alkyl Region of Fatty Acyl Residues

In a mechanism common to most bacteria, double bonds can be introduced in an oxygen-independent way into growing acyl chains during fatty acid biosynthesis (Rock 2008; Cronan and Thomas 2009) (see López-Lara and Geiger, ▶ Chap. 3, "Formation of Fatty Acids," this volume) before these unsaturated fatty acyl residues are attached to the glycerol backbone during PA biosynthesis (see Sect. 3.1). However, in a similar way as eukaryotes, some bacteria possess specific phospholipid acyl desaturases that introduce a *cis* double bond into the alkyl region of a fatty acyl residue in a glycerol-based membrane lipid (reviewed by Zhang and Rock 2008). Specifically, *B. subtilis* possesses an oxygen-dependent phospholipid acyl chain desaturase DesA (also known as Δ-5-desaturase) that introduces a *cis* double bond at the 5-position of acyl chains that are attached to existing phospholipids, whereas an analogous oxygen-dependent phospholipid acyl chain desaturase DesA (known as Δ-9-desaturase) from *P. aeruginosa* introduces a *cis* double bond at the 9-position of acyl chains in existing phospholipids (Fig. 5a).

The conversion of unsaturated fatty acyl residues on pre-existing phospholipids to cyclopropane fatty acyl residues is common in bacteria. The required methylation reaction is carried out by cyclopropane fatty acid synthase (Cfa), which uses *S*-adenosylmethionine as the methyl donor to generate the cyclopropane group (reviewed by Zhang and Rock 2008) (Fig. 5a).

Only a few bacteria have evolved a mechanism to convert *cis*-unsaturated fatty acyl residues on pre-existing phospholipids to *trans*-unsaturated fatty acyl residues. The *cis-trans* isomerase Cti catalyzing this reaction has been studied to some extent in *Pseudomonas putida* (reviewed by Zhang and Rock 2008) (Fig. 5a).

5.2 Biosynthesis of Plasmalogens

Plasmalogens, 1-*O*-alk-1′-enyl 2-acyl glycerol phospholipids and glycolipids, might have evolved first in anaerobic bacteria and are absent in most aerobic bacteria. Later on in evolution, aerobic animal cells reinvented a plasmalogen biosynthesis pathway that involves DHAP and requires molecular oxygen (reviewed by Goldfine 2010). In contrast, plasmalogen biosynthesis in anaerobic *Clostridium* species is DHAP-independent, occurs in the absence of oxygen (Goldfine 2017), and therefore must be different from the biosynthesis pathway reported for animals. It is thought that in bacteria the 1-acyl residue of glycerophospholipids is directly modified, first by reduction, converting the acyl ester linkage to a hemiacetal intermediate which, by the subsequent elimination of a water molecule, would be converted into a vinyl ether linkage thereby introducing a

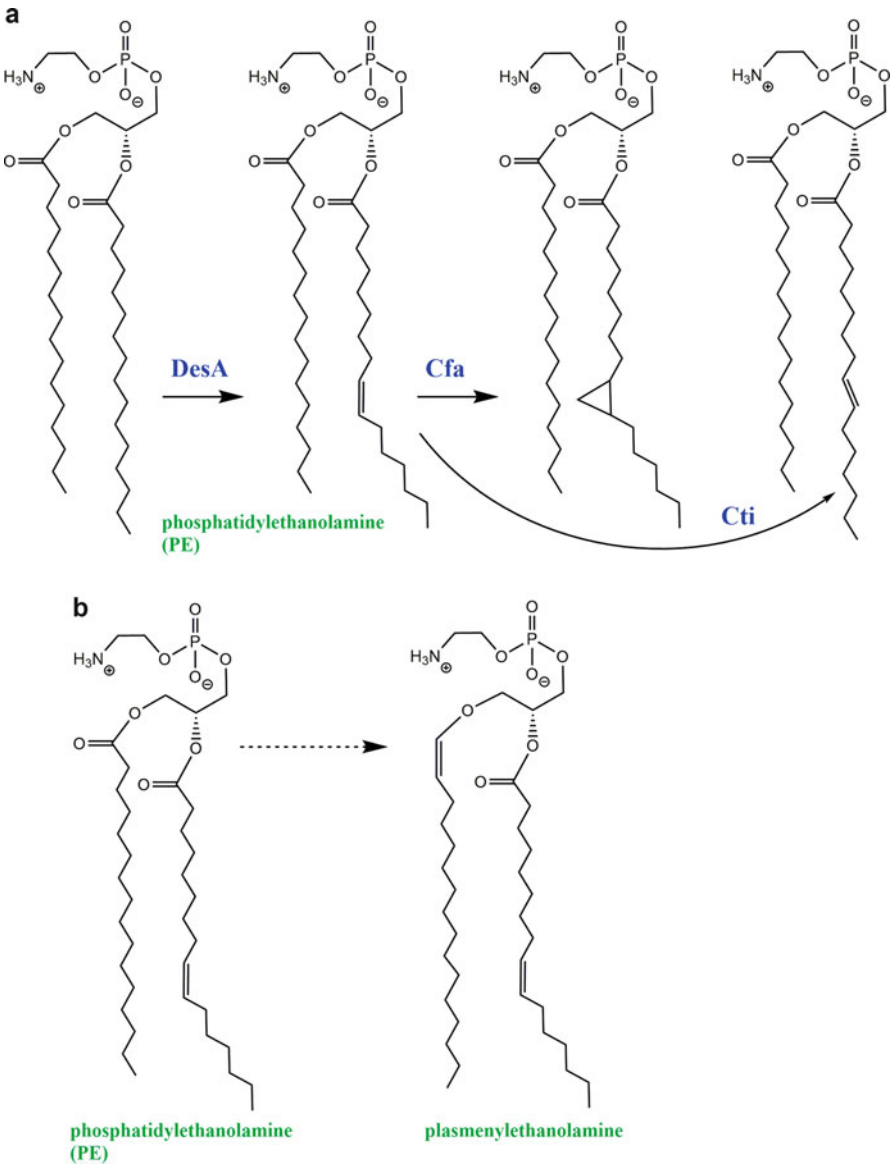


Fig. 5 Modification of acyl residues in pre-existing glycerol-based membrane lipids of bacteria (for details see text). **(a)** Modifications of pre-existing glycerol-based lipids (i.e., PE) in the alkyl region of fatty acyl residues by DesA (desaturation), Cfa (cyclopropanation), or Cti (*cis-trans* isomerization). (**b**) Biosynthesis of the plasmalogen plasmenylethanolamine. In Clostridia, plasmalogen head groups may consist of ethanolamine (as in Fig. 5b), monomethylethanolamine, glycerol, phosphatidylglycerol, or saccharide residues (not shown)

cis double bond between the 1′ and 2′ carbon of the 1-alkyl residue (Fig. 5b). In myxobacteria, a gene encoding the multifunctional enzyme ElbD is required for ether lipid biosynthesis, fruiting body formation and sporulation (Lorenzen et al. 2014). However, other structural genes or enzyme activities for bacterial plasmalogen biosynthesis are unknown to date. It has been suggested that plasmalogens permit a closer packing of membranes than diacyl phospholipids and therefore decrease lipid mobility within the bilayer (Goldfine 2010).

6 Conclusions and Research Needs

Upon phosphorus limitation, membrane phospholipids are replaced in many environmental bacteria by membrane lipids (glycolipids, sulfolipids, betaine lipids, ornithine lipids) that do not contain phosphorus in their structure. This membrane lipid remodeling is an active process which involves partial phospholipid degradation by a phospholipase C (Zavaleta-Pastor et al. 2010) and formation of the phosphorus-free membrane lipids. Obviously the question arises whether this shift in membrane lipid composition is reversible, i.e., when the physiological conditions are reversed. Is there an active degradation of phosphorus-free membrane lipids when bacteria that had been limited for phosphorus are cultivated in phosphorus-abundant culture media? Sulfolipids have been reported to serve as sulfur source in the algae *Chlamydomonas* upon sulfur limitation (Sugimoto et al. 2007). Several pathways for sulfolipid and sulfoquinovose degradation have been reported in recent years in which the externally supplied compounds can be completely degraded (Goddard-Borger and Williams 2017). However, are bacteria also capable to reconvert their intrinsic phosphorus-free membrane lipids to phospholipids again? Can ether-linked betaine lipids, such as DGTS, be degraded in phosphorus-abundant conditions?

Membrane lipids act in at least two ways. On one hand they form the lipid bilayer surrounding every cell, and on the other hand they interact with other biomolecules based on their distinct chemical nature. Bacteria produce an unusually wide spectrum of different membrane lipid classes, and for many of these lipids, important aspects on the genetics, biochemistry, and functionality are still unknown. Getting to a profound understanding of membrane protein functionality represents another bottleneck in biological research. Frequently, the function and crystallizability (in order to obtain structures) of membrane proteins depend on specific membrane lipid components and therefore make it also an imperative from this viewpoint to study bacterial membrane lipids in much more detail. Certain essential membrane lipids are made by pathways harboring enzymes unique to bacteria. Detecting specific inhibitors for these enzymes should lead to the development of next-generation antibiotics. Finally, membrane lipids of the bacterial cell surface, such as lipopolysaccharides, sphingolipids, ornithine-containing lipids, or distinct glycerol-based lipids, are of crucial importance for the interaction with eukaryotic hosts and therefore key to understand symbiotic or pathogenic processes.

Acknowledgments Research in our lab was supported by grants from Consejo Nacional de Ciencia y Tecnología-México (CONACyT-Mexico) (178359 and 253549 in Investigación Científica Básica as well as 118 in Investigación en Fronteras de la Ciencia) and from Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México (DGAPA-UNAM; PAPIIT IN202616, IN203612). We thank Angeles Moreno and Lourdes Martínez-Aguilar for their skillful technical assistance.

References

Arendt W, Groenewold MK, Hebecker S, Dickschat JS, Moser J (2013) Identification and characterization of a periplasmic aminoacyl-phosphatidylglycerol hydrolase responsible for *Pseudomonas aeruginosa* lipid homeostasis. J Biol Chem 288:24717–24730

Barák I, Muchová K, Wilkinson AJ, O'Toole PJ, Pavlendová N (2008) Lipid spirals in *Bacillus subtilis* and their role in cell division. Mol Microbiol 68:1315–1327

Benning C (1998) Biosynthesis and function of the sulfolipid sulfoquinovosyldiacylglycerol. Annu Rev Plant Physiol Plant Mol Biol 49:53–75

Benning C (2007) Questions remaining in sulfolipid biosynthesis: a historical perspective. Photosynth Res 92:199–203

Conover GM, Martinez-Morales F, Heidtman ML, Luo ZQ, Tang M, Chen C, Geiger O, Isberg RR (2008) Phosphatidylcholine synthesis is required for optimal function of *Legionella pneumophila* virulence determinants. Cell Microbiol 10:514–528

Cronan JE, Thomas J (2009) Bacterial fatty acid synthesis and its relationships with polyketide synthetic pathways. Methods Enzymol 459:395–433

Danne L, Aktas M, Gleichenhagen J, Grund N, Wagner D, Schwalbe H, Hoffknecht B, Metzler-Nolte N, Narberhaus F (2015) Membrane-binding mechanism of a bacterial phospholipid *N*-methyltransferase. Mol Microbiol 95:313–331

Dare K, Shepherd J, Roy H, Seveau S, Ibba M (2014) LysPGS formation in *Listeria monocytogenes* has broad roles in maintaining membrane integrity beyond antimicrobial peptide resistance. Virulence 5:534–546

Devers EA, Wewer V, Dombrink I, Dörmann P, Hölzl G (2011) A processive glycosyltransferase involved in glycolipid synthesis during phosphate deprivation in *Mesorhizobium loti*. J Bacteriol 193:1377–1384

Dowhan W, Bogdanov M, Mileykovskaya E (2008) Functional roles of lipids in membranes. In: Vance DE, Vance JE (eds) Biochemistry of lipids, lipoproteins and membranes, 5th edn. Elsevier, Amsterdam, pp 1–37

Ernst CM, Staubitz P, Mishra NN, Yang SJ, Hornig G et al (2009) The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog 5:e1000660

Garrett TA (2017) Major roles for minor bacterial lipids identified by mass spectrometry. Biochim Biophys Acta 1862:1319–1324

Geiger O, López-Lara IM, Sohlenkamp C (2013) Phosphatidylcholine biosynthesis and function in bacteria. Biochim Biophys Acta 1831:503–513

Goddard-Borger ED, Williams SJ (2017) Sulfoquinovose in the biosphere: occurrence, metabolism and functions. Biochem J 474:827–849

Goldfine H (2010) The appearance, disappearance and reappearance of plasmalogens in evolution. Prog Lipid Res 49:493–498

Goldfine H (2017) The anaerobic biosynthesis of plasmalogens. FEMS Lett 591:2714–2719

Gómez-Lunar Z, Hernández-González I, Rodriguez-Torres MD, Souza V, Olmedo-Álvarez G (2016) Microevolution analysis of *Bacillus coahuilensis* unveils differences in phosphorus acquisition strategies and their regulation. Front Microbiol 7:58

Gopalakrishnan AS, Chen YC, Temkin M, Dowhan W (1986) Structure and expression of the gene locus encoding the phosphatidylglycerophosphate synthase of *Escherichia coli*. J Biol Chem 261:1329–1338

Hacker S, Sohlenkamp C, Aktas M, Geiger O, Narberhaus F (2008) Multiple phospholipid *N*-methyltransferases with distinct substrate specificities are encoded in *Bradyrhizobium japonicum*. J Bacteriol 190:571–580

Henderson JC, Zimmerman SM, Crofts AA, Boll JM, Kuhns LG, Herrera CM, Trent MS (2016) The power of asymmetry: architecture and assembly of the Gram-negative outer membrane lipid bilayer. Annu Rev Microbiol 70:255–278

Hoffmann C, Leis A, Niederweis M, Plitzko JM, Engelhardt H (2008) Disclosure of the mycobacterial outer membrane. Proc Natl Acad Sci USA 105:3963–3967

Hölzl G, Dörmann P (2007) Structure and function of glycoglycerolipids in plants and bacteria. Prog Lipid Res 46:225–243

Jackson M, Crick DC, Brennan PJ (2000) Phosphatidylinositol is an essential phospholipid in mycobacteria. J Biol Chem 275:30092–30099

Jorasch P, Wolter FP, Zähringer U, Heinz E (1998) A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of *ypfP* in *Escherichia coli* and structural analysis of its reaction products. Mol Microbiol 29:419–430

Jorge CD, Borges N, Santos H (2015) A novel pathway for the synthesis of inositol phospholipids uses cytidine diphosphate (CDP)-inositol as donor of the polar head group. Environ Microbiol 17:2492–2504

Kanfer J, Kennedy EP (1964) Metabolism and function of bacterial lipids II. Biosynthesis of phospholipids in *Escherichia coli*. J Biol Chem 239:1720–1726

Kent C, Gee P, Lee SY, Bian X, Fenno JC (2004) A CDP-choline pathway for phosphatidylcholine biosynthesis in *Treponema denticola*. Mol Microbiol 51:471–481

Klement MLR, Öjemyr L, Tagscherer KE, Widmalm G, Wieslander Å (2007) A processive lipid glycosyltransferase in the small human pathogen *Mycoplasma pneumoniae*: involvement in host immune response. Mol Microbiol 65:1444–1457

Krol E, Becker A (2004) Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. Mol Genet Genomics 272:1–17

Lopez D (2015) Molecular composition of functional microdomains in bacterial membranes. Chem Phys Lipids 192:3–11

López-Lara IM, Geiger O (2017) Bacterial lipid diversity. Biochim Biophys Acta 1862:1287–1299

López-Lara IM, Sohlenkamp C, Geiger O (2003) Membrane lipids in plant-associated bacteria: their biosyntheses and possible functions. Mol Plant-Microbe Interact 16:567–579

Lorenzen W, Ahrendt T, Bozhuyuk KAJ, Bode HB (2014) A multifunctional enzyme is involved in bacterial ether lipid biosynthesis. Nature Chem Biol 10:425–427

Lu Y-J, ZhangY-M GKD, Qi J, Lee RE, Rock CO (2006) Acyl-phosphates initiate membrane phospholipid synthesis in Gram-positive pathogens. Mol Cell 23:765–772

Lu YH, Guan Z, Zhao J, Raetz CR (2011) Three phosphatidylglycerol-phosphate phosphatases in the inner membrane of *Escherichia coli*. J Biol Chem 286:5506–5518

Matsumoto K, Kusaka J, Nishibori A, Hara H (2006) Lipid domains in bacterial membranes. Mol Microbiol 61:1110–1117

Morii H, Ogawa M, Fukuda K, Taniguchi H, Koga Y (2010) A revised biosynthetic pathway for phosphatidylinositol in *Mycobacteria*. J Biochem 148:593–602

Moser R, Aktas M, Narberhaus F (2014a) Phosphatidylcholine biosynthesis in *Xanthomonas campestris* via a yeast-like acylation pathway. Mol Microbiol 91:736–750

Moser R, Aktas M, Fritz C, Narberhaus F (2014b) Discovery of a bifunctional cardiolipin/phosphatidylethanolamine synthase in bacteria. Mol Microbiol 92:959–972

Nelson DL, Cox MM (2017) Lehninger - Principles of Biochemistry, 7th edn. WH Freeman and Company, New York

Nishijima S, Asami Y, Uetake N, Yamagoe S, Ohta A, Shibuya I (1988) Disruption of the *Escherichia coli cls* gene responsible for cardiolipin synthesis. J Bacteriol 170:775–780

Raetz CRH (1986) Molecular genetics of membrane phospholipids synthesis. Annu Rev Genet 20:253–295

Raetz CRH, Newman KF (1978) Neutral lipid accumulation in the membranes of *Escherichia coli* mutants lacking diglyceride kinase. J Biol Chem 253:3882–3887

Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in Gram-negative bacteria. Annu Rev Biochem 76:295–329

Riekhof WR, Andre C, Benning C (2005) Two enzymes, BtaA and BtaB, are sufficient for betaine lipid biosynthesis in bacteria. Arch Biochem Biophys 441:96–105

Rock CO (2008) Fatty acids and phospholipids metabolism in prokaryotes. In: Vance DE, Vance JE (eds) Biochemistry of lipids, lipoproteins and membranes, 5th edn. Elsevier, Amsterdam, pp 59–96

Romantsov T, Culham DE, Caplan T, Garner J, Hodges RS, Wood JM (2017) ProP-ProP and ProP-phospholipid interactions determine the subcellular distribution of osmosensing transporter ProP in *Escherichia coli*. Mol Microbiol 103:469–482

Sahonero-Canavesi DX, López-Lara IM, Geiger O (2017) Membrane lipid degradation and lipid cycles in microbes. In: Rojo F (ed) Aerobic utilization of hydrocarbons, oils and lipids, Handbook of hydrocarbon and lipid microbiology, Springer International Publishing AG. https://doi.org/10.1007/978-3-319-39782-5_38-1

Sandoval-Calderón M, Geiger O, Guan Z, Barona-Gómez F, Sohlenkamp C (2009) A eukaryote-like cardiolipin synthase is present in *Streptomyces coelicolor* and in most actinobacteria. J Biol Chem 284:17383–17390

Shimojima M (2011) Biosynthesis and functions of the plant sulfolipid. Prog Lipid Res 50:234–239

Slavetinsky C, Kuhn S, Peschel A (2017) Bacterial aminoacyl phospholipids – biosynthesis and role in basic cellular processes and pathogenicity. Biochim Biophys Acta 1862:1310–1318

Smith AM, Harrison JS, Grube CD, Sheppe AEF, Sahara N, Ishii R, Nureki O, Roy H (2015) tRNA-dependent alanylation of diacylglycerol and phosphatidylglycerol in *Corynebacterium glutamicum*. Mol Microbiol 98:681–693

Sohlenkamp C, Geiger O (2016) Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev 40:133–159

Sohlenkamp C, López-Lara IM, Geiger O (2003) Biosynthesis of phosphatidylcholine in bacteria. Prog Lipid Res 42:115–162

Sohlenkamp C, de Rudder KE, Geiger O (2004) Phosphatidylethanolamine is not essential for growth of *Sinorhizobium meliloti* on complex culture media. J Bacteriol 186:1667–1677

Sohlenkamp C, Galindo-Lagunas KA, Guan Z, Vinuesa P, Robinson S, Thomas Oates J, Raetz CRH, Geiger O (2007) The lipid lysyl-phosphatidylglycerol is present in membranes of *Rhizobium tropici* CIAT899 and confers increased resistance to polymyxin B under acidic growth conditions. Mol Plant-Microbe Interact 20:1421–1430

Sugimoto K, Sato N, Tsuzuki M (2007) Utilization of a chloroplast membrane sulfolipid as a major internal sulfur source for protein synthesis in the early phase of sulfur starvation in *Chlamydomonas reinhardtii*. FEBS Lett 581:4519–4522

Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CR, Guan Z (2012) Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci USA 109:16504–16509

Vences-Guzmán MA, Goetting-Minesky MP, Guan Z, Castillo-Ramírez S, Córdoba-Castro LA, López-Lara IM, Geiger O, Sohlenkamp C, Fenno JC (2017) 1,2-Diacylglycerol choline phosphotransferase catalyzes the final step in the unique *Treponema denticola* phosphatidylcholine biosynthesis pathway. Mol Microbiol 103:896–912

Vinuesa P, Neumann-Silkow F, Pacios-Bras C, Spaink HP, Martínez-Romero E, Werner D (2003) Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. Mol Plant-Microbe Interact 16:159–168

Yao Y, Rock CO (2013) Phosphatidic acid synthesis in bacteria. Biochim Biophys Acta 1831:495–502

Zavaleta-Pastor M, Sohlenkamp C, Gao JL, Guan Z, Zaheer R, Finan TM, Raetz CRH, López-Lara IM, Geiger O (2010) *Sinorhizobium meliloti* phospholipase C required for lipid remodeling during phosphorus limitation. Proc Natl Acad Sci USA 107:302–307

Zhang YM, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nature Rev Microbiol 6:222–233

Ornithine Lipids and Other Amino Acid-Containing Acyloxyacyl Lipids

6

Christian Sohlenkamp

Contents

1 Introduction 110
2 Formation of Amino Acid-Containing Acyloxyacyl Lipids 110
2.1 Synthesis of Ornithine Lipid 110
2.2 Modification of OLs 113
2.3 Presence and Synthesis of Other Amino Acid-Containing Acyloxyacyl Lipids 114
3 Functions of Amino Acid-Containing Acyloxyacyl Lipids 115
4 Research Needs 118
References 118

Abstract

Ornithine lipids (OLs) are phosphorus-free membrane lipids relatively common in eubacteria, but apparently absent from archaea and eukaryotes. It has been predicted that about 50% of the sequenced bacterial species have the capacity to synthesize OLs at least under certain growth conditions. Structurally, they are composed of a 3-hydroxy fatty acid amide bound to the α-amino group of ornithine and of a second fatty acyl group ester linked to the 3-hydroxy position of the first fatty acid forming an acyloxyacyl structure. This basic structure of OLs can be modified by hydroxylations in different positions, by *N*-methylation, or by taurine transfer. The presence of OL and/or modified OLs often seems to form part of a stress response to (changing) environmental conditions. OL modification allows the bacteria to adjust membrane properties by converting already existing membrane lipids into membrane lipids with distinct properties without de novo synthesis. In addition to ornithine, other amino acids (and dipeptides) such as

C. Sohlenkamp (✉)
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico
e-mail: chsohlen@ccg.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_13

glycine, serineglycine, glutamine, and lysine have been described as headgroups of these lipids in some bacterial species.

1 Introduction

A large variety of membrane lipid structures have been shown to exist in bacteria. Usually, glycerophospholipids such as phosphatidylethanolamine (PE), phosphatidylglycerol, phosphatidylcholine, or cardiolipin are the primary components of membranes, but other amphiphilic lipids are also present in the lipid bilayer. One way to classify these alternative membrane lipids is by their structure: Some, such as diacylglycerol-based glycolipids, the sulfolipid sulfoquinovosyl diacylglycerol, or the betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS) share a diacylglycerol (DAG) backbone (Hölzl and Dörmann 2007; Geiger et al. 2010), but others such as hopanoids, ornithine lipids, or sphingolipids lack this DAG backbone (Geiger et al. 2010; Sohlenkamp and Geiger 2016). In general, the synthesis pathways of glycerophospholipids are relatively well explored, whereas often, neither the biosynthesis nor the function of the alternative membrane lipids is understood.

One important class of these alternative membrane lipids is formed by amino lipids containing an acyloxyacyl structure: α-amino acids are *N*-acylated with a primary 3-hydroxy fatty acyl residue and a secondary fatty acid, also called "piggy-back" fatty acid, is ester-bound to the hydroxyl group of the first fatty acid. The most common amino acid present in this type of lipid is ornithine (Geiger et al. 2010; Sohlenkamp and Geiger 2016) (Fig. 1). Other headgroups that have been identified in this type of lipids are lysine, glycine, glutamine, serineglycine, and taurineornithine (Moore et al. 2016). Up to now, this type of lipids has been found only in eubacteria, but not in archaea or eukaryotes. In the last 15 years, a lot has been learned about the synthesis and modification of different OLs, but we know much less about the other amino lipids. In this chapter, I will focus on the synthesis of OL and other amino acyl lipids presenting an acyloxyacyl structure and discuss what is known about their formation and possible functions.

2 Formation of Amino Acid-Containing Acyloxyacyl Lipids

2.1 Synthesis of Ornithine Lipid

Although the capacity to synthesize ornithine lipids (OLs) is widespread among eubacteria, a pathway for OL formation has been described only relatively recently by Geiger and coworkers using the nodule-forming α-proteobacterium *Sinorhizobium meliloti* as a model. A population of chemical mutants was screened under conditions of phosphate limitation to identify mutants deficient in the

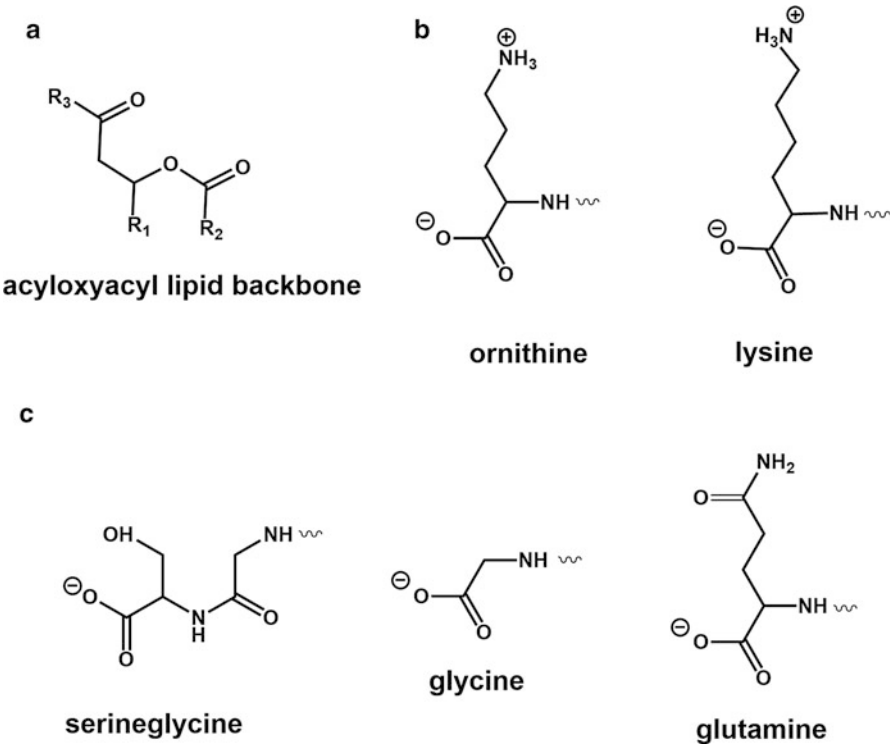


Fig. 1 Structures and headgroup variation of amino acid-containing acyloxylacyl lipids. Depending on which amino acid is present in the headgroup, the resulting lipid can be zwitterionic (ornithine, lysine) or anionic (glycine, serineglycine, glutamine). (**a**) acyloxyacyl backbone; (**b**) headgroups of zwitterionic lipids; (**c**) headgroups of anionic lipids. R_1 alkyl chain of the amide-bound 3-hydroxyfatty acid; R_2 alkyl chain of the ester-bound fatty acid; R_3 headgroups (shown in **a** and **b**)

synthesis of OL (Weissenmayer et al. 2002). Two genes encoding acyltransferases were found to be necessary to form OL in *S. meliloti*. The first step in OL synthesis is catalyzed by the *N*-acyltransferase OlsB which is responsible for the formation of lyso-OL (LOL) from ornithine and 3-hydroxyacyl-AcpP (Gao et al. 2004) (Fig. 2, reaction 1). The second step in OL synthesis is catalyzed by the *O*-acyltransferase OlsA, which is responsible for the formation of OL from LOL and acyl-AcpP (Weissenmayer et al. 2002) (Fig. 2, reaction 2). The OlsBA pathway is present mainly in α-, β-, and a few γ-proteobacteria and in some Gram-positive bacteria such as *Mycobacterium* and *Streptomyces* (Geiger et al. 2010; Sandoval-Calderón et al. 2015; Sohlenkamp and Geiger 2016). Based on the frequency of the presence of genes encoding OlsB, it has been estimated that about 25% of the sequenced bacteria have the OlsBA pathway for OL synthesis. A decade later, the bifunctional enzyme OlsF was identified in *Serratia proteamaculans* (Vences-Guzmán et al. 2015) (Fig. 2, reaction 3). The C-terminal domain of OlsF is responsible for its

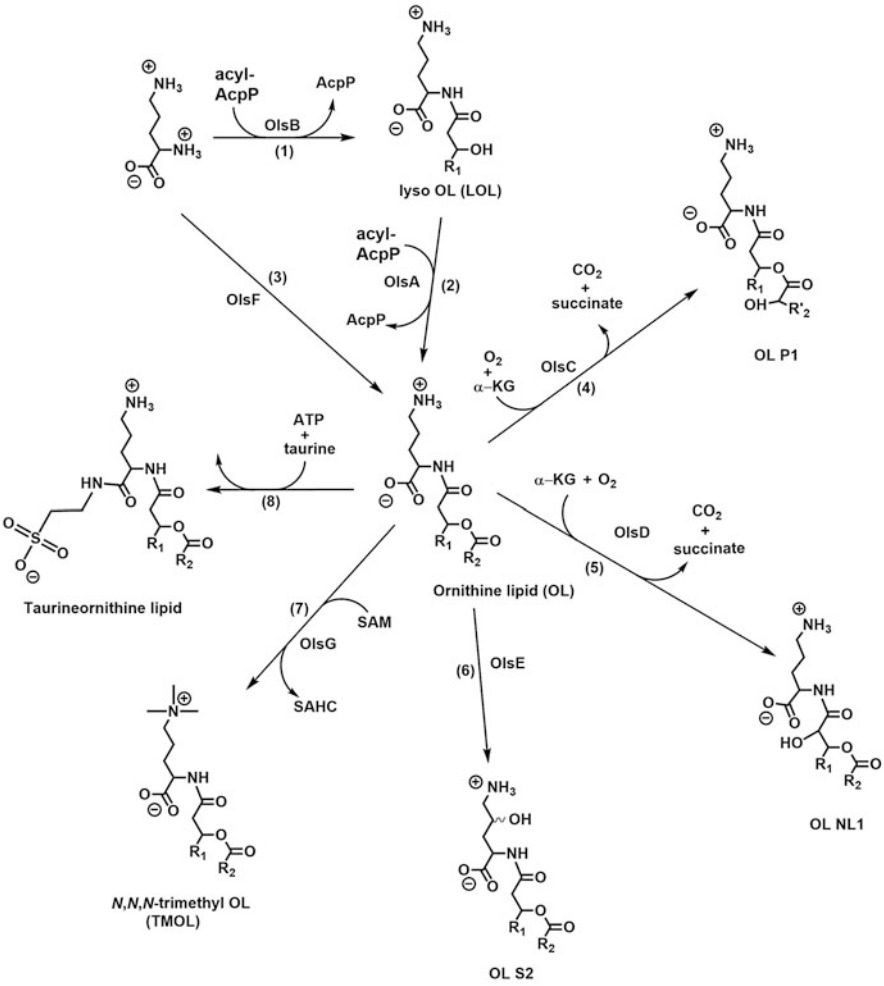


Fig. 2 Synthesis of OLs and their derivatives in bacteria. This figure shows the different known modifications of OL starting from the unmodified OL in the center of the figure. In many cases, OLs can also be subject to multiple modifications giving rise to structures not shown in the figure. The names of the OLs *S1* (substrate 1) and *S2* (substrate 2) originally described their roles as substrates in OlsC-dependent OL modifying reactions in *R. tropici*, whereas the *P1* described its role as a product of an OlsC-dependent OL modifying reaction. The lipid *NL1* (new lipid) described an unknown lipid in *B. cenocepacia*. *AcpP* constitutive acyl carrier protein; *α-KG* alpha-ketoglutarate; *SAM S*-adenosylmethionine; *SAHC S*-adenosylhomocysteine

N-acyltransferase activity and is distantly related to the *N*-acyltransferase OlsB. The N-terminal domain of OlsF is responsible for the *O*-acyltransferase reaction, but it is unrelated to the *O*-acyltransferase OlsA. Genes encoding OlsF homologues are present in about 25% of the sequenced bacterial species, mainly in γ-, δ-, and ε-proteobacteria and in bacteria of the CFB (Cytophaga-Flavobacterium-

Bacteroidetes) group. This means that about 50% of sequenced bacterial species are predicted to have the capacity to form OL at least under certain growth conditions. This number still might increase, because apparently there are OL-forming bacteria lacking genes encoding OlsBA or OlsF homologues, one example being the plantomycete *Singulisphaera acidiphila* (Moore et al. 2013; Escobedo-Hinojosa et al. 2015).

When searching sequence databases for the presence of genes encoding OlsBA or OlsF, the hits almost exclusively come from eubacterial genomes. One of the few exceptions is found in the genome sequence of the ant *Lasius niger*, where a gene encoding a good OlsF homologue can be detected. However, having a closer look at this case, it becomes clear that the contig from which this gene sequence is derived contains only bacterial sequences. The gene sequence encoding an OlsB homologue therefore most probably is derived from the genome of an endosymbiont or from contaminating bacterial DNA.

Diercks et al. (2015) showed recently that OL in *M. loti* contains 80–90% of D-ornithine. It is unclear where the D-ornithine is coming from and if D-ornithine is also present in OLs of other bacteria.

2.2 Modification of OLs

Many bacteria have the capacity to modify OL once it has been synthesized (Knoche and Shively 1972; Tahara et al. 1976b; Kawai et al. 1988; Asselineau 1991; Galbraith et al. 1999; Rojas-Jiménez et al. 2005; Lewenza et al. 2011; Moore et al. 2013; Vences-Guzmán et al. 2012; Sohlenkamp and Geiger 2016). So far hydroxylations in three different positions of OL have been described, in addition to the *N*-methylation of the δ-amino group and the modification of OL with taurine (Tahara et al. 1976b; Rojas-Jiménez et al. 2005; Gónzalez-Silva et al. 2011; Vences-Guzmán et al. 2011; Moore et al. 2013). It is not clear what the functional implications of these OL modifications might be, but one possible explanation is that modifying already existing membrane lipids allows for a quick response and adaptation to changing environmental conditions without de novo synthesis of lipids.

The 2-hydroxylation of the ester-bound fatty acid had been described already several years ago in different bacterial species such as *Gluconobacter cerinus*, *Burkholderia cenocepacia*, and *Flavobacterium* sp. (Fig. 2, reaction 4), and this is possibly the most widespread OL modification (Knoche and Shively 1972; Kawai et al. 1988; Asselineau 1991; Galbraith et al. 1999; Vences-Guzmán et al. 2012). During a search for genes involved in the response to acid stress in *Rhizobium tropici*, Rojas-Jiménez et al. (2005) identified the OL hydroxylase OlsC which is a homologue to the lipid A-hydroxylase LpxO from *Salmonella typhimurium* (Gibbons et al. 2000). Both enzymes belong to the same family of Fe^{2+}/O_2/α-ketoglutarate-dependent oxygenases. OlsC from *R. tropici* is responsible for the 2-hydroxylation of the ester-bound fatty acid in OL in this organism. The gene/enzyme responsible for introducing the same modification in *B. cenocepacia* and *Flavobacterium* sp. has not been identified yet.

Gónzalez-Silva et al. (2011) identified the OL hydroxylase OlsD from *Burkholderia cenocepacia* which is responsible for the hydroxylation of the amide-bound fatty acid (Fig. 2, reaction 5). OlsC from *R. tropici* and OlsD from *B. cenocepacia* are homologues. In addition to *Burkholderia* genomes, genes encoding OlsD homologues can be found in the genomes of *Serratia* sp. and *Mesorhizobium* sp. (Diercks et al. 2015; Vences-Guzmán et al. 2015). Diercks et al. (2015) showed that *M. loti* presents an OL hydroxylated in the 2-position of the amide-bound 3-hydroxy fatty acid.

Vences-Guzmán et al. (2011) identified the OL hydroxylase OlsE from *R. tropici* that is responsible for introducing a hydroxyl group into the ornithine headgroup of OLs (Fig. 2, reaction 6). The exact position of this hydroxylation is not known yet. OlsE belongs to the di-iron fatty acyl hydroxylase superfamily (cl01132) which includes fatty acid and carotene hydroxylases as well as sterol desaturases. Apart from *R. tropici,* OlsE activity has been detected in *Agrobacterium tumefaciens* (Vences-Guzmán et al. 2013).

Moore et al. (2013) had described a novel *N*-trimethylated OL in a few planctomycete species that had been isolated from *Sphagnum* bog. By expressing several candidate genes in an OL-forming *E. coli* strain, Escobedo-Hinojosa et al. (2015) identified the gene Sinac_1600 encoding the *N*-methyltransferase OlsG (Fig. 2, reaction 7). OlsG is a homologue of the PE *N*-methyltransferase PmtA from *S. meliloti* (de Rudder et al. 2000). Interestingly, OlsG is able to *N*-methylate PE in addition to OL when expressed in *E. coli*, thereby causing phosphatidylcholine formation.

Another OL modification that has been described a while ago in *Gluconobacter cerinus* is the ATP-dependent transfer of taurine to hydroxylated OL (Fig. 2, reaction 8) (Tahara et al. 1976b; Tahara et al. 1978). This lipid is also called cerilipin. The transferase responsible for cerilipin formation has not been identified yet, and the function of the taurineornithine lipid is not known. Recently, a draft genome of a *G. cerinus* strain has been published (Sainz et al. 2016), which should allow to search for candidate genes. Mass spectrometry data for cerilipin should also allow studying how widespread cerilipin formation is in bacteria (Moore et al. 2016).

2.3 Presence and Synthesis of Other Amino Acid-Containing Acyloxyacyl Lipids

OL is the most frequent of the amino acid-containing lipids presenting an acyloxylacyl structure. Several publications mainly from the 1970s and 1980s had shown that apart from OLs other structural homologues presenting other amino acid headgroups such as lysine, glycine, glutamine, and serineglycine exist. The presence of lysine lipids (LL) has been shown in *Agrobacterium tumefaciens*, *Pseudopedobacter saltans*, *Flavobacterium johnsoniae*, and *Rhodobacter sphaeroides* (Tahara et al. 1976a; Zhang et al. 2009; Moore et al. 2015a, 2016). In *P. saltans*, several hydroxylated versions of LL were detected (Moore et al. 2015a). The hydroxylation detected within the lysine headgroup would be the equivalent of the

hydroxylation introduced by OlsE into OL, and the 2-hydroxylation within the piggyback fatty acid of LL would be the equivalent of the hydroxylation introduced by OlsC into OL.

Glycine lipids (cytolipin) exist in *Cyclobacterium marinus*, *Pedobacter heparinus*, and *Cytophaga johnsonae* (Kawazoe et al. 1991; Batrakov et al. 1999; Moore et al. 2016). The presence of serineglycine lipids (flavolipin, L654) has been shown in *Flavobacterium meningosepticum*, *C. marinus* WH, *Porphyromonas gingivalis*, and *Porphyromonas endodontalis* (Kawai et al. 1988; Batrakov et al. 1998; Clark et al. 2013; Mirucki et al. 2014; Moore et al. 2016), and glutamine lipids are present in *Rhodobacter sphaeroides* (Zhang et al. 2009; Moore et al. 2016). Bacteria belonging to the CFB group seem to be most diverse with respect to the presence of amino acid diversity in the headgroup. Glutamine lipid is the only known amino acid-containing acyloxyacyl lipid that has not been detected in the CFB group yet.

To date, not much is known about the synthesis and distribution of these lipids. Recently, a study by Moore et al. (2016) confirms many of the earlier findings and detects many of these molecules for the first time by mass spectrometry (MS). MS technologies will allow a much quicker screening to learn about the presence of these lipids in other bacteria. It can be speculated that these lipids are synthesized by enzymes homologous to OlsBA or OlsF. For OlsF from *Flavobacterium johnsoniae*, it has been shown that upon expression in *E. coli*, lysine lipids (LLs) are formed in addition to OL (Vences-Guzmán et al. 2015). Moore et al. (2016) have also shown the presence of OL and LL in *F. johnsoniae*. Similarly, the fact that different hydroxylated versions of LL were identified suggests that hydroxylases such as OlsC or OlsE are responsible for LL hydroxylation. However, Moore et al. (2015a) could not identify good OlsC or OlsE homologues in the genome of *P. heparinus*. *Rhodobacter sphaeroides* is able to form glutamine lipids, OLs, and LLs and interestingly enough, it presents two genes encoding OlsB (OlsB1 and OlsB2) homologues (Geiger et al. 2010). OlsB1 might use lysine in addition to ornithine as substrate, whereas OlsB2 might be responsible for the formation of lyso-glutamine lipid. In a second step, these lysolipids would be converted to OL, LL, or glutamine lipid by an *O*-acyltransferase.

3 Functions of Amino Acid-Containing Acyloxyacyl Lipids

Mutants deficient in the formation and modification of OLs have been constructed in several species, but the phenotypes are not always clear and consistent. Often (but not always) the presence of (hydroxylated) OLs has been related to stress conditions. One of the best known examples for membrane remodeling occurs in some bacterial species under phosphate-limiting conditions (1), although the presence or absence of OLs can also affect the tolerance to other forms of abiotic stress such as low pH (2) or increased temperature (3). It has been suggested that trimethylated OLs are important for plantomycetes living at the anoxic/oxic interphase in *Sphagnum* bogs (4). In addition, the presence or absence of (hydroxylated) OLs can affect the

development of pathogenic or symbiotic interactions (5). Other nonstress related phenotypes related to the presence/absence of OL also have been observed (6):

1. In a few cases, such as *Pseudomonas aeruginosa*, *Sinorhizobium meliloti*, and *Serratia proteamaculans*, it has been observed that no OLs (or only minor amounts of OLs) are formed when bacteria are cultivated in complex growth media usually containing phosphate and other nutrients in excess. When cultivating these bacteria in growth media with limiting phosphate concentration, phosphorus-containing membrane lipids such as glycerophospholipids are substituted with phosphorus-free membrane lipids (Minnikin et al. 1972; Minnikin and Abdolrahimzadeh 1974; Minnikin et al. 1974; Benning et al. 1995; Geiger et al. 1999; Lewenza et al. 2011). It has been speculated that the zwitterionic PE is replaced by the zwitterionic OL (Benning et al. 1995). This membrane remodeling is regulated by the transcriptional regulator PhoB in *S. meliloti*, and in *S. proteamaculans* the *olsF* gene is also preceded by a pho box (Geiger et al. 1999; Vences-Guzmán et al. 2015). Interestingly, in other (often closely related) bacteria, OL formation is constitutive and is not restricted to conditions of phosphate limitation (Gónzalez-Silva et al. 2011; Palacios-Chaves et al. 2011; Vences-Guzmán et al. 2011, 2013; Sohlenkamp and Geiger 2016). Nevertheless, a decreased phosphate concentration in the growth medium seems to cause an increase in the OL content (Vences-Guzmán et al. 2011). It has been suggested that this remodeling allows to liberate phosphate bound within the glycerophospholipids of the membrane and to be used for other cellular processes such as nucleic acid formation. This capacity to remodel the membrane is probably an advantage for bacteria presenting different lifestyles (Zavaleta-Pastor et al. 2010). Remodeling could improve bacterial survival in phosphate-deplete habitats, whereas under conditions of sufficient phosphate, for example, in contact with a eukaryotic host, the bacterial membrane could be remodeled and allow an adaptation of the bacteria. The adaptation of phosphate-starved bacteria to phosphate-replete conditions has not been studied, and it is not clear if OLs or other phosphate-free membrane lipids are actively degraded under these conditions.
   Surprisingly, *S. meliloti* mutants deficient in OL formation grow as the wildtype under phosphate-limiting conditions. In order to observe a growth phenotype under these conditions, the OL deficiency has to be accompanied by DGTS deficiency (López-Lara et al. 2005). Similarly, a *S. proteamaculans* mutant deficient in OlsF that cannot synthesize OL grows similarly as the wildtype under phosphate-limiting conditions (Vences-Guzmán et al. 2015).
2. In different bacteria, OLs are enriched in the outer membrane (Dees and Shively 1982; Palacios-Chaves et al. 2011; Vences-Guzmán et al. 2011). For *Thiobacillus thiooxidans*, it has been suggested that the presence of OLs in the outer membrane might be related to its acid tolerance (Dees and Shively 1982). *R. tropici* accumulates increased amounts of 2-hydroxylated OL P1 (Fig. 2) when grown at pH 4.0, and *R. tropici* mutants deficient in OlsC grew much slower than the wildtype under these conditions (Vences-Guzmán et al. 2011). For sphingolipids and lipid A which

also can be hydroxylated in the 2-position, it has been speculated that the introduction of an additional hydroxyl group increases hydrogen bonding with neighboring molecules and leads to membrane stabilization (Nikaido 2003).

3. No increase in the formation of hydroxylated OLs can be observed when *R. tropici* is cultivated at 37 or 42 °C instead of 30 °C, but *R. tropici* deficient in the OL 2-hydroxylase OlsC grows much slower than the wildtype at 42 °C. Taylor et al. (1998) had observed an increase in the hydroxylated membrane lipids (which included hydroxylated OL and hydroxylated PE) in *B. cepacia* grown at 40 °C, supporting the idea that OL hydroxylation has a function at increased temperature. Again, the presence of an additional hydroxyl group might lead to membrane stabilization (see above).
4. Trimethylornithine lipids (TMOL) were recently described as abundant lipids in four isolates of *Sphagnum* wetland planctomycetes (*Gemmata*-like strain SP5, *Telmatocola sphagniphila*, *Singulisphaera rosea*, *Singulisphaera acidiphila*) (Moore et al. 2013). Later, Moore et al. (2015b) could show that TMOLs accumulated at the oxic/anoxic interphase in *Sphagnum* bogs and that the presence of TMOL correlated with an enrichment of the planctomycete community at this depth. This suggested that TMOLs were synthesized by planctomycetes in the bog as a response to changing redox conditions at the oxic/anoxic interphase (Moore et al. 2015b).
5. In some (but not all) cases, mutants deficient in OL formation or modification are affected during host-microbe interactions. *S. meliloti* mutants deficient in OL formation form functional nodules on their host plant alfalfa. In *Brucella*, OLs are dispensable for the development of pathogenicity (Palacios-Chaves et al. 2011). In contrast, *A. tumefaciens* mutants deficient in OL formation or OL modification show accelerated tumor formation during infection of potato tuber discs (Vences-Guzmán et al. 2013). *R. tropici* deficient in OL hydroxylation induces the formation of nodules on its host plant common bean that are defective in biological nitrogen fixation (Vences-Guzmán et al. 2011).
6. For OL and serineglycine lipid (flavolipin, L654), it has been published that they cause an immune response. The bacterial endotoxin lipid A presents an acyloxyacyl structure and is recognized by Toll-like receptor 4 (TLR4) as pathogen-associated molecular pattern. This acyloxyacyl structure is also present in OL and flavolipin/L654 (and the other amino acid-containing lipids discussed in this chapter). Lipid A binds to the co-receptor MD-2 (myeloid differentiation factor 2) and as a complex they bind to TLR4 in order to activate TLR4 signaling. The activation of the TLR4/MD-2 complex triggers the innate immune response of mammals and the biosynthesis of inflammatory cytokines such as TNF-α, IL-1, and IL-6 (Molinaro et al. 2015). Kawai et al. (1988) had shown that OL and flavolipin cause inflammatory immune responses measuring the formation of PGE_2, IL-1β, and TNF-α by macrophages. It was also shown that both molecules could be used as adjuvants and that they when injected into mice before exposure to lipid A prevent lethal effects of bacterial endotoxin (Kawai et al. 1991a, b, 1999, 2000a, b, 2002; Kato and Goto 1997). Earlier it had been suggested that the response to flavolipin is also transduced via the TLR4/MD-2 receptor (Gomi et al.

2002), but recently it was shown that serineglycine lipid L654 (which is structurally identical to flavolipin) is a ligand of human and mouse Toll-like receptor 2 (TLR2). The binding of L654 inhibits osteoblast differentiation, and it has been concluded that L654 and its corresponding lysolipid L430 have the potential to promote TLR2 dependent bone loss as reported in experimental periodontitis (Wang et al. 2015). Recently, L654 has been detected in human blood samples and interestingly multiple sclerosis patients had significant lower L654 levels than healthy patients (Farrokhi et al. 2013). In this context, L654 has been proposed as a microbiome-associated biomarker for multiple sclerosis (Farrokhi et al. 2013), and L654 administration to mice has been accompanied by an attenuation of autoimmune disease (Anstadt et al. 2016).

7. The presence of OLs in *R. capsulatus* is required for cytochrome maturation and optimal steady-state amounts of *c*-type cytochromes (Aygun-Sunar et al. 2006).
8. With the exception of the immunological studies with flavolipin/L654, nothing is known about the possible functions of (non-ornithine) amino acid-containing acyloxyacyl lipids.

4 Research Needs

Our knowledge about the synthesis and function of amino acid-containing acyloxyacyl lipids has advanced a lot in recent years. One important question to answer is: Why is there such a diversity of amino lipids? Using mass spectrometry, we can learn more about distribution of these amino lipids in the environment and in parallel possibly identify new lipids/structures. Once knowing what is out there, we can set out to discover the genes/enzymes involved in amino lipid formation and modification. Finally, mutants deficient in amino lipid formation or modification can be constructed to learn about the physiological roles of these lipids.

Acknowledgments Work in the laboratory was supported by grants to C.S. from SEP-CONACyT (237713) and PAPIIT-UNAM (IN202413, IN208116).

References

Anstadt EJ, Fujiwara M, Wasko N, Nichols F, Clark RB (2016) TLR tolerance as a treatment for central nervous system autoimmunity. J Immunol 197:2110–2118

Asselineau J (1991) Bacterial lipids containing amino acids or peptides linked by amide bonds. Fortschr Chem Org Naturst 56:1–85

Aygun-Sunar S, Mandaci S, Koch HG, Murria IVJ, Goldfine H, Daldai F (2006) Ornithine lipid is required for optimal steady-state amounts of *c*-type cytochromes in *Rhodobacter capsulatus*. Mol Microbiol 61:418–435

Batrakov SG, Nikitin DI, Sheichenko VI, Ruzhitsky AO (1998) A novel sulfonic-acid analogue of ceramide is the major extractable lipid of the Gram-negative marine bacterium *Cyclobacterium marinus* WH. Biochim Biophys Acta 1391:79–91

Batrakov SG, Nikitin DI, Mosezhnyi AE, Ruzhitsky AO (1999) A glycine-containing phosphorus-free lipoaminoacid from the Gram-negative marine bacterium *Cyclobacterium marinus* WH. Chem Phys Lipids 99:139–143

Benning C, Huang ZH, Gage DA (1995) Accumulation of a novel glycolipid and a betaine lipid in cells of *Rhodobacter sphaeroides* grown under phosphate limitation. Arch Biochem Biophys 317:103–111

Clark RB, Cervantes JL, Maciejewski MW, Farrokhi V, Nemati R, Yao X, Anstadt E, Fujiwara M, Wright KT, Riddle C, La Vake CJ, Salazar JC, Finegold S, Nichols FC (2013) Serine lipids of *Porphyromonas gingivalis* are human and mouse toll-like receptor 2 ligands. Infect Immun 81:3479–3489

Dees C, Shively JM (1982) Localization and quantitation of the ornithine lipid of *Thiobacillus thiooxidans*. J Bacteriol 149:798–799

Diercks H, Semeniuk A, Gisch N, Moll H, Duda KA, Hölzl G (2015) Accumulation of novel glycolipids and ornithine lipids in *Mesorhizobium loti* under phosphate deprivation. J Bacteriol 197:497–509

Escobedo-Hinojosa WI, Vences-Guzmán MA, Schubotz F, Sandoval-Calderón M, Summons RE, López-Lara IM, Geiger O, Sohlenkamp C (2015) OlsG (Sinac_1600) is an ornithine lipid *N*-methyltransferase from the planctomycete *Singulisphaera acidophila*. J Biol Chem 290:15102–15111

Farrokhi V, Nemati R, Nichols FC, Yao X, Anstadt E, Fujiwara M, Grady J, Wakefield D, Castro W, Donaldson J, Clark RB (2013) Bacterial lipodipeptide, lipid 654, is a microbiome-associated biomarker for multiple sclerosis. Clin Transl Immunol 2:e8

Galbraith L, Jonsson MH, Rudhe LC, Wilkinson SG (1999) Lipids and fatty acids of *Burkholderia* and *Ralstonia* species. FEMS Microbiol Lett 173:359–364

Gao JL, Weissenmayer B, Taylor AM, Thomas-Oates J, López-Lara IM, Geiger O (2004) Identification of a gene required for the formation of lyso-ornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. Mol Microbiol 53:1757–1770

Geiger O, Röhrs V, Weissenmayer B, Finan TM, Thomas-Oates JE (1999) The regulator gene *phoB* mediates phosphate stress-controlled synthesis of the membrane lipid diacylglycerol-*N*, *N*,*N*-trimethylhomoserine in *Rhizobium* (*Sinorhizobium*) *meliloti*. Mol Microbiol 32:63–73

Geiger O, Gónzalez-Silva N, López-Lara IM, Sohlenkamp C (2010) Amino acid-containing membrane lipids in bacteria. Prog Lipid Res 49:46–60

Gibbons HS, Lin S, Cotter RJ, Raetz CRH (2000) Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, a new Fe(II)/alpha-ketoglutarate-dependent dioxygenase homologue. J Biol Chem 275:32940–32949

Gomi K, Kawasaki K, Kawai Y, Shiozaki M, Nishijima M (2002) Toll-like receptor 4-MD-2 complex mediates the signal transduction induced by flavolipin, an amino acid-containing lipid unique to *Flavobacterium meningosepticum*. J Immunol 168:2939–2943

Gónzalez-Silva N, López-Lara IM, Reyes-Lamothe R, Taylor AM, Sumpton D, Thomas-Oates J, Geiger O (2011) The dioxygenase-encoding *olsD* gene from *Burkholderia cenocepacia* causes the hydroxylation of the amide-linked fatty acyl moiety of ornithine-containing membrane lipids. Biochemistry 50:6396–6408

Hölzl G, Dörmann P (2007) Structure and function of glycoglycerolipids in plants and bacteria. Prog Lipid Res 46:225–246

Kato H, Goto N (1997) Adjuvanticity of an ornithine-containing lipid of *Flavobacterium meningosepticum* as a candidate vaccine adjuvant. Microbiol Immunol 41:101–106

Kawai Y, Yano I, Kaneda K (1988) Various kinds of lipoamino acids including a novel serine-containing lipid in an opportunistic pathogen *Flavobacterium*. Their structures and biological activities on erythrocytes. Eur J Biochem 171:73–80

Kawai Y, Kaneda K, Morisawa Y, Akagawa K (1991a) Protection of mice from lethal endotoxemia by use of an ornithine-containing lipid or a serine-containing lipid. Infect Immun 59:2560–2566. (Erratum: Infect Immunol 1992, 60:320)

Kawai Y, Kamoshita K, Akagawa K (1991b) B-lymphocyte mitogenicity and adjuvanticity of an ornithine-containing lipid or a serine-containing lipid. FEMS Microbiol Lett 67:127–129

Kawai Y, Nakagawa Y, Matuyama T, Akagawa K, Itagawa K, Eukase K, Kusumoto S, Nishijima M, Yano I (1999) A typical bacterial ornithine-containing lipid N^{α}-(D)-[3-(hexadecanoyloxy)hexadecanoyl]-ornithine is a strong stimulant for macrophages and a useful adjuvant. FEMS Immunol Med Microbiol 23:67–73

Kawai Y, Takasuka N, Inoue K, Akagawa K, Nishijima M (2000a) Ornithine-containing lipids stimulate CD14-dependent TNF-α production from murine macrophage-like J7774.1 and RAW 264.7 cells. FEMS Immunol Med Microbiol 28:197–203

Kawai Y, Okawarab AI, Okuyama H, Kura F, Suzuki K (2000b) Modulation of chemotaxis, O_2^- production and myeloperoxidase release from human polymorphonuclear leukocytes by the ornithine-containing lipid and the serineglycine-containing lipid of *Flavobacterium*. FEMS Immunol Med Microbiol 28:205–209

Kawai Y, Watanabe M, Matsuura M, Nishijima M, Kawahara K (2002) The partially degraded lipopolysaccharide of *Burkholderia cepacia* and ornithine-containing lipids derived from some Gram-negative bacteria are useful complex lipid adjuvants. FEMS Immunol Med Microbiol 34:173–179

Kawazoe R, Okuyama H, Reichardt W, Sasaki S (1991) Phospholipids and a novel glycine-containing lipoamino acid in *Cytophaga johnsonae* Stanier strain C21. J Bacteriol 173:5470–5475

Knoche HW, Shively JM (1972) The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans*. J Biol Chem 247:170–178

Lewenza S, Falsafi R, Bains M, Rohs P, Stupak J, Sprott GD, Hancock RE (2011) The *olsA* gene mediates the synthesis of an ornithine lipid in *Pseudomonas aeruginosa* during growth under phosphate-limiting conditions, but is not involved in antimicrobial peptide susceptibility. FEMS Microbiol Lett 320:95–102

López-Lara IM, Gao JL, Soto MJ, Solares-Pérez A, Weissenmayer B, Sohlenkamp C, Verroios GP, Thomas-Oates JE, Geiger O (2005) Phosphorus-free membrane lipids of *Sinorhizobium meliloti* are not required for the symbiosis with alfalfa but contribute to increased cell yields under phosphorus-limiting conditions of growth. Mol Plant Microbe Interact 18:973–982

Minnikin DE, Abdolrahimzadeh H (1974) The replacement of phosphatidylethanolamine and acidic phospholipids by an ornithine-amide lipid and a minor phosphorus-free lipid in *Pseudomonas fluorescence* NCMB 129. FEBS Lett 43:257–260

Minnikin DE, Abdolrahimzadeh H, Baddiley J (1972) Variation of polar lipid composition of *Bacillus subtilis* Marburg with different growth conditions. FEBS Lett 27:16–18

Minnikin DE, Abdolrahimzadeh H, Baddiley J (1974) Replacement of acidic phospholipids by acidic glycolipids in *Pseudomonas diminuta*. Nature 249:268–269

Mirucki CS, Abedi M, Jiang J, Zhu Q, Wang Y-H, Safavi KE, Clark RB, Nichols FC (2014) Basic activity of *Porphyromonas endodontalis* complex lipids. J Endod 40:1342–1348

Molinaro A, Holst O, Di Lorenzo F, Callaghan M, Nurisso A, D'Errico G, Zamyatina A, Peri F, Berisio R, Jerala R, Jiménez-Barbero J, Silipo A, Martín-Santamaría S (2015) Chemistry of Lipid A: at the heart of innate immunity. Chem Eur J 21:500–519

Moore EK, Hopmans EC, Rijpstra IC, Villanueva L, Dedysh SN, Kulichevskaya IS, Wienk H, Schoutsen F, Sinninghe Damsté JS (2013) Novel mono-, di-, and trimethylornithine membrane lipids in northern wetland planctomycetes. Environ Microbiol 79:6874–6884

Moore EK, Hopmans EC, Rijpstra IC, Sánchez-Andrea I, Villanueva L, Wienk H, Schoutsen F, Stams AJM, Sinninghe Damsté JS (2015a) Lysine and novel hydroxylysine lipids in soil bacteria: amino acid membrane lipid response to temperature and pH in *Pseudopedobacter saltans*. Front Microbiol 6:637

Moore EK, Villanueva L, Hopmans EC, Rijpstra IC, Mets A, Dedysh SN, Sinninghe Damsté JS (2015b) Abundant trimethylornithine lipids and specific gene sequences are indicative of

planctomycete importance at the oxic/anoxic interface in *Sphagnum*-dominated northern wetlands. Appl Environ Microbiol 81:6333–6344

Moore EK, Hopmans EC, Rijpstra IC, Villanueva L, Sinninghe Damsté JS (2016) Elucidation and identification of amino acid containing membrane lipids using liquid chromatography/high-resolution mass spectrometry. Rapid Commun Mass Spectrom 30:739–750

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

Palacios-Chaves L, Conde-Álvarez R, Gil-Ramírez Y, Zuñiga-Ripa A, Barquero-Calvo E, Chacón-Díaz C, Chaves-Olarte E, Arce-Gorvel V, Gorvel JP, Moreno E, de Miguel MJ, Grilló MJ, Moriyón I, Iriarte M (2011) *Brucella abortus* ornithine lipids are dispensable outer membrane components devoid of a marked pathogen-associated molecular pattern. PLoS ONE 6:e16030

Rojas-Jiménez K, Sohlenkamp C, Geiger O, Martínez-Romero E, Werner D, Vinuesa P (2005) A ClC chloride channel homolog and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance. Mol Plant-Microbe Interact 18:1175–1185

de Rudder KE, López-Lara IM, Geiger O (2000) Inactivation of the gene for phospholipid *N*-methyltransferase in *Sinorhizobium meliloti*: phosphatidylcholine is required for normal growth. Mol Microbiol 37:763–772

Sainz F, Mas A, Torija MJ (2016) Draft genome sequences of *Gluconobacter cerinus* CECT 9110 and *Gluconobacter japonicus* CECT 8443, acetic acid bacteria isolated from grape must. Genome Announc 4(3):e00621–e00616. https://doi.org/10.1128/genomeA.00621-16

Sandoval-Calderón M, Nguyen DD, Kapono CA, Herron P, Dorrestein PC, Sohlenkamp C (2015) Plasticity of *Streptomyces coelicolor* membrane composition under different growth conditions and during development. Front Microbiol 6:1465

Sohlenkamp C, Geiger O (2016) Bacterial membrane lipids: Diversity in structures and pathways. FEMS Microbiol Rev 40:133–159

Tahara Y, Yamada Y, Kondo K (1976a) New lysine-containing lipid isolated from *Agrobacterium tumefaciens*. Agric Biol Chem 40:1449–1450

Tahara Y, Kameda M, Yamada Y, Kondo K (1976b) New lipid – Ornithine and taurine-containing cerilipin. Agric Biol Chem 40:243–244

Tahara Y, Shinmoto K, Yamada Y, Kondo K (1978) Enzymatic synthesis of tauro-ornithine lipid in *Gluconobacter cerinus*. Agric Biol Chem 42:205–206

Taylor CJ, Anderson AJ, Wilkinson SG (1998) Phenotypic variation of lipid composition in *Burkholderia cepacia*: a response to increased growth temperature is a greater content of 2-hydroxy acids in phosphatidylethanolamine and ornithine amide lipid. Microbiology 144:1737–1745

Vences-Guzmán MA, Guan Z, Ormeño-Orrillo E, González-Silva N, López-Lara IM, Martínez-Romero E, Geiger O, Sohlenkamp C (2011) Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899. Mol Microbiol 79:1496–1514

Vences-Guzmán MA, Geiger O, Sohlenkamp C (2012) Ornithine lipids and their structural modifications: from A to E and beyond. FEMS Microbiol Lett 335:1–10

Vences-Guzmán MA, Guan Z, Bermúdez-Barrientos JR, Geiger O, Sohlenkamp C (2013) Agrobacteria lacking ornithine lipids induce more rapid tumor formation. Environ Microbiol 15:895–906

Vences-Guzmán MA, Guan Z, Escobedo-Hinojosa WI, Bermúdez-Barrientes JR, Geiger O, Sohlenkamp C (2015) Discovery of a bifunctional acyltransferase responsible for ornithine lipid synthesis in *Serratia proteamaculans*. Environ Microbiol 17:1487–1496

Wang Y-H, Nemati R, Anstadt E, Liu Y, Son Y, Zhu Q, Yao X, Clark RB, Rowe DW, Nichols FC (2015) Serine dipeptide lipids of *Porphyromonas gingivalis* inhibit osteoblast differentiation: relationship to Toll-like receptor 2. Bone 81:654–661

Weissenmayer B, Gao JL, López-Lara IM, Geiger O (2002) Identification of a gene required for the biosynthesis of ornithine-derived lipids. Mol Microbiol 45:721–733

Zavaleta-Pastor M, Sohlenkamp C, Gao JL, Guan Z, Zaheer R, Finan TM, Raetz CRH, López-Lara IM, Geiger O (2010) *Sinorhizobium meliloti* phospholipase C required for lipid remodeling during phosphorus limitation. Proc Natl Acad Sci USA 107:302–307

Zhang X, Ferguson-Miller SM, Reid GE (2009) Characterization of ornithine and glutamine lipids extracted from cell membranes of *Rhodobacter sphaeroides*. J Am Soc Mass Spectrom 20:198–212

Bacterial Sphingolipids and Sulfonolipids

7

Otto Geiger, Jonathan Padilla-Gómez, and Isabel M. López-Lara

Contents

1 Introduction 124
2 Bacterial Sphingolipids 125
2.1 Structures of Bacterial Sphingolipids 125
2.2 Biosynthesis of Bacterial Sphingolipids 125
2.3 Localization and Transport of Bacterial Sphingolipids 130
2.4 Function of Bacterial Sphingolipids 131
3 Sulfonolipids in the *Cytophaga* Group 132
4 Conclusions and Research Needs 134
References 134

Abstract

The bacterial envelope is often composed by two membranes: the inner or cytoplasmic membrane and an outer membrane. The inner membrane consists of a lipid bilayer with phospholipids covering the inner and the outer leaflet. Although the outer membrane displays a bilayer structure as well, only the inner leaflet of the outer membrane is composed of phospholipids, whereas the outer leaflet is typically formed by lipid A-containing lipopolysaccharides in Gram-negative bacteria. However, some bacteria lack lipopolysaccharides and have sphingolipids in the outer leaflet of their outer membrane instead. Sphingolipids are considered to be typical eukaryotic membrane lipids, essential components of

O. Geiger (✉) · J. Padilla-Gómez
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico
e-mail: otto@ccg.unam.mx; jpadilla@lcg.unam.mx

I. M. López-Lara
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, Mexico
e-mail: isabel@ccg.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_12

the plasma membrane, and are crucial for signaling and organization of lipid rafts in eukaryotes.

Although there is a considerable structural diversity within bacterial sphingolipids, very little is known about their biosynthesis, transport to the outer leaflet of the outer membrane, or their evolutionary history. Whereas bacterial sphingolipids seem to be important as an outermost protective layer in some bacteria, for the survival of symbiotic *Bacteroides* in humans, as virulence factors in some pathogenic bacteria, and maybe in fruiting body formation in myxobacteria, their biological functions are poorly understood on a molecular level.

Sulfonolipids are structural analogues of sphingolipids and seem to be important for gliding motility in *Cytophaga* species, but also as crucial bacterial factors that trigger multicellularity in choanoflagellates, the closest living relatives of animals.

1 Introduction

Sphingolipids (SphLs) are membrane-forming lipids and exist in most membranes of eukaryotes. In plasma membranes, they occur mainly in the outer leaflet and they play crucial roles in signaling and organizing lipid rafts (Nelson and Cox 2017). Members of the sphingolipid family of lipids, including sphingoid bases, sphingoid base phosphates, ceramides, and complex sphingolipids, serve vital functions in cell biology and are involved in the regulation of cell division, differentiation, migration, programmed cell death, and other processes (Nelson and Cox 2017). Sphingolipid biosynthesis, regulation, and function have been studied extensively in eukaryotic microorganisms, i.e., yeast, and these findings have been reviewed in some detail (Cowart and Obeid 2006; see Ren and Hannun, "▶ Chap. 18, "Metabolism and Roles of Sphingolipids in Yeast *Saccharomyces cerevisiae*," this volume).

Many Gram-negative bacteria possess two bilayered membranes in their envelope: the inner or cytoplasmic membrane (IM) and the outer membrane (OM). The IM is composed of (glycero)phospholipids (PLs) and proteins, whereas the OM harbors a different set of proteins, PLs at its inner leaflet, and the lipid A moiety of lipopolysaccharides (LPS) at its outer leaflet (Raetz and Dowhan 1990). However, since the early 1990s, it is clear that some Gram-negative bacteria lack LPS and seem to have SphLs instead in the outer leaflet of their OM (Kawazaki et al. 1994).

Other bacteria, i.e., the genus *Acetobacter*, may have both LPS and SphLs in their OM. In *Acetobacter malorum*, an increase of ceramide can be observed in acidic conditions of growth or at elevated temperatures (Ogawa et al. 2010), which suggests that under these conditions of stress, LPS is at least partially replaced by SphLs. Bacteria of the *Bacteroides* genus are predominantly in the human intestine, and many *Bacteroides* species have in addition to LPS (Weintraub et al. 1989) distinct phosphosphingolipids (Kato et al. 1995).

Overall, SphLs seem to occur only in few bacteria, particularly some anaerobes, where they functionally replace other bacterial membrane lipids. SphLs are found in the genera *Pedobacter* (Steyn et al. 1998), *Bacteroides*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Sphingomonas*, *Sphingobacterium*, *Bdellovibrio*, *Cystobacter*,

Mycoplasma, *Flectobacillus*, *Acetobacter* (Olsen and Jantzen 2001), *Bacteriovorax* (Jayasimhulu et al. 2007), *Sorangium* (Keck et al. 2011), and *Myxococcus* (Lorenzen et al. 2014). Initially, their occurrence in bacteria was thought so unusual that often the genus name of the respective bacterium harbors the prefix "Sphingo," i.e., in *Sphingomonas* and *Sphingobacterium*. However, as outlined below, SphLs might be more widespread in bacteria than originally thought. In this chapter, we cover structures of SphLs encountered in bacteria, present knowledge on bacterial SphLs biosynthesis, localization, as well as transport, and we report on their functions. We also give a brief overview on sulfonolipids, structural analogs of SphLs.

2 Bacterial Sphingolipids

2.1 Structures of Bacterial Sphingolipids

Some Gram-negative bacteria, such as *Sphingomonas capsulata*, lack LPS in their outer membrane and instead have glycosphingolipids (glyco-SphLs) as functional replacements. In *Sphingomonas paucomobilis*, two glyco-SphLs differing in their ceramide structures are substituted with the tetrasaccharide Man-Gal-GlcN-GlcA (Kawahara et al. 1991) (Fig. 1). The chirality at carbon atoms C-2 and C-3 of the sphingoid base is D-erythro (Olsen and Jantzen 2001; Hanada 2003). The variability of glyco-SphLs in the outer bacterial membrane of the *Sphingomonadaceae* is considerable (Kinjo et al. 2008), and only some of these glyco-SphLs, such as GSL-1 (Fig. 1), are recognized by natural killer T cells which provide an innate-type immune response towards glyco-SphL-containing bacteria (Kinjo et al. 2008; Wu et al. 2006). Major molecular species of ceramides in sphingobacteria have been identified as 2-*N*-2′-hydroxy-13′-methyltetradecanoyl-15-methylhexadecasphinganine, 2-*N*-13′-methyltetradecanoyl-15-methylhexadecasphinganine, and 2-*N*-13′-methyltetradecanoyl-hexadecasphinganine (Yano et al. 1982, 1983; Naka et al. 2003). Many *Bacteroides* species have two types of phosphosphingolipids, ceramide phosphoethanolamine, and ceramide phosphoglycerol (Kato et al. 1995).

Apparently, sphingolipids seem to be common components of myxobacterial membranes as ceramides were found in *Myxococcus xanthus* and in *Cystobacter fuscus*, ceramide phosphoinositol in *M. xanthus*, ceramide phosphoethanolamine in *Myxococcus stipitatus* (Lorenzen et al. 2014), β-D-glucosylsphingenine, and even phosphonosphingolipids in *Sorangium cellulosum* (Keck et al. 2011). Also, *Bacteriovorax* (formerly *Bdellovibrio*) *stolpii*, which lives as a parasite in the periplasmic space of larger Gram-negative bacteria, possesses an ample spectrum of phosphonosphingolipids (Jayasimhulu et al. 2007).

2.2 Biosynthesis of Bacterial Sphingolipids

In eukaryotes, the biosynthesis of sphingolipids takes place in five stages (Nelson and Cox 2017). It begins with the condensation of serine and a fatty

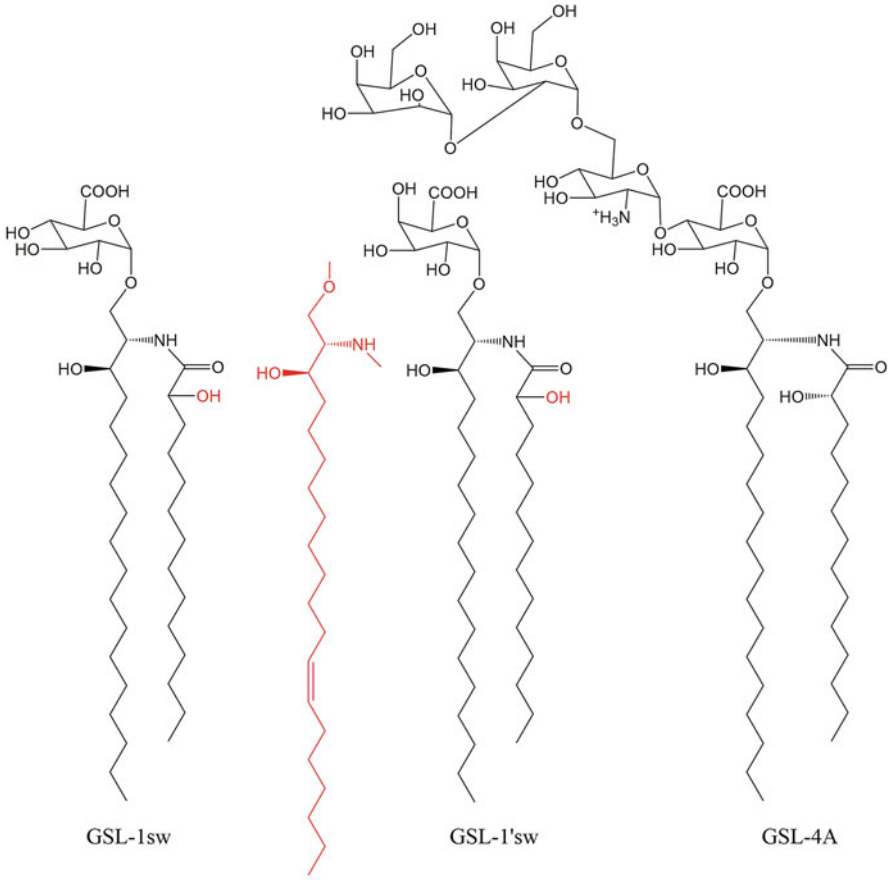


Fig. 1 Structures of sphingolipids GSL-1sw (α-glucuronosyl ceramide) and GSL-1′sw (α-galacturonosyl ceramide) from *Sphingomonas wittichii* (Kawahara et al. 2002) and GSL-4A from *Sphingomonas paucimobilis* (Kawahara et al. 1991). Structural variations in the amidified fatty acid (myristic acid or 2-OH-myristic acid) or in the dihydrosphingosine (2-amino-1,3-octadecandiol or 2-amino-*cis*-13,14-methylene-1,3-eicosandiol) residue of *S. wittichii* SphLs are highlighted

acyl-CoA to form 3-oxo-sphinganine (stage 1), followed by its reduction to sphinganine (stage 2), acylation to *N*-acylsphinganine (dihydroceramide) (stage 3), and desaturation to ceramide (stage 4) (Hanada 2003; Nieto et al. 2008). In stage 5, ceramide is modified with different polar groups to form the great diversity of sphingolipids. Although the eukaryotic genes involved in the sphingolipid biosynthetis are known (Kihara et al. 2007; Hirabayashi and Furuya 2008), little is known in bacteria. An exception is sphingolipid biosynthesis step 1 catalyzed by serine palmitoyltransferase (EC 2.3.1.50) (Fig. 2).

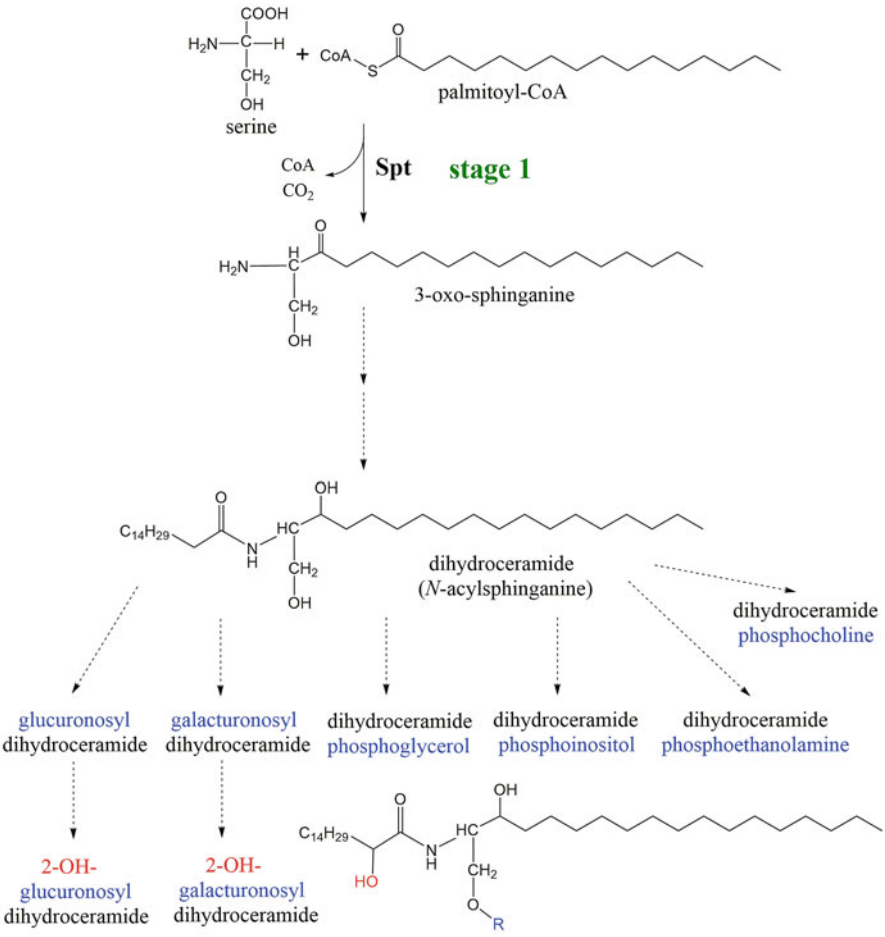


Fig. 2 Biosynthesis of sphingolipids in bacteria (see text for details). Head group modifications (R) and α-hydroxylation (2-OH) are highlighted

2.2.1 Bacterial Serine Palmitoyltransferase, an α-Oxoamine Synthase

Like other oxoamine synthases, the bacterial soluble serine palmitoyltransferase (Spt) is pyridoxal 5′- phosphate-dependent and performs a Claisen condensation between serine and the acyl-CoA thioester with a concomitant decarboxylation (Kerbarh et al. 2006). Although the Spt from *Sphingomonas* seems to be cytosolic, the Spts from *Sphingobacterium multivorum* and from *Bacteriovorax* (*Bdellovibrio*) *stolpii* are peripherically associated with the cytoplasmic side of the inner membrane (Ikushiro et al. 2001; Ikushiro et al. 2007). The *S. paucimobilis* Spt crystal structure (Yard et al. 2007) at 1.3 Å resolution shows that the enzyme is a symmetrical homodimer with two active sites composed of monomers, each consisting of three domains. The pyridoxal 5′-

phosphate cofactor is bound covalently to lysine 265 as an internal aldimine/Schiff base, and the active site is composed of residues from both subunits, located at the bottom of a deep cleft. Other common bacterial α-oxoamine synthases are 8-amino-7-oxononanoate synthase (BioF; EC 2.3.1.47), which catalyzes the formation of 8-amino-7-oxononanoate from 6-carboxyhexanoyl-CoA and L-alanine during biotin biosynthesis (Webster et al. 2000), 5-aminolevulinate synthase (HemA; EC 2.3.1.37), which catalyzes the formation of 5-aminolevulinate from succinyl-CoA and glycine during tetrapyrrole and heme biosynthesis in α-proteobacteria (Astner et al. 2005), and 2-amino-3-oxobutyrate coenzyme A ligase (Kbl; EC 2.3.1.29), which cleaves 2-amino-3-oxobutyrate into acetyl-CoA and glycine during threonine degradation (Kerbarh et al. 2006). Phylogenetic analysis of bacterial α-oxoamine synthases (Fig. 3) suggests that at least two distinct subgroups of bacterial Spts exist and that in one subgroup the encoding genes frequently form an operon with a putative acyl carrier protein gene. This finding suggests specialized acyl carrier proteins, instead of CoA, are used in some cases during the initial step of sphingolipid biosynthesis in bacteria (Geiger et al. 2010; Raman et al. 2010). Based on our analysis (Fig. 3), the ability to form SphLs is more widespread in α-proteobacteria (*Gluconobacter, Caulobacter*) than previously thought and might occur even in the β-proteobacterium *Nitrosomonas* and in several pathogenic *Escherichia coli* strains, i.e., in the enterotoxigenic *E. coli* (ETEC) B7A, but also in the host for protein expression *E. coli* BL21(DE3) (Jeong et al. 2015). Spts of the δ-proteobacteria *Bacteriovorax stolpii, Myxococcus xanthus*, or *Stigmatella aurantiaca* group with Spts of members of the *Bacteroidetes* (Wieland Brown et al. 2013) phylum (Fig. 3). Therefore, within the known bacterial phyla, genes for Spt have only been found in members of the *Proteobacteria* (α, β, γ, δ) or the *Bacteroidetes* which matches fairly well the reported occurrence of SphLs in these phyla. In eukaryotes, Spts are heterodimers consisting of distinct subunits (Ikushiro et al. 2007). All these eukaryotic subunits share a common origin (Ikushiro et al. 2007) (Fig. 3); however, they do not originate from within one of the known bacterial Spt clades (Fig. 3). Although recent work suggested that the homodimeric Spts (TgSPT1 and TgSPT2) of the eukaryotic protozoan parasite *Toxoplasma gondii* group more closely with bacterial Spts than with orthologues from animals, plants, or fungi (Mina et al. 2017), our phylogenetic tree (Fig. 3) does not support this idea. Therefore, a bacterial ancestor for eukaryotic Spts is presently not known.

2.2.2 Formation of Dihydroceramide

Although dihydroceramide is certainly formed in some bacteria, i.e., in *Acetobacter malorum* (Ogawa et al. 2010), *M. xanthus* (Lorenzen et al. 2014), or *Bacteroides fragilis* (Wieland Brown et al. 2013), it is not clear to date whether bacteria perform analogous steps as reported for stage 2 and 3 of eukaryotic sphingolipid biosynthesis in order to convert 3-oxo-sphinganine to dihydroceramide (Fig. 2).

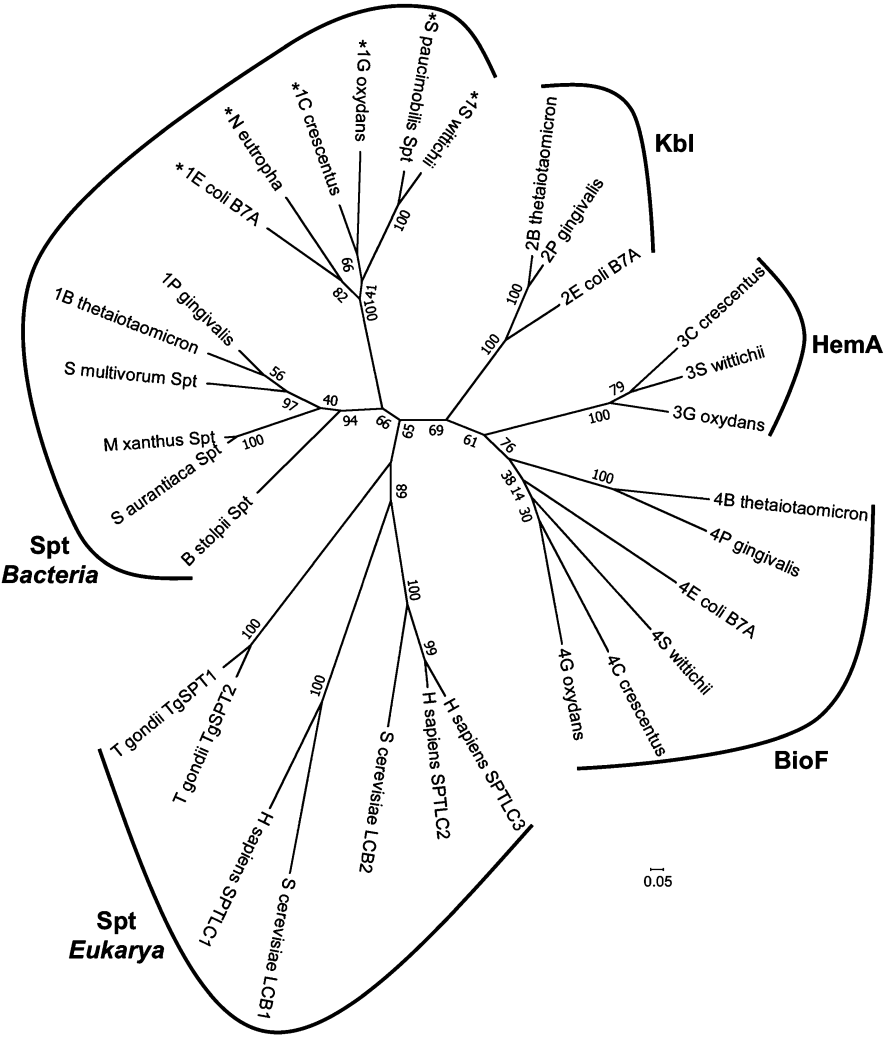


Fig. 3 Unrooted phylogenetic tree of selected bacterial and eukaryotic serine palmitoyltransferases (Spts) as well as other α-oxoamine synthases from bacteria. The amino acid sequences were aligned using the CLUSTALW program (http://www.expasy.ch/). The gap opening and extension parameters were set to 10 and 0.1, respectively. The tree was constructed using the program MEGA (http://www.megasoftware.net/) using the maximum likelihood method. Distances between sequences are expressed as 0.05 changes per amino acid residue. The number at each node represents the bootstrap value as a percentage of 500 replications. The asterisks label species in which the *spt* gene forms an operon with a putative structural gene for an acyl carrier protein. Accession numbers are as follows: *Bacteroides thetaiotaomicron* VPI-5482 (1: NP_809783; 2: NP_810284; 4: NP_810356), *Bacteriovorax stolpii* Spt (BAF73753), *Caulobacter crescentus* CB15 (1: NP_419978; 3: NP_420168; 4: NP_420387), *Escherichia coli* B7A (1: EDV60350; 2: ZP_03029941; 4: ZP_03030227), *Gluconobacter oxydans* 621H (1: AAW61792; 3: WP_011253160.1; 4: WP_011252295.1), *Homo sapiens* Spt subunits SPTLC1, SPTLC2, SPTLC3 (AAH68537, NP_004854, NP_060797), *Myxococcus xanthus* DK 1622 Spt

2.2.3 Diversification of Head Groups and α-Hydroxylation

Although it may be expected that dihydroceramide is the lipid anchor on which head group modification reactions can be performed (Fig. 2), in no case structural genes or enzymes involved have been reported in bacteria. The amidified fatty acyl residue in bacterial sphingolipids is often hydroxylated at the α- or 2-position. In the myxobacteria *Myxococcus xanthus* and *Stigmatella aurantiaca*, the fatty acid α-hydroxylases (Fah) have been identified (Ring et al. 2009) and it has been postulated that the intact sphingolipid is required in order to perform the hydroxylation (Fig. 2). Surprisingly, whereas the *S. aurantiaca* Fah forms the *R*-stereoisomer, the *M. xanthus* Fah synthesizes the *S*-isomer. Homologues of the *M. xanthus* Fah are encountered as well in genomes of the α-proteobacteria *Zymomonas mobilis* or *Gluconobacter oxydans* (Ring et al. 2009). Notably, the initially described bacterial SphLs, such as GSL-4A from *S. paucimobilis*, also display the hydroxylated *S*-isomer at the 2′-position (Kawahara et al. 1991) (Fig. 1).

2.3 Localization and Transport of Bacterial Sphingolipids

Over the last decade a number of essential components for the formation and transport of LPS have been identified in *Escherichia coli* (Whitfield and Trent 2014). The proteins involved in LPS transport (Lpt) form a complex, which bridges all the compartments of the cell reaching from the cytoplasm to the OM. Although most of the LPS biosynthesis takes place at the inner leaflet of the IM, the assembled LPS molecule is flipped to the outer leaflet of the IM by the MsbA flippase. In *E. coli*, seven proteins (LptABCDEFG) are known to be essential for cell viability and LPS transport from the outer leaflet of the IM to the outer leaflet of the OM. An ABC complex in the IM, consisting of the transmembrane proteins LptF and LptG and the ATP-binding LptB proteins, reclutes LPS from the outer leaflet of the IM. It is thought that subsequently, the LPS is transported across the periplasm through a channel formed by domains of the LptC, LptA, and LptD proteins. The carboxy-terminal domain of LptD forms a large β-barrel which is anchored in the OM and through which the LPS is moved to the outer leaflet of the outer membrane (Whitfield and Trent 2014).

It is remarkable that in SphL-producing bacteria such as *S. wittichii*, LptF and LptG homologues are encoded by genes localized in the neighborhood of SphL biosynthesis genes, such as the structural gene for Spt. In *S. wittichii*, these LptF and

Fig. 3 (continued) (ABF87747), *Nitrosomonas eutropha* C91 (WP_011633580), *Porphyromonas gingivalis* ATCC 33277 (1: WP_012458484; 2: WP_012458311; 4: WP_012457897), *Saccharomyces cerevisiae* Spt subunits LCB1, LCB2 (KZV09152, CAA98880), *Sphingobacterium multivorum* Spt (BAF73751), *Sphingomonas paucimobilis* Spt (BAB56013), *Sphingomonas wittichii* RW1 (1: WP_012050084; 3: WP_012050007; 4: WP_011952097), *Stigmatella aurantiaca* DW4/3-1 (EAU63634), and *Toxoplasma gondii* TgSPT1 (XP_002368482.1) and TgSPT2 (XP_002368481.1). For annotations of the distinct α-oxoamine synthases, see text.

LptG homologues are essential (Roggo et al. 2013). In contrast, bacteria that synthesize both LPS and SphLs have one set of genes coding for LptF and LptG homologues in the neighborhood of biosynthetic genes for LPS and another set in the neighborhood of SphL biosynthesis genes (data not shown). Therefore, it might well be that the principal transport machinery for moving LPS or SphLs from the inner surface of the IM to the outer surface of the OM is essentially the same, and that LptF/LptG homologues specific for LPS or other LptF/LptG homologues specific for SphLs would act as sorting machines and define whether LPS or SphLs are transported to the final destination in the outer leaflet of the OM.

2.4 Function of Bacterial Sphingolipids

In many Gram-negative bacteria that lack LPS in the outer leaflet of the outer membrane, SphLs seem to functionally replace them in this compartment, i.e., in *Sphingomonas* (Kawazaki et al. 1994) or *Sorangium* (Keck et al. 2011). In cases where bacteria can make both LPS and SphLs, a response to abiotic stress (acidity or elevated temperature) might be associated with an increase of SphLs or ceramide, i.e., in *A. malorum* (Ogawa et al. 2010).

In recent years, the α-proteobacterium *Caulobacter crescentus* has become one of the most-studied Gram-negative bacteria, as it serves as a model for asymmetric cell division and its regulation. Although *C. crescentus* has LPS, it also harbors genes that might be involved in the biosynthesis of SphLs (Geiger et al. 2010) (Fig. 3). In *C. crescentus*, presumptive genes for SphL biosynthesis or their transport to the OM are not essential (Christen et al. 2011); however, these genes are important for the "fitness" of the organism (Christen et al. 2011). As the phenotype of "fitness" is determined by growth of mutagenized bacterial populations to confluence and stationary phase on agar-containing solid culture media, "fitness" integrates many traits, representing a complex phenotype which is composed by several other more discernable phenotypes, i.e., affected growth, survival, competition, etc. Presently, the molecular basis for the "fitness" deficiency of potential SphL-lacking mutants is not clear.

The anaerobic *Bacteroides fragilis* is among the most abundant bacteria in the human digestive tract and is necessary as symbiotic microbe to maintain human health. Also, sphingolipids of *B. fragilis* are not required for exponential growth in culture media (An et al. 2014). However, they are essential for the survival of the bacteria in the mammalian intestine (An et al. 2011; Wieland Brown et al. 2013) and regulate the homeostasis of invariant natural killer T cells (iNKT)(An et al. 2014).

One of the mechanisms by which the innate immune system detects the invasion of pathogenic organisms is by means of Toll-like receptors (TLR) (Akira and Takeda 2004). Apparently, phosphorylated ceramides can be detected by the TLR2 receptor (Nichols et al. 2011), and α-galactosyl-ceramide is presented as lipidic antigen to iNKT cells by the CD1d protein which belongs to

the major nonpolymorphic histocompatibility complex, similar to class I (An et al. 2014). During endotoxin sensing and signaling, LPS seems to be recognized by the cluster of differentiation 14 (CD14), transferred to the myeloid differentiation factor 2 (MD-2), which in turn crosslinks TLR4 leading to a hexameric complex $(TLR4/MD\text{-}2/LPS)_2$ in the first step of the inflammatory process (Cochet and Peri 2017). However, ceramide also acts as a TLR4 agonist in a CD14-independent way (Fischer et al. 2007).

Although sphingolipids are not essential for the periodontal pathogen *Porphyromonas gingivalis*, they are important for survival in stationary phase of growth, for surviving oxidative stress, and for presenting the gingipain surface polysaccharides (Moye et al. 2016). *Porphyromonas gingivalis* phosphoglycerol dihydroceramide acts as a virulence factor enhancing osteoclastogenesis and a subsequent breakdown of bone tissue (Kanzaki et al. 2017).

During fruiting body formation in *M. xanthus*, ceramides accumulate (Arendt et al. 2015), but in spite of this correlation, the mechanistic details are not known.

In summary, bacterial sphingolipids might be essential in some cases (*Sphingomonas*, *Sorangium*), be important for survival in stationary phase of growth (*Bacteroides*, *Porphyromonas*), and provide resistance to heat (*Acetomonas*, *Bacteroides*), acid (*Acetomonas*), and oxidative stress (*Bacteroides*, *Porphyromonas*); however, molecular mechanisms are not known in any of these cases. There is clear evidence for the interaction of bacterial sphingolipids with eukaryotic signaling systems in some cases (*Sphingomonas*, *Bacteroides*), but bacterial sphingolipid signaling might be much more widespread than previously thought. The role of sphingolipids in host-microbial interactions has been reviewed recently (Heaver et al. 2018).

3 Sulfonolipids in the *Cytophaga* Group

Gram-negative bacteria of the *Cytophaga* group move by gliding and belong to the phylum *Bacteroidetes*. Major lipids in the membranes of *Cytophaga johnsonae* are sulfonolipids (SnoLs), ornithine-containing lipids (OL), and phosphatidylethanolamine (PE). SnoLs and OL are predominantly localized to the outer membrane, whereas PE is the predominant lipid of the inner membrane (Pitta et al. 1989). SnoLs contain capnine that is formed by the condensation of cysteate with fatty acyl-CoA under the release of CO_2 (White 1984; Abbanat et al. 1986) (Fig. 4a), in a reaction analogous to the one catalyzed by Spt (Yard et al. 2007). Capnine is then converted to *N*-acyl-capnine, the membrane-forming SnoL. The *N*-acylated residues vary between the *Cytophaga*, *Capnocytophaga*, and *Flexibacter* genera but include straight-chain and *iso*-fatty acids containing 14, 15, or 16 C-atoms, as well as 2-OH- (in *Flexibacter* only) and 3-OH-fatty acids (Godchaux and Leadbetter 1984). Mutants of *C. johnsonae*, deficient in gliding and SnoL biosynthesis, were

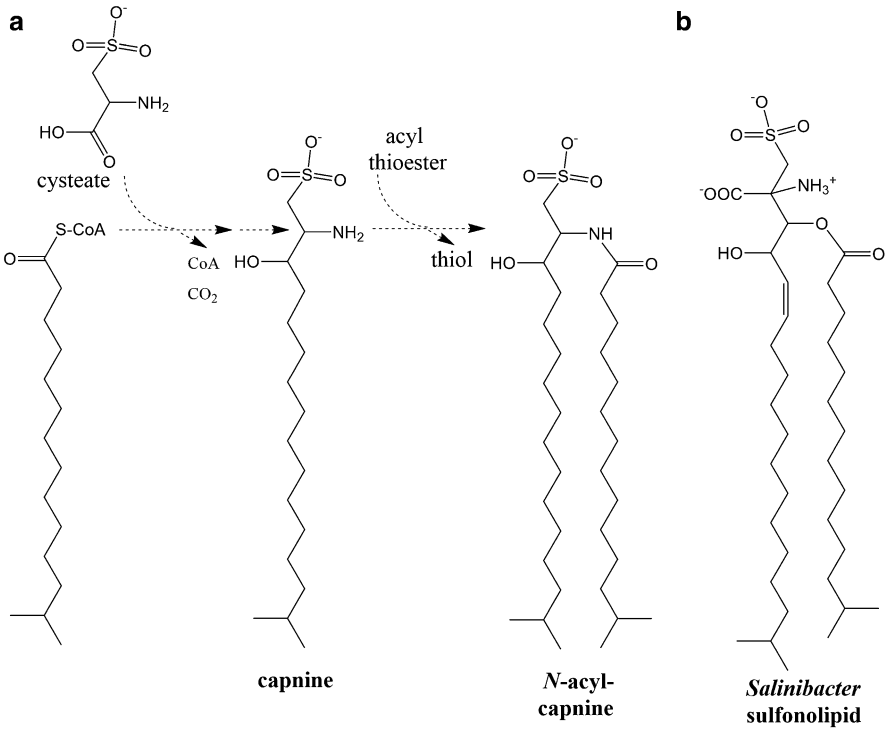


Fig. 4 Proposed pathway for sulfonolipid biosynthesis and an unusual sulfonolipid from *Salinibacter* (Reproduced from Geiger et al. 2010 with permission from Elsevier)

isolated and restoration of the SnoL content by providing cysteate resulted in recovery of the ability to glide (Abbanat et al. 1986). Therefore, SnoLs might be required for gliding motility. A structural variant of capnine exists in another member of the *Cytophaga* group, *Salinibacter ruber* (Corcelli et al. 2004). The *Salinibacter* sulfonolipid contains an extra carboxylate at carbon 2 and an *O*-acyl group at carbon 3 (Fig. 4b) that is diagnostic for this extremely halophilic bacterial genus (Corcelli et al. 2004).

In choanoflagellates, the closest living relatives of animals, multicellular rosette development might represent an initial step towards the evolution of multicellularity in animals. Formation of rosette colonies is regulated by environmental bacteria. The rosette-inducing bacterium *Algoriphagus machipongonensis* produces SnoLs termed rosette-inducing factors (Rif-1, Rif-2) (Fig. 5) which trigger the development of multicellular rosettes by the choanoflagellate *Salpingoeca rosetta* at femtomolar concentrations (Alegado et al. 2012). However, for full bioactivity, also lysophosphatidylethanolamines from *Algoriphagus* are required to synergize with rosette-inducing factors for rosette induction (Woznica et al. 2016). Notably, other *Algoriphagus* SnoLs are inactive for rosette formation, whereas the capnine IOR-1 functions as an inhibitor of rosettes (Woznica et al. 2016).

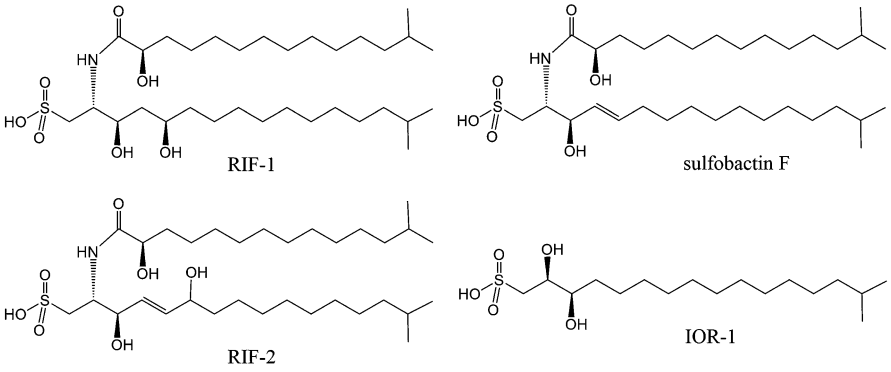


Fig. 5 Structures of sulfonolipids termed rosette-inducing factors (Rif-1, Rif-2), of an inactive sulfonolipid (sulfobactin F), and the IOR-1 capnine inhibitor of rosette formation.

4 Conclusions and Research Needs

Although sphingolipids (SphLs) are considered to be typical eukaryotic lipids, many more SphL-containing bacteria have been characterized in recent years. In bacteria, they seem to be localized to the outer leaflet of the outer membrane and in eukaryotes they seem to be enriched in the outer layer of the plasma membrane. A comparison of SphL biosynthesis enzymes is presently only possible with Spts. However, to date, it is not clear from which prokaryont the eukaryotes might have inherited the SphL biosynthetic machinery and for now the evolutionary history of SphLs remains a mystery.

So far most steps (including the corresponding structural genes and enzymes) in bacterial SphL or SnoL biosyntheses are not known, and it is unclear how SphLs or SnoLs are transported from their presumed site of biosynthesis (inner leaflet of the IM) to their presumed final destination (outer leaflet of the OM).

The functional implications of bacterial SphLs are only starting to emerge, but evidence suggests that they have important roles in protecting the bacterial producer as well as for the interaction with the abiotic and biotic environment.

Acknowledgments Research in our lab was supported by grants from Consejo Nacional de Ciencia y Tecnología-México (CONACyT-Mexico) (178,359 and 253,549 in Investigación Científica Básica as well as 118 in Investigación en Fronteras de la Ciencia) and from Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México (DGAPA-UNAM; PAPIIT IN202616, IN203612). We thank Lourdes Martínez-Aguilar for skillful technical assistance.

References

Abbanat DR, Leadbetter ER, Godchaux W III, Escher A (1986) Sulphonolipids are molecular determinants of gliding motility. Nature 324:367–369

Akira S, Takeda K (2004) Toll-like receptor signaling. Nat Rev Immunol 4:499–511

Alegado RA, Brown LA, Cao S, Dermenjian RK, Zuzow R, Fairclough SR, Clardy J, King N (2012) A bacterial sulfonolipid triggers multicellular development in the closest living relatives of animals. eLIFE 1:e00013

An D, Na C, Bielawski J, Hannun YA, Kasper DL (2011) Membrane sphingolipids as essential molecular signals for *Bacteroides* survival in the intestine. Proc Natl Acad Sci U S A 108:4666–4671

An D, Sungwhan F, Olszak T, Neves JF, Avci FY, Ertuk-Hasdemir D, Lu X, Zeissig S, Blumberg RS, Kasper DL (2014) Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. Cell 156:123–133

Arendt T, Wolff H, Bode HB (2015) Neutral and phospholipids of the *Myxococcus xanthus* lipidome during fruiting body formation and germination. Appl Environ Microbiol 81:6538–6547

Astner I, Schulze JO, van den Heuvel J, Jahn D, Schubert WD, Heinz DW (2005) Crystal structure of 5-aminolevulinate synthase, the first enzyme of heme biosynthesis, and its link to XLSA in humans. EMBO J 24:3166–3177

Christen B, Abeliuk E, Collier JM, Kalogerati VS, Passarelli B, Coller JA, Fero MJ, McAdams HH, Shapiro L (2011) The essential genome of a bacterium. Mol Syst Biol 7:528

Cochet F, Peri F (2017) The role of carbohydrates in the lipopolysaccharide (LPS)/Toll-like receptor 4 (TLR4) signalling. Int J Mol Sci 18:2318

Corcelli A, Lattanzio VMT, Mascolo G, Babudri F, Oren A, Kates M (2004) Novel sulfonolipid in the extremely halophilic bacterium *Salinibacter ruber*. Appl Environ Microbiol 70:6678–6685

Cowart LA, Obeid LM (2006) Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. Biochim Biophys Acta 1771:421–431

Fischer H, Ellström P, Ekström K, Gustafsson M, Svanborg C (2007) Ceramide as a TLR4 agonist; a putative signalling intermediate between sphingolipid receptors for microbial ligands and TLR4. Cell Microbiol 9:1239–1251

Geiger O, González-Silva N, López-Lara IM, Sohlenkamp C (2010) Amino acid-containing membrane lipids in bacteria. Prog Lipid Res 49:46–60

Godchaux IIIW, Leadbetter ER (1984) Sulfonolipids of gliding bacteria: structure of the *N*-acylaminosulfonates. J Biol Chem 259:2982–2990

Hanada K (2003) Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. Biochim Biophys Acta 1632:16–30

Heaver SL, Johnson EL, Ley RE (2018) Sphingolipids in host-microbial interactions. Curr Opin Microbiol 43:92–99

Hirabayashi Y, Furuya S (2008) Roles of L-serine and sphingolipid synthesis in brain development and neuronal survival. Prog Lipid Res 47:188–203

Ikushiro H, Hayashi H, Kagamiyama H (2001) A water-soluble homodimeric serine palmitoyltransferase from *Sphingomonas paucimobilis* EY2395T strain. Purification, characterization, cloning, and overproduction. J Biol Chem 276:18249–18256

Ikushiro H, Islam MM, Tojo H, Hayashi H (2007) Molecular characterization of membrane-associated soluble serine palmitoyltransferases from *Sphingobacterium multivorum* and *Bdellovibrio stolpii*. J Bacteriol 198:5749–5761

Jayasimhulu K, Hunt SM, Kaneshiro ES, Watanabe Y, Giner JL (2007) Detection and identification of *Bacteriovorax stolpii* UKi2 sphingophosphonolipid molecular species. J Am Soc Mass Spectrom 18:394–403

Jeong H, Kim HJ, Lee SJ (2015) Complete genome sequence of *Escherichia coli* BL21. Genome Announc 3:e00134–e00115

Kanzaki H, Movila A, Kayal R, Napimoga MH, Egashira K et al (2017) Phosphoglycerol dihydroceramide, a distinctive ceramide produced by *Porphyromonas gingivalis*, promotes RANKL-induced osteoclastogenesis by acting on non-muscle myosinII-A (Myh9), an osteoclast cell fusion regulatory factor. Biochim Biophys Acta 1862:452–462

Kato M, Muto Y, Tanaka-Bandoh K, Watanabe K, Ueno K (1995) Sphingolipid composition in *Bacteroides* species. Anaerobe 1:135–139

Kawahara K, Seydel U, Matsuura M, Danbara H, Rietschel ET, Zähringer U (1991) Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*. FEBS Lett 292:107–110

Kawahara K, Kubota M, Sato N, Tsuge K, Seto Y (2002) Occurrence of an α-galacturonosyl-ceramide in the dioxin-degrading bacterium *Sphingomonas wittichii*. FEMS Microbiol Lett 214:289–294

Kawazaki S, Moriguchi R, Sekiya K, Nakai T, Ono E, Kume K, Kawahara K (1994) The cell envelope structure of the lipopolysaccharide-lacking gram-negative bacterium *Sphingomonas paucimobilis*. J Bacteriol 176:284–290

Keck M, Gisch N, Moll H, Vorhölter FJ, Gerth K, Kahmann U, Lissel M, Lindner B, Niehaus K, Holst O (2011) Unusual outer membrane lipid composition of the gram-negative, lipopolysaccharide-lacking myxobacterium *Sorangium cellulosum* so ce56. J Biol Chem 286:12850–12859

Kerbarh O, Campopiano DJ, Baxter RL (2006) Mechanism of α-oxoamine synthases: identification of the intermediate Claisen product in the 8-amino-7-oxononanoate synthase reaction. Chem Commun 60–62

Kihara A, Mitsutake S, Mizutani Y, Igarashi Y (2007) Metabolism and biological functions of two phosphorylated sphingolipids, sphingosine 1-phosphate and ceramide 1-phosphate. Prog Lipid Res 46:126–144

Kinjo Y, Pei B, Bufali S, Raju R, Richardson SK, Imamura M, Fujio M, Wu D, Khurana A, Kawahara K, Wong CH, Howell AR, Seeberger PH, Kronenberg M (2008) Natural *Sphingomonas* glycolipids vary greatly in their ability to activate natural killer T cells. Chem Biol 15:654–664

Lorenzen W, Bozküyü KAJ, Cortina NS, Bode HB (2014) A comprehensive insight into the lipid composition of *Myxococcus xanthus* by UPLC-ESI-MS. J Lipid Res 55:2620–2633

Mina JG, Thye JK, Alqaisi AQI, Bird LE, Dods RH et al (2017) Functional and phylogenetic evidence of a bacterial origin for the first enzyme in sphingolipid biosynthesis in a phylum of eukaryotic protozoan parasites. J Biol Chem 292:12208–12219

Moye ZD, Valiuskyte K, Dewhirst FE, Nichols FC, Davey ME (2016) Synthesis of sphingolipids impacts survival of *Porphyromonas gingivalis* and the presentation of surface polysaccharides. Front Microbiol 7:1919

Naka T, Fujiwara N, Yano I, Maeda S, Doe M, Minamino M, Ikeda N, Kato Y, Watabe K, Kumazawa Y, Tomiyasu I, Kobayashi K (2003) Structural analysis of sphingophospholipids derived from *Sphingobacterium spiritivorum*, the type species of genus *Sphingobacterium*. Biochim Biophys Acta 1635:83–92

Nelson DL, Cox MM (2017) Lehninger – principles of biochemistry, 7th edn. WH Freeman and Company, New York

Nichols FC, Yao X, Bajrami B, Downes J, Finagold SM, Knee E, Gallagher JJ, Housley WJ, Clark RB (2011) Phosphorylated dihydroceramides from common human bacteria are recovered in human tissues. PLoS One 6:e16771

Nieto FL, Pescio LG, Favale NO, Adamo AM, Sterin-Speziale NB (2008) Sphingolipid metabolism is a crucial determinant of cellular fate in non-stimulated proliferating Madin-Darby canine kidney (MDCK) cells. J Biol Chem 283:25682–25691

Ogawa S, Tachimoto H, Kaga T (2010) Elevation of ceramide in *Acetobacter malorum* S24 by low pH stress and high temperature stress. J Biosci Bioeng 109:32–36

Olsen I, Jantzen E (2001) Sphingolipids in bacteria and fungi. Anaerobe 7:103–112

Pitta TP, Leadbetter ER, Godchaux W III (1989) Increase of ornithine amino lipid content in a sulfonolipid-deficient mutant of *Cytophaga johnsonae*. J Bacteriol 171:952–957

Raetz CRH, Dowhan W (1990) Biosynthesis and function of phospholipids in *Escherichia coli*. J Biol Chem 265:1235–1238

Raman MCC, Johnson KA, Clarke DJ, Naismith JH, Campopiano DJ (2010) The serine palmitoyl-transferase from *Sphingomonas wittichii* RW1: an interesting link to an unusual acyl carrier protein. Biopolymers 93:811–822

Ring MW, Schwär G, Bode HB (2009) Biosynthesis of 2-hydroxy and iso-even fatty acids is connected to sphingolipid formation in myxobacteria. Chembiochem 10:2003–2010

Roggo C, Coronado E, Moreno-Forero SK, Harshman K, Weber J, van der Meer JR (2013) Genome-wide transposon insertion scanning of environmental survival functions in the polycyclic aromatic hydrocarbon degrading bacterium *Sphingomonas wittichii* RW1. Environ Microbiol 13:2681–2695

Steyn PL, Segers P, Vancanneyt M, Sandra P, Kersters K, Joubert JJ (1998) Classification of heparinolytic bacteria into a new genus, *Pedobacter*, comprising four species: *Pedobacter heparinus* comb. nov., *Pedobacter piscium* comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. proposal of the family *Sphingobacteriaceae* fam. nov. Int J Syst Bacteriol 48:165–177

Webster SP, Alexeev D, Campopiano DJ, Watt RM, Alexeeva M, Sawyer L, Baxter RL (2000) Mechanism of 8-amino-7-oxononanoate synthase: spectroscopic, kinetic, and crystallographic studies. Biochemistry 39:516–528

Weintraub A, Zähringer U, Wollenweber HW, Seydel U, Rietschel ET (1989) Structural characterization of the lipid a component of *Bacteroides fragilis* strain NCTC9343 lipopolysaccharide. Eur J Biochem 183:425–431

White RH (1984) Biosynthesis of the sulfonolipid 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid in the gliding bacterium *Cytophaga johnsonae*. J Bacteriol 159:42–46

Whitfield C, Trent MS (2014) Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem 83:99–128

Wieland Brown LC, Penaranda C, Kashyap PC, Williams BB, Clardy J, Kronenberg M, Sonnenburg JL, Comstock LE, Bluestone JA, Fischbach MA (2013) Production of α-galactosyl ceramide by a prominent member of the human gut microbiota. PLoS Biol 11(7): e1001610

Woznica A, Cantley AM, Beemelmanns C, Freinkman E, Clardy J, King J (2016) Bacterial lipids activate, synergize, and inhibit a developmental switch in choanoflagellates. Proc Natl Acad Sci U S A 113:7894–7899

Wu D, Zajonc DM, Fujio M, Sullivan BA, Kinjo Y, Kronenberg M, Wilson IA, Wong CH (2006) Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d. Proc Natl Acad Sci U S A 103:3972–3977

Yano I, Tomiyasu I, Yabuuchi E (1982) Long chain base composition of strains of three species of *Sphingobacterium* gen. nov. FEMS Microbiol Lett 15:303–307

Yano I, Imaizumi S, Tomiyasu I, Yabuuchi E (1983) Separation and analysis of free ceramides containing 2-hydroxy fatty acids in *Sphingobacterium* species. FEMS Microbiol Lett 20:449–453

Yard BA, Carter LG, Johnson KA, Overton IM, Dorward M, Liu H, McMahon SA, Oke M, Puech D, Barton GJ, Naismith JH, Campopiano DJ (2007) The structure of serine palmitoyltransferase; gateway to sphingolipid biosynthesis. J Mol Biol 370:870–886

Phenolic Lipids Synthesized by Type III Polyketide Synthases

8

Akimasa Miyanaga and Yasuo Ohnishi

Contents

1 Introduction 140
2 Biosynthesis of Phenolic Lipids in *Azotobacter vinelandii* 140
3 Biosynthesis of Phenolic Lipids in *Streptomyces griseus* 142
4 Biosynthesis of Other Microbial Phenolic Lipids 143
5 Biosynthesis of Sorgoleone in *Sorghum bicolor* 145
6 Biosynthesis of Sporopollenin in *Arabidopsis thaliana* 146
7 Biosynthesis of Dialkylresorcinols 146
8 Research Needs 147
References 147

Abstract

Phenolic lipids, consisting of a polar aromatic ring and a hydrophobic alkyl chain, are distributed widely in bacteria, fungi, and plants, and they appear to play important roles in biological membranes. Here, recent studies of the biosynthesis of microbial and plant phenolic lipids are described. Phenolic lipids are biosynthesized by a combination of a fatty acid synthase and a type III polyketide

The authors thank the late Prof. Sueharu Horinouchi, who was a coauthor of this chapter in the first edition of this book.

A. Miyanaga (✉)
Department of Chemistry, Tokyo Institute of Technology, Tokyo, Japan
e-mail: miyanaga.a.aa@m.titech.ac.jp

Y. Ohnishi
Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
e-mail: ayasuo@mail.ecc.u-tokyo.ac.jp

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_14

synthase, which are responsible for synthesizing the alkyl chain and the aromatic ring, respectively.

1 Introduction

Phenolic lipids, which consist of a polar aromatic ring and a hydrophobic alkyl chain, are distributed widely in bacteria, fungi, and plants (Kozubek and Tyman 1999; Stasiuk and Kozubek 2010). The amphiphilic nature of phenolic lipids contributes to the formation of a stable monomolecular layer. Phenolic lipids also exhibit antimicrobial and antioxidation activities. By showing that ^{14}C-labeled acetate is efficiently incorporated into the aromatic ring of anacardic acid in the plant *Ginkgo biloba*, Gellerman et al. (1976) confirmed that a polyketide synthase (PKS) is responsible for the formation of the aromatic ring moiety of phenolic lipids.

Fatty acid synthases (FASs) and PKSs share a similar mechanism for the synthesis of fatty acids and polyketides, respectively (Smith and Tsai 2007). They possess a ketosynthase activity that catalyzes the condensation of an acyl starter substrate, with several acetate units derived from extender substrates such as malonyl-CoA. Their substrates and reaction intermediates are maintained as thioester conjugates to an acyl carrier protein (ACP) or a small molecule, coenzyme A (CoA). FAS systems perform reduction and dehydration reactions on each resulting β-keto carbon to produce an alkyl chain, whereas PKS systems omit some of the reduction and dehydration reactions and catalyze the intramolecular cyclization of the polyketide chain to generate a monocyclic or polycyclic product.

Type III PKSs, which are distributed in bacteria, fungi, and plants, consist of a homodimeric ketosynthase, which uses the same active site for both the condensation and cyclization reactions (Austin and Noel 2003). Type III PKSs can synthesize various aromatic polyketides because they differ in terms of their preferences for starter and extender substrates, the number of condensation reactions that they catalyze, and their cyclization mechanism. Recent studies showed that many type III PKSs are responsible for the biosynthesis of phenolic lipids. In this review, the biosynthesis of microbial and plant phenolic lipids is summarized. These phenolic lipids are synthesized by a combination of FASs and type III PKSs, which are responsible for synthesizing the alkyl chains and the aromatic rings, respectively. Subsequently, post-PKS tailoring enzymes diversify the chemical structures of these phenolic lipids.

2 Biosynthesis of Phenolic Lipids in *Azotobacter vinelandii*

A. vinelandii is a Gram-negative, nitrogen-fixing soil bacterium that differentiates into metabolically dormant cysts under adverse environmental conditions (Lin and Sadoff 1968). Phenolic lipids, such as alkylresorcinols and alkylpyrones (Fig. 1), are the major lipids in the cyst membrane (Reusch and Sadoff 1983). The major compound of the *A. vinelandii* phenolic lipids is 5-henicosylresorcinol, which has

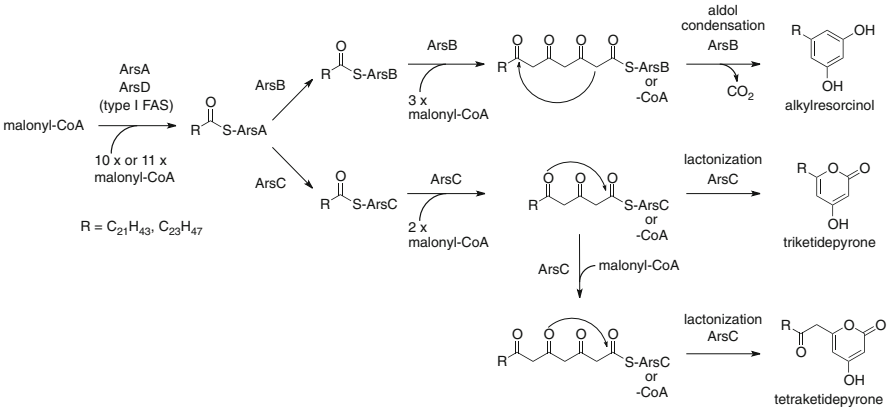


Fig. 1 Biosynthesis pathways of alkylresorcinols and alkylpyrones in *A. vinelandii*

a saturated C_{21} alkyl side chain. The phenolic lipids are synthesized by the *ars* gene cluster, which consists of two type III PKS genes, *arsB* and *arsC*, and two type I FAS genes, *arsA* and *arsD* (Funa et al. 2006).

The biosynthesis of phenolic lipids is initiated by a type I FAS consisting of ArsA and ArsD (Miyanaga et al. 2008). ArsAD catalyzes the synthesis of C_{22}, C_{24}, and C_{26} fatty acids solely from malonyl-CoA (Fig. 1). An in vitro analysis of the ArsAD reaction indicated that the fatty acid products remain attached to the ACP domain of ArsA, which is consistent with the fact that ArsA and ArsD lack a thioesterase or malonyl/palmitoyl transferase domain. Two type III PKSs, ArsB and ArsC, receive the fatty acid products directly from ArsA as starter substrates. Although ArsB and ArsC generate the same tetraketide intermediates by catalyzing three successive condensations with malonyl-CoA, ArsB and ArsC synthesize alkylresorcinols and alkylpyrones, respectively (Funa et al. 2006). The difference in catalytic properties between ArsB and ArsC depends on the cyclization mechanism. ArsB catalyzes the decarboxylative C2–C7 aldol condensation of the tetraketide intermediates to produce alkylresorcinols. The aldol condensation and thioester hydrolysis were suggested to occur before aromatization (Posehn et al. 2012). In contrast, ArsC catalyzes the C5 oxygen–C1 lactonization of the tetraketide intermediates to produce tetraketide pyrones. ArsC also catalyzes the lactonization of triketide intermediates to yield triketide pyrones. Thus, ArsB and ArsC exhibit different cyclization specificities, although they share 71% amino acid sequence identity. Mutational and crystallographic analyses suggest that a single amino acid residue at the active site cavity is a crucial determinant of the different cyclization specificities of ArsB and ArsC (Satou et al. 2013). The alkylresorcinols and alkylpyrones are the final products in *A. vinelandii* (Reusch and Sadoff 1983), which is in agreement with the fact that the *ars* gene cluster does not contain any genes encoding post-PKS tailoring enzymes.

Although ArsB and ArsC accept acyl-CoA as a starter substrate (Funa et al. 2006), the direct transfer system implies that their primary substrate should

be acyl-ACP. In *A. vinelandii*, the phenolic lipids produced by ArsB and ArsC are generated from saturated C_{22} and C_{24} fatty acids, which have longer alkyl chains than the C_{16} and C_{18} fatty acids in primary fatty acid metabolism. Therefore, the *ars* gene cluster encodes a specific type I FAS, ArsAD, for the synthesis of unusual C_{22} and C_{24} fatty acids. Because these C_{22} and C_{24} fatty acids must be used only by ArsB and ArsC, it is reasonable that ArsB and ArsC accept the long fatty acyl starter unit directly from the ACP domain of ArsA.

3 Biosynthesis of Phenolic Lipids in *Streptomyces griseus*

In the Gram-positive, filamentous bacterium *S. griseus*, phenolic lipids might be associated with the cytoplasmic membrane, and they confer resistance to β-lactam antibiotics. The phenolic lipids in *S. griseus* are synthesized by the *srs* gene cluster (Funabashi et al. 2008). This cluster consists of a type III PKS gene (*srsA*), a methyltransferase gene (*srsB*), and a flavoprotein hydroxylase gene (*srsC*). In contrast to the *ars* gene cluster, the *srs* gene cluster does not contain a type I FAS gene.

The fatty acids of *Streptomyces* are biosynthesized by a type II FAS using the amino acid degradation products 2-methylbutyryl-CoA and isobutyryl-CoA as starter units, and, therefore, they consist primarily of branched-chain fatty acids (Wallace et al. 1995). SrsA uses this branched-chain fatty acid for the biosynthesis of phenolic lipids (Funabashi et al. 2008). More specifically, SrsA presumably uses the acyl-ACP synthesized by the aforementioned type II FAS as a starter substrate. Thus, no dedicated system seems to be necessary for the preparation of the SrsA starter substrate. SrsA condenses one molecule of methylmalonyl-CoA and two molecules of malonyl-CoA with the branched starter substrate to yield 2-alkyl-3-methylresorcylic acid (Fig. 2a) (Nakano et al. 2012). Thus, SrsA acts as an alkylresorcylic acid synthase. Aldol condensation and aromatization presumably occur before hydrolysis of the thioester bond in the SrsA reaction so that the carboxyl group remains on the aromatic ring of the product. Hence, the timing of the hydrolysis of the thioester bond should differ between the ArsB and SrsA reactions. SrsA incorporates two different extender substrates in the strictly regulated order of methylmalonyl-CoA, malonyl-CoA, and malonyl-CoA.

The alkylresorcylic acid synthesized by SrsA is efficiently modified by the post-PKS tailoring enzymes SrsB and SrsC (Fig. 2a) (Nakano et al. 2012). SrsB acts as an *O*-methyltransferase to produce an alkylresorcinol methyl ether. SrsB is unable to catalyze the in vitro methylation of the alkylresorcinol that is produced by the nonenzymatic decarboxylation of the alkylresorcylic acid produced by SrsA. Moreover, no alkylresorcylic acid methyl ether has been detected in the SrsA-SrsB reaction. Based on these results, SrsB has been proposed to catalyze the decarboxylative *O*-methylation of alkylresorcylic acid. SrsC catalyzes the regiospecific hydroxylation of the alkylresorcinol methyl ether, followed by the

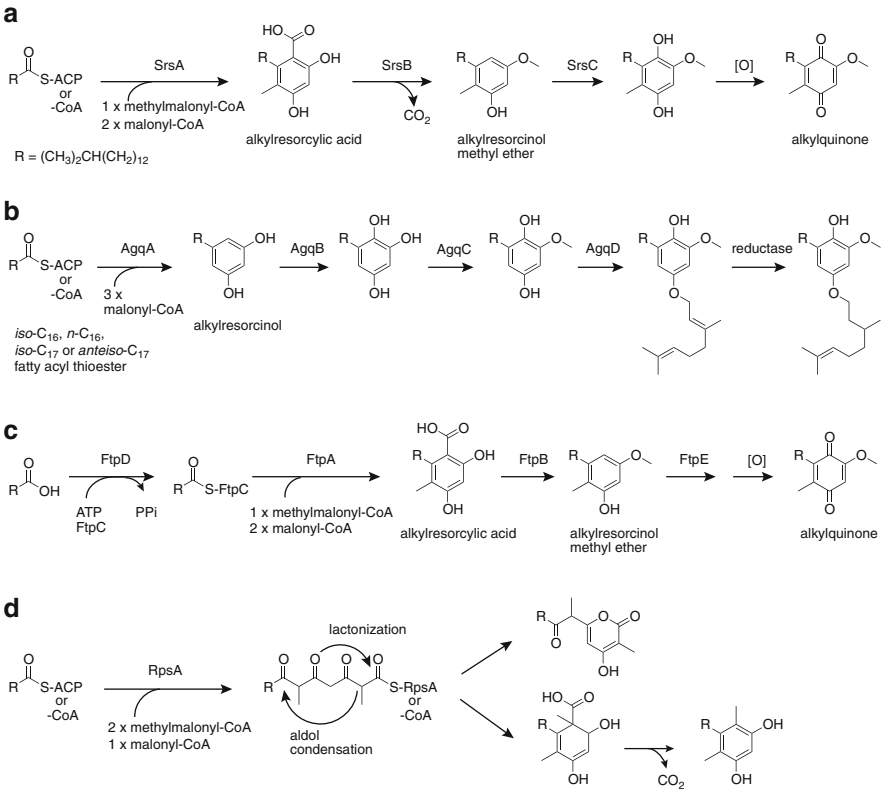


Fig. 2 Biosynthesis pathways of phenolic lipids in *S. griseus* (**a**), *A. missouriensis* (**b**), *M. xanthus* (**c**), and *R. centenum* (**d**)

nonenzymatic oxidation of the unstable hydroquinone, resulting in the formation of an alkylquinone.

4 Biosynthesis of Other Microbial Phenolic Lipids

Several bacterial and fungal type III PKSs have been shown to be involved in the biosynthesis of phenolic lipids (Katsuyama and Ohnishi 2012). Although these phenolic lipids are synthesized through similar pathways, some variations have been found, as described below.

The rare actinomycete *Actinoplanes missouriensis* produces alkyl-*O*-dihydrogeranyl-methoxyquinones from $C_{16–18}$ fatty acids. (Fig. 2b) (Awakawa et al. 2011). In the biosynthetic pathway of these prenylated phenolic lipids, the type III PKS AgqA produces alkylresorcinols from malonyl-CoA and $C_{16–18}$ fatty acyl starter substrates. The alkylresorcinols are hydroxylated by

AgqB to produce 6-alkyl-2-hydroxyhydroquinones, which are then *O*-methylated by AgqC to produce 6-alkyl-2-methoxyhydroquinones. Given that alkylresorcinols are very poor substrates for AgqC, the *O*-methylation by AgqC should occur after the hydroxylation of alkylresorcinols by AgqB. Thus, the order of *O*-methylation and hydroxylation differs from that in the Srs pathway. Finally, the UbiA-like prenyltransferase AgqD attaches a prenyl group to the C-4 hydroxy group of the 6-alkyl-2-methoxyhydroquinones to yield 6-alkyl-4-*O*-geranyl-2-methoxyhydroqinones, which are converted to 6-alkyl-4-*O*-dihydrogeranyl-2-methoxyhydroqinones by an endogenous prenyl reductase. Thus, unlike typical prenylated benzoquinones and hydroquinones, such as ubiquinones, which are derived from the shikimate pathway, *A. missouriensis* synthesizes the aromatic ring of prenylated quinone compounds through a polyketide pathway.

The myxobacterium *Myxococcus xanthus* contains the *ftp* gene cluster that is putatively involved in alkylquinone biosynthesis (Hayashi et al. 2011). The *ftp* gene cluster consists of a type III PKS gene (*ftpA*), a methyltransferase gene (*ftpB*), a standalone ACP gene (*ftpC*), an acyl-AMP ligase gene (*ftpD*), and a hydroxylase gene (*ftpE*). In vitro studies showed that FtpD adenylates a fatty acid and transfers it onto the ACP FtpC (Fig. 2c). In addition, FtpA was shown to use the resulting acyl-ACP as a starter substrate. FtpA requires two molecules of malonyl-CoA and one molecule of methylmalonyl-CoA as extender substrates to produce alkylresorcylic acids, as was reported for the SrsA reaction. Then, FtpB and FtpE catalyze the *O*-methylation and hydroxylation, respectively, of the alkylresorcylic acids to produce alkylquinones. The unique feature of this alkylquinone biosynthetic pathway is the involvement of the acyl-AMP ligase FtpD and the ACP FtpC. The *ftp* gene cluster seems to utilize a dedicated system for the preparation of the FtpA starter substrate, although the natural starter substrates of FtpA are unknown.

The type III PKS RpsA from the purple photosynthetic bacterium *Rhodospirillum centenum* has also been reported to incorporate both malonyl-CoA and methylmalonyl-CoA extender units (Fig. 2d) (Awakawa et al. 2013). Interestingly, the extender substrate preference of RpsA differs from that of SrsA and FtpA. RpsA prefers to accept two methylmalonyl-CoA units and one malonyl-CoA unit as extender substrates to produce 4-hydroxy-3-methyl-6-(1-methyl-2-oxoalkyl)pyran-2-one and 6-alkyl-4-hydroxy-1,5-dimethyl-2-oxocyclohexa-3,5-diene-1-carboxylic acid, the latter of which is nonenzymatically decarboxylated to 3-alkyl-2,4-dimethylresorcinol.

A type III PKS, 2′-oxoalkylresorcylic acid synthase (ORAS), from the filamentous fungus *Neurospora crassa* produces 2′-oxoalkylresorcylic acid (pentaketide resorcylic acid) as the main product (Funa et al. 2007). Furthermore, in vitro studies showed that the type III PKS BPKS from the phytopathogenic fungus *Botrytis cinerea* has the ability to produce hexaketide resorcylic acid (Jeya et al. 2012). ORAS and BPKS catalyze four and five condensations, respectively, with malonyl-CoA, in contrast to other phenolic lipid-producing type III PKSs that catalyze two or three condensations with extender molecules.

5 Biosynthesis of Sorgoleone in *Sorghum bicolor*

Sorgoleone, which is produced in the root hair cells of sorghum (*S. bicolor*), is probably responsible for many of the allelopathic effects of sorghum root exudates on broadleaf and grassweeds (Dayan et al. 2010). The biosynthesis of sorgoleone is initiated by fatty acid desaturases such as DES2 and DES3 (Fig. 3a) (Pan et al. 2007). They consecutively synthesize an unusual 16:3$\Delta^{9,12,15}$ fatty acyl-CoA from palmitoyl-CoA, which exists predominantly in all sorghum tissues. Although the formation of $\Delta^{9,12}$ double bonds is common, the addition of the terminal Δ^{15} vinyl double bond is characteristic of sorgoleone biosynthesis. Labeling studies showed that a PKS is involved in the biosynthesis of the quinone moiety of sorgoleone (Dayan et al. 2003). PKS activity responsible for sorgoleone biosynthesis was also detected in a sorghum root hair extract (Dayan et al. 2007). Five PKS-like sequences were identified by an expressed sequence tag analysis (Baerson et al. 2008). Among them, recombinant ARS1 and ARS2 catalyze the formation of alkylresorcinols using various fatty acyl-CoA starter units (Cook et al. 2010). In addition, RNA interference-mediated inhibition of *ARS1* and *ARS2* expression resulted in the loss of sorgoleone production. Therefore, it is likely that both ARS1 and ARS2 use a 16:3$\Delta^{9,12,15}$ fatty acyl-CoA starter unit and produce pentadecatrienyl resorcinol. This alkylresorcinol is methylated by an *O*-methyltransferase (OMT3) and subsequently hydroxylated by a hydroxylase (possibly a P450 monooxygenase) to yield dihydrosorgoleone, which is oxidized to sorgoleone (Baerson et al. 2008). Such a post-PKS tailoring system is similar to that of the phenolic lipid synthesis by the *srs* cluster.

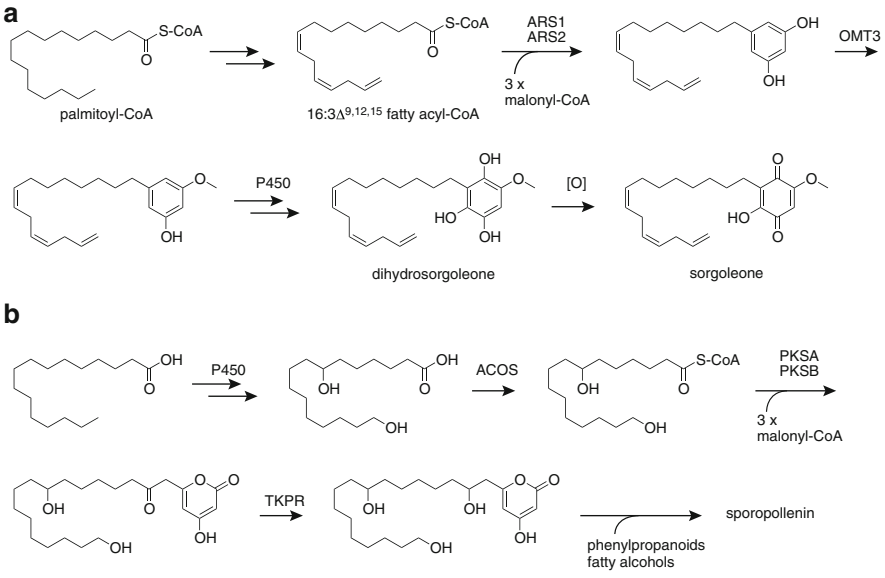


Fig. 3 Proposed biosynthesis pathways of sorgoleone (**a**) and sporopollenin (**b**) in plants

6 Biosynthesis of Sporopollenin in *Arabidopsis thaliana*

Sporopollenin is a major component of the outer exine layer of plant spores and pollen walls (Quilichini et al. 2015). The building block of sporopollenin is an alkyl pyrone derivative that consists of a polyhydroxylated long-chain fatty acid and an oxygenated aromatic ring. This building block is covalently coupled with phenylpropanoids and fatty alcohols by ether and ester linkages, resulting in a stable polymer that is resistant to degradation. In the proposed biosynthetic pathway of the building block of sporopollenin in *A. thaliana*, a fatty acid is first hydroxylated by two P450 monooxygenases, such as CYP703A2 and CYP704B1 (Morant et al. 2007; Dobritsa et al. 2009), and subsequently ligated to a CoA by an acyl-CoA synthetase (ACOS) (Fig. 3b) (de Azevedo et al. 2009). Two type III PKSs, PKSA and PKSB, condense the hydroxy fatty acyl-CoA starter unit with malonyl-CoAs to produce tetraketide hydroxyalkyl α-pyrone (Kim et al. 2010). This biosynthetic route is supported by kinetic studies showing that PKSA prefers a hydroxy fatty acyl-CoA ester as a starter substrate, rather than an unsubstituted fatty acyl-CoA ester. The resulting tetraketide α-pyrone is reduced by a tetraketide α-pyrone reductase (TKPR) to polyhydroxyalkyl α-pyrone (Grienenberger et al. 2010), which likely serves as a building block of sporopollenin.

A homologous type III PKS, *Pp*ASCL, from a bryophyte, *Physcomitrella patens*, has been functionally characterized (Colpitts et al. 2011). Similar to *Arabidopsis* PKSA, *Pp*ASCL condenses a hydroxy fatty acyl-CoA with malonyl-CoA to produce hydroxyalkyl α-pyrone, which probably serves as a building block of sporopollenin. The biosynthetic pathway of sporopollenin may be conserved in land plants.

7 Biosynthesis of Dialkylresorcinols

Bacterial 2,5-dialkylresorcinols have diverse biological activities (Schöner et al. 2015). Some dialkylresorcinols are used as bacterial signaling molecules (Brameyer et al. 2015). Although the chemical structures of dialkylresorcinols are quite similar to those of the aforementioned monoalkylresorcinols, they are produced via a different pathway (Fig. 4) (Fuchs et al. 2013). Although this review focuses on phenolic lipids that are synthesized by type III PKSs, the biosynthetic mechanism of dialkylresorcinols is described briefly for comparison. During dialkylresorcinol biosynthesis, the ketosynthase DarB is responsible for the formation of 2,5-dialkylcyclohexane-1,3-diones (CHDs) from two fatty acid-derived precursors

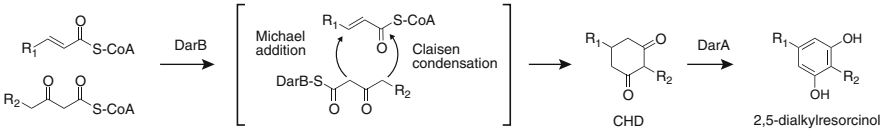


Fig. 4 Biosynthesis pathway of dialkylresorcinols

(Fuchs et al. 2013). CHDs are further oxidized to 2,5-dialkylresorcinols by the aromatase DarA. DarB catalyzes a consecutive Claisen condensation and a Michael addition of a β-ketoacyl thioester to an α,β-unsaturated acyl thioester. Thus, DarB shows a different catalytic property from type III PKSs. The phylogenetic analysis revealed that DarB represents a novel clade of ketosynthases.

8 Research Needs

Phenolic lipids, which are distributed widely in bacteria, fungi, and plants, are attractive targets for chemical and biological studies because of their various biological activities. Recent studies have revealed that type III PKSs play important roles in the biosynthesis of phenolic lipids. A variety of substrate specificities and reactivities of type III PKSs, as well as the presence of many tailoring enzymes, greatly diversify the chemical structures of phenolic lipids. Type III PKSs differ in terms of their preferences for starter and extender substrates, the number of condensation reactions that they catalyze, and their cyclization mechanism. It is still difficult to predict the substrate preference and cyclization mechanism of type III PKSs from their amino acid sequences. Further mechanistic studies are necessary to clarify the ambiguities in the reaction mechanism of type III PKSs.

References

Austin MB, Noel JP (2003) The chalcone synthase superfamily of type III polyketide synthases. Nat Prod Rep 20:79–110

Awakawa T, Fujita N, Hayakawa M, Ohnishi Y, Horinouchi S (2011) Characterization of the biosynthesis gene cluster for alkyl-*O*-dihydrogeranyl-methoxyhydroquinones in *Actinoplanes missouriensis*. Chembiochem 12:439–448

Awakawa T, Sugai Y, Otsutomo K, Ren S, Masuda S, Katsuyama Y, Horinouchi S, Ohnishi Y (2013) 4-Hydroxy-3-methyl-6-(1-methyl-2-oxoalkyl)pyran-2-one synthesis by a type III polyketide synthase from *Rhodospirillum centenum*. Chembiochem 14:1006–1013

Baerson SR, Dayan FE, Rimando AM, Nanayakkara NP, Liu CJ, Schröder J, Fishbein M, Pan Z, Kagan IA, Pratt LH, Cordonnier-Pratt MM, Duke SO (2008) A functional genomics investigation of allelochemical biosynthesis in *Sorghum bicolor* root hairs. J Biol Chem 283:3231–3247

Brameyer S, Kresovic D, Bode HB, Heermann R (2015) Dialkylresorcinols as bacterial signaling molecules. Proc Natl Acad Sci U S A 112:572–577

Colpitts CC, Kim SS, Posehn SE, Jepson C, Kim SY, Wiedemann G, Reski R, Wee AGH, Douglas CJ, Suh DY (2011) PpASCL, a moss ortholog of anther-specific chalcone synthase-like enzymes, is a hydroxyalkylpyrone synthase involved in an evolutionarily conserved sporopollenin biosynthesis pathway. New Phytol 192:855–868

Cook D, Rimando AM, Clemente TE, Schröder J, Dayan FE, Nanayakkara NP, Pan Z, Noonan BP, Fishbein M, Abe I, Duke SO, Baerson SR (2010) Alkylresorcinol synthases expressed in *Sorghum bicolor* root hairs play an essential role in the biosynthesis of the allelopathic benzoquinone sorgoleone. Plant Cell 22:867–887

Dayan FE, Kagan IA, Rimando AM (2003) Elucidation of the biosynthetic pathway of the allelochemical sorgoleone using retrobiosynthetic NMR analysis. J Biol Chem 278:28607–28611

Dayan FE, Watson SB, Nanayakkara NP (2007) Biosynthesis of lipid resorcinols and benzoquinones in isolated secretory plant root hairs. J Exp Bot 58:3263–3272

Dayan FE, Rimando AM, Pan Z, Baerson SR, Gimsing AL, Duke SO (2010) Sorgoleone. Phytochemistry 71:1032–1039

de Azevedo SC, Kim SS, Koch S, Kienow L, Schneider K, McKim SM, Haughn GW, Kombrink E, Douglas CJ (2009) A novel fatty acyl-CoA synthetase is required for pollen development and sporopollenin biosynthesis in *Arabidopsis*. Plant Cell 21:507–525

Dobritsa AA, Shrestha J, Morant M, Pinot F, Matsuno M, Swanson R, Møller BL, Preuss D (2009) CYP704B1 is a long-chain fatty acid omega-hydroxylase essential for sporopollenin synthesis in pollen of *Arabidopsis*. Plant Physiol 151:574–589

Fuchs SW, Bozhüyük KA, Kresovic D, Grundmann F, Dill V, Brachmann AO, Waterfield NR, Bode HB (2013) Formation of 1,3-cyclohexanediones and resorcinols catalyzed by a widely occurring ketosynthase. Angew Chem Int Ed 52:4108–4112

Funa N, Ozawa H, Hirata A, Horinouchi S (2006) Phenolic lipid synthesis by type III polyketide synthases is essential for cyst formation in *Azotobacter vinelandii*. Proc Natl Acad Sci U S A 103:6356–6361

Funa N, Awakawa T, Horinouchi S (2007) Pentaketide resorcylic acid synthesis by type III polyketide synthase from *Neurospora crassa*. J Biol Chem 282:14476–14481

Funabashi M, Funa N, Horinouchi S (2008) Phenolic lipids synthesized by type III polyketide synthase confer penicillin resistance on *Streptomyces griseus*. J Biol Chem 283:13983–13991

Gellerman JL, Anderson WH, Schlenk H (1976) Synthesis of anacardic acids in seeds of *Ginkgo biloba*. Biochim Biophys Acta 431:16–21

Grienenberger E, Kim SS, Lallemand B, Geoffroy P, Heintz D, Souza Cde A, Heitz T, Douglas CJ, Legrand M (2010) Analysis of TETRAKETIDE α-PYRONE REDUCTASE function in *Arabidopsis thaliana* reveals a previously unknown, but conserved, biochemical pathway in sporopollenin monomer biosynthesis. Plant Cell 22:4067–4083

Hayashi T, Kitamura Y, Funa N, Ohnishi Y, Horinouchi S (2011) Fatty acyl-AMP ligase involvement in the production of alkylresorcylic acid by a *Myxococcus xanthus* type III polyketide synthase. Chembiochem 12:2166–2176

Jeya M, Kim TS, Tiwari MK, Li J, Zhao H, Lee JK (2012) The *Botrytis cinerea* type III polyketide synthase shows unprecedented high catalytic efficiency toward long chain acyl-CoAs. Mol BioSyst 8:2864–2867

Katsuyama Y, Ohnishi Y (2012) Type III polyketide synthases in microorganisms. Methods Enzymol 515:359–377

Kim SS, Grienenberger E, Lallemand B, Colpitts CC, Kim SY, Souza Cde A, Geoffroy P, Heintz D, Krahn D, Kaiser M, Kombrink E, Heitz T, Suh DY, Legrand M, Douglas CJ (2010) LAP6/POLYKETIDE SYNTHASE A and LAP5/POLYKETIDE SYNTHASE B encode hydroxyalkyl α-pyrone synthases required for pollen development and sporopollenin biosynthesis in *Arabidopsis thaliana*. Plant Cell 22:4045–4066

Kozubek A, Tyman JH (1999) Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. Chem Rev 99:1–26

Lin LP, Sadoff HL (1968) Encystment and polymer production by *Azotobacter vinelandii* in the presence of beta-hydroxybutyrate. J Bacteriol 95:2336–2343

Miyanaga A, Funa N, Awakawa T, Horinouchi S (2008) Direct transfer of starter substrates from type I fatty acid synthase to type III polyketide synthases in phenolic lipid synthesis. Proc Natl Acad Sci U S A 105:871–876

Morant M, Jørgensen K, Schaller H, Pinot F, Møller BL, Werck-Reichhart D, Bak S (2007) CYP703 is an ancient cytochrome P450 in land plants catalyzing in-chain hydroxylation of lauric acid to provide building blocks for sporopollenin synthesis in pollen. Plant Cell 19:1473–1487

Nakano C, Funa N, Ohnishi Y, Horinouchi S (2012) The *O*-methyltransferase SrsB catalyzes the decarboxylative methylation of alkylresorcyclic acid during phenolic lipid biosynthesis by *Streptomyces griseus*. J Bacteriol 194:1544–1551

Pan Z, Rimando AM, Baerson SR, Fishbein M, Duke SO (2007) Functional characterization of desaturases involved in the formation of the terminal double bond of an unusual 16:3$\Delta^{9,12,15}$ fatty acid isolated from *Sorghum bicolor* root hairs. J Biol Chem 282:4326–4335

Posehn SE, Kim SY, Wee AGH, Suh DY (2012) Mapping the mechanism of the resorcinol ring formation catalyzed by ArsB, a type III polyketide synthase from *Azotobacter vinelandii*. Chembiochem 13:2212–2217

Quilichini TD, Grienenberger E, Douglas CJ (2015) The biosynthesis, composition and assembly of the outer pollen wall: a tough case to crack. Phytochemistry 113:170–182

Reusch RN, Sadoff HL (1983) Novel lipid components of the *Azotobacter vinelandii* cyst membrane. Nature 302:268–270

Satou R, Miyanaga A, Ozawa H, Funa N, Katsuyama Y, Miyazono KI, Tanokura M, Ohnishi Y, Horinouchi S (2013) Structural basis for cyclization specificity of two *Azotobacter* type III polyketide synthase: a single amino acid substitution reverses their cyclization specificity. J Biol Chem 288:34146–34157

Schöner TA, Kresovic D, Bode HB (2015) Biosynthesis and function of bacterial dialkylresorcinol compounds. Appl Microbiol Biotechnol 99:8323–8328

Smith S, Tsai SC (2007) The type I fatty acid and polyketide synthases: a tale of two megasynthases. Nat Prod Rep 24:1041–1072

Stasiuk M, Kozubek A (2010) Biological activity of phenolic lipids. Cell Mol Life Sci 67:841–860

Wallace KK, Zhao B, McArthur HAI, Reynolds KA (1995) In vivo analysis of straight-chain and branched-chain fatty acid biosynthesis in three actinomycetes. FEMS Microbiol Lett 131:227–234

Lipid A

9

Russell E. Bishop

Contents

1 Introduction 152
2 Endotoxin Biosynthesis 152
3 The Outer Membrane Permeability Barrier 154
4 Lipid A Modifications 154
5 L-Ara4N Cluster 155
6 EptA 156
7 PagP 156
8 LPS Transport 157
9 Research Needs 159
References 159

Abstract

Diverse lipid A structures have been observed in a multitude of Gram-negative bacteria, but the metabolic logic of lipid A biosynthesis is widely conserved. This chapter will start by describing the nine constitutive enzymes of the Raetz pathway, which catalyze conserved lipid A biosynthetic reactions that depend on cytoplasmic cofactors. Concomitant with lipid A export and assembly on the cell surface, a number of regulated covalent modifications of lipid A can occur in the extracytoplasmic compartments. The narrow phylogenetic distribution of the lipid A modification enzymes, combined with the diverse regulatory signals governing their expression, is responsible for most of the lipid A structural diversity that is observed in nature. By focusing on *E. coli* as a model system,

R. E. Bishop (✉)
Department of Biochemistry and Biomedical Sciences, Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada
e-mail: bishopr@mcmaster.ca

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_10

the general principles of lipid A biosynthesis and assembly are revealed to inform related processes that occur in more divergent organisms.

1 Introduction

In 1892, Richard Pfeiffer first defined endotoxin as a heat-stable toxic substance that was released upon disruption of microbial envelopes (Beutler and Rietschel 2003). The bioactive lipid A component of lipopolysaccharide (LPS) is arguably the most potent of the substances that fit Pfeiffer's endotoxin definition. Gram-negative bacteria can modulate the structure of lipid A in order to evade detection by the host immune system (Raetz et al. 2007). This chapter summarizes the recently elucidated pathways for the biosynthesis and transport of lipid A in *Escherichia coli*, which provides a framework for understanding lipid A structure and function in all Gram-negative bacteria.

2 Endotoxin Biosynthesis

The molecular genetics and enzymology of the conserved steps of lipid A biosynthesis are best characterized in *E. coli*, as shown in Fig. 1. The Raetz pathway begins with the key precursor molecule UDP-GlcNAc, which is also the first substrate for peptidoglycan biosynthesis (Raetz and Whitfield 2002). The first enzyme in lipid A biosynthesis is a cytoplasmic acyltransferase LpxA, which selectively transfers thioester-activated 3-OH-14:0 from acyl carrier protein (ACP) to the 3-OH of UDP-GlcNAc. The crystal structure of LpxA revealed a homotrimeric molecule, which self-associates by a distinctive left-handed parallel β-helix motif (Raetz and Roderick 1995). *E. coli* LpxA is extraordinarily selective for 3-OH-14:0-ACP as the acyl donor substrate, while the *Pseudomonas aeruginosa* LpxA prefers 3-OH-10:0-ACP. However, mutating certain key residues lining the active site cleft could modulate acyl chain selection. For example, the specificity for the G173M mutant of *E. coli* LpxA was shifted to 3-OH-10:0-ACP. In contrast, the specificity of *P. aeruginosa* LpxA could be extended to accommodate 3-OH-14:0-ACP in the corresponding M169G mutant (Wyckoff et al. 1998). These findings demonstrated the existence of precise hydrocarbon rulers in LpxAs, which can explain variations in lipid A acylation that are observed between different organisms.

The acylation of UDP-GlcNAc by LpxA is thermodynamically unfavorable, and the first committed step in lipid A biosynthesis is the subsequent deacetylation catalyzed by LpxC (EnvA). LpxC is a Zn^{2+}-dependent enzyme that is an established target for antibiotic development (Onishi et al. 1996). The crystal and NMR structures of *Aquifex* LpxC revealed two slightly different models for the mechanism of catalysis, but both include a critical role for Zn^{2+} (Coggins et al. 2003; Whittington et al. 2003). Most LpxC inhibitors are hydroxamate compounds that interact with the

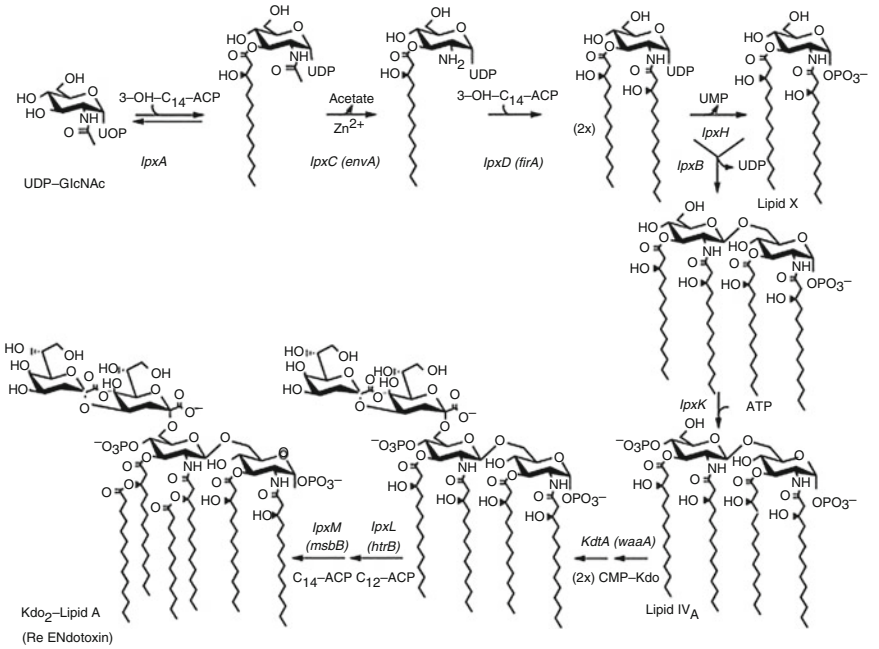


Fig. 1 The Raetz pathway for synthesis of Kdo_2-lipid A. LpxA catalyzes the addition of 3-OH-14:0 to position 3 of UDP-GlcNAc. LpxC then hydrolyzes the acetamido group at position 2, which allows LpxD to add a second 3-OH-14:0 group. LpxH cleaves the nucleotide to generate lipid X, which is then condensed with UDP-diacyl-GlcN to generate the disaccharide 1-phosphate. The 4′-kinase LpxK then generates lipid IV_A, which is converted into Kdo_2-lipid IV_A by a bifunctional Kdo transferase KdtA. Kdo_2-lipid IV_A is a substrate for the LpxL and LpxM acyltransferases, which generate the acyloxyacyl linkages at positions 2′ and 3′, respectively (Originally published in Bishop (2010), published with kind permission of ©Springer Science+ Business Media New York, 2003. All rights reserved)

catalytic Zn^{2+} ion. Current challenges are aimed at the development of inhibitors with the ability to evade efflux pumps that provide resistance, particularly in pseudomonads (Lee et al. 2016). Following deacetylation, an *N*-linked 3-OH-14:0 moiety is incorporated from ACP by LpxD (FirA) to generate UDP-2,3-diacylglucosamine (Kelly et al. 1993; Bartling and Raetz 2009). A highly selective pyrophosphatase LpxH then cleaves UDP-2,3-diacylglucosamine to form lipid X (Babinski et al. 2002; Cho et al. 2016). Next, the disaccharide synthase, LpxB, condenses UDP-2,3-diacylglucosamine and lipid X to generate the β-1′,6-linkage found in all lipid A molecules. The membrane-bound 4′ kinase LpxK then phosphorylates the disaccharide 1-phosphate to produce lipid IV_A, which is an important pharmacological agent because it functions as an endotoxin antagonist in human cell lines (Garrett et al. 1997; Emptage et al. 2012). Next, two 3-deoxy-D- *manno*-2-octulosonic acid (Kdo) sugars are incorporated by a Kdo transferase, which is encoded by the *kdtA* (*waaA)* gene, using the labile nucleotide CMP-Kdo as the

Kdo donor (Schmidt et al. 2012). The final lipid A biosynthetic steps that occur on the cytoplasmic side of the inner membrane depend on the prior addition of the Kdo sugars and involve the transfer of lauroyl (12:0) and myristoyl (14:0) groups from ACP to the distal glucosamine unit to produce acyloxyacyl linkages; the reactions are catalyzed at the at the 2'-position by LpxL (HtrB) and at the 3′-position by LpxM (MsbB), respectively (Six et al. 2008; Dovala et al. 2016). Under conditions of cold growth at 12 °C, LpxL is replaced by LpxP, which has a preference for palmitoleate (16:1cisΔ^9) in ACP (Carty et al. 1999). The incorporation of an unsaturated acyl chain into lipid A likely increases membrane fluidity under cold growth conditions. Viable mutants that lack acyloxyacyl linkages in lipid A are attenuated for virulence and reveal the importance of the lipid A acylation pattern in inflammation (Vorachek-Warren et al. 2002). All other enzymatic steps of the Raetz pathway, and those for the biosynthesis of CMP-Kdo, are essential for cell viability and, thus, provide potential targets for antibiotic development.

3 The Outer Membrane Permeability Barrier

LPS contains phosphate and acidic sugars and is therefore negatively charged. In order to reduce the electrostatic repulsion between LPS molecules at the cell surface, the bacterial outer membrane (OM) sequesters divalent cations, mainly Mg^{+2}, which neutralize the negative charges and maintain the integrity of the OM. The presence of hydrogen-bond donors and acceptors in the lipid A molecule allows for additional lateral interactions that cannot occur between phospholipid molecules (Nikaido 2003). Moreover, the six or seven saturated acyl chains of lipid A serve to reduce the fluidity of the OM bilayer compared with the inner membrane. The tight lateral interactions between LPS, combined with low membrane fluidity, provide a permeability barrier in the OM to lipophilic solutes and detergents. However, cationic antimicrobial peptides (CAMPs) can displace Mg^{+2} ions from the cell surface to promote their uptake by the bacterial cell (Hancock et al. 1995).

4 Lipid A Modifications

Considering the importance of Mg^{+2} in maintaining the OM permeability barrier, it is not surprising that Mg^{+2} limitation and CAMPs can regulate the covalent structure of lipid A by triggering the PhoP/PhoQ and PmrA/PmrB signal transduction pathways (Bader et al. 2005; Groisman 2001). Several covalent modifications of lipid A have been characterized in *E. coli* (Fig. 2). PagP is a transacylase that incorporates a palmitate chain at position 2 (Bishop et al. 2000). Moreover, the phosphate groups at positions 1 and 4′ of the lipid A disaccharide backbone can be modified with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (pEtN), which serve to reduce the overall negative charge of lipid A (Gunn et al. 1998). Lipid A modifications occur in the extracytoplasmic compartments and help to restore the OM permeability barrier and provide resistance to CAMPs.

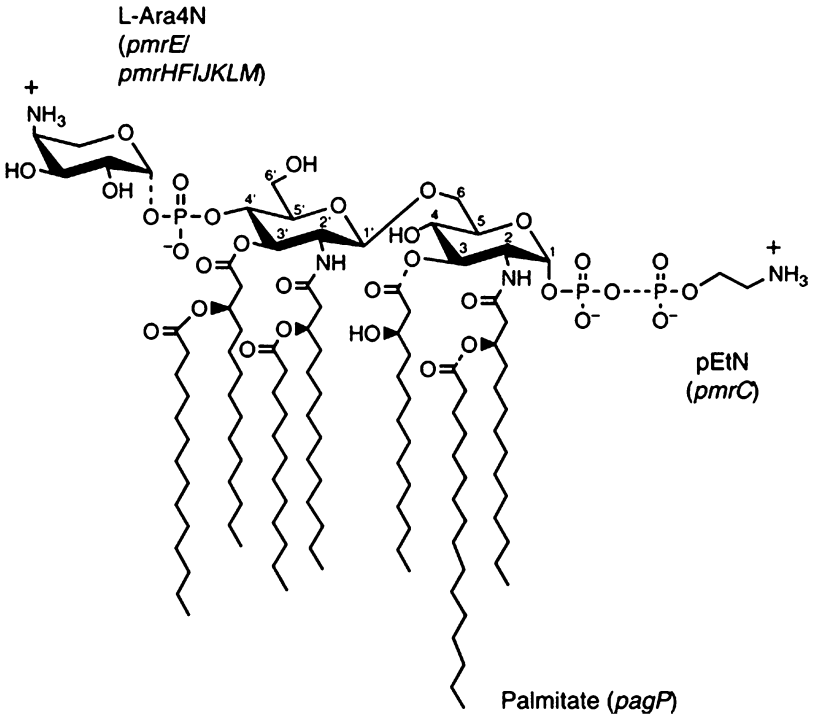


Fig. 2 Regulated covalent lipid A modifications in *E. coli*. The conserved lipid A nucleus can be modified by the addition of L-Ara4N and pEtN to the phosphate groups and by the addition of a palmitate chain at position 2. Palmitoylation of lipid A is under the direct control of PhoP/PhoQ, while PmrA/PmrB controls the phosphate modifications (Originally published in Bishop (2010), published with kind permission of ©Springer Science+Business Media New York, 2003. All rights reserved)

5 L-Ara4N Cluster

PmrA/PmrB is only one of several clusters of *pmr* genes that were originally identified in *p*oly*m*yxin-*r*esistant mutants of *E. coli*. The *pmrF* (*pbgP*) locus encodes an operon of seven open reading frames *pmrHFIJKLM*, which, together with the unlinked *pmrE* (*ugd*), are required for L-Ara4N synthesis (Gunn et al. 1998). The first step involves the conversion of UDP-glucose into UDP-glucuronic acid catalyzed by a dehydrogenase encoded by *pmrE*. Complex regulation of dehydrogenase gene expression reflects the fact that UDP-glucuronic acid is a precursor for both colanic acid-containing capsules and L-Ara4N. Next, PmrI (ArnA) catalyzes the oxidative decarboxylation of UDP-glucuronic acid to generate a novel UDP-4-keto-pyranose intermediate (Breazeale et al. 2002; Genthe et al. 2016). PmrH (ArnB) then catalyzes a transamination reaction using glutamate as the amine donor to generate UDP-L-Ara4N (Breazeale et al. 2003). The crystal structure of PmrH has verified

that a pyridoxal phosphate cofactor contributes to the catalytic mechanism (Noland et al. 2002). Interestingly, PmrI contains a second domain that formylates the 4-amine of UDP-L-Ara4N (Breazeale et al. 2005). The resultant UDP-L-Ara4-Formyl-N is transferred by PmrF (ArnC) to the membrane-anchored undecaprenyl phosphate, forming undecaprenyl phosphate-L-Ara4-Formyl-N. The formylation step may drive forward the equilibrium of the transamination step, which is thermodynamically unfavorable. The necessity of a subsequent deformylation step catalyzed by PmrJ (ArnD) is dictated by the fact that undecaprenyl phosphate-L-Ara4N is the substrate for PmrK (ArnT), which catalyzes the final transfer of L-Ara4N to lipid A at the periplasmic surface of the inner membrane (Trent et al. 2001a, b; Petrou et al. 2016). Roles for PmrL and PmrM (ArnE and ArnF) in flipping undecaprenyl phosphate-L-Ara4N across the inner membrane have been confirmed (Yan et al. 2007).

6 EptA

The pEtN-adding enzyme EptA has been cloned from *E. coli* (Lee et al. 2004), and a homologous gene from *Neisseria* has been associated with the addition of pEtN to lipid A (Cox et al. 2003; Wanty et al. 2013). The EptA-encoding gene is the upstream open reading frame that is part of the *pmrAB* operon and is also known as *pmrC* (*pagB*). The EptA active site is located on the periplasmic side of the inner membrane. Phosphatidylethanolamine is the reported pEtN donor, and several EptA homologues are likely responsible for pEtN addition to other cell envelope components including the inner core sugars of LPS (Reynolds et al. 2005). It is noteworthy that roughly one third of *E. coli* lipid A carries a diphosphate moiety instead of the monophosphate at position 1 (Touzé et al. 2008) and that the responsible phosphorylating enzyme shares with EptA the ability to generate a phosphoanhydride bond at the same position in lipid A.

7 PagP

PagP functions to transfer a palmitate chain from a phospholipid to the hydroxyl group of the *N*-linked 3-OH-14:0 chain on the proximal glucosamine unit of lipid A (Bishop 2005). PagP is regulated by PhoP/PhoQ and was the first enzyme of lipid A biosynthesis shown to be localized in the OM (Bishop et al. 2000; Jia et al. 2004). Since thioester-containing substrates are not available in the extracellular compartments, PagP uses a phospholipid as the palmitoyl donor instead. PagP was first identified in the salmonellae due to its role in providing resistance to cationic antimicrobial peptides (Guo et al. 1998) and was subsequently purified from *E. coli* (Bishop et al. 2000). In addition to these enteric pathogens, PagP homologues are present in the respiratory pathogens *Legionella pneumophila* and *Bordetella bronchiseptica*, where PagP has been shown to be necessary for disease causation in animal models of infection (Preston et al. 2003; Robey et al. 2001). PagP

homologues are also found in *Yersinia*, *Photorhabdus*, and *Erwinia* species, which adopt pathogenic lifestyles in animals, insects, and plants, respectively. The solution and crystal structures of PagP indicate that the enzyme is activated in the OM in response to perturbations of lipid asymmetry (Ahn et al. 2004; Hwang et al. 2002). Phospholipids must gain access to the PagP hydrocarbon ruler after first migrating into the OM external leaflet (Khan et al. 2007, 2009, 2010a, b; Cuesta-Seijo et al. 2010). Some evidence indicates PagP can function as a sensory transducer, which is triggered by perturbations to OM lipid asymmetry (Smith et al. 2008). PagP also displays a unique regiospecificity for the lipid A 3′-position in *Pseudomonas* (Thaipisuttikul et al. 2014) and the ability to palmitoylate the headgroup of phosphatidylglycerol in *Salmonella* (Dalebroux et al. 2014). The lysophospholipid by-product of the PagP reaction is rapidly transported to the inner membrane and reacylated (Hsu et al. 1989). PagP is the only enzyme of lipid A biosynthesis known to be located in the outer membrane of *E. coli*, but other outer membrane lipid A modification enzymes include the latent *Salmonella* lipid A deacylases PagL and LpxR, which are functional during heterologous expression in *E. coli* (Bishop 2008).

8 LPS Transport

LPS is an elaborate phospholipid that is extensively decorated with sugars, and its presence provides a barrier against harmful detergents and lipophilic antibiotics. LPS transport to the outer membrane is essential for survival and thus provides a target for new antibiotics. Structural studies have now disclosed a remarkable molecular mechanism for LPS delivery and insertion into the external leaflet of the outer membrane (Bishop 2014; Okuda et al. 2016). LPS molecules are mostly assembled by biosynthetic enzymes exposed to the cytoplasm, but the activity of several LPS-modifying enzymes in the extracytoplasmic compartments can be used to monitor the molecule on its journey to the outer membrane. First, the lipid A, along with its attached core sugars, is translocated by the ABC transporter MsbA to the external leaflet of the inner membrane. Here, a terminal O-sugar chain can be incorporated before the seven Lpt proteins of the LPS transport system take over (Fig. 3a). Next, the ABC transporter complex $LptB_2FG$ in the inner membrane detaches LPS from the external leaflet and ushers it along a protein filament, made up of LptA and LptC, which tracks through the periplasmic space. Finally, a complex between LptD and LptE in the outer membrane functions as a station to complete the track for delivering and inserting LPS into the external leaflet of the outer membrane.

LptD is built primarily from β-strands that fold into two domains: a β-jellyroll and a β-barrel. The smaller of the two, the β-jellyroll, extends away from the membrane and contains a lipophilic slot adapted to bind the lipid A while leaving the LPS sugar chain exposed. The same β-jellyroll fold is found in LptA and LptC, which interconnect to align their slots in an extended lipophilic groove that spirals across the periplasmic space between the inner and outer membranes. Thus, it seems that the LptD β-jellyroll is the terminal track for delivering LPS to the outer membrane (Fig. 3b). The structure of this LptD platform is positioned to deliver the LPS directly

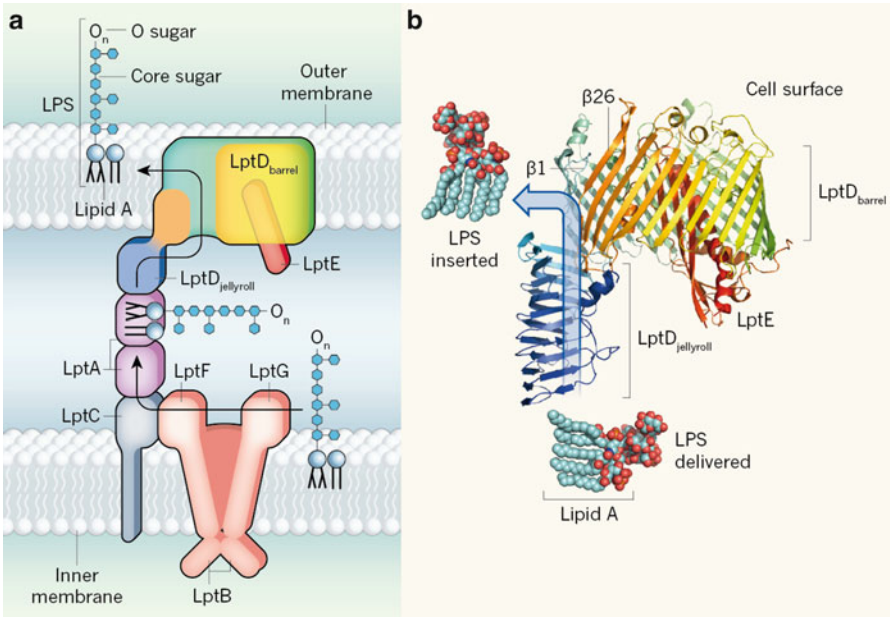


Fig. 3 Organization of the Lpt transport system and structure of the LptD–LptE complex. **a** LPS comprises a lipid A membrane anchor, a core sugar unit, and a terminal O-sugar chain. Following lipid A-core assembly in the cytoplasm and its export to the external leaflet of the inner membrane, the O-antigen can be attached before the seven Lpt proteins transport LPS from the inner to the outer membrane. **b** The crystal structure of the complex formed between LptD and LptE proteins in the outer membrane reveals the route for LPS delivery and insertion into the external leaflet (*blue arrow*). The structure shows how the sugars of LPS are positioned by the β-jellyroll region of LptD to engage first with LptE, which probably reorients the LPS into a vertical position. Delivery of the LPS into the outer membrane external leaflet then occurs by lateral migration through an opening between the first (1) and final (26) β-strands in the LptD β-barrel (First published in Nature, 511, 37–38, 2014 by Nature Publishing Group)

into the cavity of the much larger domain, the β-barrel, which is composed of 26 membrane-spanning β-strands, the greatest number of these strands ever described. The LptD β-barrel takes the shape of a kidney bean, with the lobe opposite the β-jellyroll plugged with a completely different outer membrane protein. The LptE lipoprotein is positioned almost entirely inside the LptD β-barrel so as to interact most readily with the sugar chain of the delivered LPS; it has only its lipid-modified end anchored outside the β-barrel and within the inner leaflet of the outer membrane. The other lobe, located adjacent to the β-jellyroll, remains empty on the periplasmic side so that it can transiently accommodate LPS on its passage into the external leaflet of the outer membrane. The empty lobe of the LptD β-barrel is closed at the cell surface by the L4 loop, which likely repositions itself in order to create a transitory opening where the LPS sugar chain can emerge. The LPS lipid A region then diffuses laterally into the external leaflet of the outer membrane through a weakly hydrogen-bonded gate located between β-strands 1 and 26.

LptF and LptG each display their own periplasmic β-jellyroll domains that likely help to propel LPS into the LptA–LptC filament during LptB-catalyzed ATP hydrolysis, but it remains to be determined what impels LPS to emerge from the LptD–LptE complex so it inserts into the outer membrane. LPS carries a strong net negative charge, and electrostatic repulsion between neighboring molecules might help to move them along the filament; bridging of LPS by positively charged magnesium ions in the outer membrane might then facilitate its lateral egress from LptD. Roughly 200 LptD–LptE complexes are evenly distributed over the surface of a typical bacterium, and each complex delivers about five LPS molecules to the cell surface every second. A synthetic cyclic-peptide antibiotic prevents growth of *Pseudomonas aeruginosa* by targeting its LptD and blocking LPS transport. The structures of the Lpt transport system components thus facilitate the design of better antibiotics, in addition to further clarifying the mechanism by which LPS quite literally rolls out the barrel.

9 Research Needs

Lipid A and its regulated covalent modifications exhibit profound effects on bacterial and human physiology. Novel endotoxin antagonists and immune adjuvants have already been developed from modified lipid A structures (Christ et al. 1995; Ulrich and Myers 1995). By revealing the biochemical details of lipid A structure and function, we hope to understand its role in bacterial pathogenesis and to intervene with novel treatments for infection. However, we must remind ourselves that multiple molecular subtypes of lipid A are acting in concert in the bacterial cell. The need to unravel the interactions between individual lipid A modifications will provide fertile ground for future research.

Acknowledgments Work in the author's laboratory was supported by the Canadian Institutes of Health Research. We apologize to those authors whose work could not be cited due to space limitations.

References

Ahn VE, Lo EI, Engel CK, Chen L, Hwang PM, Kay LE, Bishop RE, Privé GG (2004) A hydrocarbon ruler measures palmitate in the enzymatic acylation of endotoxin. EMBO J 23:2931–2941

Babinski KJ, Kanjilal SJ, Raetz CR (2002) Accumulation of the lipid A precursor UDP-2,3-diacylglucosamine in an *Escherichia coli* mutant lacking the *lpxH* gene. J Biol Chem 277:25947–25956

Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, Xu W, Klevit RE, Le Moual H, Miller SI (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122:461–472

Bartling CM, Raetz CR (2009) Crystal structure and acyl chain selectivity of *Escherichia coli* LpxD, the *N*-acyltransferase of lipid A biosynthesis. Biochemistry 48:8672–8683

Beutler B, Rietschel ET (2003) Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol 3:169–117

Bishop RE (2005) The lipid A palmitoyltransferase PagP: molecular mechanisms and role in bacterial pathogenesis. Mol Microbiol 57:900–912
Bishop RE (2008) Structural biology of membrane-intrinsic beta-barrel enzymes: sentinels of the bacterial outer membrane. Biochim Biophys Acta 1778:1881–1896
Bishop RE (2010) Lipid A. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer-Verlag, Berlin Heidelberg. https://doi.org/10.1007/978-3-540-77587-4_28
Bishop RE (2014) Structural biology: lipopolysaccharide rolls out the barrel. Nature 511:37–38
Bishop RE, Gibbons HS, Guina T, Trent MS, Miller SI, Raetz CR (2000) Transfer of palmitate from phospholipids to lipid A in outer membranes of Gram-negative bacteria. EMBO J 19:5071–5080
Breazeale SD, Ribeiro AA, Raetz CR (2002) Oxidative decarboxylation of UDP-glucuronic acid in extracts of polymyxin-resistant *Escherichia coli*. Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose. J Biol Chem 277:2886–2896
Breazeale SD, Ribeiro AA, Raetz CR (2003) Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose in polymyxin resistant mutants of *Escherichia coli*: an aminotransferase (ArnB) that generates UDP-4-amino-4-deoxy-L-arabinose. J Biol Chem 278:24731–24739
Breazeale SD, Ribeiro AA, McClerren AL, Raetz CR (2005) A formyltransferase required for polymyxin resistance in *Escherichia coli* and the modification of lipid A with 4-amino-4-deoxy-L-arabinose: identification and function of UDP-4-deoxy-4-formamido-L-arabinose. J Biol Chem 280:14154–14167
Carty SM, Sreekumar KR, Raetz CR (1999) Effect of cold shock on lipid A biosynthesis in *Escherichia coli*. Induction at 12 degrees C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. J Biol Chem 274:9677–9685
Cho J, Lee CJ, Zhao J, Young HE, Zhou P (2016) Structure of the essential Haemophilus influenzae UDP-diacylglucosamine pyrophosphohydrolase LpxH in lipid A biosynthesis. Nat Microbiol 1:16154
Christ WJ, Asano O, Robidoux AL, Perez M, Wang YA, Dubuc GR, Gavin WE, Hawkins LD, McGuinness PD, Mullarkey MA, Lewis MD, Kishi Y, Kawata T, Bristol JR, Rose JR, Rossignol DP, Kobayashi S, Hishinuma L, Kimura A, Asakawa N, Katayama K, Yamatsu I (1995) E5531, a pure endotoxin antagonist of high potency. Science 268:80–83
Coggins BE, Li X, McClerren AL, Hindsgaul O, Raetz CR, Zhou P (2003) Structure of the LpxC deacetylase with a bound substrate-analog inhibitor. Nat Struct Biol 10:645–651
Cox AD, Wright JC, Li J, Hood DW, Moxon ER, Richards JC (2003) Phosphorylation of the lipid A region of meningococcal lipopolysaccharide: identification of a family of transferases that add phosphoethanolamine to lipopolysaccharide. J Bacteriol 185:3270–3277
Cuesta-Seijo JA, Neale C, Khan MA, Moktar J, Tran CD, Bishop RE, Pomès R, Privé GG (2010) PagP crystallized from SDS/cosolvent reveals the route for phospholipid access to the hydrocarbon ruler. Structure 18:1210–1219
Dalebroux ZD, Matamouros S, Whittington D, Bishop RE, Miller SI (2014) PhoPQ regulates acidic glycerophospholipid content of the *Salmonella* Typhimurium outer membrane. Proc Natl Acad Sci U S A 111:1963–1968
Dovala D, Rath CM, Hu Q, Sawyer WS, Shia S, Elling RA, Knapp MS, Metzger LE 4th (2016) Structure-guided enzymology of the lipid A acyltransferase LpxM reveals a dual activity mechanism. Proc Natl Acad Sci U S A 113:E6064–E6071
Emptage RP, Daughtry KD, Pemble CW 4th, Raetz CR (2012) Crystal structure of LpxK, the 4′-kinase of lipid A biosynthesis and atypical P-loop kinase functioning at the membrane interface. Proc Natl Acad Sci U S A 109:12956–12961
Garrett TA, Kadrmas JL, Raetz CR (1997) Identification of the gene encoding the *Escherichia coli* lipid A 4′-kinase. Facile phosphorylation of endotoxin analogs with recombinant LpxK. J Biol Chem 272:21855–21864
Genthe NA, Thoden JB, Holden HM (2016) Structure of the *Escherichia coli* ArnA *N*-formyltransferase domain in complex with N(5)-formyltetrahydrofolate and UDP-Ara4N. Protein Sci 25:1555–1562

Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol 183:1835–1842

Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, Miller SI (1998) PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol 27:1171–1182

Guo L, Lim K, Poduje C, Daniel M, Gunn J, Hackett J, Miller SI (1998) Lipid A acylation and bacterial resistance against vertebrate anti-microbial peptides. Cell 95:189–198

Hancock RE, Falla T, Brown M (1995) Cationic bactericidal peptides. Adv Microb Physiol 37:135–175

Hsu L, Jackowski S, Rock CO (1989) Uptake and acylation of 2-acyl-lysophospholipids by *Escherichia coli*. J Bacteriol 171:1203–1205

Hwang PM, Choy WY, Lo EI, Chen L, Forman-Kay JD, Raetz CR, Priv GG, Bishop RE, Kay LE (2002) Solution structure and dynamics of the outer membrane enzyme PagP by NMR. Proc Natl Acad Sci U S A 99:13560–13565

Jia W, El Zoeiby A, Petruzziello TN, Jayabalasingham B, Seyedirashti S, Bishop RE (2004) Lipid trafficking controls endotoxin acylation in outer membranes of *Escherichia coli*. J Biol Chem 279:44966–44975

Kelly TM, Stachula SA, Raetz CR, Anderson MS (1993) The *firA* gene of *Escherichia coli* encodes UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase. The third step of endotoxin biosynthesis. J Biol Chem 268:19866–19874

Khan MA, Bishop RE (2009) Molecular mechanism for lateral lipid diffusion between the outer membrane external leaflet and a beta-barrel hydrocarbon ruler. Biochemistry 48:9745–9756

Khan MA, Neale C, Michaux C, Pom AR, GG PP, Woody RW, Bishop RE (2007) Gauging a hydrocarbon ruler by an intrinsic exciton probe. Biochemistry 46:4565–4579

Khan MA, Moktar J, Mott PJ, Bishop RE (2010b) A thiolate anion buried within the hydrocarbon ruler perturbs PagP lipid acyl chain selection. Biochemistry 49:2368–2379

Khan MA, Moktar J, Mott PJ, Vu M, McKie AH, Pinter T, Hof F, Bishop RE (2010a) Inscribing the perimeter of the PagP hydrocarbon ruler by site-specific chemical alkylation. Biochemistry 49:9046–9057

Lee H, Hsu FF, Turk J, Groisman EA (2004) The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. J Bacteriol 186:4124–4133

Lee CJ, Liang X, Wu Q, Najeeb J, Zhao J, Gopalaswamy R, Titecat M, Sebbane F, Lemaitre N, Toone EJ, Zhou P (2016) Drug design from the cryptic inhibitor envelope. Nat Commun 25 (7):10638

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

Noland BW, Newman JM, Hendle J, Badger J, Christopher JA, Tresser J, Buchanan MD, Wright TA, Rutter ME, Sanderson WE, Muller-Dieckmann HJ, Gajiwala KS, Buchanan SG (2002) Structural studies of *Salmonella typhimurium* ArnB (PmrH) aminotransferase: a 4-amino-4-deoxy-L-arabinose lipopolysaccharide-modifying enzyme. Structure 10:1569–1580

Okuda S, Sherman DJ, Silhavy TJ, Ruiz N, Kahne D (2016) Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model. Nat Rev Microbiol 14:337–345

Onishi HR, Pelak BA, Gerckens LS, Silver LL, Kahan FM, Chen MH, Patchett AA, Galloway SM, Hyland SA, Anderson MS, Raetz CR (1996) Antibacterial agents that inhibit lipid A biosynthesis. Science 274:980–982

Petrou VI, Herrera CM, Schultz KM, Clarke OB, Vendome J, Tomasek D, Banerjee S, Rajashankar KR, Belcher Dufrisne M, Kloss B, Kloppmann E, Rost B, Klug CS, Trent MS, Shapiro L, Mancia F (2016) Structures of aminoarabinose transferase ArnT suggest a molecular basis for lipid A glycosylation. Science 351:608–612

Preston A, Maxim E, Toland E, Pishko EJ, Harvill ET, Caroff M, Maskell DJ (2003) *Bordetella bronchiseptica* PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. Mol Microbiol 48:725–736

Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76:295–329

Raetz CR, Roderick SL (1995) A left-handed parallel beta helix in the structure of UDP-*N*-acetylglucosamine acyltransferase. Science 270:997–1000

Raetz CR, Whitfield C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71:635–700

Reynolds CM, Kalb SR, Cotter RJ, Raetz CR (2005) A phosphoethanolamine transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of *Escherichia coli* lipopolysaccharide. Identification of the *eptB* gene and Ca^{2+} hypersensitivity of an *eptB* deletion mutant. J Biol Chem 280:21202–21211

Robey M, O'Connell W, Cianciotto NP (2001) Identification of *Legionella pneumophila rcp*, a *pagP*-like gene that confers resistance to cationic antimicrobial peptides and promotes intracellular infection. Infect Immun 69:4276–4286

Schmidt H, Hansen G, Singh S, Hanuszkiewicz A, Lindner B, Fukase K, Woodard RW, Holst O, Hilgenfeld R, Mamat U, Mesters JR (2012) Structural and mechanistic analysis of the membrane-embedded glycosyltransferase WaaA required for lipopolysaccharide synthesis. Proc Natl Acad Sci U S A 109:6253–6258

Six DA, Carty SM, Guan Z, Raetz CR (2008) Purification and mutagenesis of LpxL, the lauroyltransferase of *Escherichia coli* lipid A biosynthesis. Biochemistry 47:8623–8637

Smith AE, Kim SH, Liu F, Jia W, Vinogradov E, Gyles CL, Bishop RE (2008) PagP activation in the outer membrane triggers R3 core oligosaccharide truncation in the cytoplasm of *Escherichia coli* O157:H7. J Biol Chem 283:4332–4343

Trent MS, Ribeiro AA, Doerrler WT, Lin S, Cotter RJ, Raetz CR (2001) Accumulation of a polyisoprene-linked amino sugar in polymyxin-resistant *Salmonella typhimurium* and *Escherichia coli*: structural characterization and transfer to lipid A in the periplasm. J Biol Chem 276:43132–43144

Trent MS, Ribeiro AA, Lin S, Cotter RJ, Raetz CR (2001) An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. J Biol Chem 276:43122–43131

Touzé T, Tran AX, Hankins JV, Mengin-Lecreulx D, Trent MS (2008) Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. Mol Microbiol 67:264–277

Thaipisuttikul I, Hittle LE, Chandra R, Zangari D, Dixon CL, Garrett TA, Rasko DA, Dasgupta N, Moskowitz SM, Malmström L, Goodlett DR, Miller SI, Bishop RE, Ernst RK (2014) A divergent *Pseudomonas aeruginosa* palmitoyltransferase essential for cystic fibrosis-specific lipid A. Mol Microbiol 91:158–174

Ulrich JT, Myers KR (1995) Monophosphoryl lipid A as an adjuvant. In: Powell MF, Newman MJ (eds) Vaccine design: the subunit and adjuvant approach. Plenum Press, New York, pp 495–524

Vorachek-Warren MK, Ramirez S, Cotter RJ, Raetz CR (2002) A triple mutant of *Escherichia coli* lacking secondary acyl chains on lipid A. J Biol Chem 277:14194–14205

Wanty C, Anandan A, Piek S, Walshe J, Ganguly J, Carlson RW, Stubbs KA, Kahler CM, Vrielink A (2013) The structure of the neisserial lipooligosaccharide phosphoethanolamine transferase A (LptA) required for resistance to polymyxin. J Mol Biol 425:3389–3402

Whittington DA, Rusche KM, Shin H, Fierke CA, Christianson DW (2003) Crystal structure of LpxC, a zinc-dependent deacetylase essential for endotoxin biosynthesis. Proc Natl Acad Sci U S A 100:8146–8150

Wyckoff TJ, Lin S, Cotter RJ, Dotson GD, Raetz CR (1998) Hydrocarbon rulers in UDP-*N*-acetylglucosamine acyltransferases. J Biol Chem 273:32369–32372

Yan A, Guan Z, Raetz CR (2007) An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. J Biol Chem 282:36077–36089

Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria

10

Olaf Schneewind and Dominique Missiakas

Contents

1 Introduction 164
2 Structure of LTA Molecules 164
2.1 Type I LTA 164
2.2 Type II–IV LTA 166
2.3 LTA Modifications 166
3 Biosynthesis of LTA 168
3.1 Synthesis and Assembly of Type I LTA 168
3.2 DAG Recycling During Type I LTA Synthesis 169
3.3 Synthesis and Assembly of Type IV LTA 170
4 Function of LTA 171
4.1 Contribution of LTA to Bacterial Growth and Cell Division 171
4.2 LTA Contribution to the Pathogenesis of Bacterial Infections 173
5 LTA as a Target for the Development of Anti-infective, Probiotics, and Vaccines 174
6 Frontiers of LTA Research and Research Needs 175
References 175

Abstract

Lipoteichoic acids (LTA), polymers of repeating phosphodiester-linked polyols, are found in the outer leaflet of the plasma membrane of Gram-positive bacteria. Research on LTA structure represents a large, mostly unexplored frontier. LTA biosynthesis has been studied in several model organisms, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus subtilis*, and *Bacillus anthracis*. This work led to several hypotheses of LTA function to support bacterial growth, cell division and separation, ion hemostasis, as well as envelope assembly and integrity. Molecular genetic studies also revealed

O. Schneewind · D. Missiakas (✉)
Department of Microbiology, University of Chicago, Chicago, IL, USA
e-mail: oschnee@bsd.uchicago.edu; dmissiak@bsd.uchicago.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_17

catalysts for LTA substituents with D-alanine, phosphocholine and glycolipid anchors that impact the invasive attributes of bacterial pathogens or the anti-inflammatory attributes of microbiota. Consequently, LTA is being explored as a target for the development of antibiotics, vaccines, and immune therapeutics in order to address important unmet clinical needs for the treatment of human ailments.

1 Introduction

Teichoic acid polymers were discovered by Baddiley and colleagues during studies on the function of soluble nucleotides, CDP-glycerol and CDP-ribitol (Neuhaus and Baddiley 2003 and references herein). Initially, these investigations were performed with the bacterium *Lactobacillus arabinosus*; however, it was quickly noted that several bacterial species use soluble nucleotides to polymerize polyol phosphates, which were referred to as teichoic acids for their association with the bacterial envelope (Neuhaus and Baddiley 2003 and references herein). There are two kinds of teichoic acids, wall bound and membrane bound, also known as wall teichoic acid (WTA) and lipoteichoic acid (LTA). While in some organisms both types of molecules may display the same polyol phosphate structure, WTA is tethered to peptidoglycan via murein linkage units while LTA is retained in the plasma membrane via lipid anchors (reviewed by Xia et al. 2010; Percy and Grundling 2014; Schneewind and Missiakas 2014).

Fischer classified LTA molecules as polyol phosphate derivatives with repeating phosphodiester-linked units tethered to glycolipid and distinguished four basic types (I–IV) (Fischer 1994b and references herein). Other macroamphiphiles such as the lipopolysaccharides (LPS) of Gram-negative bacteria and lipoglycans of *Corynebacterium*, *Nocardia*, and *Rhodococcus* do not contain repeating phosphodiester-linked units and thus are distinct from LTA (Fischer 1994b). This review focuses on the structure, synthesis, and function of type I and IV LTA.

2 Structure of LTA Molecules

2.1 Type I LTA

Type I LTA bears the simple structure 1,3-polyglycerol-phosphate [poly(GroP)] and is found in many genera of the phylum *Firmicutes*. Poly(GroP) of *S. aureus*, *B. subtilis*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Streptococcus agalactiae*, and *Streptococcus pyogenes* encompasses 5–50 units (Fischer 1994b) and is linked to the C-6 of the nonreducing glycosyl of the glycolipid, β-gentiobiosyldiacylglycerol (diglucosyl-diacylglycerol [Glc_2-DAG]) (Duckworth et al. 1975) (Fig. 1a). Although dihexosyl-DAG (Hex_2-DAG) is the most common

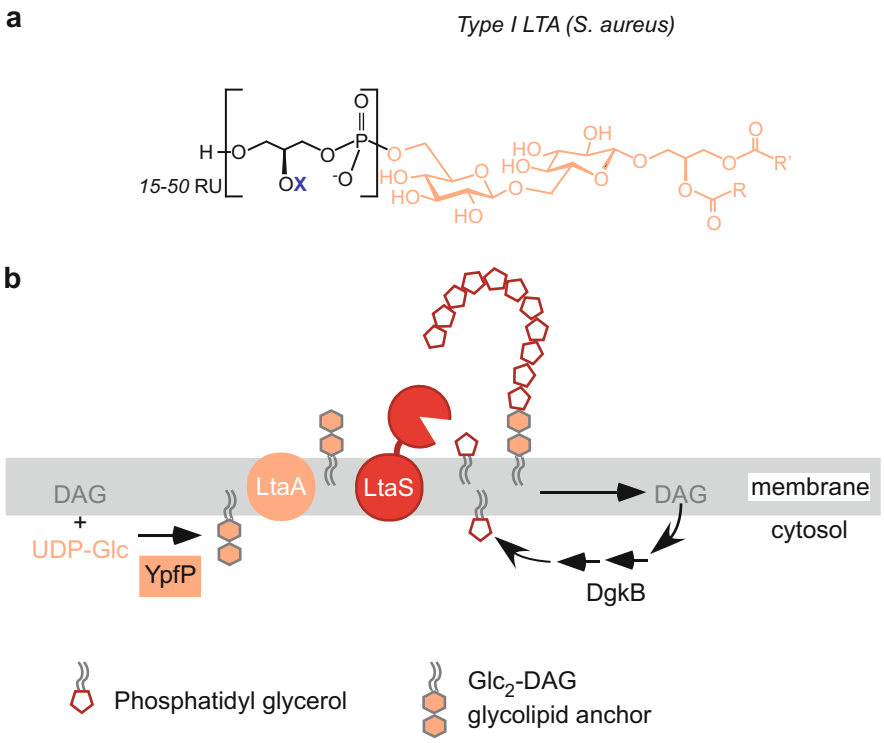


Fig. 1 Type I LTA: structure and biosynthesis pathway. (**a**) Structure of type I LTA from *S. aureus*. *Brackets* define the repeating unit (RU) and the glycolipid anchor is shown in *orange*. The 1,3-polyglycerol-phosphate RUs are substituted at the C2 position (X in *blue*) with hydrogen proton (∼15%), D-alanyl ester (∼70%), or *N*-acetylglucosamine (∼15%). (**b**) Diagram showing the biosynthetic pathway of type I LTA. In *S. aureus*, the glycolipid anchor Glc_2DAG is synthesized in a YpfP-dependent manner and transported to the outer leaflet of the membrane by LtaA. A glycerophosphate (GroP) subunit is cleaved from phosphatidylglycerol (PG) and attached to Glc_2DAG to generate $Glc_2DAG\text{-}(GroP)_1$. In *S. aureus*, this reaction is catalyzed by LtaS. Subsequent transfer of GroP onto $Glc_2DAG\text{-}(GroP)_1$ is also catalyzed by LtaS in *S. aureus*. Other bacteria (e.g., *L. monocytogenes*, *B. subtilis*) use two different enzymes whereby the first reaction is initiated by a dedicated LtaS primase. Elongation of the GroP polymer leads to the release of DAG. The DgkB enzyme initiates the recycling of DAG

glycolipid anchor, exceptions occur: *Lactobacillus gasseri* JCM 1131 uses tetrahexosyl glycerol with either two or three fatty acid chains to anchor poly(GroP) (Shiraishi et al. 2013), whereas *Streptococcus* sp. DSM 8747 uses 3-*O*-(β-D-galactofuranosyl)-1,2-DAG (Fischer 1994a). In some species, Glc_2-DAG may be substituted with either acyl or phosphatidyl leading to the production of discrete LTA variants (Fischer et al. 1983; Uchikawa et al. 1986; Dehus et al. 2011). In *L. monocytogenes*, growth temperature impacts the fatty acid composition of the LTA glycolipid anchor (Dehus et al. 2011).

Type I LTA may be found in *Actinobacteria* and *Tenericutes* along with lipoglycans, polysaccharides, that are also attached to a membrane anchor (glycolipid or phosphatidylinositol) as regular envelope constituents of acid-fast bacteria, including *Mycobacterium tuberculosis*, and otherwise lack WTA (Rahman et al. 2009a, b; Berg et al. 2007; Mishra et al. 2011; Jankute et al. 2015).

2.2 Type II–IV LTA

Type II and III LTA molecules are composed of glycosylalditol-phosphate polymers. For example, type II LTA of *Lactococcus garvieae* consists of the repeating units α-Gal(1-6)-α-Gal(1-3)-GroP polymerized onto kojibiose [α-Glc(1-2)-α-Glc(1-3)] linked to DAG; the first Glc of the lipid anchor may be acetylated (Fischer 1994b; Schmidt et al. 2011). Type III LTA of *Clostridium innocuum* is composed of α-Gal (1-3)-GroP repeats linked to the glycolipid β-GlcN(1-3)-α-Glc(1-3)-DAG where GlcN is glucosamine (Fischer 1994b; Schmidt et al. 2011).

Type IV LTA is a complex molecule that is found in *S. pneumoniae* and a few other *Streptococcus* spp. *S. pneumoniae* (pneumococcal) teichoic acids were identified with serological assays (Tillett and Francis 1930), which revealed two distinct components of the envelope: the C-polysaccharide (pneumococcal WTA) (Goebel and Adams 1943; Gotschlich and Liu 1967; Poxton et al. 1978) and the F-antigen with Forssman antigenicity and fatty acid content (pneumococcal LTA) (Briles and Tomasz 1973; Sorensen and Henrichsen 1987). The structure of the repeat units in pneumococcal WTA and LTA is identical, the pseudopentasaccharide 2-acetamido-4-amino-2,4,6-trideoxygalactose (AATGal), glucose (Glc), and ribitol phosphate (RboP) followed by two substituted GalNAc-GalNAc moieties (Fig. 2a) (Fischer 1997; Seo et al. 2008). Repeat units are polymerized via α-glycosidic linkages, whereas a β-glycosidic linkage tethers the poly to its membrane anchor, the glycolipid Glc(β1–β3)-DAG (Gisch et al. 2013). The GalNAc-GalNAc-Glc-DAG structure represents the molecular basis for the Forssman antigenicity (Seo et al. 2008).

Recently, the repeat unit of *C. difficile* LTA was revealed as [→6]-α-D-GlcNAc-(1-3)-[→*P*-6]-α-D-GlcNAc-(1-2)-GroA] where GlcNAc is *N*-acetylgalactosamine and GroA is glyceric acid. These repeat units are linked by a phosphodiester bridge between C-6 of the two GlcNAc residues. A minor fraction of LTA contains GlcN-(1-3) instead of GlcNAc-(1-3) in the repeat unit (Reid et al. 2012). The polymer is anchored through a 6–6 phosphodiester bridge to glycolipid Glc_3-DAG and represents a new type of LTA not described earlier by Fischer (Fischer 1994b). Interestingly, the structure of *C. difficile* LTA is identical to the repeat unit of a dominant cell wall antigen of *Peptostreptococcus anaerobius* (Stortz et al. 1990).

2.3 LTA Modifications

Additional chemical diversity of LTA is brought about by substituents that modify the polymer to achieve very specific properties. For example, poly-GroP type I LTA

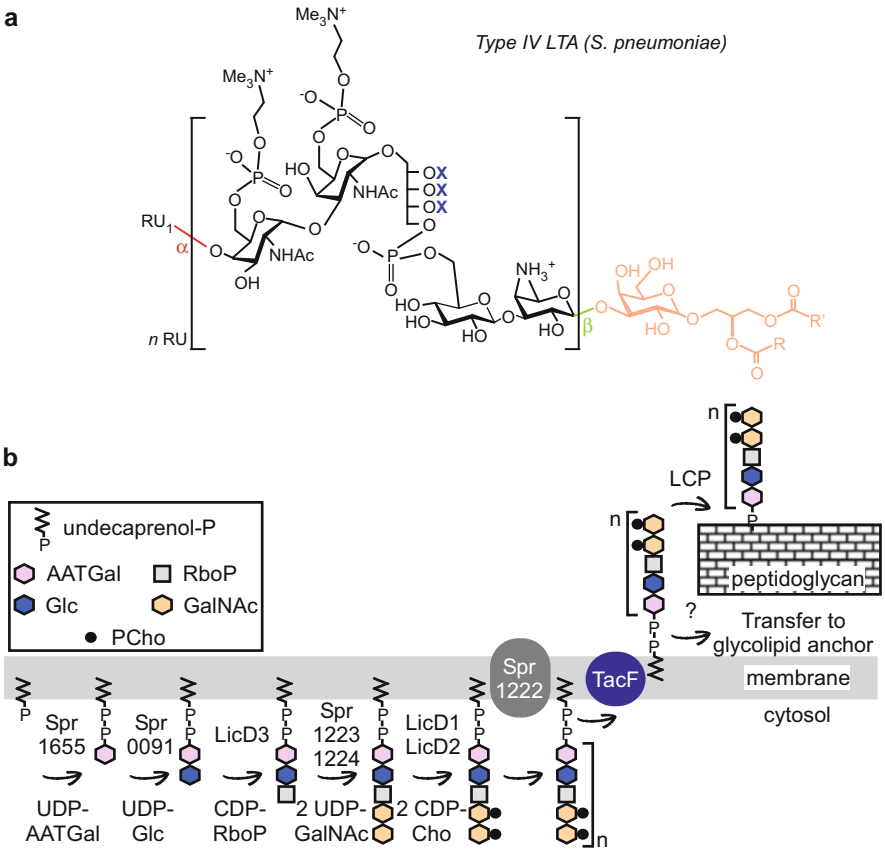


Fig. 2 Type IV LTA: structure and biosynthesis pathway. (**a**) Structure of type IV LTA of *S. pneumoniae*. *Brackets* define the repeating unit (RU). The glycolipid anchor is shown in *orange*. Substituents (X in *blue*) include hydrogen, D-alanyl, or *N*-acetylglucosamine. Substituents R, R′, and R″ in the glycolipids (*orange*) may be alkyl or branched alkyl chains. (**b**) Possible model for the biosynthetic pathway of type IV LTA. The RU is assembled onto the lipid carrier undecaprenyl-phosphate (C_{55}-P) and is initiated by Spr1655 to generate the C_{55}-PP-AATGal precursor. Subsequent reactions catalyzed by the glycosyltransferases Spr0091, LicD3, and Spr1223/1224 yield C_{55}-PP-AATGal-Glc-RboP-GalNAc-GalNAc. LicD1 and LicD2 add the PCho groups onto the GalNAc residues. Elongation of the polymer is catalyzed by Spr1222 which may then be transported to the bacterial surface by TacF. The teichoic acid polymer may be transferred onto peptidoglycan by a dedicated LCP protein to yield WTA. The mechanism of LTA assembly, i.e., transfer of the polymer from C_{55}-P onto the glycolipid anchor, is not known

may be substituted at position 2 with D-alanine esters or *N*-acetylglucosamine (GlcNAc) (Fig. 1a); the abundance of such modifications varies with growth conditions (Iwasaki et al. 1986; Morath et al. 2001). GlcNAc modification of teichoic acid has been well documented for *B. subtilis* (Arakawa et al. 1981; Iwasaki et al. 1986), while type I LTA of *Listeria monocytogenes* has been shown to be substituted with

α-Gal. The glycerol moiety of type II LTA (Gal-Gal-GroP)$_n$ may be modified with GlcNAc or GlcN, and the glycerol moiety of type III LTA (Gal-GroP)$_n$ may be modified with α-Gal; D-alanylation was not observed for these molecules (Fischer 1994b; Schmidt et al. 2011).

Type IV LTA may be modified with D-alanine on the hydroxyl groups of RboP of the terminal repeating unit; however, the polymer always carries phosphocholine (PCho) residues at position *O*-6 of both GalNAc moieties of its repeat units (Fig. 2a) (Seo et al. 2008). The PCho substituent is abundantly found in both types of teichoic acids of *S. pneumoniae* and of its close relatives, i.e., the mitis group of streptococci including *S. oralis*, *S. mitis*, *S. pseudopneumoniae*, and *S. infantis* (Young et al. 2013; Gisch et al. 2015). Glycan modification with PCho is rare but has also been reported for some capsular polysaccharides of *S. pneumoniae*, as well as for lipopolysaccharides of a few Gram-negative organisms including *Haemophilus influenzae* (Young et al. 2013).

3 Biosynthesis of LTA

We summarize here what has been learned for the synthesis and assembly of type I and type IV LTA molecules. The corresponding pathways for type II and type III LTA have not yet been studied.

3.1 Synthesis and Assembly of Type I LTA

The synthesis of type I LTA can be viewed as a simple two-step mechanism whereby two substrates, the glycolipid anchor and repeating units, are first translocated on the *trans* side of the plasma membrane and subsequently repeat units are added onto the glycolipid anchor (Fig. 1b). The enzymes responsible for this pathway were first identified and characterized using *B. subtilis* and *S. aureus* as model systems. In both organisms, synthesis of the first substrate, the glycolipid Glc$_2$-DAG, is catalyzed by the enzymes PgcA and GtaB. In this manner, glucose-6-phosphate is first converted to glucose-1-phosphate by PgcA and then to uridine diphosphate glucose (UDP-Glc) by GtaB (Forsberg et al. 1973; Pooley et al.1987; Soldo et al. 1993; Lazarevic et al. 2005). In a third step, the processive glycosyltransferase YpfP strings two UDP-Glc onto DAG to generate Glc$_2$-DAG (Jorasch et al. 1998, 2000; Kiriukhin et al. 2001). In *S. aureus*, the Glc$_2$-DAG glycolipid is translocated across the membrane by LtaA (Gründling and Schneewind 2007a). Interestingly, LtaA is not conserved among all organisms synthesizing type I LTA. For example, *ltaA* is not found in the genome of *B. subtilis* (Jorasch et al. 1998; Reichmann and Gründling 2011). Notably, *S. aureus* variants lacking *ltaA* anchor (GroP) chain onto DAG instead of Glc$_2$-DAG (Gründling and Schneewind 2007a). In *L. monocytogenes*, synthesis of the more complex glycolipid anchor requires two glycosyltransferases, LafA and LafB (LTA anchor formation protein), and the transporter LafC fulfills the function of

staphylococcal LtaA (Webb et al 2009). These genes are also found in the genomes of *E. faecalis*, *S. agalactiae*, and *S. pneumoniae* (Reichmann and Gründling 2011).

Assembly of the LTA GroP chain in *S. aureus* is catalyzed by a single enzyme LtaS, a transmembrane protein with a large extracytoplasmic domain that cleaves GroP from phosphatidylglycerol (PG) for elongation of GroP polymers (Gründling and Schneewind 2007b) (Fig. 1b). In *L. monocytogenes*, a related primase LtaP catalyzes the first addition of GroP on the lipid anchor, and subsequent elongation of the chain is catalyzed by LtaS (Webb et al. 2009). *B. subtilis* and *B. anthracis* encode four LtaS-like proteins but presumably produce one type I LTA molecule. Thus, the importance of multiple different LtaS enzymes for the synthesis of LTA is not fully understood (Gründling and Schneewind 2007b; Schirner et al. 2009; Wörmann et al. 2011; Garufi et al. 2012).

The addition of D-alanine to type I LTA is achieved by the concerted activity of the Dlt proteins that include the D-alanine-activating ligase (DtlA) and the D-alanine carrier protein (DltC) (Baddiley and Neuhaus 1960; Perego et al. 1995; Neuhaus and Baddiley 2003). Two models have been proposed to explain the function of DltB and DltD (Baddiley and Neuhaus 1960; Perego et al. 1995; Neuhaus and Baddiley 2003). The most recent investigations support a model whereby DltB transfers D-alanine from DltC to undecaprenyl-phosphate followed by flipping of the lipid-linked intermediate across the membrane. DltD, on the *trans* side of the membrane, then transfers D-alanine from the lipid carrier to LTA (Baddiley and Neuhaus 1960; Perego et al. 1995; Neuhaus and Baddiley 2003; Reichmann et al. 2013). It seems noteworthy that type I LTA is also thought to serve as an intermediate for the D-alanyl esterification of WTA. In vitro and in vivo pulse-chase experiments support a model where D-alanyl esters are transferred from LTA to WTA, presumably in a catalyst-independent manner (Haas et al. 1984; Koch et al. 1985).

Studying *L. monocytogenes*, Gründling and colleagues developed a model for the glycosylation of type I LTA based (Mancuso and Chiu 1982; Yokoyama et al. 1988; Percy and Grundling 2014; Percy et al. 2016). The model proposes that a transmembrane glycosyltransferase first links UDP-Gal to undecaprenyl-P, while another glycosyltransferase catalyzes transport across the membrane and transfer onto LTA (Mancuso and Chiu 1982; Yokoyama et al. 1988; Percy and Grundling 2014). Gründling and colleagues note that this assembly mechanism is reminiscent of the pathway for 4-amino-4-deoxy-l-arabinose (r-Ara4N) modification of lipopolysaccharide in *E. coli* and used a bioinformatics approach to identify GtlA (Lmo0933) as the *L. monocytogenes* initiating glycosyltransferase of LTA synthesis (Percy et al. 2016).

3.2 DAG Recycling During Type I LTA Synthesis

Polymerization of type I LTA consumes large amounts of PG, which serves as a substrate for LtaS-mediated production of poly-GroP and release of DAG. Labeling experiments revealed that the staphylococcal PG pool is turned over twice per generation in order to produce LTA molecules with approximately 25 GroP subunits accounting for one ninth of total membrane lipids (Koch et al. 1984). DAG kinase

(DgkB) phosphorylates DAG to yield phosphatidic acid, a precursor of PG synthesis (Taron et al. 1983) (Fig. 1b). Deletion of the *dgkB* gene in *B. subtilis* has been shown to result in loss of viability (Matsuoka et al. 2011). Presumably, this phenotype can be rescued by deleting either one of the housekeeping *ltaS*-like genes, *yflE* or *yfnI*, albeit that deletion of *yfnI* does not correlate with a reduced DAG content as would be expected if the toxicity of the *dgkB* mutation was due solely to the accumulation of DAG (Matsuoka et al. 2011). There are no reports to suggest that assembled LTA molecules can be recycled and LTA molecules may be released from the microbial surface into the extracellular medium during bacterial division. Indeed, released LTA molecules have been observed to form micelles in vitro and have been proposed to stimulate mammalian immune responses and exert inflammatory properties (Wicken et al. 1986).

3.3 Synthesis and Assembly of Type IV LTA

In *S. pneumoniae* and mitis group streptococci, LTA and WTA polymers share the same repeating unit: AATGal-Glc-RboP-GalNAc-GalNAc (Fig. 2a). It is assumed, but not experimentally demonstrated, that the same pathway is used for the polymerization of this repeat unit in a manner that is reminiscent of WTA synthesis in *S. aureus* and *B. subtilis* (Xia et al. 2010). Briefly, repeat units are thought to be synthesized on the lipid carrier undecaprenyl-phosphate (C_{55}-P) on the *cis* side of the plasma membrane via a sequence of event that is initiated by Spr1655, a proposed sugar transferase, utilizing soluble UDP-AATGal to generate the insoluble C_{55}-PP-AATGal and UMP (Denapaite et al. 2012) (Fig. 2b). Next, the glycosyltransferase Spr0091 has been proposed to transfer Glc onto C_{55}-PP-AATGal, utilizing UDP-Glc substrate to generate C_{55}-PP-AATGal-Glc. LicD3 (Spr1225) phosphotransferase is thought to require CDP-RboP substrate, whereas Spr1223 and Spr1224 each utilize UDP-GalNAc for the synthesis of C_{55}-PP-AATGal-Glc-RboP-GalNAc-GalNAc (Denapaite et al. 2012). Finally, LicD1 and LicD2 modify the GalNAc residues of the repeat unit precursor with PCho (Zhang et al. 1999; Denapaite et al. 2012) (Fig. 2b).

Spr1222 has been designated to polymerize C_{55}-PP-AATGal-Glc-RboP-GalNAc (PCho)-GalNAc(PCho) (Denapaite et al. 2012). Spr1222 is a member of the Wzy transmembrane protein family, which includes the well-characterized O-antigen polysaccharide polymerase; this enzyme links O-antigen repeat units via glycosidic linkage to lipid A, thereby completing the synthesis of LPS. TacF (Spr1150), a member of the family of 14 transmembrane-helix transporters, is thought to translocate Spr1222-assembled teichoic acid polymers to the *trans* side of the plasma membrane (Denapaite et al. 2012).

Bioinformatics evidence supports the hypothesis that a member of the LytR-CpsA-Psr (LCP) protein family (Spr1759) may tether WTA precursor to peptidoglycan (Denapaite et al. 2012), as occurs in *B. subtilis* and *S. aureus* (Kawai et al. 2011; Chan et al. 2013). However, it is not clear how TA polymers are transferred onto their glycolipid anchors (Fig. 2b). Members of the LCP family of proteins have been shown to transfer C_{55}-P-linked polymer precursors onto peptidoglycan (Kawai

et al. 2011; Eberhardt et al. 2012; Chan et al. 2013, 2014; Liszewski Zilla et al. 2015). *Actinomyces oris* LCP-like protein appears to represent an exception, as this protein is thought to promote the glycosylation of the GspA surface protein (Wu et al. 2014). If LCP proteins were involved in transferring teichoic acid precursors onto glycolipids, such reactions would certainly expand the currently appreciated spectrum of substrates for these enzymes.

It is interesting that *S. pneumoniae* does not synthesize choline and can only grow in media supplemented with this molecule (Rane and Subbarow 1940; Tomasz 1967). As occurs in *Haemophilus influenzae*, extracellular choline is transported into pneumococci by LicB permease, where LicA phosphorylates choline is to yield PCho, which is subsequently activated into CDP-choline by the cytidylyltransferase LicC (Fan et al. 2003; Gehre et al. 2008). Choline-independent growth variants have been isolated, and genetic suppressors mapped to the proposed TacF transporter (Damjanovic et al. 2007). It has been suggested that suppression occurs by increasing the substrate repertoire of TacF to allow for transport of teichoic acid precursors lacking choline (Damjanovic et al. 2007). Furthermore, the *tacF* gene cannot be deleted in pneumococci, presumably due to the accumulation of TA precursors and the depletion of C_{55}-P, which is essential for peptidoglycan synthesis and bacterial growth (Song et al. 2005; Denapaite et al. 2012).

D-alanylation of type IV LTA is also mediated by the DltABCD, as already described for type I LTA. It is interesting that only some but not all *S. pneumoniae* isolates carry the *dltXABCD* gene operon (Denapaite et al. 2012). The commonly used laboratory strain R6 carries a point mutation in the *dlt* operon, which abolishes the D-alanylation of pneumococcal teichoic acids (Kovacs et al. 2006).

4 Function of LTA

4.1 Contribution of LTA to Bacterial Growth and Cell Division

It has been difficult to define the physiological functions of TA as mutations that abrogate the synthesis of either WTA or LTA molecules could not be defined until the discovery of the enzymes that are responsible for the synthesis and assembly of these molecules. In *S. aureus* and *B. subtilis*, *tagO* mutants lacking WTA continue to replicate; however, staphylococcal mutants lacking WTA are enlarged and do not divide in the same manner as wild type (Weidenmaier et al. 2004; D'Elia et al. 2006; Campbell et al. 2011). *B. subtilis* cannot tolerate the simultaneous deletion of *tagO* and *ltaS* genes (Schirner et al. 2009). Further, deletion of *ltaS* genes leads to severe growth and cell division defects in several microbes, including *S. aureus*, *L. monocytogenes*, *B. subtilis*, and *B. anthracis* (Gründling and Schneewind 2007b; Schirner et al. 2009; Webb et al. 2009; Wörmann et al. 2011; Garufi et al. 2012). In *B. subtilis ltaS* mutants, the filamentation phenotype was attributed to mislocalization of FtsZ, a key cell division protein that otherwise assembles at division sites (Schirner et al. 2009). In *S. aureus*, a direct interaction between cell division proteins and LTA synthesis proteins has been documented (Reichmann and

Gründling 2011). Careful examination of *S. aureus* mutants with a deleted *ltaS* gene revealed that viability required the acquisition of compensatory mutations and that viability could be improved by maintaining *ltaS* mutants under osmotically stabilizing conditions (high concentrations of NaCl or sucrose) or by lowering the growth temperature (Gründling and Schneewind 2007b; Oku et al. 2009; Corrigan et al. 2011). Thus, LTA synthesis is essential for *S. aureus* growth, and growth of *ltaS* mutants can be restored by increases in cellular cyclic di-adenosine monophosphate (c-di-AMP), a signaling molecule promoting increased transport of potassium and other ions (Oku et al. 2009; Corrigan et al. 2011, 2013). Together these observations suggest that LTA may act as an osmoprotectant (Percy and Grundling 2014). In their recent review, Percy and Grundling have highlighted similarities between the synthesis pathways of LTA and osmoregulated periplasmic glycans of Gram-negative bacteria. Formerly referred to as membrane-derived oligosaccharides, osmoregulated periplasmic glycans accumulate under low-osmolarity conditions in the periplasm of Gram-negative bacteria to counteract swelling of the cell. These glycans are modified with GroP residues obtained from PG by MdoB, an enzyme that shares structural and functional features of LtaS (Percy and Grundling 2014 and references herein). Recently, it was reported that spontaneous suppressors of the slow growing phenotype of a *S. aureus clpX* mutant mapped to *ltaS*. A majority of suppressor alleles was the result of genetic changes that abolished LtaS synthesis (Baek et al. 2016). The absence of the chaperone ClpX partly alleviated the septum placement defects of cells lacking LTA, yet the double *clpX ltaS* mutant remained otherwise large in size and displayed reduced autolytic activity as is also observed upon depletion of *ltaS* (Baek et al. 2016). Phenotypic suppression was found to be independent of c-di-AMP; however, it is not known why LTA becomes nonessential for growth upon loss of the cytosolic ClpX chaperone (Baek et al. 2016).

Owing to the extensive modifications of LTA polymers, teichoic acids act as zwitterionic molecules and have been proposed to modulate ion homeostasis (Neuhaus and Baddiley 2003). D-alanylation of LTA has been proposed to promote Mg^{2+} ion scavenging, to localize autolysins to discrete positions within the bacterial envelope during cell division-associated peptidoglycan separation, and to restrict the activity of autolysins and cell wall-active antibiotics (Cleveland et al. 1975; Lambert et al. 1977; Peschel et al. 2000; Schlag et al. 2010). Thus, in addition to its roles during cell division and an osmoprotectant, the esterified forms of LTA contribute to maintaining the integrity of the bacterial envelope.

Substituted LTA has been proposed to bind and retain proteins in the envelope of Gram-positive bacteria, in particular proteins carrying GW modules, an approximately 80-amino-acid-long domain carrying the conserved glycine-tryptophan motif (Jonquieres et al. 1999). For example, the GW module is present in staphylococcal autolysins AtlA and AtlE from *S. aureus* and *S. epidermidis*, and a structural model for their interaction with LTA was recently proposed (Zoll et al. 2012). However, in *L. monocytogenes*, it has also been shown that mutants lacking the ability to glycosylate and D-alanine esterify LTA continue to bind the GW module proteins InlB and Ami, pointing to the possibility that GW proteins may interact with peptidoglycan (Percy et al. 2016).

Choline-modified LTA has been shown to enable the deposition of LytB glucosaminidase and LytA autolysin near the septum of dividing pneumococci, suggesting type IV LTA also promotes the localization of specific factors during bacterial cell division and separation (Mosser and Tomasz 1970). Because autolysins contribute to cell division and biofilm formation (Heilmann et al. 1997), it is not surprising that bacteria with reduced amounts of LTA display pleiotropic phenotypes (Fedtke et al. 2007).

4.2 LTA Contribution to the Pathogenesis of Bacterial Infections

It is not possible to assess the contribution of LTA toward bacterial pathogenesis by infecting animals with variants that are unable to synthesize LTA; as is outlined above, *ltaS* variants divide more slowly than wild type and display pleiotropic phenotypes impacting cell division and growth that undoubtedly impact the pathogenesis of bacterial infections without necessarily contributing unique virulence traits. Nonetheless, it has been shown that *ypfP* mutant staphylococci with altered LTA membrane anchor structure are affected for their ability to invade human brain microvascular endothelial cells, which led to the proposal that LTA may mediate bacterial invasion of endothelial cells as a means for microbial exit from the bloodstream and entry into the central nervous system (Sheen et al. 2010).

D-alanylation of LTA is also important for maintaining the integrity of the bacterial envelope (autolysis and cation homeostasis). Nevertheless, experimental work with *dlt* mutations in *L. monocytogenes* and *S. aureus* implicated D-alanylation of TA as an important virulence trait (Abachin et al. 2002; Xia et al. 2010). *dlt* mutations affect bacterial adhesion to and invasion of host cells in *L. monocytogenes* (Abachin et al. 2002) and group A streptococci (Kristian et al. 2005), as well as biofilm formation in enterococci (Fabretti et al. 2006). *dlt* mutants also display diminished resistance to cell wall-active antibiotics, suggesting that D-alanylation of TAs represents an intrinsic resistance trait for antibiotics (Peschel et al. 2000; Weidenmaier and Peschel 2008). Modifications of TA polymers with D-alanyl esters or *N*-acetylglucosamine have been shown to be important for resistance toward host cationic antimicrobial peptides (Peschel et al. 1999; Abachin et al. 2002; Kristian et al. 2005).

LTA has been proposed to activate the immune system of infected hosts and promote inflammation by acting as ligands for Toll-like receptors (TLR2/TLR6) and their cofactors (CD14 and CD36) (Nilsen et al. 2008). Additional pattern recognition receptors implicated recognizing LTA include the macrophage type I scavenger receptor (Greenberg et al. 1996) and the platelet-activating factor receptor, which is thought to trigger mucin production by epithelial cells (Lemjabbar and Basbaum 2002). Nonetheless, the stimulatory activity of LTA for pattern recognition receptors is at least three orders of magnitude less potent than that of LPS (Ginsburg 2002). Chemical synthesis of LTA has been used to rigorously define the contributions of the glycolipid anchor, the polymeric repeats, or polymer substituents of LTA for their roles in stimulating the immune system of vertebrates and to address the concern that

LTA purified from bacteria may be contaminated with compounds already known to activate immune responses (peptidoglycan and lipoprotein) (Henneke et al. 2005; Schmidt et al. 2011; Rockel and Hartung 2012). This work suggested that chemically synthesized LTA activates the lectin pathway of complement but not TLR2 (Schmidt et al. 2011).

5 LTA as a Target for the Development of Anti-infective, Probiotics, and Vaccines

LtaS, the catalyst of type I LTA synthesis, encompasses five transmembrane domains as well as a C-terminal catalytic domain and is found in bacteria but not in mammalian organisms (Gründling and Schneewind 2007b). The unique presence of LTA and LtaS in the envelope of bacteria, the display of the catalytic domain of LtaS on the bacterial surface, and the requirement of LTA synthesis for bacterial growth and cell division are inclusion criteria for high-value targets of antibiotic development. A small-molecule inhibitor of LtaS, designated compound 1771, was identified as an antibiotic for Gram-positive bacteria with polyglycerol-phosphate LTA (Richter et al. 2013). While spontaneous suppressors of staphylococcal *ltaS* mutations can be readily isolated, spontaneous mutants that are resistant to compound 1771 were not observed, which suggests that compound 1771 may affect another target in addition to LtaS (Richter et al. 2013). Further, the effectiveness of an antibiotic targeting LtaS would be limited to bacteria synthesizing type I LTA. Nonetheless, the discovery of compound 1771 validates LTA synthesis as a target for the discovery and development of antibiotics that may be useful as therapeutics for infectious diseases caused by antibiotic-resistant Gram-positive bacteria.

Probiotics, the formulation of microbes for gastrointestinal uptake benefitting microbiota hemostasis, have been used as preventives and therapeutics of autoimmune and inflammatory diseases. The LTA pathway has been targeted to affect the inflammatory attributes of the probiotics. For example, intestinal colonization of mice with *Lactobacillus acidophilus* revealed that compared to wild type, *ltaS* mutant lactobacilli downregulate production of inflammatory cytokines (IL-12 and TNFα) while increasing the production of anti-inflammatory cytokine (IL-10), thereby mitigating the effects of experimentally induced colitis (Mohamadzadeh et al. 2011). This result suggests that the LTA synthesis pathway may be exploited for the development of probiotic therapies of intestinal and other inflammatory disorders (Lebeer et al. 2012).

Finally, LTA has been targeted as a vaccine protective antigen. Immunization of animals with type I LTA has been reported to elicit the production of antibodies with opsonic properties for bacteria expressing the corresponding LTA molecules (Theilacker et al. 2006, 2012; Kodali et al. 2015). Further, a monoclonal antibody that binds type I LTA of *S. aureus* was developed and in preclinical trials was demonstrated to promote opsonophagocytic killing of staphylococci as well as prevention of staphylococcal sepsis (Weisman et al. 2011). Unfortunately, a clinical trial examining intravenous administration of LTA-specific antibody for protection

against staphylococcal sepsis in very-low birth weight neonates did not achieve the designated endpoints (Weisman et al. 2011). Other efforts of developing LTA as a vaccine antigen are under way, for example, by improving the immunogenicity of LTA through the use of synthetic derivatives or the conjugation of LTA to proteins with adjuvant function (Oberli et al. 2011; Cox et al. 2013; Broecker et al. 2016).

6 Frontiers of LTA Research and Research Needs

Although the structure of some LTA molecules has been known for several decades, the catalysts and biosynthesis pathways for type I and IV LTA were only recently revealed and are still not completed. Much additional work is needed to uncover the biosynthesis of type II and III LTA and to reveal the structural complexity of LTA from different Gram-positive bacteria. Taken together this work will provide much needed insight on the physiological functions of LTA for bacterial growth, cell division, ion hemostasis, and integrity of bacterial microbial envelope. It can also lead to the development of antibiotics specifically targeting LTA synthesis. Key LTA functions during microbial associations with vertebrate microbiota or as invasive pathogens appear to rely on LTA substituents such as D-Ala, PCho, or glycolipid anchor moieties. Exploring the impact of structural LTA modifications may provide new insights into the pathogenesis of bacterial infections and the protective functions of microbiota toward autoimmune and inflammatory diseases. Finally, vertebrate adaptive immune responses toward LTA, for example, antibodies triggering opsonophagocytosis, may be used to develop preventive vaccines or immune therapeutics for infectious diseases caused by Gram-positive bacteria.

References

Abachin E, Poyart C, Pellegrini E et al (2002) Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. Mol Microbiol 43:1–14

Arakawa H, Shimada A, Ishimoto N et al (1981) Occurrence of ribitol-containing lipoteichoic acid in *Staphylococcus aureus* H and its glycosylation. J Biochem 89:1555–1563

Baddiley J, Neuhaus FC (1960) The enzymic activation of D-alanine. Biochem J 75:579–587

Baek KT, Bowman L, Millership C et al (2016) The cell wall polymer lipoteichoic acid becomes nonessential in *Staphylococcus aureus* cells lacking the ClpX chaperone. MBio 7. https://doi.org/10.1128/mBio.01228-16

Berg S, Kaur D, Jackson M et al (2007) The glycosyltransferases of *Mycobacterium tuberculosis* – roles in the synthesis of arabinogalactan, lipoarabinomannan, and other glycoconjugates. Glycobiology 17:35–56R

Briles EB, Tomasz A (1973) Pneumococcal Forssman antigen. A choline-containing lipoteichoic acid. J Biol Chem 248:6394–6397

Broecker F, Martin CE, Wegner E et al (2016) Synthetic lipoteichoic acid glycans are potential vaccine candidates to protect from *Clostridium difficile* infections. Cell Chem Biol 23:1014–1022

Campbell J, Singh AK, Santa Maria JP Jr (2011) Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in *Staphylococcus aureus*. ACS Chem Biol 6:106–116

Chan YG, Frankel MB, Dengler V et al (2013) *Staphylococcus aureus* mutants lacking the LytR-CpsA-Psr family of enzymes release cell wall teichoic acids into the extracellular medium. J Bacteriol 195:4650–4659

Chan YG, Kim HK, Schneewind O et al (2014) The capsular polysaccharide of *Staphylococcus aureus* is attached to peptidoglycan by the LytR-CpsA-Psr (LCP) family of enzymes. J Biol Chem 289:15680–15690

Cleveland RF, Höltje JV, Wicken AJ et al (1975) Inhibition of bacterial wall lysins by lipoteichoic acids and related compounds. Biochem Biophys Res Commun 67:1128–1135

Corrigan RM, Abbott JC, Burhenne H (2011) c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. PLoS Pathog 7:e1002217

Corrigan RM, Campeotto I, Jeganathan T et al (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. Proc Natl Acad Sci U S A 110:9084–9089

Cox AD, St Michael F, Aubry A et al (2013) Investigating the candidacy of a lipoteichoic acid-based glycoconjugate as a vaccine to combat *Clostridium difficile* infection. Glycoconj J 30:843–855

D'Elia MA, Millar KE, Beveridge TJ et al (2006) Wall teichoic acid polymers are dispensable for cell viability in *Bacillus subtilis*. J Bacteriol 188:8313–8316

Damjanovic M, Kharat AS, Eberhardt A et al (2007) The essential *tacF* gene is responsible for the choline-dependent growth phenotype of *Streptococcus pneumoniae*. J Bacteriol 189:7105–7111

Dehus O, Pfitzenmaier M, Stuebs G et al (2011) Growth temperature-dependent expression of structural variants of *Listeria monocytogenes* lipoteichoic acid. Immunobiology 216:24–31

Denapaite D, Bruckner R, Hakenbeck R et al (2012) Biosynthesis of teichoic acids in *Streptococcus pneumoniae* and closely related species: lessons from genomes. Microb Drug Resist 18:344–358

Duckworth M, Archibald AR, Baddiley J (1975) Lipoteichoic acid and lipoteichoic acid carrier in *Staphylococcus aureus* H. FEBS Lett 53:176–179

Eberhardt A, Hoyland CN, Vollmer D et al (2012) Attachment of capsular polysaccharide to the cell wall in *Streptococcus pneumoniae*. Microb Drug Resist 18:240–255

Fabretti F, Theilacker C, Baldassarri L et al (2006) Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect Immun 74:4164–4171

Fan X, Pericone CD, Lysenko E et al (2003) Multiple mechanisms for choline transport and utilization in *Haemophilus influenzae*. Mol Microbiol 50:537–548

Fedtke I, Mader D, Kohler T et al (2007) A *Staphylococcus aureus ypfP* mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity. Mol Microbiol 65:1078–1091

Fischer W (1994a) Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*. Med Microbiol Immunol 183:61–76

Fischer W (1994b) Lipoteichoic acids and lipoglycans. In: Ghuysen JM, Hakenbeck R (eds) New comprehensive biochemistry. Elsevier Science, Amsterdam, pp 199–215

Fischer W (1997) Pneumococcal lipoteichoic and teichoic acid. Microb Drug Resist 3:309–325

Fischer W, Koch HU, Haas R (1983) Improved preparation of lipoteichoic acids. Eur J Biochem 133:523–530

Forsberg CW, Wyrick PB, Ward JB et al (1973) Effect of phosphate limitation on the morphology and wall composition of *Bacillus licheniformis* and its phosphoglucomutase-deficient mutants. J Bacteriol 113:969–984

Garufi G, Hendrickx AP, Beeri K et al (2012) Synthesis of lipoteichoic acids in *Bacillus anthracis*. J Bacteriol 194:4312–4321

Gehre F, Leib SL, Grandgirard D et al (2008) Essential role of choline for pneumococcal virulence in an experimental model of meningitis. J Intern Med 264:143–154

Ginsburg I (2002) Role of lipoteichoic acid in infection and inflammation. Lancet Infect Dis 2:171–179

Gisch N, Kohler T, Ulmer AJ et al (2013) Structural reevaluation of *Streptococcus pneumoniae* lipoteichoic acid and new insights into its immunostimulatory potency. J Biol Chem 288:15654–15667

Gisch N, Schwudke D, Thomsen S et al (2015) Lipoteichoic acid of *Streptococcus oralis* Uo5: a novel biochemical structure comprising an unusual phosphorylcholine substitution pattern compared to Streptococcus pneumoniae. Sci Rep 5:16718

Goebel WF, Adams MH (1943) The immunological properties of the heterophile antigen and somatic polysaccharide of Pneumococcus. J Exp Med 77:435–449

Gotschlich EC, Liu TY (1967) Structural and immunological studies on the pneumococcal C polysaccharide. J Biol Chem 242:463–470

Greenberg JW, Fischer W, Joiner KA (1996) Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. Infect Immun 64:3318–3325

Gründling A, Schneewind O (2007a) Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. J Bacteriol 189:2521–2530

Gründling A, Schneewind O (2007b) Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. Proc Natl Acad Sci USA 104:8478–8483

Haas R, Koch HU, Fischer W (1984) Alanyl turnover from lipoteichoic acid to teichoic acid in *Staphylococcus aureus*. FEMS Microbiol Lett 21:27–31

Heilmann C, Hussain M, Peters G et al (1997) Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Mol Microbiol 24:1013–1024

Henneke P, Morath S, Uematsu S et al (2005) Role of lipoteichoic acid in the phagocyte response to group B streptococcus. J Immunol 174:6449–6455

Iwasaki H, Shimada A, Ito E (1986) Comparative studies of lipoteichoic acids from several *Bacillus strains*. J Bacteriol 167:508–516

Jankute M, Cox JA, Harrison J et al (2015) Assembly of the mycobacterial cell wall. Annu Rev Microbiol 69:405–423

Jonquieres R, Bierne H, Fiedler F et al (1999) Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. Mol Microbiol 34:902–914

Jorasch P, Wolter FP, Zahringer U et al (1998) A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of *ypfP* in *Escherichia coli* and structural analysis of its reaction products. Mol Microbiol 29:419–430

Jorasch P, Warnecke DC, Lindner B et al (2000) Novel processive and nonprocessive glycosyltransferases from *Staphylococcus aureus* and *Arabidopsis thaliana* synthesize glycolipids, glycophospholipids, glycosphingolipids, and glycosylsterols. Eur J Biochem 267:3770–3783

Kawai Y, Marles-Wright J, Cleverley RM et al (2011) A widespread family of bacterial cell wall assembly proteins. EMBO J 30:4931–4941

Kiriukhin MY, Debabov DV, Shinabarger DL et al (2001) Biosynthesis of the glycolipid anchor in lipoteichoic acid of *Staphylococcus aureus* RN4220: role of YpfP, the diglucosyldiacylglycerol synthase. J Bacteriol 183:3506–3514

Koch HU, Haas R, Fischer W (1984) The role of lipoteichoic acid biosynthesis in membrane lipid metabolism of growing *Staphylococcus aureus*. Eur J Biochem 138:357–363

Koch HU, Doker R, Fischer W (1985) Maintenance of D-alanine ester substitution of lipoteichoic acid by reesterification in *Staphylococcus aureus*. J Bacteriol 164:1211–1217

Kodali S, Vinogradov E, Lin F et al (2015) A Vaccine approach for the prevention of infections by multidrug-resistant *Enterococcus faecium*. J Biol Chem 290:19512–19526

Kovacs M, Halfmann A, Fedtke I et al (2006) A functional dlt operon, encoding proteins required for incorporation of d-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. J Bacteriol 188:5797–5805

Kristian SA, Datta V, Weidenmaier C et al (2005) D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. J Bacteriol 187:6719–6725

Lambert PA, Hancock IC, Baddiley J (1977) Occurrence and function of membrane teichoic acids. Biochim Biophys Acta 472:1–12

Lazarevic V, Soldo B, Medico N et al (2005) *Bacillus subtilis* alpha-phosphoglucomutase is required for normal cell morphology and biofilm formation. Appl Environ Microbiol 71:39–45

Lebeer S, Claes IJ, Vanderleyden J (2012) Anti-inflammatory potential of probiotics: lipoteichoic acid makes a difference. Trends Microbiol 20:5–10

Lemjabbar H, Basbaum C (2002) Platelet-activating factor receptor and ADAM10 mediate responses to *Staphylococcus aureus* in epithelial cells. Nat Med 8:41–46

Liszewski Zilla M, Chan YG, Lunderberg JM et al (2015) LytR-CpsA-Psr enzymes as determinants of *Bacillus anthracis* secondary cell wall polysaccharide assembly. J Bacteriol 197:343–353

Mancuso DJ, Chiu TH (1982) Biosynthesis of glucosyl monophosphoryl undecaprenol and its role in lipoteichoic acid biosynthesis. J Bacteriol 152:616–625

Matsuoka S, Hashimoto M, Kamiya Y et al (2011) The *Bacillus subtilis* essential gene *dgkB* is dispensable in mutants with defective lipoteichoic acid synthesis. Genes Genet Syst 86:365–376

Mishra AK, Driessen NN, Appelmelk BJ et al (2011) Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction. FEMS Microbiol Rev 35:1126–1157

Mohamadzadeh M, Pfeiler EA, Brown JB et al (2011) Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci U S A 108(Suppl 1):4623–4630

Morath S, Geyer A, Hartung T (2001) Structure-function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*. J Exp Med 193:393–397

Mosser JL, Tomasz A (1970) Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J Biol Chem 245:287–298

Neuhaus FC, Baddiley J (2003) A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev 67:686–723

Nilsen NJ, Deininger S, Nonstad U et al (2008) Cellular trafficking of lipoteichoic acid and toll-like receptor 2 in relation to signaling: role of CD14 and CD36. J Leukoc Biol 84:280–291

Oberli MA, Hecht ML, Bindschadler P et al (2011) A possible oligosaccharide-conjugate vaccine candidate for *Clostridium difficile* is antigenic and immunogenic. Chem Biol 18:580–588

Oku Y, Kurokawa K, Matsuo M et al (2009) Pleiotropic roles of polyglycerolphosphate synthase of lipoteichoic acid in growth of *Staphylococcus aureus* cells. J Bacteriol 191:141–151

Percy MG, Grundling A (2014) Lipoteichoic acid synthesis and function in gram-positive bacteria. Annu Rev Microbiol 68:81–100

Percy MG, Karinou E, Webb AJ et al (2016) Identification of a lipoteichoic acid glycosyltransferase enzyme reveals that GW-domain-containing proteins can be retained in the cell wall of *Listeria monocytogenes* in the absence of lipoteichoic acid or its modifications. J Bacteriol 198:2029–2042

Perego M, Glaser P, Minutello A et al (1995) Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis.* Identification of genes and regulation. J Biol Chem 270:15598–15606

Peschel A, Otto M, Jack RW et al (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274:8405–8410

Peschel A, Vuong C, Otto M et al (2000) The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. Antimicrob Agents Chemother 44:2845–2847

Pooley HM, Paschoud D, Karamata D (1987) The *gtaB* marker in *Bacillus subtilis* 168 is associated with a deficiency in UDPglucose pyrophosphorylase. J Gen Microbiol 133:3481–3493

Poxton IR, Tarelli E, Baddiley J (1978) The structure of C-polysaccharide from the walls of Streptococcus pneumoniae. Biochem J 175:1033–1042

Rahman O, Dover LG, Sutcliffe IC (2009a) Lipoteichoic acid biosynthesis: two steps forwards, one step sideways? Trends Microbiol 17:219–225

Rahman O, Pfitzenmaier M, Pester O et al (2009b) Macroamphiphilic components of thermophilic actinomycetes: identification of lipoteichoic acid in Thermobifida fusca. J Bacteriol 191:152–160

Rane L, Subbarow Y (1940) Nutritional requirements of the pneumococcus: I. Growth factors for types I, II, V, VII, VIII. J Bacteriol 40:695–704
Reichmann NT, Gründling A (2011) Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in gram-positive bacteria of the phylum Firmicutes. FEMS Microbiol Lett 319:97–105
Reichmann NT, Cassona CP, Grundling A (2013) Revised mechanism of D-alanine incorporation into cell wall polymers in gram-positive bacteria. Microbiology 159:1868–1877
Reid CW, Vinogradov E, Li J et al (2012) Structural characterization of surface glycans from Clostridium difficile. Carbohydr Res 354:65–73
Richter GS, Elli D, Kim HK et al (2013) Small molecule inhibitor of lipoteichoic acid synthesis is an antibiotic for gram-positive bacteria. Proc Natl Acad Sci U S A 110:3531–3536
Rockel C, Hartung T (2012) Systematic review of membrane components of gram-positive bacteria responsible as pyrogens for inducing human monocyte/macrophage cytokine release. Front Pharmacol 3:56
Schirner K, Marles-Wright J, Lewis RJ et al (2009) Distinct and essential morphogenetic functions for wall- and lipo-teichoic acids in *Bacillus subtilis*. EMBO J 28:830–842
Schlag M, Biswas R, Krismer B et al (2010) Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. Mol Microbiol 75:864–873
Schmidt RR, Pedersen CM, Qiao Y et al (2011) Chemical synthesis of bacterial lipoteichoic acids: an insight on its biological significance. Org Biomol Chem 9:2040–2052
Schneewind O, Missiakas D (2014) Lipoteichoic acids, phosphate containing polymers in the envelope of gram-positive bacteria. J Bacteriol 196:1133–1142
Seo HS, Cartee RT, Pritchard DG et al (2008) A new model of pneumococcal lipoteichoic acid structure resolves biochemical, biosynthetic, and serologic inconsistencies of the current model. J Bacteriol 190:2379–2387
Sheen TR, Ebrahimi CM, Hiemstra IH et al (2010) Penetration of the blood-brain barrier by *Staphylococcus aureus*: contribution of membrane-anchored lipoteichoic acid. J Mol Med (Berl) 88:633–639
Shiraishi T, Yokota S, Morita N et al (2013) Characterization of a *Lactobacillus gasseri* JCM 1131T lipoteichoic acid with a novel glycolipid anchor structure. Appl Environ Microbiol 79:3315–3318
Soldo B, Lazarevic V, Margot P et al (1993) Sequencing and analysis of the divergon comprising *gtaB*, the structural gene of UDP-glucose pyrophosphorylase of *Bacillus subtilis* 168. J Gen Microbiol 139:3185–3195
Song JH, Ko KS, Lee JY et al (2005) Identification of essential genes in *Streptococcus pneumoniae* by allelic replacement mutagenesis. Mol Cells 19:365–374
Sorensen UB, Henrichsen J (1987) Cross-reactions between pneumococci and other streptococci due to C polysaccharide and F antigen. J Clin Microbiol 25:1854–1859
Stortz CA, Cherniak R, Jones RG et al (1990) Polysaccharides from *Peptostreptococcus anaerobius* and structure of the species-specific antigen. Carbohydr Res 207:101–120
Taron DJ, Childs WC 3rd, Neuhaus FC (1983) Biosynthesis of D-alanyl-lipoteichoic acid: role of diglyceride kinase in the synthesis of phosphatidylglycerol for chain elongation. J Bacteriol 154:1110–1116
Theilacker C, Kaczynski Z, Kropec A et al (2006) Opsonic antibodies to *Enterococcus faecalis* strain 12030 are directed against lipoteichoic acid. Infect Immun 74:5703–5712
Theilacker C, Kropec A, Hammer F et al (2012) Protection against *Staphylococcus aureus* by antibody to the polyglycerolphosphate backbone of heterologous lipoteichoic acid. J Infect Dis 205:1076–1085
Tillett WS, Francis T (1930) Serological reactions in pneumonia with a non-protein somatic fraction of Pneumococcus. J Exp Med 52:561–571
Tomasz A (1967) Choline in the cell wall of a bacterium: novel type of polymer-linked choline in Pneumococcus. Science 157:694–697
Uchikawa K, Sekikawa I, Azuma I (1986) Structural studies on lipoteichoic acids from four *Listeria* strains. J Bacteriol 168:115–122

Webb AJ, Karatsa-Dodgson M, Gründling A (2009) Two-enzyme systems for glycolipid and polyglycerolphosphate lipoteichoic acid synthesis in *Listeria monocytogenes*. Mol Microbiol 74:299–314

Weidenmaier C, Peschel A (2008) Teichoic acids and related cell-wall glycopolymers in gram-positive physiology and host interactions. Nat Rev Microbiol 6:276–287

Weidenmaier C, Kokai-Kun JF, Kristian SA et al (2004) Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. Nat Med 10:243–245

Weisman LE, Thackray HM, Steinhorn RH et al (2011) A randomized study of a monoclonal antibody (pagibaximab) to prevent staphylococcal sepsis. Pediatrics 128:271–279

Wicken AJ, Evans JD, Knox KW (1986) Critical micelle concentrations of lipoteichoic acids. J Bacteriol 166:72–77

Wörmann ME, Corrigan RM, Simpson PJ et al (2011) Enzymatic activities and functional interdependencies of *Bacillus subtilis* lipoteichoic acid synthesis enzymes. Mol Microbiol 79:566–583

Wu C, Huang IH, Chang C et al (2014) Lethality of sortase depletion in *Actinomyces oris* caused by excessive membrane accumulation of a surface glycoprotein. Mol Microbiol 94:1227–1241

Xia G, Kohler T, Peschel A (2010) The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. Int J Med Microbiol 300:148–154

Yokoyama K, Araki Y, Ito E (1988) The function of galactosyl phosphorylpolyprenol in biosynthesis of lipoteichoic acid in *Bacillus coagulans*. Eur J Biochem 173:453–458

Young NM, Foote SJ, Wakarchuk WW (2013) Review of phosphocholine substituents on bacterial pathogen glycans: synthesis, structures and interactions with host proteins. Mol Immunol 56:563–573

Zhang JR, Idanpaan-Heikkila I, Fischer W et al (1999) Pneumococcal *licD2* gene is involved in phosphorylcholine metabolism. Mol Microbiol 31:1477–1488

Zoll S, Schlag M, Shkumatov AV et al (2012) Ligand-binding properties and conformational dynamics of autolysin repeat domains in staphylococcal cell wall recognition. J Bacteriol 194:3789–3802

Mycolic Acids: From Chemistry to Biology

11

Mamadou Daffé, Annaïk Quémard, and Hedia Marrakchi

Contents

1 Introduction 182
2 Structures of Mycolic Acids 184
3 Mycolic Acid Conformational Packing into the Cell Wall 186
4 Mycolic Acid Biosynthetic Machinery and Regulation 187
4.1 Biosynthesis of the Mycolic Acid Precursors 187
4.2 Meromycolic Chain-Modifying Enzymes 189
4.3 Mycolic Acid Elongation Complexes 192
4.4 Mycolic Condensation and Transfer Onto Trehalose 193
4.5 Translocation, Formation of the Mycolate-Containing Compounds, and Recycling 194
4.6 Mycolic Acid Biosynthesis Regulation 195
5 Mycolic Acid Biogenesis as a Target for TB Drug Discovery 197
5.1 Isoniazid and Ethionamide 197
5.2 Thiacetazone and Isoxyl 198
5.3 Thiolactomycin (TLM) 199
5.4 Novel Molecules Targeting MA Biosynthesis and Translocation 199
6 Some Biological Functions of Mycolic Acids 200
6.1 Immunostimulatory Activity of Mycolic Acid-Containing Compounds 201
6.2 Mycolic Acid Types and Virulence 201
6.3 Mycolic Acids with Respect to Cording and Biofilm Formation 202
7 Research Needs 204
References 206

M. Daffé (✉) · A. Quémard · H. Marrakchi
Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, France
e-mail: mamadou.daffe@ipbs.fr; annaik.quemard@ipbs.fr; hedia.marrakchi@ipbs.fr

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_18

Abstract

Mycolic acids are exceptionally long-chain fatty acids that are major and specific lipid components of the cell envelope of members of the *Corynebacteriales* order, which includes the causative agents of both tuberculosis and leprosy. These acids participate to the composition of the recently discovered "outer membrane," a component unexpected for these Gram-positive microorganisms. Many proteins involved in mycolic acid biosynthesis and transport are essential for the mycobacterial survival and represent both validated targets and highly relevant candidates for the development of novel antimycobacterial agents, in the alarming context of multidrug-resistant tuberculosis.

1 Introduction

Originally, mycolic acids (MAs) were defined as C_{60}–C_{90} 2-alkyl 3-hydroxy fatty acids typifying the cell envelope of mycobacteria (Marrakchi et al. 2014; Quemard 2016), a group of microorganisms that may cause severe health-threatening diseases such as tuberculosis and leprosy. Structurally similar molecules with varying chain lengths have been later found in phylogenetically close microorganisms grouped in the *Corynebacteriales* order. MAs are found either linked to the cell wall arabinogalactan, the polysaccharide which, together with peptidoglygan, forms the insoluble cell-wall skeleton (Daffé and Draper 1998), or as esters of trehalose (or other polyols that may be present in the bacterial environment). Both forms are assumed to participate to the remarkable architecture and impermeability of the mycobacterial cell envelope as major lipid components of the two leaflets of the outer membrane, also called mycomembrane, unexpectedly found in these Gram-positive bacteria (Hoffmann et al. 2008; Sani et al. 2010; Zuber et al. 2008) (Fig. 1). In addition, MA-containing compounds have been associated with many physiological properties of mycobacteria such as their characteristic serpentine-like growth, called "cording," biofilm formation, foamy macrophage formation in TB granulomas, and more largely in the pathogenicity process (Daffé and Draper 1998; Goren and Brennan 1979; Verschoor et al. 2012).

MA synthesis inhibition is one of the primary effects of the frontline and most efficient antitubercular drug isoniazid (INH) and several other inhibitors of the mycobacterial growth (Marrakchi et al. 2014; Quemard 2016). Accordingly, this metabolic pathway represents a niche of potential targets for the development of new antimycobacterial drugs. As such, their study has attracted much interest, especially in the alarming context of the emergence of drug-resistant tuberculosis. This chapter summarizes the current knowledge in the chemistry of mycolic acids, the biosynthetic pathways generating these compounds and their regulation, as well as their traffic through the cell envelope. It also describes some key biological roles played by these unique molecules.

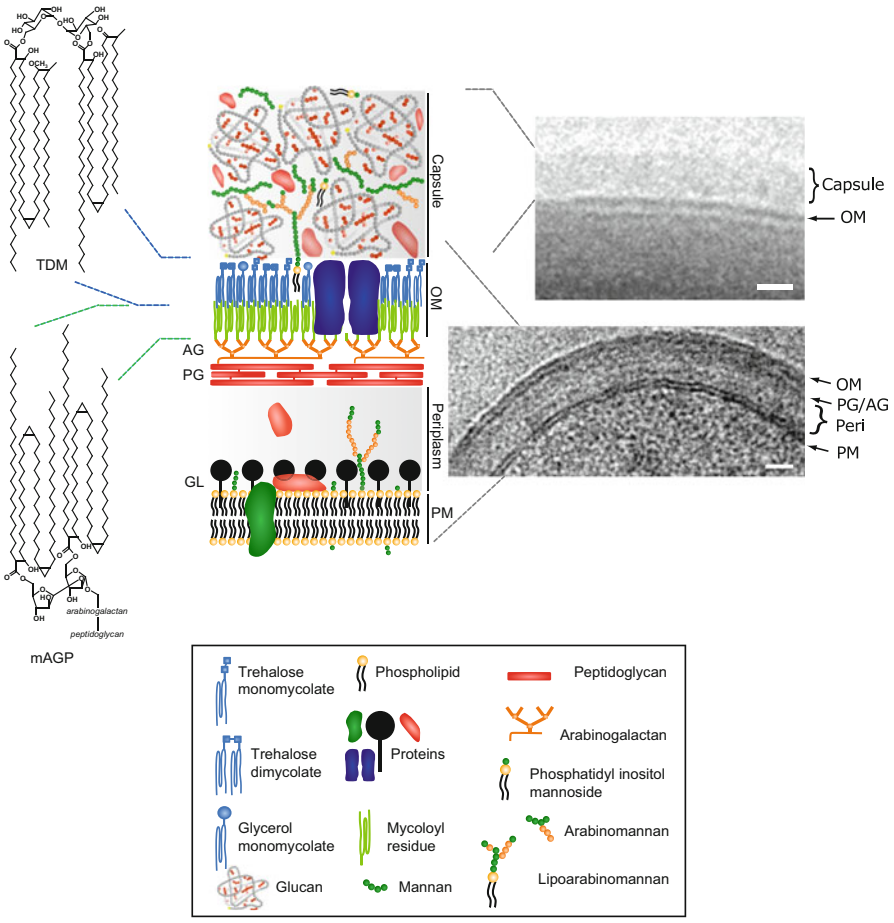


Fig. 1 Model, constituents and ultrastructure of the mycobacterial cell envelope. The outermost layer of the cell envelope, also called capsule in the case of pathogenic mycobacteria (Daffé and Draper 1998), is mainly composed of polysaccharides (a glycogen-like glucan, arabinomannan, and mannan) and proteins. Transmission electron microscopy (*EM*) micrographs of mycobacteria whole cell: *upper panel*: cryoEM showing native capsule, adapted from Sani et al. (2010); *lower panel*: cryo-electron microscopy of vitreous sections (CEMOVIS), adapted from Zuber et al. (2008), where the bilayer aspect of both the plasma membrane (*PM*) and the outer membrane (*OM*) is clearly visible. Scale bars: 20 nm. The OM, also called "mycomembrane," represents a permeability barrier. Its inner leaflet is formed by a parallel arrangement of mycoloyl chains (in *green*) linked to arabinogalactan (*AG*), which in turn is covalently attached to peptidoglycan (*PG*), thus forming the mycoloyl-arabinogalactan-peptidoglycan complex (*mAGP*). The outer leaflet of the OM (in *blue*) is presumably composed of free lipids (noncovalently linked to the mAGP), which include trehalose dimycolate (*TDM*). Chemical representations (*left*) of TDM and mAGP show the very long-chains of mycolic acids that pack upon folding into "W-shape" to fit in a conventional membrane thickness of 7–8 nm. The periplasm (Peri) above the PM contains a granular layer (*GL*) composed of proteins

2 Structures of Mycolic Acids

MAs are the longest fatty acids (FA) ever found in nature. They occur in all members of the *Corynebacteriales* order examined to date, with a very few exceptions (e.g., *Corynebacterium amycolatum* and *C. kroppenstedtii*). Mycobacteria produce specimens among the longest ones (C_{60}–C_{90}) (Fig. 2) but even longer molecules, called "ultra/extra-long-chain mycolic acids" (XL-MAs), were recently discovered in the *Segniliparus* genus and certain mycobacterial strains (Hong et al. 2012; Laneelle et al. 2013; Slama et al. 2016; Watanabe et al. 2001). MAs have been regarded as genus- and species-specific compounds and, consequently, were largely used as taxonomic markers (Daffé et al. 1983; Marrakchi et al. 2014); they consist in chains of 22 to 38 carbon atoms in *Corynebacterium*, 30 to 36 in *Hoyosella* and *Amycolicicoccus*, 34 to 38C in *Dietzia*, 34 to 52C in *Rhodococcus*, 46 to 60C in *Nocardia*, 46 to 66C in *Gordonia*, 64 to 78C in *Tsukamurella*, 60 to 90C in *Mycobacterium*, and up to 100C in *Segniliparus* (Marrakchi et al. 2014).

The first structure of mycolic acids has been described in 1950 (Asselineau and Lederer 1950) as α-branched, *β*-hydroxylated long-chain fatty acids, a feature that confers to the molecule the property to be cleaved at high temperature into a "mero"aldehyde main chain, also called "meromycolic" chain, and a "fatty acid," a reaction similar to a reverse Claisen type condensation. The lengths of the released fatty acids range from C_8 to C_{18} in *Corynebacterium* and up to C_{26} in the *M. tuberculosis* complex and *Mycobacterium xenopi* (Daffé et al. 1983). The stereochemistry of the centers at positions 2 and 3 has been shown to be conserved in all mycolic acid-containing genera, as 2*R*, 3*R* (Fig. 2) (Asselineau and Asselineau 1966; Asselineau et al. 1970a).

Structural elucidation of MAs has been addressed through the application of combined analytical techniques, notably thin-layer chromatography (TLC), gas chromatography, high pressure liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy. The structures of MAs of genera other than mycobacteria are relatively simple, being composed only of homologous series with various numbers of double bonds, up to six for some *Tsukamurella* species, borne by the meromycolic chain (Marrakchi et al. 2014; Tomiyasu and Yano 1984). In contrast, MAs of mycobacteria display a large diversity of chain lengths and chemical functions, located at defined positions, the so-called proximal and distal (relative to the carboxyl group of MA), that define different classes, leading to complex TLC patterns (Daffé et al. 1983; Laneelle et al. 2015). The major homologues of the most apolar mycolic acids, referred to as α-MAs, contain 75–83 carbon atoms and generally two *cis* cyclopropyl groups, as found in *M. tuberculosis*, or two double bonds (of *cis* or *trans* configuration) encountered in many nontuberculous mycobacterial species such as *M. smegmatis*, and possibly a mixture of double bond and cyclopropyl group, located in the meromycolic chain (Fig. 2). A small fraction of α-MAs, XL-MAs, contains an extra unsaturated hydrocarbon segment, as observed in some strains of the *M. tuberculosis* complex (Watanabe et al. 2001; Slama et al. 2016), as well as in the *Segniliparus* genus (Laneelle et al. 2013). Polyunsaturated α-MAs were shown to represent a significant portion of the MAs in *M. fallax* (Rafidinarivo et al. 1985). MAs with 60–62 carbon atoms, known as α′-mycolic acids, and typical of *M. smegmatis*, contain one *cis* double bond (Fig. 2).

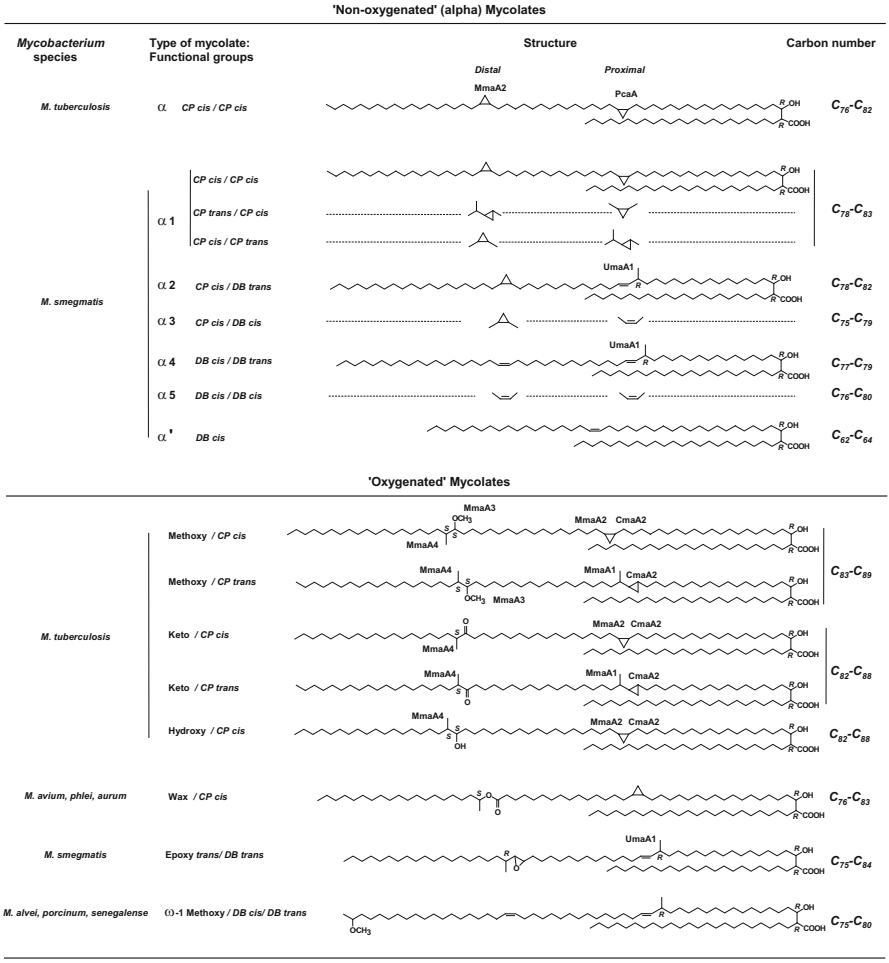


Fig. 2 Structural features of mycobacterial mycolic acids (MAs). Detailed structures of known types of α-MAs from *M. tuberculosis* and *M. smegmatis* and representative types of "oxygenated" MAs from various mycobacterial species. The proximal and distal positions (relative to the carboxyl group of MA) on the various functions are indicated. The configuration of double bonds (*DB*) and cyclopropanes (*CP*) is either *trans* (with an adjacent methyl branch) or *cis*. The established functions of the MA methyl transferases (MA-MTs) are indicated. The stereochemistry of the asymmetric carbon atoms (*S* or *R*) is indicated. PcaA: proximal cyclopropane mycolic acid methyl transferase (MA-MT); MmaA1: *trans* cyclopropane MA-MT; MmaA2: *cis* cyclopropane MA-MT; CmaA2: cyclopropane MA-MT; MmaA3: methoxy MA-MT; MmaA4 (Hma): hydroxy MA-MT; UmaA1 ("unidentified MA-MT"): proximal *trans* double bond MA-MT. For each mycolate type, the carbon numbers of the major homologues, as determined by MALDI-MS and NMR (Laval et al. 2001, 2008), are indicated

Moreover, in addition to the hydroxyl and carboxylic groups of the mycolic motif, MAs from most mycobacteria examined so far contain oxygen functions located in the distal part of the meromycolic chain, and defining the keto-, methoxy-, wax ester-, epoxy-, and hydroxy-types of MAs (Fig. 2); these so-called oxygenated MAs are absent from a few nontuberculous mycobacterial species such as *M. chelonae* and *M. abscessus* (Daffé et al. 1983). The additional oxygen functions occurring in mycobacterial MAs are typified by an adjacent methyl branch. It is noteworthy that in *M. tuberculosis*, the oxygenated MAs, i.e., keto-, methoxy-, and hydroxy-MAs, contain around 84–88 carbon atoms, 4–6 carbons longer than the α-MAs from the same strains (Laval et al. 2001; Watanabe et al. 2001). In sharp contrast, the chain lengths of epoxy- and wax ester-MAs (Fig. 2) found in non-tuberculous mycobacterial species, in which keto-, methoxy-, and/or hydroxy-MAs have never been detected, are similar to those of α-MAs of the same strains (Laval et al. 2001), suggesting a biosynthetic filiation between α- and oxygenated MAs in the latter species.

3 Mycolic Acid Conformational Packing into the Cell Wall

The implication of the MAs in the cell wall permeability barrier was known long ago. Mycobacteria are extremely impermeable to small molecules, including nutrients, such as glucose and glycerol, and antibiotics, contributing to the intrinsic resistance of mycobacteria to hydrophobic drugs (Jarlier and Nikaido 1994; Liu et al. 1996). This low permeability was correlated with the extremely hydrophobic surface attributed mainly to the high amount of MAs in the cell wall (Fig. 1). Indeed, analyses of mutants in which specific genes that encode proteins involved in the biosynthesis and transfer of MAs show correlation between the permeability of mycobacterial strains with the amounts and nature of their MAs (Draper 1998; Jackson et al. 1999).

The structural features of mycobacterial MAs at distal and proximal positions of the meromycolic chains, e.g., double bonds, cyclopropyl groups, and oxygenated functions (see Sect. 2) (Fig. 2), are essential for their physiological roles in maintaining the cell wall architecture. The importance of the fine structure of MAs was illustrated by studying the conformational behavior of different types of MAs by Langmuir monolayers experiments. According to the MA type, α or oxygenated, folded or extended conformations were adopted, depending on temperature (Villeneuve et al. 2005). Double bonds or cyclopropane rings are regularly spaced, around 14–17 methylene units, and methyl branches are found at both proximal and distal positions adjacent to the transunsaturations (Fig. 2). As demonstrated in monolayer studies (Villeneuve et al. 2005, 2007), the regular spacing facilitates folding and packing. Furthermore, the *trans* cyclopropanes, which cause a decrease in the cell wall fluidity (Liu et al. 1996), facilitate the folding of the oxygenated MAs into W-form conformations with the four hydrocarbon chains in parallel, allowing tight packing of the MAs in *M. tuberculosis* H37Rv (Villeneuve et al. 2013). In the *M. tuberculosis* avirulent strain H37Ra, the absence of *trans*-cyclopropane rings

prevents the oxygenated MAs from adopting stable fully folded W-form conformations. This folding ability is critical in allowing MAs to be accommodated within the dimensions of the mycobacterial outer membrane (Fig. 1), whose thickness is surprisingly conventional as revealed by cryo-electron microscopy (Hoffmann et al. 2008; Zuber et al. 2008).

The overall MA chain length is also a major determinant of cell wall fluidity (Liu et al. 1996). Thus, the presence of XL-MAs in *M. tuberculosis* might be one of the factors contributing to the extremely low permeability of its mycomembrane. It has been proposed that the XL-MAs might adopt a "tripleU-form" conformation where the additional segment of the meromycolic chain folds parallel to the other hydrocarbon chains, facilitating MA packing (Slama et al. 2016).

4 Mycolic Acid Biosynthetic Machinery and Regulation

4.1 Biosynthesis of the Mycolic Acid Precursors

MAs are the products of a mixed fatty acid synthase (FAS)/polyketide synthase (PKS) biosynthesis pathway (Fig. 3). Mycobacteria have the unique property of possessing two FAS systems (Bloch 1977), the "eukaryotic-type" FAS-I for de novo synthesis and the "bacterial-type" FAS-II for fatty acid (FA) elongation. A single gene (*fas, Rv2524c*) encodes the multidomain FAS-I protein required for the de novo synthesis cycle, where the FAs produced are long-chain acyl-CoAs with a bimodal distribution, C_{16}–C_{18} and C_{24}–C_{26}, which is a unique feature of the mycobacterial FAS-I among those of *Corynebacteriales* (Bloch and Vance 1977). The mycobacterial FAS-II elongates the C_{16}–C_{18} FAs to yield C_{18}–C_{30} acyl-ACPs in vitro and is incapable of de novo FA synthesis from acetyl-CoA, unlike the type II synthases of other bacteria (Odriozola et al. 1977). It is believed that the mycobacterial FAS-II system produces full-size meromycolic chains in vivo. The synthesis proceeds through the elongation of enzyme-bound intermediates, covalently linked to the mycobacterial acyl carrier protein AcpM (Rv2244), by iterative cycles, each comprising four main steps (Bloch 1977) (Fig. 3) to yield the precursors of the meromycolic chains. On the other hand, the long-chain C_{24}–C_{26} acyl-CoAs made by FAS-I subsequently constitute, after carboxylation by an acyl-CoA carboxylase (ACCase), the α-chain of MAs. The decarboxylative condensation of the latter with the activated meromycolic chain generates mature MAs (see Sect. 4.4) (Fig. 3).

Within FAS-II, InhA, MabA, HadB, and KasA are essential proteins (Bhatt et al. 2005; Parish et al. 2007; Sacco et al. 2007; Vilcheze et al. 2000), consistent with the essentiality of MA biosynthesis. In contrast, neither HadC nor KasB are essential (Bhatt et al. 2007a; Gao et al. 2003; Slama et al. 2016). Despite the high sequence similarity between KasA and KasB on the one hand, and HadA and HadC subunits of HadAB and HadBC enzymes on the other hand, they show different chain-length specificities (Kremer et al. 2002; Schaeffer et al. 2001). Several lines of evidence

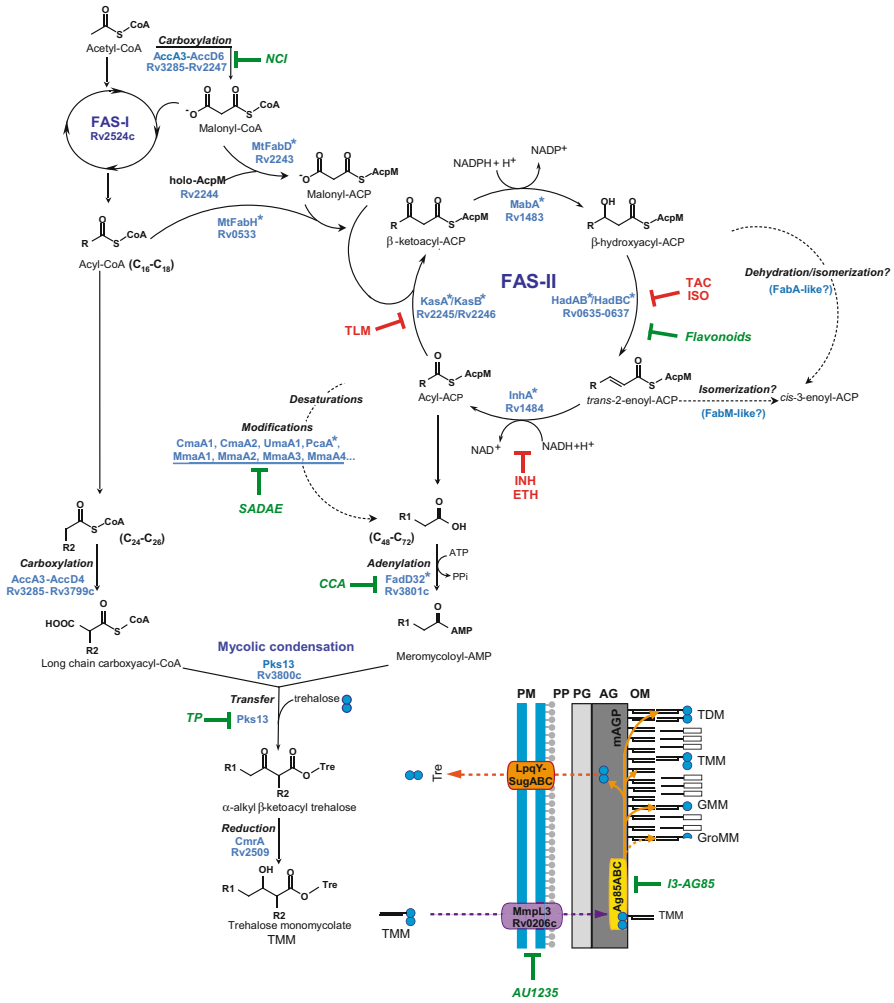


Fig. 3 Biosynthesis, inhibition, and regulation of mycobacterial mycolic acids (MAs). The biosynthetic pathway of MAs is initiated with the de novo synthesis of fatty acids operated by the mycobacterial fatty acid synthase (FAS)-I, followed by their elongation catalyzed by FAS-II system. The *β*-ketoacyl-ACP synthase III (MtFabH) links FAS-I to FAS-II by catalyzing the first condensation between acyl-CoA precursors (produced by FAS-I) and the extender unit malonyl-ACP to form *β*-ketoacyl-ACPs. The iterative FAS-II elongation cycles include four main steps. The meromycolic chains are differentiated through the introduction of functional groups by a family of dedicated methyltransferases, most likely from *cis* double bonds first introduced by unknown mechanisms (possibly desaturations, dehydration-isomerization, or simple isomerization). The carboxylation of acyl-CoAs (the FAS-I products) provides the alkylmalonate at the origin of the α-branch of MAs. Activation of both substrates (meromycolic acid and acyl-CoA) occurs prior to their condensation that yields α-alkyl β-ketoester of trehalose, itself likely reduced to form the mature mycolic acid under the form of a *t*rehalose *m*ono*m*ycolate (*TMM*). The latter is transported through the plasma membrane by MmpL3. The mycoloyltransferases (Ag85 complex) associated to the mycomembrane synthesize biologically active mycolate-containing compounds, the mAGP

have indicated that HadAB and KasA enzymes are involved in the initial extension steps catalyzed by FAS-II, while HadBC and KasB enzymes are implicated primarily in the late extension steps leading to the formation of full length meromycolic chains (Bhatt et al. 2007a; Gao et al. 2003; Sacco et al. 2007; Slama et al. 2016). This biosynthetic scheme is supported by the differential distribution of the proteins among the members of the *Corynebacteriales* order (Sacco et al. 2007). For genera producing long meromycolic chains, like *Hoyosella*, *Gordonia,* orthologues of *kasA, kasB*, and *hadABC* genes are detected in these organisms, while *kasB* and *hadC* orthologues are absent from genera producing shorter meromycolic chains like *Rhodococcus* and *Nocardia* (Laneelle et al. 2012; Sacco et al. 2007). Interestingly, in *Segniliparus,* a genus in which very long MAs are synthesized, two copies of *hadB,* the gene encoding the catalytic domain of the dehydratases, exist in the genome (Laneelle et al. 2013). Consistent with this scheme, the deletion of the *hadC* gene of *M. tuberculosis* strongly inhibited the synthesis of the oxygenated MAs and blocked the formation of the XL-MAs bearing a fourth cyclopropanated meromycolic segment (Slama et al. 2016). Interestingly, mutants in putative additional mycobacterial dehydratases of *M. smegmatis* and *M. abscessus* have been constructed and shown to be affected in their MA profiles, suggesting that extra dehydratases of FAS-II might exist (Carrere-Kremer et al. 2015; Halloum et al. 2016).

4.2 Meromycolic Chain-Modifying Enzymes

4.2.1 Possible Origins of Double Bonds Present in the Meromycolic Chain

The presence of double bonds is observed in all MAs, from the simplest C_{32}–C_{34} corynomycolic acids to the most complex mycobacterial MAs. In corynebacteria, which are devoid of FAS-II system, unsaturated MAs result from the condensation of two FAs with at least one molecule of oleic acid (C18:1Δ9). The mechanisms by which the other *Corynebacteriales* introduce double bonds in their longer chain MAs are still under investigation. Several mechanisms have been described for the formation of unsaturated FAs in bacteria. First, the aerobic desaturation performed

Fig. 3 (continued) complex, TDM, GMM, and possibly GroMM. The released free trehalose is recycled by the LpqY-SugA-SugB-SugC ABC transporter. The proteins regulated by Serine-Threonine Protein Kinases are indicated by an asterisk (*). Selective antibiotics or antituberculous drugs targeting this pathway are indicated in *red*, and known inhibitors in *green* (and italics): NCI, NCI-172033; INH, Isoniazid; ETH, Ethionamide; TLM, Thiolactomycin; ISO, Isoxyl; TAC, Thiacetazone; CCA, 4,6-diaryl-5,7-dimethyl coumarin; TP, Thiophene compound; AU1235, 1-(2-adamantyl)-3-(2,3,4-trifluorophenyl)urea; I3-AG85: 2-amino-6-propyl-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile. Tre, trehalose; PM, plasma membrane; PP, periplasm; PG: peptidoglycan; AG: arabinogalactan; OM, outer membrane. TDM: trehalose dimycolate; GMM: glucose monomycolate; GroMM: glycerol monomycolate (Adapted from Gavalda et al. (2014), Marrakchi et al. (2014))

on a yet-synthesized long chain and requiring molecular oxygen leads to the formation of oleic acid ($C_{18:1}\Delta 9$) from stearic acid (C_{18}) through the action of the Δ9 desaturase, reaction which is performed by DesA3 (Rv3329c) desaturase in mycobacteria (Phetsuksiri et al. 2003). Such a mechanism has been proposed for the formation of the *cis* double bond at the distal position in α-MAs (Asselineau et al. 1970b). Consistent with this, a recent survey suggests the involvement of the putative desaturase DesA1 in the introduction of one double bond at an as yet undefined position in the α-MAs of *M. smegmatis* (Singh et al. 2016). The function of the third mycobacterial putative desaturase DesA2 remains unknown.

An alternative mechanism is the anaerobic desaturation reaction performed by the 3-hydroxydecanoyl-ACP dehydratase (FabA) of *Escherichia coli* FAS-II system. This dehydratase/isomerase enzyme generates *cis*-3-enoyl intermediates (Fig. 3), which are subsequently not reduced but further elongated by the *β*-ketoacyl-ACP synthase I (FabB) to long-chain monounsaturated FAs, thus diverting the nascent acyl-chain into the unsaturated fatty acid synthetic pathway (Rock and Cronan 1996). Several groups of bacteria, including mycobacteria, lack both FabA and FabB enzymes, although they synthesize unsaturated fatty acids and/or have an anaerobic metabolism. In *Streptococcus pneumoniae*, a novel enzyme was discovered, FabM, a *trans*-2-*cis*-3-enoyl-ACP isomerase, able to divert fatty acid synthesis to the unsaturated mode (Marrakchi et al. 2002). Candidate FabM-like proteins exist in the *M. tuberculosis* genome and may function in a FabM-like manner for the formation of the *cis* double bond of the meromycolic chain, followed by the elongation of the *cis*-3-enoyl FA (Fig. 3) (Bloch 1969; Etemadi 1967).

In the case of the mycolic acids produced by the *Nocardia* genus, the "nocardomycolic acids," a mechanism of head-to-tail condensation of palmitic acid after an ω-oxidation step, has been proposed based on structural studies and labeling experiments, consistent with the double bond location at ω16 (Bordet and Michel 1969; Tarnok and Rohrscheidt 1976). Similarly, the structural relationship between different types of mycolic acids from mycobacteria and the location of the motifs at similar positions in the meromycolic chains led to the hypothesis that the building of the latter could be the result of condensation between three common fatty acids (C_{20}, C_{16}, C_{22}). The position of double bonds or oxygenated groups would be determined by the length of the FA precursors and by the presence or absence of a methyl group (Asselineau et al. 2002). Is it noteworthy that among the *cis* double bond-containing mycolates, the shortest α′-MAs are remarkable in their invariable chain lengths of $C_{62:1}$–$C_{64:1}$ (Fig. 2) regardless of the species where they are found, e.g., *M. smegmatis*, *M. abscessus*, or the new genus *Segniliparus* (Laneelle et al. 2013). The fact that no further modifications (cyclopropane, methyl branch, oxygenated functions) are observed in the α′-MAs suggests a distinct pathway for their synthesis.

4.2.2 Insertion of Cyclopropane Functions and Methyl Groups

It is not clear yet at which precise step of the MA biosynthesis pathway the chemical functions are introduced in the meromycolic chain, although an array of data suggest that at least some of them are formed during the FAS-II elongation cycles

(Asselineau et al. 1970b; Qureshi et al. 1984; Takayama and Qureshi 1978). These modifications are operated by a family of paralogous *S*-adenosylmethionine (SAM)-dependent MA methyltransferases (MTs), which have been thoroughly reviewed (Marrakchi et al. 2014). It is believed that these enzymes adopt a catalytic mechanism similar to the classical scheme described for the introduction of cyclopropanes catalyzed by the *E. coli* cyclopropane synthase (CPS) and of methyl branches and which is based on the modification of a *cis* double bond by the methyl groups derived from SAM (Lederer 1969). Indeed, among the eight homologous SAM-dependent MTs present in the genome of *M. tuberculosis* (Cole et al. 1998), four MTs, namely CmaA1, CmaA2, PcaA (proximal cyclopropanation of alpha-MAs, formerly UmaA2), and MmaA2, display very strong sequence similarities with the *E. coli* CPS, suggesting a common reaction mechanism. Inactivation experiments of the *M. tuberculosis* MT genes have established their respective functions (Fig. 2) and have corrected some false interpretations concluded from protein overexpression experiments (reviewed in Marrakchi et al. 2014).

Nevertheless, some questions are still pending, especially regarding the specific MA-MTs responsible for introducing methyl groups at the proximal position and adjacent to *trans* cyclopropanes in the oxygenated MAs of the *M. tuberculosis* complex species and to double bonds in the α- and epoxy-MAs of *M. smegmatis*. While the *umaA1* orthologue in *M. smegmatis*, the MSMEG0913 protein, has been shown to catalyze the formation of a *trans* cyclopropyl group or double bond with an adjacent methyl branch at the proximal position of both α- and epoxy-MAs (Fig. 2), the *umaA1* (Rv0469) knock-out mutant exhibits no change in the MA profile in *M. tuberculosis* although the mutant strain exhibited a hypervirulence phenotype in the SCID mouse infection model (Laval et al. 2008).

4.2.3 Introduction of Oxygenated Functions

The biosynthesis of both keto- and methoxy-MAs (Fig. 2), two classes of MAs found in members of the *M. tuberculosis* complex and selective slow-growers, is intimately linked to that of the common hydroxymycolate precursor, which is catalyzed by the MT Hma (MmaA4, Rv0642c), a SAM-dependent enzyme. The proposed mechanism is the transfer of a methyl branch onto a *cis* double bond followed by the addition of an adjacent hydroxy group from a water molecule (Dubnau et al. 1997; Yuan and Barry 1996). The *M. tuberculosis hma* mutant no longer produces keto- or methoxy-MAs or their hydroxylated precursor (Dubnau et al. 2000), while still able to synthesize an unusual α-MAs bearing a *cis* double bond in the distal position (Dinadayala et al. 2003). The occurrence and precise position of this distal double bond puts forth this ethylenic mycolate as the possible substrate of Hma. A recent study has proposed the *M. tuberculosis* Rv0132c protein as the missing hydroxyl-MA dehydrogenase (HMAD) that would convert the hydroxyl-MAs synthesized by Hma into keto-MAs (Fig. 2) (Purwantini and Mukhopadhyay 2013). However, this work has been performed using the heterologous co-expression of *M. tuberculosis hma* and *Rv0132c* genes in *M. smegmatis*, a species that has oxygenated MAs distinct from those of *M. tuberculosis* (Fig. 2). As heterologous expression of MA biosynthesis genes may lead to misinterpretation of

their function (Glickman et al. 2001), the validation of Rv0132c function awaits the phenotypic analysis of a *M. tuberculosis* knock-out mutant. The hydroxyl-MAs or their meromycolic precursors are the substrates of MmaA3 (Rv0643c) to yield methoxy-MAs. Interestingly, the gene that encodes the MmA3 protein is either truncated or mutated in mycobacterial strains devoid of methoxy-MAs (e.g., *M. leprae*, or the Pasteur strain of *M. bovis* BCG) (Dubnau et al. 1998).

The proteins involved in the biosynthesis of other oxygenated functions such as wax-, epoxy-, and ω-1 methoxylated MAs (Fig. 2) remain to be discovered. Nevertheless, from structural data, it is possible to formulate a few hypotheses. The wax-mycolates have been shown to derive from the keto-MAs by Baeyer-Villiger oxidation, which consists in the insertion of molecular oxygen that converts the ketone to the corresponding ester (Toriyama et al. 1982), as postulated earlier (Etemadi and Gasche 1965; Laneelle and Laneelle 1970). Flavin-containing Baeyer-Villiger monoxygenases are present in the *M. tuberculosis* and other mycobacterial genomes.

Based on structural analyses, notably investigating the global chain length as well as the stereochemistry of the asymmetric carbon bearing the methyl branch adjacent to the oxygenated function (Fig. 2), it appears that no structural relationship can be established between the epoxy- and the keto-MAs. In contrast, it is reasonable to postulate that the formation of epoxy-MAs could result from the transformation of an α-mycolate-type (ethylenic) intermediate having a *trans* double bond at the distal position with an adjacent methyl branch (Dinadayala et al. 2003).

The ω-1 position of the methoxy group found in a class of MAs of some rapid grower mycobacterial species (Fig. 2) raises a new question. As no methyl branch adjacent to the oxygen function is found in this compound, it is tempting to speculate that the precursor of such a product could be an intermediate with a terminal double bond. In this respect, it is interesting to mention that a careful analysis of MAs from *M. smegmatis* had shown the presence of minor amounts of MAs with a terminal double bond (Wong and Gray 1979). Furthermore, *M. smegmatis* was shown to perform ω-1 oxidation of hydrocarbons (Rehm and Reiff 1981).

4.3 Mycolic Acid Elongation Complexes

Based on genetic and biochemical interaction experiments between the various FAS-II components, a working model has been proposed in which different specialized FAS-II complexes would be interconnected (Veyron-Churlet et al. 2004) and interact with MA-MTs (Cantaloube et al. 2011). This model predicts the occurrence of at least three specialized FAS-II complexes involved in MA synthesis, each complex being constituted by a "FAS-II core" (formed by InhA, MabA, MtFabD) associated to specific condensing (Kas) enzyme and dehydratase (Had) heterodimer, thus defining their substrate specificity. An "initiation" FAS-II complex (I-FAS-II), containing MtFabH, might allow the channeling of acyl-CoA from FAS-I to FAS-II during their condensation with malonyl-ACP (Fig. 3). This step would be followed by a type-I "elongation" complex (E1-FAS-II) formed by the core proteins and KasA

plus HadAB, which would elongate medium-length acyl-ACP chains that could serve as substrates for a type-II elongation complex (E2-FAS-II). The latter, comprising the core and KasB plus HadBC, could end the synthesis of the meromycolic chain. This end-product of E2-FAS-II might be used in the terminal condensation reaction catalyzed by Pks13, which is assumed to be part of a "termination" FAS-II (T-FAS-II) complex. Interestingly, these complexes also colocalized with cell division enzymes at the poles and septa of the growing mycobacteria (Carel et al. 2014).

4.4 Mycolic Condensation and Transfer Onto Trehalose

The ultimate biosynthesis steps of the MA biosynthesis are performed by the mycolic condensation system (Fig. 3). The latter catalyzes the condensation between a meromycolic chain provided by the FAS-II system and a fatty acyl chain provided by the FAS-I system (Gavalda et al. 2009), generating a C_{60}–C_{100} α-alkyl β-ketoacyl intermediate. This highly insoluble molecule is transferred onto a polar carrier, trehalose (Gavalda et al. 2014). The reduction of the β-ketone function of the lipid moiety (Lea-Smith et al. 2007) results in the formation of the α-alkyl β-hydroxy mycolic motif, generating a mature mycoloyl chain (Fig. 3). A candidate gene cluster, *fadD32-pks13-accD4,* was identified for these steps, highly conserved in *Corynebacteriales* (Portevin et al. 2004). Phenotypic studies of corynebacterial deletion mutants and mycobacterial conditional mutants have demonstrated the requirement of the three genes for mycolic acid production and their essentiality in *M. smegmatis* (Portevin et al. 2004, 2005). The different reaction steps catalyzed by the type-I polyketide synthase Pks13 (Rv3800c) and its partner protein FadD32 have been thoroughly deciphered (Gavalda et al. 2009; Leger et al. 2009). In contrast to known PKSs, which universally use short building blocks such as malonyl-CoA and methyl-malonyl-CoA, Pks13 appears as an atypical PKS performing a single Claisen-type condensation cycle between exceptionally long starter units (meromycolic chains) and extender units (carboxylated C_{22}–C_{26} acyl chain) (Fig. 3) (Gavalda et al. 2009).

Regarding the role of FadD32 (FAAL32, Rv3801c), the protein first catalyzes the activation of the very long-chain mero-MAs produced by FAS-II into meromycoloyl-AMPs (Fig. 3) (Leger et al. 2009; Trivedi et al. 2004), a feature consistent with the protein crystal structures showing the long fatty acyl chain of the co-crystallized substrate analog buried in a hydrophobic tunnel close to the enzyme active site (Guillet et al. 2016). Then, FadD32 loads the meromycoloyl chain onto the P-pant arm of the N-terminal ACP domain of Pks13, thereby displaying a unique acyl-ACP ligase function, where the final acceptor is the P-pant arm of an ACP unit (Gavalda et al. 2009; Leger et al. 2009), in agreement with the identification of a putative P-pant binding site (Li et al. 2015).

Pks13 was shown to load the extender unit onto its C-terminal ACP domain via its acyltransferase (AT) domain (Gavalda et al. 2009). The latter plays the gatekeeper role, selecting the appropriate long-chain 2-carboxyacyl-CoA substrates using a dedicated channel evidenced by crystallography (Bergeret et al. 2012). Data strongly

suggest that the long chain acyl-CoA carboxylase (ACCase) that provides the extender units to Pks13 includes at least AccA3 and AccD4 (Fig. 3) (Gande et al. 2004; Oh et al. 2006; Portevin et al. 2005). Yet the question of a putative involvement of additional subunits, AccD5 and AccE5, has been raised (Bazet Lyonnet et al. 2014; Gago et al. 2006; Oh et al. 2006; Portevin et al. 2005). After transfer of the carboxyacyl chain onto the C-terminal ACP domain of Pks13, the keto synthase domain achieves its condensation with the meromycoloyl chain to produce an α-alkyl β-ketoacyl chain (Fig. 3) (Gavalda et al. 2009). Given the high hydrophobicity of Pks13 products, it was hypothesized that they would not be released as free acids but directly transferred onto a hydrophilic acceptor unit by discrete unknown mycoloyltransferases (Takayama et al. 2005). In contrast to this model, we have discovered that the thioesterase (TE)-like domain of Pks13 itself catalyzes the cleavage of the thioester bond with the condensation products. Following an unprecedented mechanism, the TE-like domain subsequently performs the intermolecular transfer of these chains onto trehalose, leading to the formation of α-alkyl β-ketoacyl trehalose (Fig. 3) (Gavalda et al. 2014). Consistently, a putative trehalose-binding pocket was identified in the catalytic site of the TE-like domain (Gavalda et al. 2014). The mature MA chains are formed after the reduction of Pks13 products by the CmrA protein (Lea-Smith et al. 2007). *Corynebacterium* and *M. smegmatis cmrA* deletion mutants produce α-alkyl β-ketoacyl trehalose (Bhatt et al. 2008; Lea-Smith et al. 2007), suggesting that the reduction step occurs on this trehalose derivative, leading to the synthesis of trehalose monomycolate (TMM) (Fig. 3).

4.5 Translocation, Formation of the Mycolate-Containing Compounds, and Recycling

Independent surveys have recently indicated that the inhibition of mycobacterial MmpL3 protein (mycobacterial membrane protein large 3, Rv0206c) activity and the conditional mutation of the *mmpL3* essential gene abolish the translocation of MAs, resulting in a decrease in trehalose dimycolate (TDM) and cell-wall-bound MAs, both anchored in the outer membrane and in intracellular accumulation of TMM (Grzegorzewicz et al. 2012a; Tahlan et al. 2012; Varela et al. 2012). The MmpL3 transporter, which belongs to the resistance-nodulation-division (RND) superfamily, is responsible for the export of mycoloyl chains, most likely under the form of TMM (Fig. 3). Interestingly, the nonessential MmpL11 (Rv0202c) transporter, whose gene is located in the vicinity of *mmpL3*, would also play a role in the translocation of MA- or mero-MA-containing compounds, the monomeromycoloyl diacylglycerol (MMDAG) and a newly identified mycolate ester wax (Pacheco et al. 2013). MmpL3 and MmpL11 must be involved in two independent transport apparatuses since *mmpL11* inactivation has no impact on the TDM and cell-wall-linked MA contents. Its essentiality, both in vitro and during infection in mice (Li et al. 2016), underlines the preponderant role of the MmpL3-dependent export pathway over that of MmpL11 for the physiology of mycobacteria. The three active mycoloyltransferases of the antigen 85 complex (Belisle et al. 1997; Puech et al. 2002),

Ag85A, Ag85B, and Ag85C (FbpABC, Rv3804c, Rv1886c, Rv0129c), whose respective specific roles remain unclear, perform the ultimate steps of the mycolate-containing compound production (for review, see Tang et al. 2012). In corynebacteria, part of the mycoloyltransferases (De Sousa-D'Auria et al. 2003) presumably remains associated to the outer membrane (Marchand et al. 2012). Ag85ABC complex uses the TMM as a donor of mycoloyl chains, which they transfer onto selected acceptors for the biosynthesis of the mAGP complex and the mycolate-containing lipids, including TDM, used for the outer membrane biogenesis (Fig. 3) (see Sect. 3). In *M. tuberculosis,* the extracellular trehalose released during these reactions might be recycled by the LpqY-SugABC ATP-binding cassette (ABC) transporter that mediates trehalose retrograde transport (Kalscheuer et al. 2010) (Fig. 3). Thus, the latter step important for *M. tuberculosis* virulence might be needed for further MA biosynthesis specially during the infectious process since the trehalose is absent in the mammal hosts. The newly synthesized TDM can be hydrolyzed by a serine esterase (MSMEG_1529, TDM hydrolase) discovered in *M. smegmatis*, thus releasing free MAs (Ojha et al. 2010). This enzymatic activity, also detected in *M. tuberculosis*, is necessary for an efficient biofilm growth, which requires the formation of a free MA-rich extracellular matrix (see Sect. 6.3 and Fig. 4). Free MAs can also be released from the mAGP complex by the action of Lysin B (LysB) esterase produced by mycobacteriophage D29 in *M. smegmatis* (Payne et al. 2009). Importantly, the mutation of the *mce1* operon in *M. tuberculosis* produces an accumulation of MAs in the cell wall or the culture supernatant, with a concomitant upregulation of genes involved in MA transport and metabolism (Cantrell et al. 2013; Forrellad et al. 2014; Queiroz et al. 2015). As *mce1* operon is negatively regulated during mouse infection (Uchida et al. 2007), a model is emerging where free MAs would also be recycled by the cell wall putative ABC lipid importer Mce1 to be used as substrates of lipid biosynthesis and/or as carbon sources under starvation conditions, according to the concept that pathogenic mycobacteria switch their metabolism from carbohydrate to lipid pathways during their intracellular life (Forrellad et al. 2014). The free MAs might also serve as a barrier against host aggression or contribute to the organization of the bacilli in biofilms (Cantrell et al. 2013).

4.6 Mycolic Acid Biosynthesis Regulation

Mycobacterial lipids are essential structural constituents of the cell wall and their biosynthetic pathways are rigorously regulated at both genetic and biochemical levels. In *M. tuberculosis*, transcriptional regulators of MA metabolism have been identified, $FadR_{Mt}$ (Rv0494) and MabR (Rv2242), both functioning as negative regulators of FAS-II genes (Biswas et al. 2013; Mondino et al. 2013; Salzman et al. 2010). MabR functions as a repressor of *fabD-acpM-kasA-kasB-accD6* gene cluster essential for MA synthesis, while also affecting the expression of *fas*, which encodes the multifunctional FAS-I enzyme that supports phospholipid and triglyceride synthesis and provides the FAS-II system with precursors.

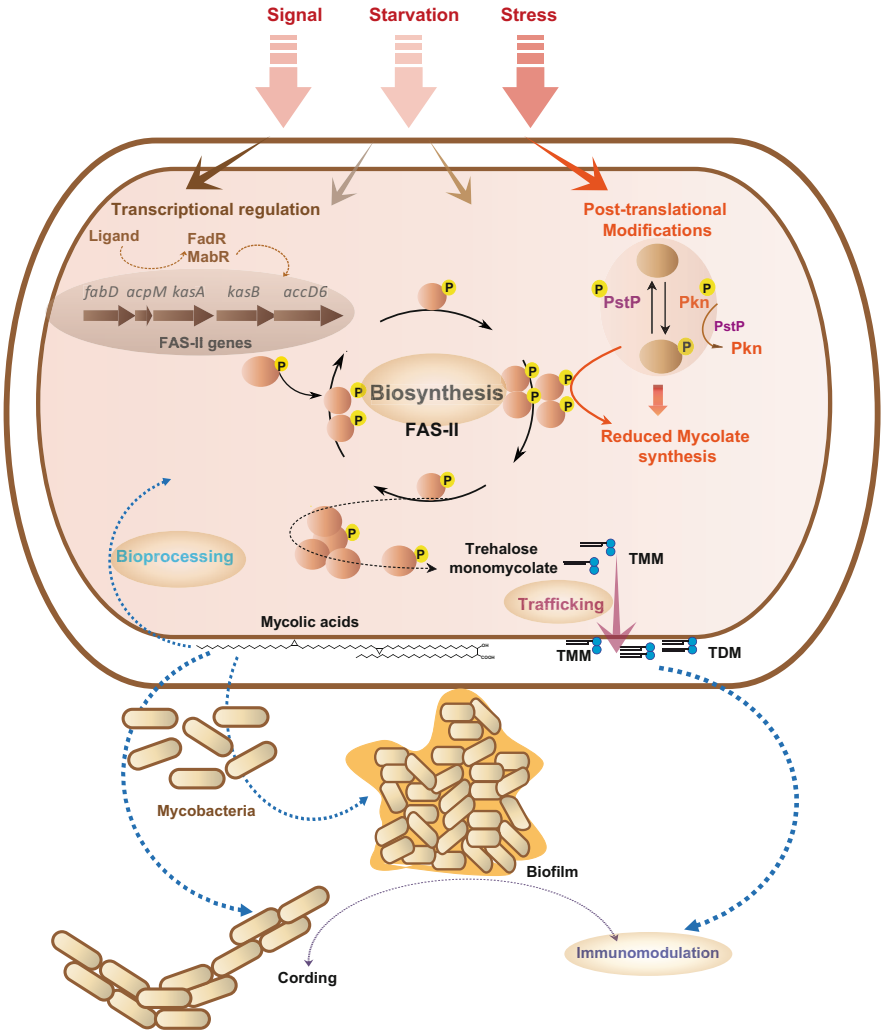


Fig. 4 Schematic representation of mycolic acid (MA) regulation, trafficking, and MA contribution to cell assembly. Under stress conditions, mycobacterial cells sense various signals and cues which promote their adaptation to environmental changes though numerous regulation processes. Amongst these, MA metabolism is down-regulated by transcriptional regulators (FadR, MabR) acting on genes encoding FAS-II proteins. On the other hand, post-translational modifications mediated by *S*er/*T*hr *P*rotein *K*inase (*STPK*)-dependent phosphorylation of MA biosynthesis enzymes allow a direct and tight control of MA biosynthesis. Auto-phosphorylation of the mycobacterial STPKs (*Pkn*) and subsequent phosphorylation of their substrates are reversible; the dephosphorylation, which adds another level of regulation, is operated by the Ser/Thr protein phosphatase (*PstP*). Mycobacteria have the capacity to assemble into serpentine-like structures called "cords." They are also able to form biofilms, where the bacilli are embedded in a mycolic acid-rich matrix. These structures of mycobacterial populations would play a role during the infectious process. During the latter, the ability of pathogenic mycobacteria to potentiate the host immune response is critical for the virulence and persistence of the pathogen. The lipid components of the mycobacterial envelope, and specifically the MA-containing compounds, play determinant immunomodulator roles

On the other hand, protein phosphorylation by serine/threonine protein kinases (STPKs), which can be reversed by protein phosphatases, is emerging as a major post-translational regulatory mechanism of fundamental biological processes in mycobacteria, including MA biosynthesis (Av-Gay and Everett 2000; Greenstein et al. 2005; Wehenkel et al. 2008), with repercussions on the physiology and virulence of *M. tuberculosis.* In MA metabolism, many proteins have been identified as substrates for the STPKs (Figs. 3 and 4) and their phosphorylation was found to modulate their enzymatic activity to tightly control MA synthesis (Bhatt et al. 2007a; Molle and Kremer 2010). Phosphorylation of enzymes of the *M. tuberculosis* FAS-II system (Figs. 3 and 4), KasA, KasB, InhA, MabA, and the dehydratases HadAB and HadBC were found to down-regulate their respective activities (Molle and Kremer 2010; Slama et al. 2011; Vilcheze et al. 2014). Similarly, phosphorylation of the methyl transferase PcaA inhibits MA cyclopropanation, thus modulating intracellular survival of mycobacteria (Corrales et al. 2012). The proportion of phosphorylated HadAB and HadBC clearly increases at the stationary growth phase, suggesting that mycobacteria use this regulatory mechanism to tightly control mycolic acid production under nonreplicating conditions (Slama et al. 2011). It was recently shown that the activity of FadD32, a key enzyme of the mycolic condensation complex (see Sect. 4.4, Fig. 3), is also down-regulated by phosphorylation (Le et al. 2016). Identification of numerous STPK substrates brings to light the various combinations by which the bacillus can regulate its MA synthesis to promote adaptation to environmental changes. In a recent survey, our group has shown that when mycobacteria encounter starvation conditions during infection, this leads to a global down-regulation, partially mediated by the stringent response, of genes required for MA biosynthesis and transport, likely to slow down this highly energy-consuming process during nutrient scarcity (Jamet et al. 2015a). Therefore, at least two levels of regulation put forth a sophisticated manner in which MA metabolism is tightly regulated in order to efficiently adapt lipid composition to the different conditions that the microorganism faces during the complex life cycle within the host (Fig. 4).

5 Mycolic Acid Biogenesis as a Target for TB Drug Discovery

The MA biosynthesis and transport pathways represent one of the "Achilles' heels" of the tubercle bacillus. Indeed, several drugs, specifically used in the chemotherapy of tuberculosis as well as novel candidates which are in the tuberculosis drug development pipeline, target these pathways. We report below the modes of action of some of these molecules.

5.1 Isoniazid and Ethionamide

Isoniazid (INH) is a first-line drug introduced in the TB chemotherapy in the early 1950s. The drug has been shown to directly inhibit the mycobacterial MA production (Quemard et al. 1991; Takayama et al. 1972), resulting in an alteration of the bacterial poles and subsequently amounting to profound morphologic changes (Bardou et al.

1996; Vilcheze et al. 2000). INH is activated by the catalase-peroxydase KatG leading to the formation of intermediate and highly reactive radicals (Johnsson and Shultz 1994; Quémard et al. 1996; Sinha 1983; Zhang et al. 1992). Ethionamide (ETH), a second-line TB prodrug structurally related to INH, is activated by a distinct enzyme, the NADPH-specific flavin adenine dinucleotide–containing monooxygenase EthA (or EtaA) (DeBarber et al. 2000; Hanoulle et al. 2006). The overexpression or mutation of the *inhA* gene confers resistance to both INH and ETH (Banerjee et al. 1994), strongly suggesting that the InhA enzyme is the primary target of both drugs. InhA was shown to be involved in the mycolic acid biosynthesis (Banerjee et al. 1994) and to catalyze the 2-*trans*-enoyl-ACP reduction step of the FAS-II system (Marrakchi et al. 2000; Quemard et al. 1995). Mutations in InhA conferring resistance to INH and ETH map to the NADH-binding pocket and induce a loss of affinity of InhA for its cofactor (Basso et al. 1998; Dessen et al. 1995; Quemard et al. 1995), consistent with the requirement of NADH for an efficient binding of INH and ETH to InhA (Quémard et al. 1996). Crystallographic studies showed that activated INH and ETH covalently bind to the nicotinamide ring of NADH within the InhA active site, generating inhibitory adducts (Rozwarski et al. 1998; Wang et al. 2007). The KasA enzyme of the FAS-II system had also been proposed as being the primary target of INH (Mdluli et al. 1996), but a thorough study has clearly shown that this was not the case (Kremer et al. 2003).

5.2 Thiacetazone and Isoxyl

Thiacetazone (TAC) and isoxyl (ISO, thiocarlide) are structurally related thiourea prodrugs. TAC differs from other TB drugs in being entirely bacteriostatic (Mitchison 1998). Introduced in 1946, it was progressively replaced by other molecules because of its relatively high toxicity. ISO was also successfully used for the clinical treatment of tuberculosis during the 1960s but fell from clinical use due to low bioavailability and untoward absorption kinetics. Both TAC and ISO must be activated via *S*-oxidation of their thiocarbonyl moiety by the monooxygenase EthA that also activates ETH (Dover et al. 2007; Kordulakova et al. 2007). They possess multiple targets within mycobacteria, such as MA methyltransferases, epoxide hydrolases, and the stearoyl-CoA Δ9 desaturase (Alahari et al. 2007; Brown et al. 2011; Phetsuksiri et al. 2003). These enzymes, however, unlikely constitute primary targets because of their dispensability for growth. In contrast, ISO and TAC treatments induce a complete inhibition of the essential MA biosynthesis, suggesting that their primary target(s) reside within this pathway (Winder 1982). Yet their precise mode of action has long remained unknown. The recent observation of the accumulation of long chain 3-hydroxyacids in TAC- or ISO-treated *M. tuberculosis* pointed to the (3*R*)-hydroxyacyl-ACP dehydration step of the FAS-II cycle as the possible target (Grzegorzewicz et al. 2012b). Consistent with this, overexpression of *hadABC* genes encoding the FAS-II dehydratases or certain missense mutations in *hadA* or *hadC* conferred resistance to both drugs (Belardinelli and Morbidoni 2012; Gannoun-Zaki et al. 2013; Grzegorzewicz et al. 2012b). It was later shown that

both ISO and TAC active forms covalently react with a specific cysteine residue (Cys61) of the HadA subunit, resulting in the inhibition of HadAB enzyme activity (Grzegorzewicz et al. 2015). Frameshift and missense mutations affecting Hma and MmaA2 were also found in *M. tuberculosis* ISO and TAC spontaneous resistant mutants. The favored hypothesis is that these MA-MTs would play a role in the action of these antibiotics via protein-protein interactions with FAS-II dehydratases (Grzegorzewicz et al. 2012b).

5.3 Thiolactomycin (TLM)

Most of the interest was driven towards the natural molecule produced by *Nocardia*, thiolactomycin (TLM), which is specific for type-II synthases and exhibit broad activity towards Gram-negative and Gram-positive bacteria, and towards important pathogens, including *Plasmodium falciparum* (Kremer et al. 2000). TLM was shown to inhibit MA biosynthesis in vitro and retains good antimycobacterial activity in vivo (Douglas et al. 2002). TLM, composed of a thiolactone ring, competes malonate in binding in the same region of the active site of the condensing enzymes. The drug targets both *M. tuberculosis* KasA and KasB, the former being the more sensitive. Yet analogs of TLM may represent better inhibitors of the mycobacterial KasA enzyme (Kapilashrami et al. 2013).

5.4 Novel Molecules Targeting MA Biosynthesis and Translocation

In the context of MDR and XDR tuberculosis, targeting the MA pathway targeted by a frontline antibiotic (INH) is an effective strategy for anti-TB drug discovery by potentially bypassing existing resistances to current pathway inhibitors. This is particularly illustrated by the recent identification of inhibitors of the essential mycolic condensation enzymes Pks13/FadD32 and MA translocation and transfer enzymes MmpL3/Ag85 (Fig. 3).

The operon *fadD32-pks13-accD4* is essential for the viability of mycobacteria (Portevin et al. 2004, 2005) and was established both as a vulnerable target (Carroll et al. 2011) and "druggable" (Galandrin et al. 2013). Recently, the 4,6-diaryl-5,7-dimethyl coumarins, with the most potent CCA34 compound, were shown to kill *M. tuberculosis* by inhibiting FadD32 activity and effectively blocking bacterial replication in vitro and in animal models of TB (Stanley et al. 2013). Thiophene (TP) compounds bind to the N-ACP domain of Pk13 interfering with the activity of the enzyme and MA synthesis, thus leading to mycobacterial cell death (Wilson et al. 2013). A benzofuran compound, killing mycobacterial cells and discovered by combining high-throughput screening (HTS) with whole-genome sequencing (WGS) of resistant isolates, interferes with the function of the thioesterase domain of Pks13 (Ioerger et al. 2013).

The above HTS campaign with WGS has also identified a compound that inhibits the inner membrane transporter MmpL3 (Rv0206c). The latter is also targeted by the adamantyl urea derivative AU1235, the pyrrole derivative BM112 and the TB drug candidate SQ109, which all have potent bactericidal activities against *M. tuberculosis* (Grzegorzewicz et al. 2012a). These molecules abolish the translocation of trehalose monomycolates (TMM) to the envelope, resulting in a decrease in TDM and cell-wall-bound MAs (see Sect. 4.5, Fig. 3) (Grzegorzewicz et al. 2012a; La Rosa et al. 2012; Tahlan et al. 2012). These compounds, like others, inhibit Mmpl3 in replicating *M. tuberculosis* bacilli most likely by dissipating the transmembrane electrochemical proton gradient of the inner membrane where MmpL3 is located, which explains why such a large diversity of pharmacophores display inhibitory activities against MmpL3 (Li et al. 2014). Recently, optimized analogs of rimonabant, a cannabinoid receptor modulator structurally related to BM112, were also found to be potent inhibitors of *M. tuberculosis* growth (Ramesh et al. 2016). Moreover, a phenotypic screening against the pathogen *M. abscessus* revealed a piperidinol derivative as a potentially MmpL3-binding inhibitor (Dupont et al. 2016). Importantly, a new optimized indolecarboxamide likely targeting MmpL3 shows excellent activities against sensitive, MDR and XDR *M. tuberculosis* strains and in the TB aerosol lung infection model, and works in synergy with rifampin (Stec et al. 2016).

The mycoloyltransferase Ag85C protein was validated as a drug target by detailed characterization of an Ag85C-binding molecule, a benzothiophene derivative (I3-AG85), that exhibits an antibacterial activity towards *M. tuberculosis* growing both in vitro and within macrophages (Warrier et al. 2012) by blocking Ag85-mediated TDM synthesis with no effect on cell wall-bound MAs.

EthR (Rv3855) is a TetR-type repressor of the EthA enzyme, the common activator of ETH, ISO, and TAC (see above). Thus, counteracting EthR function would potentially result in an increased sensitivity of *M. tuberculosis* to these drugs. Thus, different series of ETH boosters were successfully developed (for review, see North et al. 2014), like for example *N*-phenylphenoxyacetamide derivatives and a family of 1,2,4-oxadiazole compounds (Flipo et al. 2012). More recently, a fragment-based rational drug design approach was used to develop small inhibitors filling the entire binding pocket of EthR (Nikiforov et al. 2016).

6 Some Biological Functions of Mycolic Acids

Several aspects of the biological activities of MAs have been reviewed (Glickman 2008; Vergne and Daffe 1998; Vander Beken et al. 2011; Verschoor et al. 2012). This chapter will thus recall some of them and focus on two aspects, namely the immunostimulatory activity of MA-containing compounds and their contribution to mycobacterial virulence.

6.1 Immunostimulatory Activity of Mycolic Acid-Containing Compounds

Infection with *M. tuberculosis* gives rise to granulomatous inflammation at infection sites and a powerful induction of T-cell responses. Immunostimulatory activity of lipids associated with the mycobacterial cell wall has been recognized for several decades and exploited in a large variety of different adjuvant preparations. Several compounds from the mycobacterial cell wall have been implicated in mediating host cell immune activation, among which trehalose dimycolate or TDM (cord factor). Numerous roles have been associated with TDM, greatly depending on the ways of mixing the glycolipid with other lipids (Vergne and Daffe 1998). These include the inhibition of phagosome-lysosome fusion and acidification of phagosomes (Indrigo et al. 2002, 2003), tissue damage and necrosis (Hunter et al. 2009) by inducing a high level of the proinflammatory cytokines TNF-alpha, IL-6, and IL-12 when used for stimulation of bone marrow dentritic cells.

Glycerol monomycolate (GroMM), delivered in cationic liposomes, was shown to be particularly efficient in TH1-inducing adjuvant formulation effective against tuberculosis. This mycobacterial antigen stimulates CD1b-stimulated $CD4^+$ T cells, and both the hydroxyl group of glycerol and the MA lengths were shown to be critical for triggering the T-cell responses. The stereochemistry of the molecule, e.g., the (*R*)-1-*O*-mycoloyl-glycerol was more stimulatory than the (*S*)-1-*O*-mycoloyl-glycerol, as well as the chain lengths of MA were found to play an important role in T-cell responses (Layre et al. 2009). Similarly, in glucose monomonomycolate (GMM) presented on CD1b-restricted T cells, the precise structures of natural GMM, including the glucose, the linkage of the glucose to the MA and the *R,R*-stereochemistry of the hydroxyl part of the mycolate, have been shown to dictate the T-cell recognition (Moody et al. 1997, 2000).

Immunological properties have also been reported for chemical structures related to the arabinogalactan termini (Rombouts et al. 2012), but these substances may occur only after the killing and degradation of the bacilli.

6.2 Mycolic Acid Types and Virulence

M. tuberculosis synthesizes α-, keto-, and methoxy-MAs as the main classes of MA (Daffé et al. 1983) (Fig. 2). Both the quantity and fine structure of MAs are critical for the survival and pathogenicity of the tubercle bacilli and related pathogenic mycobacterial species. For instance, the deletion of the in vitro most active mycoloyltransferase Ag85C (Belisle et al. 1997), resulting in a strain producing 40%-less MA compared to its isogenic parental *M. tuberculosis* strain, was less virulent in mice and more permeable (Jackson et al. 1999). The deletion of the *hma* gene, which led to abolishing the production of the oxygenated keto- and methoxy-MAs, although not affecting the total amount of MAs, resulted in a less virulent and less permeable strain of *M. tuberculosis* (Dubnau et al. 2000). Similarly, cyclopropanation of α-MA at the proximal position was shown to impact both the formation of "cords" and the virulence

of the tubercle bacilli (Glickman et al. 2000). *Trans*-cyclopropanation of MAs on trehalose dimycolate has been shown to suppress *M. tuberculosis*-induced inflammation and virulence (Rao et al. 2006). Moreover, *kasB* mutants of both *M. marinum* and *M. tuberculosis*, which produce MAs shorter by up to six carbon atoms, exhibit an increased permeability of their cell walls and a severe defect in resisting host defense mechanisms and antibiotic action (Gao et al. 2003).

M. tuberculosis is an obligate human parasite able to develop in alveolar macrophages. Accumulation of lipid droplets in the macrophages of individuals developing a post-primary infection, gives a foamy aspect to these macrophages. In alveolar foamy macrophages, the bacilli were mainly found within lipid droplets. The formation of foamy macrophages (FM), a granuloma-specific population characterized by its high lipid content, was studied comparatively in mycobacteria with different MA composition, i.e., in *M. tuberculosis*, *M. smegmatis*, and recombinant strains of the latter species overexpressing the *hma* gene responsible for the introduction of ketomycolic acids. Only bacteria containing oxygenated mycolic acids induced the formation of FMs. Oxygenated MAs triggered the differentiation of human monocyte-derived macrophages into FMs, which might constitute a shelter for persisting bacilli (Peyron et al. 2008).

Taking advantage from the nonessentiality of the *hadC* gene of *M. tuberculosis* (Jamet et al. 2015b; Slama et al. 2016), we have addressed its contribution to the virulence of the tubercle bacillus, through the knock-out of the gene in the virulent H37Rv variant. This resulted in a MA profile similar to that of the avirulent strain H37Ra that has a point mutation in this gene, i.e., a strong reduction of the global content of the oxygenated MAs (methoxy- and keto-MAs, Fig. 2), reminiscent of *M. tuberculosis* Δ*kasB* phenotype (Bhatt et al. 2007b), and inhibition of the formation of extra-long MAs bearing a fourth cyclopropanated meromycolic segment (Quemard 2016; Slama et al. 2016). This strongly affected the virulence in mice, the cording capacity, and the sensitivity to rifampicin. Furthermore, the deletion of *hadA* and *hadC* genes of *M. smegmatis*, which also triggered an alteration of the MA profile, had a dramatic effect on the bacterial physiology and fitness (Jamet et al. 2015b). Similarly, in *M. abscessus* the deletion of MAB_4780, a dehydratase that is distinct from the HadBC enzyme, led to a decreased level of α-MA with a concomitant lack of cording and an increased susceptibility to the drug thiacetazone compared with the wild type strain (Halloum et al. 2016). The mutant had also an attenuated virulence in macrophages as well as in zebrafish embryos, with only a few infectious foci and no induction of the formation of granulomas (Halloum et al. 2016). The mutation has a role in preventing phagosome-lysosome fusion, a key strategy that is used by pathogenic mycobacteria to avoid exposure to lysosomal hydrolases, thus promoting survival.

6.3 Mycolic Acids with Respect to Cording and Biofilm Formation

In a liquid medium without detergent, *M. tuberculosis* cells form microscopic structures that resemble cords, a phenomenon known as cord formation, or cording,

and considered as a virulence factor in the *M. tuberculosis* complex. In the 1950s, cording was related to TDM that, consequently, was named the "cord factor." The fine structure of the mycoloyl chains in the TDM plays a determinant role in the cording capacity, as illustrated by phenotypic analyses of mutants (see some examples in Sect. 6.2) (Glickman 2008). However, the subsequent demonstration of the occurrence of TDM in all the mycobacterial species studied so far, except *M. leprae* where only TMM was clearly identified, suggested that it was not the only factor required for cording. Consistent with this, modern techniques of microbial genetics have revealed that cording can be affected by mutations in genes not directly involved in TDM biosynthesis. Natural or laboratory mutants of the *M. tuberculosis* complex that were unable to form microscopic cords showed impaired virulence compared to the original cording strains (Ferrer et al. 2009; Glickman 2008). A strong correlation exists between microscopic cords, rough colonial morphology, and increased persistence of mycobacteria inside macrophages (Julian et al. 2010).

Many bacterial cells have the ability to form biofilms that enable them to exist as communities and to adhere tightly to surfaces. Biofilm formation requires a switch from a motile to a sessile life style, and generation of an extracellular matrix. The biofilms of pathogenic microorganisms, including *M. tuberculosis*, are a major medical issue, because they are difficult to eradicate due to the presence in these structures of drug-tolerant bacteria (Ojha et al. 2008). The discovery that virulent tubercle bacilli grew on the surface of a liquid medium, forming veils that spread uniformly over the entire surface of the liquid medium, and climbed up the sides of the glass container, was first described by Koch in 1884 and later by others as a typical characteristic of *M. tuberculosis* complex strains. The natural tendency of *M. tuberculosis* to form biofilms has been exploited long ago for the production of large amounts of cell biomasses that are necessary for chemical characterization of the bacterial cell constituents. Indeed, historically, *M. tuberculosis* was grown as a surface pellicle, a biofilm-like structure, at the liquid-air interface of some synthetic media, like the Sauton's medium. These in vitro biofilms were shown to be rich in free MAs released by enzymatic hydrolysis of TDM or cell lysis due to the long-time culture needed for biomass production (Ojha et al. 2010). A relationship between MA biosynthesis and biofilm formation has been demonstrated by inactivation of the gene GroEL1, a dedicated chaperone involved in MA biosynthesis. GroEL1 modulates synthesis of MAs specifically during biofilm formation and physically associates with KasA. Biofilm is associated with elevated synthesis of C56–C58 fatty acids (Ojha et al. 2005). In the same context, the deletion of *groEL* (also known as *cpn60.1* or *hsp60*) in *M. bovis* BCG led to the loss of its capacity to form biofilm, accompanied by changes in lipid composition, mainly in MAs (with 2–4 carbon atoms shorter) and phthiocerol dimycocerosates (Wang et al. 2011).

The types of mycolic acids also play a role in the biofilm formation, as typified by the essentiality of the keto-MAs (see Fig. 2) in these structures. By deletion of the *mmaA4* (or *hma*) gene, the resulting mutant devoid of keto-MAs was shown to be both pellicle-defective and highly sensitive to rifampicin under planktonic growth. It was proposed that when incorporated within the wild type pellicle biofilm, the cells

were protected from the bactericidal activity of the antibiotic (Sambandan et al. 2013). The FAS-II enzymes KasA/KasB and HadBC are also important in biofilm formation, consistent with their roles in the elongation of the meromycolic chain of MAs (Gao et al. 2003; Jamet et al. 2015b; Slama et al. 2016).

During the past decade, considerable progress has been made in recognizing the importance of biofilms in chronic infections and understanding the biochemical and cellular processes that lead to biofilm formation in vitro, although many questions remain. In the particular case of tuberculosis, the relationship between biofilm formation and pathogenesis has not been clearly established. Progress will require technologies that will enable sophisticated analyses of the biochemistry and cell biology of biofilm-forming microorganisms in vivo, as well as ongoing development of animal models that faithfully mimic chronic infections.

7 Research Needs

Despite the progress in the past decades in deciphering the biosynthesis pathway leading to mycolic acids, the whole biosynthesis is not fully known. Many relevant questions remain to be solved. Among these:

(i) In mycobacteria, all mycolates contain two positions, i.e., distal and proximal, that initially contain double bonds, subsequently modified into *cis* cyclopropane, *trans* double bond, transcyclopropane, or oxygenated function with an adjacent methyl branch. A very recent survey suggests the involvement of the desaturase DesA1 in the formation of one double bond in α-MAs of *M. smegmatis* (Singh et al. 2016). However, the exact mechanisms underlying the introduction of both double bonds in all types of meromycolic chain and the proteins catalyzing these desaturation/dehydration-isomerization steps remain to be discovered.

(ii) In *M. tuberculosis*, the characterization of monounsaturated fatty acids ranging from C_{24} to C_{30} with a double bond localized exactly at the position expected for the elongation of a Δ5 tetracosenoic acid precursor as well as other data suggest that at least some of the modifications are introduced during the growth of the meromycolic chain (Asselineau et al. 1970b; Qureshi et al. 1984; Takayama and Qureshi 1978; Yuan et al. 1998). Yet conclusive data are still lacking.

(iii) Several enzymes of the *M. tuberculosis* FAS-II system have been shown to be down-regulated by STPK-mediated phosphorylation (Molle and Kremer 2010), a growth phase-dependent phenomenon (Slama et al. 2011). However, the precise STPKs regulating this process, the timing, and its consequences on the bacterial physiology are unclear. A few transcriptional regulators of MA metabolism have also been identified. To face this fragmented information, a complete picture of the mechanisms of regulation of the MA metabolism involved in adapting the production level and the fine structure of these envelope components in response to environmental conditions must be

established. The physiological advantages gained by post-translational modifications or transcriptional regulation and how the different regulatory mechanisms may be coordinated, in a synergistic or antagonistic way, need future exploration.

(iv) Recently, MmpL3 was identified as the transporter of the TMM, product of the mycolic condensation system, through the plasma membrane (Grzegorzewicz et al. 2012a), while MmpL11 was shown to export distinct MA derivatives (Pacheco et al. 2013). Thus, MA biosynthesis is very likely to occur on the cytoplasmic side of the plasma membrane. The mechanisms by which the MA derivatives are routed from the periplasmic side of the inner membrane transporters MmpL3 and MmpL11 to the envelope's outer layers (like the mycomembrane) or the bacterial surface have not been investigated yet.

(v) The existence of protein-protein interactions between known FAS-II enzymes (Veyron-Churlet et al. 2004, 2005) and MA methyl transferases led to propose a model with interconnected specialized complexes (Cantaloube et al. 2011). The atomic structures of many individual proteins of MA biosynthesis and transfer have been solved, but besides that of the FAS-I system (Boehringer et al. 2013; Ciccarelli et al. 2013), the 3D structures of the MA biosynthesis and export machineries remain unknown. This should represent a future field of investigation that may open new avenues for antituberculosis inhibition strategies like the development of molecules blocking the assembly of protein complexes.

(vi) The role of biofilm growth during the colonization of the host by *M. tuberculosis* and other pathogenic mycobacteria is another outstanding question. For example, the *M. tuberculosis* pilus (MTP), which contributes to biofilm formation, has an impact on lesion architecture in infected lungs (Mann et al. 2016). Moreover, it has been shown that *M. tuberculosis* biofilms harbor an important drug-tolerant population that persists despite exposure to high antibiotic levels (Ojha et al. 2008). The mechanisms that enable these MA-rich mycobacterial biofilms to resist the action of antibiotics constitute a fundamental issue both in terms of antimycobacterial chemotherapies and prevention against nosocomial infections.

The interdisciplinary efforts to understand both the basic biology and pathogenesis of *M. tuberculosis* will enable substantial progress in developing new avenues for therapeutic interventions in the coming years.

Acknowledgments The authors are grateful to their colleagues for fruitful collaborations and discussions, and for sharing unpublished material. We acknowledge funding from the European Union (NM4TB, grant LSHP-CT-2005-018923; TB-Drug grant LSHP-CT-2006-037217; SysteMTb HEALTH-2009-2.1.2-1 241587), the Agence Nationale de la Recherche (XPKS-MYCO, grant 09-BLAN-0298-03; FASMY, grant ANR-14-CE16-0012), the Région Midi-Pyrénées (MYCA, FEDER grant 34249), the France-Argentina ECOS-MINCyT cooperation program (grant A11B04) and the "Vaincre la Mucoviscidose" association (IC0716, France).

References

Alahari A, Trivelli X, Guerardel Y, Dover LG, Besra GS, Sacchettini JC, Reynolds RC, Coxon GD, Kremer L (2007) Thiacetazone, an antitubercular drug that inhibits cyclopropanation of cell wall mycolic acids in mycobacteria. PLoS One 2:e1343

Asselineau C, Asselineau J (1966) Stéréochimie de l'acide corynomycolique. Bull Soc Chim Fr 6:1992–1999

Asselineau J, Lederer E (1950) Structure of the mycolic acids of mycobacteria. Nature 166:782–783

Asselineau C, Tocanne G, Tocanne JF (1970a) Stéréochimie des acides mycoliques. Bull Soc Chim Fr 4:1455–1459

Asselineau CP, Lacave CS, Montrozier HL, Prome JC (1970b) Structural relations between unsaturated mycolic acids and short-chain unsaturated acids synthesized by *Mycobacterium phlei*. Metabolic implications. Eur J Biochem 14:406–410

Asselineau C, Asselineau J, Laneelle G, Laneelle MA (2002) The biosynthesis of mycolic acids by mycobacteria: current and alternative hypotheses. Prog Lipid Res 41:501–523

Av-Gay Y, Everett M (2000) The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. Trends Microbiol 8:238–244

Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, de Lisle G, Jacobs WR Jr (1994) *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 263:227–230

Bardou F, Quemard A, Dupont MA, Horn C, Marchal G, Daffe M (1996) Effects of isoniazid on ultrastructure of *Mycobacterium aurum* and *Mycobacterium tuberculosis* and on production of secreted proteins. Antimicrob Agents Chemother 40:2459–2467

Basso LA, Zheng R, Musser JM, Jacobs WR Jr, Blanchard JS (1998) Mechanisms of isoniazid resistance in *Mycobacterium tuberculosis*: enzymatic characterization of enoyl reductase mutants identified in isoniazid-resistant clinical isolates. J Infect Dis 178:769–775

Bazet Lyonnet B, Diacovich L, Cabruja M, Bardou F, Quemard A, Gago G, Gramajo H (2014) Pleiotropic effect of AccD5 and AccE5 depletion in acyl-coenzyme A carboxylase activity and in lipid biosynthesis in mycobacteria. PLoS One 6:e99853

Belardinelli JM, Morbidoni HR (2012) Mutations in the essential FAS II beta-hydroxyacyl ACP dehydratase complex confer resistance to thiacetazone in *Mycobacterium tuberculosis* and *Mycobacterium kansasii*. Mol Microbiol 86:568–579

Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science 276:1420–1422

Bergeret F, Gavalda S, Chalut C, Malaga W, Quemard A, Pedelacq JD, Daffe M, Guilhot C, Mourey L, Bon C (2012) Biochemical and structural study of the atypical acyltransferase domain from the mycobacterial polyketide synthase Pks13. J Biol Chem 287:33675–33690

Bhatt A, Kremer L, Dai AZ, Sacchettini JC, Jacobs WR Jr (2005) Conditional depletion of KasA, a key enzyme of mycolic acid biosynthesis, leads to mycobacterial cell lysis. J Bacteriol 187:7596–7606

Bhatt A, Molle V, Besra GS, Jacobs WR Jr, Kremer L (2007a) The *Mycobacterium tuberculosis* FAS-II condensing enzymes: their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. Mol Microbiol 64:1442–1454

Bhatt A, Fujiwara N, Bhatt K, Gurcha SS, Kremer L, Chen B, Chan J, Porcelli SA, Kobayashi K, Besra GS, Jacobs WR Jr (2007b) Deletion of kasB in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. Proc Natl Acad Sci U S A 104:5157–5162

Bhatt A, Brown AK, Singh A, Minnikin DE, Besra GS (2008) Loss of a mycobacterial gene encoding a reductase leads to an altered cell wall containing beta-oxo-mycolic acid analogs and accumulation of ketones. Chem Biol 15:930–939

Biswas RK, Dutta D, Tripathi A, Feng Y, Banerjee M, Singh BN (2013) Identification and characterization of Rv0494: a fatty acid-responsive protein of the GntR/FadR family from *Mycobacterium tuberculosis*. Microbiology 159:913–923

Bloch K (1969) Enzymatic synthesis of monounsaturated fatty acids. Acc Chem Res 2:193–202
Bloch K (1977) Control mechanisms for fatty acid synthesis in *Mycobacterium smegmatis*. Adv Enzymol Relat Areas Mol Biol 45:1–84
Bloch K, Vance D (1977) Control mechanisms in the synthesis of saturated fatty acids. Annu Rev Biochem 46:263–298
Boehringer D, Ban N, Leibundgut M (2013) 7.5-A cryo-em structure of the mycobacterial fatty acid synthase. J Mol Biol 425:841–849
Bordet C, Michel G (1969) Structure and biogenesis of high molecular weight lipids from *Nocardia asteroides*. Bull Soc Chim Biol (Paris) 51:527–548
Brown JR, North EJ, Hurdle JG, Morisseau C, Scarborough JS, Sun D, Kordulakova J, Scherman MS, Jones V, Grzegorzewicz A, Crew RM, Jackson M, McNeil MR, Lee RE (2011) The structure-activity relationship of urea derivatives as anti-tuberculosis agents. Bioorg Med Chem 19:5585–5595
Cantaloube S, Veyron-Churlet R, Haddache N, Daffe M, Zerbib D (2011) The *Mycobacterium tuberculosis* FAS-II dehydratases and methyltransferases define the specificity of the mycolic acid elongation complexes. PLoS One 6:e29564
Cantrell SA, Leavell MD, Marjanovic O, Iavarone AT, Leary JA, Riley LW (2013) Free mycolic acid accumulation in the cell wall of the mce1 operon mutant strain of *Mycobacterium tuberculosis*. J Microbiol 51:619–626
Carel C, Nukdee K, Cantaloube S, Bonne M, Diagne CT, Laval F, Daffe M, Zerbib D (2014) *Mycobacterium tuberculosis* proteins involved in mycolic acid synthesis and transport localize dynamically to the old growing pole and septum. PLoS One 9:e97148
Carrere-Kremer S, Blaise M, Singh VK, Alibaud L, Tuaillon E, Halloum I, van de Weerd R, Guerardel Y, Drancourt M, Takiff H, Geurtsen J, Kremer L (2015) A new dehydratase conferring innate resistance to thiacetazone and intra-amoebal survival of *Mycobacterium smegmatis*. Mol Microbiol 96:1085–1102
Carroll P, Faray-Kele MC, Parish T (2011) Identifying vulnerable pathways in *Mycobacterium tuberculosis* by using a knockdown approach. Appl Environ Microbiol 77:5040–5043
Ciccarelli L, Connell SR, Enderle M, Mills DJ, Vonck J, Grininger M (2013) Structure and conformational variability of the *Mycobacterium tuberculosis* fatty acid synthase multienzyme complex. Structure 21:1251–1257
Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Barrell BG (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537–544
Corrales RM, Molle V, Leiba J, Mourey L, de Chastellier C, Kremer L (2012) Phosphorylation of mycobacterial PcaA inhibits mycolic acid cyclopropanation: consequences for intracellular survival and for phagosome maturation block. J Biol Chem 287:26187–26199
Daffé M, Draper P (1998) The envelope layers of mycobacteria with reference to their pathogenicity. Adv Microb Physiol 39:131–203
Daffé M, Laneelle MA, Asselineau C, Levy-Frebault V, David H (1983) Taxonomic value of mycobacterial fatty acids: proposal for a method of analysis. Ann Microbiol (Paris) 134B:241–256
De Sousa-D'Auria C, Kacem R, Puech V, Tropis M, Leblon G, Houssin C, Daffe M (2003) New insights into the biogenesis of the cell envelope of corynebacteria: identification and functional characterization of five new mycoloyltransferase genes in *Corynebacterium glutamicum*. FEMS Microbiol Lett 224:35–44
DeBarber AE, Mdluli K, Bosman M, Bekker LG, Barry CE 3rd (2000) Ethionamide activation and sensitivity in multidrug-resistant *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 97:9677–9682
Dessen A, Quemard A, Blanchard JS, Jacobs WR Jr, Sacchettini JC (1995) Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. Science 267:1638–1641

Dinadayala P, Laval F, Raynaud C, Lemassu A, Laneelle MA, Laneelle G, Daffe M (2003) Tracking the putative biosynthetic precursors of oxygenated mycolates of *Mycobacterium tuberculosis.* Structural analysis of fatty acids of a mutant strain deviod of methoxy- and ketomycolates. J Biol Chem 278:7310–7319

Douglas JD, Senior SJ, Morehouse C, Phetsukiri B, Campbell IB, Besra GS, Minnikin DE (2002) Analogues of thiolactomycin: potential drugs with enhanced anti-mycobacterial activity. Microbiology 148:3101–3109

Dover LG, Alahari A, Gratraud P, Gomes JM, Bhowruth V, Reynolds RC, Besra GS, Kremer L (2007) EthA, a common activator of thiocarbamide-containing drugs acting on different mycobacterial targets. Antimicrob Agents Chemother 51:1055–1063

Draper P (1998) The outer parts of the mycobacterial envelope as permeability barriers. Front Biosci 3:D1253–D1261

Dubnau E, Laneelle MA, Soares S, Benichou A, Vaz T, Prome D, Prome JC, Daffe M, Quemard A (1997) *Mycobacterium bovis* BCG genes involved in the biosynthesis of cyclopropyl keto- and hydroxy-mycolic acids. Mol Microbiol 23:313–322

Dubnau E, Marrakchi H, Smith I, Daffe M, Quemard A (1998) Mutations in the cmaB gene are responsible for the absence of methoxymycolic acid in *Mycobacterium bovis* BCG Pasteur. Mol Microbiol 29:1526–1528

Dubnau E, Chan J, Raynaud C, Mohan VP, Laneelle MA, Yu K, Quemard A, Smith I, Daffe M (2000) Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. Mol Microbiol 36:630–637

Dupont C, Viljoen A, Dubar F, Blaise M, Bernut A, Pawlik A, Bouchier C, Brosch R, Guerardel Y, Lelievre J, Ballell L, Herrmann JL, Biot C, Kremer L (2016) A new piperidinol derivative targeting mycolic acid transport in *Mycobacterium abscessus*. Mol Microbiol 101:515–529

Etemadi AH (1967) Structural and biogenetic correlations of mycolic acids in relation to the phylogenesis of various genera of Actinomycetales. Bull Soc Chim Biol (Paris) 49:695–706

Etemadi AH, Gasche J (1965) On the biogenetic origin of 2-eicosanol and 2-octadecanol of *Mycobacterium avium*. Bull Soc Chim Biol (Paris) 47:2095–2104

Ferrer NL, Gomez AB, Soto CY, Neyrolles O, Gicquel B, Garcia-Del Portillo F, Martin C (2009) Intracellular replication of attenuated *Mycobacterium tuberculosis* phoP mutant in the absence of host cell cytotoxicity. Microbes Infect 11:115–122

Flipo M, Willand N, Lecat-Guillet N, Hounsou C, Desroses M, Leroux F, Lens Z, Villeret V, Wohlkonig A, Wintjens R, Christophe T, Kyoung Jeon H, Locht C, Brodin P, Baulard AR, Deprez B (2012) Discovery of novel *N*-phenylphenoxyacetamide derivatives as EthR inhibitors and ethionamide boosters by combining high-throughput screening and synthesis. J Med Chem 55:6391–6402

Forrellad MA, McNeil M, Santangelo Mde L, Blanco FC, Garcia E, Klepp LI, Huff J, Niederweis M, Jackson M, Bigi F (2014) Role of the Mce1 transporter in the lipid homeostasis of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 94:170–177

Gago G, Kurth D, Diacovich L, Tsai SC, Gramajo H (2006) Biochemical and structural characterization of an essential acyl coenzyme A carboxylase from *Mycobacterium tuberculosis*. J Bacteriol 188:477–486

Galandrin S, Guillet V, Rane RS, Leger M, Radha N, Eynard N, Das K, Balganesh TS, Mourey L, Daffe M, Marrakchi H (2013) Assay development for identifying inhibitors of the mycobacterial FadD32 activity. J Biomol Screen 18:576–587

Gande R, Gibson KJ, Brown AK, Krumbach K, Dover LG, Sahm H, Shioyama S, Oikawa T, Besra GS, Eggeling L (2004) Acyl-CoA carboxylases (accD2 and accD3), together with a unique polyketide synthase (Cg-pks), are key to mycolic acid biosynthesis in Corynebacterianeae such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. J Biol Chem 279:44847–44857

Gannoun-Zaki L, Alibaud L, Kremer L (2013) Point mutations within the fatty acid synthase type II dehydratase components HadA or HadC contribute to isoxyl resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 57:629–632

Gao LY, Laval F, Lawson EH, Groger RK, Woodruff A, Morisaki JH, Cox JS, Daffe M, Brown EJ (2003) Requirement for kasB in Mycobacterium mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Mol Microbiol 49:1547–1563

Gavalda S, Leger M, van der Rest B, Stella A, Bardou F, Montrozier H, Chalut C, Burlet-Schiltz O, Marrakchi H, Daffe M, Quemard A (2009) The Pks13/FadD32 crosstalk for the biosynthesis of mycolic acids in *Mycobacterium tuberculosis*. J Biol Chem 284:19255–19264

Gavalda S, Bardou F, Laval F, Bon C, Malaga W, Chalut C, Guilhot C, Mourey L, Daffe M, Quemard A (2014) The polyketide synthase Pks13 catalyzes a novel mechanism of lipid transfer in mycobacteria. Chem Biol 21:1660–1669

Glickman MS (2008) Cording, cord factors, and trehalose dimycolate. In: Daffé M, Reyrat JM (eds) The mycobacterial cell envelope. ASM Press, Washington, DC, pp 63–73

Glickman MS, Cox JS, Jacobs WR Jr (2000) A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. Mol Cell 5:717–727

Glickman MS, Cahill SM, Jacobs WR (2001) The *Mycobacterium tuberculosis* cmaA2 gene encodes a mycolic acid trans-cyclopropane synthetase. J Biol Chem 276:2228–2233

Goren MB, Brennan PJ (1979) Mycobacterial lipids: chemistry and biological activities. In: Youmans GP (ed) Tuberculosis. The WB Saunders Co, Philadelphia, pp 63–193

Greenstein AE, Grundner C, Echols N, Gay LM, Lombana TN, Miecskowski CA, Pullen KE, Sung PY, Alber T (2005) Structure/function studies of Ser/Thr and Tyr protein phosphorylation in *Mycobacterium tuberculosis*. J Mol Microbiol Biotechnol 9:167–181

Grzegorzewicz AE, Pham H, Gundi VA, Scherman MS, North EJ, Hess T, Jones V, Gruppo V, Born SE, Kordulakova J, Chavadi SS, Morisseau C, Lenaerts AJ, Lee RE, McNeil MR, Jackson M (2012a) Inhibition of mycolic acid transport across the *Mycobacterium tuberculosis* plasma membrane. Nat Chem Biol 8:334–341

Grzegorzewicz AE, Kordulakova J, Jones V, Born SE, Belardinelli JM, Vaquie A, Gundi VA, Madacki J, Slama N, Laval F, Vaubourgeix J, Crew RM, Gicquel B, Daffe M, Morbidoni HR, Brennan PJ, Quemard A, McNeil MR, Jackson M (2012b) A common mechanism of inhibition of the *Mycobacterium tuberculosis* mycolic acid biosynthetic pathway by isoxyl and thiacetazone. J Biol Chem 287:38434–38441

Grzegorzewicz AE, Eynard N, Quemard A, North EJ, Margolis A, Lindenberger JJ, Jones V, Kordulakova J, Brennan PJ, Lee RE, Ronning DR, McNeil MR, Jackson M (2015) Covalent modification of the FAS-II dehydratase by Isoxyl and Thiacetazone. ACS Infect Dis 1:91–97

Guillet V, Galandrin S, Maveyraud L, Ladeveze S, Mariaule V, Bon C, Eynard N, Daffe M, Marrakchi H, Mourey L (2016) Insight into structure-function relationships and inhibition of the fatty acyl-AMP ligase (FadD32) orthologs from mycobacteria. J Biol Chem 291:7973–7989

Halloum I, Carrere-Kremer S, Blaise M, Viljoen A, Bernut A, Le Moigne V, Vilcheze C, Guerardel Y, Lutfalla G, Herrmann JL, Jacobs WR Jr, Kremer L (2016) Deletion of a dehydratase important for intracellular growth and cording renders rough *Mycobacterium abscessus* avirulent. Proc Natl Acad Sci U S A 113:E4228–E4237

Hanoulle X, Wieruszeski JM, Rousselot-Pailley P, Landrieu I, Locht C, Lippens G, Baulard AR (2006) Selective intracellular accumulation of the major metabolite issued from the activation of the prodrug ethionamide in mycobacteria. J Antimicrob Chemother 58:768–772

Hoffmann C, Leis A, Niederweis M, Plitzko JM, Engelhardt H (2008) Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc Natl Acad Sci U S A 105:3963–3967

Hong S, Cheng TY, Layre E, Sweet L, Young DC, Posey JE, Butler WR, Moody DB (2012) Ultralong C100 mycolic acids support the assignment of *Segniliparus* as a new bacterial genus. PLoS One 7:e39017

Hunter RL, Armitige L, Jagannath C, Actor JK (2009) TB research at UT-Houston – a review of cord factor: new approaches to drugs, vaccines and the pathogenesis of tuberculosis. Tuberculosis (Edinb) 89:S18–S25

Indrigo J, Hunter RL Jr, Actor JK (2002) Influence of trehalose 6,6′-dimycolate (TDM) during mycobacterial infection of bone marrow macrophages. Microbiology 148:1991–1998

Indrigo J, Hunter RL Jr, Actor JK (2003) Cord factor trehalose 6,6′-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. Microbiology 149:2049–2059

Ioerger TR, O'Malley T, Liao R, Guinn KM, Hickey MJ, Mohaideen N, Murphy KC, Boshoff HI, Mizrahi V, Rubin EJ, Sassetti CM, Barry CE 3rd, Sherman DR, Parish T, Sacchettini JC (2013) Identification of new drug targets and resistance mechanisms in *Mycobacterium tuberculosis*. PLoS One 8:e75245

Jackson M, Raynaud C, Laneelle MA, Guilhot C, Laurent-Winter C, Ensergueix D, Gicquel B, Daffe M (1999) Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. Mol Microbiol 31:1573–1587

Jamet S, Quentin Y, Coudray C, Texier P, Laval F, Daffe M, Fichant G, Cam K (2015a) Evolution of mycolic acid biosynthesis genes and their regulation during starvation in *Mycobacterium tuberculosis*. J Bacteriol 197:3797–3811

Jamet S, Slama N, Domingues J, Laval F, Texier P, Eynard N, Quemard A, Peixoto A, Lemassu A, Daffe M, Cam K (2015b) The non-essential mycolic acid biosynthesis genes hadA and hadC contribute to the physiology and fitness of *Mycobacterium smegmatis*. PLoS One 10:e0145883

Jarlier V, Nikaido H (1994) Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol Lett 123:11–18

Johnsson K, Shultz PG (1994) Mechanistic studies of the oxidation of isoniazid by the catalase peroxidase from *Mycobacterium tuberculosis*. J Am Chem Soc 116:7425–7426

Julian E, Roldan M, Sanchez-Chardi A, Astola O, Agusti G, Luquin M (2010) Microscopic cords, a virulence-related characteristic of *Mycobacterium tuberculosis*, are also present in nonpathogenic mycobacteria. J Bacteriol 192:1751–1760

Kalscheuer R, Weinrick B, Veeraraghavan U, Besra GS, Jacobs WR Jr (2010) Trehalose-recycling ABC transporter LpqY-SugA-SugB-SugC is essential for virulence of *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 107:21761–21766

Kapilashrami K, Bommineni GR, Machutta CA, Kim P, Lai CT, Simmerling C, Picart F, Tonge PJ (2013) Thiolactomycin-based beta-ketoacyl-AcpM synthase A (KasA) inhibitors: fragment-based inhibitor discovery using transient one-dimensional nuclear overhauser effect NMR spectroscopy. J Biol Chem 288:6045–6052

Kordulakova J, Janin YL, Liav A, Barilone N, Dos Vultos T, Rauzier J, Brennan PJ, Gicquel B, Jackson M (2007) Isoxyl activation is required for bacteriostatic activity against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 51:3824–3829

Kremer L, Douglas JD, Baulard AR, Morehouse C, Guy MR, Alland D, Dover LG, Lakey JH, Jacobs WR Jr, Brennan PJ, Minnikin DE, Besra GS (2000) Thiolactomycin and related analogues as novel anti-mycobacterial agents targeting KasA and KasB condensing enzymes in *Mycobacterium tuberculosis*. J Biol Chem 275:16857–16864

Kremer L, Dover LG, Carrere S, Nampoothiri KM, Lesjean S, Brown AK, Brennan PJ, Minnikin DE, Locht C, Besra GS (2002) Mycolic acid biosynthesis and enzymic characterization of the beta-ketoacyl-ACP synthase A-condensing enzyme from *Mycobacterium tuberculosis*. Biochem J 364:423–430

Kremer L, Dover LG, Morbidoni HR, Vilcheze C, Maughan WN, Baulard A, Tu SC, Honore N, Deretic V, Sacchettini JC, Locht C, Jacobs WR Jr, Besra GS (2003) Inhibition of InhA activity, but not KasA activity, induces formation of a KasA-containing complex in mycobacteria. J Biol Chem 278:20547–22055

La Rosa V, Poce G, Canseco JO, Buroni S, Pasca MR, Biava M, Raju RM, Porretta GC, Alfonso S, Battilocchio C, Javid B, Sorrentino F, Ioerger TR, Sacchettini JC, Manetti F, Botta M, De Logu A, Rubin EJ, De Rossi E (2012) MmpL3 is the cellular target of the antitubercular pyrrole derivative BM212. Antimicrob Agents Chemother 56:324–331

Laneelle MA, Laneelle G (1970) Structure of mycolic acids and an intermediate in the biosynthesis of dicarboxylic mycolic acids. Eur J Biochem 12:296–300

Laneelle MA, Launay A, Spina L, Marrakchi H, Laval F, Eynard N, Lemassu A, Tropis M, Daffe M, Etienne G (2012) A novel mycolic acid species defines two novel genera of the Actinobacteria, *Hoyosella* and *Amycolicicoccus*. Microbiology 158:843–855

Laneelle MA, Eynard N, Spina L, Lemassu A, Laval F, Huc E, Etienne G, Marrakchi H, Daffe M (2013) Structural elucidation and genomic scrutiny of the C60–C100 mycolic acids of *Segniliparus rotundus*. Microbiology 159:191–203

Laneelle MA, Nigou J, Daffe M (2015) Lipid and lipoarabinomannan isolation and characterization. Methods Mol Biol 1285:77–103

Laval F, Laneelle MA, Deon C, Monsarrat B, Daffe M (2001) Accurate molecular mass determination of mycolic acids by MALDI-TOF mass spectrometry. Anal Chem 73:4537–4544

Laval F, Haites R, Movahedzadeh F, Lemassu A, Wong CY, Stoker N, Billman-Jacobe H, Daffe M (2008) Investigating the function of the putative mycolic acid methyltransferase UmaA: divergence between the *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* proteins. J Biol Chem 283:1419–1427

Layre E, Collmann A, Bastian M, Mariotti S, Czaplicki J, Prandi J, Mori L, Stenger S, De Libero G, Puzo G, Gilleron M (2009) Mycolic acids constitute a scaffold for mycobacterial lipid antigens stimulating CD1-restricted T cells. Chem Biol 16:82–92

Le NH, Molle V, Eynard N, Miras M, Stella A, Bardou F, Galandrin S, Guillet V, Andre-Leroux G, Bellinzoni M, Alzari P, Mourey L, Burlet-Schiltz O, Daffe M, Marrakchi H (2016) Ser/Thr phosphorylation regulates the Fatty Acyl-AMP Ligase activity of FadD32, an essential enzyme in mycolic acid biosynthesis. J Biol Chem 291:22793–22805

Lea-Smith DJ, Pyke JS, Tull D, McConville MJ, Coppel RL, Crellin PK (2007) The reductase that catalyzes mycolic motif synthesis is required for efficient attachment of mycolic acids to arabinogalactan. J Biol Chem 282:11000–11008

Lederer E (1969) Some problems concerning biological C-alkylation reactions and phytosterol biosynthesis. Q Rev Chem Soc 23:453–481

Leger M, Gavalda S, Guillet V, van der Rest B, Slama N, Montrozier H, Mourey L, Quemard A, Daffe M, Marrakchi H (2009) The dual function of the *Mycobacterium tuberculosis* FadD32 required for mycolic acid biosynthesis. Chem Biol 16:510–519

Li W, Upadhyay A, Fontes FL, North EJ, Wang Y, Crans DC, Grzegorzewicz AE, Jones V, Franzblau SG, Lee RE, Crick DC, Jackson M (2014) Novel insights into the mechanism of inhibition of MmpL3, a target of multiple pharmacophores in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 58:6413–6423

Li W, Gu S, Fleming J, Bi L (2015) Crystal structure of FadD32, an enzyme essential for mycolic acid biosynthesis in mycobacteria. Sci Rep 5:15493

Li W, Obregon-Henao A, Wallach JB, North EJ, Lee RE, Gonzalez-Juarrero M, Schnappinger D, Jackson M (2016) Therapeutic potential of the *Mycobacterium tuberculosis* mycolic acid transporter, MmpL3. Antimicrob Agents Chemother 60:5198–5207

Liu J, Barry CE 3rd, Besra GS, Nikaido H (1996) Mycolic acid structure determines the fluidity of the mycobacterial cell wall. J Biol Chem 271:29545–29551

Mann KM, Pride A, Flentie K, Kimmey J, Weiss L, Stallings C (2016) Analysis of the contribution of MTP and the predicted Flp pilus genes to *Mycobacterium tuberculosis* pathogenesis. Microbiology 162:1784–1796

Marchand CH, Salmeron C, Bou Raad R, Meniche X, Chami M, Masi M, Blanot D, Daffe M, Tropis M, Huc E, Le Marechal P, Decottignies P, Bayan N (2012) Biochemical disclosure of the mycolate outer membrane of *Corynebacterium glutamicum*. J Bacteriol 194:587–597

Marrakchi H, Laneelle G, Quemard A (2000) InhA, a target of the antituberculous drug isoniazid, is involved in a mycobacterial fatty acid elongation system, FAS-II. Microbiology 146:289–296

Marrakchi H, Choi KH, Rock CO (2002) A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*. J Biol Chem 277:44809–44816

Marrakchi H, Laneelle MA, Daffe M (2014) Mycolic acids: structures, biosynthesis, and beyond. Chem Biol 21:67–85

Mdluli K, Sherman DR, Hickey MJ, Kreiswirth BN, Morris S, Stover CK, Barry CE 3rd (1996) Biochemical and genetic data suggest that InhA is not the primary target for activated isoniazid in *Mycobacterium tuberculosis*. J Infect Dis 174:1085–1090

Mitchison DA (1998) Basic concepts in the chemotherapy of tuberculosis. In: Gangadharam PRJ, Jenkins PA (ed) Mycobacteria: II chemotherapy. Chapman & Hall Medical Microbiology Series, Springer US, pp 15–43

Molle V, Kremer L (2010) Division and cell envelope regulation by Ser/Thr phosphorylation: *Mycobacterium* shows the way. Mol Microbiol 75:1064–1077

Mondino S, Gago G, Gramajo H (2013) Transcriptional regulation of fatty acid biosynthesis in mycobacteria. Mol Microbiol 89:372–387

Moody DB, Reinhold BB, Guy MR, Beckman EM, Frederique DE, Furlong ST, Ye S, Reinhold VN, Sieling PA, Modlin RL, Besra GS, Porcelli SA (1997) Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. Science 278:283–286

Moody DB, Guy MR, Grant E, Cheng TY, Brenner MB, Besra GS, Porcelli SA (2000) CD1b-mediated T cell recognition of a glycolipid antigen generated from mycobacterial lipid and host carbohydrate during infection. J Exp Med 192:965–976

Nikiforov PO, Surade S, et al (2016) A fragment merging approach towards the development of small molecule inhibitors of Mycobacterium tuberculosis EthR for use as ethionamide boosters. Org Biomol Chem 14(7):2318–2326

North EJ, Jackson M, Lee RE (2014) New approaches to target the mycolic acid biosynthesis pathway for the development of tuberculosis therapeutics. Curr Pharm Des 20:4357–4378

Odriozola JM, Ramos JA, Bloch K (1977) Fatty acid synthetase activity in *Mycobacterium smegmatis*. Characterization of the acyl carrier protein-dependent elongating system. Biochim Biophys Acta 488:207–217

Oh TJ, Daniel J, Kim HJ, Sirakova TD, Kolattukudy PE (2006) Identification and characterization of Rv3281 as a novel subunit of a biotin-dependent acyl-CoA Carboxylase in *Mycobacterium tuberculosis* H37Rv. J Biol Chem 281:3899–3908

Ojha A, Anand M, Bhatt A, Kremer L, Jacobs WR Jr, Hatfull GF (2005) GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. Cell 123:861–873

Ojha AK, Baughn AD, Sambandan D, Hsu T, Trivelli X, Guerardel Y, Alahari A, Kremer L, Jacobs WR Jr, Hatfull GF (2008) Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. Mol Microbiol 69:164–174

Ojha AK, Trivelli X, Guerardel Y, Kremer L, Hatfull GF (2010) Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. J Biol Chem 285:17380–17389

Pacheco SA, Hsu FF, Powers KM, Purdy GE (2013) MmpL11 protein transports mycolic acid-containing lipids to the mycobacterial cell wall and contributes to biofilm formation in *Mycobacterium smegmatis*. J Biol Chem 288:24213–24222

Parish T, Roberts G, Laval F, Schaeffer M, Daffe M, Duncan K (2007) Functional complementation of the essential gene fabG1 of *Mycobacterium tuberculosis* by *Mycobacterium smegmatis* fabG, but not *Escherichia coli* fabG. J Bacteriol 189:3721–3728

Payne K, Sun Q, Sacchettini J, Hatfull GF (2009) Mycobacteriophage Lysin B is a novel mycolylarabinogalactan esterase. Mol Microbiol 73:367–381

Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, Daffe M, Emile JF, Marchou B, Cardona PJ, de Chastellier C, Altare F (2008) Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence. PLoS Pathog 4:e1000204

Phetsuksiri B, Jackson M, Scherman H, McNeil M, Besra GS, Baulard AR, Slayden RA, DeBarber AE, Barry CE 3rd, Baird MS, Crick DC, Brennan PJ (2003) Unique mechanism of action of the thiourea drug isoxyl on *Mycobacterium tuberculosis*. J Biol Chem 278:53123–53130

Portevin D, De Sousa-D'Auria C, Houssin C, Grimaldi C, Chami M, Daffe M, Guilhot C (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. Proc Natl Acad Sci U S A 101:314–319

Portevin D, de Sousa-D'Auria C, Montrozier H, Houssin C, Stella A, Laneelle MA, Bardou F, Guilhot C, Daffe M (2005) The acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth: identification of the carboxylation product and determination of the acyl-CoA carboxylase components. J Biol Chem 280:8862–8874

Puech V, Guilhot C, Perez E, Tropis M, Armitige LY, Gicquel B, Daffe M (2002) Evidence for a partial redundancy of the fibronectin-binding proteins for the transfer of mycoloyl residues onto the cell wall arabinogalactan termini of *Mycobacterium tuberculosis*. Mol Microbiol 44:1109–1122

Purwantini E, Mukhopadhyay B (2013) Rv0132c of *Mycobacterium tuberculosis* encodes a coenzyme F420-dependent hydroxymycolic acid dehydrogenase. PLoS One 8:e81985

Queiroz A, Medina-Cleghorn D, Marjanovic O, Nomura DK, Riley LW (2015) Comparative metabolic profiling of mce1 operon mutant vs wild-type *Mycobacterium tuberculosis* strains. Pathog Dis 73:ftv066

Quemard A (2016) New insights into the mycolate-containing compound biosynthesis and transport in mycobacteria. Trends Microbiol 24:725–738

Quemard A, Lacave C, Laneelle G (1991) Isoniazid inhibition of mycolic acid synthesis by cell extracts of sensitive and resistant strains of *Mycobacterium aurum*. Antimicrob Agents Chemother 35:1035–1039

Quemard A, Sacchettini JC, Dessen A, Vilcheze C, Bittman R, Jacobs WR Jr, Blanchard JS (1995) Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*. Biochemistry (Mosc) 34:8235–8241

Quémard A, Dessen A, Sugantino M, Jacobs WR Jr, Sacchettini JC, Blanchard JS (1996) Binding of catalase-peroxidase-activated isoniazid to wild-type and mutant *Mycobacterium tuberculosis* enoyl-ACP reductases. J Am Chem Soc 118:1561–1562

Qureshi N, Sathyamoorthy N, Takayama K (1984) Biosynthesis of C30 to C56 fatty acids by an extract of *Mycobacterium tuberculosis* H37Ra. J Bacteriol 157:46–52

Rafidinarivo E, Prome JC, Levy-Frebault V (1985) New kinds of unsaturated mycolic acids from *Mycobacterium fallax* sp. nov. Chem Phys Lipids 36:215–228

Ramesh R, Shingare RD, Kumar V, Anand ABS, Veeraraghavan S, Viswanadha S, Ummanni R, Gokhale R, Srinivasa Reddy D (2016) Repurposing of a drug scaffold: identification of novel sila analogues of rimonabant as potent antitubercular agents. Eur J Med Chem 122:723–730

Rao V, Gao F, Chen B, Jacobs WR Jr, Glickman MS (2006) Trans-cyclopropanation of mycolic acids on trehalose dimycolate suppresses *Mycobacterium tuberculosis* -induced inflammation and virulence. J Clin Invest 116:1660–1667

Rehm HJ, Reiff I (1981) Mechanisms and occurence of microbial oxidation of long-chain alkanes. Adv Biochem Eng 19:175–215

Rock CO, Cronan JE (1996) *Escherichia coli* as a model for the regulation of dissociable (type II) fatty acid biosynthesis. Biochim Biophys Acta 1302:1–16

Rombouts Y, Brust B, Ojha AK, Maes E, Coddeville B, Elass-Rochard E, Kremer L, Guerardel Y (2012) Exposure of mycobacteria to cell wall-inhibitory drugs decreases production of arabinoglycerolipid related to Mycolyl-arabinogalactan-peptidoglycan metabolism. J Biol Chem 287:11060–11069

Rozwarski DA, Grant GA, Barton DH, Jacobs WR Jr, Sacchettini JC (1998) Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. Science 279:98–102

Sacco E, Covarrubias AS, O'Hare HM, Carroll P, Eynard N, Jones TA, Parish T, Daffe M, Backbro K, Quemard A (2007) The missing piece of the type II fatty acid synthase system from *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 104:14628–14633

Salzman V, Mondino S, Sala C, Cole ST, Gago G, Gramajo H (2010) Transcriptional regulation of lipid homeostasis in mycobacteria. Mol Microbiol 78:64–77

Sambandan D, Dao DN, Weinrick BC, Vilcheze C, Gurcha SS, Ojha A, Kremer L, Besra GS, Hatfull GF, Jacobs WR Jr (2013) Keto-mycolic acid-dependent pellicle formation confers tolerance to drug-sensitive *Mycobacterium tuberculosis*. MBio 4:e00222–13

Sani M, Houben EN, Geurtsen J, Pierson J, de Punder K, van Zon M, Wever B, Piersma SR, Jimenez CR, Daffe M, Appelmelk BJ, Bitter W, van der Wel N, Peters PJ (2010) Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. PLoS Pathog 6:e1000794

Schaeffer ML, Agnihotri G, Volker C, Kallender H, Brennan PJ, Lonsdale JT (2001) Purification and biochemical characterization of the *Mycobacterium tuberculosis* beta-ketoacyl-acyl carrier protein synthases KasA and KasB. J Biol Chem 276:47029–47037

Singh A, Varela C, Bhatt K, Veerapen N, Lee OY, Wu HH, Besra GS, Minnikin DE, Fujiwara N, Teramoto K, Bhatt A (2016) Identification of a desaturase involved in mycolic acid biosynthesis in *Mycobacterium smegmatis*. PLoS One 11:e0164253

Sinha BK (1983) Enzymatic activation of hydrazine derivatives. A spin-trapping study. J Biol Chem 258:796–801

Slama N, Leiba J, Eynard N, Daffe M, Kremer L, Quemard A, Molle V (2011) Negative regulation by Ser/Thr phosphorylation of HadAB and HadBC dehydratases from *Mycobacterium tuberculosis* type II fatty acid synthase system. Biochem Biophys Res Commun 412:401–406

Slama N, Jamet S, Frigui W, Pawlik A, Bottai D, Laval F, Constant P, Lemassu A, Cam K, Daffe M, Brosch R, Eynard N, Quemard A (2016) The changes in mycolic acid structures caused by hadC mutation have a dramatic effect on the virulence of *Mycobacterium tuberculosis*. Mol Microbiol 99:794–807

Stanley SA, Kawate T, Iwase N, Shimizu M, Clatworthy AE, Kazyanskaya E, Sacchettini JC, Ioerger TR, Siddiqi NA, Minami S, Aquadro JA, Schmidt Grant S, Rubin EJ, Hung DT (2013) Diarylcoumarins inhibit mycolic acid biosynthesis and kill *Mycobacterium tuberculosis* by targeting FadD32. Proc Natl Acad Sci U S A 110:11565–11570

Stec J, Onajole OK, Lun S, Guo H, Merenbloom B, Vistoli G, Bishai WR, Kozikowski AP (2016) Indole-2-carboxamide-based MmpL3 inhibitors show exceptional antitubercular activity in an animal model of tuberculosis infection. J Med Chem 59:6232–6247

Tahlan K, Wilson R, Kastrinsky DB, Arora K, Nair V, Fischer E, Barnes SW, Walker JR, Alland D, Barry CE 3rd, Boshoff HI (2012) SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 56:1797–1809

Takayama K, Qureshi N (1978) Isolation and characterization of the monounsaturated long chain fatty acids of *Mycobacterium tuberculosis*. Lipids 13:575–579

Takayama K, Wang L, David HL (1972) Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2:29–35

Takayama K, Wang C, Besra GS (2005) Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. Clin Microbiol Rev 18:81–101

Tang X, Deng W, Xie J (2012) Novel insights into *Mycobacterium* antigen Ag85 biology and implications in countermeasures for *M. tuberculosis*. Crit Rev Eukaryot Gene Expr 22:179–187

Tarnok I, Rohrscheidt E (1976) Biochemical background of some enzymatic tests used for the differentiation of mycobacteria. Tubercle 57:145–150

Tomiyasu I, Yano I (1984) Separation and analysis of novel polyunsaturated mycolic acids from a psychrophilic, acid-fast bacterium, Gordona aurantiaca. Eur J Biochem 139:173–180

Toriyama S, Izaizumi S, Tomiyasu I, Masui M, Yano I (1982) Incorporation of ^{18}O into long-chain secondary alkohols derived from ester mycolic acids in *Mycobacterium phlei*. Biochim Biophys Acta 712:427–429

Trivedi OA, Arora P, Sridharan V, Tickoo R, Mohanty D, Gokhale RS (2004) Enzymic activation and transfer of fatty acids as acyl-adenylates in mycobacteria. Nature 428:441–445

Uchida Y, Casali N, White A, Morici L, Kendall LV, Riley LW (2007) Accelerated immunopathological response of mice infected with *Mycobacterium tuberculosis* disrupted in the mce1 operon negative transcriptional regulator. Cell Microbiol 9:1275–1283

Vander Beken S, Al Dulayymi JR, et al (2011) Molecular structure of the Mycobacterium tuberculosis virulence factor, mycolic acid, determines the elicited inflammatory pattern. Eur J Immunol 41(2):450–460

Varela C, Rittmann D, Singh A, Krumbach K, Bhatt K, Eggeling L, Besra GS, Bhatt A (2012) MmpL genes are associated with mycolic acid metabolism in mycobacteria and corynebacteria. Chem Biol 19:498–506

Vergne I, Daffe M (1998) Interaction of mycobacterial glycolipids with host cells. Front Biosci 3: d865–d876

Verschoor JA, Baird MS, Grooten J (2012) Towards understanding the functional diversity of cell wall mycolic acids of *Mycobacterium tuberculosis*. Prog Lipid Res 51:325–339

Veyron-Churlet R, Guerrini O, Mourey L, Daffe M, Zerbib D (2004) Protein-protein interactions within the Fatty Acid Synthase-II system of *Mycobacterium tuberculosis* are essential for mycobacterial viability. Mol Microbiol 54:1161–1172

Veyron-Churlet R, Bigot S, Guerrini O, Verdoux S, Malaga W, Daffe M, Zerbib D (2005) The biosynthesis of mycolic acids in *Mycobacterium tuberculosis* relies on multiple specialized elongation complexes interconnected by specific protein-protein interactions. J Mol Biol 353:847–858

Vilcheze C, Morbidoni HR, Weisbrod TR, Iwamoto H, Kuo M, Sacchettini JC, Jacobs WR Jr (2000) Inactivation of the inhA-encoded fatty acid synthase II (FASII) enoyl-acyl carrier protein reductase induces accumulation of the FASI end products and cell lysis of *Mycobacterium smegmatis*. J Bacteriol 182:4059–4067

Vilcheze C, Molle V, Carrere-Kremer S, Leiba J, Mourey L, Shenai S, Baronian G, Tufariello J, Hartman T, Veyron-Churlet R, Trivelli X, Tiwari S, Weinrick B, Alland D, Guerardel Y, Jacobs WR Jr, Kremer L (2014) Phosphorylation of KasB regulates virulence and acid-fastness in *Mycobacterium tuberculosis*. PLoS Pathog 10:e1004115

Villeneuve M, Kawai M, Kanashima H, Watanabe M, Minnikin DE, Nakahara H (2005) Temperature dependence of the Langmuir monolayer packing of mycolic acids from *Mycobacterium tuberculosis*. Biochim Biophys Acta 1715:71–80

Villeneuve M, Kawai M, Watanabe M, Aoyagi Y, Hitotsuyanagi Y, Takeya K, Gouda H, Hirono S, Minnikin DE, Nakahara H (2007) Conformational behavior of oxygenated mycobacterial mycolic acids from *Mycobacterium bovis* BCG. Biochim Biophys Acta 1768:1717–1726

Villeneuve M, Kawai M, Horiuchi K, Watanabe M, Aoyagi Y, Hitotsuyanagi Y, Takeya K, Gouda H, Hirono S, Minnikin DE (2013) Conformational folding of mycobacterial methoxy- and ketomycolic acids facilitated by alpha-methyl trans-cyclopropane groups rather than cis-cyclopropane units. Microbiology 159:2405–2415

Wang F, Langley R, Gulten G, Dover LG, Besra GS, Jacobs WR Jr, Sacchettini JC (2007) Mechanism of thioamide drug action against tuberculosis and leprosy. J Exp Med 204:73–78

Wang XM, Lu C, Soetaert K, S'Heeren C, Peirs P, Laneelle MA, Lefevre P, Bifani P, Content J, Daffe M, Huygen K, De Bruyn J, Wattiez R (2011) Biochemical and immunological characterization of a cpn60.1 knockout mutant of *Mycobacterium bovis* BCG. Microbiology 157:1205–1219

Warrier T, Tropis M, Werngren J, Diehl A, Gengenbacher M, Schlegel B, Schade M, Oschkinat H, Daffe M, Hoffner S, Eddine AN, Kaufmann SH (2012) Antigen 85C inhibition restricts *Mycobacterium tuberculosis* growth through disruption of cord factor biosynthesis. Antimicrob Agents Chemother 56:1735–1743

Watanabe M, Aoyagi Y, Ridell M, Minnikin DE (2001) Separation and characterization of individual mycolic acids in representative mycobacteria. Microbiology 147:1825–1837

Wehenkel A, Bellinzoni M, Grana M, Duran R, Villarino A, Fernandez P, Andre-Leroux G, England P, Takiff H, Cervenansky C, Cole ST, Alzari PM (2008) Mycobacterial Ser/Thr protein kinases and phosphatases: physiological roles and therapeutic potential. Biochim Biophys Acta 1784:193–202

Wilson R, Kumar P, Parashar V, Vilcheze C, Veyron-Churlet R, Freundlich JS, Barnes SW, Walker JR, Szymonifka MJ, Marchiano E, Shenai S, Colangeli R, Jacobs WR Jr, Neiditch MB, Kremer L, Alland D (2013) Antituberculosis thiophenes define a requirement for Pks13 in mycolic acid biosynthesis. Nat Chem Biol 9:499–506

Winder FG (1982) Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of the mycobacteria. In: Ratledge C, Stanford J (eds) The biology of the mycobacteria. Academic, London, pp 354–438

Wong MY, Gray GR (1979) Structures of the homologous series of monoalkene mycolic acids from *Mycobacterium smegmatis*. J Biol Chem 254:5741–5744

Yuan Y, Barry CE (1996) A common mechanism for the biosynthesis of methoxy and cyclopropyl mycolic acids in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 93:12828–12833

Yuan Y, Mead D, Schroeder BG, Zhu Y, Barry CE 3rd (1998) The biosynthesis of mycolic acids in *Mycobacterium tuberculosis*. Enzymatic methyl(ene) transfer to acyl carrier protein bound meromycolic acid in vitro. J Biol Chem 273:21282–21290

Zhang Y, Heym B, Allen B, Young D, Cole S (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 358:591–593

Zuber B, Chami M, Houssin C, Dubochet J, Griffiths G, Daffe M (2008) Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. J Bacteriol 190:5672–5680

Lipid Intermediates in Bacterial Peptidoglycan Biosynthesis

12

Hélène Barreteau, Didier Blanot, Dominique Mengin-Lecreulx, and Thierry Touzé

Contents

1 Introduction 218
2 Biosynthesis of Lipid I by MraY 220
3 Biosynthesis of Lipid II by MurG 222
4 Modifications of Lipid Intermediates 222
5 Flipping of Lipid II 224
6 Synthesis and Recycling of the Lipid Carrier 225
7 Antibacterials Targeting PG Lipid Intermediates and C_{55}-P (Re)generation 228
8 Research Needs 230
References 231

Abstract

Peptidoglycan constitutes one of the major "Achilles heels" of bacteria because it is an essential component for cell integrity, and its metabolism is the target for a great number of antibacterials of different natures, e.g., antibiotics such as β-lactams and vancomycin, host immune system antimicrobial peptides, and bacteriocins. Peptidoglycan synthesis requires the translocation, across the plasma membrane, of the polymer building block, a disaccharide-pentapeptide. This event is performed via the attachment of the subunit to a lipid carrier, undecaprenyl-phosphate. Lipid intermediates called lipids I and II are generated through the sequential transfer of *N*-acetylmuramoyl-pentapeptide and *N*-acetylglucosamine moieties from nucleotide precursors to the lipid carrier by MraY and MurG transglycosylases, respectively. The last membrane

H. Barreteau · D. Blanot · D. Mengin-Lecreulx · T. Touzé (✉)
Group Bacterial Cell Envelopes and Antibiotics, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris Sud, Université Paris-Saclay, Gif-sur-Yvette, France
e-mail: helene.barreteau@i2bc.paris-saclay.fr; didier.blanot@gmail.com; dominique.mengin-lecreulx@i2bc.paris-saclay.fr; thierry.touze@i2bc.paris-saclay.fr

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_11

intermediate, lipid II (undecaprenyl-pyrophosphate-*N*-acetylmuramoyl(-pentapeptide)-*N*-acetylglucosamine), can be further enzymatically modified through the addition of functional groups, amino acids, or peptides, before being flipped towards the outer leaflet of the plasma membrane where the final transfer of the peptidoglycan subunits to the growing polymer is catalyzed by penicillin-binding proteins. The integral membrane proteins FtsW, MurJ, and AmJ are thought to play a major role in the translocation process; however, the exact mechanism and the role of these molecular determinants is yet to be established. The lipid carrier is generated via a pathway involving two steps, first a polymerization reaction of isopentenyl-pyrophosphate catalyzed by the essential cytosoluble UppS enzyme, yielding undecaprenyl-pyrophosphate, followed by a dephosphorylation step ensured by a yet unknown enzyme. At each final transfer of a subunit to the elongating peptidoglycan, the lipid carrier is released in the pyrophosphate form, which is recycled to guarantee the high rate of polymer synthesis. Several integral membrane undecaprenyl-pyrophosphate phosphatases, from two distinct protein families and having their active site facing the extracytoplasmic side, have been identified, BacA and PAP2 enzymes. These enzymes can readily dephosphorylate the released lipid carrier precursor. Thereafter, the lipid is flipped back to the inner side of the membrane, by a yet unknown mechanism, in order to be reused as a glycan acceptor for a new round of peptidoglycan polymerization.

1 Introduction

Peptidoglycan (PG) is an essential component of the cell envelope of almost all bacteria. It is a complex heteropolymer composed of long glycan chains made up of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked by β1→4 bonds. Moreover, the glycan chains are cross-linked by short peptides attached to the D-lactoyl group of each MurNAc residue (Vollmer et al. 2008). Its main function is to preserve cell integrity by withstanding the inner osmotic pressure. It also contributes to the maintenance of a defined cell shape and is involved in the processes of cell elongation and division (den Blaauwen et al. 2008). Since the inhibition of its biosynthesis or its degradation generally causes cell lysis, PG constitutes an attractive target for the design of new antibacterial agents (Bugg et al. 2011). The biosynthesis of this polymer (Fig. 1a) is a three-stage process, which takes place in the cytoplasm (synthesis of nucleotide precursors), at the plasma membrane (synthesis of lipid intermediates and their translocation), and in the extracytoplasmic space (PG polymerization) (Lovering et al. 2012). The second stage can be summarized as follows: first, the 1-phospho-MurNAc-pentapeptide moiety from UDP-MurNAc-pentapeptide is transferred to the membrane polyprenol carrier undecaprenyl-phosphate (C_{55}-P), yielding lipid I. Thereafter, the addition of a GlcNAc residue from UDP-GlcNAc to lipid I leads to the formation of lipid II which in many bacteria, especially Gram-positive species, is enzymatically

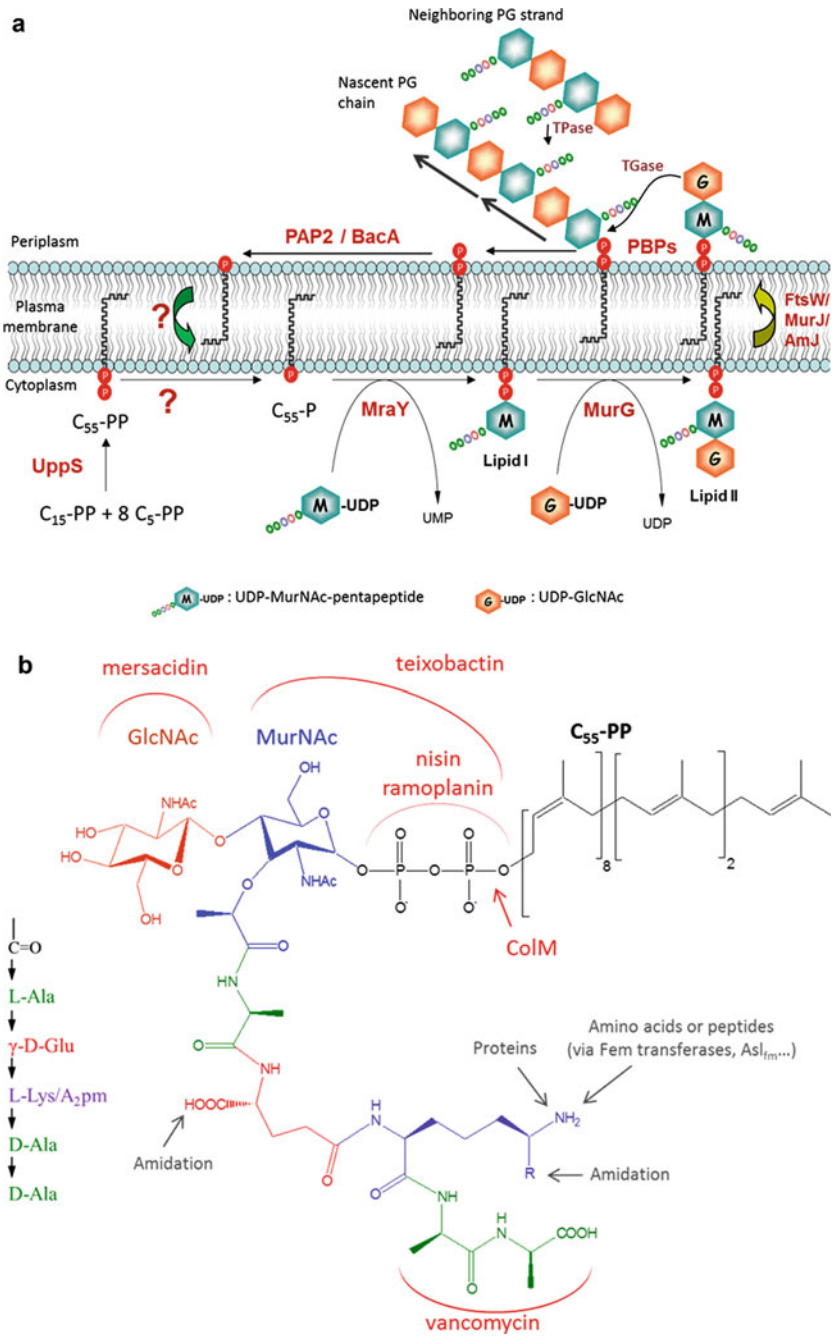


Fig. 1 (**a**) Schematic representation of PG biosynthesis and C_{55}-P metabolism. (**b**) Chemical structure of lipid II. Possible enzymatic modifications and antibacterial acting sites are indicated. TPase and TGase: transpeptidase and transglycosylase reactions, respectively. *A_2pm* diaminopimelic acid, *ColM* colicin M, *R* H (L-Lys) or COOH (A_2pm)

modified (Fig. 1b). The disaccharide-pentapeptide moiety is then translocated through the membrane to reach the outer face, where the transglycosylation (TGase) and transpeptidation (TPase) reactions (stage 3), catalyzed by penicillin-binding proteins (PBPs), take place. The TGase reaction releases the lipid carrier in the undecaprenyl pyrophosphate form (C_{55}-PP) that is actively recycled. The whole biosynthesis of PG has been the subject of several reviews (Barreteau et al. 2008; Bouhss et al. 2008; Sauvage et al. 2008; van Heijenoort 2010; Teo and Roper 2015). Here, we essentially focus on the metabolisms of the lipid I, lipid II, and C_{55}-P.

2 Biosynthesis of Lipid I by MraY

The formation of lipid I from UDP-MurNAc-pentapeptide and C_{55}-P is catalyzed by MraY, which belongs to the superfamily of polyprenyl phosphate *N*-acetylhexosamine 1-phosphate transferases (PNPT) (Price and Momany 2005), including enzymes involved in the biosynthesis of other cell surface polymers (WecA, TagO, etc.). MraY is an integral membrane enzyme with ten transmembrane segments and periplasmic N- and C-terminal ends (Bouhss et al. 1999). Cytoplasmic loops (especially the so-called loop E) are involved in substrate recognition and catalysis. Owing to its integral membrane nature, MraY proved to be difficult to purify. Nevertheless, orthologs from *Bacillus subtilis*, *Escherichia coli*, and some other species were purified using either suitable detergents or nanodisc-containing cell-free systems (Bouhss et al. 2004; Henrich et al. 2016). Crystal structures of MraY from *Aquifex aeolicus* have been solved as the apoenzyme (Fig. 2a) (Chung et al. 2013) or in complex with muraymycin D2 (MD2; Fig. 2b) (Chung et al. 2016), a naturally occurring ribosamino-uridine inhibitor which is competitive toward the nucleotide substrate (Tanino et al. 2011). Both the apoenzyme and the enzyme-inhibitor complex crystallized as a dimer with a hydrophobic tunnel at the dimer interface large enough to accommodate phospholipids. After binding MD2, MraY undergoes large conformational rearrangements near the active site, especially at the level of loop E (Fig. 2b). A two-step catalytic mechanism with an enzyme-phospho-MurNAc-pentapeptide covalent intermediate was first proposed from experiments carried out with crude membrane extracts (van Heijenoort 2010). However, recent data obtained with the pure enzyme are rather in favor of a one-step mechanism without covalent intermediate (Fig. 2c), consistent with a random bi-bi model (Al-Dabbagh et al. 2016; Liu et al. 2016). Site-directed mutagenesis and examination of the crystal structure helped identifying certain active site residues. Three aspartyl and one histidyl residues are particularly important for catalysis: D98, D99, D231, and H289 in *B. subtilis* and D117, D118, D265, and H324 in *A. aeolicus*. One of the aspartyl residues (D231/265) interacts with an essential Mg^{2+} ion which binds the pyrophosphate bridge of the nucleotide substrate (Chung et al. 2013). Another residue (D98/117) deprotonates a hydroxyl group of the lipid substrate prior to the nucleophilic attack of UDP-MurNAc-pentapeptide by C_{55}-P (Al-Dabbagh et al.

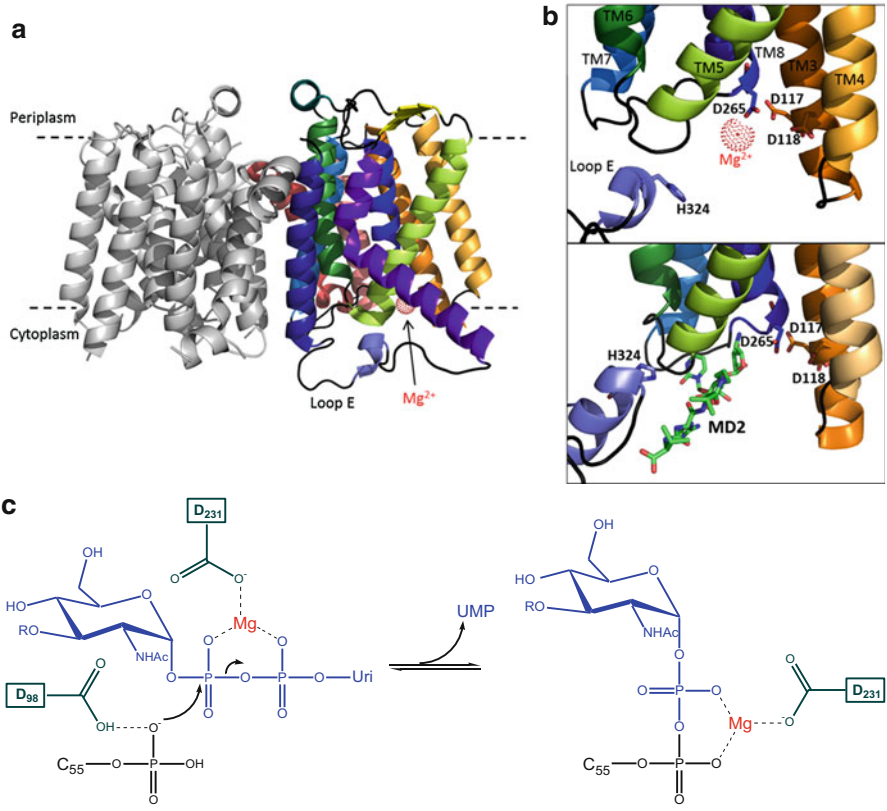


Fig. 2 (**a**) Structure of MraY from *A. aeolicus* (PDB entry, 4J72). Only one protomer is *colored*; the other one is shown in *grey*. Each transmembrane segment is colored from the *red* (N-terminus) to the *blue* (C-terminus). (**b**) Zoom at MraY active site in the apoenzyme (*up*) and in the complex with the MD2 inhibitor (*down*) (PDB entry, 5CKR). (**c**) Proposed one-step MraY reaction mechanism. The amino acid numbering is that of the *B. subtilis* sequence. *Uri* uridine, C_{55} undecaprenyl chain, *R* D-lactoyl-pentapeptide

2008). Surprisingly, MD2 interacts neither with the three aspartyl residues nor with the Mg^{2+} ion, suggesting that the mode of binding of this nucleoside antibiotic differs from that of UDP-MurNAc-pentapeptide (Chung et al. 2016). MraY is the target of many natural or synthetic nucleoside inhibitors belonging to the tunicamycin, ribosamino-uridine, uridylpeptide, and capuramycin classes (Dini 2005). However, owing to toxicity problems, none of them is of clinical use. MraY is also inhibited by the lysis protein E from bacteriophage ΦX174 (Zheng et al. 2009), as well as by cationic antimicrobial peptides containing the RWxxW motif (Bugg et al. 2016).

3 Biosynthesis of Lipid II by MurG

The formation of lipid II from lipid I and UDP-GlcNAc is catalyzed by MurG, which belongs to the glycosyltransferase B superfamily (Ünligil and Rini 2000). MurG is associated to the inner face of the plasma membrane (Bupp and van Heijenoort 1993). Its purification (Crouvoisier et al. 1999; Ha et al. 1999) and site-directed mutagenesis of invariant amino acids (Crouvoisier et al. 2007) have been reported for the *E. coli* enzyme, and the structures of the orthologs from *E. coli* and *Pseudomonas aeruginosa* have been solved (Brown et al. 2013; Ha et al. 2000; Hu et al. 2003). Crystal structures reveal that MurG contains two domains separated by a deep cleft (Fig. 3a). The C-terminal domain harbors the UDP-GlcNAc binding site, while lipid I is presumably bound by the N-terminal domain (Ha et al. 2000). The width of the cleft is reduced upon UDP-GlcNAc binding, the enzyme adopting a more closed conformation (Hu et al. 2003). MurG obeys an ordered bi-bi mechanism in which UDP-GlcNAc binds first (Chen et al. 2002). An as yet unidentified residue is believed to deprotonate the C4 hydroxyl group of the MurNAc moiety of lipid I, thereby generating an oxyanion which attacks the C1 of GlcNAc of the nucleotide substrate to form an oxycarbenium-ion-like transition state. Finally, the reaction results in the inversion of the anomeric configuration of GlcNAc in lipid II (Fig. 3b). MurG is inhibited by uridine-linked transition-state mimics (Trunkfield et al. 2010) as well as by compounds derived from the screening of chemical libraries (Hu et al. 2004). Recently, steroid-like compound murgocil was found to selectively hinder PG synthesis in *Staphylococci* through MurG inhibition (Mann et al. 2013).

4 Modifications of Lipid Intermediates

PG is subjected to many types of modifications leading to an important variability of its chemical structure within the bacterial world, which can be involved in resistance towards various antibacterial agents or modulate bacterial recognition by host immune systems. These modifications occur at various steps of its biosynthesis, but those undergone at the level of lipids I and II are especially amidation and extra amino acids or protein attachment (Fig. 1b). For example, the LtsA protein from *Corynebacterium glutamicum* is responsible for the amidation of PG *meso*-A_2pm residues (Levefaudes et al. 2015). LtsA catalyzes the transfer of an amino group from L-glutamine onto the carboxyl group of lipid II *meso*-A_2pm. Amidation can also occur on the glutamate residue of lipid II, as in *Staphylococcus aureus* where the glutamine amidotransferase-like protein GatD and the Mur ligase homolog MurT in concert catalyze the formation of D-isoglutamine at position 2 of the peptide stem (Figueiredo et al. 2012; Münch et al. 2012). PG is the point of covalent attachment of cell envelope proteins (Dramsi et al. 2008). In Gram-negative bacteria, Braun's lipoprotein seems to be the unique PG-linked protein (Braun and Rehn 1969). This 58 amino acid-long triacylated protein is anchored in the outer membrane. In *E. coli*, the side chain amine of the C-terminal lysine residue of Braun's lipoprotein is linked to the α-carboxyl group of the *meso*-A_2pm residue of lipid II, and the

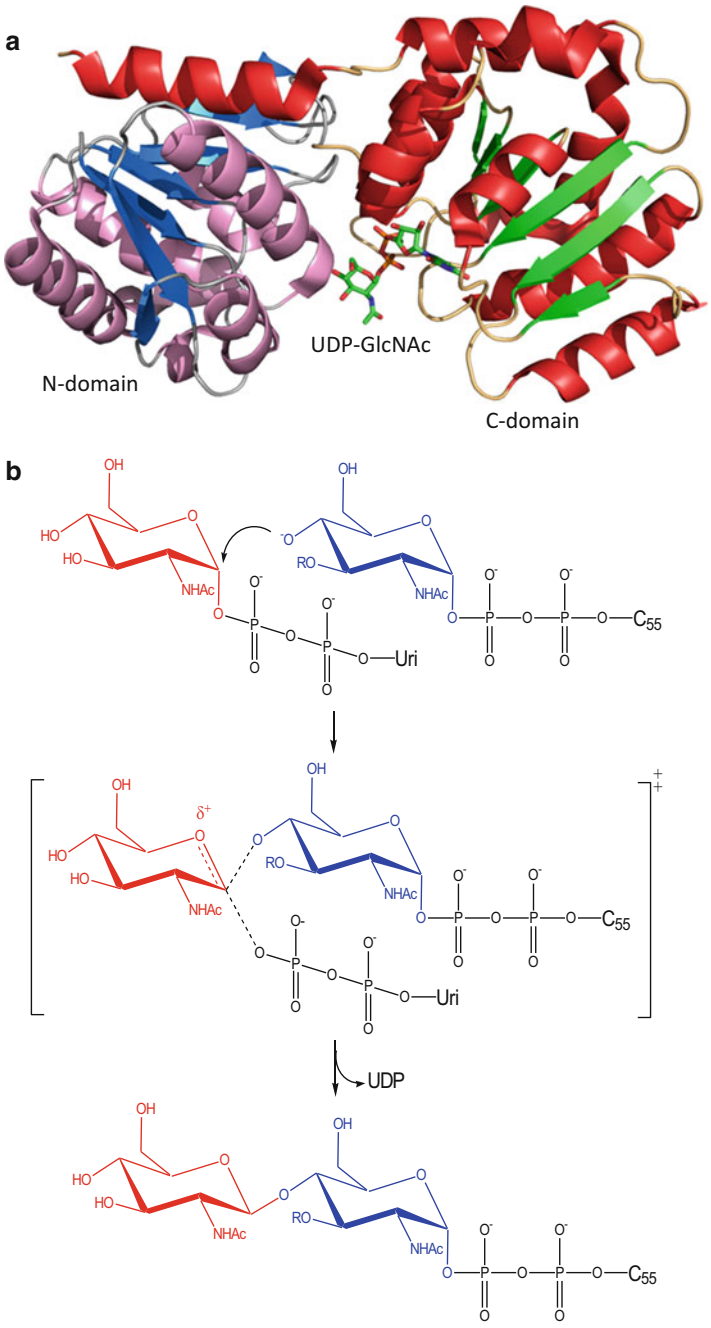


Fig. 3 (**a**) Structure of the *E. coli* MurG:UDP-GlcNAc complex (PDB entry, 1NLM). (**b**) Reaction mechanism of MurG. C_{55} undecaprenyl chain, *Uri* uridine, *R* D-lactoyl-pentapeptide

formation of this bond is catalyzed by one of the four L,D-transpeptidase activities identified in this species (Magnet et al. 2007). The major modifications of lipid II are performed in Gram-positive bacteria by different enzymes responsible for the addition of interpeptide bridges required for PG polymerization (transpeptidation) in these species. The Fem transferases synthesize interpeptide bridges from activated glycine or L-amino acids as aminoacyl-tRNAs (Mainardi et al. 2008). For instance, FemX, FemA, and FemB peptidyltransferases from *S. aureus* synthesize a penta-glycine interpeptide bridge by successive incorporations of a first glycine residue (FemX), the next two glycines (FemA), and finally the last two glycines (FemB) to the ε-amino group of L-Lys of lipid II peptide stem (Schneider et al. 2004). In *Enterococcus faecium*, the interpeptide bridge consists of a single D-amino acid (D-aspartic acid), the addition of which is performed by Asl_{fm}, an enzyme of the ATP-grasp superfamily; activation occurs through the formation of β-aspartyl phosphate in an ATP-dependent reaction (Bellais et al. 2006). The amino group of the interpeptide bridge of lipid II from Gram-positive bacteria is also the site of attachment of specific proteins. This covalent attachment is ensured by the sortase family of transpeptidases, which have been shown to be essential in pathogenesis (Bradshaw et al. 2015).

5 Flipping of Lipid II

Once synthesized and possibly modified, lipid II is translocated toward the outer leaflet of the plasma membrane to allow the transfer of the PG building block (disaccharide-peptide) to the growing polymer by the PBPs (TGase and TPase reactions) (Fig. 1a). The knowledge of the molecular determinant(s) and the mechanism of this flipping event represent the "Holy Grail" in this research field, and recent progresses have opened intense debates as several protein "flippase" candidates have arisen. The integral membrane FtsW protein and its orthologs (RodA, SpoVE) from the SEDS (shape, elongation, division, and sporulation) superfamily have long been considered as playing a central role in this process because of their presence in virtually all PG-containing bacteria, their essentiality, and their interaction with other PG biosynthesis enzymes in the so-called elongasome and divisome complexes (Boyle et al. 1997; Fraipont et al. 2011). Moreover, Mohammadi et al. have reported biochemical evidence for a flippase activity of FtsW after its reconstitution into lipid II-containing liposomes (Mohammadi et al. 2011). In their in vitro assays, a fluorescent 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) analogue of lipid II was used, which emphasized that the presence of FtsW in liposomes enhances the translocation of NBD-lipid II from one leaflet of the bilayer to the other, as shown by a sharp increase in fluorescence extinction with a fluorescence quencher. These data, supporting a direct role of FtsW in flipping lipid II, did not put an end to the story as MurJ (or MviN), another contender for this activity, was strengthened. The integral membrane protein MurJ, which is essential for PG biosynthesis in *E. coli*, belongs to the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily (Hvorup et al. 2003; Inoue et al. 2008; Ruiz 2008). Wzx, another member of this

superfamily, mediates, by an as yet unknown mechanism, the translocation of C_{55}-PP-linked intermediates in the biosynthesis pathways of enterobacterial common antigen (ECA), O-antigen from lipopolysaccharides (LPS), capsule, and other cell surface polymers (Islam and Lam 2013). Sham et al. recently designed an elegant in vivo assay to demonstrate the involvement of MurJ in lipid II translocation (Sham et al. 2014). They expressed in Δ*murJ E. coli* cells a functional cysteine-containing variant of MurJ which can be inhibited by a sulfhydryl-reactive reagent. Then, they showed that upon MurJ inhibition, the newly synthesized lipid II was no more accessible to the colicin M toxin, a lipid II-degrading enzyme acting exclusively at the periplasmic side of the membrane (see below), strongly suggesting an arrest of lipid II translocation in these conditions. The authors concluded that MurJ was the essential lipid II flippase and that other factors catalyzing this event in *E. coli* were therefore unlikely to exist. Nevertheless, the support of MurJ as a general lipid II flippase was hindered by the fact that MurJ was not essential in *B. subtilis* and that no flippase activity was observed in Mohammadi et al.'s in vitro assay in MurJ-containing liposomes (Fay and Dworkin 2009; Mohammadi et al. 2011). The nonessentiality of MurJ in *B. subtilis* let the MurJ's supporters to argue that an alternate lipid II flippase could exist in this bacterium. A search for such an ancillary translocase highlighted a synthetic lethal phenotype caused by the inactivation of MurJ together with AmJ (alternate to MurJ), another membrane protein with no sequence similarity with MurJ, FtsW, or other transporters (Meeske et al. 2015). Furthermore, AmJ from *B. subtilis* was able to support the growth of MurJ-inactivated *E. coli* cells, undoubtedly showing that MurJ and AmJ display a redundant function. As to whether FtsW and/or MurJ/AmJ are themselves part of the translocase or function as essential partners or regulators of the latter and the mechanism by which this event occurs remain largely to be deciphered, and this exciting debate seems to be far from being closed (Ruiz 2016).

6 Synthesis and Recycling of the Lipid Carrier

C_{55}-P is a general lipid carrier for cell surface polymers subunits, which are synthesized in the cytoplasm and must be transported throughout the plasma membrane (e.g., PG, teichoic acids, LPS O-antigen) (Manat et al. 2014). Therefore, a unique C_{55}-P pool must be shared by several metabolic pathways in a single cell, implying a fine-tuned synchronization in the synthesis of these various polymers. The (re)generation and delivery of this essential lipid should thus be tightly controlled. However, to date, little is known about these regulation processes. C_{55}-P originates from the dephosphorylation of its precursor, C_{55}-PP, itself being generated by de novo synthesis or released after each TGase reaction at the periplasmic side of the membrane (Fig. 1a). The de novo synthesis of C_{55}-PP is catalyzed by the essential cytosoluble UppS enzyme belonging to the *cis* prenyltransferase family (Teng and Liang 2012). UppS performs eight sequential condensations of the five-carbon building block, the homoallylic isopentenyl pyrophosphate substrate (C_5-PP), with the allylic 15-carbon-chain *trans*, *trans*-farnesyl pyrophosphate

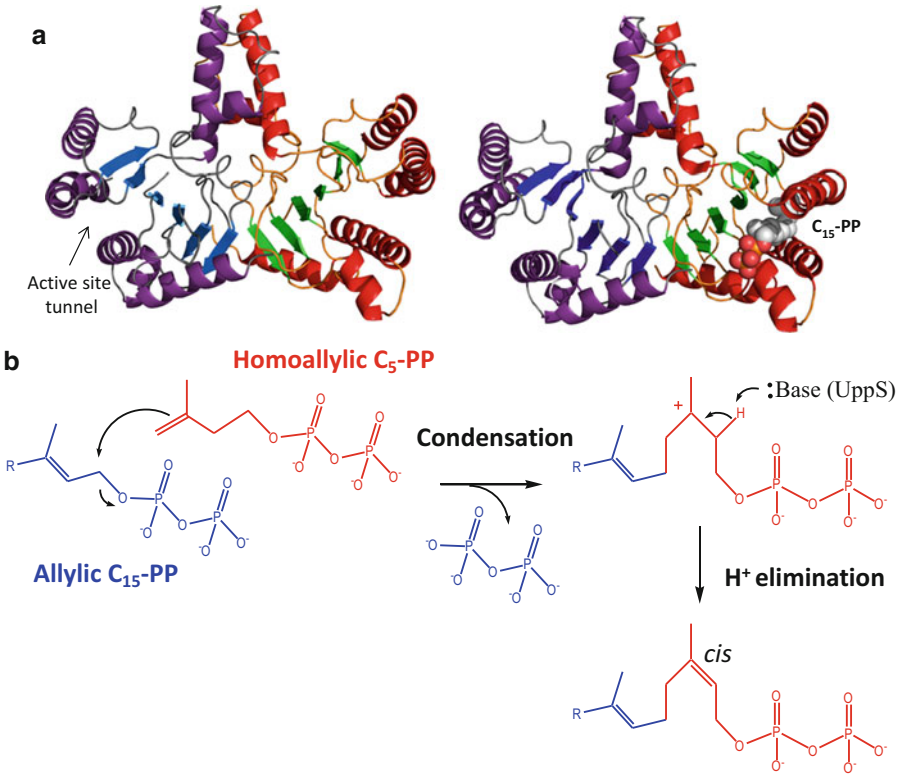


Fig. 4 (**a**) On the *left*, structure of UppS from *E. coli* in the apo-form (open conformation) (PDB entry, 1UEH). On the *right*, structure of UppS in complex with C_{15}-PP (closed conformation) (PDB entry, 1V7U). One monomer is represented with *purple* α-helices and *blue* β-strands and the other monomer is represented with *red* α-helices and *green* β-strands. (**b**) Mechanism of condensation by the *cis*-prenyltransferase UppS

(C_{15}-PP), yielding di-*trans*, octa-*cis*-C_{55}-PP. Several structures of UppS in the apo-form (Fujihashi et al. 2001) and in complex with C_{15}-PP, C_5-PP and the Mg^{2+} ion cofactor (Guo et al. 2005), together with several mutagenesis and kinetic studies (Teng and Liang 2012) have provided considerable knowledge of this catalytic process. As an essential enzyme, UppS is another potential target for novel antibacterials; therefore, UppS inhibitors are searched through different approaches (Jukič et al. 2016). UppS forms a dimer, with each protomer enclosing a deep hydrophobic cleft which accommodates the isoprenoid carbon tail during its elongation (Fig. 4a). UppS binds first the allylic C_{15}-PP substrate, triggering a conformational change that allows this binary complex to further bind C_5-PP, whose phosphate groups interact with an aspartyl residue from the active-site tunnel entrance (*E. coli* UppS D29 residue) via a magnesium bridge. A concerted mechanism was proposed, in which the release of the pyrophosphate group from the allylic substrate and the nucleophilic attack of C_{15}-PP C1-atom by C_5-PP C4-atom, ending in the formation of a new

double bond, occur simultaneously (i.e., no carbocation intermediate being formed) (Fig. 4b). The catalytic aspartyl D26 residue was then proposed to play a central role by controlling the migration of Mg^{2+} from C_5-PP to C_{15}-PP, where the metal ion may then facilitate the C_{15}-PP pyrophosphate group dissociation, concomitantly to the nucleophilic attack. The length of the final product is controlled by a molecular ruler mechanism, where bulky residues from the bottom of the tunnel block further condensation reaction via steric hindrance (Ko et al. 2001). Therefore, the newly synthesized C_{55}-PP pushes away a so-called entrance loop, which allows the relaxation of the active-site tunnel ready for a new cycle of synthesis.

The dephosphorylation of C_{55}-PP represents the ultimate step in the formation of the carrier lipid, which occurs in the course of de novo synthesis and also recycling (Fig. 1a). In contrast to the previous step that involves the single and essential UppS enzyme, the dephosphorylation of C_{55}-PP can be catalyzed by four different integral membrane enzymes in *E. coli* (El Ghachi et al. 2004, 2005). These enzymes belong to two distinct protein families: BacA, which constitutes a novel family of phosphatases present in a majority of bacteria, and three members of the ubiquitous PAP2 family (phosphatidic acid phosphatases of type 2) named PgpB, YbjG, and LpxT (formerly YeiU). Multiple genes inactivation is then required to elicit a lethal phenotype, raising the question of the significance of such a redundancy of enzymes for a single function. BacA requires a divalent metal ion for activity, with Ca^{2+} providing the highest activity as observed in vitro (Chang et al. 2014; Manat et al. 2015). BacA enzymes display two regions with a strong degree of conservation, which likely harbor active site residues. Mutagenesis studies have shown that BacA requires, especially, three amino acid residues belonging to these conserved regions (S24, E21, and R174) in order to display its activity both in vivo (functional complementation assay) and in vitro (kinetic analysis) (Manat et al. 2015). BacA membrane topology studies strongly suggested that these catalytic residues are facing the periplasm (Fig. 5). The PAP2 enzymes form a large family of integral membrane and soluble phosphatases with various substrates and physiological functions (Sigal et al. 2005). Interestingly, PgpB displays a dual function as being involved, in addition to C_{55}-P metabolism, in the synthesis of phosphatidylglycerol through phosphatidylglycerol phosphate dephosphorylation (Dillon et al. 1996).

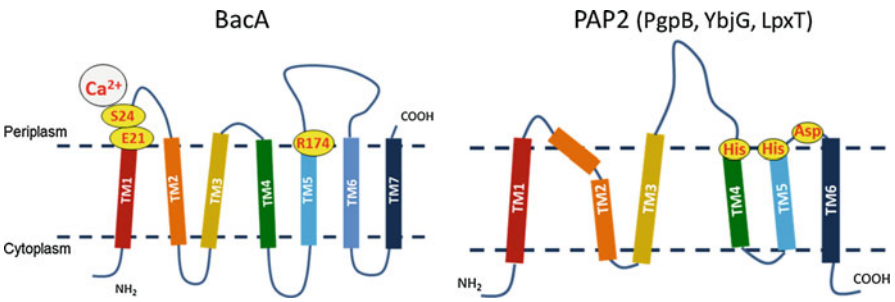


Fig. 5 Schematic representation of the C_{55}-PP phosphatases from *E. coli*

All PAP2 enzymes likely share a similar catalytic mechanism, which has been proposed to rely on a catalytic triad with one aspartyl and two histidyl residues. Based on biochemical and structural studies of different PAP2 enzymes, the catalysis should occur via a nucleophilic attack of the phosphate anhydride bond of C_{55}-PP by a catalytic histidine, leading to the formation of a phospho-histidine enzyme intermediate and the release of C_{55}-P (Ishikawa et al. 2000). The aspartyl residue may be involved in a charge relay system allowing this nucleophilic attack, while the additional histidine may protonate the leaving C_{55}-P product. In a second step, the phosphate group is released through its transfer to a water molecule, generating inorganic phosphate, or to another specific acceptor molecule. In such a way, LpxT enzyme catalyzes the transfer of a phosphate group from C_{55}-PP onto the lipid A moiety from LPS (Touzé et al. 2008a). Even though the physiological role of the resulting phosphorylated lipid A is yet unknown, LpxT takes part in a complex network of LPS modifications which are involved in bacterial adaptation to various conditions (Herrera et al. 2010; Kato et al. 2012). This unexpected phosphotransferase activity further suggests that other C_{55}-PP phosphatases may also exhibit similar activities with specific acceptor molecules (Manat et al. 2014). Interestingly, the active site residues of PAP2-type C_{55}-PP phosphatases are also oriented towards the periplasm, as demonstrated by several biochemical studies and recently confirmed by the 3D structure of PgpB (Fan et al. 2014; Tatar et al. 2007; Touzé et al. 2008b). The periplasmic orientation of the active sites of BacA and PAP2 suggests that these enzymes are rather involved in the recycling of C_{55}-PP. Indeed, the latter lipid is released at the outer leaflet of the plasma membrane as a product of TGase reactions, and it is therefore readily accessible to BacA and PAP2 enzymes. In contrast, it is unclear how the de novo synthesized C_{55}-PP, present at the inner leaflet after its synthesis by UppS, is dephosphorylated. As to whether a yet unidentified C_{55}-PP phosphatase with a cytoplasm-oriented active site exists or whether the C_{55}-PP flips toward the outer leaflet in order to be dephosphorylated needs further investigations. Another open issue concerns the mechanism by which the lipid carrier is translocated back to the inner leaflet, once dephosphorylated at the periplasmic side, in order to be reused as sugar acceptor for another cycle of PG (and other polymers) synthesis.

7 Antibacterials Targeting PG Lipid Intermediates and C_{55}-P (Re)generation

Lipid II is a well-validated drug target, especially in Gram-positive bacteria, where it is relatively well accessible from the outside due to the absence of an outer membrane. Many antibacterials bind lipid II in a noncovalent way. They are usually polar and of small size, and most of them are cyclic peptides, depsipeptides, or post-translationally modified peptides. Vancomycin is probably the most famous lipid II-targeting antibacterial, and it is also the only one of clinical use. This glycopeptide tightly binds to the D-Ala-D-Ala moiety of lipid II peptide stem, thereby inhibiting PG polymerization (TPase reactions) (Perkins

1969). The glycodepsipeptide ramoplanin, which is currently in the late stage of clinical development for the treatment of *Clostridium difficile* infections, has been known for a long time to inhibit TGase reactions. Recent studies have proposed ramoplanin to form membrane amphipathic symmetric dimers sequestrating lipid II, possibly at the level of its pyrophosphoryl moiety (Hamburger et al. 2009). Other antibacterials, such as a few lantibiotics and proteins from the colicin M family, target lipid II on various epitopes. Among the lantibiotics, which are gene-encoded peptides produced by some strains of bacilli (Willey and van der Donk 2007), the type-A nisin exhibits a dual mode of action by interfering with PG synthesis and also by disrupting the electric potential of the plasma membrane. Nisin uses the pyrophosphate moiety of lipid II as a docking site, which improves its efficiency to form pores in the membrane (Breukink and de Kruijff 2006). The type-B lantibiotic mersacidin is thought to bind to the GlcNAc moiety of lipid II, thereby inhibiting TGase reactions without pore formation (Brötz et al. 1998). Despite the fact that the molecular details of the latter interaction are yet to be resolved, an NMR study revealed conformational changes of mersacidin upon lipid II binding, suggesting a conformational adaptability of this class of antibiotic that might be central to their mode of action (Hsu et al. 2003). The colicin M protein and its orthologs display a unique phosphodiesterase enzymatic activity towards lipid II, releasing undecaprenol and pyrophospho-disaccharide-pentapeptide as dead end products, thus leading to cell death (El Ghachi et al. 2006). Unlike the other lipid II-targeting compounds, these proteins are produced by *Escherichia*, *Pseudomonas*, *Pectobacterium*, and *Burkholderia* genera and are active against a narrow range of related species (Barreteau et al. 2009; Grinter et al. 2012). This specificity is due to their mode of access to the periplasm of the target cell that relies on specific interactions with proteins used as receptors and translocation machineries (Cascales et al. 2007). In the search for new antibiotics, the exploitation of uncultured bacteria could be a fruitful approach. For example, the Gram-negative soil bacterium *Eleftheria terrae* produces a 1.2-kDa compound named teixobactin, which inhibits the growth of methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, and *Mycobacterium tuberculosis*, but is not active against Gram-negative bacteria (Ling et al. 2015). Even though it also interacts in vitro with lipid I and C_{55}-PP, the formal mechanism of action of teixobactin was shown to rely on an interaction with the pyrophosphoryl and MurNAc moieties of lipid II.

The (re)generation of the lipid carrier is also the target of antibacterial compounds. Bacitracin, a mixture of related cyclic peptides produced by some strains of *B. subtilis*, tightly binds to the pyrophosphoryl moiety of C_{55}-PP. The sequestration of C_{55}-PP prevents an efficient production of the active form of lipid carrier, C_{55}-P, thus inhibiting PG biosynthesis (Siewert and Strominger 1967; Stone and Strominger 1971). In *E. coli*, the overexpression of C_{55}-PP phosphatases-encoding genes was proven to overcome bacitracin cytotoxicity (El Ghachi et al. 2004). C_{55}-P was also shown to be the target of friulimycin, produced by *Actinoplanes friuliensis* (Schneider et al. 2009), and of laspartomycin C, produced by *Streptomyces viridochromogenes* (Kleijn et al. 2016). Both lipopeptides are branched with C_{14}

and C_{15} fatty acids, respectively, and they act by C_{55}-P sequestration, preventing lipid II formation.

8 Research Needs

The biosynthesis of PG lipid intermediates by the essential MraY and MurG enzymes is now well decrypted, numerous kinetic and structural studies providing good knowledge of these enzymatic steps. Nevertheless, substrate recognition and mechanistic details are further required to get an in-depth comprehension of these reactions, which requires higher resolution, substrate-liganded protein structures. This knowledge offers a framework for the study of mechanisms of action of yet identified inhibitors and the design or the high-throughput screening of novel molecules for therapeutic applications. The enzymatic modifications of lipid II, which are bacterial strain-specific, represent an important field of investigation. These modifications are either essential, such as the addition of peptide bridges in Gram-positive bacteria, or required for adaptation to certain environmental conditions (e.g., resistance towards PG-targeting antibacterials and modulation of host immune response). The physiological functions of these modifications, the enzymatic processes underlying these structural changes, and their regulation need more research efforts. The design of antibacterials directed against these enzymatic steps can be an interesting approach to target specific pathogens without disturbing the whole microbiota. As already mentioned, the mechanism of lipid II translocation across the plasma membrane is the subject of a passionate controversy with several contenders as the true flippase. Whether these different candidates participate in that process and the way the latter is conducted remain largely to be deciphered through more genetic, biochemical, and structural studies. It will be of particular interest to highlight the role of the isoprenoid lipid, which is a universal glycan carrier. It is conceivable that the particular structure of this lipid may play a decisive role in the translocation event. This step is considered as an attractive drug target because it may be readily accessible from the extracytoplasmic side of the membrane. The (re) generation of the lipid carrier constitutes another interesting field of research especially with recent findings in the function and properties of the multiple C_{55}-PP phosphatases. The emphasized LpxT phosphotransferase reaction suggests that other PAP2 enzymes may perform comparable reactions with specific acceptor molecules. The physiological functions of such reactions, including the LpxT-dependent lipid A modification, will have to be further addressed. The role of the multiplicity of C_{55}-PP phosphatases in a single bacterium, their regulation, and their mechanism of action will also have to be examined. The fact that all C_{55}-PP phosphatases yet identified exhibit a periplasm-oriented active site suggests their involvement in lipid carrier recycling, raising the question of the de novo synthesis pathway which requires a similar enzymatic reaction. Finally, the mechanism of translocation of C_{55}-P back to the inner leaflet of the plasma membrane, to be reused, is another open question.

References

Al-Dabbagh B, Henry X, El Ghachi M, Auger G, Blanot D, Parquet C, Mengin-Lecreulx D, Bouhss A (2008) Active site mapping of MraY, a member of the polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate transferase superfamily, catalyzing the first membrane step of peptidoglycan biosynthesis. Biochemistry 47:8919–8928

Al-Dabbagh B, Olatunji S, Crouvoisier M, El Ghachi M, Blanot D, Mengin-Lecreulx D, Bouhss A (2016) Catalytic mechanism of MraY and WecA, two paralogues of the polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate transferase superfamily. Biochimie 127:249–257

Barreteau H, Kovač A, Boniface A, Sova M, Gobec S, Blanot D (2008) Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol Rev 32:168–207

Barreteau H, Bouhss A, Fourgeaud M, Mainardi JL, Touzé T, Gérard F, Blanot D, Arthur M, Mengin-Lecreulx D (2009) Human- and plant-pathogenic *Pseudomonas* species produce bacteriocins exhibiting colicin M-like hydrolase activity towards peptidoglycan precursors. J Bacteriol 191:3657–3664

Bellais S, Arthur M, Dubost L, Hugonnet JE, Gutmann L, van Heijenoort J, Legrand R, Brouard JP, Rice L, Mainardi JL (2006) Asl_{fm}, the D-aspartate ligase responsible for the addition of D-aspartic acid onto the peptidoglycan precursor of *Enterococcus faecium*. J Biol Chem 281:11586–11594

den Blaauwen T, de Pedro MA, Nguyen-Distèche M, Ayala JA (2008) Morphogenesis of rod-shaped sacculi. FEMS Microbiol Rev 32:321–344

Bouhss A, Mengin-Lecreulx D, Le Beller D, van Heijenoort J (1999) Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. Mol Microbiol 34:576–585

Bouhss A, Crouvoisier M, Blanot D, Mengin-Lecreulx D (2004) Purification and characterization of the bacterial MraY translocase catalyzing the first membrane step of peptidoglycan biosynthesis. J Biol Chem 279:29974–29980

Bouhss A, Trunkfield AE, Bugg TD, Mengin-Lecreulx D (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiol Rev 32:208–233

Boyle DS, Khattar MM, Addinall SG, Lutkenhaus J, Donachie WD (1997) *ftsW* is an essential cell-division gene in *Escherichia coli*. Mol Microbiol 24:1263–1273

Bradshaw WJ, Davies AH, Chambers CJ, Roberts AK, Shone CC, Acharya KR (2015) Molecular features of the sortase enzyme family. FEBS J 282:2097–2114

Braun V, Rehn K (1969) Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *Escherichia coli* cell wall. The specific effect of trypsin on the membrane structure. Eur J Biochem 10:426–438

Breukink E, de Kruijff B (2006) Lipid II as a target for antibiotics. Nat Rev Drug Discov 5:321–332

Brötz H, Bierbaum G, Leopold K, Reynolds PE, Sahl HG (1998) The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrob Agents Chemother 42:154–160

Brown K, Vial SC, Dedi N, Westcott J, Scally S, Bugg TD, Charlton PA, Cheetham GM (2013) Crystal structure of the *Pseudomonas aeruginosa* MurG:UDP-GlcNAc substrate complex. Protein Pept Lett 20:1002–1008

Bugg TD, Braddick D, Dowson CG, Roper DI (2011) Bacterial cell wall assembly: still an attractive antibacterial target. Trends Biotechnol 29:167–173

Bugg TD, Rodolis MT, Mihalyi A, Jamshidi S (2016) Inhibition of phospho-MurNAc-pentapeptide translocase (MraY) by nucleoside natural product antibiotics, bacteriophage ΦX174 lysis protein E, and cationic antibacterial peptides. Bioorg Med Chem 24:6340–6347

Bupp K, van Heijenoort J (1993) The final step of peptidoglycan subunit assembly in *Escherichia coli* occurs in the cytoplasm. J Bacteriol 175:1841–1843

Cascales E, Buchanan SK, Duché D, Kleanthous C, Lloubès R, Postle K, Riley M, Slatin S, Cavard D (2007) Colicin biology. Microbiol Mol Biol Rev 71:158–229

Chang HY, Chou CC, Hsu MF, Wang AH (2014) Proposed carrier lipid-binding site of undecaprenyl pyrophosphate phosphatase from *Escherichia coli*. J Biol Chem 289:18719–18735

Chen L, Men H, Ha S, Ye XY, Brunner L, Hu Y, Walker S (2002) Intrinsic lipid preferences and kinetic mechanism of *Escherichia coli* MurG. Biochemistry 41:6824–6833

Chung BC, Zhao J, Gillespie RA, Kwon DY, Guan Z, Hong J, Zhou P, Lee SY (2013) Crystal structure of MraY, an essential membrane enzyme for bacterial cell wall synthesis. Science 341:1012–1016

Chung BC, Mashalidis EH, Tanino T, Kim M, Matsuda A, Hong J, Ichikawa S, Lee SY (2016) Structural insights into inhibition of lipid I production in bacterial cell wall synthesis. Nature 533:557–560

Crouvoisier M, Mengin-Lecreulx D, van Heijenoort J (1999) UDP-*N*-acetylglucosamine:*N*-acetylmuramoyl-(pentapeptide) pyrophosphoryl undecaprenol *N*-acetylglucosamine transferase from *Escherichia coli*: overproduction, solubilization, and purification. FEBS Lett 449:289–292

Crouvoisier M, Auger G, Blanot D, Mengin-Lecreulx D (2007) Role of the amino acid invariants in the active site of MurG as evaluated by site-directed mutagenesis. Biochimie 89:1498–1508

Dillon DA, Wu WI, Riedel B, Wissing JB, Dowhan W, Carman GM (1996) The *Escherichia coli pgpB* gene encodes for a diacylglycerol pyrophosphate phosphatase activity. J Biol Chem 271:30548–30553

Dini C (2005) MraY inhibitors as novel antibacterial agents. Curr Top Med Chem 5:1221–1236

Dramsi S, Magnet S, Davison S, Arthur M (2008) Covalent attachment of proteins to peptidoglycan. FEMS Microbiol Rev 32:307–320

El Ghachi M, Bouhss A, Blanot D, Mengin-Lecreulx D (2004) The *bacA* gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. J Biol Chem 279:30106–30113

El Ghachi M, Derbise A, Bouhss A, Mengin-Lecreulx D (2005) Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. J Biol Chem 280:18689–18695

El Ghachi M, Bouhss A, Barreteau H, Touzé T, Auger G, Blanot D, Mengin-Lecreulx D (2006) Colicin M exerts its bacteriolytic effect via enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors. J Biol Chem 281:22761–22772

Fan J, Jiang D, Zhao Y, Liu J, Zhang XC (2014) Crystal structure of lipid phosphatase *Escherichia coli* phosphatidylglycerophosphate phosphatase B. Proc Natl Acad Sci U S A 111:7636–7640

Fay A, Dworkin J (2009) *Bacillus subtilis* homologs of MviN (MurJ), the putative *Escherichia coli* lipid II flippase, are not essential for growth. J Bacteriol 191:6020–6028

Figueiredo TA, Sobral RG, Ludovice AM, Almeida JM, Bui NK, Vollmer W, de Lencastre H, Tomasz A (2012) Identification of genetic determinants and enzymes involved with the amidation of glutamic acid residues in the peptidoglycan of *Staphylococcus aureus*. PLoS Pathog 8:e1002508

Fraipont C, Alexeeva S, Wolf B, van der Ploeg R, Schloesser M, den Blaauwen T, Nguyen-Distèche M (2011) The integral membrane FtsW protein and peptidoglycan synthase PBP3 form a subcomplex in *Escherichia coli*. Microbiology 157:251–259

Fujihashi M, Zhang YW, Higuchi Y, Li XY, Koyama T, Miki K (2001) Crystal structure of *cis*-prenyl chain elongating enzyme, undecaprenyl diphosphate synthase. Proc Natl Acad Sci U S A 98:4337–4342

Grinter R, Milner J, Walker D (2012) Ferredoxin containing bacteriocins suggest a novel mechanism of iron uptake in *Pectobacterium* spp. PLoS ONE 7:e33033

Guo RT, Ko TP, Chen AP, Kuo CJ, Wang AH, Liang PH (2005) Crystal structures of undecaprenyl pyrophosphate synthase in complex with magnesium, isopentenyl pyrophosphate, and farnesyl thiopyrophosphate: roles of the metal ion and conserved residues in catalysis. J Biol Chem 280:20762–20774

Ha S, Chang E, Lo MC, Men H, Park P, Ge M, Walker S (1999) The kinetic characterization of *Escherichia coli* MurG using synthetic substrate analogues. J Am Chem Soc 121:8415–8426

Ha S, Walker D, Shi Y, Walker S (2000) The 1.9 Å crystal structure of *Escherichia coli* MurG, a membrane-associated glycosyltransferase involved in peptidoglycan biosynthesis. Protein Sci 9:1045–1052

Hamburger JB, Hoertz AJ, Lee A, Senturia RJ, McCafferty DG, Loll PJ (2009) A crystal structure of a dimer of the antibiotic ramoplanin illustrates membrane positioning and a potential lipid II docking interface. Proc Natl Acad Sci U S A 106:13759–13764

van Heijenoort J (2010) Lipid intermediates in bacterial peptidoglycan biosynthesis. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology, 1st edn. Springer, Berlin/Heidelberg, pp 435–444

Henrich E, Ma Y, Engels I, Münch D, Otten C, Schneider T, Henrichfreise B, Sahl HG, Dötsch V, Bernhard F (2016) Lipid requirements for the enzymatic activity of MraY translocases and *in vitro* reconstitution of the lipid II synthesis pathway. J Biol Chem 291:2535–2546

Herrera CM, Hankins JV, Trent MS (2010) Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. Mol Microbiol 76:1444–1460

Hsu ST, Breukink E, Bierbaum G, Sahl HG, de Kruijff B, Kaptein R, van Nuland NA, Bonvin AM (2003) NMR study of mersacidin and lipid II interaction in dodecylphosphocholine micelles: conformational changes are a key to antimicrobial activity. J Biol Chem 278:13110–13117

Hu Y, Chen L, Ha S, Gross B, Falcone B, Walker D, Mokhtarzadeh M, Walker S (2003) Crystal structure of the MurG:UDP-GlcNAc complex reveals common structural principles of a superfamily of glycosyltransferases. Proc Natl Acad Sci USA 100:845–849

Hu Y, Helm JS, Chen L, Ginsberg C, Gross B, Kraybill B, Tiyanont K, Fang X, Wu T, Walker S (2004) Identification of selective inhibitors for the glycosyltransferase MurG via high-throughput screening. Chem Biol 11:703–711

Hvorup RN, Winnen B, Chang AB, Jiang Y, Zhou XF, Saier MH Jr (2003) The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily. Eur J Biochem 270:799–813

Inoue A, Murata Y, Takahashi H, Tsuji N, Fujisaki S, Kato J (2008) Involvement of an essential gene, *mviN*, in murein synthesis in *Escherichia coli*. J Bacteriol 190:7298–7301

Ishikawa K, Mihara Y, Gondoh K, Suzuki E, Asano Y (2000) X-ray structures of a novel acid phosphatase from *Escherichia blattae* and its complex with the transition-state analog molybdate. EMBO J 19:2412–2423

Islam ST, Lam JS (2013) Wzx flippase-mediated membrane translocation of sugar polymer precursors in bacteria. Environ Microbiol 15:1001–1015

Jukič M, Rožman K, Gobec S (2016) Recent advances in the development of undecaprenyl pyrophosphate synthase inhibitors as potential antibacterials. Curr Med Chem 23:464–482

Kato A, Chen HD, Latifi T, Groisman EA (2012) Reciprocal control between a bacterium's regulatory system and the modification status of its lipopolysaccharide. Mol Cell 47:897–908

Kleijn LH, Oppedijk SF, 't Hart P, van Harten RM, Martin-Visscher LA, Kemmink J, Breukink E, Martin NI (2016) Total synthesis of laspartomycin C and characterization of its antibacterial mechanism of action. J Med Chem 59:3569–3574

Ko TP, Chen YK, Robinson H, Tsai PC, Gao YG, Chen AP, Wang AH, Liang PH (2001) Mechanism of product chain length determination and the role of a flexible loop in *Escherichia coli* undecaprenyl-pyrophosphate synthase catalysis. J Biol Chem 276:47474–47482

Levefaudes M, Patin D, de Sousa-d'Auria C, Chami M, Blanot D, Hervé M, Arthur M, Houssin C, Mengin-Lecreulx D (2015) Diaminopimelic acid amidation in Corynebacteriales: new insights into the role of LtsA in peptidoglycan modification. J Biol Chem 290:13079–13094

Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A, Schaberle TF, Hughes DE, Epstein S, Jones M, Lazarides L, Steadman VA, Cohen DR, Felix CR, Fetterman KA, Millett WP, Nitti AG, Zullo AM, Chen C, Lewis K (2015) A new antibiotic kills pathogens without detectable resistance. Nature 517:455–459

Liu Y, Rodrigues JP, Bonvin AM, Zaal EA, Berkers CR, Heger M, Gawarecka K, Swiezewska E, Breukink E, Egmond MR (2016) New insight into the catalytic mechanism of bacterial MraY from enzyme kinetics and docking studies. J Biol Chem 291:15057–15068

Lovering AL, Safadi SS, Strynadka NC (2012) Structural perspective of peptidoglycan biosynthesis and assembly. Annu Rev Biochem 81:451–478

Magnet S, Arbeloa A, Mainardi JL, Hugonnet JE, Fourgeaud M, Dubost L, Marie A, Delfosse V, Mayer C, Rice LB, Arthur M (2007) Specificity of L,D-transpeptidases from Gram-positive bacteria producing different peptidoglycan chemotypes. J Biol Chem 282:13151–13159

Mainardi JL, Villet R, Bugg TD, Mayer C, Arthur M (2008) Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. FEMS Microbiol Rev 32:386–408

Manat G, Roure S, Auger R, Bouhss A, Barreteau H, Mengin-Lecreulx D, Touzé T (2014) Deciphering the metabolism of undecaprenyl-phosphate: the bacterial cell-wall unit carrier at the membrane frontier. Microb Drug Resist 20:199–214

Manat G, El Ghachi M, Auger R, Baouche K, Olatunji S, Kerff F, Touzé T, Mengin-Lecreulx D, Bouhss A (2015) Membrane topology and biochemical characterization of the *Escherichia coli* BacA undecaprenyl-pyrophosphate phosphatase. PLoS ONE 10:e0142870

Mann PA, Müller A, Xiao L, Pereira PM, Yang C, Ho Lee S, Wang H, Trzeciak J, Schneeweis J, Dos Santos MM, Murgolo N, She X, Gill C, Balibar CJ, Labroli M, Su J, Flattery A, Sherborne B, Maier R, Tan CM, Black T, Onder K, Kargman S, Monsma FJ Jr, Pinho MG, Schneider T, Roemer T (2013) Murgocil is a highly bioactive staphylococcal-specific inhibitor of the peptidoglycan glycosyltransferase enzyme MurG. ACS Chem Biol 8:2442–2451

Meeske AJ, Sham LT, Kimsey H, Koo BM, Gross CA, Bernhardt TG, Rudner DZ (2015) MurJ and a novel lipid II flippase are required for cell wall biogenesis in *Bacillus subtilis*. Proc Natl Acad Sci USA 112:6437–6442

Mohammadi T, van Dam V, Sijbrandi R, Vernet T, Zapun A, Bouhss A, Diepeveen-de Bruin M, Nguyen-Distèche M, de Kruijff B, Breukink E (2011) Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. EMBO J 30:1425–1432

Münch D, Roemer T, Lee SH, Engeser M, Sahl HG, Schneider T (2012) Identification and *in vitro* analysis of the GatD/MurT enzyme-complex catalyzing lipid II amidation in *Staphylococcus aureus*. PLoS Pathog 8:e1002509

Perkins HR (1969) Specificity of combination between mucopeptide precursors and vancomycin or ristocetin. Biochem J 111:195–205

Price NP, Momany FA (2005) Modeling bacterial UDP-HexNAc: polyprenol-P HexNAc-1-P transferases. Glycobiology 15:29R–42R

Ruiz N (2008) Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. Proc Natl Acad Sci USA 105:15553–15557

Ruiz N (2016) Filling holes in peptidoglycan biogenesis of *Escherichia coli*. Curr Opin Microbiol 34:1–6

Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 32:234–258

Schneider T, Senn MM, Berger-Bächi B, Tossi A, Sahl HG, Wiedemann I (2004) *In vitro* assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of *Staphylococcus aureus*. Mol Microbiol 53:675–685

Schneider T, Gries K, Josten M, Wiedemann I, Pelzer S, Labischinski H, Sahl HG (2009) The lipopeptide antibiotic friulimicin B inhibits cell wall biosynthesis through complex formation with bactoprenol phosphate. Antimicrob Agents Chemother 53:1610–1618

Sham LT, Butler EK, Lebar MD, Kahne D, Bernhardt TG, Ruiz N (2014) Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. Science 345:220–222

Siewert G, Strominger JL (1967) Bacitracin: an inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in the biosynthesis of the peptidoglycan of bacterial cell walls. Proc Natl Acad Sci USA 57:767–773

Sigal YJ, McDermott MI, Morris AJ (2005) Integral membrane lipid phosphatases/phosphotransferases: common structure and diverse functions. Biochem J 387:281–293

Stone KJ, Strominger JL (1971) Mechanism of action of bacitracin: complexation with metal ion and C_{55}-isoprenyl pyrophosphate. Proc Natl Acad Sci USA 68:3223–3227

Tanino T, Al-Dabbagh B, Mengin-Lecreulx D, Bouhss A, Oyama H, Ichikawa S, Matsuda A (2011) Mechanistic analysis of muraymycin analogues: a guide to the design of MraY inhibitors. J Med Chem 54:8421–8439

Tatar LD, Marolda CL, Polischuk AN, van Leeuwen D, Valvano MA (2007) An *Escherichia coli* undecaprenyl-pyrophosphate phosphatase implicated in undecaprenyl phosphate recycling. Microbiology 153:2518–2529

Teng KH, Liang PH (2012) Undecaprenyl diphosphate synthase, a *cis*-prenyltransferase synthesizing lipid carrier for bacterial cell wall biosynthesis. Mol Membr Biol 29:267–273

Teo AC, Roper DI (2015) Core steps of membrane-bound peptidoglycan biosynthesis: recent advances, insight and opportunities. Antibiotics (Basel) 4:495–520

Touzé T, Blanot D, Mengin-Lecreulx D (2008a) Substrate specificity and membrane topology of *Escherichia coli* PgpB, an undecaprenyl pyrophosphate phosphatase. J Biol Chem 283:16573–16583

Touzé T, Tran AX, Hankins JV, Mengin-Lecreulx D, Trent MS (2008b) Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. Mol Microbiol 67:264–277

Trunkfield AE, Gurcha SS, Besra GS, Bugg TD (2010) Inhibition of *Escherichia coli* glycosyltransferase MurG and *Mycobacterium tuberculosis* Gal transferase by uridine-linked transition state mimics. Bioorg Med Chem 18:2651–2663

Ünligil UM, Rini JM (2000) Glycosyltransferase structure and mechanism. Curr Opin Struct Biol 10:510–517

Vollmer W, Blanot D, de Pedro MA (2008) Peptidoglycan structure and architecture. FEMS Microbiol Rev 32:149–167

Willey JM, van der Donk WA (2007) Lantibiotics: peptides of diverse structure and function. Annu Rev Microbiol 61:477–501

Zheng Y, Struck DK, Young R (2009) Purification and functional characterization of ΦX174 lysis protein E. Biochemistry 48:4999–5006

Marinobacter as a Model Organism for Wax Ester Accumulation in Bacteria

13

Carolann M. Knutson, Eric M. Lenneman, and Brett M. Barney

Contents

1 Introduction: Features of *Marinobacter* 238
2 Heterologous Expression and Utilization of Enzymes from *M. aquaeolei* VT8 239
3 Transcriptional Studies During Wax Ester Accumulation 242
4 Enzymatic Redundancy of Key Wax Ester Biosynthesis Genes 245
5 Fatty Acyl-CoAs and Fatty Acyl-ACPs, Intermediate Fatty Acids, and the Role of Thioesterases 246
6 Selectivity Versus Availability and the Implications in Biosynthetic Strategies 248
7 Posttranslational Regulation of Enzymes Involved in Wax Ester Biosynthesis 253
8 Research Needs 253
References 255

Abstract

Wax esters are derived from the esterification of a long-chain fatty alcohol with a fatty acid. Wax esters are a diverse type of neutral lipid that is utilized by all domains of life to serve a wide variety of functions. The model bacterium *Marinobacter aquaeolei VT8* has become an ideal model organism for studying wax ester biosynthesis, as it naturally accumulates wax esters in addition to having many other desirable features. While the pathway of wax ester biosynthesis has been mostly elucidated, there are still potential gaps in our understanding of how the two independent pathways of fatty acid biosynthesis and wax ester biosynthesis are linked. *M. aquaeolei* VT8 has also become a primary source of a number of key enzymes from the wax ester biosynthesis pathway that are either

C. M. Knutson · E. M. Lenneman · B. M. Barney (✉)
Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN, USA
e-mail: knuts623@umn.edu; emlenneman@gmail.com; bbarney@umn.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_19

studied in the laboratory for purposes of characterization or have been transferred to other model species for use in developing alternative biosynthetic routes to novel products. Studies of global transcriptional regulation during wax ester biosynthesis are also providing us with a view of how organisms that evolved to accumulate wax esters naturally could be used as a template to inform decisions as we attempt to move these pathways into foreign hosts. Understanding the in vivo flow of substrates through the wax ester biosynthesis from de novo fatty acid biosynthesis to the final wax ester product is a key requirement to improving biosynthetic approaches for producing wax esters.

1 Introduction: Features of *Marinobacter*

Marinobacter aquaeolei VT8 is a marine bacterium of the γ-proteobacteria class which was initially isolated from an oil-producing well on an offshore platform near the coast of Vietnam (Huu et al. 1999). Cosmopolitan in nature, *Marinobacter* strains have been found in a range of environments, but are generally associated with marine environments such as the oceans or brackish water. Specific *Marinobacter* strains have been studied due to their ability to both degrade long-chain hydrocarbons and accumulate intracellular wax esters when provided sufficient carbon under growth-limiting conditions (Gauthier et al. 1992; Huu et al. 1999; Singer et al. 2011). *Marinobacter* have also been studied for the production of unique biosurfactants (Nakano et al. 2012) and siderophores (Homann et al. 2009). Particular species of *Marinobacter* have been found to participate in unique associations with dinoflagellates and coccolithophores, including an association that seems linked to the siderophore vibrioferrin, which is susceptible to photolysis (Amin et al. 2009). In this association, it is suggested that *Marinobacter* produces this photosensitive siderophore to facilitate iron uptake, which is more readily taken up by the algae, while the algae provide organic compounds to support the bacterium, resulting in a mutualistic relationship. *Marinobacter adhaerens* can attach to the surface of certain diatoms and produces an exopolymer that helps to facilitate the formation of aggregates and the production of marine snow (Gardes et al. 2010). *Marinobacter* has also been found to be a dominant member of the bacterial communities associated with green algae (Lupette et al. 2016) and has been routinely found among bacterial communities associated with large oil spills (Kostka et al. 2011). They produce important enzymes which could be beneficial in industrial applications (Pérez et al. 2011; Shanmughapriya et al. 2010; Wahlen et al. 2009) and that have been shown to play an essential role in the function of certain microbial fuel cells and microbial solar cells (Strycharz-Glaven et al. 2013) which are linked to the ability of *Marinobacter* to oxidize iron (Bonis and Gralnick 2015; Strycharz-Glaven et al. 2013).

Our laboratory selected *M. aquaeolei* VT8 as a model system for the study of neutral lipid accumulation in a bacterium, following a comparison of several strains that contained key enzymes related to wax ester production. While some other bacteria can also accumulate neutral lipids, *M. aquaeolei* VT8 has several

advantageous features that have made it an attractive target to study. In *M. aquaeolei* VT8, wax esters can be synthesized from simple carbohydrates such as those constituting various intermediates of the citric acid cycle (Barney et al. 2012). It is also halotolerant and capable of growth at salt concentrations in excess of 3 M NaCl, which can be used to limit contamination without requiring antibiotics (Huu et al. 1999). *M. aquaeolei* VT8 is genetically tractable (Lenneman et al. 2013), and our laboratory has developed several plasmids for heterologous gene expression. While quite a few laboratories have sought to incorporate the genes from *M. aquaeolei* VT8 into heterologous hosts, we have focused our attention on understanding the processes that this bacterium uses to naturally accumulate wax esters using the internal pathways that evolved naturally to perform this function.

In this chapter, we will highlight a number of fascinating aspects of *Marinobacter* in relationship to wax ester biosynthesis. This will include:

- A survey of enzymes isolated from *M. aquaeolei* VT8 and incorporated into foreign biosynthetic schemes
- Important information gained from transcriptional studies that are shining an additional light on how the cell regulates or selects key enzymes that might have multiple pathways to a specific product
- How intracellular intermediates are transferred between fatty acid biosynthesis and wax ester biosynthesis
- Aspects related to selectivity and availability of many of these enzymes that have very broad substrate ranges

We will conclude by highlighting several features that should be a focus of future studies in this interesting model system.

2 Heterologous Expression and Utilization of Enzymes from *M. aquaeolei* VT8

M. aquaeolei VT8 has been an ideal source of proteins for enzymatic studies (Barney et al. 2012, 2013; Hofvander et al. 2011; Wahlen et al. 2009; Wei et al. 2013; Willis et al. 2011). Our laboratory initiated our work on *M. aquaeolei* VT8 by searching for an enzyme capable of reducing a fatty aldehyde to a fatty alcohol (Fig. 1), an enzyme which had *not yet* been identified in a bacterium and was viewed as a missing component of the bacterial wax ester biosynthesis pathway (Kalscheuer et al. 2006b). We also sought to isolate and characterize an enzyme with homology to a previously characterized fatty acyl-coenzyme A (acyl-CoA) reductase from *Acinetobacter* (Reiser and Somerville 1997). At the time, it was proposed that these two enzymes likely constituted a two-part pathway to produce fatty alcohols from the fatty acyl-CoA pool. We isolated the first enzyme from *M. aquaeolei* VT8 – Maqu_2220 – and confirmed that this enzyme had fatty aldehyde reductase activity (Wahlen et al. 2009). We also isolated the fatty acyl-CoA reductase (FACoAR) – Maqu_2507 – and determined that this enzyme performed the two-step reduction of

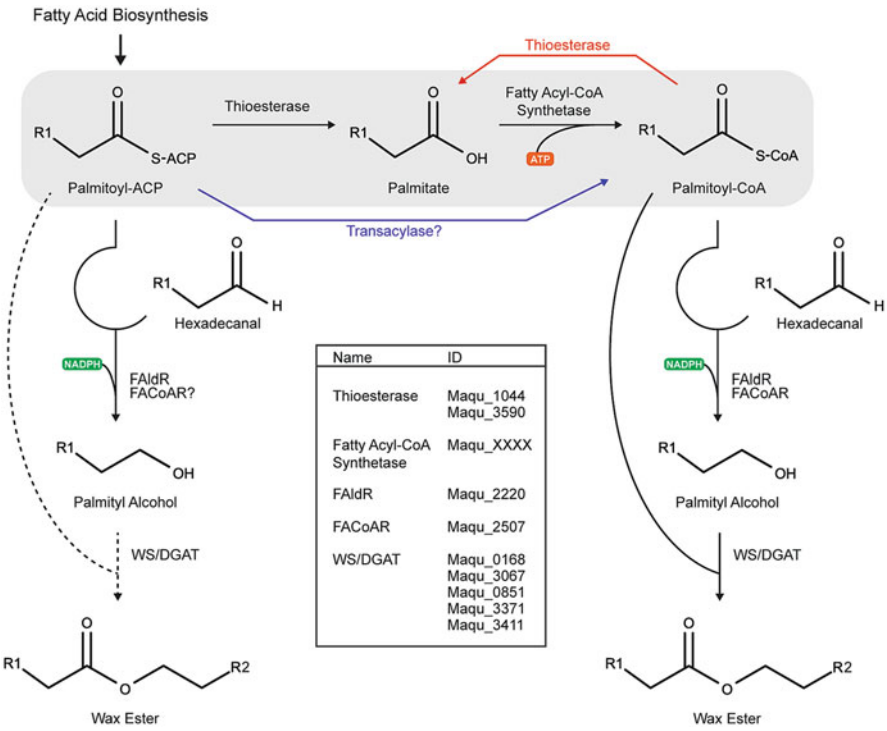


Fig. 1 Wax ester biosynthesis pathway of *Marinobacter aquaeolei* VT8 from fatty acid biosynthesis to the terminal wax ester product. Shown are several perspectives on how the wax ester biosynthesis pathway could be linked to fatty acid biosynthesis. Putative pathways that bridge these pathways include alternative reactions that have not been robustly demonstrated for the wax ester synthase (WS/DGAT) shown as *dashed lines*. A potential specific transacylase could shuttle substrates between these pathways (shown in *blue*). Reactions representing activities that have been characterized in vitro with purified enzymes are shown in *black*. Additional activities of specific thioesterases which would be detrimental and self-defeating are also shown (in *red*), as these would lead to a potential futile cycle that would simply consume energy. There are many potential genes from *M. aquaeolei* VT8 that could yield products which catalyze the reactions of fatty acyl-CoA synthetase, so this is still considered to *require further characterization*.

a fatty acyl-CoA all the way to a fatty alcohol and did not release a fatty aldehyde, as was described for the homologous enzyme from *Acinetobacter* (Reiser and Somerville 1997), indicating that FACoAR (Maqu_2507) alone was sufficient to reduce fatty acyl-CoA substrates to fatty alcohols. In addition to the reduction of fatty acyl-CoA, Maqu_2507 was also able to reduce fatty aldehydes independently to fatty alcohols, as was found for Maqu_2220 (Willis et al. 2011). A later report determined that Maqu_2220 could also reduce fatty acyl-CoA or fatty acyl-acyl carrier protein (ACP) to fatty alcohol, similar to our findings for Maqu_2507 (Hofvander et al. 2011; Willis et al. 2011). Finally, gene deletion studies in *M. aquaeolei* VT8 targeting the genes coding for both Maqu_2220 and Maqu_2507 demonstrated that either enzyme is capable of providing the indigenous fatty alcohols required

to support wax ester biosynthesis by themselves (Fig. 1) and that both genes had to be disrupted to dramatically decrease wax ester biosynthesis (Lenneman et al. 2013). The wax ester production phenotype was rescued in this double gene substituted strain by including exogenous fatty alcohols as a substrate in the growth, and the use of non-native fatty alcohols proved to be an effective tool for tracking the fates of these substrates into the wax ester pathway (Lenneman et al. 2013). The activity of both Maqu_2220 and Maqu_2507 was very high, with the fatty acyl-CoA substrate showing several orders of magnitude higher activity than what was reported for a similar enzyme obtained from *Acinetobacter* (Reiser and Somerville 1997; Willis et al. 2011). We further continued our studies of enzymes involved in wax ester biosynthesis by exploring a range of wax ester synthases from *M. aquaeolei* VT8 and several other strains (Barney et al. 2012). A comparison of five different wax ester synthases from four different species identified one wax ester synthase with a significantly higher level of activity than what was found for the other enzymes tested. Maqu_0168 from *M. aquaeolei* VT8 had sufficient activity to pursue mutagenesis studies and also resulted in a very pure protein following a two-stage affinity purification strategy that relied upon the fusion of a maltose-binding protein to improve the solubility of the wax ester synthase.

In addition to the three core classes of enzymes described above that are involved in wax ester biosynthesis, our group has also isolated a range of additional enzymes from this species that were able to be purified to homogeneity with high levels of activity when heterologously expressed in *Escherichia coli*. These enzymes include thioesterases, fatty aldehyde dehydrogenases, a medium alcohol dehydrogenase (Wei et al. 2013), leucine dehydrogenase, two potential acyl carrier proteins, and several additional enzymes. In most all of these cases, these enzymes have been isolated with very high activity and have been purified to near homogeneity in one- or two-step processes using affinity purification approaches.

Success in the cloning and heterologous expression of *M. aquaeolei* VT8 enzymes in *E. coli* demonstrates the potential application of this strain as a source of enzymes with biotechnological relevance. Many other homologs of the same enzymes from other species were either not successful, had little activity, or suffered from degradation during the purification. Others have sought to obtain enzymes from various halotolerant *Marinobacter* species previously (Pérez et al. 2011; Shanmughapriya et al. 2010), specifically because halophiles are often an ideal source of stable enzymes, and more than 15 crystal structures of proteins cloned or isolated from *M. aquaeolei* VT8 are currently deposited in the protein data bank (Berman et al. 2000; Vetting et al. 2015; Vorobiev et al. 2012). In addition to expressing and isolating *M. aquaeolei* VT8 enzymes for biochemical studies, efforts to utilize these enzymes in biosynthetic routes in other species have also had widespread success. The fatty aldehyde reductase and fatty acyl-ACP/CoA reductase (Maqu_2220) has been shown to be very effective at producing fatty alcohols in a wide variety of organisms (Liu et al. 2013; Wang et al. 2016; Yao et al. 2014). Recent efforts to produce wax esters have also begun to tap *Marinobacter*-derived enzymes for the wax ester synthase as well and have demonstrated the ability of these

enzymes to utilize a very broad range of substrates in vivo (Aslan et al. 2015; Shi et al. 2012; Teo et al. 2015).

3 Transcriptional Studies During Wax Ester Accumulation

The ability to study whole-cell gene regulation in a native wax-ester-accumulating organism offers a unique opportunity to evaluate how nature has evolved specific organisms to perform this biosynthetic process efficiently. A first step to pursuing such studies is to first establish tightly controlled conditions under which wax esters are accumulated and compare these with similar conditions where wax esters are not actively synthesized. The use of batch cultures that cycle through the various stages of wax production and degradation is an ideal platform for such studies, especially when coupled with RT-qPCR or RNA-Seq experiments. The formation of wax esters in neutral-lipid-accumulating bacteria can be induced by environmental conditions in which there is a high carbon to nitrogen ratio. It is believed that without nitrogen available for the production of amino acids and DNA, the organism is presented with conditions which limit replication (Barney et al. 2012; Wältermann et al. 2005). Under these conditions, the bacteria will start to sequester wax esters in insoluble lipid inclusions (Wältermann et al. 2005). These neutral lipid bodies are also thought to help with isolating the lipids from the aqueous portion of the cell, thus protecting other cellular components such as membranes from any disruption (Alvarez et al. 2002).

M. aquaeolei VT8 accumulates wax esters when grown on a simple defined medium containing organic acids such as citrate as a sole carbon source, and goes through a wax ester accumulation cycle consisting of three different stages relevant to wax ester biosynthesis: (1) exponential growth with no wax ester accumulation, (2) wax ester accumulation (anabolic processes), (3) and wax ester degradation (catabolic processes). Fig. 2a shows typical gas chromatography/flame ionization detection (GC-FID) chromatograms obtained from the extraction of dried *M. aquaeolei* VT8 cells as described previously (Barney et al. 2012; Lenneman et al. 2013).

During the exponential growth stage, *M. aquaeolei* VT8 cells achieve maximal growth with sufficient amounts of nitrogen to support replication until the time when all the available nitrogen in the media is consumed. If an excess of carbon is still available at this stage, the wax ester accumulation stage begins shortly after that, reaching a maximum quantity at about 100 h (in the experiment shown). Following this, the culture enters a final stage where wax esters in the cell begin to decline to very low levels after about 150 h. Results shown in Fig. 2b are arbitrarily fit to a Gaussian curve for the sake of visualization only and are not meant to indicate that the wax ester accumulation cycle behaves precisely in this manner. The important aspect of such a fit is that it allows us to separate each batch culture into three discreet growth stages. Comparisons between these stages then enable us to determine if specific genes are transcribed at elevated or diminished rates during each stage.

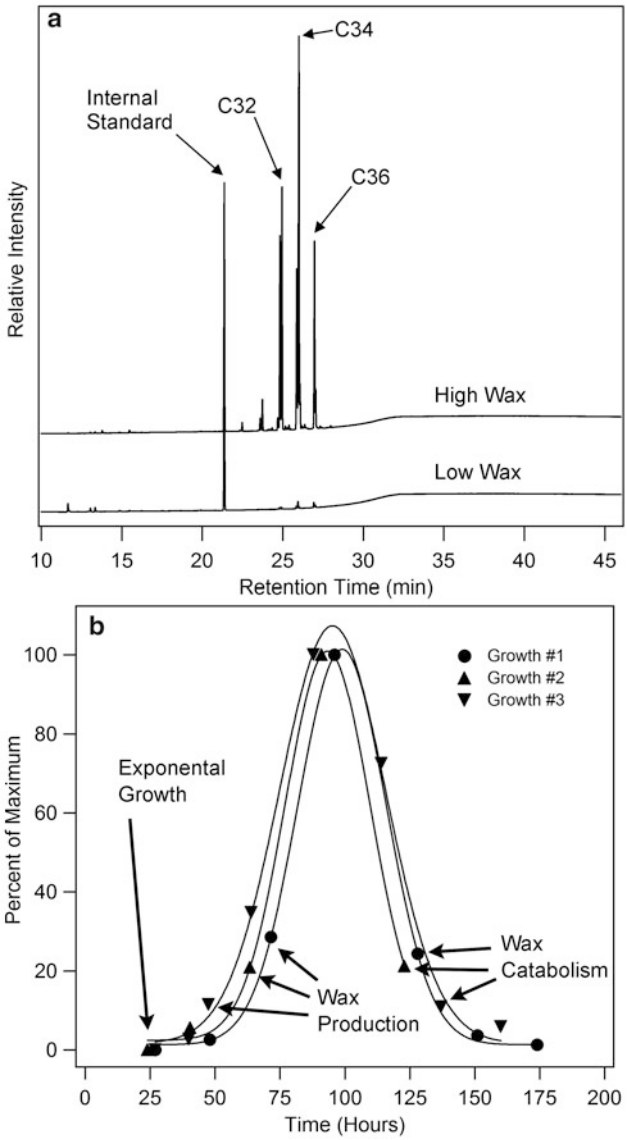


Fig. 2 Characteristic GC/FID chromatograms (**a**) and stages important to wax ester biosynthesis (**b**). The number of carbons atoms in each wax ester is indicated.

Based on the results obtained for transcriptional studies using both RT-qPCR and RNA-Seq approaches, we have been able to develop a picture of mRNA levels in *M. aquaeolei* VT8 under these various stages of growth (Lenneman 2013). These results allowed us to compare mRNA levels of the two potential enzymes capable of producing fatty alcohols (Lenneman et al. 2013), which was key to revealing that

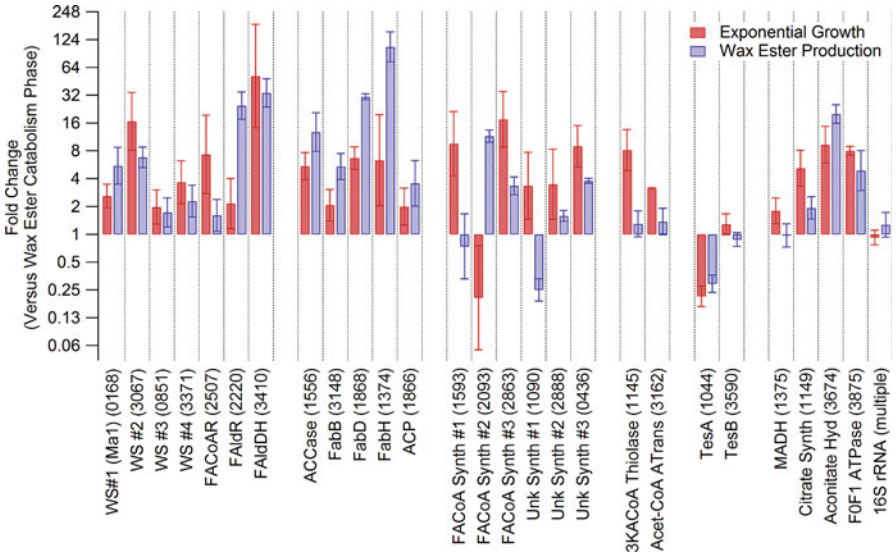


Fig. 3 Transcriptional analysis of wax ester synthesis genes compared to select genes from additional pathways. Results obtained as described in Lenneman (2013). Y-axis is plotted as a Log2 scale.

one of these genes seems to play a greater role in this natural process, even though either enzyme is capable of independently performing this role in *M. aquaeolei* VT8.

Specific genes were targeted in these initial studies to facilitate a better understanding of the proposed pathway and identify the enzymes involved in wax ester biosynthesis in *M. aquaeolei* VT8. This pathway has been described by others and ourselves (Barney et al. 2012; Manilla-Pérez et al. 2010; Wahlen et al. 2009; Wältermann et al. 2005, 2007; Willis et al. 2011), but details related to the transcription of these genes in *M. aquaeolei* VT8 had not been presented as a complete data set. Fig. 3 shows the initial genes targeted as part of these studies (Lenneman 2013), which include all of the genes believed to be essential to produce wax esters from the fatty acyl-CoA pool, as has been proposed by others (Stöveken and Steinbüchel 2008; Wältermann et al. 2005). Since the precursors for fatty acyl-CoAs in this study would have to be derived from de novo fatty acid synthesis, we also selected several genes from both fatty acid biosynthesis and fatty acid metabolism pathways, using the KEGG database as a primary template to identify specific genes in *M. aquaeolei* VT8. In addition, representative genes were selected from central metabolism that would be expected to participate in the citric acid cycle or additional housekeeping genes, and several genes were selected which we thought would be transcribed at constant levels in the cell, including the gene for recombinase A, which was utilized as the primary reference gene (Takle et al. 2007).

4 Enzymatic Redundancy of Key Wax Ester Biosynthesis Genes

An especially interesting facet of wax ester production in *M. aquaeolei* VT8 is tied to the amount of redundancy within this pathway. Many of the key genes involved in wax ester biosynthesis and accumulation in *M. aquaeolei* VT8 seem to have multiple closely related homologs or alternatives that are also able to catalyze the same reaction. This feature appears to be species specific, where model *Acinetobacter* and *Psychrobacter* strains contain only a single wax ester synthase, *M. aquaeolei* VT8 and strains of *Rhodococcus jostii* RHA1 have two or more of these genes present (Bakermans et al. 2006; Barbe et al. 2004; McLeod et al. 2006; Singer et al. 2011). *M. aquaeolei* VT8 contains at least four, and perhaps as many as five unique genes that share similarity to the initially characterized wax ester synthase gene from *Acinetobacter* (Kalscheuer and Steinbüchel 2003). Studies with isolated enzymes have confirmed activity for at least two of these wax ester synthases, while the other three have not been isolated in an active state (Barney et al. 2012; Holtzapple and Schmidt-Dannert 2007). Similar to the approach taken for fatty alcohol biosynthesis in *M. aquaeolei* VT8, transcriptional levels have been measured for genes coding these wax ester synthases from *M. aquaeolei* VT8 (Lenneman 2013). These results indicate that the wax ester synthases with confirmed in vitro activity (Maqu_0168 and Maqu_3067) show similar levels of elevated transcription during wax ester accumulation, though these levels are relatively minor (less than eightfold), and increases in transcription are significantly lower than what is seen for other target genes as part of this initial evaluation. The Maqu_0851 and Maqu_3371 wax ester synthases showed little change in transcription during this experiment (Fig. 3). However, while these genes have not been isolated in an active state when heterologously expressed in *Escherichia coli*, this does not rule out the possibility that these genes produce an active enzyme in the native host.

The genes involved in the production of fatty alcohols appear to trade off roles for the two growth states shown in Fig. 3. The FACoAR (Maqu_2507) is transcribed to a higher degree during exponential growth but drops during wax ester accumulation, while FAldR (Maqu_2220) does just the opposite, showing highest transcription during wax ester accumulation. In a general sense, the production of fatty alcohol seems to be more highly regulated, at the transcriptional level, than wax ester synthase. A similar connection might be drawn from posttranslational regulation of the wax ester synthase, which seems to be activated by high levels of fatty alcohol, but inhibited by elevated levels of fatty acyl-CoA (see below). In addition to the genes for the two enzymes capable of reducing fatty aldehydes to fatty alcohols (FACoAR and FAldR), another gene coding for a fatty aldehyde dehydrogenase, which would oxidize fatty aldehydes back to their corresponding fatty acids, was also found to be highly transcribed during both exponential growth and wax ester accumulation (Lenneman et al. 2013). This gene may play a role in assuring that toxic by-products of wax ester biosynthesis do not accumulate within the cell.

As expected, many of the genes selected due to their potential role in fatty acid biosynthesis (coding for the ACCase, FabB, FabD, FabH, and acyl carrier protein)

were seen to increase in transcription during wax ester accumulation (Fig. 3). Two genes that should play a role in degrading fatty acids (coding for 3-ketoacyl-CoA thiolase and acetyl-CoA acetyltransferase) showed a drop in transcription during wax ester accumulation. Genes involved in the citric acid cycle (coding for citrate synthase and aconitase) were both elevated during exponential growth, while aconitase was also highly transcribed during wax ester accumulation.

5 Fatty Acyl-CoAs and Fatty Acyl-ACPs, Intermediate Fatty Acids, and the Role of Thioesterases

The putative pathway for the production of wax esters often assumes that precursors are derived from a pool of available fatty acids within the cell. The wax ester biosynthetic pathway requires that these fatty acids are first activated to a fatty acyl-CoA substrate, which can then feed into the divergent pathway that would yield a fatty alcohol, which is then combined with a second fatty acyl-CoA substrate to synthesize the final wax ester product. This pathway is supported by in vitro studies with isolated enzymes that constitute this pathway (Fig. 1) (Barney et al. 2012; Hofvander et al. 2011; Kalscheuer and Steinbüchel 2003; Lenneman et al. 2013; Wahlen et al. 2009; Willis et al. 2011). This may be a feasible and logical pathway for catabolic, degradative processes resulting in the conversion of lipids obtained from the external environment into wax esters. However, in the indigenous route of *M. aquaeolei* VT8 growing on citrate as a sole carbon source, where the precursors of wax esters flow from the anabolic process of fatty acid biosynthesis, the requirement for fatty acyl-CoA substrates poses a potentially wasteful and unnecessary step. Specifically, the final step of fatty acid biosynthesis, following the last stage of elongation of the activated acid, would result in a long-chain fatty acyl-ACP, such as palmitoyl-ACP, which must be hydrolyzed to form the fatty acid (Fig. 1). Hydrolysis of palmitoyl-ACP is likely accomplished by an acyl-ACP hydrolase (3.1.2.2) or an alternative thioesterase with broad specificity that can act on both fatty acyl-CoA and fatty acyl-ACP substrates. Once this step is completed, the free acid would need to be reactivated to the fatty acyl-CoA substrate to again prime the fatty acid for use by the wax ester synthase. This step costs additional energy and would also release the activated fatty acid from the fatty acyl-ACP as an intermediate. In addition to shuttling the growing acyl group between the various enzymes of the fatty acid biosynthesis pathway, the ACP also assists in keeping this very hydrophobic substrate soluble. Thus, a pathway requiring that the activated substrate be hydrolyzed and then activated again, means that the hydrophobic substrate would be released during this intermediate state. Finally, the enzyme that hydrolyzed the fatty acyl-ACP would need to be very specific for this substrate and not act upon the new fatty acyl-CoA substrate formed by the fatty acyl-CoA synthetase following this process. This absolute requirement of a very specific thioesterase that acts upon fatty acyl-ACP but not fatty acyl-CoA presents a bit of a paradox.

There are several alternative routes that nature could follow to avoid the issues associated with wasting energy for a potentially futile step within the pathway linking fatty acid biosynthesis to wax ester biosynthesis or that would circumvent some of the problems listed above. The first would be to identify an enzyme functioning within the pathway that can transfer the fatty acid from the fatty acyl-ACP directly to free coenzyme A without requiring additional energy from the hydrolysis of ATP. Several examples can be found in the literature for an enzyme that can transfer the activated acid from CoA to ACP (Han et al. 1998; Hoffmann et al. 2002; Kervabon et al. 1977; Williamson and Wakil 1966), and for certain substrates, these reactions have been shown to be reversible (Williamson and Wakil 1966). In some of these cases, the activated acids are much smaller substrates (Han et al. 1998; Williamson and Wakil 1966), while others have been shown to utilize larger substrates (Hoffmann et al. 2002; Kervabon et al. 1977). The β-ketoacyl-acyl carrier protein synthase III (FabH) is one enzyme that demonstrates this acyl-coenzyme A (acyl-CoA):ACP transacylase activity in addition to the more prominent role it plays in fatty acid biosynthesis (Han et al. 1998). However, it is not clear how well FabH can catalyze this reaction for larger substrates, such as those required by *M. aquaeolei* VT8 for wax ester biosynthesis, and how reversible this reaction would be. While no direct evidence currently exists for the participation of FabH from *M. aquaeolei* VT8 in a transacylase reaction, the gene for this enzyme is among those with the highest transcriptional levels found during wax ester accumulation (Fig. 3), though this may be primarily attributed to the participation in early steps in fatty acid biosynthesis. Based on the dual roles that FabH would be playing within the cell, simple experiments to disrupt *fabH* within *M. aquaeolei* VT8 are doubtful to be successful, due to the importance of this pathway for cell growth.

A second alternative to overcome the ACP/CoA substrate requirement issue is to identify an enzyme that hydrolyzes only fatty acyl-ACP but has limited activity with fatty acyl-CoA substrates. This is perhaps the least favorable alternative, as it would result in the wasteful consumption of ATP energy as described above, and it seems more unlikely that an enzyme capable of hydrolyzing an acyl-ACP substrate would not also hydrolyze an acyl-CoA substrate, as the reaction and substrates would be so similar. Of the two potential thioesterases that have been targeted in other systems (Cho and Cronan 1995; Youngquist et al. 2013), little change in transcription was found for the genes coding the two most likely enzymes from *M. aquaeolei* VT8 that might catalyze this reaction, TesA and TesB (Fig. 3).

The final alternative would be for all of the enzymes that compose the wax ester pathway to utilize not only fatty acyl-CoA substrates but also fatty acyl-ACP substrates. This alternative has already been reported for the fatty aldehyde reductase (Maqu_2220) but has not yet been rigorously demonstrated in vitro for wax ester synthase. One primary question associated with this is whether heterologous sources of ACP are really suitable as substrates for wax ester synthase and other enzymes or whether the native ACPs from each organism might be required to test this requirement under the optimal conditions. Since the production of native ACPs requires several precarious steps and a significant amount of effort, many labs often utilize heterologous sources of ACPs, such as those obtained from *Escherichia coli* or even

higher plants, for use in assays to test for activity with these alternative substrates (Broadwater and Fox 1999; Hofvander et al. 2011; Kuo and Ohlrogge 1984; Shanklin 2000). Though the conservation between ACPs is reasonably high, there are still some substantial differences (conservation for the ACPs between *M. aquaeolei* VT8 and *E. coli* MG1655 is approximately 82% identical and 89% similar). This difference means that for one to be certain of substrate preferences for the wax ester biosynthetic enzymes, one would need to be certain to utilize the native ACP from the same host. Support for this alternative pathway where fatty acyl-ACP is the substrate for these enzymes can be found not only for certain enzymes obtained from *M. aquaeolei* VT8 but also for the acyltransferase reactions found in cyanobacteria, which have been shown to use fatty acyl-ACPs as the substrate to provide fatty aldehydes for the production of alkanes (Coursolle et al. 2015; Schirmer et al. 2010).

6 Selectivity Versus Availability and the Implications in Biosynthetic Strategies

As part of our efforts to better understand the biochemical features of the enzymes that constitute the wax ester biosynthesis pathway, we have developed assays to test for substrate specificity. In one particular assay, by limiting one of the two substrates for the wax ester synthase (the fatty acyl-CoA) while providing equimolar quantities of a variety of different substrates to serve as the second substrate (fatty alcohols differing in carbon chain length), we can generate a substrate preference profile for the non-limiting substrates (Barney et al. 2012, 2013, 2015). We have used these assays to provide a rapid glimpse of optimal substrate preferences of wax ester synthase enzymes and also to quickly scan mutations that we selected to probe for the residues which line a putative substrate binding site for alterations in the substrate selectivity profile. Our studies of specific residues that may play a role in the selectivity of the fatty alcohol active site of the wax ester synthase are important because they represent the first site-specific mutagenesis studies of *non-catalytic* site residues in this enzyme that can alter substrate profiles, and not just overall enzyme activity. Additionally, many of the sites we have identified are a significant distance – in relation to the primary protein sequence – from the catalytic residues that have been determined to play an important role in the activity of this enzyme (Barney et al. 2013, 2015; Stöveken et al. 2009). It should be noted that mutagenesis studies are limited for many of the key enzymes involved in wax ester biosynthesis, as there is not currently an available wax ester synthase structure to assist in the rational selection of residues for altered activity studies. For this reason, we are left to probe specific residues based on indirect evidence and suspected fold homology based on the structures of related enzymes, even though sequence conservation between these models is extremely poor (Barney et al. 2013).

A recent benefit of studying the enzymes involved in wax ester biosynthesis from *M. aquaeolei* VT8 is that many other groups are adopting these enzymes in their synthetic biology approaches to produce novel products in other organisms.

For this reason, a broad range of in vivo data is being reported based on applications with other organisms. These studies have resulted in some contradictory conclusions on the application of these enzymes. One laboratory has reported that they find no differences in the product profiles when utilizing non-native *M. aquaeolei* VT8 wax ester synthases in foreign hosts such as *Arabidopsis* in their in vivo studies when making comparisons to our results from in vitro studies using purified enzymes in the laboratory under tightly controlled conditions (Aslan et al. 2015). This lack of change in the specific products has been cited as evidence that wax ester synthase enzymes do not have similar activities in vivo to what is found in vitro. It could be argued that these findings are a misinterpretation of the in vivo results. While these enzymes do demonstrate significant activity with alternative substrates in the laboratory, as reported by ourselves and in many other reports from other laboratories (Barney et al. 2012; Kalscheuer et al. 2006a; Stöveken and Steinbüchel 2008; Teo et al. 2015), efficient production of alternative products in any model organism requires not only an enzyme with the ability – or improved ability – to catalyze that reaction but also the availability of those substrates produced by upstream processes within the cell. This has been demonstrated previously by providing the cell with extraneous substrates for a specific reaction (Lenneman et al. 2013) or by unintentionally providing a substrate such as butanol (Kalscheuer et al. 2006b) or by incorporating additional non-native pathways to produce substrates such as ethanol (Kalscheuer et al. 2006a) or isoamyl alcohol (Teo et al. 2015). Our analysis when providing isoamyl alcohol shows that this substrate is effective at producing isoamyl esters in *M. aquaeolei* VT8 and has been a target substrate for direct studies of residues altering selectivity toward this substrate in the wax ester synthase (Barney et al. 2015). Thus, any evaluation of the potential of a heterologous enzyme in a non-native host requires not only a measure of the products under normal growth conditions but also a measure of differences when alternative substrates are provided to those enzymes in a state and at levels that would be sufficient to yield the expected products.

In the model organism *M. aquaeolei* VT8, it is possible to supplement the medium provided to this bacterium with extraneously supplied alcohols, similar to what has been done inadvertently in *E. coli* (Kalscheuer et al. 2006b). Examples in which *M. aquaeolei* VT8 was grown under wax ester-accumulating conditions and provided with fatty alcohols such as pentadecanol (Lenneman et al. 2013) or isoamyl alcohol (Barney et al. 2015) have demonstrated that the wax ester product profiles in these strains can be altered quite significantly by including non-native medium and fatty alcohols. It is important to note that this production of novel wax esters within *M. aquaeolei* VT8 requires not only substrate availability to the wax ester synthase but also relies on the efficient transport of these exogenous alcohols into the cell from the extracellular space. In addition to the need to transfer these alcohols across the membrane and into the cell, these substrates could also be targeted by other enzymes within the cell to be used as either a carbon source to meet the energy needs of the organism or to flow into other elements of the wax ester biosynthesis pathway.

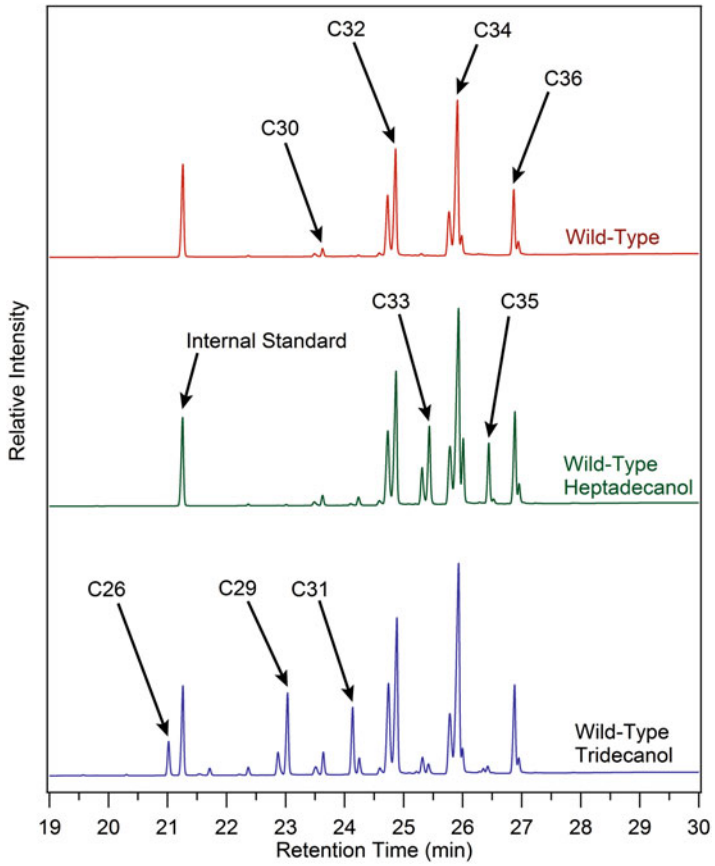


Fig. 4 GC/FID chromatogram of wax esters obtained from *Marinobacter aquaeolei* VT8 when the exogenous alcohols heptadecanol or tridecanol were provided to the culture during wax ester accumulation. Extractions and analysis performed as described in Barney et al. (2012) and Lenneman et al. (2013).

As shown in Fig. 4, *M. aquaeolei* VT8 was grown using citrate as the sole carbon source under conditions that induce the accumulation of wax esters within the cell, yielding a typical wax ester profile (Barney et al. 2012). The predominant fatty acids found within *M. aquaeolei* VT8 wax esters are C16, C16:1, and C18:1. A similar chain length is found for the fatty alcohols, as these are believed to be derived from the same pool as the activated fatty acids. Thus, the overall size distribution of wax esters is made up of C32 (derived from two C16 components with differences in the degree of saturation), C34 (derived from one C16 and one C18 component with differences in the degree of saturation), and C36 (derived from two C18 components, where the predominant member would be C18:1 in both cases (Barney et al. 2012)). The presence of odd numbered fatty acids or alcohols is very minimal in *M. aquaeolei* VT8. This fact enables us to provide odd numbered fatty alcohols to

the growth medium and track these substrates as they are incorporated into the wax esters. Thus, the addition of C17 fatty alcohol (heptadecanol) results in three new peaks, one C35 peak (derived from the C17 fatty alcohol and the predominant C18:1 fatty acid) and two C33 peaks (derived from the C17 fatty alcohol and either the C16 or C16:1 peaks derived from the two dominant forms of C16 acids found in *M. aquaeolei* VT8). The relative amounts of these are similar to what is found for the indigenous wax esters that are also produced under these conditions, indicating that transport of C17 fatty alcohol is sufficient to produce this non-native wax ester. A similar experiment was performed using C13 fatty alcohol, which is more similar to the fatty alcohols that we find to be the most favorable in vitro substrate for our model wax ester synthase (Maqu_0168 (Barney et al. 2012)), and illustrates that inclusion of this fatty alcohol also yields a shifted wax ester profile containing the expected C29 and C31 wax esters in addition to the native wax esters. An additional peak found at C26 indicates that some of the fatty alcohol is being converted to a C13 fatty acid to feed into the wax ester biosynthesis pathway and produce this new wax ester when combined with the C13 fatty alcohol. A similar feature was seen previously when adding C15 fatty alcohol (Lenneman et al. 2013). Additional peaks found at C33 indicate that some of the fatty alcohol is also being directed into fatty acid biosynthesis pathways, resulting in the addition of two carbon units to produce other odd numbered fatty acids or fatty alcohols from this feedstock. Importantly, these experiments all indicate that while the wax ester synthase and the fatty acyl-CoA and fatty aldehyde reductases (or fatty acyl reductases, FARs) are capable of producing a broad range of different products (Barney et al. 2012; Wahlen et al. 2009; Willis et al. 2011), the indigenous products found within the cell are primarily determined by the available substrates and provided to these downstream enzymes by the upstream processes of fatty acid biosynthesis.

A final benefit of using the selectivity assays we have developed is related to the ability to scan a large range of substrates very rapidly and provide a substrate profile in a single experiment. Trying to determine these sometimes subtle differences, or even screen for major changes using a series of specific activity assays with differing substrates, would be extremely tedious. The use of our selectivity assays provides a rapid and highly reproducible result (Fig. 5). To further assist in our enzymatic characterizations of many of these key enzymes in the wax ester biosynthesis pathway, we also employ the use of a maltose-binding protein fusion, which is used to improve the solubility of these enzymes (Kapust and Waugh 1999). While we have found the maltose-binding protein to be an indispensable asset in our purifications, this alteration of the solubility of these enzymes could also be of concern, especially with regard to the typical substrates, which can be quite insoluble. As a demonstration of the consistency of the selectivity protocols we have described previously (Barney et al. 2012, 2015) and also to confirm that the inclusion of the maltose-binding protein fusion is not altering these results versus alternative approaches, we have also constructed fusions using the small metal-binding protein (SmbP), another protein with a very high solubility that also behaves as a natural his-tagged fusion, related to the formation of a "histidine zipper" that arises for a repeating motif found in this protein (Barney et al. 2004).

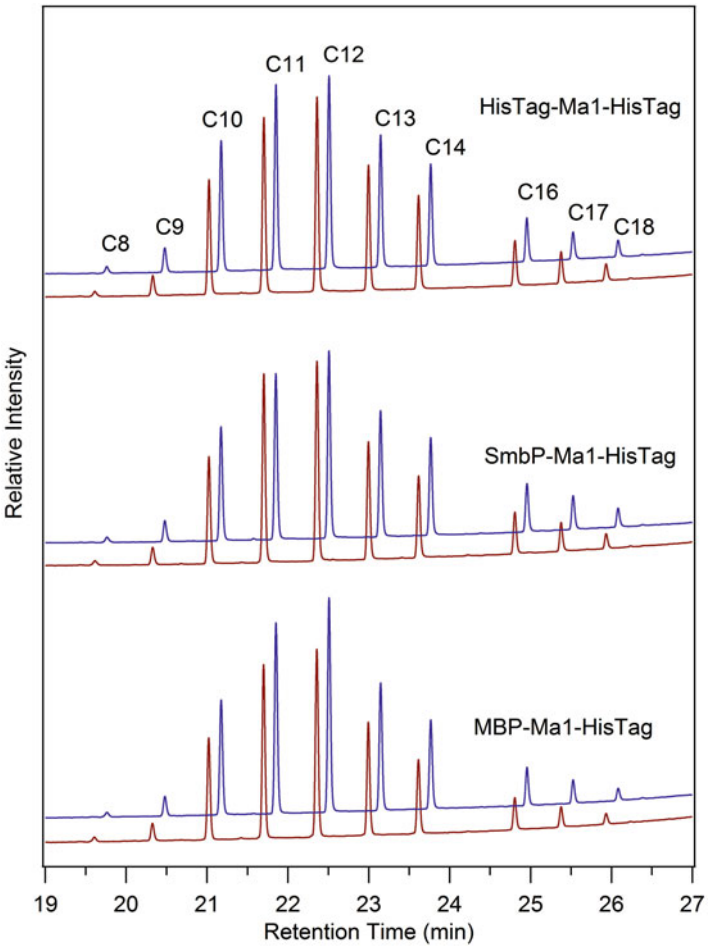


Fig. 5 GC/FID chromatograms of three different forms of the Ma1 (Maqu_0168) wax ester synthase incubated with palmitoyl-CoA and a range of fatty alcohol substrates. Each assay is shown as duplicates with the second sample offset by 10 s to show assay reproducibility. Assays were run as described in Barney et al. (2012).

Additionally, we have also isolated a fusion of the Maqu_0168 wax ester synthase containing conventional his-tags on both the N-terminus and the C-terminus. All three enzymes were subjected to the same selectivity assay to determine if the addition of the solubility fusions had altered the substrate profile. As demonstrated in Fig. 5, each of the assays yields a very similar and very reproducible result. The robustness of this assay is one of the principal benefits of this technique that has allowed us to pursue mutagenesis studies with these model enzymes and identify specific residues that might govern access to these sites in the process.

7 Posttranslational Regulation of Enzymes Involved in Wax Ester Biosynthesis

Many of the enzymes constituting the wax ester biosynthesis pathway in *M. aquaeolei* VT8 have revealed interesting activity profiles in vitro. For instance, the activity found for the wax ester synthase Ma1 (Maqu_0168) was highly susceptible to the order in which substrates were added to the reaction (Barney et al. 2012). If the wax ester synthase was first incubated with palmitoyl-CoA before the addition of the fatty alcohol, then the activity decreased by nearly tenfold to what was obtained if the enzyme was first incubated with the fatty alcohol. Subjecting the enzyme to both substrates at the same time (by adding the enzyme last) resulted in an intermediate activity. The implications of this finding could shed some light on the importance of substrate pools in the cell, as low intracellular levels of fatty alcohols could severely limit the activity of this enzyme if a similar trend was seen in vivo. Assays with the FACoAR (Maqu_2507) revealed a strong substrate inhibition pattern for palmitoyl-CoA, with maximal activity found for this enzyme in the low μM concentration range, but quickly dropping in activity if the substrate concentration provided in the assay was greater than 10 μM. This inhibition would subside once the enzyme had converted sufficient levels of the substrate to bring the substrate levels back down to the low μM range. It is important to note that this feature may simply be related to the critical micelle concentration of this substrate, which could fall in a similar range (Constantinides and Steim 1985). However, it is also possible that this is not an artifact, and is actually related to substrate inhibition, as this result is only seen for FACoAR, while studies using the wax ester synthase (Maqu_0168) with similar concentrations of palmitoyl-CoA as a substrate did not suffer from inhibition at elevated levels of this substrate. Taken together, these results seem to indicate that cellular levels of substrates might be very tightly regulated, and attempts to modify the regulation of these genes in this or other species could be problematic if a specific intermediate was to accumulate within the cell. Such potential problems should be considered by anyone attempting to produce specific products using biosynthetic schemes that require any of these enzymes.

8 Research Needs

Enzymatic Characterization While quite a bit of effort has been put into biosynthetic strategies that utilize many of the key enzymes that constitute the wax ester biosynthetic pathway from various wax-ester-accumulating bacteria, there remains a significant lack of detailed biochemical characterization of these enzymes. The enzymes obtained from *M. aquaeolei* VT8 represent an opportunity to extend our knowledge of these enzymes as they are quite amenable to further characterization and are easy to purify and quite stable. Mutagenesis studies have already revealed that these enzymes could be altered to improve their characteristics for specific applications, which could continue to be a fruitful line of research into the future. These enzymes are also ideal for studies related to posttranslational regulation, as

several exhibit interesting features of either activation or inhibition based on substrate levels in vitro.

A Structural Basis for Rational Experimentation Perhaps the greatest contribution to the studies of the enzymes that result in wax ester production in bacteria would be the addition of high-resolution structures of these enzymes. Such information would allow more precise targeting of residues within these proteins that could be altered to change their substrate profiles (Barney et al. 2013, 2015) and allow us to determine why some enzymes catalyze one-step reductions of fatty acyl-CoA to a fatty aldehyde, while others are able to perform the second step as well and reduce the aldehyde to the fatty alcohol (Hofvander et al. 2011; Reiser and Somerville 1997; Willis et al. 2011). To date, attempts to crystallize these enzymes have not been successful but are being actively pursued by several laboratories.

Use of Native Acyl Carrier Proteins for In Vitro Studies Successful studies of the wax ester biosynthesis pathway require a careful comparison of all potential substrates for these enzymes. Since fatty acyl-ACP is among the most logical of substrates for these enzymes, more focus and efforts directed toward using native ACPs derived from the same host as the enzymes that utilize these substrates would be an important pursuit moving forward. Since so many of these enzymes have been isolated from *M. aquaeolei* VT8, both successfully and with high activity, the isolation of ACP from *M. aquaeolei* VT8 is among the top priorities.

Utilizing RNA-Seq to Identify Genes that Play a Role in Wax Biosynthesis Several experiments have already provided a wealth of information related to key genes that are playing a role in the production of wax esters in *M. aquaeolei* VT8. Further, more complex studies that identify not only the genes involved in the anabolic biosynthesis of the wax esters but also look at the catabolic utilization of these wax esters for energy could also inform potential efforts to increase yields or alter product profiles. These studies could then be linked to gene-targeting studies to increase the production of genes that improve synthesis, while deleting or diminishing the transcription of genes that degrade the wax esters.

Expanding Perspectives of Marinobacter As more reports accumulate in the primary literature related to the fascinating roles of various species of *Marinobacter* and their importance in a broad range of environmental processes, it is clear that the community of researchers that study these organisms need to expand their interactions to take on a more global view (Singer et al. 2011). Indeed our own laboratory has focused primarily on wax ester biosynthesis, even while our group has additional interests in the roles of siderophores within the environment, which is also a fascinating aspect of potential mutualistic relationships found within certain species of *Marinobacter* (Amin et al. 2009; Homann et al. 2009; Villa et al. 2014). Developing a tighter community of researchers focused on *Marinobacter* would be potentially beneficial to all of those who work with this organism.

Acknowledgments This work was supported by grants from the National Science Foundation to B.M.B. (Award Numbers 0968781 and CBET-1437758). Further support was provided through generous start-up funds through the University of Minnesota.

References

Alvarez HM, Luftmann H, Silva RA, Cesari AC, Viale A, Wältermann M, Steinbüchel A (2002) Identification of phenyldecanoic acid as a constituent of triacylglycerols and wax ester produced by *Rhodococcus opacus* PD630. Microbiology 148:1407–1412

Amin SA, Green DH, Hart MC, Küpper FC, Sunda WG, Carrano CJ (2009) Photolysis of iron-siderophore chelates promotes bacterial-algal mutualism. Proc Natl Acad Sci U S A 106:17071–17076

Aslan S, Hofvander P, Dutta P, Sun CX, Sitbon F (2015) Increased production of wax esters in transgenic tobacco plants by expression of a fatty acid reductase: wax synthase gene fusion. Transgenic Res 24:945–953

Bakermans C, Ayala-del-Río HL, Ponder MA, Vishnivetskaya T, Gilichinsky D, Thomashow MF, Tiedje JM (2006) *Psychrobacter cryohalolentis* sp. nov. and *Psychrobacter arcticus* sp. nov., isolated from Siberian permafrost. Int J Syst Evol Microbiol 56:1285–1291

Barbe V, Vallenet D, Fonknechten N, Kreimeyer A, Oztas S, Labarre L, Cruveiller S, Robert C, Duprat S, Wincker P, Ornston LN, Weissenbach J, Marlière P, Cohen GN, Médigue C (2004) Unique features revealed by the genome sequence of *Acinetobacter* sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Res 32:5766–5779

Barney BM, LoBrutto R, Francisco WA (2004) Characterization of a small metal binding protein from *Nitrosomonas europaea*. Biochemistry 43:11206–11213

Barney BM, Wahlen BD, Garner E, Wei JS, Seefeldt LC (2012) Differences in substrate specificities of five bacterial wax ester synthases. Appl Environ Microbiol 78:5734–5745

Barney BM, Mann RL, Ohlert JM (2013) Identification of a residue affecting fatty alcohol selectivity in wax ester synthase. Appl Environ Microbiol 79:396–399

Barney BM, Ohlert JM, Timler JG, Lijewski AM (2015) Altering small and medium alcohol selectivity in the wax ester synthase. Appl Microbiol Biotechnol 99:9675–9684

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE (2000) The protein data bank. Nucleic Acids Res 28:235–242

Bonis BM, Gralnick JA (2015) *Marinobacter subterrani*, a genetically tractable neutrophilic Fe(II)-oxidizing strain isolated from the Soudan iron mine. Front Microbiol 6:11

Broadwater JA, Fox BG (1999) Spinach holo-acyl carrier protein: overproduction and phosphopantetheinylation in *Escherichia coli* BL21(DE3), in vitro acylation, and enzymatic desaturation of histidine-tagged isoform I. Protein Expr Purif 15:314–326

Cho HS, Cronan JE (1995) Defective export of a periplasmic enzyme disrupts regulation of fatty-acid synthesis. J Biol Chem 270:4216–4219

Constantinides PP, Steim JM (1985) Physical properties of fatty acyl-CoA. Critical micelle concentrations and micellar size and shape. J Biol Chem 260:7573–7580

Coursolle D, Lian JZ, Shanklin J, Zhao HM (2015) Production of long chain alcohols and alkanes upon coexpression of an acyl-ACP reductase and aldehyde-deformylating oxygenase with a bacterial type-I fatty acid synthase in *E. coli*. Mol BioSyst 11:2464–2472

Gardes A, Kaeppel E, Shehzad A, Seebah S, Teeling H, Yarza P, Glockner FO, Grossart HP, Ullrich MS (2010) Complete genome sequence of *Marinobacter adhaerens* type strain (HP15), a diatom-interacting marine microorganism. Stand Genomic Sci 3:97–107

Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P, Bertrand JC (1992) *Marinobacter hydrocarbonoclasticus* gen-Nov, sp-Nov, a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. Int J Syst Bacteriol 42:568–576

Han L, Lobo S, Reynolds KA (1998) Characterization of β-ketoacyl-acyl carrier protein synthase III from *Streptomyces glaucescens* and its role in initiation of fatty acid biosynthesis. J Bacteriol 180:4481–4486

Hoffmann N, Amara AA, Beermann BB, QS Q, Hinz HJ, BHA R (2002) Biochemical characterization of the *Pseudomonas putida* 3-hydroxyacyl ACP: CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis. J Biol Chem 277:42926–42936

Hofvander P, Doan TTP, Hamberg M (2011) A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. FEBS Lett 585:3538–3543

Holtzapple E, Schmidt-Dannert C (2007) Biosynthesis of isoprenoid wax ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: identification and characterization of isoprenoid coenzyme a synthetase and wax ester synthases. J Bacteriol 189:3804–3812

Homann VV, Edwards KJ, Webb EA, Butler A (2009) Siderophores of *Marinobacter aquaeolei*: petrobactin and its sulfonated derivatives. Biometals 22:565–571

Huu NB, Denner EBM, Ha DTC, Wanner G, Stan-Lotter H (1999) *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well. Int J Syst Bacteriol 49:367–375

Kalscheuer R, Steinbüchel A (2003) A novel bifunctional wax ester synthase/acyl-CoA: diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. J Biol Chem 278:8075–8082

Kalscheuer R, Stölting T, Steinbüchel A (2006a) Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152:2529–2536

Kalscheuer R, Stöveken T, Luftmann H, Malkus U, Reichelt R, Steinbüchel A (2006b) Neutral lipid biosynthesis in engineered *Escherichia coli*: jojoba oil-like wax esters and fatty acid butyl esters. Appl Environ Microbiol 72:1373–1379

Kapust RB, Waugh DS (1999) *Escherichia coli* Maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci 8:1668–1674

Kervabon A, Albert B, Etemadi AH (1977) Subunit composition and some properties of palmitoyl-CoA-ACP-transacylase of *Mycobacterium smegmatis*. Biochimie 59:363–374

Kostka JE, Prakash O, Overholt WA, Green SJ, Freyer G, Canion A, Delgardio J, Norton N, Hazen TC, Huettel M (2011) Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater horizon oil spill. Appl Environ Microbiol 77:7962–7974

Kuo TM, Ohlrogge JB (1984) A novel, general radioimmunoassay for acyl carrier proteins. Anal Biochem 136:497–502

Lenneman EM (2013) The utilization of algicidal bacteria for improved lipid extractions and insights into neutral lipid production in a wax ester accumulating bacterium, Univeristy of Minnesota

Lenneman EM, Ohlert JM, Palani NP, Barney BM (2013) Fatty alcohols for wax esters in *Marinobacter aquaeolei* VT8: two optional routes in the wax biosynthesis pathway. Appl Environ Microbiol 79:7055–7062

Liu A, Tan X, Yao L, Lu X (2013) Fatty alcohol production in engineered *E. coli* expressing *Marinobacter* fatty acyl-CoA reductases. Appl Microbiol Biotechnol 97:7061–7071

Lupette J, Lami R, Krasovec M, Grimsley N, Moreau H, Piganeau G, Sanchez-Ferandin S (2016) *Marinobacter* dominates the bacterial community of the *Ostreococcus tauri* phycosphere in culture. Front Microbiol 7:14

Manilla-Pérez E, Lange AB, Hetzler S, Steinbüchel A (2010) Occurrence, production, and export of lipophilic compounds by hydrocarbonoclastic marine bacteria and their potential use to produce bulk chemicals from hydrocarbons. Appl Microbiol Biotechnol 86:1693–1706

McLeod MP, Warren RL, Hsiao WW, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D, Dosanjh M, Hara H, Petrescu A, Morin RD, Yang G, Stott JM, Schein JE, Shin H, Smailus D, Siddiqui AS, Marra MA, Jones SJ, Holt R, Brinkman FS, Miyauchi K, Fukuda M, Davies JE, Mohn WW, Eltis LD (2006) The complete genome of *Rhodococcus sp.* RHA1 provides insights into a catabolic powerhouse. Proc Natl Acad Sci U S A 103:15582–15587

Nakano M, Iehata S, Tanaka R, Maeda H (2012) Extracellular neutral lipids produced by the marine bacteria *Marinobacter* sp. Biocontrol Sci 17:69–75

Pérez D, Martín S, Fernández-Lorente G, Filice M, Guisán JM, Ventosa A, García MT, Mellado E (2011) A novel halophilic lipase, LipBL, showing high efficiency in the production of eicosapentaenoic acid (EPA). PLoS One 6:11

Reiser S, Somerville C (1997) Isolation of mutants of *Acinetobacter calcoaceticus* deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme a reductase. J Bacteriol 179:2969–2975

Schirmer A, Rude MA, Li X, Popova E, del Cardayre SB (2010) Microbial biosynthesis of alkanes. Science 329:559–562

Shanklin J (2000) Overexpression and purification of the *Escherichia coli* inner membrane enzyme acyl-acyl carrier protein synthase in an active form. Protein Expr Purif 18:355–360

Shanmughapriya S, Kiran GS, Selvin J, Thomas TA, Rani C (2010) Optimization, purification, and characterization of extracellular mesophilic alkaline cellulase from sponge-associated *Marinobacter* sp. MSI032. Appl Biochem Biotechnol 162:625–640

Shi SB, Valle-Rodríguez JO, Khoomrung S, Siewers V, Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. Biotechnol Biofuels 5

Singer E, Webb EA, Nelson WC, Heidelberg JF, Ivanova N, Pati A, Edwards KJ (2011) Genomic potential of *Marinobacter aquaeolei*, a biogeochemical "opportunitroph". Appl Environ Microbiol 77:2763–2771

Stöveken T, Steinbüchel A (2008) Bacterial acyltransferases as an alternative for lipase-catalyzed acylation for the production of oleochemicals and fuels. Angew Chem Int Ed Eng 47:3688–3694

Stöveken T, Kalscheuer R, Steinbüchel A (2009) Both histidine residues of the conserved HHXXXDG motif are essential for wax ester synthase/acyl-CoA: diacylglycerol acyltransferase catalysis. Eur J Lipid Sci Technol 111:112–119

Strycharz-Glaven SM, Glaven RH, Wang Z, Zhou J, Vora GJ, Tender LM (2013) Electrochemical investigation of a microbial solar cell reveals a nonphotosynthetic biocathode catalyst. Appl Environ Microbiol 79:3933–3942

Takle GW, Toth IK, Brurberg MB (2007) Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. BMC Plant Biol 7

Teo WS, Ling H, Yu AQ, Chang MW (2015) Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid short- and branched-chain alkyl esters biodiesel. Biotechnol Biofuels 8:9

Vetting MW, Al-Obaidi N, Zhao SW, San Francisco B, Kim J, Wichelecki DJ, Bouvier JT, Solbiati JO, Vu H, Zhang XS, Rodionov DA, Love JD, Hillerich BS, Seidel RD, Quinn RJ, Osterman AL, Cronan JE, Jacobson MP, Gerlt JA, Almo SC (2015) Experimental strategies for functional annotation and metabolism discovery: targeted screening of solute binding proteins and unbiased panning of metabolomes. Biochemistry 54:909–931

Villa JA, Ray EE, Barney BM (2014) *Azotobacter vinelandii* Siderophore can provide nitrogen to support the culture of the green algae *Neochloris oleoabundans* and *Scenedesmus* sp. BA032. FEMS Microbiol Lett 351:70–77

Vorobiev SM, Neely H, Yu B, Seetharaman J, Xiao R, Acton TB, Montelione GT, Hunt JF (2012) Crystal structure of a catalytically active GG(D/E)EF diguanylate cyclase domain from *Marinobacter aquaeolei* with bound c-di-GMP product. J Struct Funct Genom 13:177–183

Wahlen BD, Oswald WS, Seefeldt LC, Barney BM (2009) Purification, characterization, and potential bacterial wax production role of an NADPH-dependent fatty aldehyde reductase from *Marinobacter aquaeolei* VT8. Appl Environ Microbiol 75:2758–2764

Wältermann M, Hinz A, Robenek H, Troyer D, Reichelt R, Malkus U, Galla HJ, Kalscheuer R, Stöveken T, von Landenberg P, Steinbüchel A (2005) Mechanism of lipid-body formation in prokaryotes: how bacteria fatten up. Mol Microbiol 55:750–763

Wältermann M, Stöveken T, Steinbüchel A (2007) Key enzymes for biosynthesis of neutral lipid storage compounds in prokaryotes: properties, function and occurrence of wax ester synthases/acyl-CoA: diacylglycerol acyltransferases. Biochimie 89:230–342

Wang W, Wei H, Knoshaug E, Van Wychen S, Xu Q, Himmel ME, Zhang M (2016) Fatty alcohol production in *Lipomyces starkeyi* and *Yarrowia lipolytica*. Biotechnol Biofuels 9:12

Wei J, Timler JG, Knutson CM, Barney BM (2013) Branched-chain 2-keto acid decarboxylases derived from *Psychrobacter*. FEMS Microbiol Lett 346:105–112

Williamson IP, Wakil SJ (1966) Studies on the mechanism of fatty acid synthesis. XVII. Preparation and general properties of acetyl coenzyme a and malonyl coenzyme A-acyl carrier protein transacylases. J Biol Chem 241:2326–2332

Willis RM, Wahlen BD, Seefeldt LC, Barney BM (2011) Characterization of a fatty acyl-CoA reductase from *Marinobacter aquaeolei* VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol. Biochemistry 50:10550–10558

Yao L, Qi FX, Tan XM, Lu XF (2014) Improved production of fatty alcohols in cyanobacteria by metabolic engineering. Biotechnol Biofuels 7:9

Youngquist JT, Schumacher MH, Rose JP, Raines TC, Politz MC, Copeland MF, Pfleger BF (2013) Production of medium chain length fatty alcohols from glucose in *Escherichia coli*. Metab Eng 20:177–186

Vitamin Formation from Fatty Acid Precursors

14

Michael F. Dunn

Contents

1 Introduction 260
2 Lipoic Acid Synthesis 260
2.1 Synthesis of Octanoyl-ACP 261
2.2 Lipoylation of LA-Dependent Enzymes Occurs by Two Pathways 263
3 Synthesis of Biotin 264
3.1 Production of the Pimelate Moiety 264
3.2 Assembly of the Biotin Ring Structures 268
3.3 Attachment of Biotin to Biotin-Dependent Enzymes and Regulation of Biotin Biosynthesis 268
4 Research Needs 269
References 270

Abstract

Enzymes that require biotin or lipoic acid cofactors for their activity occur in all domains of life and play essential roles in metabolism. The de novo synthesis of these vitamins depends on the production of fatty acid precursors and has been most extensively characterized in *Escherichia coli* and *Bacillus* species. The octanoyl-acyl carrier protein precursor for lipoic acid is synthesized in reactions catalyzed by the fatty acid biosynthesis (Fab) enzymes. The octanoyl moiety is linked to the lipoyl domains of lipoic acid-dependent enzymes and then converted to lipoate by lipoyl synthase. For biotin biosynthesis, both the BioC-BioH pathway in *E. coli* and the BioI pathway in *Bacillus* species rely on Fab enzymes

M. F. Dunn (✉)
Programa de Genómica Funcional de Procariontes, Centro de Ciencias Genómicas, Universidad Nacional Autonoma de México, Av. Universidad s/n, Col. Chamilpa, Cuernavaca, Morelos, Mexico
e-mail: mike@ccg.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_24

to produce the pimeloyl-acyl carrier protein required for biotin production. This review presents an overview of the biosynthetic pathways for biotin and lipoic acid, with an emphasis on the role of fatty acid metabolism in their synthesis.

1 Introduction

Fatty acids (FAs) are aliphatic hydrocarbons with a carboxylic acid group on carbon number 1 and a methyl group at the other end of the chain (see octanoic acid in Fig. 1a as an example). FAs are synthesized by organisms in all domains of life and play vital roles in membrane structure, energy storage, and as metabolic precursors (Cronan 2014; Dibrova et al. 2014; López-Lara and Geiger 2010; Sohlenkamp and Geiger 2016). This review presents a brief overview of how bacterial FA synthesis (FAS) participates in the production of the vitamins biotin and lipoic acid (LA) (Fig. 1), with emphasis on *Escherichia coli* and *Bacillus* species. Bacterial FAS is covered in detail in two chapters in this handbook: ▶ Chap. 3, "Formation of Fatty Acids" by López-Lara and Geiger and ▶ Chap. 21, "Fatty Acid Synthesis and Regulation" by López-Lara. Details of biotin and LA pathway enzyme mechanisms, structures, and regulation are described in recent reviews (Cronan 2014, 2016; Cronan and Lin 2011; Lin and Cronan 2011; Rock 2009).

Biotin and LA are prosthetic groups for selected metabolic enzymes, where they are covalently attached to a specific domain or subunit of the enzymes requiring them. Both cofactors act as "swinging arms" to shuttle reaction intermediates between different active sites of their cognate enzymes (Perham 2000). Examples of biotin- and LA-dependent enzymes and the de novo synthesis of these cofactors are described in the following sections.

2 Lipoic Acid Synthesis

The structure of LA is that of the eight-carbon saturated FA octanoic acid into which sulfur atoms are inserted at carbons 6 and 8 (Fig. 1A). Pyruvate dehydrogenase (PDH) is a well-characterized example of a LA-dependent enzyme. It catalyzes the oxidative decarboxylation of pyruvate to produce acetyl coenzyme A (acetyl-CoA), which serves as the acetate donor for FAS (Fig. 2) and in many other vital metabolic functions. PDH is a complex consisting of multiple copies of subunits, designated E1 (pyruvate dehydrogenase), E2 (dihydrolipoyl transacetylase), and E3 (dihydrolipoyl dehydrogenase). E2 contains the bound LA cofactor and forms the structural core of the enzyme by binding noncovalently to E1 and E3. The E2 lipoyl group (Fig. 1a) acts as a "swinging arm" to transfer acetate between the E1 and E3 active sites of the enzyme. First, subunit E1 decarboxylates pyruvate, and the resulting hydroxyethyl group is bound to the thiamine pyrophosphate (TPP) cofactor of the E1 subunit, forming hydroxyethyl TPP (E1-TPP-CHOH-CH_3). The

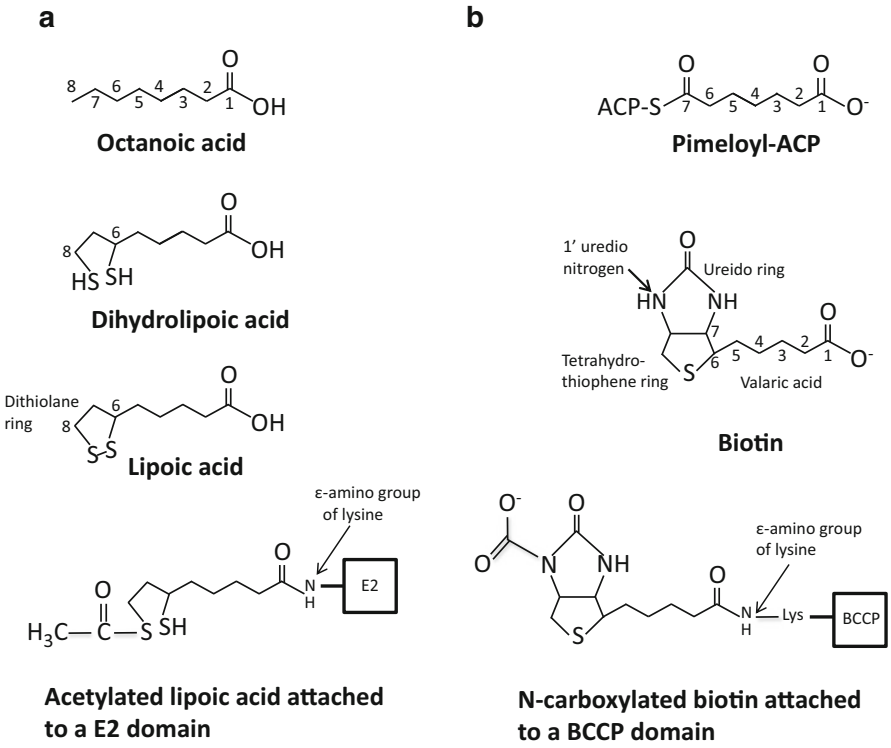


Fig. 1 Structural features of biotin and lipoic acid. (**a**) Lipoic acid (*LA*) and related structures. The C6 and C8 carbon atoms indicate those that are bound to sulfur in the dithiolane ring of LA. The structure at the bottom of the figure is the specific example of acetyl-modified LA attached to the E2 domain of pyruvate dehydrogenase. (**b**) Biotin and related structures. Pimeloyl-ACP donates the majority of carbon atoms (numbered) to biotin. The 1′ uredio nitrogen of biotin is the site of carboxylation, as shown in the structure of carboxylated biotin linked to the BCCP domain of a biotin-dependent enzyme

hydroxyethyl group is oxidized to an acetyl group that is then transferred to one of the LA sulfur atoms, forming acetyl-LA (Fig. 1a). The acetyl group is then transferred to CoA to produce acetyl-CoA. Finally, the reduced LA is reoxidized to reform the dithiolane ring (Fig. 1a) in an FAD-dependent reaction catalyzed by the E3 subunit (Perham 2000; Rock 2009).

2.1 Synthesis of Octanoyl-ACP

LA biosynthesis requires octanoic acid, a C8:0 FA formed in the normal course of bacterial FAS (Fig. 1a). Bacterial FAS systems are comprised of Fab (fatty acid biosynthesis) enzymes. Acyl carrier protein (ACP) shuttles FAs from one Fab enzyme to another and also prevents them from being degraded during their

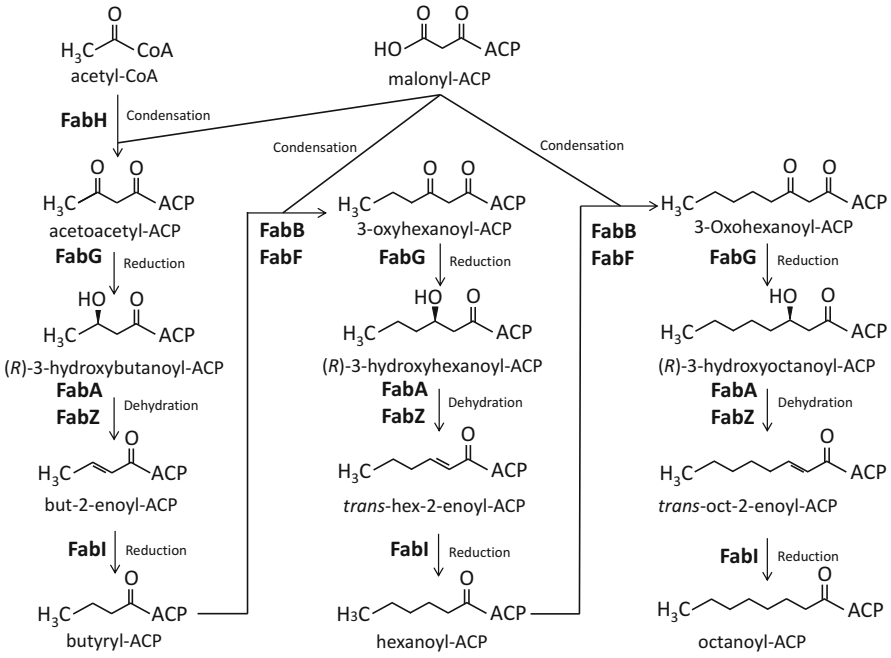


Fig. 2 Reactions of FAS producing the octanoyl-ACP precursor of lipoic acid in *E. coli* K-12 MG1655, based on the KEGG database (Kanehisa et al. 2016). See Sect. 2.1 for details. Abbreviations: *ACP* acyl carrier protein, *FabH* 3-oxoacyl-ACP synthase III, *FabG* 3-oxoacyl-ACP reductase, *FabA and FabZ* 3-hydroxyacyl-ACP dehydratases, *FabI* enoyl-ACP reductase I

synthesis (Chan and Vogel 2010; Janßen and Steinbüchel 2014; López-Lara and Geiger 2010). ACP contains a covalently bound 4′-phosphopantetheine prosthetic group to which fatty acyl groups are linked by a thioester bond between the carboxyl group of the FA and the sulfhydryl group of the 4′-phosphopantetheine (Janßen and Steinbüchel 2014).

The first reaction of FAS is catalyzed by the biotin-dependent enzyme acetyl-CoA carboxylase (ACC; see Sect. 3), which adds CO_2 to acetyl-CoA to form malonyl-CoA. Next, the CoA on malonyl-CoA is exchanged with ACP to form malonyl-ACP in a reversible reaction catalyzed by malonyl-CoA/ACP transacylase (FabD). A condensation reaction catalyzed by 3-oxoacyl-ACP synthase III (FabH) combines malonyl-ACP with acetyl-CoA to yield acetoacetyl-ACP (Fig. 2). The four-carbon acetoacetyl-CoA produced by FabH is subjected to a reduction, a dehydration, and a second reduction by Fab enzymes to yield butyryl-ACP (Fig. 2). Two more rounds of FAS comprised of condensation-reduction-dehydration-reduction reactions each add two carbon units to the acyl chain, resulting in the production of octanoyl-ACP (Janßen and Steinbüchel 2014; Qui et al. 2001; Rock and Jackowski 2002) (Fig. 2).

2.2 Lipoylation of LA-Dependent Enzymes Occurs by Two Pathways

Two different pathways have evolved in *E. coli* for the lipoylation of LA-dependent enzymes (Morris et al. 1995) (Fig. 3). The scavenging pathway starts with the passive uptake of LA from the environment (Cronan 2016), after which lipoate-protein ligase (LplA) attaches it to lipoyl domains (LDs) in a two-step reaction that involves an activated lipoyl-AMP intermediate. The activated lipoyl moiety is then transferred to a specific lysine residue of the LD and AMP is released (Cronan 2014; Rock 2009) (Fig. 3). In *Bacillus subtilis*, the LplA ortholog is designated LplJ (Martin et al. 2011).

In the *E. coli* pathway for the de novo synthesis of LA, octanoyl-ACP/protein *N*-octanoyltransferase (LipB) cleaves the ACP from the octanoyl-ACP produced by FAS (Fig. 2) and binds the octonate moiety (Fig. 3). The octanoyl-LipB intermediate then donates the octanoyl moiety to the LD to form octanoyl-LD. Finally, lipoyl synthase (LipA) catalyzes the SAM-dependent insertion of two sulfur atoms, derived from the LipA [Fe-S] cluster, into the LD-bound octanoyl moiety, converting it to a lipoyl-LD. In *B. subtilis*, LipM performs the same function as the *E. coli* LipB (Cronan 2014; Jordan and Cronan 1997; Martin et al. 2011; Morris et al. 1995).

LA synthesis is unusual in that it requires the octanoyl moiety precursor to be covalently linked to a LD. This in situ synthesis of the lipoyl moiety contrasts with the production of biotin (Sect. 3) and numerous other enzyme cofactors, which are

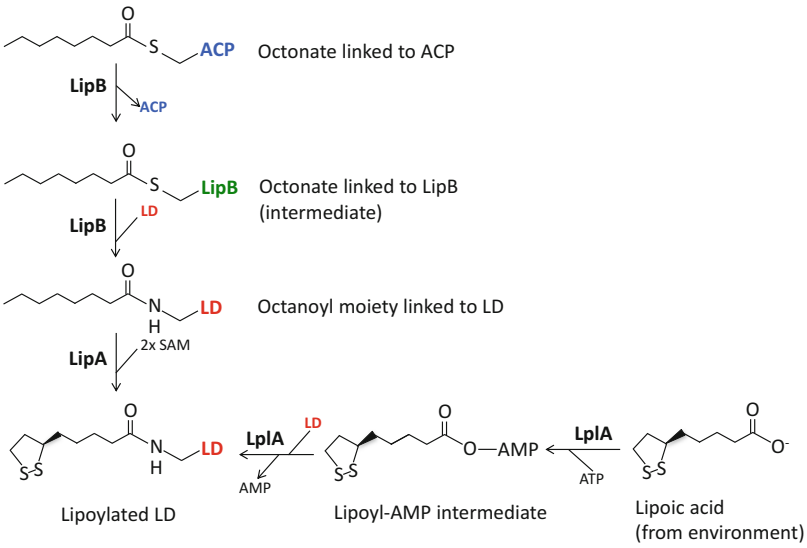


Fig. 3 The de novo synthesis of lipoic acid (*LA*) from octanoyl-ACP (*left* hand of figure) and the scavenging pathway utilizing exogenous LA (*bottom* of figure). See Sect. 2.2 for details. Abbreviations: *ACP* acyl carrier protein, *LD* lipoyl domain (The figure is modified from Cronan (2016))

fully synthesized prior to their attachment to their cognant enzymes (Sect. 3) (Cronan 2014; Jordan and Cronan 1997; Morris et al. 1995).

In *E. coli*, neither *lipA* nor *lipB* appears to be transcriptionally regulated. This may be because the sulfur insertions catalyzed by LipA occur only when the octanoyl moiety is linked to a LD, and so the supply of subunits requiring lipoylation determines the quantity of LA synthesized (Feng and Cronan 2014). In contrast, the expression of *lipA* and *lipB* in *Shewanella oneidensis*, which form an operon, is regulated by glucose in a cyclic adenosine monophosphate-dependent manner (Zhang et al. 2015).

3 Synthesis of Biotin

E. coli contains a single biotin-dependent enzyme, acetyl-CoA carboxylase (ACC), which is essential for FAS and thus viability (Beckett 2007; Cronan 2014). ACCs in prokaryotes are comprised of three subunits: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT) (Tong 2013). ACC catalyzes a two-step reaction that adds CO_2 (from bicarbonate, HCO_3^-) to acetyl-CoA to produce malonyl-CoA:

1. ACC-biotin + CO_2 + MgATP – >ACC-biotin-CO_2^- + MgADP + Pi + H^+
2. ACC-biotin-CO_2^- + CH_3-CO-SCoA – >ACC-biotin + ^-O_2C-CH_2-CO-S-CoA

In step 1, the BC domain catalyzes the ATP-dependent carboxylation of the biotin attached to a specific lysine residue in the BCCP domain (Fig. 1b). In step 2, the activated CO_2 is transferred from biotin to acetyl-CoA in a reaction catalyzed by the CT domain. The valeryl side chain of biotin (Fig. 1b) is important in its ability to interact with the BC and CT domains (Tong 2013).

To satisfy their biotin requirements, bacteria can use specific transporters to obtain the vitamin from the environment or synthesize it de novo. Some bacteria lack complete biotin synthesis pathways and are able to obtain biotin only from external sources, while others are capable of its synthesis and lack transporters. Some bacteria can use both options (Feng et al. 2015; Guillén-Navarro et al. 2005; Rodionov et al. 2002; Satiaputra et al. 2016).

The four final steps of biotin biosynthesis (Sect. 3.2) have been extensively studied and are well conserved in different organisms (Beckett 2007; Lin 2012). In contrast, knowledge of how the pimelate moiety precursor for these final steps is produced is much more recent, as described below.

3.1 Production of the Pimelate Moiety

The pimelate moiety (pimeoyl-ACP) is the source of carbons 1 through 7 of biotin (Fig. 1b). The BioI and BioC-BioH pathways are distinct routes for producing the pimelate moiety that occur in different bacteria, and both are dependent on FAS (Cronan and Lin 2011) (Fig. 4). The BioI pathway occurs principally in *B. subtilis* and related bacteria. BioI is an oxygen-dependent cytochrome P450 family protein

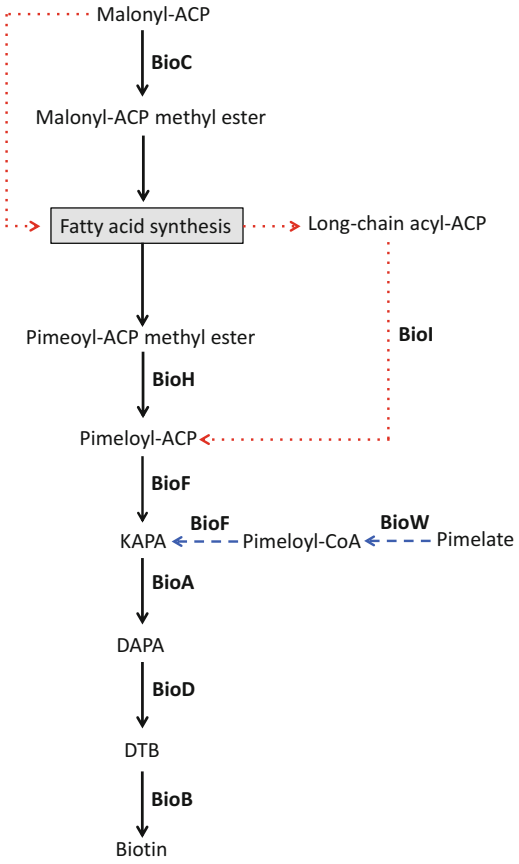


Fig. 4 A schematic view of showing enzymatic alternatives for biotin production. Fatty acid synthesis (*shaded box*) plays a central role in the BioI (*dotted arrows*) and BioC-BioH pathways (*solid arrows*), but not in the BioW-BioF pathway (*dashed arrows*). See Sects. 3.1 and 3.2 for details

that produces pimeloyl-ACP by the cleavage of saturated and unsaturated long-chain fatty acyl-ACPs produced by the FAS system (Stok and De Voss 2000) (Fig. 4). The FA chain of these substrates and the 4′-phosphopantetheine prosthetic group of ACP fit into the hydrophobic substrate-binding cavity of BioI. A sharp bend is induced in the acyl chain that places its C7 and C8 atoms in close proximity to the heme iron of the active site. Catalysis is thought to occur by the successive hydroxylation of C7 and C8, followed by the oxidative cleavage of the carbon-carbon bond between them (Cronan and Lin 2011; Cryle and Schlichting 2008). The pimeloyl-ACP thus produced is converted to biotin in four steps described in the next section. The heterologous overexpression of *Bacillus* BioI in *E. coli bioC* or *bioH* mutants (Figs. 4 and 5) permits their growth in biotin-free medium (Bower et al. 1996).

Despite decades of biochemical and genetic characterization of biotin biosynthesis in *E. coli*, the roles of BioC and BioH (Fig. 4) in producing the pimelate moiety were only recently defined. BioC is a *O*-methyltransferase that methylates the carboxyl group of malonyl-ACP to form malonyl-ACP methyl ester, using *S*-adenosyl-L-methionine (SAM) as a methyl group donor (Fig. 5). Replacement of

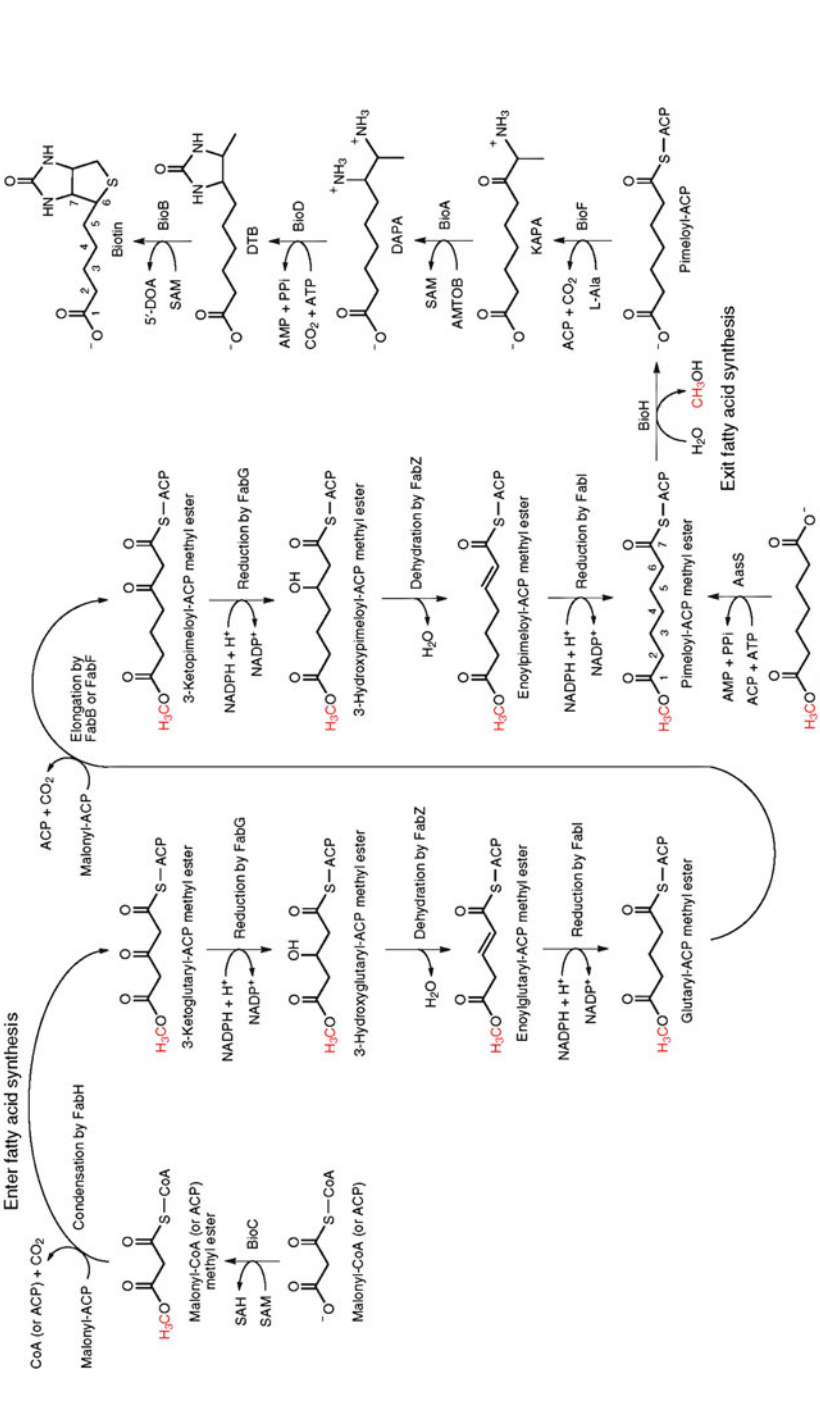


Fig. 5 Biotin biosynthesis by the BioC-BioH pathway showing the FAS reactions using methyl esterified FA intermediates and the formation of the bicyclic rings. See Sect. 3.2 for details (The figure is reproduced from Lin et al. 2010 and used by permission of the Nature Publishing Group)

the carboxyl group with a methyl ester group removes the molecule's negative charge and mimics the methyl groups normally present on FA chains. The methyl ester modification channels the BioC reaction product into biotin biosynthesis and allows it to enter the hydrophobic active sites of the FAS enzymes: three rounds of FAS result in the production of pimeloyl-ACP methyl ester (Bi et al. 2016; Lin 2012; Lin and Cronan 2011; Lin et al. 2010) (Fig. 5).

Much of what we know about BioC comes from the characterization of the *Bacillus cereus* enzyme, overexpressed in *E. coli* and obtained in purified form. This is because the BioC from *E. coli* and several other bacteria has proved intractable to efficient purification. The *B. cereus* BioC is able to functionally complement an *E. coli bioC* mutant's biotin auxotrophy, indicating that the *Bacillus* BioC is able to utilize FA substrates linked to the *E. coli* ACP. Malonyl-ACP, rather than malonyl-CoA, is by far the preferred methyl accepting substrate for the *Bacillus* BioC. Interestingly, the overexpression of the *Bacillus* BioC in *E. coli* leads to a shutdown of FA production and abolished growth. This is because the high-level expression of BioC converts too much of the available malonyl-ACP to its methyl ester, which is not a substrate for the normal FAS pathway (Lin 2012; Lin and Cronan 2012). In wild-type *B. cereus* and *E. coli*, BioC catalytic activity appears to be very low. This and the regulation of *bioC* transcriptional expression by BirA (Sect. 3.3) in *E. coli* (and possibly *B. cereus*) probably act to allow the enzyme to compete against FabH for malonyl-CoA without overly depleting the malonyl-CoA pool and reducing the synthesis of normal FAs (Lin 2012; Lin et al. 2010; Lin and Cronan 2012; Rodionov et al. 2002). It is reasonable to suspect that BioI in *Bacillus* is controlled in a similar manner to prevent the excess conversion of long-chain acyl-ACPs to pimeloyl-ACP (Stok and De Voss 2000).

The *E. coli* carboxylesterase BioH then removes the methyl group from pimeloyl-ACP methyl ester, allowing it to exit FAS and providing the substrate for the later stages of biotin biosynthesis (Fig. 5). BioH is much more catalytically active than BioC and the enzymes involved in biotin ring synthesis (Sect. 3.2). Possibly, BioH has not yet been completely integrated with the other enzymes of biotin synthesis, as evidenced by its location outside of the biotin gene operon and its lack of regulation by BirA (Sect. 3.3) (Lin 2012).

Some bacteria contain BioC but lack BioH, which is replaced by any one of several nonorthologous esterases (Bi et al. 2016; Feng et al. 2014; Rodionov et al. 2002; Shapiro et al. 2012). A possible reason that this disparate group of esterases can replace BioH in different bacteria is that the ester hydrolysis producing pimeloyl-ACP is a relatively unchallenging enzymatic reaction, in comparison to the chemically complex ring formation reactions catalyzed by the more evolutionary conserved BioFADB enzymes described in the next section (Lin 2012; Shapiro et al. 2012).

As a sole or additional (along with BioI) route for pimelate moiety synthesis, *B. subtilis* and a few other bacterial species produce pimeloyl-CoA synthetase (BioW) (Fig. 4). This enzyme converts pimelic acid, probably obtained from the environment, to pimeloyl-CoA, which is used by BioF to produce 7-keto-8-aminopelargonic acid (KAPA), an early intermediate in the ring closure reactions of biotin biosynthesis (Ploux et al. 1992; Rodionov et al. 2002) (Fig. 4).

3.2 Assembly of the Biotin Ring Structures

The second phase of biotin biosynthesis requires four enzymes to assemble the vitamin's bicyclic rings (Figs. 1 and 5). In the first reaction, KAPA synthase (BioF) decarboxylatively condenses L-alanine and pimeloyl-ACP to form KAPA, resulting in the cleavage of the pimelate thioester bond and release of CO_2. This results in the seven-carbon pimeloyl-ACP (Fig. 1) being extended with two carbons and a nitrogen derived from L-alanine, which constitute the C8, C9, and N8 atoms of biotin (Lin 2012) (Fig. 5).

KAPA is transaminated at C7 to produce 7,8-diaminopelargonic acid (DAPA) in the reaction catalyzed by DAPA synthase (BioA), which is unique among characterized enzymes in using SAM, the common methyl donor in enzyme reactions, as the amino donor. BioA and BioF are both pyridoxal 5′-phosphate (PLP)-dependent enzymes that belong to the same aminotransferase family and probably evolved from a common ancestor. The BioA reaction introduces the N7 amino group required for ureido ring formation. Dethiobiotin (DTB) synthetase (BioD) converts DAPA to DTB by the ATP-dependent insertion of CO_2 between N7 and N8 of DAPA to form the biotin ureido ring (Figs. 1 and 5). The final reaction of biotin synthesis is catalyzed by biotin synthase (BioB), an iron-sulfur enzyme. BioB inserts sulfur, obtained from its own $[2Fe\text{-}2S]^{2+}$ cluster, between the C6 methylene and C9 methyl groups of DTB to form the tetrathiophane ring of biotin (Lin 2012) (Figs. 1 and 5).

3.3 Attachment of Biotin to Biotin-Dependent Enzymes and Regulation of Biotin Biosynthesis

BirA (biotin retention protein A) is a bifunctional protein acting as a transcriptional repressor of most of the biotin genes and a biotin-protein ligase (BPL) that attaches biotin to the BCCP subunit or domain of biotin-dependent enzymes. BirAs are also referred to as class II BPLs. Class I BPLs contain only the domains required for ligating biotin to apo-BCCPs and lack the N-terminal DNA-binding domain present in the class II proteins, so they do not function as transcriptional regulators (Satiaputra et al. 2016). BPLs are ubiquitous in bacteria, although only a small portion of these are of the class II (bifunctional) type (Rodionov et al. 2002).

The ability of BirA to both ligate biotin to apo-BCCPs and regulate transcription are functionally linked and have been extensively studied in *E. coli*. In the first reaction of biotin ligation, biotinyl-5′-AMP is formed from biotin and ATP. This compound, rather than biotin itself, acts as the BirA corepressor of transcription. BirA::biotinyl-5′-AMP is able to dimerize and interact with the biotin operator regions to repress biotin gene transcription. Rapidly growing *E. coli* cells require a relatively high level of ACC activity for membrane FA synthesis. In these cells, biotin is in great demand because apo-BCCP levels are high. BirA::bio-5′-AMP functions as a biotinylating enzyme and not as a transcriptional repressor. In slowly dividing cells, BirA::bio-5′-AMP accumulates since there are few apo-BCCP

domains to biotinylate and thus acts as a transcriptional repressor (Beckett 2007; Cronan 2014; Satiaputra et al. 2016).

Alternative modes of biotin gene regulation occur in some bacteria having class I BPLs. BioR is a GntR family transcriptional regulator that represses the expression of biotin biosynthesis and/or transport genes in *Paracoccus denitrificans*, *Brucella melitensis*, and *Agrobacterium tumefaciens* (Feng et al. 2015; Saitputra et al. 2016). BioQ is a TetR family transcriptional repressor of biotin synthesis and transport in *Corynebacterium glutamicum* and of biotin biosynthesis in *Mycobacterium* species. However, the biotin metabolite ligand with which BioR or BioQ DNA binding is modulated has not been identified (Feng et al. 2015; Satiaputra et al. 2016). However, *Mycobacterium smegmatis* wild-type cultures grown with increasing concentrations of exogenous biotin showed reduced expression of their *bioF*, *bioD*, and *bioB* genes. This biotin-mediated repression does not occur in a *bioQ* deletion strain, suggesting that BioQ is able to directly or indirectly sense biotin. Similarly, the repression of biotin biosynthesis by exogenous biotin has been reported in the *A. tumefaciens* wild type, but does not occur in a *bioR* mutant strain (Satiaputra et al. 2016). A general two-protein "cross-talk" model in which class I BPLs and BioQ or BioR act together to coordinate biotin biosynthesis, apo-BCCP biotinylation, and biotin transport has been proposed, but many details are lacking (Beckett 2007; Satiaputra et al. 2016).

4 Research Needs

Unanswered questions on the reaction mechanisms, regulation, and structural characteristics of several enzymes involved in biotin and LA synthesis have been raised in several reviews (Cronan 2014, 2016; Cronan and Lin 2011; Lin and Cronan 2011; Rock 2009). For example, one long-standing area of doubt centers on whether the BioB is inactivated following each catalysis, since it donates sulfur from its own $[2Fe\text{-}2S]^{2+}$ cluster. Various proposals have been made that might allow reconstitution of the cluster to permit multiple turnovers (Choi-Rhee and Cronan 2005). Because very few molecules of biotin are required for the growth of *E. coli*, catalytic efficiency of BioB and other enzymes of the pathway may not be a physiological necessity (Choi-Rhee and Cronan 2005; Cronan 2014).

BioC acts as a "gatekeeper" that directs the entry of carbon skeletons into the FAS system for biotin biosynthesis. In *E. coli*, BioC's catalytic inefficiency and the transcriptional control by *bioC* by BirA probably act to limit its consumption of malonyl-ACP, which is also required for normal FAS. Are the BioCs in bacteria that lack dual function BPLs subject to a distinct genetic or biochemical regulation? Another area to explore is how biotin biosynthesis and the partitioning of biotin to different enzymes is controlled in bacteria that produce multiple biotin-dependent enzymes, especially those having only class I BPLs (Guillén-Navarro et al. 2005).

During LA synthesis in *E. coli*, *lipB* is not transcriptionally regulated. What controls the enzyme's production or activity to prevent it from withdrawing too

much of the octanoyl-ACP that is needed in further elongation reactions for the synthesis of membrane FAs?

An interesting and unanswered evolutionary conundrum is how did biotin synthesis evolve? Because FAS is required for making biotin, and the biotin-dependent ACC is required for FAS, biotin is required for its own synthesis. One suggestion is that early in evolution, malonyl-CoA was produced by an ancestral enzyme (Cronan and Lin 2011).

Given the diversity of many of the enzymes involved in biotin and LA synthesis in different bacteria, additional bioinformatic analysis of genome sequence data, including that obtained in metagenomic studies, would significantly advance our knowledge of the taxonomic distribution of the known pathways for the synthesis of these cofactors and might uncover completely novel pathways.

References

Beckett D (2007) Biotin sensing: universal influence of biotin status on transcription. Annu Rev Genet 41:443–464

Bi H, Zhu L, Jia J, Cronan JE (2016) A biotin biosynthesis gene restricted to *Helicobacter*. Sci Rep 6:21162. https://doi.org/10.1038/srep21162

Bower S, Perkins JB, Yocum RR, Howitt CL, Rahaim P, Pero J (1996) Cloning, sequencing, and characterization of the *Bacillus subtilis* biotin biosynthetic operon. J Bacteriol 178:4122–4130

Chan DI, Vogel HJ (2010) Current understanding of fatty acid biosynthesis and the acyl carrier protein. Biochem J 430:1–19

Choi-Rhee E, Cronan JE (2005) Biotin synthase is catalytic *in vivo*, but catalysis engenders destruction of the protein. Chem Biol 12:461–468

Cronan JE (2014) Biotin and lipoic acid: synthesis, attachment, and regulation. EcoSal Plus. https://doi.org/10.1128/ecosalplus ESP-0001-2012

Cronan JE (2016) Assembly of lipoic acid on its cognate enzymes: an extraordinary and essential biosynthetic pathway. Microbiol Mol Biol Rev 80:429–450

Cronan JE, Lin S (2011) Synthesis of the α, ω-dicarboxylic acid precursor of biotin by the canonical fatty acid biosynthetic pathway. Curr Opinion Chem Biol 15:407–413

Cryle MJ, Schlichting I (2008) Structural insights from a P450 carrier protein complex reveal how specificity is achieved in the $P450_{BioI}$ ACP complex. Proc Natl Acad Sci U S A 105:15696–15701

Dibrova DV, Galperin MY, Mulkidjanian AY (2014) Phylogenomic reconstruction of archeal fatty acid metabolism. Environ Microbiol 16:907–918

Feng Y, Cronan JE (2014) PdhR, the pyruvate dehydrogenase repressor, does not regulate lipoic acid synthesis. Res Microbiol 165:429–438

Feng Y, Napier BA, Manandhar M, Henke SK, Weiss DS, Cronan JE (2014) A *Francisella* virulence factor catalyzes an essential reaction of biotin biosynthesis. Mol Microbiol 91:300–314

Feng Y, Kumar R, Ravcheev DA, Zhang H (2015) *Paracoccus denitrificans* possesses two BioR homologs having a role in the regulation of biotin metabolism. Microbiol Open 4:644–659

Guillén-Navarro K, Encarnación S, Dunn MF (2005) Biotin biosynthesis, transport and utilization in rhizobia. FEMS Microbiol Lett 246:159–165

Janßen HJ, Steinbüchel A (2014) Fatty acid synthesis in *Escherichia coli* and its application towards the production of fatty acid based biofuels. Biotechnol Biofuels 7:7

Jordan SW, Cronan JE (1997) A new metabolic link. The acyl carrier protein of lipid synthesis donates lipoic acid to the pyruvate dehydrogenase complex in *Escherichia coli* and mitochondria. J Biol Chem 272:17903–17906

Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K (2016) KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res 45(D1):D353–D361
Lin S (2012) Biotin synthesis in *Escherichia coli*. PhD thesis, University of Illinois at Urbana-Champaign, 140 pp
Lin S, Cronan JE (2011) Closing in on complete pathways of biotin biosynthesis. Mol BioSyst 7:1811–1821
Lin S, Cronan JE (2012) The BioC *O*-methyltransferase catalyzes methyl esterification of malonyl-acyl carrier protein, an essential step in biotin synthesis. J Biol Chem 287:37010–37020
Lin S, Hanson RE, Cronan JE (2010) Biotin synthesis begins by hijacking the fatty acid synthetic pathway. Nat Chem Biol 6:682–688
López-Lara IM, Geiger O (2010) Formation of fatty acids. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 385–393
Martin N, Christensen QH, Mansilla MC, Cronan JE, de Mendoza D (2011) A novel two-gene requirement for the octanoyltransfer reaction of *Bacillus subtilis* lipoic acid biosynthesis. Mol Microbiol 80:335–349
Morris TW, Reed KE, Cronan JE Jr (1995) Lipoic acid metabolism in *Escherichia coli*: the *lplA* and *lipB* genes define redundant pathways for ligation of lipoyl groups to apoprotein. J Bacteriol 177:1–10
Perham RN (2000) Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. Annu Rev Biochem 69:961–1004
Ploux O, Soularue P, Marquet A, Gloeckler R, Lemoine Y (1992) Investigation of the first step of biotin biosynthesis in *Bacillus sphaericus*. Biochem J 287:685–690
Qui X, Janson CA, Smith WW, Head M, Lonsdale J, Konstantinidis AK (2001) Refined structures of ß-ketoacyl-acyl carrier protein synthase III. J Mol Biol 307:341–356
Rock CO (2009) Opening a new path to lipoic acid. J Bacteriol 191:6782–6784
Rock CO, Jackowski S (2002) Forty years of bacterial fatty acid synthesis. Biochem Biophys Res Commun 292:1155–1166
Rodionov DA, Mironov AA, Gelfand MS (2002) Conservation of the biotin regulon and the BirA regulatory signal in eubacteria and Archaea. Genome Res 12:1507–1516
Satiaputra J, Shearwin KE, Booker GW, Polyak SW (2016) Mechanisms of biotin-regulated gene expression in microbes. Synth Syst Biotechnol 1:17–24
Shapiro MM, Chakravartty V, Cronan JE (2012) Remarkable diversity in the enzymes catalyzing the last step in synthesis of the pimelate moiety of biotin. PLoS One 7(11):e49440. https://doi.org/10.1371/journal.pone.0049440
Sohlenkamp C, Geiger O (2016) Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev 40:139–159
Stok JE, De Voss J (2000) Expression, purification, and characterization of BioI: a carbon-carbon bond cleaving cytochrome P450 involved in biotin biosynthesis in *Bacillus subtilis*. Arch Biochem Biophys 384:351–360
Tong L (2013) Structure and function of biotin-dependent carboxylases. Cell Mol Life Sci 70:863–891
Zhang H, Luo Q, Gao H, Feng Y (2015) A new regulatory mechanism for bacterial lipoic acid synthesis. Microbiol Open 4:282–300

Functional Roles of Non-membrane Lipids in Bacterial Signaling

15

María J. Soto, N. Calatrava-Morales, and Isabel M. López-Lara

Contents

1 Introduction 274
2 Fatty Acids (DSF Family) 276
3 Fatty Acid Esters 279
4 Acyl-Based Molecules 279
4.1 *N*-Acylhomoserine Lactones (AHL) 279
4.2 γ-Butyrolactones (GBL) 280
5 Alkyl-Based Molecules 282
5.1 Alkyl-Quinolones (AQ) 282
5.2 Dialkylresorcinols 282
6 Alkane-Derived Signals (CAI-1 Family) 284
7 Bacterial Volatile Compounds (BVC) 285
8 Research Needs 287
References 287

Abstract

Bacteria produce some lipids and lipid-related compounds that function as signals for intercellular communication among prokaryotes or even in inter-kingdom communication (i.e., between prokaryotes and eukaryotes). Most of these lipidic signals participate in quorum-sensing regulation, a process that is dependent on cell density and enables a coordinated response within the pop-

M. J. Soto (✉) · N. Calatrava-Morales
Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain
e-mail: mariajose.soto@eez.csic.es; nieves.calatrava@eez.csic.es

I. M. López-Lara
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, Mexico
e-mail: isabel@ccg.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_16

ulation. The number and variety of bacterial non-membrane lipids that have been found to function as molecular signals is increasing and includes unsaturated fatty acids, fatty acid esters, acyl-based molecules such as *N*-acylhomoserine lactones and γ-butyrolactones, alkyl-based compounds such as quinolones and dialkylresorcinols, or alkane-derived signals such as α-hydroxyketones. Most of these signals are amphipathic and can diffuse through membranes and some of them are volatile. They are synthesized from common metabolites including intermediates of lipid metabolism and are recognized by membrane-bound or cytosolic receptors that trigger specific signal transduction responses. These signals regulate important bacterial traits such as motility, production of antimicrobials, expression of virulence factors, and biofilm formation. Some of these bacterial lipids induce immune responses in eukaryotic organisms.

1 Introduction

Lipids play multiple and important roles in bacterial life. They are part of different components of the bacterial cell envelope such as membrane phospholipids and other membrane lipids, lipoproteins, or the lipid A part of lipopolysaccharides. In addition to this primary structural role, lipids are also used to synthesize cofactors required in several enzymatic reactions, biosurfactants that impact colonization of ecological niches, or energy storage components. Perhaps the function less frequently associated with lipids, but focusing much attention in the last decades, is the role that some bacterial lipids and lipid-related compounds play as signaling molecules to communicate with individuals of the same or different species or even with eukaryotic organisms.

Few bacterial lipidic signals are mainly known for their role in inter-kingdom signaling. This is the case of lipochitooligosaccharides (also known as Nod factors) produced by rhizobia to trigger nodule organogenesis on their respective host plants. On the contrary, the majority of lipid-related signals produced by bacteria are recognized for having a role in quorum-sensing (QS) regulation. QS is a cell-to-cell communication process that allows bacteria to control gene expression in response to changes in the population density, altering collective behaviors that are crucial for survival to their changing environments. This regulatory process involves the production, release, and detection of extracellular diffusible molecules of different chemical classes, which are called autoinducers (AI) (Fig. 1) (Fuqua and Greenberg 2002; Ng and Bassler 2009).

N-acylhomoserine lactones (AHL) are the best known and most common class of lipidic AI which are used by Gram-negative bacteria. However, the number and types of lipid-related signals that have been shown to participate in intercellular communication controlling important bacterial phenotypes have been increasing during the last years. These signaling molecules are synthesized from common metabolites

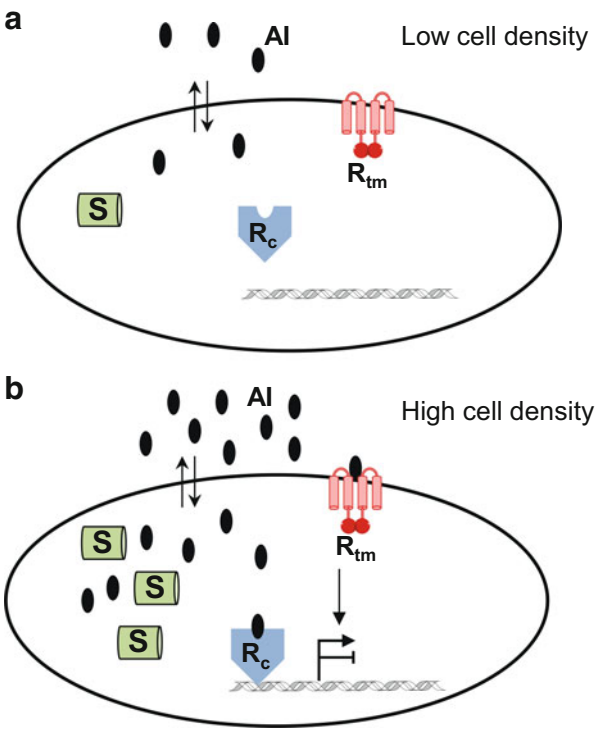


Fig. 1 General quorum sensing model based on autoinducers used in bacteria. (**a**) At low cell density, the autoinducer synthase (*S*) produces a basal level of autoinducer (*AI*). (**b**) At high cell density, the AI signal reaches a threshold and binds to a transmembrane receptor (R_{tm}) or cytosolic receptor (R_c), and transcription of genes is regulated

including intermediates of lipid metabolism, either with a single signal synthase or through a series of enzymatic reactions. After synthesis, AI are released into the extracellular environment, and when the concentration of the AI is above a certain threshold, the signal is recognized by specific receptors that reside either in the inner membrane or in the cytoplasm. Signal recognition elicits a signal transduction cascade that leads to increased AI production and a high cell density gene expression program. Many bacteria use several QS circuits which can be interconnected, enabling intra- and interspecies communication (Papenfort and Bassler 2016). These complex networks permit a faster and more coordinated adaptation of the bacterial population to the changing environment. Interestingly, some of these signaling molecules are also involved in inter-kingdom communication.

In this chapter we will describe several hydrophobic signaling compounds identified in bacteria with a recognized role in cell-to-cell communication. We will focus mainly on AI, making reference to their structure, biosynthesis, and recognition by their cognate receptors. We will describe some of the bacterial traits they regulate, and point out whether they participate in cross-kingdom communication. Finally, we will also refer to an increasing group of bacterial volatile compounds that function as airborne signals which can modulate at distance different bacterial traits and participate in inter-kingdom communication.

2 Fatty Acids (DSF Family)

Several Gram-negative bacteria comprising many phytopathogenic bacteria, as well as opportunistic animal pathogens, employ fatty acids in cell–cell signaling. Recognized as a unique chemical class of QS signals, these molecules are involved in intraspecies, interspecies, and even cross-kingdom communication. They regulate important bacterial phenotypes such as growth, virulence, antibiotic tolerance, motility, polymer production, biofilm formation, and persistence.

Fatty acid signals of the *D*iffusible *S*ignal *F*actor (DSF) family are *cis*-2-unsaturated fatty acids of different chain lengths and branching (Ryan and Dow 2011; Ryan et al. 2015). The *cis*-unsaturated double bond at the 2-position is the signature for the DSF family and a key structural feature for activity. The paradigm of these QS signals is the 11-methyl-*cis*-2-dodecenoic acid (Fig. 2a), which was the first DSF molecule to be identified from the plant pathogen *Xanthomonas campestris* pv. *campestris* (Xcc). Subsequently, additional signals exhibiting different chain length, lineal or branched, or with additional double bonds were described in bacteria such as *Burkholderia cenocepacia* (*cis*-2-dodecenoic acid or BDSF; Fig. 2a), *Pseudomonas aeruginosa* (*cis*-2-decenoic acid), *Xylella fastidiosa* (*cis*-2-tetradecenoic acid or XfDSF1 and *cis*-

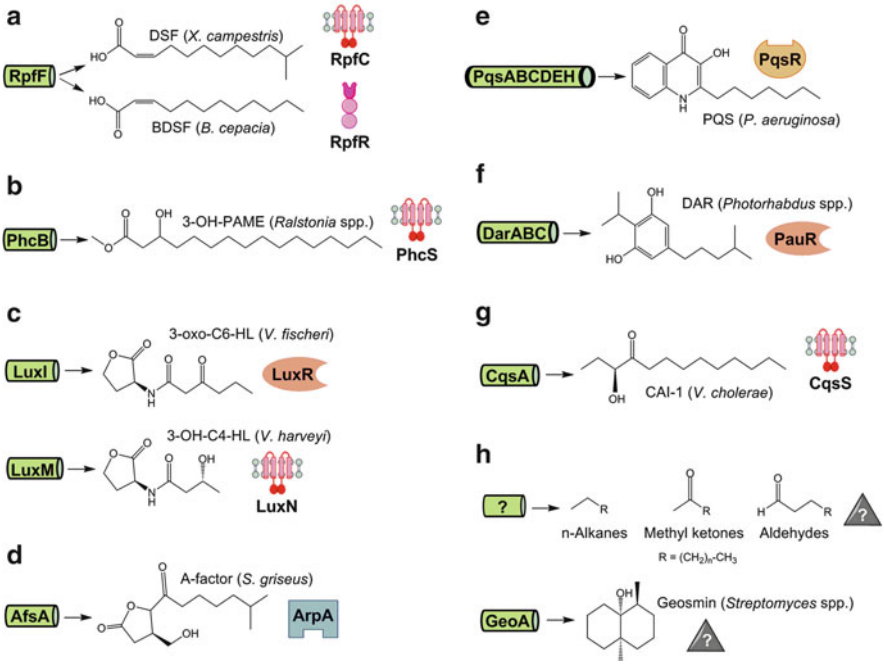


Fig. 2 Representative structures of bacterial lipid-related signals, key enzymes involved in their synthesis (*cylinders*), and their cognate receptors (for details see text). (**a**) Fatty acids. (**b**) Fatty acid esters. (**c**) *N*-acylhomoserine lactones. (**d**) γ-butyrolactones. (**e**) Alkyl-quinolones. (**f**) Dialkylresorcinol. (**g**) α-hydroxyketones. (**h**) Bacterial volatile compounds

2-hexadecenoic acid or XfDSF2), and *Xanthomonas oryzae* (11-methyldodeca-*cis*, *cis*-2,5-dienoic acid or CDSF). It is now known that, with the exception of *P. aeruginosa*, each of these bacteria produces multiple DSF family signals, although each genus seems to be most responsive to the major signal that it produces. The amounts and relative proportions of these signals are influenced by culture medium composition. Thus, Xcc mainly produces BDSF in the presence of carbohydrates, whereas branched chain amino acids are required for biosynthesis of DFS-like signals containing methyl ramifications (Zhou et al. 2015a).

The *rpf* (*r*egulator of *p*athogenicity *f*actor) gene cluster encodes proteins that control synthesis, perception, and transduction of the DSF family signals. In Xcc, biosynthesis of these signaling molecules requires RpfF and the classic fatty acid synthesis elongation cycle (Zhou et al. 2015a). RpfF is a unique crotonase that shows both thioesterase and dehydratase activities on intermediates of fatty acid synthesis, acyl–acyl carrier protein (acyl-ACP) substrates (Bi et al. 2012). RpfF first catalyzes the formation of a double bond between carbons 2 and 3 of a 3-hydroxyacyl moiety and then hydrolyzes the thioester bond with ACP to release a free acid (Fig. 3a). RpfB is a fatty acyl-CoA ligase that activates a wide range of long-chain fatty acids to their CoA thioesters and

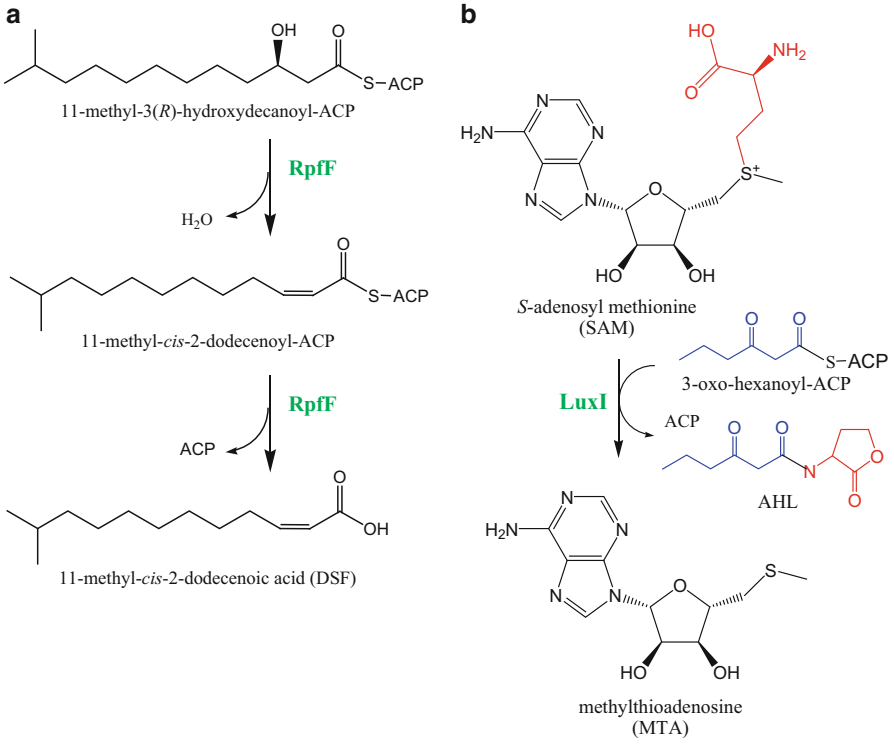


Fig. 3 Biosynthesis of DSF (**a**) and of AHL (**b**). (**a**) The RpfF enzyme performs two reactions, a dehydration followed by a thioester cleavage, resulting in DSF formation. (**b**) Lux I synthase condenses the homoserine lactone ring provided by SAM to the fatty acyl moiety

was originally thought to be involved in DSF synthesis or processing. However, it has been shown that the role played by RpfB in pathogenesis is to counteract the thioesterase activity of the DSF synthase RpfF (Bi et al. 2014). More recently, it has been reported that RpfB represents a DSF family signal turnover system, which could be used by different bacterial cells to exit from the QS mode (Zhou et al. 2015b).

Two different core pathways have been involved in DSF perception (Ryan et al. 2015): (i) the RpfCG two-component system identified in *Xanthomonas* spp., and (ii) the cytosolic RpfR protein first identified in *Burkholderia* spp. Accessory DSF-sensing systems have also been described in these bacteria. The RpfCG two-component system comprises the complex sensor kinase RpfC and the response regulator RpfG. RpfC is a dual-function protein that not only transduces the DSF family signal to its cognate response regulator RpfG using a conserved phosphorelay mechanism but also negatively controls DSF family signal production through a specific protein–protein interaction with the DSF synthase RpfF. RpfG is a phosphodiesterase that is activated by RpfC upon recognition of DSF-like signals, leading to the enzymatic degradation of intracellular second messenger cyclic dimeric guanosine monophosphate (c-di-GMP). In *Burkholderia* spp., DSF-like signals can be detected by RpfR, a cytosolic protein that exhibits an N-terminal PER-ARNT-SIM (PAS) domain and GGDEF and EAL domains. PAS domains are sensory modules, typically sensing oxygen tension, redox potential, or light intensity, although they can also mediate protein–protein interactions or bind small ligands (Henry and Crosson 2011). GGDEF and EAL domains, named after their conserved residues, are implicated in the synthesis and degradation, respectively, of c-di-GMP. Binding of BDSF to the N-terminal PAS domain leads to c-di-GMP degradation. Thus in different bacteria sensing of a DSF family signal is linked to c-di-GMP turnover. This second messenger plays a major role in controlling lifestyle changes of many bacteria, including transition from the motile to the sessile or biofilm state or from the virulent state in acute infections to the less virulent but more resistant state characteristic of chronic infectious diseases.

In many bacteria, DSF family-mediated signaling contributes to bacterial virulence by increasing the production of extracellular enzymes and polysaccharides, the formation of surface-associated bacterial communities or biofilms, and antibiotic tolerance. In addition to intraspecies signaling, DSF family signals have been implicated in interspecies and inter-kingdom signaling, in which they modulate the behavior of other microorganisms that do not produce the signal. For example, although *P. aeruginosa* does not synthesize DSF or BDSF, it is capable of sensing these molecules, with consequences for bacterial behavior (biofilm formation, increased resistance to polymyxin, persistence in mouse). In other bacteria such as *Bacillus*, *Staphylococcus*, and *Escherichia coli*, DSF perception leads to inhibition of biofilm formation or susceptibility to antibiotics. Finally, the DSF family of signal molecules has also been implicated in inter-kingdom signaling. DSF, BDSF, and CDSF can all act to prevent the morphological yeast-hyphal transition of the fungal pathogen *Candida albicans*. More recently, DSF produced by Xcc has been shown to elicit plant defenses: callose deposition, plant cell death, and induction of the PR1

defense gene (Kakkar et al. 2015). Indeed, treatment with DSF leads to a decrease in Xcc virulence and disease severity.

3 Fatty Acid Esters

Long-chain fatty acid methyl esters, specifically methyl 3-hydroxypalmitate (3-OH-PAME) (Fig. 2b) and methyl 3-hydroxymyristate (3-OH-MAME), have been identified as diffusible volatile factors responsible for activating virulence gene expression in different strains of the phytopathogen *Ralstonia solanacearum*, a soilborne β-proteobacterium that causes a lethal bacterial wilt affecting many economically important plants (Flavier et al. 1997; Kai et al. 2015). These fatty acid-derived signals function as AI of a genus-specific QS system referred to as "*phc* QS." Synthesis of 3-OH-PAME and 3-OH-MAME is mediated by PhcB, an *S*-adenosylmethionine (SAM)-dependent methyl transferase which is thought to convert 3-hydroxyacyl-ACP intermediates in the synthesis of fatty acids to the corresponding 3-hydroxyacyl methyl ester.

The *phcB* gene forms an operon together with the two-component system genes *phcS/phcR*, coding for the histidine kinase sensor and response regulator, respectively. Genetic data indicate that the three genes function together to regulate expression of the *phcA* gene, which codes for a LysR-type transcriptional regulator that controls expression of key virulence factors in *Ralstonia* spp. such as exopolysaccharide, plant cell wall-degrading enzymes, and ralfuranones. When the concentration of the fatty acid ester reaches threshold levels, the PhcS/PhcR two-component system activates PhcA, which in turn induces the production of virulence factors.

4 Acyl-Based Molecules

4.1 *N*-Acylhomoserine Lactones (AHL)

These are the best studied QS signal molecules and represent the main group of AI used by Gram-negative bacteria for cell-to-cell communication. AHL participate in the coordinated control of a wide variety of cell density-dependent behaviors, such as motility, biofilm formation, production of virulence factors, bioluminescence emission, or nodulation (Galloway et al. 2011). These molecules share a common structure based on a homoserine lactone ring, which is *N*-acylated with a 4–18 carbon acyl chain that can contain modifications such as several degrees of unsaturation, or the presence of different substituents (3-oxo, 3-hydroxy groups) at carbon 3 (Fig. 2c). This chemical composition endows AHL with an amphipathic nature, making them able to diffuse through cell membranes.

The first AHL-based QS system discovered was that of the marine bacterium *Vibrio fischeri*, which is considered the prototype of QS in Gram-negative bacteria. It comprises the AI *N*-3-oxohexanoyl-HSL (Fig. 2c), the LuxI synthase, and the LuxR receptor. The LuxI synthase catalyzes AHL production from two substrates: SAM,

which provides the homoserine lactone ring moiety, and an appropriate acyl-ACP, which is the precursor of the fatty acyl side chain (Fig. 3b). AHL are sensed by specific LuxR-type receptors, which are cytosolic transcriptional regulators.

There are some remarkable exceptions to the canonical synthesis/recognition model. For example, the plant-associated photosynthetic bacterium *Rhodopseudomonas palustris* uses the plant-derived metabolite *p*-coumarate instead of intermediates in the bacterial fatty acid synthesis for the acyl chain of the AHL 4-coumaroyl-HSL (Schaefer et al. 2008). Besides LuxI-type synthases, two other families of synthases, called LuxM (Fig. 2c) and HdtS, can participate in the synthesis of AHL. The first one functions in a similar way to the LuxI-type synthases except for the utilization of acyl-coenzyme A instead of acyl-ACP as substrates. On the contrary, HdtS synthases can use both types of thioester intermediates, although the molecular mechanism is still unknown. Concerning AHL recognition, some atypical receptors have been described. The LuxN receptor of *V. harveyi* is a two-component membrane-bound histidine kinase that phosphorylates cytosolic transcription factors upon binding to its cognate AHL (Fig. 2c). Furthermore, transcriptional repression associated to AHL-LuxR complexes has been reported.

In addition to cell–cell communication, AHL have also been involved in inter-kingdom signaling. These molecules can be recognized by plants leading to specific responses (Mathesius et al. 2003). Interestingly, some AHL are able to prime plants for enhanced defense against pathogens (Schenk et al. 2014).

4.2 γ-Butyrolactones (GBL)

These acyl-based molecules are produced by Gram-positive soil bacteria of the genus *Streptomyces*. The structure of γ-butyrolactones (GBLs) consists of a common lactone ring of 3*R*–hydroxymethyl-1,4-butyrolactone linked to a variable fatty acid side chain which is generally species-specific (Fig. 2d) (Biarnes-Carreras et al. 2015; Niu et al. 2016). These compounds induce the production of antibiotics and other secondary metabolites and control morphological differentiation in *Streptomyces* spp., phenotypes that require a strictly coordinated population communication (Takano 2006; Biarnes-Carrera et al. 2015). Autoregulatory factor, or A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), is a representative of the GBL family (Fig. 2d). It regulates production of streptomycin in *Streptomyces griseus*. The protein AfsA is the key enzyme in A-factor biosynthesis. This enzyme transfers the β-ketoacyl group from 8-methyl-3-oxononanoyl-ACP to the hydroxyl group of dihydroxyacetone phosphate (DHAP), forming a fatty acid ester. Subsequent dephosphorylation, aldol condensation, and reduction reactions are required to generate A-factor (Fig. 4) (Niu et al. 2016).

A-factor, which can freely diffuse across cell membranes, is recognized by a cytoplasmic receptor protein belonging to the TetR family of transcriptional regulators, ArpA. In the absence of A-factor, this protein acts as a repressor of AdpA, an AraC/XylS-like master regulator. As the concentration of A-factor increases with the population growth, it binds to ArpA, releasing repression of *adpA* transcription. This

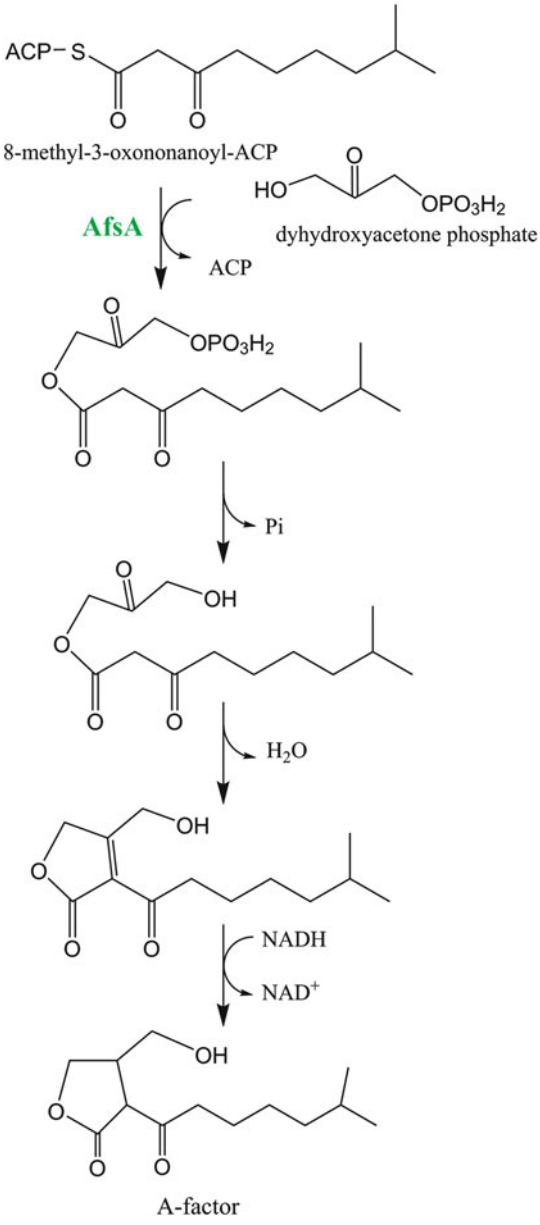


Fig. 4 Biosynthesis of A-factor. AfsA transfers the β-ketoacyl group from 8-methyl-3-oxononanoyl-ACP to dihydroxyacetone phosphate forming a fatty acid ester. Subsequent dephosphorylation, aldol condensation, and reduction reactions are required to generate A-factor

master regulator is ultimately responsible for the transition to the antibiotic-producing stage of *S. griseus*, among other phenotypes. Homologues to *afsA* and *arpA* genes have been found in all sequenced species of *Streptomyces* although variations in the biosynthetic and regulatory pathways have also been identified (Niu et al. 2016).

5 Alkyl-Based Molecules

5.1 Alkyl-Quinolones (AQ)

Quinolones are fused heterocyclic compounds that form the active structure of a wide range of potent broad-spectrum antimicrobial agents (Heeb et al. 2011). An important class of quinolones are the 2-alkyl-4(1*H*)-quinolones (AQ) produced by *P. aeruginosa*. Initially known for their antimicrobial activity against Gram-positive and Gram-negative bacteria, the function of some of these compounds in intercellular signaling has focused much attention. AQ such as 2-heptyl-3-hydroxy-4(1*H*)-quinolone (termed the *Pseudomonas* Quinolone Signal or PQS) (Fig. 2e) (Pesci et al. 1999) and its biological precursor 2-heptyl-4(1*H*)-quinolone (HHQ) are used as QS signal molecules by pathogenic Gram-negative bacteria, including *P. aeruginosa*, and certain *Burkholderia* and *Alteromonas* strains. AQ signaling pathways in these species control production of several virulence determinants including biofilm formation. Because of its hydrophobic nature, a high proportion is associated with the bacterial outer membrane and with membrane vesicles.

AQ are derived from condensation reactions of anthranilate with malonyl-CoA and octanoyl-CoA. The *pqsR*, *pqsABCDE*, and *pqsH* genes are required for AQ biosynthesis in *P. aeruginosa*. PqsR is a LysR-type transcriptional regulator that, after binding the autoinducer (PQS or HHQ), activates expression of the biosynthetic *pqs* operon. The first step in the biosynthesis involves the activation of anthranilate by PqsA, an anthranilate coenzyme A ligase (Fig. 5). PqsD shares some sequence similarity with the *E. coli* initiation condensing enzyme FabH and catalyzes the decarboxylating condensation of anthraniloyl-CoA and malonyl-CoA resulting in 2-aminobenzoylacetyl-CoA. Subsequently, CoA is hydrolyzed by the action of the thioesterase PqsE. PqsB and PqsC, which are similar to β-keto-acyl-ACP synthases involved in fatty acid synthesis and form a heterodimer, perform another decarboxylating condensation using octanoyl-CoA leading to the PQS precursor HHQ (Drees et al. 2016). The *pqsH* gene encodes a predicted FAD-dependent monooxygenase that hydroxylates the 3-′-carbon atom of HHQ in the final step of PQS biosynthesis (Fig. 5). Besides autoinduction, PQS synthesis is subject to complex regulation in which additional QS systems as well as nutrient availability play important roles.

A remarkable property of PQS is its ability to chelate ferric iron, a function which is mediated by the 3′-hydroxy group present on the molecule. HHQ is unable to do so. Addition of PQS to *P. aeruginosa* cultures upregulates genes involved in the production of the siderophores pyoverdine and pyochelin, which are produced in response to iron starvation. Although PQS may trap iron in the cell membrane, it is unlikely that it can act as a siderophore per se.

5.2 Dialkylresorcinols

Since the initial identification of bacterial dialkylresorcinols (DAR) in 1975, several biological activities were associated with these compounds including antimicrobial

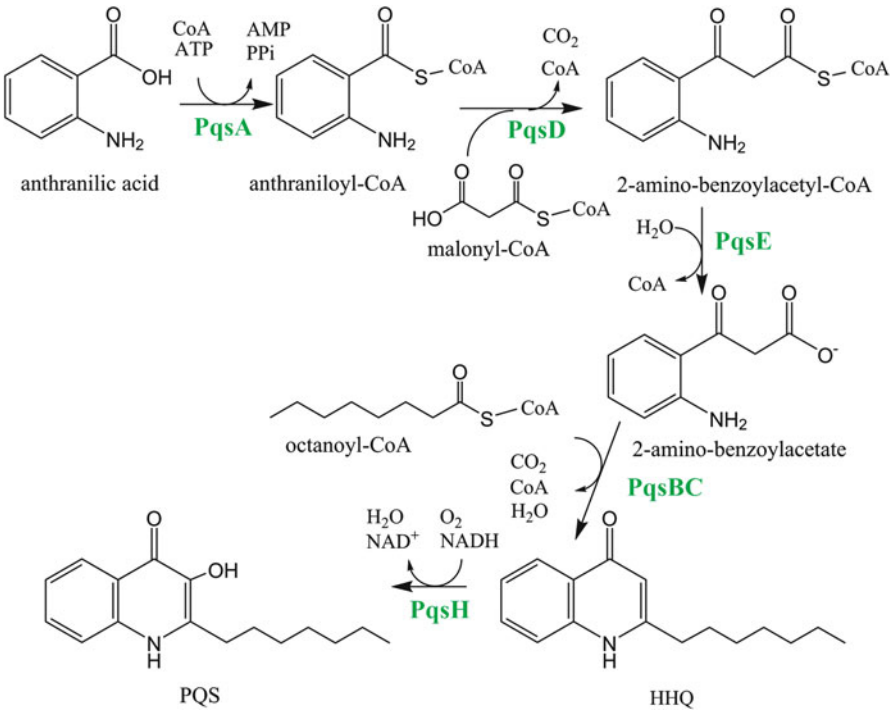


Fig. 5 Biosynthesis pathway for PQS. PqsA activates anthranilic acid to anthraniloyl-CoA. PqsD condenses malonyl-CoA and anthraniloyl-Co-A to form 2-amino-benzoylacetyl-CoA. After hydrolytic release of CoA, PqsBC condenses octanoyl-CoA with 2-amino-benzoylacetate to form HHQ. PqsH hydroxylates HHQ to obtain PQS

activity, protection from free radical-induced lipid peroxidation, or plant protection effect against pathogenic fungi (reviewed in Schöner et al. 2015). Recently, DAR have been described as signaling molecules that participate in QS regulation in the insect and human pathogen *Photorhabdus asymbiotica* (Brameyer et al. 2015). These molecules are sensed by PauR, a LuxR-type receptor that does not sense AHL. Upon detection of the signal, PauR activates expression of the *Photorhabdus* clumping factor (*pcf*) locus, leading to PCF-dependent cell clumping that contributes to the pathogenicity of *P. asymbiotica* toward insect larvae. Production of the signal requires the *darABC* biosynthetic gene cluster that comprises the ACP-encoding *darC*, the 3-ketoacyl-ACP synthase III (KAS III)-encoding *darB*, and the aromatase-encoding gene *darA*. DarB catalyzes the cyclization of a β-keto-acyl precursor with an α,β-unsaturated acyl precursor to form the 4-carboxy-cyclohexanedione (4-carboxy-CHD). Due to a possible spontaneous hydrolysis and decarboxylation, CHD is formed. In the final step of the biosynthesis, DarA oxidizes either 4-carboxy-CHD, CHD, or both to the corresponding DAR derivative. The synthesis pathway is widespread, and often present in human pathogens, which might suggest that DAR are widespread signaling molecules.

6 Alkane-Derived Signals (CAI-1 Family)

A group of aquatic γ-proteobacteria that includes the opportunistic pathogens *Vibrio cholerae* and *Legionella pneumophila*, which cause cholera and Legionnaires' pneumonia, respectively, are able to produce and detect α-hydroxyketones (AHK) as QS signaling molecules (Tiaden et al. 2010). The *C*holera *A*uto*I*nducer-*1* (CAI-1; 3-hydroxytridecane-4-one) was the first molecule of this group to be identified (Fig. 2g) (Miller et al. 2002). We now know that CAI-1 is only one member of a family of structurally similar signals made by diverse proteobacteria. The family comprises ketones in which the carbonyl group is at position 4 that vary at carbons 2 and 3 and in the length of their acyl groups. C3 can have a hydroxy or amino group, and the bonds between C2 and C3 can be saturated or unsaturated. The "*Legionella a*utoinducer-*1*" (LAI-1) is 3-hydroxypentadecane-4-one (Spirig et al. 2008). Both CAI-1 and LAI-1 are diffusible, volatile AI.

AHK signaling regulates important phenotypes in *Vibrio* and *Legionella* including virulence, formation of biofilms, pathogen–host cell interactions, expression of a genomic "fitness island," and natural competence for DNA uptake. At low cell density, *V. cholerae* activates the expression of virulence factors and forms biofilms. At high cell density, the accumulation of CAI-1 represses production of the canonical virulence factor TCP (toxin co-regulated pilus), while motility and competence are induced (Ng and Bassler 2009). *Legionella* quorum sensing (*lqs*) mutants are severely defective for host cell uptake and intracellular replication, produce extracellular filaments, are impaired for motility, and show enhanced natural competence for DNA acquisition (Tiaden and Hilbi 2012; Schell et al. 2016). Therefore, CAI-1 and LAI-1 regulate the two different lifestyles exhibited by these bacteria characterized by the transition between environmental and host-associated niches. Recently, LAI-1 was shown to be involved in inter-kingdom communication, modulating host cell migration.

The Lqs system comprises the membrane-bound sensor kinases LqsS and LqsT, the prototypic response regulator LqsR, and the autoinducer synthase LqsA (Spirig et al. 2008). The Cqs system comprises the sensor kinase CqsS and the autoinducer synthase CqsA. In contrast to *L. pneumophila*, *Vibrio* spp. utilize the Cqs system together with elements of different QS systems, such as the LuxU and LuxO proteins (Papenfort and Bassler 2016). CqsS and LqsS belong to the class of six transmembrane helix two-component sensor histidine kinases that couple the detection of the AI molecules via a receptor domain at the N-terminus to signal transduction modules at the C-terminal part of the protein.

CqsA and LqsA are 45% identical, and both enzymes are predicted to be related to pyridoxal-5′-phosphate (PLP)-dependent aminotransferases. SAM was identified as a biosynthetic substrate for CqsA. CqsA couples SAM and decanoyl-CoA to produce the novel compound 3-aminotridec-2-en-4-one (Ea-CAI-1) (Fig. 6). The unstable enamine Ea-CAI-1 is converted to CAI-1 via the spontaneous conversion into tridecane-3,4-dione (DK-CAI-1) followed by an NADPH-dependent reduction catalyzed by a dehydrogenase (reviewed in Tiaden and Hilbi 2012). CqsA and LqsA exhibit relaxed specificity regarding the acyl-CoA tail, and therefore these proteins can produce different CAI-1- and LAI-1-related signals.

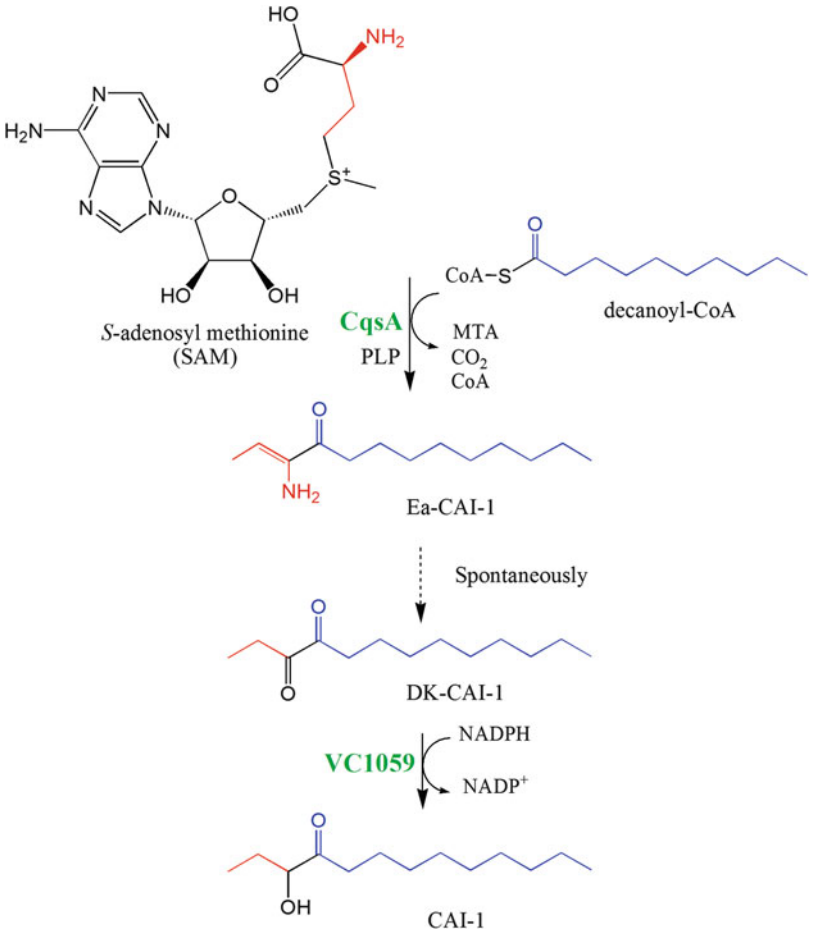


Fig. 6. Biosynthesis of CAI-1. SAM and decanoyl-CoA are the substrates for CqsA, resulting in the formation of Ea-CAI-1. It is suggested that conversion of Ea-CAI-1 to DK-CAI-1 occurs spontaneously. VC1059 is the enzyme responsible for conversion of DK-CAI-1 to CAI-1

Homologues to the *lqs*/*cqs* gene clusters have been identified in a number of environmental bacteria, indicating that AHK signaling might be widely used for cell–cell signaling.

7 Bacterial Volatile Compounds (BVC)

Some lipidic compounds produced by bacteria are volatile, i.e., molecules of low molecular weight (<300 Da) and high vapor pressure (>0.01 kPa at 20 °C) that can readily evaporate and diffuse. This group includes several fatty acid derivatives and terpenoids (Fig. 2h). The former are generated from intermediates of the fatty acid

biosynthesis or degradation pathways, through different processes (Schulz and Dickschat 2007). Thus, a decarboxylation step reduces polarity and water solubility, yielding compounds such as alkanes, 1-alkenes, or methyl ketones. Another possibility to form BVC is the reduction of the carboxyl group to aldehydes and 1-alkanols. Hydrocarbons and acids occur less often than ketones and alcohols. Methyl ketones with an odd number of carbon atoms (acetone to pentadecan-2-one) are derived from even-numbered β-keto acids by decarboxylation, and occur in many bacteria. In contrast, even-numbered methyl ketones arise from fatty acids with an odd number of carbons, and are therefore rarer. Unbranched aldehydes are also relatively rare, most probably due to their high reactivity. Long-chain aliphatic alcohols (i.e., 1-octanol, 1-decanol, and 1-dodecanol) are produced through β- or α-oxidation of fatty acid derivatives. In contrast, terpenoids are derived from the terpene building units dimethylallyl pyrophosphate and isopentenyl pyrophosphate, which can arise either from the mevalonate pathway or the deoxyxylulose phosphate pathway. Only monoterpenes (C_{10}), sesquiterpenes (C_{15}), and their derivatives or degradation products have been reported from bacterial volatile blends (Schulz and Dickschat 2007). An example of BVC belonging to this chemical group is the earthy-odor compound geosmin (Fig. 2h), a noncanonical terpenoid ($C_{12} \neq C_{5n}$) which is produced by several bacterial species, through the action of a bifunctional sesquiterpene cyclase that catalyzes tandem cyclization and fragmentation reactions utilizing the C_{15} substrate farnesyl diphosphate to yield geosmin, acetone, and inorganic pyrophosphate. Another example is albaflavenone, a sesquiterpene with powerful earthy camphor-like aroma, which is produced exclusively by *Streptomyces* spp.

Different and interesting functions have been assigned to some BVC (Audrain et al. 2015). The fatty acid esters 3-OH-PAME and 3-OH-MAME produced by *Ralstonia* spp. and the α-hydroxyketones (AHKs) produced by *Vibrio* and *Legionella* spp. have a signaling role as AI in QS regulation. Given the relevance of these molecules in controlling the virulence of important plant and animal pathogens, they have been described in separate sections of this chapter (see Sects. 3 and 6). Effects on bacterial growth and development have also been reported associated to different lipidic BVC. For example, production of geosmin correlates with sporulation in *Streptomyces albidoflavus*. Albaflavenone exhibits antibacterial activity against *Bacillus subtilis*, and volatile short-chain fatty acids produced through fermentation by intestinal bacteria inhibit growth of several enteropathogens. Compounds such as 1-butanol, hexadecane, and several methyl ketones (2-butanone, 2-heptanone, 2-nonanone, and 2-undecanone) affect biofilm formation in different bacteria. The influence of some BVC on bacterial pathogenicity occurs by increasing the production of virulence factors, like the effect caused by 2,3-butanediol in *P. aeruginosa*. Receptors and downstream signaling components for these BVC are mostly unknown. Interestingly, lipidic volatiles also contribute to inter-kingdom interactions, modulating host defense responses, and they can induce plant resistance to environmental stresses and pathogens. 2,3-Butanediol emitted from plant growth-promoting rhizobacteria induces systemic resistance to drought and also to bacterial pathogens. Long-chain volatiles such as tridecane and

hexadecane produced by *Paenibacillus polymyxa* can protect *Arabidopsis thaliana* plants via induced systemic resistance against several phytopathogens (Park et al. 2013).

8 Research Needs

Given the effects of several bacterial lipid-related signals on the virulence and biofilm formation of important plant and animal pathogens, research in this field will potentially lead to clinical and biotechnological applications. It is crucial to obtain fundamental insights on the metabolism of all of these signals. Identification of the key enzymes involved in their synthesis and turnover, deciphering at the molecular level their mechanism of action, or identifying additional regulatory mechanisms that control their expression would allow the development of new strategies to manipulate intercellular communication. Likewise, research on the signal receptors and downstream signaling cascades would help to rationally design drugs. Last but not least, understanding how these bacterial signals are recognized by eukaryotic organisms will increase our knowledge about potential host reactions to bacteria. This will also facilitate the development of biotechnological strategies for enhanced protection against harmful pathogens.

Acknowledgments Work on MJS's lab was funded by Grant BIO2013-42801-P. NCM was supported by an FPU fellowship from the Spanish Ministry of Education and Science. Work on IMLL's lab was funded by Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México (DGAPA-UNAM; PAPIIT IN202616).

References

Audrain B, Farag MA, Ryu CM, Ghigo JM (2015) Role of bacterial volatile compounds in bacterial biology. FEMS Microbiol Rev 39:222–233

Bi H, Christensen QH, Feng Y, Wang H, Cronan JE (2012) The *Burkholderia cenocepacia* BDSF quorum sensing fatty acid is synthesized by a bifunctional crotonase homologue having both dehydratase and thioesterase activities. Mol Microbiol 83:840–855

Bi H, Yu Y, Dong H, Wang H, Cronan JE (2014) *Xanthomonas campestris* RpfB is a fatty acyl-CoA ligase required to counteract the thioesterase activity of the RpfF Diffusible Signal Factor (DSF) synthase. Mol Microbiol 93:262–275

Biarnes-Carrera M, Breitling R, Takano E (2015) Butyrolactone signalling circuits for synthetic biology. Curr Opin Chem Biol 28:91–98

Brameyer S, Kresovic D, Bode HB, Heermann R (2015) Dialkylresorcinols as bacterial signaling molecules. Proc Natl Acad Sci U S A 112:572–577

Drees SL, Li C, Prasetya F, Saleem M, Dreveny I, Williams P, Hennecke U, Emsley J, Fetzner S (2016) PqsBC, a condensing enzyme in the biosynthesis of the *Pseudomonas aeruginosa* quinolone signal: crystal structure, inhibition, and reaction mechanism. J Biol Chem 291:6610–6624

Flavier AB, Clough SJ, Schell MA, Denny TP (1997) Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. Mol Microbiol 26:251–259

Fuqua C, Greenberg EP (2002) Listening in on bacteria: acylhomoserine lactone signalling. Nat Rev Mol Cell Biol 3:685–695

Galloway WRJD, Hodgkinson JT, Bowden SD, Welch M, Spring DR (2011) Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. Chem Rev 111:28–67

Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M (2011) Quinolones: from antibiotics to autoinducers. FEMS Microbiol Rev 35:247–274

Henry JT, Crosson S (2011) Ligand-binding PAS domains in a genomic, cellular, and structural context. Annu Rev Microbiol 65:261–286

Kai K, Ohnishi H, Shimatani M, Ishikawa S, Mori Y, Kiba A, Ohnishi K, Tabuchi M, Hikichi Y (2015) Methyl 3-hydroxymyristate, a diffusible signal mediating *phc* quorum sensing in *Ralstonia solanacearum*. Chembiochem 16:2309–2318

Kakkar A, Nizampatnam NR, Kondreddy A, Pradhan BB, Chatterjee S (2015) *Xanthomonas campestris* cell-cell signalling molecule DSF (diffusible signal factor) elicits innate immunity in plants and is suppressed by the exopolysaccharide xanthan. J Exp Bot 66:6697–6714

Mathesius U, Mulders S, Gao M, Teplitski M, Caetano-Anolles G, Rolfe BG et al (2003) Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. Proc Natl Acad Sci U S A 100:1444–1449

Miller MB, Skorupski K, Lenz DH, Taylor RK, Bassler BL (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. Cell 110:303–314

Ng WL, Bassler BL (2009) Bacterial quorum-sensing network architectures. Annu Rev Genet 43 (1):97–222

Niu G, Chater KF, Tian Y, Zhang J, Tan H (2016) Specialised metabolites regulating antibiotic biosynthesis in *Streptomyces* spp. FEMS Microbiol Rev 40:554–573

Papenfort K, Bassler BL (2016) Quorum sensing signal-response systems in Gram-negative bacteria. Nat Rev Microbiol 14:576–588

Park HB, Lee B, Kloepper JW, Ryu CM (2013) One shot-two pathogens blocked: exposure of *Arabidopsis* to hexadecane, a long chain volatile organic compound, confers induced resistance against both *Pectobacterium carotovorum* and *Pseudomonas syringae*. Plant Signal Behav 8: e24619

Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 96:11229–11234

Ryan RP, Dow JM (2011) Communication with a growing family: diffusible signal factor (DSF) signaling in bacteria. Trends Microbiol 19:145–152

Ryan RP, An SQ, Allan JH, McCarthy Y, Dow JM (2015) The DSF family of cell-cell signals: an expanding class of bacterial virulence regulators. PLoS Pathog 11(7):e1004986

Schaefer AL, Greenberg EP, Oliver CM, Oda Y, Huang JJ, Bittan-Banin G, Peres CM, Schmidt S, Juhaszova K, Sufrin JR, Harwood CS (2008) A new class of homoserine lactone quorum-sensing signals. Nature 454:595–599

Schell U, Simon S, Sahr T, Hager D, Albers MF, Kessler A, Fahrnbauer F, Trauner D, Hedberg C, Buchrieser C, Hilbi H (2016) The alpha-hydroxyketone LAI-1 regulates motility, Lqs-dependent phosphorylation signalling and gene expression of *Legionella pneumophila*. Mol Microbiol 99:778–793

Schenk ST, Hernandez-Reyes C, Samans B, Stein E, Neumann C, Schikora M, Reichelt M, Mithofer A, Becker A, Kogel KH, Schikora A (2014) *N*-acyl-homoserine lactone primes plants for cell wall reinforcement and induces resistance to bacterial pathogens via the salicylic acid/oxylipin pathway. Plant Cell 26:2708–2723

Schöner TA, Kresovic D, Bode H (2015) Biosynthesis and function of bacterial dialkylresorcinol compounds. Appl Microbiol Biotechnol 99:8323–8328

Schulz S, Dickschat JS (2007) Bacterial volatiles: the smell of small organisms. Nat Prod Rep 24:814–842

Spirig T, Tiaden A, Kiefer P, Buchrieser C, Vorholt JA, Hilbi H (2008) The *Legionella* autoinducer synthase LqsA produces an α-hydroxyketone signaling molecule. J Biol Chem 283:18113–18123

Takano E (2006) Gamma-butyrolactones: *Streptomyces* signalling molecules regulating antibiotic production and differentiation. Curr Opin Microbiol 9:287–294

Tiaden A, Spirig T, Hilbi H (2010) Bacterial gene regulation by α-hydroxyketone signaling. Trends Microbiol 18:288–297

Tiaden A, Hilbi H (2012) α-Hydroxyketone synthesis and sensing by *Legionella* and *Vibrio*. Sensors 12:2899–2919

Zhou L, Yu Y, Chen X, Diab AA, Ruan L, He J, Wang H, He YW (2015a) The multiple DSF-family QS signals are synthesized from carbohydrate and branched-chain amino acids via the FAS elongation cycle. Sci Rep 5:13294

Zhou L, Wang XY, Sun S, Yang LC, Jiang BL, He YW (2015b) Identification and characterization of naturally occurring DSF-family quorum sensing signal turnover system in the phytopathogen *Xanthomonas*. Environ Microbiol 17:4646–4658

Formation of Lipochitin Oligosaccharide Signaling Molecules

16

V. Poinsot and F. Couderc

Contents

1 Introduction 292
2 Biosynthesis of LCOs in the Rhizobial Model 293
2.1 Synthesis of the Oligochitin Backbone 294
2.2 *N*-Decoration 296
2.3 *O*-Decorations of LCOs 299
2.4 Regulation of the Nod Genes 301
2.5 Secretion 302
2.6 Topology and Time Course of the Biosynthesis 303
3 Biosynthesis in Other Models (Fungi or Non-rhizobial Bacteria) 305
3.1 Presence of the *NodBC*-like Genes 305
3.2 Other Synthesis of the CO Backbone: Chitin Hydrolases 307
3.3 Existence of *NodA*-like Genes 308
4 Conclusion and Research Needs 308
References 309

Abstract

Lipochitinoligosaccharides (LCOs) are made up of an *N*-acetylglucosamine backbone of four to five monosaccharide units bearing at it nonreducing end an *N*-linked fatty acyl chain. They are partly secreted as signaling molecules and are playing key roles in plant-root symbioses. However, these molecules are synthesized at the inner membrane and accumulated there. Therefore, they can be considered as membrane-associated lipids. We summarize in this chapter the outstanding contributions performed in the 1990s to the biosynthesis of these compounds in the rhizobium background, where the LCOs are named Nod factors. Recent work provided a new perspective to the time course of this complex biosynthetic pathway, and we will particularly elaborate it here. Finally,

V. Poinsot (✉) · F. Couderc
Laboratoire IMRCP UMR5623 UPS/CNRS, Université Paul Sabatier, Toulouse, France
e-mail: poinsot@chimie.ups-tlse.fr; couderc@chimie.ups-tlse.fr

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_64

since Nod factor-like molecules were attested to be synthesized by the mycorrhiza, the actual trend is to understand their synthesis in fungi and to extend their inventory within this reign. Even if significant efforts are deployed nowadays, only little has been published on this topic. We aim to summarize these contributions to open up new avenues of investigation enabled by rapid genomic analysis.

1 Introduction

Due to their environmental and agronomical importance, studies on root endosymbiosis are widespread. Two systems are especially investigated, the nitrogen-fixing symbiosis and mycorrhization. The first one involves bacteria belonging to the rhizobia family and leguminous plants. Rhizobia comprise alpha-proteobacteria genera (*Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium*, and *Bradyrhizobium*) but also members of the beta-proteobacteria (Moulin et al. 2001). The word rhizobia implies therefore rather the ability to be a root endosymbiont than a phylogenetic assignment. The second endosymbiosis involves the arbuscular mycorrhizal (AM) fungi and a wide spectrum of plants.

Contrary to the AM fungi, rhizobia can be cultivated in absence of any host. Moreover, the genetic manipulation of the bacteria is far easier than of mycorrhizal fungi. As such, the molecular events in nitrogen fixation are far better understood (for review see Perret et al. 2000; D'Haeze and Holsters 2002; and Fraysse et al. 2003). The maximum number of publications in this field was generated at the end of the 1990s (close to 100). However, investigations are still ongoing mainly on the plant perception mechanisms (for review: Gough and Jacquet 2013) and on the infection negative autoregulation involving plant and bacteria (Kassaw et al. 2015).

In the nitrogen fixing symbiosis, the interaction between the partners starts with a chemotaxis. Actually, the plant attractiveness correlates to the spectrum of secreted phenolic substances. Flavonoid (2-phenyl-1,4-benzopyrone) concentration increases in the rhizosphere when the compatible partner is found (Schmidt et al. 1994). Rhizobia respond to them through NodD activation of specific nodulation genes and also catabolize them (Rao and Cooper 1994). These nodulation genes encode nodulation factors which were identified as being lipochitin oligosaccharides (LCOs). LCOs activate plant organogenesis and provoke the setting up and occupation of the nodule as a symbiotic organ. A complex recognition protocol exists between the partners, and the polysaccharides play there an important role (for review: Fraysse et al. 2003). The infection process (number of nodules and infection of them) is strictly regulated (Kassaw et al. 2015) as this process is symbiotic and not parasitic.

As Nod factors are required for the early steps of the interaction and are the basis for the host-specific recognition, their structure, biosynthesis, role, and transduction were investigated in detail.

Only 5 years after the determination of the Nod factor structure, parallels were made between the molecular basis of the nitrogen fixation and the arbuscular mycorrhization processes (Gianinazzi-Pearson and Denarié 1997). However, it was only demonstrated in 2011 that arbuscular mycorrhizal (AM) fungi produce mycorrhization factors identified as being LCOs (Maillet et al. 2011). Later on, it was demonstrated that the two symbioses share part of the signal transduction pathway *in planta* (Oldroyd 2013). Since then, several publications reported on the role of chitinoligosaccharides (COs) and on the mixtures of the two: COs and LCOs (e.g., Genre et al. 2013).

Based on the structural homology, *nod*-like genes were sought in endo- and ectomycorrhiza. However, no common *nodABC*-like genes have been reported so far in any endomycorrhizal fungus, and only one publication exists on putative fungal *nodABC*-like genes of the ectomycorrhizal (ECM) fungi *Laccaria bicolor* and *Tuber melanosporum* (Garcia et al. 2015).

In this chapter, we will describe in detail the biosynthesis of complex LCOs in rhizobia, and we will explore whether the absence of common *nod* genes in endomycorrhiza could be circumvented by other biosynthetic pathways.

2 Biosynthesis of LCOs in the Rhizobial Model

The oligosaccharide part of the LCOs consists of β-1,4-linked *N*-acetyl-D-glucosamine units corresponding to a chitin fragment. The backbone is always substituted on the amine of the nonreducing end by an acyl chain. This acyl chain reflects usually the fatty acyl pool that composes the phospholipids of the strain. An exception is the α,β-unsaturated fatty acyl residue detected in rhizobial strains nodulating the galegoïds, and which requires a specific biosynthesis. The length of the *N*-acetyl-D-glucosamine (GlcNAc) core varies between three and six, and the number of supplementary substituents can range from few (*R. leguminosarum* bv. *viciae*) to several (*Sinorhizobium fredii* sp. NGR234) (Fig. 1).

Several proteins are encoded by the called *nod*, *nol*, or *noe* genes, and many of these are involved in the biosynthesis of LCOs. Identification of bacterial genes required for nodulation started toward the end of the 1980s applying a transposon mutagenesis approach. Nodulation genes are mostly located on a large symbiotic plasmid (pSym) and sometimes in several loci of the chromosome (*R. loti*, *S. fredii* sp. NGR234). Nod is the name given to the common genes involved in the LCO synthesis. Noe corresponds to *no*dulation *e*nzymes involved in the host specificity (formerly named hsn for *h*ost-*s*pecific *n*odulation). All rhizobia studied to date contain the *nodABC* genes and a range of copies of various other *nod* genes. *nodABC* products can assemble on their own the LCO structure. The three encoded proteins are named NodABC. NodC assembles the chitin skeleton, NodB deacetylates the amide-linked acetate at the nonreducing end of this chain, and NodA transfers an acyl to the amine thus liberated. Other genes are strain specific (e.g., *nodFEL* or *nodSU*). Most of these genes are organized in operons and are preceded by

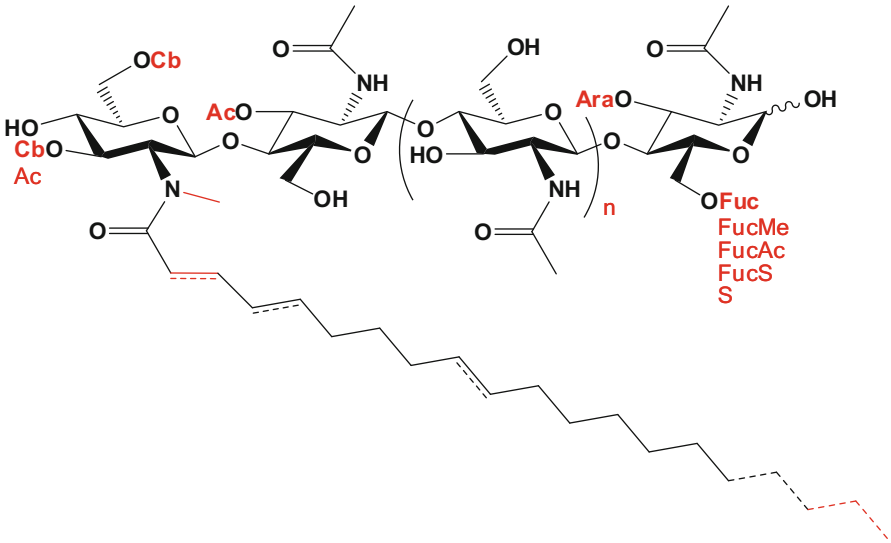


Fig. 1 Nod factor's general structure

promoters that are presenting a NodD-binding consensus sequence (*nod*-box) (Fisher et al. 1988).

2.1 Synthesis of the Oligochitin Backbone

2.1.1 Biosynthesis of GlcNAc Subunits (NodM)

The precursor of the chitin backbone synthesis is UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc). In *E. coli*, its biosynthesis involves proteins encoded by *glm* genes. The first step in this process is the transformation of fructose phosphate into glusosamine-6-P, involving *glmS* (Dutka-Malen et al. 1988). In rhizobia, within the *nod* proteins encoded by the nodulation genes, NodM presents sequence similarity to some phosphoribosyl transferases, especially to GlmS. Mutations in the *nodM* gene result in the reduction of the LCO production (Spaink et al. 1992). This suggests that other genes, such as *glmS*, can partly compensate for this defect. Therefore, an efficient production of UDP-GlcNAc by GlmS and/or NodM is a requisite for adequate LCO production (Fig. 2).

2.1.2 Synthesis of the Oligochitin Chain (NodC)

Chitin is a linear arrangement of β-1,4-linked GlcNAc units. The nodulation protein NodC presents sequence similarity with chitin synthase of *Saccharomyces cerevisiae* and as such is the putative LCO backbone builder (Bulawa and Wasco 1991). *nodC* was cloned and expressed in *E. coli*. The resulting strain secreted β-1,4-linked GlcNAc oligomers with a polymerization degree (DP) ranging from two to five (Kamst et al. 1995). The synthesis of polysaccharides often requests a lipid anchor,

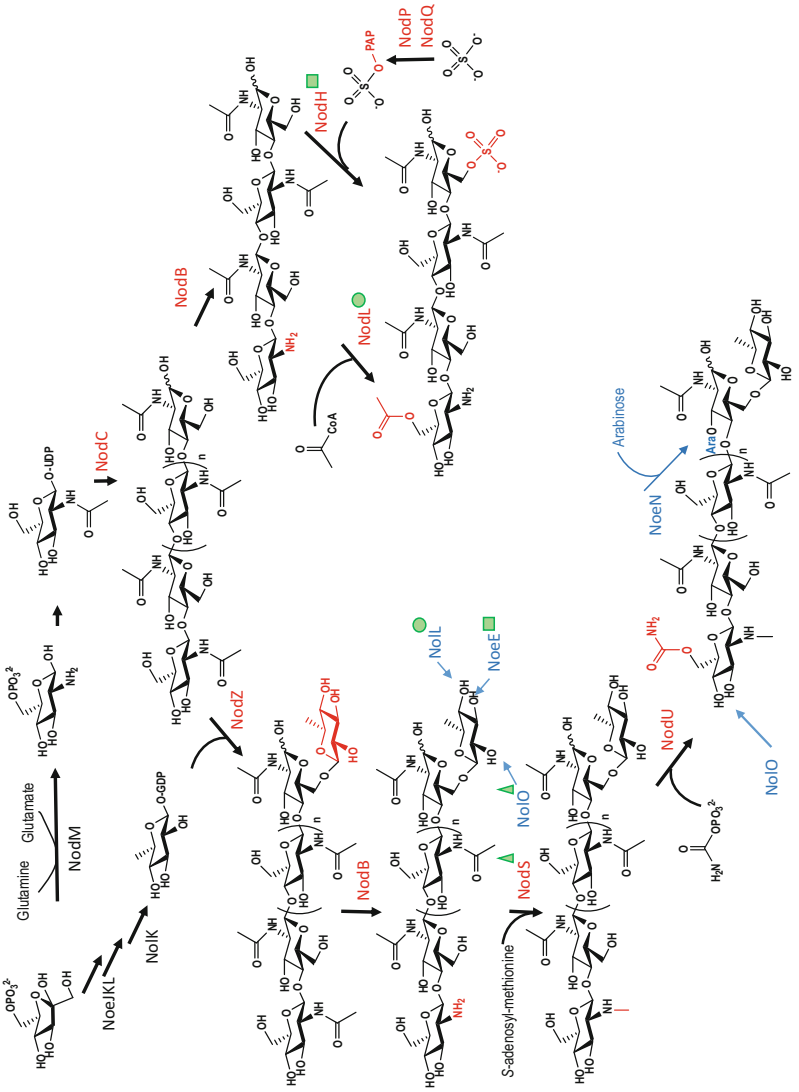


Fig. 2 Synthesis of the saccharidic backbone

and due to the inner membrane localization of the NodC protein (Barny and Downie 1993), such lipid-linked intermediates were searched but never identified. The elongation of the chain was demonstrated to take place at the nonreducing end (Kamst et al. 1999). The authors proposed a model where UDP-GlcNAc binds to NodC followed directly by the hydrolysis of the UDP subunit and a translocation of the free GlcNAc to the elongation site where it is bound to the nonreducing end of the growing chain. When the size of the chain is appropriate (DP4 or DP5 preferentially), the chain dissociates from the NodC-binding site. Interestingly, the chain length is strain dependent. Exchanging the *nodC* gene between different strains affect the DP of the produced Nod factors (Debellé et al. 1995).

2.1.3 Deacetylation of the Chitin Backbone (NodB)

The common feature of all the LCOs is related to the presence of an acyl chain on the amine of nonreducing terminal glucosamine (GlcN). To access this, the nonreducing end first needs to be deacetylated to enable the coupling between the so-liberated amine and a fatty acyl chain. In the common *nod* gene locus, *nodB* precedes *nodC*. Its sequence was found to be similar to the chitin deacetylase of *Mucor rouxii* (Kafetzopoulos et al. 1993). When *nodBC* are cloned in *E. coli* (or in rhizobia lacking the pSym), chitin oligosaccharides deacetylated at nonreducing end are produced (Spaink et al. 1994). NodB is the only product of the three canonical genes to have been studied in vitro. In vitro, NodB deacetylates chitin oligosaccharides but not GlcNAc (John et al. 1993). It has been reported that the deacetylation occurs directly after the chitin chain oligomerization by NodC. Actually, numerous LCO-modifying enzymes seem to prefer monodeacylated chitooligomers as substrate (Bloemberg et al. 1995; Schultze et al. 1995; Geelen et al. 1995).

2.2 *N*-Decoration

2.2.1 *N*-Methylation (NodS)

Numerous rhizobial strains produce Nod factors bearing a *N*-methyl group at the nonreducing end (e.g., *R. etli*, *M. loti*, *Rhizobium tropici*; *Sinorhizobium* sp. *NGR234*; *A. caulinodans*; *B. elkanii*) (for review: D'Haeze and Holsters 2002). By cloning the *nodABCS* genes of *A. caulinodans* and expressing them in the *E. coli* background, *nodS* was discovered to code for this *N*-methyl transferase (Mergaert et al. 1995). NodS uses *S*-adenosyl-methionine as methyl donor (Geelen et al. 1995). NodS accepts in vitro deacetylated chitooligomers ranging from DP2 to 6 but prefers the pentasaccharide as substrate (Jabbouri et al. 1995).

2.2.2 *N*-Acylation

Fatty Acyl Chain Synthesis (NodFE)

Contrarily to the higher animals, bacteria synthesize their fatty acids with a number of monofunctional enzymes. In prokaryotes, a small protein is linked to the elongating acyl chain via a 4′-phosphopantetheine group. This was highlighted as being

also the rule in the rhizobia (Geiger et al. 1991). These acyl carrier proteins (ACP) are involved in the synthesis and in the transport of the fatty acids. ACPs operate similarly to the coenzyme A (CoA). However, a protein offers more recognition possibilities for the enzymes and therefore is more suitable for a specific biosynthetic purpose. Constitutive ACPs have been detected in *Rhizobium leguminosarum* and in *Sinorhizobium meliloti* (López-Lara and Geiger 2000).

Usually, in bacteria, the fatty acid synthesis occurs by the repetitive sequential action of four types of enzymes (Figure 3). First the malonyl-CoA is transferred to the ACP (FabD). The β-keto-acyl synthase transfers the malonyl-ACP unit to the elongating chain producing a β-keto-acyl-ACP (FabB). The carbonyl at the 3-position is then reduced by the β-keto-acyl-reductase (FabG). The resulting alcohol group is then removed by a β-hydroxy-acyl dehydrase (FabZ) and converted into an enol group which is reduced to a saturated acyl group by the enoyl reductase (FabI). In bacteria, the so-produced acyl acts again as a malonyl acceptor, if bound to an ACP. For a recent review, see Byers and Gong (2007).

Most of the published Nod factor structures attest that the fatty acids bound to the nonreducing end correspond to those present in the membrane (principally palmitic, stearic, and vaccenic acids). Therefore, no specific biosynthesis has to be encoded by the nodulation genes. An exception exists for all the rhizobia nodulating the galegoïd group. In these symbiotic interactions, the produced Nod factors carry α,β-unsaturated fatty acids.

Two host-specific *nod* genes, *nodF* and *nodE*, are crucial for the generation of α,β-unsaturated fatty acids. Actually, *S. meliloti (Sm)* synthesizes Nod factors bearing C16:2 fatty acyls. When mutated in *nodF* or *nodE, S. meliloti* produces only Nod factors bearing a vaccenic acid (C18:1), and no C16:2 fatty acids can be detected. *R. leguminosarum* bv. *trifolii* synthesizes acyls with 18 and 20 carbons, whereas *S. meliloti* produces principally 16 carbon chains. The combination of *SmNodF* and *RlNodE* was therefore constructed in the *R. leguminosarum* background. The resulting strain produced the *R. leguminosarum* acyl chain profile. This indicates that NodE is the determinant for the acyl chain length, not NodF (Demont et al. 1993). NodFE are sufficient to synthesize completely α,β-unsaturated fatty acids before their transfer to the chitin chain (Geiger et al. 1994). NodF is clearly a member of the ACP family as it carries 4′-phosphopantetheine (Geiger et al. 1991). As NodE is essential for the α,β-unsaturated acyl chain synthesis, it is considered as being the malonyl-condensation enzyme, but its precise biochemical function remains unknown. As the fatty acids are α,β-unsaturated, it makes sense to hypothesize that NodF-bounded enoyls are no more recognized by the enoyl reductase (FabI) and that NodE contrarily to FabB has the capacity to elongate enoyls. In vitro, FabD (the malonyl-CoA acyl transferase of *E. coli*) is able to transfer a malonyl to NodF (first step of the fatty acid biosynthesis), but this one cannot be elongated by FabB. This points out that the biosynthesis of the α,β-unsaturated fatty acids probably starts with the common pathway involving Fab enzymes. Depending on the strains, C12-, C14-, and/or C16-ACP chains (medium-chain unsaturated or not) are then condensed to a malonyl-NodF unit. The resulting chain can no more be reduced by FabI and is then elongated by NodE for one to three cycles more.

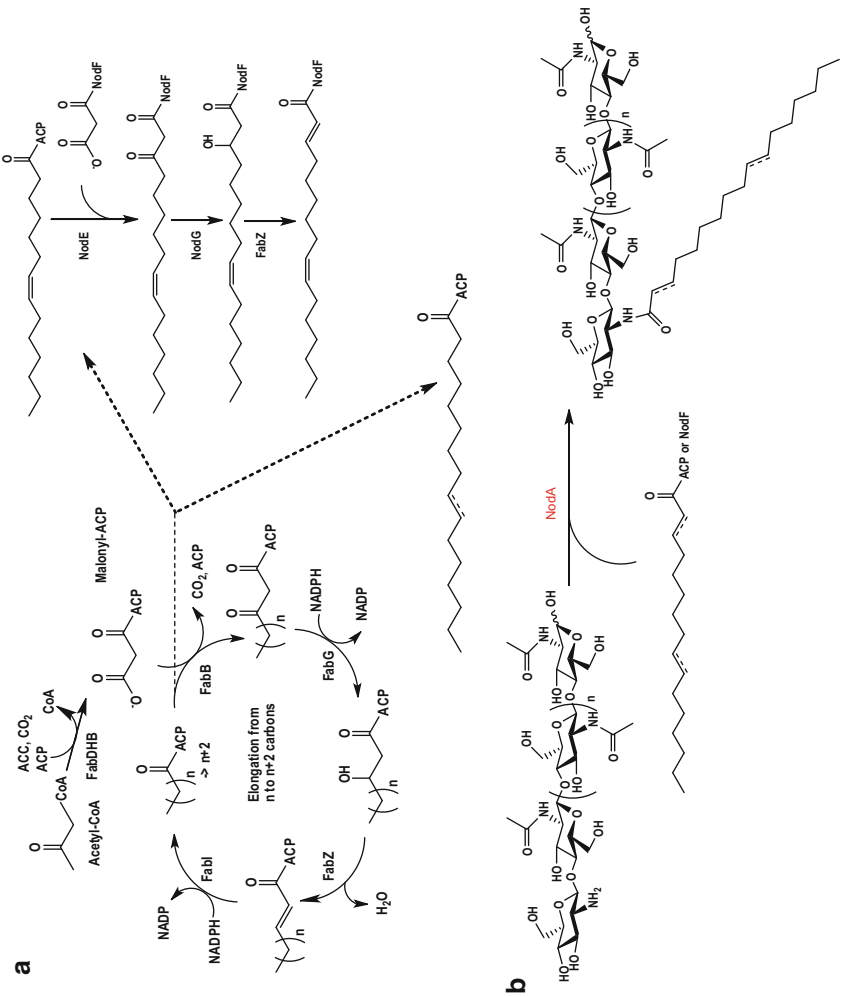


Fig. 3 Synthesis and transfer of the acyl chain

S. meliloti and *Mesorhizobium* sp. N33's pSym contain a supplementary *nodG* gene. Due to its sequence homology with *fabG, nodG was* proposed to code for a β-oxo-acyl-ACP reductase (Slabas et al. 1992; López-Lara and Geiger 2001). A mutation in *nodG* changes the relative amount of C16:1, C16:2, and C16:3 meaning that NodG is probably a more efficient β-keto-acyl-NodF reductase than FabG (Demont et al. 1993).

N-Acylation of the Deacetylated Chitin Backbone (NodA)

By applying a genetic approach, it was reported that *nodA* encodes for the transfer of the fatty acyl chains to the free amine of the nonreducing end (Atkinson et al. 1994). Although NodA is thought to be an acyl transferase, transferring an acyl group from an acyl-ACP to the free amine of the nonreducing end of LCOs, no enzyme assay for NodA has been developed to date. As the distribution of phospholipid-type fatty acids can differ from the one bound to the Nod factors, NodA is supposed to selectively transfer the α,β-unsaturated acyl chains – when present – (Geiger et al. 1998), maybe due to a preferred interaction with the NodF ACP. However, it is important to notice that the unsaturation degree of the fatty acids seems to be affected by the oxygenation of the strains (van der Drift et al. 1996). *nodA* of *Bradyrhizobium japonicum* (Bj) and *S. fredii* (Sf) cause the transfer of common fatty acids to the chitin backbone. *nodA* of *R. leguminosarum* bv. *viciae* and *S. meliloti* provoke the transfer of polyunsaturated fatty acyl residues. When *nodA* of the two first strains (Bj or Sf) are transferred to the two others (Rl or Sm), no *nodFE*-dependent fatty acyl groups are relocated. *nodAB* from *S. meliloti* were cloned and expressed in *E. coli*. Di- to tetra-sulfated chitooligomers were added to become the acceptors for the acyl chain. Only tetramers were identified as carrying an *N*-acyl residues (Atkinson et al. 1994).

NodA acts as an acceptor and a donor of fatty acyl groups, specifically on a free saccharide amine, and therefore NodA is an *N*-acyl transferase. Even if NodA is encoded by the "common *nod* genes," its preference for both the acceptors and the donors of the acyls can turn it to be a host-specific determinant (Ritsema et al. 1996).

2.3 *O*-Decorations of LCOs

2.3.1 At the Reducing end of LCOs

Fucosylation (NolK, NoeL, NodZ, and Substitution NoeE and NolL)

Several strains belonging to various rhizobial genera present at their reducing end the supplementary deoxysugar fucose (e.g., *Sinorhizobium fredii*, *Azorhizobium caulinodans*, *Bradyrhizobium japonicum*, *Rhizobium etli*) (for review: D'Haeze and Holsters 2002). More recently, the rice host *Rhizobium* IRBG74 was reported as producing also fucosylated Nod factors (Poinsot et al. 2016). All these strains have *nodZ* as common gene. Introducing *nodZ* in *R. leguminosarum* bv. *viciae* results in the synthesis of fucosylated Nod factors normally absent (López-Lara et al. 1996). When overexpressed in *E. coli*, NodZ transfers fucose from GDP-fucose

to the 6-*O*-position of the reducing-end saccharide (Quinto et al. 1997). Another gene *nolK* is determinative for the LCO fucosylation (Mergaert et al. 1996). NolK and NoeL are supposed to be involved in the GDP-fucose synthesis, due to their homologies to proteins converting GDP-D-mannose into GDP-fucose. NoeL is similar to a mannose-4,6-dehydratase and NolK a keto-deoxy-mannose epimerase (Aoyama et al. 1994). Two other genes are present in the *nodZ* locus but are not essential for the fucosylation.

The fucose residue presents frequently supplementary substituents such as a 2-*O*-methyl (*B. elkanii*, *S. fredii*, *B. japonicum*) or a 4-*O*-acetyl (*M. loti*, *R. etli*) (for review: D'Haeze and Holsters 2002). In *S.* sp. NGR234, in addition to 2-*O*-Me and 4-*O*-Ac, the fucose carries also a 3-*O*-sulfate (Price et al. 1996).

By applying a mutation approach to strain *Sinorhizobium* sp. NGR234, it was highlighted that *noeI* is controlling the fucose methylation (Jabbouri et al. 1998), *nolL* the fucose acetylation (Berk et al. 1999), and *noeE* the sulfation of the fucosyl residue (Perret et al. 2000). Although *noeE* shows sequence similarity with *nodH* (the chitin sulfotransferase, see the next paragraph), their specificities are different. NodH transfers the sulfate group to the both, the reducing end GlcNAc and the Fuc residue, whereas NoeE is more selective and modifies the fucose residue only (Quesada-Vincens et al. 1998).

Sulfation (NodH and NodPQ)

With the exception of NoeE, NodH is the only enzyme characterized as a sulfotransferase within the nodulation genes (Ehrhardt et al. 1995). This sulfation is specific to the 6-*O*-position of the reducing end GlcNAc and occurs after the core oligosaccharide synthesis (Schultze et al. 1995). NodH (as well as NoeE) is a 3′-phosphoadenosine-5′-phosphosulfate transferase. This sulfate donor is encoded by the *nodPQ* genes (Schwedock and Long 1990; Schwedock et al. 1994). Less specific than NoeE, NodH transfers the sulfate to the fucose and to the 6-*O* of the GlcNAc at the reducing end (Perret et al. 2000).

Arabinosylation (NoeCHOP)

Arabinosylation is not frequently reported (D'Haeze and Holsters 2002). Actually only *A. caulinodans*, *S. saheli, S. teranga*, and *R.* sp. IRBG74 (Poinsot et al. 2016) are known to produce such a decoration. The arabinosylation occurs in addition to fucosylation. Therefore, the Nod factors carry either arabinose, fucose, or both (except IRBG74 where arabinose is observed with difficulty in the absence of fucose). The nodulation enzyme NoeC and/or the downstream genes are essential for the arabinosylation of *A. caulinodans* (Mergeart et al. 1996). *noeP* was demonstrated to cause arabinosylation in strain R. sp. IRBG74.

Acetylation (NodX)

The acetylation of the reducing end GlcNAc was reported only in one strain, *R. leguminosarum* TOM (host of *Pisum sativum*). It is the result of the *nodX* gene activity (Firmin et al. 1993). Interestingly, NodX shows homologies with acetyl

transferases involved in the modifications of EPS and LPS but not with NodL that acetylates GlcNAc at the nonreducing end (see Sect. 2.3.2.2).

2.3.2 At the Nonreducing End

Acetylation (NodL, NoeT)

6-*O*-acetylation at the nonreducing end was reported for *R. elkanii*, *B. japonicum*, *M. loti*, *S. meliloti*, and *R. leguminosarum* bv. *trifolii* and *viciae* strains (for review see D'Haeze and Holsters 2002). NodL is the nodulation protein involved in this modification of the core. The sequence of *nodL* is indeed similar to two acetyl transferase encoding genes in *E. coli* (*lacA* and *cysE*). Moreover, a mutation in *nodL* produces LCOs lacking the 6-*O*-acetyl group (Ardoundel et al. 1995). Acetyl-CoA was identified as being the acetyl donor (Bloemberg et al. 1994). NodL recognizes every chitin oligomer as acceptor with a light preference for the de-acetylated COs (Bloemberg et al. 1995).

Acetylation of the *N*-acetylglucosamine next to the nonreducing end was first found for the Nod factors of two *R. galegae* strains (*Neorhizobium galegae* HAMBI, *R. leguminosarum* bv. *trifolii*) with different geographical origins (Yang et al. 1999). Such an observation was also made on R. sp. BR816 (Snoek et al. 2001). *noeT* is involved in the 3-*O* acetylation of the penultimate sugar of the nonreducing end (Österman et al. 2014).

Carbamoylation (NodU, NolO)

Carbamoylations have been reported in several strains at different positions of the nonreducing end. *M. loti* secretes carbamoylated Nod factors. The position of the carbamoylation has been tentatively located on 4-*O* by methylation analysis (López-Lara et al. 1995). The structures of carbamoylated Nod factors secreted by *R. etli* were claimed as identical to the ones of *M. loti* (Cardenas et al. 1995). *S.* sp. NGR234 secretes a wide variety of Nod factors, with zero, one, or two carbamoyl groups at the reducing terminal residue. When mutated in the *nodU* or the *nolO* genes, doubly carbamoylated Nod factors were no more synthesized (Jabbouri et al. 1998). As these genes encode for different carbamoyl transferases, the mono-carbamoylated species formed by these mutant strains were not identical. A detailed mass spectrometric analysis demonstrated that the first carbamoylation encoded by *nodU* is located on 6-*O*, and the second one encoded by *nolO* is located on the 3-*O*, even for *M. loti* and *R. etli* (Treilhou et al. 2000).

2.4 Regulation of the Nod Genes

The regulation of the *nod* gene expression depends on the strains and is mostly mediated by NodD proteins (Schlaman and Phillips 1998). Multiple sequences of *nodD* were identified. *nodD* genes are seen as the first level of host specificity, as the products of the different *nodD* have species-specific ways to respond to the different flavonoid groups (Dénarié et al. 1992). However, they share a conserved ligand-

interacting domain at the C-terminal end of the DNA binding motif (Györgypàl and Kondorosi 1991). They bind to a highly conserved DNA motif (47 bp) called *nod* boxes (Fisher et al. 1988). The number of *nodD* gene copies varies from one to five depending in the strain (van Rijn et al. 1993). Even if NodD binds on its own to these boxes, flavonoids are necessary for the *nod* gene expression (Fisher et al. 1988). Therefore, NodD can be considered as a plant exudate sensor and as a promoter of *nod* gene expression. These proteins are part of the cytoplasmic membrane where the flavonoids accumulate (Schlaman et al. 1989). NodD1 was demonstrated to be an activator of the *nodABC* locus. The *nod* gene regulation is even more complicated due to the involvement of other LysR family members noted SyrM. *syrM* shows homology with *nodD*. SyrM and NodD3 can induce the expression of the *nod* genes in absence of plant signals (Demont et al. 1993). They constitute a self-amplifying regulatory system for *nod* gene expression in the developing nodule, without flavonoids (Swanson et al. 1993; Demont et al. 1994). *nod* gene expression using *nod*-box promoters is not the unique upregulation system. In *B. japonicum*, two genes, *nodV* (plant sensor) and *nodW* (*nod* gene activator) are also involved in the flavonoid gene regulation. They are essential for isoflavone-mediated gene activation (Loh and Stacey 2003). Nothing is known so far about the way they operate. Surprisingly, *nolX* in *S. fredii* is not associated to a *nod* box but is *nodD*- and flavonoid-dependently activated (Bellato et al. 1996). Finally, abiotic stress (like acidic or saline conditions) induces the Nod factor synthesis without the presence of plant signals (Guasch-Vidal et al. 2013).

The *nod* gene expression requires also negative control for an optimal nodulation process. The *nodABC* genes of *R. leguminosarum* bv. *viciae* are indeed not expressed in the bacteroides (Schlaman and Phillips 1998). In the *Sinorhizobium* genus, the 13 kDa protein NolR was found to bind the promoter regions of the *nod* genes and to repress *nodD*. The *nodABC* expressions are repressed as a consequence of this direct attachment to their overlapping promoter regions (Kiss et al. 1998). NodD2 was also described as a repressor of the *nodABC* expression (Fellay et al. 1998). In *B. japonicum*, other *nod* genes like *nolA* or genes like *nwsB* also play specific regulatory roles (Sadowsky et al. 1991).

NodD1 is considered to be the principal activator of *nod* gene transcription. 19 *nod*-boxes are identified in the plasmid of the wide host-range bacterium *Sinorhizobium* sp. NGR234. *NodD1* regulates five of them, promoting *nodABCIJnolOnoeI*, *nodSU*, *nodZ*, *noeE*, and *nolL* (Freiberg et al.1997). The occurrence of the supplementary activators probably allows triggering the expression of the dispersed nodulation loci. The repressors (*NolR* or *nolA* and *NodVW*) allow to modulate this expression (for review: see Pueppke 1996).

2.5 Secretion

Little is known concerning the LCOs' presence in the rhizosphere. However, LCOs produced in vitro are mainly encountered in the spent medium, indicating that the LCO are secreted by the bacteria. The presence of pivotal *nodABC* genes, as outlined

above, is common to all rhizobia. Other *nod* genes are reported in the same locus. *NodIJ* are part of them. Their sequences are similar to the ones of genes encoding for ATP-binding cassette transport systems (Vazquez et al. 1993). Studies on *R. leguminosarum* and *A. caulinodans* have demonstrated that NodI and NodJ are both required for an efficient secretion of LCOs. A mutation in *nodIJ* leads to the reduction of the LCO secretion but only in the first hours after gene induction. Lateron, alternative secretion systems seem to take over. Moreover, using an *E. coli nodABCSU* construction, it was suggested that the efficiency of the transport of LCOs by NodIJ is dependent of its structure (Fernandez-Lopez et al. 1996). ABC-export systems consist in a construction of accessory proteins (membrane or periplasmic proteins) associated with a hydrophobic membrane-spanning protein, acting often as dimers (inner membrane proteins) and an ATP binding protein (for review Pohl et al. 2005). NodI has clearly ATP-binding motifs. NodJ presents sequence homologies with membrane-spanning proteins and has a leucine zipper dimerization domain. Finally NodT has been found as a possible accessory protein in *Rhizobium leguminosarum* bv. viciae (Rivilla et al. 1995).

Although nodulation factors are secreted by the presented specific system, they also are enriched on the bacterial surface (Orgambide et al. 1995).

2.6 Topology and Time Course of the Biosynthesis

2.6.1 Topology

The study of the individual gene products did not allow to identify the topological organization of the LCO biosynthesis. NodC is located at the inner membrane (Barny and Downie 1993) as well as NodD (Schlaman et al. 1989). NodH was reported as being membrane associated (Schmidt et al. 1991). However, NodB, NodL, and NodS were reported as being cytosolic (Schmidt et al. 1988; Bloemberg et al. 1994; Geelen et al. 1995). The sequence of NodX presents a hydrophobic domain making it a probable membrane protein (Firmin et al. 1993). The localization of NodA remains unclear. Due to the lipophilic properties of the acyl chain, LCOs are expected to insert into the inner membrane where further modifications can occur. These observations allowed only to assess that the enzymatic process starts and ends at the inner membrane.

2.6.2 Time Course of the Biosynthesis

Only few things were known about the time course of the Nod factor biosynthesis before the publication led by Poinsot et al. (2016). Only NodL and NodS were supposed to modify the chitin oligosaccharide before acylation by NodA (Bloemberg et al. 1994; Geelen et al. 1995).

A complete in vivo biosynthetic study was published, based on directed mutations of the *Agrobacterium Rhizobium* sp. IRGB74 genome (Poinsot et al. 2016). This strain is able to colonize rice in addition to the *Sesbania* species. Its genome annotation suggested the production of various and complex Nod factors. Over 40 different LCO structures were detected using mass spectrometry. The variety of

substitutions and of their combinations opened the opportunity to investigate the time course of the Nod factor biosynthesis. The authors developed an interesting approach by studying in parallel (using a mass spectrometric approach) the chitin oligomers (COs) and LCOs produced by several *nod* gene mutants.

R. sp. IRBG74 LCOs are strictly *N*-methylated. Their *N*-acyl residues are saturated or polyunsaturated and range from C16 to C20. These Nod factors are also carbamoylated, fucosylated, and arabinosylated.

In the absence of any mutation, *R*. sp. IRBG74 secretes COs and *N*-deacetylated COs (dCOs). Both are produced in equal quantities but represent only 10% of the secreted LCOs. COs and dCOs present different compositions. COs are nude or bear only a fucose residue. The nude COs range from two to five GlcNAc units, and the fucosylated COs count only four or five GlcNAc residues. The dCOs (ranging up to six GlcNAc) are strictly *N*-methylated, mostly fucosylated and carbamoylated. No arabinose could be detected on either of the two: COs and dCOs.

The authors confirmed the gene annotation in the *Agrobacterium* background. Actually, the *nodB* mutant produced only COs with the same profile as the wild-type strain (wt). The *nodA* mutant produced no LCOs. The COs and dCOS produced by the Δ*nodA* mutant remained similar to the wild-type ones. The *nodE* mutant produced only the usual C16 and C18 acyl residues. *nodU* inactivation abolished solely the carbamoylation.

The first difference with previous publications is related to *the nodZ* mutant. The directed mutagenesis of the *R*. sp. IRBG74 *nodZ* did not suppress but only reduced the fucosylation by 90%. This mutation also suppressed completely the arabinosylation. The second unexpected result is related to the *nodS* mutant. The *nodS* deletion abolished the *N*-methylation but increased the arabinosylation and severely decreased the carbamoylation of the LCOs.

However, the novelty lies primarily in the important fucosylation ratio of the COs in the wild-type exudates. This indicates that the fucosylation occurs directly after the action of NodC. As no other substitution was demonstrated on COs, but as methylation and carbamoylation occurred on dCOs, it was deducted that NodB acts directly after NodC and NodZ. Considering that all the carbamoylated structures are strictly *N*-methylated, the carbamoylation has to follow the methylation. This hypothesis is reinforced by the fact that the *nodS* mutation drastically decreases the carbamoylation of the LCOs. Finally, as no arabinose was found on COs and dCOs, the arabinosylation requests the presence of both the fucose and the acyl chain. The arabinosylation occurs as such very late in the overall process.

As the sulfation and the *O*-acetylation are not reported for the *R*. sp. IRBG74 Nod factors, the same approach was applied to the *S. meliloti* background. The *nodB*-directed mutant produces only nude COs. The *nodA* mutant secreted sulfated and acetylated dCOs. This indicates that sulfation and acetylation occur after NodB action, but before the acylation by NodA.

It remains unknown whether the *nod* proteins act as an enzyme complex.

3 Biosynthesis in Other Models (Fungi or Non-rhizobial Bacteria)

As called to mind in the introduction, the ability to establish root nodule symbiosis seems to result from the evolution of the old arbuscular mycorrhization (AM) by recruitment mechanisms developed by the rhizobia (Kisner and Parniske 2002). These processes are based on the synthesis and perception of LCO. The LCOs are perceived by plant kinases, and they activate a signaling pathway common to the both nitrogen-fixing and AM symbioses. It was published that AM fungi produce also LCO signals named Myc factors (Maillet et al. 2011). Recently, in a genomic analysis of proteobacteria, it was considered that *Actinobacteria* could be involved in this evolution (Persson et al. 2015). Frankia are nitrogen-fixing *Actinobacteria* that can enter in root symbioses with actinorhizal plants. Frankia were classified in three clades. Frankia of cluster I and III are cultivable, contrarily to cluster II. LCOs could never be identified in cultures of Frankia. The recently sequenced genome of the uncultivable *Candidatus Frankia* datiscae Dg1 contains the canonical *nodABC* genes, arranged in two operons (*nodB2C* and *nodB1A*). Putative *nodIJ* (>45% homology) are found directly downstream from $nodB_2C$. Even if *Streptomyces bottropensis* (*Actinobacteria*) presents the *nodABC* genes organized in a single operon, it does not produce nodule symbiosis. Finally, based on genomic data, two ectomycorhiza (ECM) *Laccaria bicolor* and *Tuber melanosporum* were reported as having potential homologues of the *nodABC* genes. It remains unclear if these are used for LCO synthesis or for cell wall remodeling (Garcia et al. 2015).

In summary, LCOs or genes encoding for these compounds are not only found in rhizobia but also in phylogenetically distant bacteria and even in more distant organisms, such as in AM and ECM fungi. Therefore, a question remains open: Can LCO biosynthesis occur in several organisms? We made a database analysis of the ubiquity of the canonical *nod* genes in the fungi reign. We chose to compare the sequence of the predicted or established fungus protein with the NodA sequence of *R. leguminosarum* without focusing on biovars, because NodA is conserved in this genus. These rhizobia synthesize, beside the major alpha-beta unsaturated acyl chains, simple LCOs bearing classic fatty acyls (saturated or mid chain unsaturated)) (Table 1).

3.1 Presence of the *NodBC*-like Genes

3.1.1 *NodC*: Chitin-Oligomer Synthase Using UDP-GlcNAc

Surprisingly, *nodC* seems to be the least ubiquitous of the three canonic *nodABC* genes in fungi. Only 18 strains exhibit putative *nodC* genes in their genome. They belong to two genera, of two orders and divisions: *Mucorales* (10) and *Ascomycetes* (8). Mucorales corresponds to the division of the AM fungi.

Table 1 NodABC Data Bank research for fungi

Protein	Division	Order	Family/genus	Binominal name
NodC like (18)	**Mucorales (10)**	**Rhizopodaceae (5)**	Rhizopus (2)	***R. microsporus [2e^{-06}, 27%], R. delemar [4e^{-07}, 23%].***
	Ascomycetes (8)	**Saccharomycetales (1)**	**Saccharomycetaceae (1)** Saccharomyces (1)	***S. cerevisiae [6e^{-19}, 25%]***
NodB like (695)	**Mucorales (17)**	**Rhizopodaceae (5)**	Rhizopus (5)	***R. microsporus [5e^{-22}; 35%], R. delemar [9e^{-16}; 33%],*** *R. oryzae, R. stolonifer, R. circinans*
	Ascomycetes (507)	**Saccharomycetales (197)** **Pezizales (3)**	**Saccharomycetaceae (145)** Saccharomyces (117) Lachancea (3) Kluyveromyces (5) **Phaffomycetaceae (5)** **Debaryomycetaceae (37)** Debaryomyces (2) Scheffersomyces (1) **Tuberaceae (2)** Tuber **Pyronemataceae (5)** Pyronema	***S. cerevisiae[1e^{-20}; 31%],*** *S. eubayanus, S. kudriavzevii, S. sp. "boulardii" L. lanzarotensis,* ***L thermotolerans^{b} [2e^{-15}; 35%],*** *L. quebecensis K. dobzhanskii, K. lactis,* ***K. marxianus^{b} [1e^{-14}; 30%]*** *Cyberlindnera fabianii,* ***Cyberlindnera jadinii^{b} [7e^{-16}; 27%]****, Komagataella phaffii, Wickerhamomyces ciferrii D. fabryi,* ***D. hansenii^{b} [4e^{-18}; 33%]****, S. stipitis* ***T. melanosporum^{b} [3e^{-18}; 34%]*** *P. omphalodes*
	Basidiomycetes (132)	**Agaricales (24)**	**Tricholomataceae**	***Laccaria bicolor [6e^{-15}; 31%]****, Laccaria amethystina*

(*continued*)

Table 1 (continued)

Protein	Division	Order	Family/genus	Binominal name
NodA like[a] (794)	**Mucorales (9)**	**Rhizopodaceae (5)**	Rhizopus (2)	***R. microsporus* [0.068; 67%],, *R. delemar* [0.89; 43%],**
	Ascomycetes (25)	**Saccharomycetales (10)**	**Saccharomycetaceae (5)** Saccharomyces (2) Lachancea (2) Kluyveromyces (1) **Phaffomycetaceae (3)** Cyberlindnera (1) Komagataella (2) **Debaryomycetaceae (2)** Scheffersomyces (1) Debaryomyces (1)	***S. cerevisiae [0.71; 45%]*** ***L. thermotolerans[b] [5.1; 50%]*** ***K. marxianus[b] [5.8; 21%]*** ***C. jadinii[b] [no significant homology]*** *K. pastoris* ***D. hansenii[b] [no significant homology]***
	Basidiomycetes (122)	**Agaricales (21)**	**Tricholomataceae (2)** Laccaria	***L. bicolor[b] [7.4; 38%],*** *L. amethystina*

Comparison trees of NodA, NodB, NodC-like proteins or oligochitinase and *N*-acyltransferase in fungi

() number of different species referenced in NCBI Protein Data Base resource. Bold fungi, the fungi who may have the same NodA, NodB, NodC proteins, [E value, Identity] the homology calculation with BLAST compare to *Rhizobium leguminosarum (accession* WP003592722) one of the two rhizobium models

[a]NodA or *N*-acyltransferase

[b]Indicate the fungi exhibiting the 3 putative nodABC genes. In Bold, the fungi presenting only *nodB* and *nodA*

3.1.2 *NodB*: Nonreducing End-Specific Chitin Deacetylase

Chitin deacetylases are widespread in the fungi orders. Homologies to *nodB* are found close to 700 times, but we do not know so far anything about their specificity. A key question is raised here about NodB-like proteins: Are they really specific of the nonreducing end or do they contribute to strategies disabling the action of chitinases or are they involved in cell lysis? Again the majority of the homologies is encountered in the *Mucorales* and *Ascomycetes* divisions.

3.2 Other Synthesis of the CO Backbone: Chitin Hydrolases

As *nodC* was the most stringent gene, we focused to find out if a tetra- or pentamer backbone could be obtained by degrading a chitin polymer. Of course, as chitin degradation is requested in all fungi's metabolisms, more than 10,000 hits were

obtained. Again nothing indicates that some of them are capable to generate specifically tetra- or pentamers, lending little predictability to this approach.

3.3 Existence of *NodA*-like Genes

Of course, 1000 of matches are occurring in the data bases as we are searching for ACP proteins. We aimed to become more specific by selecting only the *N*-acyl carriers presenting homologies to NodA. More than 700 *N*-acyl transferases were identified, but only 34 in the two *Mucorales* (9) and *Ascomycetes* (25) divisions. Extremely low E-factors and high identity are highlighted as we compare the sequence of NodA from *R. leguminosarum* with the sequences of several other rhizobia NodA. This indicates a very good conservation of NodA in this family. Disappointingly, the correlations found between the NodA-like fungal protein and *R. leguminosarum* NodA are not significant. This could point out that the *N*-acyl transferases found in fungi do probably not transfer fatty acyls to the nonreducing end of COs. However, we know that this is far more complex due to the fact that absolutely no similarity between the NodA protein of *R. leguminosarum* and the NodA-like one of *Rhizophagus irregularis* was found, even if the last is known for producing LCOs.

In summary, potential *N*-acyl transferase and *nodB* and *nodC* genes are frequently found in the fungal reign. Correlations with NodA are in the best case unsatisfying. Only two divisions seem to have the occurrence of the three together: the *Mucorales* and the *Ascomycetes*. It would be particularly relevant to investigate some of the strains belonging to them in order to see if they are really able to produce LCOs. Two of them are *Mucorales*: *R. microspores, R. delemar*, and one belongs to *Ascomycetes*: *S. cerevisae*.

4 Conclusion and Research Needs

Considering rhizobia, even if the LCOs biosynthesis was investigated in details, the role of the gene products was mostly found out by the study of mutants, not by cloning, expression, or of a functional study of an individual gene product. Therefore, studies on individual enzymes involved in LCO biosynthesis are mostly lacking. Only partial knowledge is established so far concerning the functioning of NodE, NodC, and NodA.

The second observation is that the LCOs analyzed are always produced in liquid cultures and often in minimal media. These conditions are far away from the natural ones. It was reported that culture conditions can influence the level of LCO production and affect some specific biosynthetic steps. Moreover, flavonoids alone do not allow to activate all the genes. So one important point that needs clarification is whether the LCO produced in the laboratory reflect the most efficient and abundant LCOs produced in soil. It will be highly relevant to analyze the LCOs produced by

bacteria in the soil and in contact with the host plant. Such approaches might be accessible with the mass spectrometric systems commercialized to date.

Finally, as we highlighted in the part 3 of this chapter, LCOs are produced by AM fungi but without identifying convincing *nodABC* genes. On the other hand, convincing homologies (E value $< e^{-10}$, Identity >30%) to *nodABC* genes are sometimes identified in other fungi which, however, lack LCO production. As the LCO signaling pathway of plant/microbe interaction is common between AM and rhizobia symbioses, such signaling could exist in other host-invited interactions (e.g., ectomycorrhiza). Therefore, a consequent work has to be considered by researching LCOs across the genera, families, and reigns and in correlating their presence to biosynthetic genes by exploiting the huge genomic databases constructed over the past 10 years.

References

Aoyama K, Haase A, Reeves PR (1994) Evidence for effect of the random genetic drift on G+C content after lateral transfer of fucose pathway genes to *Escherichia coli* K-12. Mol Biol Evol 11:829–838

Ardoundel M, Lortet G, Maillet F, Roche P, Truchet G, Promé JC, Rosenberg C (1995) In *Rhizobium meliloti*, the operon associated with the *nod* box n5 comprises *nodL*, *noeA* and *noeB*, three host-range genes specifically required for the nodulation of particular *Medicago* species. Mol Microbiol 17:687–699

Atkinson EM, Palcic MM, Hindsgaul O, Long SR (1994) Biosynthesis of *Rhizobium meliloti* lipooligosaccharide Nod factors: NodA is required for an *N*-acyltransferase activity. Proc Natl Acad Sci U S A 91:8418–8422

Barny MA, Downie JA (1993) Identification of NodC protein in the inner but not in the outer membrane of *Rhizobium leguminosarum*. Mol Plant-Microbe Interact 6:669–672

Bellato CM, Balatti PA, Pueppke SG, Krishnan H (1996) Proteins from cells of *Rhizobium fredii* bind to DNA sequences preceding *nolX*, a flavonoid inducible nod gene that is not associated with a *nod* box. Mol Plant-Microbe Interact 9:457–463

Berk S, Perret X, Quesada-Vincens D, Promé JC, Broughton WJ, Jabbouri S (1999) *nolL* of strain *Rhizobium* sp. NGR234 is required for *O*-acetyl-transferase activity. J Bacteriol 181:957–964

Bloemberg GV, Thomas-Oates JE, Lugtenberg BJJ, Spaink HP (1994) Nodulation protein NodL of *Rhizobium leguminosarum O*-acetylates lipo-oligosaccharides, chitin fragments and *N*-acetyl glucosamine *in vitro*. Mol Microbiol 11:793–804

Bloemberg GV, Lagas R, van Leeuwen S, van der Marel G, van Boom JH, Lugtenberg BJJ, Spaink HP (1995) Substrate specificity and kinetic studies of nodulation protein NodL of *Rhizobium leguminosarum*. Biochemistry 34:12712–12720

Bulawa CE, Wasco W (1991) Chitin and nodulation. Nature 353:710

Byers DM, Gong H (2007) Acyl carrier protein: structure-function relationships in a conserved multifunctional protein family. Biochem Cell Biol 85:649–662

Cardenas L, Dominguez J, Quinto C, Lopez-Lara IM, Lugtemberg BJJ, Spaink HP, Rademaker GJ, Haverkamp J, Thomas-Oates JE (1995) Isolation, chemical structures and biological activities of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*. Plant Mol Biol 29:453–464

D'Haeze W, Holsters M (2002) Nod factor structure, response and perception during initiation of nodule development. Glycobiology 12:79R–105R

Debellé F, Roche P, Plazanet C, Maillet F, Pujol C, Ardourel M, Demont N, Rosenberg C, Truchet G, Promé JC, Dénarié J (1995) The genetics of *Rhizobium* host-range control: allelic

and non-allelic variation. In: Tikhonovich IA (ed) Nitrogen fixation: fundamentals and applications. Kluwer, Dordrecht, pp 275–280

Demont N, Debellé F, Aurelle H, Dénarié J, Promé JC (1993) Role of the *Rhizobium meliloti nodF* and *nodE* genes in the biosynthesis of lipo-oligosaccharidic nodulation factors. J Biol Chem 268:20134–20142

Demont N, Ardourel M, Maillet F, Promé D, Ferro M, Promé JC, Dénarié J (1994) The *Rhizobium meliloti* regulatory *nodD3* and *syrM* genes control the synthesis of a particular class of nodulation factors *N*-acylated by (omega-1)-hydroxylated fatty acids. EMBO J 13:2139–2149

Dénarié J, Debellé F, Rosenberg C (1992) Signaling and host range variation in nodulation. Annu Rev Microbiol 46:497–531

van der Drift KMGM, Spaink HP, Bloemberg BJJ, Haverkamp J, Thomas-Oates JE (1996) *Rhizobium leguminosarum* bv. *trifolii* produces Lipo-chitin oligosaccharides with *nodE*-dependent highly unsaturated fatty acyl moieties : an electrospray ionization and collision-induced dissociation tandem mass spectrometric study. J Biol Chem 271:22563–22569

Dutka-Malen S, Mazodier P, Badet B (1988) Molecular cloning and overexpression of the glucosamine synthase gene from *Escherichia coli*. Biochimie 70:287–290

Ehrhardt DW, Atkinson EM, Faull KF, Freedberg DI, Sutherlin DP, Amstrong R, Long SR (1995) In vitro sulfotransferase activity of NodH, a nodulation protein of *Rhizobium meliloti* required for host-specific nodulation. J Bacteriol 177:6237–6245

Fellay R, Hanin M, Montorzi G, Frey J, Freiberg C, Golinowski W, Staehelin C, Broughton WJ, Jabbouri S (1998) *nodD*2 of *Rhizobium* sp. NGR234 is involved in the repression of the *nodABC* operon. Mol Microbiol 27:1039–1050

Fernandez-Lopez M, D'Haeze W, Mergeart P, Verplancke C, Promé JC, van Montagu M, Holster M (1996) Role of NodI and NodJ in lipo chitooligosaccharide secretion in *Azorhizobium caulinodans*. Mol Microbiol 20:993–1000

Firmin JL, Wilson KE, Carlson RW, Davies AE, Dowie JA (1993) Resistance to nodulation of cv Afghanistan peas is overcome by *nodX* which mediates an *O*-acetylation of *the Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. Mol Microbiol 10:351–360

Fisher RF, Egelhoff TT, Long SR (1988) Specific binding proteins from *Rhizobium meliloti* cell-free extracts containing nod to DNA sequences upstream of inducible nod genes. Genes Dev 2:282–293

Fraysse N, Couderc F, Poinsot V (2003) Surface polysaccharides involvement in establishing the *Rhizobium*-legume symbiosis. Revue Eur J Biochem 270:1365–1380

Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosentahl A, Perret X (1997) Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387:394–401

Garcia K, Delaux PM, Cope KR, Ané JM (2015) Molecular signals required for the establishment and maintenance of ectomycorrhizal symbioses. New Phytol 208:79–87

Geelen D, Leyman B, Mergaert P, Klarskov K, van Montagu M, Geremia R, Holsters M (1995) NodS is an S-adenosyl-L-methionine-dependent methyl transferase that methylates chitooligosaccharides deacetylated at the non-reducing end. Mol Microbiol 17:387–397

Geiger O, Spaink HP, Kennedy EP (1991) Isolation of the rhizobium leguminosarum NodF nodulation protein: NodF carries a 4′-phosphopantetheine prosthetic group. J Bacteriol 173:2872–2878

Geiger O, Thomas-Oates JE, Glushka J, Spaink HP, Lugtenberg BJJ (1994) Phospholipids of *Rhizobium* contain *nodE*-determined highly unsaturated fatty acid moieties. J Biol Chem 269:11090–11097

Geiger O, Glushka J, Lugtenberg BJJ, Spaink HP, Thomas-Oates JE (1998) NodFE-dependent fatty acids that lack an alpha-beta unsaturation are subject to differential transfer, leading to novel phospholipids. Mol Plant-Microbe Interact 11:33–44

Genre A, Chabaud M, Balzergue C, Puech-Pagès V, Novero M, Rey T, Fournier J, Rochange S, Bécard G, Bonfante P, Barker DG (2013) Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. New Phytol 198:190–202

Gianinazzi-Pearson V, Dénarié J (1997) Red carpet genetic programmes for root endosymbiosis. Trends in Plant Sci 2:371–372

Gough C, Jacquet C (2013) Nod factor perception protein carries weight in biotic interactions. Trends Plant Sci 18:566–574

Guasch-Vidal B, Estévez J, Dardanelli MS, Soria-Díaz ME, de Córdoba FF, Balog CI, Manyani H, Gil-Serrano A, Hensbergen PJ, Deelder AM, Megías M, van Brussel AA (2013) High NaCl concentrations induce the nod genes of *Rhizobium tropici* CIAT899 in the absence of flavonoid inducers. Mol Plant-Microbe Interact 26:451–460

Györgypàl Z, Kondorosi A (1991) Homology of the ligand-binding regions of *Rhizobium* symbiotic regulatory protein NodD and vertebrate nuclear receptors. Mol Gen Genet 226:337–340

Jabbouri S, Fellay R, Talmont F, Kamalaprija P, Burger U, Relic B, Promé JC, Broughton WJ (1995) Involvement of NodS in *N*-methylation and nod U in 6-carbamoylation of *Rhizobium* sp NGR234 nod factors. J Biol Chem 270:22968–22973

Jabbouri S, Relić B, Hanin M, Kamalaprija P, Burger U, Promé D, Promé JC, Broughton WJ (1998) nolO and noeI (HsnIII) of *Rhizobium* sp. NGR234 are involved in 3-*O*-carbamoylation and 2-*O*-methylation of Nod factors. J Biol Chem 273:12047–12055

John M, Röhrig H, Schmidt J, Wienecke U, Schell J (1993) *Rhizobium* NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. Proc Natl Acad Sci U S A 90:625–629

Kafetzopoulos D, Thieros G, Vournakis JN, Bouriotis V (1993) The primary structure of a fungal chitin deacetylase reveals the function for two bacterial gene products. Proc Natl Acad Sci U S A 90:8005–8008

Kamst E, van der Drift KMGM, Thomas-Oates JE, Lugtenberg BJJ, Spaink HP (1995) Mass spectrometric analysis of chitin oligosaccharides produced by NodC protein in *Escherichia coli*. J Bacteriol 177:6282–6285

Kamst E, Bakkers J, Quaedvlieg NEM, Pilling J, Kijne JW, Lugtenberg BJJ, Spaink HP (1999) Chitin oligosaccharide synthesis by the bacterial NodC protein and zebrafish DG42 starts at the reducing terminal residue. Biochemistry 38:4045–4052

Kassaw T, Bridges W, Frugoli J (2015) Multiple Autoregulation of nodulation (AON) signals identified through split root analysis of *Medicago truncatula sunn* and *rdn1* mutants. Plants 4:209–224

Kisner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7:511–518

Kiss E, Mergaert P, Olàh B, Kereszt A, Staehelin C, Davies AE, Downie JA, Kondorosi A, Kondorosi E (1998) Conservation of *noIR* in the *Sinorhizobium* and *Rhizobium* genera of the *Rhizobiaceae* Family. Mol Plant-Microbe Interact 12:1186–1195

Loh J, Stacey G (2003) Nodulation gene regulation in *Bradyrhizobium japonicum*: a unique integration of global regulatory circuits. Appl Environ Microbiol 69:10–17

López-Lara IM, Geiger O (2000) Expression and purification of four different rhizobial acyl carrier proteins. Microbiology 146:839–849

López-Lara IM, Geiger O (2001) The nodulation protein NodG shows the enzymatic activity of an 3-oxoacyl-acyl carrier protein reductase. Mol Plant-Microbe Interact 14:349–357

López-Lara IM, van den Berg JD, Thomas-Oates JE, Glushka J, Lugtenberg BJ, Spaink HP (1995) Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*. Mol Microbiol 15:627–638

López-Lara IM, Blog-Tip L, Quinto C, Garcia ML, Stacey G, Bloemberg GV, Lamers GEM, Lugtenberg BJJ, Thomas-Oates JE, Spaink HP (1996) NodZ of Bradyrhizobium extends the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation signals. Mol Microbiol 21:397–408

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, Martinez EA, Driguez H, Bécard G, Dénarié J (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469:58–63

Mergaert P, D'Haeze W, Geelen D, Promé D, van Montagu M, Geremia R, Promé JC, Holsters M (1995) Biosynthesis of *Azorhizobium caulinodans* nod factors: study of the activity of the NodABC proteins by expression of the genes in *Escherichia coli*. J Biol Chem 270:29217–29223

Mergeart P, D'Haeze W, Fernandez-Lopez M, Geelen D, Goethals K, Promé JC, von Montagu M, Holsters M (1996) Fucosylation and arabinosylation of nod factors in *Azorhizobium caulinodans*: involvements of nolK, nodZ as well as noeC and or downstream genes. Mol Microbiol 21:409–419

Moulin L, Munive A, Dreyfus B, Boivin-Masson C (2001) Nodulation of legumes by members of beta-subclass of proteobacteria. Nature 411:948–950

Oldroyd GED (2013) Speak, friend and enter: signaling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol 11:252–263

Orgambide GG, Lee J, Hollingworth RI, Dazzo FB (1995) Structurally diverse chitooligosaccharide Nod factors accumulate primarily in membranes of wild type *Rhizobium leguminosarum biovar trifolii*. Biochemistry 34:3832–3840

Österman J, Marsh J, Laine PK, Zeng Z, Alatalo E, Sullivan JT, Young JPW, Thomas-Oates J, Paulin L, Lindström K (2014) Genome sequencing of two *Neorhizobium galegae* strains reveals a *noeT* gene responsible for the unusual acetylation of the nodulation factors. BMC Genomics 15:500

Perret X, Staehelin C, Broughton W (2000) Molecular basis of symbiotic promiscuity. Microbiol Mol Biol Rev 64:180–201

Persson T, Battenberg K, Demin IV, Vigil-stenman T, Vanden Heuvel B, Puijic P et al (2015) Candidatus Frankia Datiscae Dg1, the *Actinobacterial* microsymbiont of *Dastica glomerata*, expresses the canonical nod genes nodABC in symbiosis with its host plant. PLoS ONE 10: e0127630. https://doi.org/10.1371/journal.pone.0127630

Pohl A, Devaux PF, Herrmann A (2005) Function of prokaryotic and eukaryotic ABC proteins in lipid transport. Biochim Biophys Acta 1733:29–52

Poinsot V, Crook MB, Erdn S, Maillet F, Bascaules A, Ané JM (2016) New insights into Nod factor biosynthesis: analyses of chitooligomers and lipo-chitooligomers of *Rhizobium* sp. IRBG mutants. Carbohydr Res 434:83–93

Price NPJ, Talmont F, Wieriszeski JM, Promé D, Promé JC (1996) Structural determination of symbiotic nodulation factors from the broad host-range *Rhizobium* species NGR234. Carbohydr Res 289:115–136

Pueppke SG (1996) The genetic and biochemical basis for nodulation of legumes by rhizobia. Crit Rev Biotechnol 16:1–51

Quesada-Vincens D, Hanin M, Fellay R, Broughton WJ, Jabbouri S (1998) In vitro sulfotransferase activity of *noeE*, a nodulation protein of rhizobium sp. NGR234. Mol Plant-Microbe Interact 11:592–600

Quinto C, Wijfjes AHM, Bloemberg GV, Blog-Tip L, Lopès-Lara IM, Lugtenberg BJJ, Thomas-Oates JE, Spaink HP (1997) Bacterial nodulation protein NolZ is a chitin oligosaccharide fucosyl transferase which can also recognize related substrates of animal origin. Proc Natl Acad Sci U S A 94:4336–4341

Rao JR, Cooper JE (1994) Rhizobia catabolize nod gene inducing flavonoids via C-ring fission mechanisms. J Bacteriol 176:5409–5413

Ritsema T, Wijfjes AHM, Lugtenberg BJJ, Spaink HP (1996) *Rhizobium* nodulation protein NodA is a host-specific determinant of the transfer of fatty acids in nod factor biosynthesis. Mol Gen Genet 251:44–51

Rivilla R, Sutton JM, Downie JA (1995) *Rhizobium leguminosarum* nodT is related to a family of outer-membrane transport proteins that includes TolC, PrtF, CyaE and AprF. Gene 161:27–31

Sadowsky MJ, Cregan PB, Gottfert M, Sharma A, Gerhold D, Rodriguez-Quinones F, Keyser HH, Hennecke H, Stacey G (1991) The bradyrhizobium japonicum nolA gene and its involvement in the genotype-specific nodulation of soybeans. Proc Natl Acad Sci U S A 88:637–641

Schlaman HRM, Phillips DA (1998) Genetic organization and transcriptional regulation of Rhizobial nodulation genes. In Spaink HP, Kondorosi E, Hooykaas HH (eds) The *Rhizobiaceae* Springer, Dordrecht, 361–386

Schlaman HRM, Spaink HH, Okker RJK, Lugtenberg BJJ (1989) Subcellular localization of the nod gene product in *Rhizobium leguminosarum*. J Bacteriol 171:4686–4693

Schmidt J, Wingender R, John M, Wieneke U, Schell J (1988) *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stimulates mitosis of plant cells. Proc Natl Acad Sci U S A 83:9581–9585

Schmidt J, John M, Wieneke U, Stacey G, Röhring H, Schell J (1991) Studies of the function of *Rhizobium* meliloti nodulation genes. In: Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions. Kluwer, Dordrecht, pp 150–155

Schmidt PE, Broughton WJ, Werner D (1994) Nod factor of *Bradyrhizobium japonicum and Rhizobium sp NGR234* induce flavonoid accumulation in soybean root exudate. Mol Plant-Microbe Interact 7:384–390

Schultze M, Staehlin C, Röhrig H, John M, Schmidt J, Kondorosi E, Schell J, Kondorosi A (1995) In vitro sulfotransferase activity or *Rhizobium meliloti* NodH protein: Lipochitooligosaccharide nodulation signal are sulfated after synthesis of the core structure. Proc Natl Acad Sci U S A 92:2706–2709

Schwedock JS, Long SR (1990) ATP sulphurylase activity of *nodP* and *nodQ* gene products of *Rhizobium meliloti*. Nature 348:644–647

Schwedock JS, Liu CX, Leyh TS, Long SR (1994) *Rhizobium meliloti* NodP and NodQ form a multifunctional sulfate-activating complex requiring GTP for activity. J Bacteriol 176:7055–7064

Slabas AR, Chase D, Nishida I, Murata N, Sidebottom C, Safford R, Sheldon PS, Kekwick RG, Hardie DG, Mackintosh RW (1992) Molecular cloning of higher-plant 3-oxoacyl-(acyl carrier protein) reductase. Sequence identities with the *nodG*-gene product of the nitrogen-fixing soil bacterium *Rhizobium meliloti*. Biochem J 283:321–326

Snoek C, Luyten E, Poinsot V, Savagnac A, Vanderleyden J, Promé JC (2001) *Rhizobium* sp. BR816 produces a complex mixture of known and novel lipooligosaccharide molecules. Mol Plant-Microbe Interact 14:678–684

Spaink HP, Aarts A, Stacey G, Bloemberg GV, Lugtenberg BJJ, Kennedy EP (1992) Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin layer chromatography. Mol Plant-Microbe Interact 5:72–80

Spaink HP, Wijfjes AHM, van der Drift KMGM, Haverkamp J, Thomas-Oates JE, Lugtenberg BJJ (1994) Structural identification of metabolites produced by the NodB and NodC proteins of *Rhizobium leguminosarum*. Mol Microbiol 13:821–831

Swanson JA, Mulligan JT, Long SR (1993) Regulation of *syrM* and *nodD3* in *Rhizobium meliloti*. Genetics 134:435–444

Treilhou M, Ferro M, Monteiro C, Poinsot V, Jabbouri S, Kanony C, Promé D, Promé JC (2000) Differentiation of *O*-acetyl and *O*-Carbamoyl esters of *N*-acetyl-glucosamine by decomposition of their oxonium ions. Application to the structure of the nonreducing terminal residue of Nod factors. J Am Soc Mass Spectrom 11:301–311

van Rijn P, Feys B, Verreth C, Vanderleyden J (1993) Multiple copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816. J Bacteriol 175:438–447

Vazquez M, Santana O, Quinto C (1993) The NodI and NodJ proteins from *Rhizobium* and *Bradyrhizobium* strains are similar to capsular polysaccharide secretion proteins from gram negative bacteria. Mol Microbiol 5:71–75

Yang GP, Debellé F, Savagnac A, Ferro M, Schiltz O, Maillet F, Promé D, Treilhou M, Vialas C, Lindström K, Promé JC (1999) Structure of the *Mesorhizobium huakii* and *Rhizobium galegea* Nod factors: a cluster of phylogenetically related legumes are nodulated by rhizobia producing Nod factors with α,β-unsaturated *N*-acyl substitutions. Mol Microbiol 34:227–237

Metabolism and Regulation of Glycerolipids in Yeast

17

Vanina Zaremberg, Suriakarthiga Ganesan, and Brittney N. Shabits

Contents

1 Introduction 316
2 Glycerolipid Metabolism in Yeast: Membrane Proliferation Versus Fatty Acid Storage 317
3 De Novo Glycerolipid Biosynthesis: Initial Acylation Steps to Produce Phosphatidic Acid 319
4 Interconversion of Phosphatidic Acid and Diacylglycerol-Phosphatidate Phosphatase and DAG Kinase 325
4.1 Discovery and Characterization of the Yeast Phosphatidate Phosphatase Pah1 325
4.2 Regulation of Pah1 by Phosphorylation 326
4.3 Phosphatidic Acid as Master Regulator of Glycerolipid Synthesis: Ino2-Ino4-Opi1 Circuit 328
5 Phosphatidic Acid Conversion to CDP-DAG and Mitochondrial Phospholipid Transport 329
5.1 ER-Mitochondria Encounter Structure (ERMES) 330
5.2 Mitochondrial Transport of PA by Ups1-Mdm35 331
5.3 Mitochondrial Transport of PS and PE 332
6 Research Needs 333
References 334

Abstract

Bilayer-forming phospholipids and storage lipids like triacylglycerol are all part of the lipid class known as glycerolipids. Research conducted in the model organism *Saccharomyces cerevisiae* has been at the forefront of the identification of the enzymes involved in the metabolism of glycerolipids and its regulation. The initial two sequential acylations of glycerol 3-phosphate using acyl-CoA as acyl donor to produce phosphatidic acid (PA) are common steps in the de novo

V. Zaremberg (✉) · S. Ganesan · B. N. Shabits
Department of Biological Sciences, University of Calgary, Calgary, AB, Canada
e-mail: vzarembe@ucalgary.ca; ganesans@ucalgary.ca; bnshabit@ucalgary.ca

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_20

synthesis of glycerolipids. PA represents a critical branching point in this pathway as it can either be (1) dephosphorylated to produce diacylglycerol (DAG) for the synthesis of triacylglycerol or phospholipids through the Kennedy pathways or (2) be converted to CDP-DAG for the synthesis of phospholipids. PA has surfaced as not only the precursor for all glycerolipids but also as a potent signalling lipid able to integrate cellular cues to balance synthesis of phospholipids for membrane expansion versus storage in the form of triacylglycerol. The PA-dependent regulatory circuit Ino2-Ino4-Opi1 that controls expression of enzymes involved in glycerolipid synthesis downstream of the PA branching point has been very well characterized in yeast. Less is known about the regulation of the upstream initial steps leading to PA synthesis. Emerging research points to the production of different pools of PA by the acyltransferases in charge of the initial acylation steps in concert with other pathways that produce and use acyl-CoAs. In addition, recent discoveries on the movement of PA between organelles further indicate these pools are mobile. Although elucidation of lipid pool partitioning remains a major challenge in the field, current technology combined with the tractability of yeast warrant future progress on this topic.

1 Introduction

Glycerolipids include storage lipids like triacylglycerides (TAG) and membrane lipids encompassing the category of glycerophospholipids, commonly known as phospholipids. The bilayer-forming nature of phospholipids results in this lipid class representing the most abundant components of eukaryotic membranes. In addition to serving structural roles, many phospholipids have the potential of becoming signaling molecules, informing the cell of changes in environmental conditions and energy status. These lipids serve as platforms for the organization of regulatory complexes that participate in adaptive responses that may allow cell survival at one end or cell death at the other. The yeast *Saccharomyces cerevisiae* has been used for decades as an excellent model organism for studying glycerolipid metabolism and its regulation. While the characterization of the proteins that participate in the pathways that metabolize glycerolipids in yeast is still being refined, and new enzymes or new activities for old enzymes are being identified, the picture is quite complete with very few gaps in knowledge. Major progress in understanding how lipid metabolic systems are spatiotemporally regulated has been made in recent years. Increasing evidence indicates that separation of lipid pools within cells is critical for channeling of cellular processes, as well as for controlling the bioactive and signalling properties of many lipid species. Elucidation of lipid pool partitioning remains a major challenge in the field.

Since completion of its entire genome in 1996, yeast has been at the vanguard of the development of functional genomic, transcriptomic, proteomic, and metabolomic approaches. This has resulted in a vast amount of high-throughput data collection, with yeast currently representing the organism with the most

comprehensive and well-curated experimental dataset available to the research community (Cherry et al. 2012). New levels of understanding of the physiological roles of lipids and their remodeling as part of the cell adaptation to physiological changes are emerging as consequence of the use of these systems biology endeavors.

In this chapter, we will provide an overview of recent progress made regarding the biochemistry, cell biology, and systems biology of the initial steps in glycerophospholipid metabolism leading to phosphatidic acid synthesis and its regulation in yeast. Gaps in knowledge and controversies will be highlighted, hoping the material will be suitable for both the beginner and expert alike.

2 Glycerolipid Metabolism in Yeast: Membrane Proliferation Versus Fatty Acid Storage

Typical growth of yeast in rich medium, with glucose as carbon source, is initiated with a temporary lag phase where cells reprogram their metabolism to start proliferation. Following is the exponential phase, where fermentation of glucose to ethanol fuels growth. Once glucose is depleted, cells enter a transitory quiescent phase known as the diauxic shift. During this phase metabolism is adjusted to support growth in the post-diauxic phase by using cellular respiration of ethanol and fatty acid oxidation. Finally, cells enter the quiescent stationary phase and growth ceases (Fig. 1a). Several adjustments and modulation of lipid metabolism occur during the physiological adaptations observed throughout the growth of yeast in rich medium. Notoriously, the ratio between storage and membrane lipids oscillates across all growth stages (Kohlwein et al. 2013; Casanovas et al. 2015). Mobilization of neutral lipids (TAG and sterol esters) dominates during proliferation, when membrane lipids are required for secretory traffic and plasma membrane expansion. On the other hand, accumulation of storage lipids increases when cells enter quiescent phases (Fig. 1b). These fluctuations in glycerolipid synthesis and turnover are exquisitely regulated at many levels, including gene expression controlled by phospholipid precursors, compartmentalization, and posttranslational modifications of enzymes (Henry et al. 2012).

Although a striking structural diversity of lipids characterizes eukaryotic membranes, laboratory strains of *S. cerevisiae* display a discrete complement of acyl chains in the glycerolipids, mostly comprised of C16:0, C16:1, C18:0, and C18:1 (Ejsing et al. 2009). Interestingly, the total level of saturated fatty acid (SFA) moieties in the yeast lipidome builds up until the mid exponential phase, followed by an increase in the total level of monounsaturated fatty acid (MUFA) moieties until growth reaches the post-diauxic phase (Fig. 1c). These changes correlate with the abundance of the only yeast desaturase (Δ9) Ole1. During exponential phase when SFAs are high, the expression of *OLE1* is high while expression decreases as MUFAs increase (Casanovas et al. 2015).

It is well known that the synthesis of lipids is spatially restricted, and localization of biosynthetic systems is in fact the first determinant of the distinct lipid compositions of organelles (van Meer et al. 2008) (Fig. 2). De novo synthesis of glycerolipids

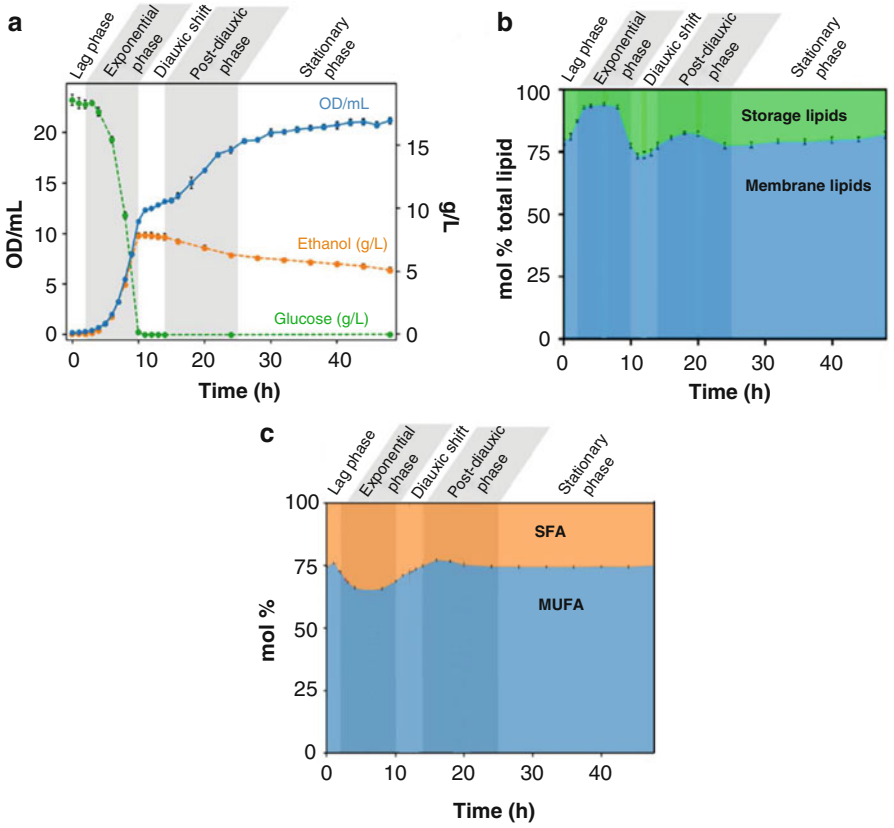


Fig. 1 *Fluctuations in storage and membrane lipids and acyl chain composition during yeast growth*. (**a**) Cell density (OD/ml) and extracellular glucose and ethanol concentrations. (**b**) Temporal dynamics of storage and membrane lipids. Shaded areas correspond to phases of proliferation. (**c**) Temporal profile of saturated (SFA) and monounsaturated (MUFA) fatty acid moieties in the yeast lipidome. All results are expressed as the mean ± SD of five biological replicates (Adapted from Casanovas et al. 2015)

(Fig. 3) occurs mostly in the endoplasmic reticulum (ER) and is initiated by two consecutive acylation steps using acyl-CoA as acyl donor, to produce phosphatidic acid (PA) from glycerol 3-phosphate or dihydroxyacetone phosphate (Sect. 3). PA represents a critical branching point in glycerolipid metabolism where flux toward synthesis of triacylglycerol (TAG) for storage in lipid droplets (LDs), versus synthesis of phospholipids for membrane expansion, is adjusted by the conversion of PA to diacylglycerol (DAG) or its activated form CDP-DAG, respectively. PA is not only a precursor of all glycerolipids but also a master regulator of the activity of both DAG and CDP-DAG biosynthetic branches involved in the production of TAG and phospholipids (Sect. 4). PA made in the ER can be translocated to mitochondria by a recently discovered protein complex composed of the mitochondrial proteins Ups1 and Mdm35 (Sect. 5). This pool of PA is used for the biosynthesis of the

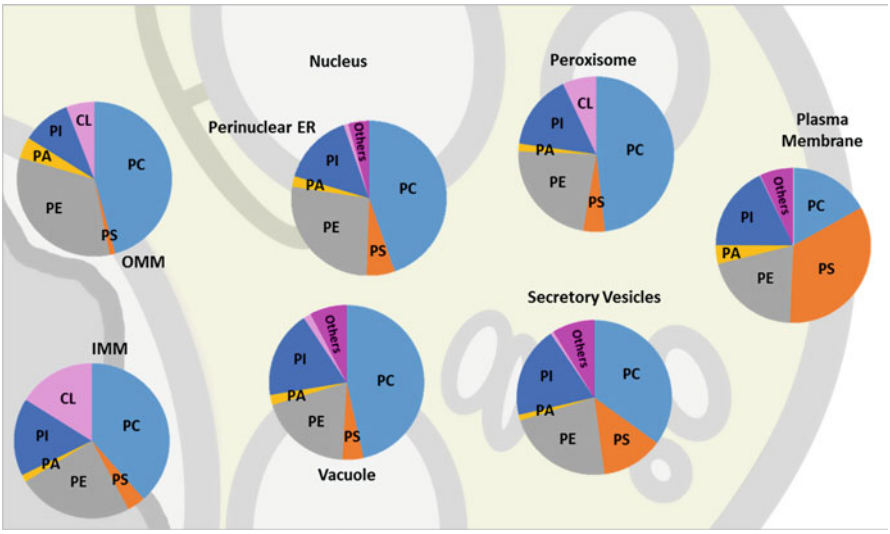


Fig. 2 *Glycerophospholipid composition of yeast intracellular membranes*. The distribution of glycerophospholipids in the indicated yeast compartments was obtained from Zinser et al. (1991). *PC* phosphatidylcholine, *PI* phosphatidylinositol, *PE* phosphatidylethanolamine, *PS* phosphatidylserine, *PA* phosphatidic acid, *CL* cardiolipin, *IMM* inner mitochondrial membrane, *OMM* outer mitochondrial membrane. Others include lysophospholipids and diacylglycerol

mitochondrial lipids phosphatidylglycerol (PG) and cardiolipin (CL) via a CDP-DAG branch of glycerolipid metabolism.

3 De Novo Glycerolipid Biosynthesis: Initial Acylation Steps to Produce Phosphatidic Acid

De novo synthesis of glycerolipids is initiated by glycerol 3-phosphate acyltransferases (GPATs), which transfer a fatty acid from fatty acyl-CoA to the *sn*-1 position of glycerol-3-phosphate (G3-P) to produce lysophosphatidic acid (LysoPA) (Table 1 and Fig. 4). This represents the committed and rate-limiting step in the biosynthesis of PA. Two genes coding for the yeast GPATs have been identified, *SCT1/GAT2* and *GPT2/GAT1* (Zheng and Zou 2001; Zaremberg and McMaster 2002). Although deletion of both *SCT1* and *GPT2* is lethal, deletion of either gene alone causes no growth defects on rich media (Zaremberg and McMaster 2002).

Both Sct1 and Gpt2 are dual substrate-specific *sn*-1 acyltransferases that can use G3-P as well as dihydroxyacetone phosphate (DHAP), although Sct1 prefers G3-P over DHAP (Zheng and Zou 2001). The purpose of the DHAP shunt in yeast is not clear as *S. cerevisiae* does not possess a peroxisomal pathway for the synthesis lipids. It is well known peroxisomal DHAP acyltransferases are involved in the

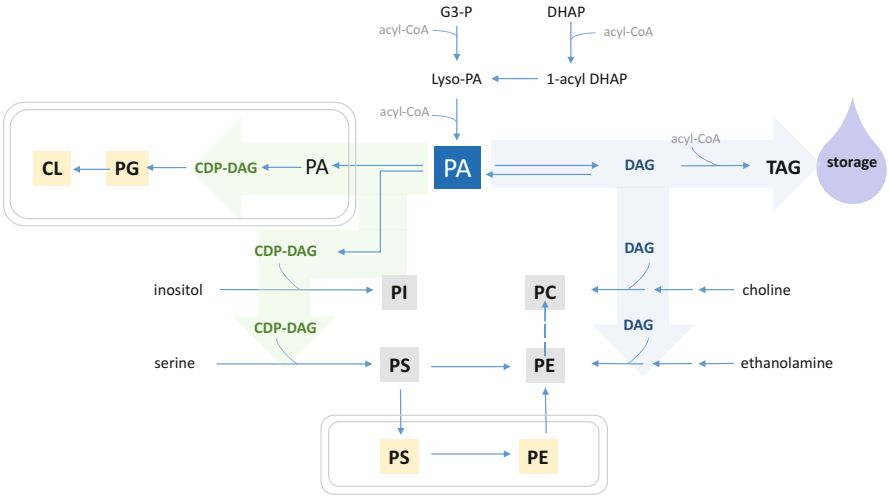
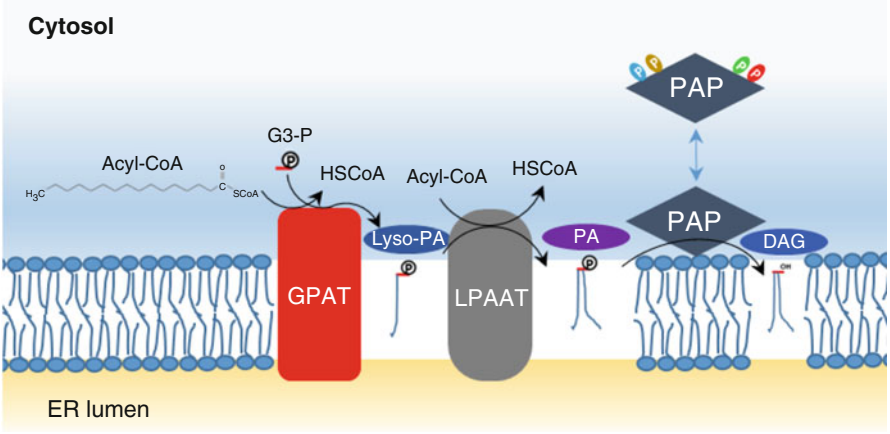


Fig. 3 *De novo synthesis of glycerolipids in yeast*. De novo synthesis of glycerolipids in yeast is initiated by the sequential acylation of glycerol-3-phosphate, G3-P, or dihydroxyacetone phosphate, DHAP, to produce lysophosphatidic acid, LysoPA, and then phosphatidic acid, PA. PA represents a branching point as it can be converted to diacylglycerol, DAG, feeding the Kennedy pathways (*blue* pathways) or to CDP-DAG (*green* pathways). Mitochondrial pathways are represented within double-membrane-enclosed compartments. Lipid droplet (*purple*) is the storage compartment for triacylglycerol, TAG. *PC* phosphatidylcholine, *PI* phosphatidylinositol, *PE* phosphatidylethanolamine, *PS* phosphatidylserine, *PG* phosphatidylglycerol, *CL* cardiolipin

synthesis of ether glycerolipids in mammals, but these lipids have not been described so far in Fungi. A recent phylogenetic analysis of acyltransferases in Fungi and Metazoa identified a family of fungal putative peroxisomal acyltransferases (absent in *S. cerevisiae*), potentially indicative of an undescribed metabolic capacity in this lineage (Smart et al. 2014).

The two yeast GPATs are microsomal (ER) enzymes differentially enriched in ER domains (Bratschi et al. 2009). The ER forms a network with points of contact with every organelle of the cell, and it is possible that Sct1 and Gpt2 associate with sites where inter-organelle transport of lipids occurs. GPAT activity has been measured in purified yeast LDs, and mass spectrometry analysis has identified Gpt2 (but not Sct1) among the proteins enriched in this compartment (Athenstaedt and Daum 1997; Daum et al. 2011; Wang 2015). Interestingly, association of Gpt2 with purified LDs depends on carbon source as it was detected in glucose but not in oleate-growing cells (Daum et al. 2011; Marr et al. 2012). In fact, ER-derived structures enriched in Gpt2 that tightly associate with LDs induced by growth on oleate have been observed (Marr et al. 2012), supporting recent models of LD biogenesis in which the ER bilayer juxtaposes the monolayer of LDs (Mishra et al. 2016). Cells lacking Gpt2 (but not Sct1) fail to grow on oleate, a phenotype known to be linked to TAG synthesis and peroxisomal function in yeast. The evidence supports a role of Gpt2 in the control of flux of oleate into TAG as cells lacking Gpt2 (*gpt2Δ*) cannot

Table 1 Initial steps in de novo glycerolipid synthesis



GPATs/ DHAPATs	LPAATs	PAP and LPPs[t]
Sct1/Gat2 (*YBL011W*)[a] Dual substrate (G-3P/DHAP) *sn*-1 acyltransferase. Prefers G3-P and 16 carbon fatty acyl-CoAs[b] Integral membrane protein localized to the ER[c] Phosphorylated[c] Synthetic lethality with *GPT2*	*Slc1* (*YDL052C*) LysoPA *sn*-2 acyltransferase, with preference for C18:1, C14:0, C12:0, and C10:0 acyl-CoAs[g] Integral membrane protein localized to ER and lipid droplets[h] Proposed topology positions part of catalytic motifs facing ER lumen[i] Gain of function allele allows for incorporation of C26 FAs to LysoPA or LysoPI[j,k] Synthetic lethality with *ALE1*	*Pah1*/Smp2 (*YMR165C*) Lipin-like, Mg^{2+}-dependent phosphatidate phosphatase (PAP), involved in de novo synthesis of phospholipids and TAG Phosphorylated protein is soluble and localizes to the cytosol and nucleus. Dephosphorylation by Nem1-Spo7 phosphatase complex mediates its recruitment to the ER membrane
Gpt2/Gat1 (*YKR067W*) Dual substrate (G-3P/DHAP) *sn*-1 acyltransferase[b] Integral membrane protein localized to ER and lipid droplets[c,d,e] Proposed topology positions part of catalytic motifs facing ER lumen[f] Phosphorylated[c] Synthetic lethality with *SCT1*	*Ale1*/Slc4, Lpt1, Lca1 (*YOR175C*) Broad-specificity lysophospholipid *sn*-2 acyltransferase; part of MBOAT family of membrane-bound *O*-acyltransferases; key component of Lands´ cycle. Highest activity towards LysoPC and medium-chain, monounsaturated acyl-CoA[l,m,n,o,p] Integral membrane protein localized to ER and enriched in the MAM[m] Proposed topology positions part of catalytic motifs facing	*App1* (*YNL094W*) Mg^{2+}-dependent lipid phosphate phosphatase (LPP), involved in endocytosis Prefers PA as substrate over diacylglycerol pyrophosphate and lysoPA Soluble protein localized to actin cortical patches and actin cytoskeleton

(*continued*)

Table 1 (continued)

GPATs/ DHAPATs	LPAATs	PAP and LPPs[t]
	ER lumen[k] Phosphorylated Synthetic lethality with *SLC1*	
	Loa1/Vps66 (*YPR139C*) Proposed LysoPA *sn*-2 acyltransferase, with preference for oleoyl-CoA[q] Integral membrane protein localized to the ER and lipid droplets[q,r]	*Dpp1*/Zrg1 (*YDR284C*) Mg^{2+}-independent LPP, utilizes PA, diacylglycerol pyrophosphate and lysoPA as substrates Integral membrane protein localized to vacuole
	Ict1 (*YLR099C*) Proposed soluble LysoPA *sn* − 2 acyltransferase, with preference for oleoyl-CoA, which is upregulated on isooctane treatment[s]	*Lpp1* (*YDR503C*) Mg^{2+}-independent LPP, utilizes PA, diacylglycerol pyrophosphate and lysoPA as substrates Integral membrane protein localized to Golgi

[a]Systematic gene name in italics and parenthesis. *GPAT* Glycerol 3-phosphate acyltransferase; *LPAAT* lysoPA acyltransferase; *PAP* phosphatidate phosphatase; *LPP* lipid phosphate phosphatase References: [b]Zheng and Zou 2001; [c]Bratschi et al. 2009; [d]Daum et al. 2011; [e]Marr et al. 2012; [f]Pagac et al. 2012; [g]Shui et al. 2010; [h]Athenstaedt and Daum 1997; [i]Pagac et al. 2011; [j]Nagiec et al. 1993; [k]Vionnet et al. 2011; [l]Jasieniecka-Gazarkiewicz et al. 2016; [m]Riekhof et al. 2007b; [n]Jain et al. 2007; [o]Tamaki et al. 2007; [p]Benghezal et al. 2007; [q]Ayciriex et al. 2012; [r]Daum et al. 2011; [s]Ghosh et al. 2008; [t]Pascual and Carman 2013

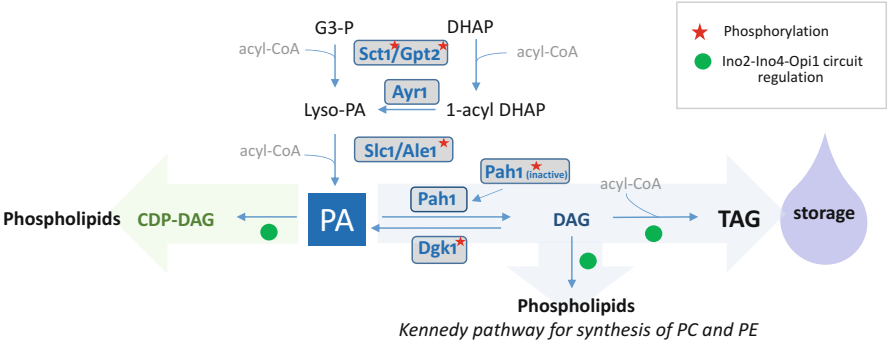


Fig. 4 *De novo synthesis of phosphatidic acid in yeast*. Names of yeast enzymes catalyzing the initial steps in PA synthesis and its interconversion to DAG are indicated. Enzymes known to be regulated through phosphorylation are denoted by the *red star*. Downstream DAG and CDP-DAG branches are regulated by the Ino2-Ino4-Opi1 circuit (*green dot*)

use oleate as carbon source (Marr et al. 2012). The oleate sensitivity of *gpt2* mutants could be related to the substrate specificity displayed by Sct1 (which is the only GPAT present in the *gpt2Δ* mutant). Determination of in vitro substrate specificities indicated that Gpt2 had a broad fatty acyl-CoA specificity profile, while Sct1 had a

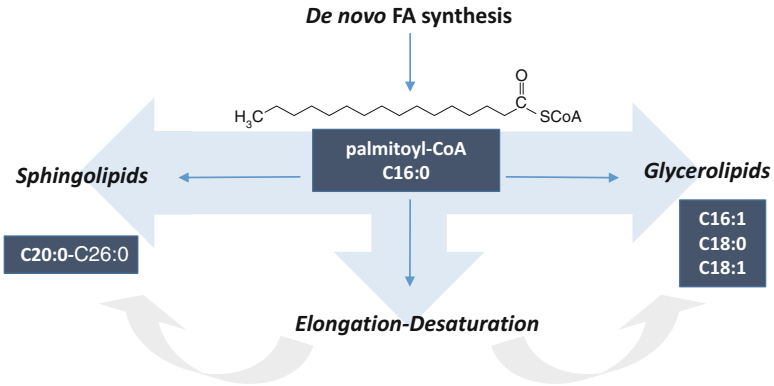


Fig. 5 *Competition for palmitoyl-CoA between de novo synthesis of glycerolipids and other pathways*. Palmitoyl-CoA is the main product of the de novo synthesis of fatty acids in yeast and is used by acyltransferases in the glycerolipid biosynthetic pathway to make PA, by fatty acid-modifying enzymes and is also a precursor for the synthesis of sphingolipids

marked preference for 16 carbon fatty acyl chains (Zheng and Zou 2001). This substrate preference is relevant in vivo, as it has been shown that Sct1 competes with the only yeast desaturase (Δ9) Ole1 for the common substrate palmitoyl-CoA (Fig. 5) (De Smet et al. 2012). In fact, competition of the GPATs for the palmitoyl-CoA pool produced from the de novo fatty acid synthesis with the sphingolipid biosynthetic pathway could be also anticipated (Fig. 5). In addition, deletion of Sct1 (but not Gpt2) was shown to revert the aberrant nuclear envelope morphology that results from lack of the PA phosphatase Pah1 (Han et al. 2008a).

Therefore, several lines of evidence support the idea that distinct pools of PA varying in acyl chain length and saturation are controlled at the GPAT step. These PA pools can regulate the flux of fatty acids for storage into TAG or for membrane proliferation depending on nutritional conditions or in response to membrane stress.

Sct1 and Gpt2 are phosphoproteins and their phosphorylation status can be distinguished by polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE). Phosphorylation of yeast GPATs responds to growth phase, carbon source, and cellular GPAT imbalance (Bratschi et al. 2009; Marr et al. 2012). An intricate phosphorylation pattern involving more than 20 phospho-residues mostly localized to a serine rich region in the carboxy-end of Gpt2 has been uncovered by mass spectrometry analysis of the yeast phosphoproteome and immunoaffinity purified Gpt2, while only six putative phospho-residues have been detected in Sct1 (Cherry et al. 2012 and Zaremberg unpublished results). How phosphorylation regulates the yeast GPATs is still unknown.

The second step in the de novo synthesis of PA and downstream glycerolipids is catalyzed by LysoPA acyltransferases (LPAATs) (Table 1 and Fig. 4). Slc1 was the first *sn*-2-specific, acyl-CoA-dependent LPAAT identified in yeast (Nagiec et al. 1993; Athenstaedt and Daum 1997). Slc1 substrate preference includes 18:1, 14:0, 12:0, and 10:0 fatty acids (Shui et al. 2010). When *SLC1* was knocked out, LPAAT

activity was still present, and it was later discovered that this redundant LPAAT activity was due, at least, to another lysophospholipid acyltransferase, Ale1 (also known as Slc4, Lpt1, or Lca1) (Jain et al. 2007; Tamaki et al. 2007; Riekhof et al. 2007b; Benghezal et al. 2007). The double deletion of *ALE1* and *SLC1* in *S. cerevisiae* is synthetic lethal, indicating no other yeast protein can compensate for the loss of this essential activity (Jain et al. 2007; Tamaki et al. 2007; Riekhof et al. 2007b; Benghezal et al. 2007). Ale1 is an integral membrane protein, enriched in sites of contact between the ER and mitochondria and is a member of the membrane-bound *O*-acyltransferase family (Riekhof et al. 2007b). It has been shown that PA generated by either Slc1 or Ale1 can be used similarly for further glycerophospholipid biosynthesis (Benghezal et al. 2007).

Ale1 is a broad-specificity lysophospholipid acyltransferase and it acylates LysoPC with the highest activity. Ale1 also has activity toward LysoPA, LysoPE, LysoPS, LysoPI, and LysoPG (Jain et al. 2007; Riekhof et al. 2007a, b; Tamaki et al. 2007; Benghezal et al. 2007; Jasieniecka-Gazarkiewicz et al. 2016). Ale1 preferentially transfers medium-chain (6–14 carbons), monounsaturated acyl chain from an acyl-CoA to the *sn*-2 position of these lysophospholipids (Jain et al. 2007; Tamaki et al. 2007; Riekhof et al. 2007b). As Ale1 is the main LysoPC acyltransferase, it is a key component in the Lands' cycle of PC remodelling (Chen et al. 2007). The topology of Slc1 and Ale1 have been studied, suggesting that the most conserved residues and catalytic motifs of both proteins are actually facing the ER lumen, and not the cytosol, suggesting that PA may be produced in the luminal leaflet of the ER (Pagac et al. 2011). Interestingly, it has been recently suggested that Slc1 can operate reversibly on the products of the acylation in vitro, although it is unlikely that this backward reaction occurs in vivo (Jasieniecka-Gazarkiewicz et al. 2016).

In addition to Slc1 and Ale1, two other yeast proteins have been identified as LPAATs. Loa1 (Vps66) is an integral membrane LPAAT, specific for oleoyl-CoA (Ayciriex et al. 2012), localized to ER and LDs (Daum et al. 2011; Ayciriex et al. 2012). Loa1 has a role in LD biogenesis, as cells lacking *LOA1* (*loa1Δ*) have an increased number of smaller LDs, while the overexpression of *LOA1* results in a lower number of LDs than wild-type cells that are larger in size (Ayciriex et al. 2012). The *loa1Δ* mutants show a decrease in the overall cellular TAG content with an increase in sterol esters and phospholipids. Therefore, it has been proposed that Loa1 might be an enzyme preferentially channeling oleic acid into the TAG biosynthetic pathway (Ayciriex et al. 2012). Loa1 has also been shown to be involved in medium-chain fatty acid (MCFAs, containing 6–14 carbons) homeostasis, as *loa1Δ* cells have increased neutral lipid MCFA content (Froissard et al. 2015).

The only soluble lysophosphatidic acid acyl transferase, Ict1, has a preference for oleoyl-CoA over palmitoyl- or stearoyl-CoA (Ghosh et al. 2008). Specifically, *ICT1* expression is induced during isooctane exposure, allowing for increased PA synthesis and therefore increased phospholipid synthesis and tolerance to this organic solvent stress condition via membrane repair (Miura et al. 2000; Ghosh et al. 2008). Ict1 has homologues in both mammals and plants.

LPAAT activity by two yeast lipases, Tgl4 and Tgl5, has also been recently reported, suggesting these enzymes play a dual function in lipid metabolism

contributing to both anabolic and catabolic processes (Rajakumari and Daum 2010a; Rajakumari and Daum 2010b).

Thus, the prominent redundancy of the second acylation step in the synthesis of PA provides a high level of versatility in the flux of diverse fatty acids into phospholipids and TAG. The distinct acyl-CoA preference and localization of the acyltransferases catalyzing this step further supports the existence of unique PA pools in the cell.

4 Interconversion of Phosphatidic Acid and Diacylglycerol-Phosphatidate Phosphatase and DAG Kinase

PA can be dephosphorylated by PA phosphatases to make DAG, and DAG in turn can be converted back to PA by the activity of DAG kinases. Given how relevant these lipids are in signalling pathways and as precursors of glycerolipids, the activity of these enzymes is highly regulated. These interconversions between PA and DAG regulate the flux of fatty acids into either membrane phospholipids or TAG for storage and are therefore critical for maintenance of lipid homeostasis.

Four phosphatases capable of dephosphorylating PA have been identified to date in yeast, named App1, Dpp1, Lpp1, and Pah1 (Toke et al. 1998a, b; Han et al. 2006; Chae and Carman 2013) (Table 1 and Fig. 4). Only Pah1 is specific for PA (phosphatidate phosphatase, PAP) and plays a key role in lipid synthesis, while the other three enzymes are lipid phosphate phosphatases (LPP) that can use PA among other substrates, playing mostly signalling roles (Pascual and Carman 2013). App1 localizes to actin patches, while Dpp1 and Lpp1 localize to the vacuole and Golgi apparatus, respectively (Pascual and Carman 2013). Pah1 on the other hand has been found associated with the nucleus, ER, nucleus-vacuolar junction (NVJ), vacuole, and LDs (Santos-Rosa et al. 2005; Pascual and Carman 2013; Barbosa et al. 2015).

Until 2008, it was believed yeast did not have a DAG kinase. The reason for the elusive nature of this enzymatic activity was that assays were typically performed in the presence of ATP, until the group lead by George Carman (Rutgers University, USA) discovered that the uncharacterized yeast protein known at the time as Hsd1 and renamed Dgk1 is a unique DAG kinase that uses CTP instead (Han et al. 2008b). Dgk1 contains four transmembrane domains and localizes mostly in the ER. Dgk1 is the only known DAG kinase in yeast and is structurally different from the mammalian enzymes, which are highly redundant soluble proteins in inactive state that translocate to membranes upon activation (Han et al. 2008a; Sakane et al. 2016).

4.1 Discovery and Characterization of the Yeast Phosphatidate Phosphatase Pah1

The PA dephosphorylation reaction was first characterized using chicken liver and rat tissues in the late 1950s, and the name phosphatidic acid phosphatase (PAP) was proposed for this enzyme (Smith et al. 1957). Succeeding studies showed that this

PAP activity is dependent on Mg^{2+}, and most of these enzymes are soluble as opposed to membrane bound (Hosaka and Yamashita 1984). In the late 1980s, limited amount of enzyme was successfully purified from *S. cerevisiae* by Carman's group, and was used for kinetic and enzymological studies, but attempts to identify its sequence failed (Carman 2011). Further characterization showed that this enzyme activity is important for regulation of lipid metabolism in yeast (Carman 2011). Serendipitously in 2005, the same group recovered an old sample of the purified PA phosphatase, and sequencing by mass spectrometry conclusively revealed its sequence leading to the identification of the gene encoding the yeast PAP, *PAH1* (Han et al. 2006). Further characterization of the *PAH1* gene revealed its evolutionary relationship to a mammalian gene of unknown function at the time named Lipin1 (Han et al. 2006). Therefore, identification of Pah1 in yeast expedited the characterization of Lipin1 as a PA phosphatase in mammalian cells (Carman 2011). This discovery allowed detailed characterization of PAP enzymes in both yeast and mammals, and the scientific community began to understand their important roles in lipid metabolism.

Although cells lacking Pah1 (*pah1Δ*) are viable, they are unable to grow on non-fermentable carbon sources, display shorter chronological lifespan, have fragmented vacuoles, aberrant nuclear/ER membrane expansion, increased levels of phospholipids, and are sensitive to high temperatures and fatty acid-induced lipotoxicity (Irie et al. 1993; Fakas et al. 2011; Sasser et al. 2012; Barbosa et al. 2015; Park et al. 2015).

Interestingly, while deletion of *DGK1* suppresses most phenotypes displayed by *pah1Δ* cells, it does not reverse the temperature sensitivity of this mutant and fatty acid-induced toxicity observed in these cells. This suggests that TAG production is indeed essential to redirect the accumulated fatty acids into lipid droplets, thus reducing the toxic effect caused by these free fatty acids (Fakas et al. 2011). Suppression of *pah1Δ* phenotypes by deletion of *DGK1* also shows that Dgk1 has access to a DAG pool that is not generated by Pah1 and is most probably independent of TAG lipolysis as well.

4.2 Regulation of Pah1 by Phosphorylation

Pah1 is highly regulated at the posttranslational level by phosphorylation, which controls its membrane recruitment, PAP activity, and proteasome-mediated degradation (Karanasios et al. 2010; Su et al. 2014a, b; Hsieh et al. 2015). To date, five protein kinases have been shown to use Pah1 as substrate, including protein kinase A (PKA), protein kinase C (PKC), Pho80-Pho85, Cdc28-cyclin B kinase, and casein kinase II (CKII) (Fig. 6). On the other hand, Pah1 is dephosphorylated by the Nem1/Spo7 protein phosphatase complex allowing nuclear/ER membrane recruitment of Pah1 (Karanasios et al. 2010; Pascual and Carman 2013; Su et al. 2014a, b; Hsieh et al. 2016). Nem1/Spo7 is a membrane-bound complex, and to be dephosphorylated, Pah1 needs to be recruited to the membrane for activation (Su et al. 2014b). The carboxy-end acidic tail of Pah1 is important for its interaction with the Nem1/Spo7 complex

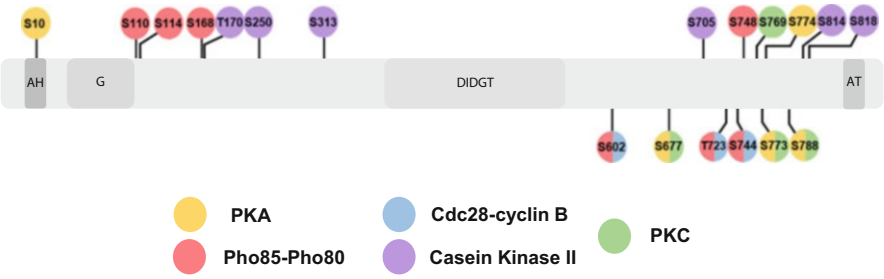


Fig. 6 *Regulation of Pah1 through phosphorylation*. Diagram shows Ser (S) and Thr (T) residues of Pah1 that can be phosphorylated by PKA (*yellow*), Pho85-Pho80 (*red*), Cdc28-cyclin B (*blue*), casein kinase II (*purple*), and PKC (*green*). Single residues that can be phosphorylated by two different kinases display more than one color. Amphipathic helix (AH) in the amino terminal is required for membrane recruitment of Pah1. The N-terminal conserved lipin homologue (NLIP) domain containing the conserved glycine residue (G) and the DIDGT motif within the haloacid dehalogenase (HAD)-like domain are required for catalytic activity of Pah1. The acidic tail (AT) in the carboxy terminal is necessary for interaction of Pah1 with the Nem1-Spo7 phosphatase complex

(Karanasios et al. 2013). Nem1/Spo7 specifically dephosphorylates Pah1 when pre-phosphorylated by Pho80-Pho85 (Su et al. 2014b). Dephosphorylation of Pah1 then enables a short amphipathic helix located at the amino-terminus of the protein to anchor Pah1 onto the nuclear/ER membrane where it finds its substrate (Karanasios et al. 2010). A functional helix is required for the enhancement of the activity of Pah1 by dephosphorylation, both in vitro and in vivo (Karanasios et al. 2010).

Phosphorylation by Pho80-Pho85, PKA, and Cdc28-Cyclin B prevent translocation of Pah1 to the membrane retaining it in the cytosol (Su et al. 2012; Su et al. 2014a; Su et al. 2014b).

Phosphorylation of Pah1 by CKII has a minor effect on subcellular localization and activity of Pah1 (Hsieh et al. 2016). CKII phosphorylates and prevents activation of Pah1 moderately compared to Pho80-Pho85 kinase which strongly inhibits Pah1 activity (Hsieh et al. 2016). The expression of a Pah1 mutant carrying combined mutations which mimic its phosphorylation by CKII and lack of phosphorylation by Pho85-Pho80 resulted in an increase in TAG content and lipid droplet number in cells expressing the Nem1-Spo7 phosphatase complex (Hsieh et al. 2016). Interestingly, CKII also phosphorylates Dgk1 regulating its function in the production of PA (Qiu et al. 2016).

PKC phosphorylation of Pah1 has a minor effect on its activity. Pah1 pre-phosphorylated with PKA has a reduced level of PKC phosphorylation, and this is attributed to overlapping phosphorylation sites by these two kinases on Pah1 (Su et al. 2014a). Pho80-Pho85 prephosphorylation of Pah1 also exerts a similar inhibitory effect on PKC phosphorylation despite having distinct phosphorylation sites on Pah1 (Su et al. 2014a).

Pah1 stability is mainly controlled by the ubiquitin-independent degradation pathway mediated by the 20S proteasome in yeast (Hsieh et al. 2015). Pho80-Pho85 and PKA phosphorylation of Pah1 prevent its degradation, while

phosphorylation by PKC has the opposite effect in the absence of Pho80-Pho85 phosphorylation (Su et al. 2014a; Hsieh et al. 2015). When dephosphorylated by Nem1/Spo7, Pah1 is highly susceptible to degradation (Hsieh et al. 2015).

The kinases that can act on Pah1 identified so far respond to a wide range of cues related to the cell cycle, nutrient availability, and stress conditions. This highlights the importance of the PA to DAG conversion step in maintenance of a balance between membrane expansion during proliferative phases of growth when nutrients are available versus early quiescent phases when lipid storage and LD biogenesis predominate. Pah1 has therefore emerged as a versatile sensor that adjusts its localization and activity in response to multiple signals (Barbosa et al. 2015).

4.3 Phosphatidic Acid as Master Regulator of Glycerolipid Synthesis: Ino2-Ino4-Opi1 Circuit

PA operates at different levels through recruitment of specific effector proteins to the ER membrane. The most iconic example of the role of PA as master regulator of glycerolipid synthesis comes from studies performed on the Ino2-Ino4-Opi1 yeast circuit, and the reader is directed to excellent recent reviews written on this topic (Shin and Loewen 2011; Henry et al. 2012). We will briefly highlight in this section key cellular strategies and concepts arising from the study of this circuit and its regulation.

The heteromeric Ino2p/Ino4p basic helix-loop-helix transcription activator binds to inositol/choline-responsive elements (UAS_{INO}), inducing expression of phospholipid biosynthetic genes in response to inositol depletion. Opi1 is a transcriptional repressor that directly interacts with Ino2 inhibiting the expression of genes activated by this factor. UAS_{INO}-containing genes include those coding for enzymes in the CDP-DAG, and Kennedy pathways for the synthesis of phospholipids and TAG, all located downstream of the PA branching point (Figs. 4 and 7). In addition, the de novo fatty acid synthesis pathway that produces mainly palmitoyl-CoA is also regulated by the Ino2-Ino4-Opi1 circuit. Interestingly, the genes coding for the enzymes catalyzing the initial acylations and PA/DAG interconversion steps in the de novo glycerolipid biosynthetic pathway do not contain the UAS_{INO} element in their promoters. In fact, the PA pool localized to the ER resulting from these first steps regulates this circuit through recruitment of the transcriptional repressor Opi1. When PA levels are high, Opi1 is tethered to the ER, preventing its translocation to the nucleus. Recruitment of Opi1 by PA in the ER is cooperative and also depends on the presence of the ER integral membrane protein Scs2 (Loewen et al. 2004). Upon exogenous addition of the lipid precursor inositol, PA is rapidly consumed for PI synthesis via the CDP-DAG pathway, and Opi1 is released from the ER membrane moving to the nucleus where it represses the expression of genes under Ino2/4 transcriptional activation (Loewen et al. 2004; Carman and Henry 2007). A recent study reported that Opi1 can be differentially regulated by varying the acyl chain composition of PA as it preferentially binds saturated PA species with shorter acyl chains (Hofbauer et al. 2014). It is worth noting that phosphorylation by PKC, PKA,

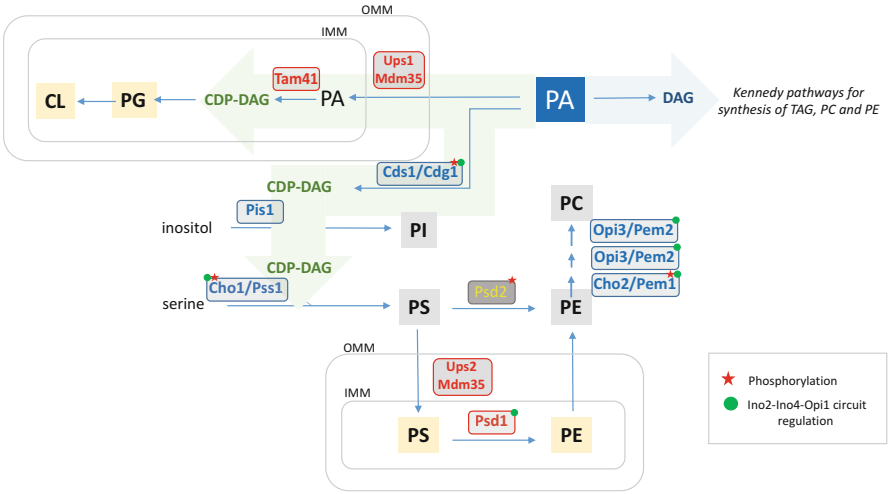


Fig. 7 *Conversion of PA to CDP-DAG and downstream pathways for de novo synthesis of phospholipids*. Names of yeast enzymes (name/alias) catalyzing the conversion of phosphatidic acid, PA, to CDP-DAG and downstream steps are indicated. Font color represents localization: *blue*, endoplasmic reticulum; *red*, mitochondrial; *yellow* Golgi/vacuole. A *red star* denotes regulation through phosphorylation and a *green dot* regulation by the Ino2-Ino4-Opi1 circuit. Mitochondrial pathways are represented within double-membrane-enclosed compartments. *OMM* outer mitochondrial membrane, *IMM* inner mitochondrial membrane

and CKII controls the activity of Opi1 in concert with regulation of Pah1 (Carman and Kersting 2004; Chang and Carman 2006). Thus, these kinases regulate the pivotal conversion of PA to DAG and recruitment of Opi1 to the ER membrane, fine-tuning the expression of enzymes in the glycerolipid metabolic pathways downstream of the PA branching point, in response to nutrients, growth phase, or stress conditions.

5 Phosphatidic Acid Conversion to CDP-DAG and Mitochondrial Phospholipid Transport

The conversion of PA to CDP-DAG leads to the branching of phospholipid synthesis into several different pathways (Fig. 7). Two cellular compartments are involved in this conversion catalyzed by CDP-DAG synthase using CTP: the ER and mitochondria. The CDP-DAG synthase Cds1 provides CDP-DAG in the ER for the synthesis of phosphatidylinositol (PI) and phosphatidylserine (PS) (Henry et al. 2012). PS synthesis in the ER by the PS synthase Cho1 in yeast differs from the mammalian pathways where PS is made from preexisting phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by a head group exchange mechanism.

A different CDP-DAG pool is required for the synthesis of the mitochondrial phospholipids phosphatidylglycerol (PG) and cardiolipin (CL). Prior to 2013, it was

believed that Cds1 was the only CDP-DAG synthase in yeast and was present in both the ER and mitochondria. However, it was discovered that Cds1 is found exclusively in the ER, and a novel CDP-DAG synthase, Tam41, was shown to convert PA to CDP-DAG in the inner mitochondrial membrane (IMM) (Tamura et al. 2013). Interestingly, both Cds1 and Tam41 are highly conserved, although not with each other. Cds1 and Tam41 represent a perfect example of convergent evolution, where two unique proteins independently evolved to have the same function but in single organelles of the cell. Concurrently with the discovery of Tam41 involvement in the production of CDP-DAG in mitochondria was the identification of the protein complex Ups1-Mdm35 responsible for the transport the substrate PA to the IMM where Tam41 resides (Connerth et al. 2012). Our knowledge on the molecular details governing PA transport within mitochondria has grown immensely in the last years and will be discussed next, highlighting the regulatory role that transport of lipids between ER contact sites and mitochondria have on glycerolipid synthesis.

5.1 ER-Mitochondria Encounter Structure (ERMES)

The exchange of lipids, such as PS and PE, between the ER and the mitochondria occurs mostly at the contact sites between mitochondrial-associated membranes of the ER and the mitochondrial outer membrane (OMM) (Vance 1990; Achleitner et al. 1999). This lipid exchange is almost entirely protein-dependent; however, close membrane contacts may help with spontaneous transfer (Tatsuta et al. 2014). The ER-mitochondria encounter structure (ERMES) has been widely studied as a molecular tether between the two organelles and is highly conserved and unique to fungi (Kornmann et al. 2009). The role of ERMES as a molecular tether has been widely shown; however, additional functions such as the involvement of ERMES in the transport of phospholipids between organelles remain controversial. Three of the proteins in ERMES (Mmm1, Mdm12, and Mdm34) contain domains similar to tubular lipid-binding (TULIP) domains, which are known to bind lipids and other hydrophobic ligands for transfer in a wide range of organisms, including yeast (Kopec et al. 2010). These TULIP-like domains were identified to be synaptotagmin-like mitochondrial lipid-binding protein (SMP) domains, found in proteins that are localized to membrane contact sites (Kopec et al. 2010; AhYoung et al. 2015). The presence of these domains suggests that ERMES plays a role in inter-organelle lipid binding and transport. In support of this conclusion, the structure of ERMES was recently solved, revealing evidence that ERMES does indeed bind phospholipids and is important not only for tethering the two organelles but also for their transport between ER and mitochondria. It was shown that the SMP domains of Mdm12 and Mmm1 associate as a heterotetramer, creating a hydrophobic tunnel which can accommodate a phospholipid, with preference for PC (AhYoung et al. 2015). Most recently, in vitro experiments revealed that phospholipid transport between ER and mitochondria actually rely on membrane intactness, not energy sources, and that ERMES has an effect on PS movement to the mitochondria, but not PE movement to ER (Kojima et al. 2016). This is in contrast to earlier reports that concluded that ERMES is not responsible for

the transport of PS to the mitochondria (Nguyen et al. 2012; Voss et al. 2012). It was suggested that this discrepancy is most likely due to growth defects caused by the complete absence of ERMES components and the fact that the export of PE from the mitochondria to the ER was not previously considered (Kojima et al. 2016).

5.2 Mitochondrial Transport of PA by Ups1-Mdm35

The discovery of a conserved mitochondrial PA transporter further advanced the knowledge of phospholipid movement within the yeast cell. Ups1, an intermembrane space (IMS) protein, was shown to have the ability to bind and transport PA from the mitochondrial OMM to the IMM (Connerth et al. 2012). Ups1 was shown to regulate the accumulation of CL within the mitochondria (Osman et al. 2009; Tamura et al. 2009; Connerth et al. 2012). In cells lacking Ups1, the levels of the PS decarboxylase Psd1 were reduced resulting in the conversion of PS to PE to be significantly diminished as well (Tamura et al. 2012b). The overall lowered levels of Psd1 were shown to be caused by a defect in the import of mitochondrial proteins when Ups1 is not present, due to a decrease in the membrane potential across the IMM (Tamura et al. 2009, 2012b). Ups1 is only able to transport PA across the IMS with the presence of another IMS protein, Mdm35 (Connerth et al. 2012). The assembly of Ups1 and Mdm35 is not only required for proper Ups1 folding, solubility, and accumulation in the IMS but also allows Ups1 to make it across the IMS without being degraded by the *i*-AAA protease Yme1 (Potting et al. 2010; Connerth et al. 2012). Mdm35 contains two Cx_9C sequence motifs and is able to bind another IMS protein, Ups2, as well as Ups1 (Potting et al. 2010). This critical interaction is evolutionarily conserved in mammalian cells with TRIAP1 and PRELI being the orthologues of Mdm35 and Ups1, respectively (Potting et al. 2013).

It was suggested that Ups1 first binds to and extracts PA from the OMM, which then combines with Mdm35 to form the full complex, although only the Ups1-Mdm35 complex could be purified in the soluble fraction (Connerth et al. 2012). The Ups1-Mdm35 complex then crosses the IMS with PA in tow to the IMM. Once there, the binding of the complex to the IMM destabilizes the complex and causes the Mdm35 to dissociate. PA is then released into the IMM, which can then be used as a precursor for the synthesis of CL. It appears that the Ups1-Mdm35 complex is directional and functions as a negative feedback operation; less PA is transported to the IMM when there are high levels of CL present there. It has been shown through in vitro studies that endogenous levels of CL in a membrane mimicking the IMM (10–20%) prevent the dissociation of Ups1 from the membrane, causing it to be degraded and therefore preventing more PA from being transferred and halting the levels of CL (Connerth et al. 2012). This PA transport is independent of acyl chains and can be bidirectional; however, the conversion of PA to CL in the IMM provides directionality (Connerth et al. 2012). Interestingly, Ups1, and not Mdm35, was shown to bind other negatively charged phospholipids but only transports PA (Connerth et al. 2012). Although Ups1 is required for the accumulation of CL in the mitochondria, even in the absence of Ups1, the IMM still contained CL

(Connerth et al. 2012). This finding suggests an alternative pathway for CL synthesis in the IMM, perhaps using precursor lipids synthesized in other organelles; however, this has yet to be elucidated (Tatsuta and Langer 2016).

Most recently, the structure of the Ups1-Mdm35 complex has been revealed. The original structure modelling suggested a strong similarity to phosphatidylinositol transfer proteins (PITPs), even though there is almost no homology at the amino acid level (Connerth et al. 2012; Miliara et al. 2015). The full structure of the Ups1-Mdm35 complex, with and without PA, has been solved (Watanabe et al. 2015). Mdm35 contains three α-helices that assemble into a clamp-like structure which wrap Ups1 to produce a stable complex, allowing PA transfer only when a heterodimer is formed (Yu et al. 2015). This heterodimer creates a deep pocket, allowing PA to insert headfirst, and a flexible Ω-loop "lid" which is required for PA binding and transfer (Watanabe et al. 2015; Yu et al. 2015). Three distinct "sections" were identified to be critical in both the extraction and binding of PA from the OMM and the transfer of PA across the IMS: (1) a positively charged, hydrophilic patch at the bottom of the pocket available for interaction with the head group of PA, (2) hydrophobic residues lining the inner wall of the pocket and lid for recognition of the tail, and (3) the lid, either opened or closed, which allows PA to enter or prevents it from leaving (Watanabe et al. 2015; Yu et al. 2015). Despite all this new knowledge, it remains elusive as to how Ups1-Mdm35 complex recognizes PA and moves it across the IMS. A structure containing the complex bound to CL has not been solved yet; however, it appears that the pocket may be too small to fit all four acyl chains of CL (Yu et al. 2015). A possible reason may be that when CL levels are high in the IMM, two of the four acyl chains of CL may bind to the complex, while the other two remain in the membrane, preventing Ups1 from leaving and therefore causing it to be degraded, resulting in the negative feedback loop mentioned above (Yu et al. 2015).

5.3 Mitochondrial Transport of PS and PE

As mentioned above, Ups1 is not the only binding partner of Mdm35 in the IMS; Ups2 is also part of the conserved family of Ups1-/PRELI-like proteins (Osman et al. 2009; Potting et al. 2010). There is also Ups3, which is believed to be redundant with Ups2 (Osman et al. 2009). Unlike Ups1 and 2, Ups3 was not found to form complex with Mdm35, although Mdm35 is required for the import of all three Ups proteins (Potting et al. 2010; Tamura et al. 2010). All three Ups proteins are located in the IMS and only share 25% sequence identity (Yu et al. 2015).

In yeast cells, most PE are synthesized in the mitochondria via decarboxylation by Psd1, using imported PS from the ER (Trotter et al. 1993; Choi et al. 2005). The newly made PE can then be transported back to the ER, where it can then be methylated to form PC by the PE-methyltransferases Cho2 and Opi3 (Kodaki and Yamashita 1987). This pathway differs from mammalian cells where most PE is synthesized in the ER and is then transported to the mitochondria (Scharwey et al. 2013). In yeast, it has been shown that PS is transported to the IMM, independently from the following decarboxylation step to make PE (Tamura et al. 2012b). Unlike

the other decarboxylase, Psd2 in the Golgi/vacuole, Psd1 was shown not to be responsible for the transport of PS between mitochondrial membranes (Tamura et al. 2012b). It has, however, been recently shown that Psd1 can also decarboxylate PS that is in the OMM, bypassing the need to transport it to the IMM, when the two mitochondrial membranes are in close proximity (Aaltonen et al. 2016). This Psd1 activity is dependent on mitochondrial contact sites (MICOS) for tethering the OMM and IMM close enough to each other. MICOS/MitOS (mitochondrial organizing structure)/MINOS (mitochondrial inner membrane organizing system) are names for the same thing: IMM-associated complexes that are found to be enriched at the mitochondrial contact sites and are critical in mitochondrial cristae formation and morphology (von der Malsburg et al. 2011; Hoppins et al. 2011; Harner et al. 2011).

Early results found that deletion of Ups2 leads to a decrease in PE levels, while deletion of Ups1 lead to a decrease in CL (Osman et al. 2009; Tamura et al. 2009). This was originally explained by the idea that Ups1 and Ups2 acted antagonistically, where Ups1 was responsible for the export of PE from the IMM to the OMM while Ups2 acted oppositely, preferring to keep PE in the IMM (Tamura et al. 2012a, b). It was found instead, however, that PE levels decreased without Ups2 because Ups2 is the transporter for PS to the IMM, and therefore without it, less PE should be made (Miyata et al. 2016). Additionally, when both *UPS1* and *UPS2* are deleted, CL levels return back to normal, suggesting some sort of coordination between PE and CL (Osman et al. 2009; Tamura et al. 2009; Tamura et al. 2012a). This may be due to Mdm31, an integral IMM protein which was shown to be required to maintain CL levels in the mitochondria in Ups1-deleted cells (Tamura et al. 2012a).

Most recently, Ups2-Mdm35 was revealed to be a PS-specific transfer complex, similar to how Ups1-Mdm35 transfers PA, as described above (Aaltonen et al. 2016; Miyata et al. 2016). Ups2 was found to bind negatively charged phospholipids such as PS, PA, and CL; however, it was shown to specifically transport PS only (Aaltonen et al. 2016). This contrasts with the work from Miyata et al. which suggests that the Ups2-Mdm35 complex can also bind and transport PA in vitro (Miyata et al. 2016). Similarly to the Ups1-Mdm35 complex, Mdm35 mostly dissociated when Ups2 was bound to the membrane, and unlike the Ups1-Mdm35 complex, PS transfer was increased when CL, PI, or PG were present in the acceptor membrane (Aaltonen et al. 2016). Interestingly, it was also found that Psd1 can convert OMM PS into PE in juxtaposed membranes; thus, PE synthesis by Psd1 can occur independent of PS transfer by Ups2-Mdm35 (Aaltonen et al. 2016). This was only possible due to MICOS, as it helped to bring the two membranes close together. Overall, these results suggest a novel role for MICOS in PE metabolism, controlling PE production in the mitochondria and promoting PC synthesis in the ER from mitochondrial PE.

6 Research Needs

We are just beginning to elucidate how lipid metabolic systems are spatiotemporally regulated. Most enzymes in the glycerolipid biosynthetic pathways are integral membrane proteins, and their location and topology have not been conclusively

elucidated. Interestingly the emerging topologies for the acyltransferases participating in the sequential acylation of glycerol 3-phosphate suggest their catalytic domains are facing the luminal side of the ER, therefore challenging the current belief that availability of acyl-CoA pools and catalysis occur on the cytosolic side of this organelle. In addition, many of these enzymes seem to be able to have dual roles in anabolic and catabolic steps of lipid metabolism, adding more complexity to the study of their function and regulation.

Proteomic studies in addition to directed investigations have unveiled an intricate regulation of the glycerolipid biosynthetic pathways by posttranslational modifications. Most enzymes are regulated through phosphorylation, and it is imperative to identify the kinases, phosphatases, and the role that phosphorylation plays in the regulation of each of them. A comprehensive picture is emerging from studies conducted on the regulation of PA phosphatase Pah1 by phosphorylation and is expected that same kinases and phosphatases act in concert phosphorylating other proteins in the glycerolipid biosynthetic pathways.

A major challenge in the field is the study of lipid pools. Increasing evidence points to the importance of the acyl tails of signalling lipids on the outcome of the signal transduction triggered by the interaction of these lipids with downstream effector proteins. In addition, movement of specific pools of lipid precursors from the ER to other organelles started to get unveiled, but our knowledge is still limited with respect to the molecular mechanisms governing lipid compositions of precise membrane compartments. Lipidomic approaches combined with subcellular fractionation as well as the use of lipid probes for microscopy approaches should aid in the study of the processes governing lipid pool dynamics.

Given the relative simplicity of the yeast lipidome; the level of conservation of the enzymes metabolizing, regulating, and transporting lipids; and the comprehensive and well-curated experimental dataset available to the research community, yeast will continue to be an appealing and efficient model to tackle these challenges.

Acknowledgments This work was supported by an NSERC Discovery grant and NSERC Discovery Accelerator Supplement to VZ; an Eyes High International Doctoral Scholarship to SG and, an NSERC-CGSM award to BNS.

References

Aaltonen MJ, Friedman JR, Osman C et al (2016) MICOS and phospholipid transfer by Ups2–Mdm35 organize membrane lipid synthesis in mitochondria. J Cell Biol 213. https://doi.org/10.1083/jcb.201602007

Achleitner G, Gaigg B, Krasser A et al (1999) Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. Eur J Biochem 264:545–553

AhYoung AP, Jiang J, Zhang J et al (2015) Conserved SMP domains of the ERMES complex bind phospholipids and mediate tether assembly. Proc Natl Acad Sci U S A 112:E3179–E3188. https://doi.org/10.1073/pnas.1422363112

Athenstaedt K, Daum G (1997) Biosynthesis of phosphatidic acid in lipid particles and endoplasmic reticulum of *Saccharomyces cerevisiae*. J Bacteriol 179:7611–7616

Ayciriex S, Le Guédard M, Camougrand N et al (2012) YPR139c/LOA1 encodes a novel lysophosphatidic acid acyltransferase associated with lipid droplets and involved in TAG homeostasis. Mol Biol Cell 23:233–246. https://doi.org/10.1091/mbc.E11-07-0650

Barbosa AD, Sembongi H, Su W-M et al (2015) Lipid partitioning at the nuclear envelope controls membrane biogenesis. Mol Biol Cell 26:3641–3657. https://doi.org/10.1091/mbc.E15-03-0173

Benghezal M, Roubaty C, Veepuri V et al (2007) SLC1 and SLC4 encode partially redundant acyl-coenzyme a 1-acylglycerol-3-phosphate O-acyltransferases of budding yeast. J Biol Chem 282:30845–30855. https://doi.org/10.1074/jbc.M702719200

Bratschi MW, Burrowes DP, Kulaga A et al (2009) Glycerol-3-phosphate acyltransferases Gat1p and Gat2p are microsomal phosphoproteins with differential contributions to polarized cell growth. Eukaryot Cell 8:1184–1196. https://doi.org/10.1128/EC.00085-09

Carman GM (2011) The discovery of the fat-regulating phosphatidic acid phosphatase gene. Front Biol (Beijing) 6:172–176. https://doi.org/10.1007/s11515-011-0910-7

Carman GM, Henry SA (2007) Phosphatidic acid plays a central role in the transcriptional regulation of glycerophospholipid synthesis in *Saccharomyces cerevisiae*. J Biol Chem 282:37293–37297. https://doi.org/10.1074/jbc.R700038200

Carman GM, Kersting MC (2004) Phospholipid synthesis in yeast: regulation by phosphorylation. Biochem Cell Biol 82:62–70. https://doi.org/10.1139/o03-064

Casanovas A, Sprenger RR, Tarasov K et al (2015) Quantitative analysis of proteome and lipidome dynamics reveals functional regulation of global lipid metabolism. Chem Biol 22:412–425. https://doi.org/10.1016/j.chembiol.2015.02.007

Chae M, Carman GM (2013) Characterization of the yeast actin patch protein App1p phosphatidate phosphatase. J Biol Chem 288:6427–6437. https://doi.org/10.1074/jbc.M112.449629

Chang Y-F, Carman GM (2006) Casein kinase II phosphorylation of the yeast phospholipid synthesis transcription factor Opi1p. J Biol Chem 281:4754–4761. https://doi.org/10.1074/jbc.M513064200

Chen Q, Kazachkov M, Zheng Z, Zou J (2007) The yeast acylglycerol acyltransferase LCA1 is a key component of Lands cycle for phosphatidylcholine turnover. FEBS Lett 581:5511–5516. https://doi.org/10.1016/j.febslet.2007.10.061

Cherry JM, Hong EL, Amundsen C et al (2012) Saccharomyces genome database: the genomics resource of budding yeast. Nucleic Acids Res 40:D700–D705. https://doi.org/10.1093/nar/gkr1029

Choi J-Y, Wu W-I, Voelker DR (2005) Phosphatidylserine decarboxylases as genetic and biochemical tools for studying phospholipid traffic. Anal Biochem 347:165–175. https://doi.org/10.1016/j.ab.2005.03.017

Connerth M, Tatsuta T, Haag M et al (2012) Intramitochondrial transport of phosphatidic acid in yeast by a lipid transfer protein. Science. https://doi.org/10.1126/science.1225625

Daum G, Wagner B, Arrey TN et al (2011) Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim Biophys Acta Mol Cell Biol Lipids 1811:1165–1176

De Smet CH, Vittone E, Scherer M et al (2012) The yeast acyltransferase Sct1p regulates fatty acid desaturation by competing with the desaturase Ole1p. Mol Biol Cell 23:1146–1156. https://doi.org/10.1091/mbc.E11-07-0624

Ejsing CS, Sampaio JL, Surendranath V et al (2009) Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proc Natl Acad Sci U S A 106:2136–2141. https://doi.org/10.1073/pnas.0811700106

Fakas S, Qiu Y, Dixon JL et al (2011) Phosphatidate phosphatase activity plays key role in protection against fatty acid-induced toxicity in yeast. J Biol Chem 286:29074–29085. https://doi.org/10.1074/jbc.M111.258798

Froissard M, Canonge M, Pouteaux M et al (2015) Lipids containing medium-chain fatty acids are specific to post-whole genome duplication Saccharomycotina yeasts. BMC Evol Biol 15:97. https://doi.org/10.1186/s12862-015-0369-2

Ghosh AK, Ramakrishnan G, Rajasekharan R (2008) YLR099C (ICT1) encodes a soluble acyl-CoA-dependent lysophosphatidic acid acyltransferase responsible for enhanced phospholipid

synthesis on organic solvent stress in *Saccharomyces cerevisiae*. J Biol Chem 283:9768–9775. https://doi.org/10.1074/jbc.M708418200

Han G-S, Wu W-I, Carman GM (2006) The *Saccharomyces cerevisiae* Lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. J Biol Chem 281:9210–9218. https://doi.org/10.1074/jbc.M600425200

Han G-S, O'Hara L, Carman GM, Siniossoglou S (2008a) An unconventional diacylglycerol kinase that regulates phospholipid synthesis and nuclear membrane growth. J Biol Chem 283:20433–20442. https://doi.org/10.1074/jbc.M802903200

Han G-S, O'Hara L, Siniossoglou S, Carman GM (2008b) Characterization of the yeast DGK1-encoded CTP-dependent diacylglycerol kinase. J Biol Chem 283:20443–20453. https://doi.org/10.1074/jbc.M802866200

Harner M, Körner C, Walther D et al (2011) The mitochondrial contact site complex, a determinant of mitochondrial architecture. EMBO J 30:4356–4370. https://doi.org/10.1038/emboj.2011.379

Henry SA, Kohlwein SD, Carman GM (2012) Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. Genetics 190:317–349. https://doi.org/10.1534/genetics.111.130286

Hofbauer HF, Schopf FH, Schleifer H et al (2014) Regulation of gene expression through a transcriptional repressor that senses acyl-chain length in membrane phospholipids. Dev Cell 29:729–739. https://doi.org/10.1016/j.devcel.2014.04.025

Hoppins S, Collins SR, Cassidy-Stone A et al (2011) A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. J Cell Biol 195:323–340. https://doi.org/10.1083/jcb.201107053

Hosaka K, Yamashita S (1984) Partial purification and properties of phosphatidate phosphatase in *Saccharomyces cerevisiae*. Biochim Biophys Acta 796:102–109

Hsieh L-S, Su W-M, Han G-S, Carman GM (2015) Phosphorylation regulates the ubiquitin-independent degradation of yeast Pah1 phosphatidate phosphatase by the 20S proteasome. J Biol Chem 290:11467–11478. https://doi.org/10.1074/jbc.M115.648659

Hsieh L-S, Su W-M, Han G-S, Carman GM (2016) Phosphorylation of yeast Pah1 Phosphatidate phosphatase by casein kinase II regulates its function in lipid metabolism. J Biol Chem 291:9974–9990. https://doi.org/10.1074/jbc.M116.726588

Irie K, Takase M, Araki H, Oshima Y (1993) A gene, SMP2, involved in plasmid maintenance and respiration in *Saccharomyces cerevisiae* encodes a highly charged protein. Mol Gen Genet 236:283–288

Jain S, Stanford N, Bhagwat N et al (2007) Identification of a novel lysophospholipid acyltransferase in *Saccharomyces cerevisiae*. J Biol Chem 282:30562–30569. https://doi.org/10.1074/jbc.M706326200

Jasieniecka-Gazarkiewicz K, Demski K, Lager I et al (2016) Possible role of different yeast and plant Lysophospholipid:acyl-CoA Acyltransferases (LPLATs) in acyl remodelling of phospholipids. Lipids 51:15–23. https://doi.org/10.1007/s11745-015-4102-0

Karanasios E, Han G-S, Xu Z et al (2010) A phosphorylation-regulated amphipathic helix controls the membrane translocation and function of the yeast phosphatidate phosphatase. Proc Natl Acad Sci 107:17539–17544. https://doi.org/10.1073/pnas.1007974107

Karanasios E, Barbosa AD, Sembongi H et al (2013) Regulation of lipid droplet and membrane biogenesis by the acidic tail of the phosphatidate phosphatase Pah1p. Mol Biol Cell 24:2124–2133. https://doi.org/10.1091/mbc.E13-01-0021

Kodaki T, Yamashita S (1987) Yeast phosphatidylethanolamine methylation pathway. Cloning and characterization of two distinct methyltransferase genes. J Biol Chem 262:15428–15435

Kohlwein SD, Veenhuis M, van der Klei IJ (2013) Lipid droplets and peroxisomes: key players in cellular lipid homeostasis or a matter of fat – store 'em up or burn 'em down. Genetics 193:1–50. https://doi.org/10.1534/genetics.112.143362

Kojima R, Endo T, Tamura Y (2016) A phospholipid transfer function of ER-mitochondria encounter structure revealed in vitro. Sci Rep 6:30777. https://doi.org/10.1038/srep30777

Kopec KO, Alva V, Lupas AN (2010) Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria. Bioinformatics 26:1927–1931. https://doi.org/10.1093/bioinformatics/btq326

Kornmann B, Currie E, Collins SR et al (2009) An ER-mitochondria tethering complex revealed by a synthetic biology screen. Science 325:477–481. https://doi.org/10.1126/science.1175088

Loewen CJR, Gaspar ML, Jesch SA et al (2004) Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. Science 304:1644–1647. https://doi.org/10.1126/science.1096083

Marr N, Foglia J, Terebiznik M et al (2012) Controlling lipid fluxes at glycerol-3-phosphate acyltransferase step in yeast: unique contribution of Gat1p to oleic acid-induced lipid particle formation. J Biol Chem 287:10251–10264. https://doi.org/10.1074/jbc.M111.314112

Miliara X, Garnett JA, Tatsuta T et al (2015) Structural insight into the TRIAP1/PRELI-like domain family of mitochondrial phospholipid transfer complexes. EMBO Rep 16:824–835. https://doi.org/10.15252/embr.201540229

Mishra S, Khaddaj R, Cottier S et al (2016) Mature lipid droplets are accessible to ER luminal proteins. J Cell Sci 129:3803–3815. https://doi.org/10.1242/jcs.189191

Miura S, Zou W, Ueda M, Tanaka A (2000) Screening of genes involved in isooctane tolerance in *Saccharomyces cerevisiae* by using mRNA differential display. Appl Environ Microbiol 66:4883–4889. https://doi.org/10.1128/AEM.66.11.4883-4889.2000

Miyata N, Watanabe Y, Tamura Y et al (2016) Phosphatidylserine transport by Ups2–Mdm35 in respiration-active mitochondria. J Cell Biol 214:77–88. https://doi.org/10.1083/jcb.201601082

Nagiec MM, Wells GB, Lester RL, Dickson RC (1993) A suppressor gene that enables *Saccharomyces cerevisiae* to grow without making sphingolipids encodes a protein that resembles an *Escherichia coli* fatty acyltransferase. J Biol Chem 268:22156–22163

Nguyen TT, Lewandowska A, Choi JY et al (2012) Gem1 and ERMES do not directly affect phosphatidylserine transport from ER to mitochondria or mitochondrial inheritance. Traffic 13:880–890. https://doi.org/10.1111/j.1600-0854.2012.01352.x

Osman C, Haag M, Potting C et al (2009) The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. J Cell Biol 184:583–596. https://doi.org/10.1083/jcb.200810189

Pagac M, de la Mora HV, Duperrex C et al (2011) Topology of 1-acyl-sn-glycerol-3-phosphate acyltransferases SLC1 and ALE1 and related membrane-bound *O*-acyltransferases (MBOATs) of *Saccharomyces cerevisiae*. J Biol Chem 286:36438–36447. https://doi.org/10.1074/jbc.M111.256511

Pagac M, Vazquez HM, Bochud A et al (2012) Topology of the microsomal glycerol-3-phosphate acyltransferase Gpt2p/Gat1p of *Saccharomyces cerevisiae*. Mol Microbiol 86:1156–1166. https://doi.org/10.1111/mmi.12047

Park Y, Han G-S, Mileykovskaya E et al (2015) Altered lipid synthesis by lack of yeast Pah1 phosphatidate phosphatase reduces chronological life span. J Biol Chem 290:25382–25394. https://doi.org/10.1074/jbc.M115.680314

Pascual F, Carman GM (2013) Phosphatidate phosphatase, a key regulator of lipid homeostasis. Biochim Biophys Acta Mol Cell Biol Lipids 1831:514–522. https://doi.org/10.1016/j.bbalip.2012.08.006

Potting C, Wilmes C, Engmann T et al (2010) Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35. EMBO J 29:2888–2898. https://doi.org/10.1038/emboj.2010.169

Potting C, Tatsuta T, König T et al (2013) TRIAP1/PRELI complexes prevent apoptosis by mediating intramitochondrial transport of phosphatidic acid. Cell Metab 18:287–295. https://doi.org/10.1016/j.cmet.2013.07.008

Qiu Y, Hassaninasab A, Han G-S, Carman GM (2016) Phosphorylation of Dgk1 diacylglycerol kinase by casein kinase II regulates phosphatidic acid production in *Saccharomyces cerevisiae*. J Biol Chem 291. https://doi.org/10.1074/jbc.M116.763839

Rajakumari S, Daum G (2010a) Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Mol Biol Cell 21:501–510. https://doi.org/10.1091/mbc.E09-09-0775

Rajakumari S, Daum G (2010b) Multiple functions as lipase, steryl ester hydrolase, phospholipase, and acyltransferase of Tgl4p from the yeast *Saccharomyces cerevisiae*. J Biol Chem 285:15769–15776. https://doi.org/10.1074/jbc.M109.076331

Riekhof WR, Wu J, Gijón MA et al (2007a) Lysophosphatidylcholine metabolism in *Saccharomyces cerevisiae*: the role of P-type ATPases in transport and a broad specificity acyltransferase in acylation. J Biol Chem 282:36853–36861. https://doi.org/10.1074/jbc.M706718200

Riekhof WR, Wu J, Jones JL, Voelker DR (2007b) Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*. J Biol Chem 282:28344–28352. https://doi.org/10.1074/jbc.M705256200

Sakane F, Mizuno S, Komenoi S (2016) Diacylglycerol kinases as emerging potential drug targets for a variety of diseases: an update. Front Cell Dev Biol 4:82. https://doi.org/10.3389/fcell.2016.00082

Santos-Rosa H, Leung J, Grimsey N et al (2005) The yeast lipin Smp2 couples phospholipid biosynthesis to nuclear membrane growth. EMBO J 24:1931–1941. https://doi.org/10.1038/sj.emboj.7600672

Sasser T, Qiu Q-S, Karunakaran S et al (2012) Yeast lipin 1 orthologue pah1p regulates vacuole homeostasis and membrane fusion. J Biol Chem 287:2221–2236. https://doi.org/10.1074/jbc.M111.317420

Scharwey M, Tatsuta T, Langer T (2013) Mitochondrial lipid transport at a glance. J Cell Sci 126:5317–5323. https://doi.org/10.1242/jcs.134130

Shin JJ, Loewen CJ (2011) Putting the pH into phosphatidic acid signaling. BMC Biol 9:85. https://doi.org/10.1186/1741-7007-9-85

Shui G, Guan XL, Gopalakrishnan P et al (2010) Characterization of substrate preference for Slc1p and Cst26p in *Saccharomyces cerevisiae* using lipidomic approaches and an LPAAT activity assay. PLoS One 5:e11956. https://doi.org/10.1371/journal.pone.0011956

Smart HC, Mast FD, Chilije MFJ et al (2014) Phylogenetic analysis of glycerol 3-phosphate acyltransferases in opisthokonts reveals unexpected ancestral complexity and novel modern biosynthetic components. PLoS One 9:e110684. https://doi.org/10.1371/journal.pone.0110684

Smith SW, Weiss SB, Kennedy EP (1957) The enzymatic dephosphorylation of phosphatidic acids. J Biol Chem 228:915–922

Su W-M, Han G-S, Casciano J, Carman GM (2012) Protein kinase A-mediated phosphorylation of Pah1p phosphatidate phosphatase functions in conjunction with the Pho85p-Pho80p and Cdc28p-Cyclin B kinases to regulate lipid synthesis in yeast. J Biol Chem 287:33364–33376. https://doi.org/10.1074/jbc.M112.402339

Su W-M, Han G-S, Carman GM (2014a) Cross-talk phosphorylations by protein kinase C and Pho85p-Pho80p protein kinase regulate Pah1p phosphatidate phosphatase abundance in *Saccharomyces cerevisiae*. J Biol Chem 289:18818–18830. https://doi.org/10.1074/jbc.M114.581462

Su W-M, Han G-S, Carman GM (2014b) Yeast Nem1-Spo7 protein phosphatase activity on Pah1 phosphatidate phosphatase is specific for the Pho85-Pho80 protein kinase phosphorylation sites. J Biol Chem 289:34699–34708. https://doi.org/10.1074/jbc.M114.614883

Tamaki H, Shimada A, Ito Y et al (2007) LPT1 encodes a membrane-bound *O*-acyltransferase involved in the acylation of lysophospholipids in the yeast *Saccharomyces cerevisiae*. J Biol Chem 282:34288–34298. https://doi.org/10.1074/jbc.M704509200

Tamura Y, Endo T, Iijima M, Sesaki H (2009) Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. J Cell Biol 185:1029–1045. https://doi.org/10.1083/jcb.200812018

Tamura Y, Iijima M, Sesaki H (2010) Mdm35p imports ups proteins into the mitochondrial intermembrane space by functional complex formation. EMBO J 29:2875–2887. https://doi.org/10.1038/emboj.2010.149

Tamura Y, Onguka O, Aiken Hobbs AE et al (2012a) Role for two conserved intermembrane space proteins, Ups1p and Up2p, in intra-mitochondrial phospholipid trafficking. J Biol Chem 287:15205–15218. https://doi.org/10.1074/jbc.M111.338665

Tamura Y, Onguka O, Itoh K et al (2012b) Phosphatidylethanolamine biosynthesis in mitochondria: phosphatidylserine (PS) trafficking is independent of a PS decarboxylase and intermembrane space proteins Ups1p and Ups2p. J Biol Chem 287:43961–43971. https://doi.org/10.1074/jbc.M112.390997

Tamura Y, Harada Y, Nishikawa SI et al (2013) Tam41 is a CDP-diacylglycerol synthase required for cardiolipin biosynthesis in mitochondria. Cell Metab 17:709–718. https://doi.org/10.1016/j.cmet.2013.03.018

Tatsuta T, Langer T (2017) Intramitochondrial phospholipid trafficking. Biochim Biophys Acta 1862(1):81–89. https://doi.org/10.1016/j.bbalip.2016.08.006

Tatsuta T, Scharwey M, Langer T (2014) Mitochondrial lipid trafficking. Trends Cell Biol 24:44–52. https://doi.org/10.1016/j.tcb.2013.07.011

Toke DA, Bennett WL, Dillon DA et al (1998a) Isolation and characterization of the *Saccharomyces cerevisiae* DPP1 gene encoding diacylglycerol pyrophosphate phosphatase. J Biol Chem 273:3278–3284

Toke DA, Bennett WL, Oshiro J et al (1998b) Isolation and characterization of the *Saccharomyces cerevisiae* LPP1 gene encoding a Mg^{2+}-independent phosphatidate phosphatase. J Biol Chem 273:14331–14338

Trotter PJ, Pedretti J, Voelker DR (1993) Phosphatidylserine decarboxylase from *Saccharomyces cerevisiae*. Isolation of mutants, cloning of the gene, and creation of a null allele. J Biol Chem 268:21416–21424

van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9:112–124. https://doi.org/10.1038/nrm2330

Vance JE (1990) Phospholipid synthesis in a membrane fraction associated with mitochondria. J Biol Chem 265:7248–7256

Vionnet C, Roubaty C, Ejsing CS et al (2011) Yeast cells lacking all known ceramide synthases continue to make complex sphingolipids and to incorporate ceramides into glycosylphosphatidylinositol (GPI) anchors. J Biol Chem 286:6769–6779. https://doi.org/10.1074/jbc.M110.176875

von der Malsburg K, Müller JM, Bohnert M et al (2011) Dual role of mitofilin in mitochondrial membrane organization and protein biogenesis. Dev Cell 21:694–707. https://doi.org/10.1016/j.devcel.2011.08.026

Voss C, Lahiri S, Young BP et al (2012) ER-shaping proteins facilitate lipid exchange between the ER and mitochondria in *S. cerevisiae*. J Cell Sci 125:4791–4799. https://doi.org/10.1242/jcs.105635

Wang C-W (2015) Lipid droplet dynamics in budding yeast. Cell Mol Life Sci 72:2677–2695. https://doi.org/10.1007/s00018-015-1903-5

Watanabe Y, Tamura Y, Kawano S, Endo T (2015) Structural and mechanistic insights into phospholipid transfer by Ups1-Mdm35 in mitochondria. Nat Commun 6:7922. https://doi.org/10.1038/ncomms8922

Yu F, He F, Yao H et al (2015) Structural basis of intramitochondrial phosphatidic acid transport mediated by Ups1-Mdm35 complex. EMBO Rep 16:813–823. https://doi.org/10.15252/embr.201540137

Zaremberg V, McMaster CR (2002) Differential partitioning of lipids metabolized by separate yeast glycerol-3-phosphate acyltransferases reveals that phospholipase D generation of phosphatidic acid mediates sensitivity to choline-containing lysolipids and drugs. J Biol Chem 277:39035–39044. https://doi.org/10.1074/jbc.M207753200

Zheng Z, Zou J (2001) The initial step of the glycerolipid pathway: identification of glycerol 3-phosphate/dihydroxyacetone phosphate dual substrate acyltransferases in *Saccharomyces cerevisiae*. J Biol Chem 276:41710–41716. https://doi.org/10.1074/jbc.M104749200

Zinser E, Sperka-Gottlieb CD, Fasch EV et al (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. J Bacteriol 173:2026–2034

Metabolism and Roles of Sphingolipids in Yeast *Saccharomyces cerevisiae*

18

Jihui Ren and Yusuf A. Hannun

Contents

1 Introduction 342
2 Sphingolipid Metabolism in Yeast 344
2.1 Sphingolipid Biosynthesis 344
2.2 Sphingolipid Breakdown 347
3 Functions of Yeast Sphingolipids 349
3.1 Function of Yeast Ceramides 349
3.2 Function of Complex Sphingolipids 352
3.3 Function of Yeast LCBs 352
4 Research Needs 354
References 355

Abstract

Sphingolipids are major membrane components of all eukaryotic cells. They are also important bioactive molecules involved in a plethora of essential cellular processes that are implicated in various human diseases. Most bioactive sphingolipids also serve as intermediate products of the sphingolipid metabolic network. It is thus critical to understand sphingolipid metabolic pathways in order to dissect sphingolipid function at single-species level. Here, we review in detail the biosynthetic and degradation pathways of sphingolipids in the yeast *Saccharomyces cerevisiae*, a model organism whose sphingolipid composition is rather simple but with conserved metabolic pathways and regulation mechanisms as higher eukaryotes. The functions of yeast sphingoid bases, ceramides,

J. Ren · Y. A. Hannun (✉)
Stony Brook Cancer Center, Stony Brook University, Health Science Center, Stony Brook, NY, USA
e-mail: Jihui.Ren@stonybrookmedicine.edu; Yusuf.Hannun@stonybrookmedicine.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_21

and complex sphingolipids are also discussed. Together, this knowledge provides a foundation to understand sphingolipid metabolism, their physiological roles, their potential as therapeutic targets for human diseases, and the major challenges the field is facing.

1 Introduction

Sphingolipids were first discovered as the major component of brain extract in 1865, and their chemical composition was revealed decades later (Carter et al. 1947; Kanfer and Hakomori 1983). Until the mid-1980s, sphingolipids had been regarded as mere structural components of eukaryotic cell membranes. The discovery of sphingosine as a specific inhibitor of protein kinase C suggested the possible role of sphingolipids as second messengers and as bioactive molecules (Hannun et al. 1986; Merrill et al. 1986; Wilson et al. 1986). These studies marked the start of a new era where the investigation of the physiological relevance of sphingolipid metabolism in cell took the center stage. Sphingolipids are now appreciated as important bioactive molecules involved in a plethora of essential biological processes such as cell growth, apoptosis, differentiation, migration, angiogenesis, and inflammation (Hannun and Obeid 2008).

In addition to the sphingolipidoses, inherited disorders caused by defective sphingolipid degradation in the lysosome, altered sphingolipid metabolism is also associated with a variety of other prevalent human diseases including type 2 diabetes, cancer, inflammation, and neurological disorders such as Alzheimer's disease and hereditary sensory and autonomic neuropathy type I (HSAN1) (Bejaoui et al. 2002; Kolter and Sandhoff 2006; Pralhada Rao et al. 2013).

Despite the rapid progress in deciphering the role of sphingolipids at both the cellular and organism levels, major challenges still exist. One of these challenges relates to the fact that individual bioactive sphingolipids are connected by complex metabolic pathways, and it is difficult to alter one species without affecting the level of its precursors or downstream products, which makes it hard to pinpoint the function of any specific species. This problem is further compounded by the compartmentalization of sphingolipid metabolism such that there are recognized complements of sphingolipid enzymes that reside in each of the major subcellular compartments (nucleus, endoplasmic reticulum (ER), mitochondria, Golgi, lysosomes, plasma membrane, and extracellularly). Understanding sphingolipids is complicated even further by the diverse classes of sphingolipid derivatives. The blueprint of the structure of sphingolipids is fairly simple (Fig. 1). It is composed of three moieties: a long-chain base (LCB) containing hydroxyl and amino groups, a long-acyl-chain amide linked to the LCB amino group, and a head group attached to the hydroxyl group at C1 position of the LCB. It is the modifications to this basic structure that give rises to diverse species with variations in head groups, acyl-chain length, position and number of hydroxyl groups, and degree of unsaturation of both the LCB and the acyl-chain. Sphingolipids contain several chiral centers, and among all the possible stereoisomers, natural sphingolipids only occur in certain configuration

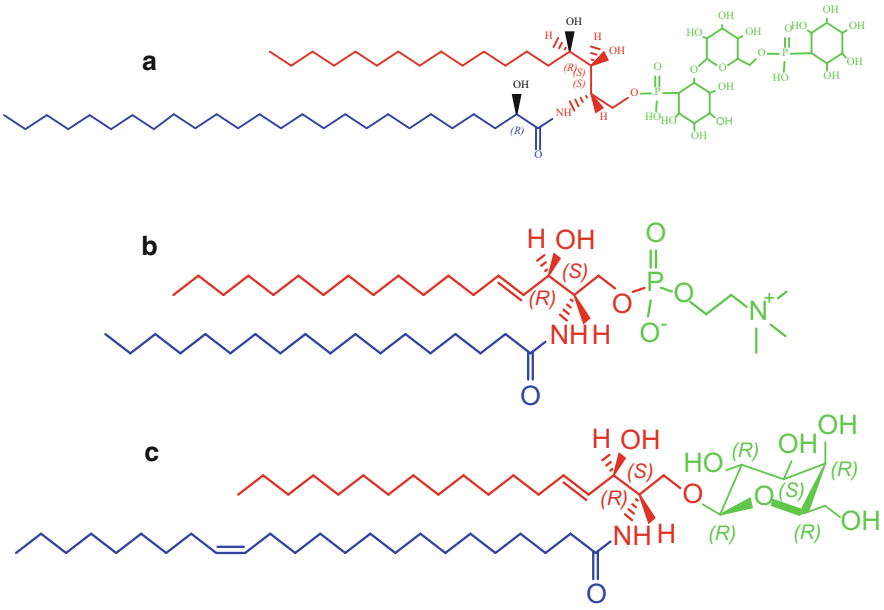


Fig. 1 Sphingolipid structure. The long chain base (*LCB*) backbone is shown in *red*. Acylation of LCB forms ceramide; the amide-linked acyl chain to the LCB amino group is shown in *blue*. The addition of head group to ceramide forms complex sphingolipids; the head group attached to the C1 hydroxyl group is shown in *green*. Hydroxyl groups that decorate both the LCB backbone and the acyl chain are shown in *black*. Examples of different sphingolipids are shown in (**a**) *S. cerevisiae* α-OH-C26-M(IP)2C (Mannose-(inositol-P)$_2$-ceramide); (**b**) structure of porcine brain sphingomyelin and (**c**) C24:1 Galactosyl (β) ceramide

as shown in Fig. 1. Unique biological properties have been found to associate with each stereoisomer.

The sphingolipid composition of yeast *Saccharomyces cerevisiae* is much simpler than that of humans and thus serves as an excellent model to study sphingolipid metabolism and their cellular roles. The unicellular nature, fast growth cycle, and ease of genetic manipulations make the yeast *S. cerevisiae* a powerful working model in investigating sphingolipid metabolism and function. Indeed, many of the enzymes in sphingolipid metabolic pathway were first discovered in yeast followed by the cloning of their counterparts in other fungi, plant, and animal kingdoms. Studies in *S. cerevisiae* have not only helped elucidate the function of individual genes in the sphingolipid metabolic pathways but have also pioneered in the use of systems biology in dissecting specific sphingolipid functions (Cowart et al. 2010b; Montefusco et al. 2013). The goal of this review is to provide students and researchers who are interested in the sphingolipid field with an introduction to the major sphingolipid metabolic pathways in *Saccharomyces cerevisiae*, their functions, and their potential connections to higher organisms.

2 Sphingolipid Metabolism in Yeast

2.1 Sphingolipid Biosynthesis

De novo sphingolipid synthesis begins at the cytosolic leaflet of the ER where serine palmitoyl transferase (SPT) catalyzes the first and rate-limiting step of condensation of serine with palmitoyl-CoA to produce 3-ketodihydrosphingosine (Dickson and Lester 1999) (Fig. 2). Yeast SPT belongs to a subfamily of pyridoxal 5′-phosphate enzymes known as α-oxoamine synthases, and it is composed of the catalytic subunit – a heterodimer of two homologous subunits Lcb1 and Lcb2 – and a small hydrophobic subunit, Tsc3, which is required for optimal enzyme activity (Gable et al. 2000; Nagiec et al. 1994). Homologues of LCB1/2 are found in all organisms that synthesize sphingolipids including mammals (Hanada et al. 1997). Mutations in the gene encoding the human SPT Lcb1b subunit (SPTLC1) are associated with HSAN1 (Bejaoui et al. 2002). In these cases, the mutant SPT shows substrate preference for alanine and glycine over serine, resulting in the formation of alternative (noncanonical) sphingolipids, and this altered amino acid selectivity of SPT is proposed to cause the neuropathy (Gable et al. 2010; Penno et al. 2010). Two low molecular weight proteins, ssSPTa and ssSPTb, have been identified in mammalian cells. Although they share no sequence homology to Tsc3, they are regarded as its functional homologue since they interact with hLCB1/hLCB2 and stimulate SPT activity (Han et al. 2009).

Yeast studies have also disclosed additional layers of regulation of sphingolipid synthesis through modulating SPT activity. Orm1/2 are homologous ER proteins functioning as negative regulators of yeast SPT, which were purified in the same SPT complex (Han et al. 2010). The inhibitory effect of Orm1/2 on SPT activity correlates with their phosphorylation/dephosphorylation status, which is controlled by Pkh-Ypk and Cdc55-PP2A pathways (Roelants et al. 2011; Sun et al. 2012). Pkh1/2 can be activated by TORC2 (target of rapamycin signaling complex 2) in an Slm1-/2-dependent manner (Berchtold et al. 2012). Slm1/2 are homologous plasma membrane (PM) proteins that bind both PI(4,5)P2 and TORC2 (Tabuchi et al. 2006). Slm1/2 was proposed to facilitate Pkh1 phosphorylation by TORC2 through recruiting Pkh1 to PM (Niles et al. 2012; Niles and Powers 2012). SPT activity can also be upregulated through inhibition of TORC1 (target of rapamycin signaling complex 1) which leads to Orm1 phosphorylation by Npr1 (Shimobayashi et al. 2013). ORM1/2 are conserved in humans (ORMDL1/2/3), and their human homologues show the same interaction and inhibitory effect on human SPT. Their inhibitory effect on SPT is also controlled by their phosphorylation status. The study of ORMs is of significant clinical interest since polymorphism of ORMDL3 is associated with childhood asthma (Breslow et al. 2010). Yeast cells with lack of Orm1/2 show increased unfolded protein response, and this defect can be corrected by inhibition of sphingolipid biosynthesis, suggesting the role of sphingolipids in protein quality control in the ER (Han et al. 2010).

After the initial condensation reaction catalyzed by SPT, 3-ketodihydrosphingosine is reduced to dihydrosphingosine (DHS or sphinganine) by the enzyme 3-ketodihydrosphingosine reductase, Tsc10 (Beeler et al. 1998). Like LCB1/2, TSC10 is an essential

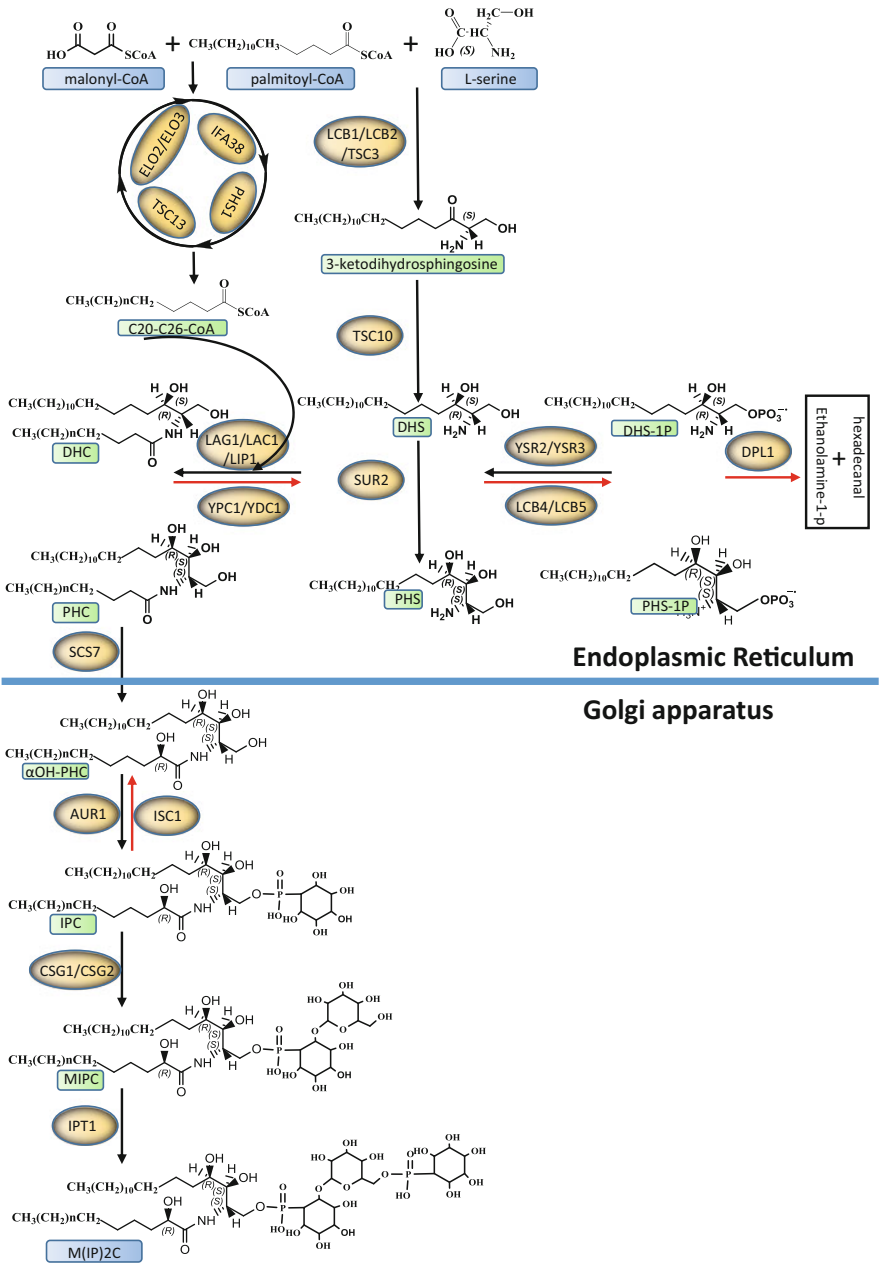


Fig. 2 Overview of yeast sphingolipid metabolism. The initial substrates and final products of the sphingolipid biosynthetic pathway are highlighted with *blue rectangles*. The intermediate products are highlighted in *green rectangles*. Genes catalyzing each step are indicated in *gold capsules*. *Black arrows* show the steps in the biosynthetic pathway and the *red arrows* points to the reactions that lead to sphingolipid catabolism

gene, and its deletion causes cell death unless the growth medium is supplemented with dihydrosphingosine or phytosphingosine. TSC10 homologues in both human (hFVT-1) and mouse (mFVT-1) have been cloned and can functionally substitute for yeast TSC10 (Kihara and Igarashi 2004). A point mutation in bovine 3-ketodihydrosphingosine reductase, *FVT1*, was proposed as the candidate causal mutation for bovine spinal muscular atrophy (Krebs et al. 2007).

The C4 position of DHS can be hydroxylated by yeast hydroxylase, Sur2p, to form phytosphingosine (PHS) (Grilley et al. 1998). SUR2 is not an essential gene, and both DHS and PHS exist in yeast although the functional difference between the two has yet to be defined. However, some studies suggest that PHS is the more biologically active LCBs in yeast and have reported that PHS shows specific inhibition of yeast growth and nutrient uptake (Chung et al. 2001).

The next step in yeast sphingolipid synthesis is the *N*-acylation of LCBs to form dihydro- and phytoceramide. The reaction is catalyzed by Lag1 and Lac1, two apparently redundant homologues that comprise the major ceramide synthase (or also called sphingoid base *N*-acyl transferase) activity (Guillas et al. 2001; Schorling et al. 2001). Homologous genes of LAG1/LAC1 have been identified in almost all eukaryotes. LASSs/CerSs are the mammalian homologue of LAG1/LAC1, and they are *bona fide* ceramide synthases (Levy and Futerman 2010). Six members have been identified in the CerS/LASS family, and they are distinguished from each other by their preference to acyl-chains of different length. A third subunit, Lip1, is found to co-purify with Lag1p/Lac1 in yeast and is required for the yeast ceramide synthase activity (Vallee and Riezman 2005); however no mammalian homologue of Lip1 has been identified, and overexpression of Lip1 cannot regulate ceramide synthesis in mammals (Levy and Futerman 2010). Production of ceramide shares similar regulatory mechanism as LCB biosynthesis. Ypk1 was mentioned before to promote LCB production by alleviating negative regulation of SPT by Orm1/2. The activity of Lag1/Lac1 is also regulated by TORC2-dependent protein kinase Ypk1 phosphorylation and dephosphorylation by Ca2+/calmodulin-dependent protein phosphatase (PP2B) (Muir et al. 2014). Both Slm1/2, PH domain-containing proteins, and TORC2 subunit Avo1 have been suggested to recruit Ypk1/2 to TORC2 complex upon stress response (Berchtold et al. 2012; Liao and Chen 2012; Niles et al. 2012).

Notably, while ceramide in mammalian cells shows great diversity due to acyl-chains of different length linked to the amino group of LCB backbone as well as variations in hydroxylation and unsaturation, there is less diversity of ceramide species in yeast. One of the distinguishing features of yeast ceramides is the preponderant incorporation of very long-chain fatty acids (VLCFAs) into ceramide, such that yeast sphingolipids contain a significant amount of VLCFA of 26 carbons. The elongation from long-chain fatty acid (LCFA) of C14–C18 acyl-CoA to VLCFA of C22–C26 is catalyzed by the VLCFA synthase complex consisting of fatty acid elongase Elo2/Elo3(Oh et al. 1997), beta-keto reductase Ifa38p (Han et al. 2002), 3-hydroxyacyl-CoA dehydratase Phs1p (Denic and Weissman 2007; Schuldiner et al. 2005), and enoyl-reductase Tsc13p (Kohlwein et al. 2001). In this reaction, two carbons are added to the starting acyl-CoA after each cycle of elongation. In

contrast, in mammalian cells there are seven elongases, and ELOVL1-7 and VLCFA are incorporated into both sphingolipids and glycerophospholipids (Kihara 2012). Another unique feature of yeast sphingolipids is the abundance of hydroxylated products. Other than the Sur2p-catalyzed formation of C4-hydroxyl on the sphingoid base of phytoceramide, the C2 position of the fatty acid chain can be hydroxylated by the yeast hydroxylase Scs7p to form α-hydroxy- dihydro-(phyto)ceramide (Dale Haak 1997). Mammalian homologues of these hydroxylases do exist, and hydroxylated sphingolipids are also detected especially in the nervous system, although at present their function is largely unknown (Hama 2010). Finally, in addition to the hydroxyl status, the other major difference between yeast and mammalian ceramide is the formation of a double bond between sphingoid base carbon C4 and C5 by human DEGS1 desaturase. In contrast, no dihydroceramide desaturase has been identified in *S. cerevisiae.*

Once dihydro-/phytoceramides are synthesized, they are transported from the ER to the Golgi network where they can be further processed to form complex sphingolipids. This is mediated through vesicular membrane transport as well as in a non-vesicular way through direct contact between ER and Golgi networks in yeast (Funato and Riezman 2001). In mammalian cells ceramide can also be transported by ceramide transfer (CERT) protein (Hanada et al. 2003; Kumagai et al. 2005). Complex sphingolipids are formed from ceramide through modification of the 1-OH head group of the sphingoid base. In mammals this head group can be modified with either sugar residues to form glycosphingolipids or with phosphocholine to form sphingomyelin. In contrast, the final sphingolipid product in yeast is mannose-(inositol-P)$_2$-ceramide (M(IP)2C), which is formed in three steps involving four enzymes. These genes do not exist in mammals and thus present great potential as antifungal drug targets. Aur1p catalyzes the formation of inositol phosphoryl-ceramide (IPC) by transferring the phosphoinositol head group from phosphatidylinositol to dihydro-/phytoceramides (Nagiec et al. 1997). Notably, the by-product of this reaction is DAG, itself an important lipid signaling molecular that functions at the interface of glycerophospholipid metabolism and membrane trafficking. Phosphatidylinositol, the donor of the head group, is also a key signaling molecule. IPC is further mannosylated to mannose-inositol-phosphoceramide (MIPC) by the MIPC synthase complex containing the catalytic subunit, Csg1p, and the regulatory subunit, Csg2p (Beeler et al. 1997). Lastly M(IP)$_2$C is formed by the addition of a second phosphoinositol moiety by inositolphospho transferase, Ipt1p(Dickson et al. 1997b). Once synthesized, M(IP)2C is then transported to the plasma membrane, and it is the most abundant sphingolipid in yeast.

2.2 Sphingolipid Breakdown

The de novo synthesis of yeast sphingolipids starts in the ER by the condensation of serine and palmitoyl-CoA and ends at the plasma membrane where M(IP)2C predominantly resides. The multi-step biosynthesis pathway also generates important intermediate products such as LCBs and ceramides that have their own functions

in the cell. In reverse of the biosynthetic pathway, complex sphingolipids can be broken down to ultimately generate non-sphingolipid catabolites: fatty aldehydes and ethanolamine phosphate. These reactions are highlighted by red arrows in Fig. 2.

The first enzyme involved in sphingolipid breakdown is ISC1, which encodes a phospholipase C type activity that cleaves the polar head groups from yeast sphingolipids (Sawai et al. 2000). Isc1p is able to hydrolyze all three of the complex sphingolipids IPC, MIPC, and M(IP)2C back into dihydro-/phytoceramides, and it is the only enzyme known thus far in yeast that can do this. Isc1 normally resides in the ER but is predominantly located in mitochondria during the diauxic shift when cells change from fermentative to respiratory growth (Vaena de Avalos et al. 2004).The mammalian homologues of Isc1 are nSMase1 and nSMase2 (neutral sphingomyelinase), which catalyze the breakdown of sphingomyelin into ceramide. The role of nSMase1 in mammalian sphingomyelin metabolism however is questionable as its preferred substrates are lysoPAF and lysoPC. nSMase2 is activated by a host of extracellular signals to produce ceramide which functions as bioactive regulatory molecule in processes like inflammation signaling and apoptosis (Chatterjee 1999). nSMase2 thus plays important roles in human pathologies such as cancer metastasis and atherosclerosis (Hwang et al. 2015). Mammalian systems also have acid sphingomyelinase, and mutations in human acid sphingomyelinase, SMPD1, cause type A and type B Niemann-Pick disease (Schuchman 2010).

Yeast ceramides are in turn broken down to LCBs by Ypc1p or Ydc1p. Ypc1p preferentially hydrolyzes phytoceramide to generate phytosphingosine, whereas Ydc1p shows preference for dihydroceramide to generate dihydrosphingosine (Mao et al. 2000a, b). Significant ceramide synthase activity has also been detected for Ypc1 but no Ydc1. Ypc1 and Ydc1 also show functional differences with the deletion of only YDC1 being able to reduce yeast thermotolerance. Biochemically, both Ypc1 and Ydc1 function optimally at alkaline pH, and their mammalian homologues, alkaline ceramidases (ACERS 1–3), have been cloned and studied. They demonstrate similar functions to break down mammalian ceramides into LCBs (Mao et al. 2001). A mutation in ACER3 has been identified in patients with leukodystrophy (Edvardson et al. 2016). The loss of ACER3 activity and the accompanied abnormal accumulation of sphingolipids were proposed to be the underlining mechanism behind a certain form of leukodystrophy. Neutral and acid ceramidase are also present in mammalian cells, with mutations in human acid ceramidase associated with lipid storage disorder, Farber disease (Park and Schuchman 2006).

Yeast LCBs can be phosphorylated at the 1-OH group to form LCB phosphates by cytosolic LCB kinases Lcb4/5, products of two paralogs that arose from whole-genome duplication (Nagiec et al. 1998). The LCB4/5 are not essential genes in yeast, but their mammalian counterparts, SK1 and SK2, are key regulators of cell proliferation and angiogenesis through generation of sphingosine-1-phosphate (Hla et al. 2001). Mammalian sphingosine-1-phosphate exerts its extracellular signaling roles by binding to the G-protein-coupled Edg receptor family (S1PR 1–5). Intracellular targets of sphingosine-1-phosphate have also been suggested (Strub et al. 2010). There is limited information on the function of dihydro-/phyto-sphingosine-

1-phosphate in yeast other than its possible role in regulating genes required for mitochondrial respiration through the HAP complex transcription factor (Cowart et al. 2010b). Also no receptors of LCB phosphate have been discovered in yeast. LCB phosphate in yeast can be dephosphorylated by phosphatases encoded by YSR2 and YSR3 (Mao et al. 1997; Qie et al. 1997). Mammalian sphingosine-1-phosphate phosphatases (SPPases) are the functional homologues of yeast Ysr2p (Mandala et al. 2000). The phosphatase controls the balance between LCB and LCB-1-phosphate.

Dihydro-/phyto-sphingosine-1-phosphate can be cleaved by sphingosine-1-phosphate lyase, Dpl1p, to generate hexadecanal/hydroxy-hexadecanal and ethanolamine-1-phosphate, which are the end products of the sphingolipid catabolism pathway (Percy et al. 1984). This is an irreversible process and the only exit point from the sphingolipid metabolic network. The end products can be reused by the cell through various metabolic pathways to synthesize complex metabolites including phosphatidylethanolamine (Saba et al. 1997). Misregulation of mammalian sphingosine-1-P lyase, SPL, has been identified in metastatic tumor tissues including colon and ovarian cancer specimen of human patients (Fyrst and Saba 2008; Ikeda et al. 2004).

3 Functions of Yeast Sphingolipids

Sphingolipids, along with glycerophospholipids and cholesterol, are major components of cell membranes. Beyond a structural role, sphingolipids have specific functions in several fundamental cellular processes that in turn are integral players in human diseases such as cancer, diabetes, inflammation, and neural degenerate diseases. The bioactive sphingolipids include the end products of the sphingolipid biosynthetic pathway, the complex sphingolipids, as well as major intermediate products such as ceramide, LCBs, and their phosphorylated derivatives. Ceramides and sphingosine-1-P are the best known and studied bioactive sphingolipids, with ceramide largely thought to function predominantly as an antiproliferative signal, while sphingosine-1-P acts as a pro-growth signal (Hannun and Obeid 2008). The signaling pathways mediated through ceramide were first demonstrated in mammalian cells and later proved to be conserved in the yeast *S. cerevisiae*. Both complex sphingolipids and LCBs in yeast have been implicated in specific functions which will be described below. In contrast, no DHS-1P or PHS-1-P receptors have been identified, and there is so far limited information about the definitive functions of these lipids in yeast.

3.1 Function of Yeast Ceramides

The establishment of ceramide as a bioactive molecule in mammalian cells stemmed from the following observations: (1) activation of various cell types such as HL-60 leukemic cells and MCF7 breast cancer cells by a number of extracellular reagents

such as Vitamin D3, TNFα, and α-interferon results in early accumulation of ceramide; (2) exogenously added ceramide analogs specifically induce similar cell responses to those caused by ligands (Dressler et al. 1992; Hannun et al. 1993; Kim et al. 1991; Kolesnick 1991; Obeid et al. 1993); (3) moreover, interfering with ceramide formation often obliterates or diminishes the respective action of those extracellular agents. Subsequent to these observations, considerable research has identified ceramide-mediated cell responses including the cell cycle arrest, induction of differentiation, and initiation of apoptosis (Hannun 1994). The immediate ceramide effectors are proposed to include both the ceramide-activated protein kinases (CAPK) and ceramide-activated protein phosphatase (CAPP). The presumptive CAPKs include JNK, PKCζ, and C-Raf, and the CAPPs include PP2A and PP1 (Hannun and Obeid 2008; Huwiler et al. 1996; Kolesnick and Golde 1994; Kolesnick 1991; Liu et al. 1994). In addition, ceramide has been shown to activate the protease cathepsin D. Most evidence favors a key role for CAPPs in mediating the actions of ceramides on a number of targets including Akt, PKC, ß-catenin, Bcl2, and many others. Yet more questions remain including the detailed mechanisms such as ceramide production upon specific stimuli, how ceramide interacts with its immediate downstream effectors, and what is the nature of the signaling pathways downstream of ceramide effectors. The yeast *S. cerevisiae* is a useful model system to study the mechanism of ceramide function for three major reasons. Firstly, the generation of ceramide through both the biosynthetic pathway and the hydrolytic pathway from complex sphingolipid degradation is conserved between yeast and mammalian systems. Secondly, various ceramide effectors, including both CAPK and CAPP, have been found in yeast. Finally, many of the major signaling pathways related to ceramide functions are conserved between yeast and mammalian systems.

The role of ceramide in yeast was first explored by adding C2-ceramide, a cell-permeable analogue of ceramide to the yeast culture where it was found to inhibit yeast cell growth. Importantly, a ceramide-dependent serine/threonine phosphatase activity in the yeast lysate was demonstrated and found to be required for the growth inhibition (Dobrowsky and Hannun 1993; Fishbein et al. 1993). Later it was shown that exogenously added ceramide induces a G1 arrest in yeast, and this effect was mediated by Sit4, a yeast phosphatase belonging to the PP2A family (Nickels and Broach 1996). This PP2A phosphatase complex is composed of one catalytic subunit, Sit4, and two regulatory subunits, Cdc55 and Tpd3. Notably, the inhibitory effect of ceramide on yeast cell growth was greatly reduced in mutants of any subunit suggesting that Sit4 is a direct downstream target of ceramide in mediating its inhibitory effect on cell growth.

Additionally, the observation that activation of the RAS pathway is synthetically lethal with reduced Sit4 activity suggests the possible antagonism of ceramide and RAS pathway. Yeast pheromone-induced cell cycle arrest at G1/S is also mediated through ceramide, which induces MAP kinase transcription (Villasmil et al. 2016).

Ceramide has also been shown to associate with the yeast genotoxic response. Yeast mutants defective in ceramide synthesis (*lag1Δlac1Δ*) or salvage (*isc1Δ*) show sensitivity to DNA-damaging reagents methyl methane sulfonate (MMS) and

hydroxyurea (HU). This sensitivity reveals a new role of yeast ceramide in G2/M regulation through its effect on Swe1 and the G2/M checkpoint cyclin-dependent kinase Cdc28. As with the G1 arrest above, this process also depends on the PP2A phosphatase Sit4. Importantly, C18:1-phytoceramide was subsequently identified as the specific ceramide species that mediates HU resistance in yeast (Matmati et al. 2009, 2013). The existence of a sphingolipid-PP2A-Swe1-Cdc28 pathway is also supported by analysis of cell cycle delay in tgl3Δtgl4Δ, a mutant defective in yeast neutral lipid lipolysis. Supplementation of PHS rescues the cell cycle delay in tgl3Δtgl4Δ mutant through Swe1 phosphorylation of Cdc28 in a PP2A- and Cdc55-dependent manner (Chauhan et al. 2015).

Yeast ceramides are also important regulators of mitochondria function as supported by the following evidence: (1) the change in location of ISC1 from ER to mitochondria during the switch from fermentation to respiration at diauxic shift (Kitagaki et al. 2007; Vaena de Avalos et al. 2004); (2) yeast lacking ISC1 demonstrates a failure of growth at diauxic shift, oxidative stress, shortened chronical life span, and impaired mitochondrial dynamics (Almeida et al. 2008; Kitagaki et al. 2009); and (3) these phenotypes are mediated by ceramide signaling through Sit4 and Hog1, known as ceramide-responsive phosphatases and MAP kinase (Teixeira et al. 2015).

Ceramide is also required for stable membrane association of glycosylphosphatidylinositol (GPI)-anchored proteins to the ER and their transport from the ER to Golgi (Watanabe et al. 2002). This was demonstrated by the defective ER-to-Golgi transport of a GPI-anchored protein, Gap1, in lcb1–100, a yeast mutant with impaired sphingolipid biosynthesis. This transport defect can be rescued by exogenous PHS but not in the presence of ceramide synthesis inhibitor australifungin. It is possible that the transport defect is due to defective GPI anchor lipid remodeling process, by which the lipid moiety of the GPI anchor is replaced with ceramide, caused by the lack of ceramide in the mutant (Bosson et al. 2009).

Despite the significant progress that has been made, major challenges still exist in determining specific signaling roles of distinct ceramide species. These challenges emanate from the following factors: (1) ceramide comprises actually a class of related molecules with variations in the acyl-chain length, degree of unsaturation, and different hydroxylation modifications. The mechanisms that regulate the generation of each subspecies are not clear yet and thus impede to study their functions through manipulation of individual enzymes. (2) Most of the ceramide-dependent functions so far are delineated from manipulation of individual enzymes of ceramide metabolism with the assumption that the functional changes are caused by the direct substrate or product of that enzyme. Sphingolipid metabolism is in fact composed of a highly connected network, and the manipulation of one enzyme is associated with a broad change of sphingolipid metabolites, and their contribution to the functional change caused by disturbing individual enzyme is mostly neglected. This is an area where yeast genetics has been of great value. A few studies have actually aimed at "triangulating" the actual lipid mediator by performing studies in deletion mutants in the various enzymes of ceramide metabolism. For example, such an approach has allowed the implication of ceramide and not the other sphingolipids in the action of

HU. (3) Notwithstanding, only limited functions have been identified to be associated with individual ceramides from studies testing the role of individual genes in the sphingolipid metabolism pathway. Montefusco et al. proposed a systematic approach that integrates measuring lipidomics and transcriptomic response to systematically perturbed ceramide metabolism (Montefusco et al. 2013). The study revealed new roles of ceramide such as the association between long-chain dihydroceramides and ion metabolism and very long-chain dihydroceramide with oxidative stress response.

3.2 Function of Complex Sphingolipids

Complex sphingolipids in yeast include IPC, MIPC, and $M(IP)_2C$. They are the major components of the plasma membrane where they constitute approximately 30% of the total phospholipid content (Patton and Lester 1991). Surprisingly, however, none of the yeast complex sphingolipids have been found to be essential for cell survival as is demonstrated by the normal growth of csg1 and csg2 deletion mutants that do not synthesize MIPC or its downstream product $M(IP)_2C$. Indeed, while treatment of yeast with aureobasidin A, an inhibitor of the IPC synthase Aur1p, is able to cause cell death, this lethality is reversed by the deletion of both LAG1 and LAC1. This suggests that it is the accumulation of ceramide rather than lack of complex sphingolipids that caused cell death after aureobasidin A treatment (Schorling et al. 2001).

The functions of yeast complex sphingolipids have also been investigated using a yeast strain in which AUR1 was deleted (Epstein et al. 2012). The lethality caused by ceramide accumulation is this strain is overcome by replacing the endogenous yeast ceramide synthases, Lag1p and Lac1p, with cotton CerS, GhLag1. The strain produces predominately C18 instead of the presumably toxic C26 sphingolipids. The lack of complex sphingolipids in this strain causes defects in cytokinesis as well as accumulation of lipid droplets. However, the underlying mechanisms by which complex sphingolipids regulate these processes have yet to be defined.

Finally, accumulation of IPC is associated with regulation of cellular Ca^{2+} homeostasis. This is demonstrated by the failure of cgs1/cgs2 mutants to grow in medium with a high Ca^{2+} concentration and the identification of suppressor mutants that can reverse the Ca^{2+} sensitivity of cgs1/2 mutant by inhibiting the IPC synthesis (Beeler et al. 1994, 1997, 1998; Schneiter 1999; Zhao et al. 1994). However, the mechanism of how IPC regulates Ca^{2+} homeostasis in yeast remains unknown.

3.3 Function of Yeast LCBs

Long-chain sphingoid bases (LCBs), DHS and PHS in yeast, are required for proper actin organization, which is critical for the internalization step of endocytosis (Munn and Riezman 1994; Zanolari et al. 2000). This physiological function of LCB was first established using lcb1-100, a yeast mutant that is defective in the first step of sphingolipid biosynthesis at non-permissive temperatures. In this study, significant

defects in endocytosis were observed when sphingolipid synthesis was blocked. Moreover, the blocking of endocytosis in lcb1-100 was abrogated by supplementation of exogenous LCB, DHS, or PHS, even when LCB-1-P and ceramide synthesis are abolished. Subsequent studies established that the signaling pathway downstream of LCB was composed of Pkh1/2, two kinases that are homologous to mammalian 3-phosphoinositide dependent kinase-1 (PDK1), and their downstream effectors PKC1, Sch9, and YPK1, homologues of the mammalian serum- and glucocorticoid-induced kinase SGKp (deHart et al. 2002; Friant et al. 2000, 2001; Liu et al. 2005; Sun et al. 2000). Indeed, Pkh1/2 was shown to be activated by nanomolar concentration of sphingoid bases, and overexpression of Pkh1/2 as well as their downstream effectors, Pkc1 and Ypk1, was shown to suppress the LCB requirement for endocytosis.

Sphingoid bases from de novo synthesis have also been established as potential second messengers in the heat stress response, the ability of yeast to adapt to growth at elevated temperatures as high as 39 °C. Complex physiological changes occur during the yeast response to heat stress. These changes include gaining of thermotolerance through generation of trehalose, induction of heat shock proteins, decreased nutrient import, transient arrest of the cell cycle at G0/G1, and finally recovery of normal growth state even at the elevated temperature. Strikingly, a ~10-fold increase of C20-PHS and C20-DHS was detected immediately after the temperature shift from 30 °C to 39 °C (Jenkins et al. 1997), and subsequent studies implicated DHS/PHS in many relevant heat stress pathways. For example, DHS/PHS can cause trehalose accumulation through activation of TPS1, a gene encoding a subunit of trehalose synthase (Dickson et al. 1997a); inhibit amino acid transport via downregulation of nutrient permease, such as Fur4, through ubiquitin-mediated protein degradation (Chung et al. 2000; Skrzypek et al. 1998); and induce cell cycle arrest at G0/G1 through Gln3 (Jenkins and Hannun 2001). These roles of DHS/PHS are supported by two lines of evidence: (1) the response to heat stress is abolished when the sphingoid generation is impaired by genetically inactivating specific genes or pharmaceutically inhibiting the specific enzyme activity and (2) exogenous addition of DHS/PHS is able to cause similar effects as heat stress. However, despite the possible downstream effectors of sphingoid bases identified for each physiological change associated with the yeast heat stress response, there is still a lack of information about the direct downstream targets of sphingoid base and if and how the identified LCB responsive kinase Pkh1/2 coordinates these different processes.

Indeed, a more broader analysis found that the expression level of ~1,500 out of a total of ~6,000 yeast genes changes during the heat stress response as measured by microarray analysis (Gasch et al. 2000). Of those, ~200 genes showed different transcription responses to heat stress between wild-type yeast and the *lcb1–100*, which is unable to produce sphingolipids under heat stress. This suggests that the regulation of these genes is sphingolipid dependent, and thus they function as potential targets of bioactive sphingolipids in yeast. Strikingly, these genes function in diverse cellular pathways including cell cycle control, protein synthesis regulation, amino acid metabolism, and tRNA synthesis (Cowart et al. 2003). This approach was validated by detailed analyses of the genes identified from the

microarray experiment. CHA1, a gene encoding serine/threonine dehydratase, was identified as one of the genes expressed differently between wild-type and lcb1-100 strains upon heat stress. It was suggested that serine-induced CHA1 expression, a major mechanism to attenuate serine levels in yeast, is mediated through the synthesis of sphingoid bases DHS or PHS, thus revealing the primordial role of sphingolipids in regulating amino acid metabolism (Montefusco et al. 2012). Sphingoid bases have also been implicated in the regulation of cap-dependent translation initiation (Meier et al. 2006). Reduced synthesis of heat shock proteins was observed in lcb1-100 cells upon heat stress. This was mediated by depletion of eIF4G, the cap-dependent translation initiation factor. The eIF4G level was shown to be partially controlled by the sphingoid base-responsive Pkh-Ypk pathway (Meier et al. 2006). Increased levels of yeast sphingoid bases are also required for mRNA "P-body" formation in response to heat stress, and that implicates a role of sphingolipids in translation initiation (Cowart et al. 2010a).

Finally, sphingolipid metabolism has also been associated with yeast aging as shown by the phenotypic expression of changed chronical life span (CLS) in various mutants in the sphingolipid metabolism pathway. Most strikingly, the mutants *lag1Δ* and YPC1 overexpression in yeast show decreased CLS (D'Mello et al. 1994; Yi et al. 2016). However, as the levels of both ceramide and sphingoid base change simultaneously in the two mutants, it is difficult to dissect the functional difference between ceramide and sphingoid bases on yeast aging. Some studies suggest that lack of ceramide is the primary effect underlying yeast CLS because of the decreased CLS observed in isc1Δ, where the gene deletion directly caused decreased ceramide level due to failed hydrolysis from IPC (Almeida et al. 2008; Barbosa et al. 2016). However, other studies suggest the accumulation of sphingoid bases as the major cause of CLS in yeast as manipulation of sphingoid base levels in these cells independently of ceramide is able to cause similar defects in yeast CLS (Yi et al. 2016). Nonetheless the identification of the direct downstream sphingolipid target in yeast aging will ultimately pinpoint the functional sphingolipid species in the process.

4 Research Needs

Here, we have reviewed the basic sphingolipid metabolic pathway in yeast and detailed the known functions of bioactive sphingolipids in yeast biology. As can be seen, the metabolism of sphingolipids is tightly regulated and integrated with major yeast signaling pathways (Dickson 2008; Olson et al. 2016). It is worth noting that sphingolipids represent a vast family of lipids including all the metabolic intermediates and a large number of derivatives that arise from modification to the basic structures. Moreover, with the advance of new techniques such as high-resolution mass spectrometry, previously unknown and unappreciated sphingolipid species are being discovered, leading to further questions about the enzymes that generate these species, the mechanism of their regulation, and their cellular function.

Our current knowledge on roles of sphingolipids is primarily based on the identification of bioactive sphingolipid targets. Further investigation of sphingolipid

function using system biology coupled with yeast genetics and biochemical approaches will not only reveal more sphingolipid targets but also establish more specific mechanisms between sphingolipids and their targets and downstream responses at the single-species level.

Lastly, our understanding of sphingolipid function will advance greatly with more detailed characterization of the physical interaction between sphingolipids and their direct targets, including the identification and characterization of novel sphingolipid-interacting domains.

Acknowledgments The authors would like to acknowledge Drs. Cungui Mao, Christopher Clarke, and Cosima Rhein for their critical discussions and valuable comments. Work related to this review in Dr. Hannun's laboratory was supported by grant R35 GM118128.

References

Almeida T, Marques M, Mojzita D, Amorim MA, Silva RD, Almeida B, Rodrigues P, Ludovico P, Hohmann S, Moradas-Ferreira P et al (2008) Isc1p plays a key role in hydrogen peroxide resistance and chronological lifespan through modulation of iron levels and apoptosis. Mol Biol Cell 19:865–876

Barbosa AD, Pereira C, Osorio H, Moradas-Ferreira P, Costa V (2016) The ceramide-activated protein phosphatase Sit4p controls lifespan, mitochondrial function and cell cycle progression by regulating hexokinase 2 phosphorylation. Cell Cycle 15:1620–1630

Beeler T, Bacikova D, Gable K, Hopkins L, Johnson C, Slife H, Dunn T (1998) The *Saccharomyces cerevisiae* TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca2+-sensitive csg2Delta mutant. J Biol Chem 273:30688–30694

Beeler TJ, Fu D, Rivera J, Monaghan E, Gable K, Dunn TM (1997) SUR1 (CSG1/BCL21), a gene necessary for growth of *Saccharomyces cerevisiae* in the presence of high Ca2+ concentrations at 37 degrees C, is required for mannosylation of inositolphosphorylceramide. Mol Gen Genet MGG 255:570–579

Beeler T, Gable K, Zhao C, Dunn T (1994) A novel protein, CSG2p, is required for Ca2+ regulation in *Saccharomyces cerevisiae*. J Biol Chem 269:7279–7284

Bejaoui K, Uchida Y, Yasuda S, Ho M, Nishijima M, Brown RH Jr, Holleran WM, Hanada K (2002) Hereditary sensory neuropathy type 1 mutations confer dominant negative effects on serine palmitoyltransferase, critical for sphingolipid synthesis. J Clin Invest 110:1301–1308

Berchtold D, Piccolis M, Chiaruttini N, Riezman I, Riezman H, Roux A, Walther TC, Loewith R (2012) Plasma membrane stress induces relocalization of Slm proteins and activation of TORC2 to promote sphingolipid synthesis. Nat Cell Biol 14:542–547

Bosson R, Guillas I, Vionnet C, Roubaty C, Conzelmann A (2009) Incorporation of ceramides into *Saccharomyces cerevisiae* glycosylphosphatidylinositol-anchored proteins can be monitored in vitro. Eukaryot Cell 8:306–314

Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, Shevchenko A, Ejsing CS, Weissman JS (2010) Orm family proteins mediate sphingolipid homeostasis. Nature 463:1048–1053

Carter HE, Haines WJ et al (1947) Biochemistry of the sphingolipides; preparation of sphingolipides from beef brain and spinal cord. J Biol Chem 169:77–82

Chatterjee S (1999) Neutral sphingomyelinase: past, present and future. Chem Phys Lipids 102:79–96

Chauhan N, Visram M, Cristobal-Sarramian A, Sarkleti F, Kohlwein SD (2015) Morphogenesis checkpoint kinase Swe1 is the executor of lipolysis-dependent cell-cycle progression. Proc Natl Acad Sci U S A 112:E1077–E1085

Chung N, Jenkins G, Hannun YA, Heitman J, Obeid LM (2000) Sphingolipids signal heat stress-induced ubiquitin-dependent proteolysis. J Biol Chem 275:17229–17232

Chung N, Mao C, Heitman J, Hannun YA, Obeid LM (2001) Phytosphingosine as a specific inhibitor of growth and nutrient import in *Saccharomyces cerevisiae*. J Biol Chem 276:35614–35621

Cowart LA, Gandy JL, Tholanikunnel B, Hannun YA (2010) Sphingolipids mediate formation of mRNA processing bodies during the heat-stress response of *Saccharomyces cerevisiae*. Biochem J 431:31–38

Cowart LA, Okamoto Y, Pinto FR, Gandy JL, Almeida JS, Hannun YA (2003) Roles for sphingolipid biosynthesis in mediation of specific programs of the heat stress response determined through gene expression profiling. J Biol Chem 278:30328–30338

Cowart LA, Shotwell M, Worley ML, Richards AJ, Montefusco DJ, Hannun YA, Lu X (2010) Revealing a signaling role of phytosphingosine-1-phosphate in yeast. Mol Syst Biol 6:349

D'Mello NP, Childress AM, Franklin DS, Kale SP, Pinswasdi C, Jazwinski SM (1994) Cloning and characterization of LAG1, a longevity-assurance gene in yeast. J Biol Chem 269:15451–15459

deHart AK, Schnell JD, Allen DA, Hicke L (2002) The conserved Pkh-Ypk kinase cascade is required for endocytosis in yeast. J Cell Biol 156:241–248

Denic V, Weissman JS (2007) A molecular caliper mechanism for determining very long-chain fatty acid length. Cell 130:663–677

Dickson RC (2008) Thematic review series: sphingolipids. New insights into sphingolipid metabolism and function in budding yeast. J Lipid Res 49:909–921

Dickson RC, Lester RL (1999) Yeast sphingolipids. Biochim Biophys Acta 1426:347–357

Dickson RC, Nagiec EE, Skrzypek M, Tillman P, Wells GB, Lester RL (1997) Sphingolipids are potential heat stress signals in Saccharomyces. J Biol Chem 272:30196–30200

Dickson RC, Nagiec EE, Wells GB, Nagiec MM, Lester RL (1997) Synthesis of mannose-(inositol-P)2-ceramide, the major sphingolipid in *Saccharomyces cerevisiae*, requires the IPT1 (YDR072c) gene. J Biol Chem 272:29620–29625

Dobrowsky RT, Hannun YA (1993) Ceramide-activated protein phosphatase: partial purification and relationship to protein phosphatase 2A. Adv Lipid Res 25:91–104

Dressler KA, Mathias S, Kolesnick RN (1992) Tumor necrosis factor-alpha activates the sphingomyelin signal transduction pathway in a cell-free system. Science 255:1715–1718

Edvardson S, Yi JK, Jalas C, Xu R, Webb BD, Snider J, Fedick A, Kleinman E, Treff NR, Mao C et al (2016) Deficiency of the alkaline ceramidase ACER3 manifests in early childhood by progressive leukodystrophy. J Med Genet 53:389–396

Epstein S, Castillon G. A, Qin Y, Riezman H (2012) An essential function of sphingolipids in yeast cell division. Mol Microbiol 84:1018–32

Fishbein JD, Dobrowsky RT, Bielawska A, Garrett S, Hannun YA (1993) Ceramide-mediated growth inhibition and CAPP are conserved in *Saccharomyces cerevisiae*. J Biol Chem 268:9255–9261

Friant S, Lombardi R, Schmelzle T, Hall MN, Riezman H (2001) Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. EMBO J 20:6783–6792

Friant S, Zanolari B, Riezman H (2000) Increased protein kinase or decreased PP2A activity bypasses sphingoid base requirement in endocytosis. EMBO J 19:2834–2844

Funato K, Riezman H (2001) Vesicular and nonvesicular transport of ceramide from ER to the Golgi apparatus in yeast. J Cell Biol 155:949–959

Fyrst H, Saba JD (2008) Sphingosine-1-phosphate lyase in development and disease: sphingolipid metabolism takes flight. Biochim Biophys Acta 1781:448–458

Gable K, Gupta SD, Han G, Niranjanakumari S, Harmon JM, Dunn TM (2010) A disease-causing mutation in the active site of serine palmitoyltransferase causes catalytic promiscuity. J Biol Chem 285:22846–22852

Gable K, Slife H, Bacikova D, Monaghan E, Dunn TM (2000) Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. J Biol Chem 275:7597–7603

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257

Grilley MM, Stock SD, Dickson RC, Lester RL, Takemoto JY (1998) Syringomycin action gene SYR2 is essential for sphingolipid 4-hydroxylation in *Saccharomyces cerevisiae*. J Biol Chem 273:11062–11068

Guillas I, Kirchman PA, Chuard R, Pfefferli M, Jiang JC, Jazwinski SM, Conzelmann A (2001) C26-CoA-dependent ceramide synthesis of *Saccharomyces cerevisiae* is operated by Lag1p and Lac1p. EMBO J 20:2655–2665

Haak D, Gable K, Beeler T, Dunn T (1997) Hydroxylation of Saccharomyces cerevisiae ceramides requires Sur2p and Scs7p. J Biol Chem 272:29704–29710

Hama H (2010) Fatty acid 2-Hydroxylation in mammalian sphingolipid biology. Biochim Biophys Acta 1801:405–414

Han G, Gable K, Kohlwein SD, Beaudoin F, Napier JA, Dunn TM (2002) The *Saccharomyces cerevisiae* YBR159w gene encodes the 3-ketoreductase of the microsomal fatty acid elongase. J Biol Chem 277:35440–35449

Han G, Gupta SD, Gable K, Niranjanakumari S, Moitra P, Eichler F, Brown RH Jr, Harmon JM, Dunn TM (2009) Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. Proc Natl Acad Sci U S A 106: 8186–8191

Han S, Lone MA, Schneiter R, Chang A (2010) Orm1 and Orm2 are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control. Proc Natl Acad Sci U S A 107:5851–5856

Hanada K, Hara T, Nishijima M, Kuge O, Dickson RC, Nagiec MM (1997) A mammalian homolog of the yeast LCB1 encodes a component of serine palmitoyltransferase, the enzyme catalyzing the first step in sphingolipid synthesis. J Biol Chem 272:32108–32114

Hanada K, Kumagai K, Yasuda S, Miura Y, Kawano M, Fukasawa M, Nishijima M (2003) Molecular machinery for non-vesicular trafficking of ceramide. Nature 426:803–809

Hannun YA (1994) The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269:3125–3128

Hannun YA, Obeid LM (2008) Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol 9:139–150

Hannun YA, Loomis CR, Merrill AH Jr, Bell RM (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. J Biol Chem 261:12604–12609

Hannun YA, Obeid LM, Wolff RA (1993) The novel second messenger ceramide: identification, mechanism of action, and cellular activity. Adv Lipid Res 25:43–64

Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ (2001) Lysophospholipids – receptor revelations. Science 294:1875–1878

Huwiler A, Brunner J, Hummel R, Vervoordeldonk M, Stabel S, van den Bosch H, Pfeilschifter J (1996) Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. Proc Natl Acad Sci U S A 93:6959–6963

Hwang SY, Kim TH, Lee HH (2015) Neutral sphingomyelinase and breast cancer research. J Menopausal Med 21:24–27

Ikeda M, Kihara A, Igarashi Y (2004) Sphingosine-1-phosphate lyase SPL is an endoplasmic reticulum-resident, integral membrane protein with the pyridoxal 5′-phosphate binding domain exposed to the cytosol. Biochem Biophys Res Commun 325:338–343

Jenkins GM, Hannun YA (2001) Role for de novo sphingoid base biosynthesis in the heat-induced transient cell cycle arrest of *Saccharomyces cerevisiae*. J Biol Chem 276:8574–8581

Jenkins GM, Richards A, Wahl T, Mao C, Obeid L, Hannun Y (1997) Involvement of yeast sphingolipids in the heat stress response of *Saccharomyces cerevisiae*. J Biol Chem 272:32566–32572

Kanfer JN, Hakomori S-i (1983) Sphingolipid biochemistry. Plenum Press, New York

Kihara A (2012) Very long-chain fatty acids: elongation, physiology and related disorders. J Biochem 152:387–395

Kihara A, Igarashi Y (2004) FVT-1 is a mammalian 3-ketodihydrosphingosine reductase with an active site that faces the cytosolic side of the endoplasmic reticulum membrane. J Biol Chem 279:49243–49250

Kim MY, Linardic C, Obeid L, Hannun Y (1991) Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation. J Biol Chem 266:484–489

Kitagaki H, Cowart LA, Matmati N, Montefusco D, Gandy J, de Avalos SV, Novgorodov SA, Zheng J, Obeid LM, Hannun YA (2009) ISC1-dependent metabolic adaptation reveals an indispensable role for mitochondria in induction of nuclear genes during the diauxic shift in *Saccharomyces cerevisiae*. J Biol Chem 284:10818–10830

Kitagaki H, Cowart LA, Matmati N, Vaena de Avalos S, Novgorodov SA, Zeidan YH, Bielawski J, Obeid LM, Hannun YA (2007) Isc1 regulates sphingolipid metabolism in yeast mitochondria. Biochim Biophys Acta 1768:2849–2861

Kohlwein SD, Eder S, Oh CS, Martin CE, Gable K, Bacikova D, Dunn T (2001) Tsc13p is required for fatty acid elongation and localizes to a novel structure at the nuclear-vacuolar interface in *Saccharomyces cerevisiae*. Mol Cell Biol 21:109–125

Kolesnick RN (1991) Sphingomyelin and derivatives as cellular signals. Prog Lipid Res 30:1–38

Kolesnick R, Golde DW (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell 77:325–328

Kolter T, Sandhoff K (2006) Sphingolipid metabolism diseases. Biochim Biophys Acta 1758:2057–2079

Krebs S, Medugorac I, Rother S, Strasser K, Forster M (2007) A missense mutation in the 3-ketodihydrosphingosine reductase FVT1 as candidate causal mutation for bovine spinal muscular atrophy. Proc Natl Acad Sci U S A 104:6746–6751

Kumagai K, Yasuda S, Okemoto K, Nishijima M, Kobayashi S, Hanada K (2005) CERT mediates intermembrane transfer of various molecular species of ceramides. J Biol Chem 280:6488–6495

Levy M, Futerman AH (2010) Mammalian ceramide synthases. IUBMB Life 62:347–356

Liao HC, Chen MY (2012) Target of rapamycin complex 2 signals to downstream effector yeast protein kinase 2 (Ypk2) through adheres-voraciously-to-target-of-rapamycin-2 protein 1 (Avo1) in *Saccharomyces cerevisiae*. J Biol Chem 287:6089–6099

Liu J, Mathias S, Yang Z, Kolesnick RN (1994) Renaturation and tumor necrosis factor-alpha stimulation of a 97-kDa ceramide-activated protein kinase. J Biol Chem 269:3047–3052

Liu K, Zhang X, Lester RL, Dickson RC (2005) The sphingoid long chain base phytosphingosine activates AGC-type protein kinases in *Saccharomyces cerevisiae* including Ypk1, Ypk2, and Sch9. J Biol Chem 280:22679–22687

Mandala SM, Thornton R, Galve-Roperh I, Poulton S, Peterson C, Olivera A, Bergstrom J, Kurtz MB, Spiegel S (2000) Molecular cloning and characterization of a lipid phosphohydrolase that degrades sphingosine-1- phosphate and induces cell death. Proc Natl Acad Sci U S A 97:7859–7864

Mao C, Xu R, Bielawska A, Obeid LM (2000) Cloning of an alkaline ceramidase from *Saccharomyces cerevisiae*. An enzyme with reverse (CoA-independent) ceramide synthase activity. J Biol Chem 275:6876–6884

Mao C, Xu R, Bielawska A, Szulc ZM, Obeid LM (2000) Cloning and characterization of a *Saccharomyces cerevisiae* alkaline ceramidase with specificity for dihydroceramide. J Biol Chem 275:31369–31378

Mao C, Wadleigh M, Jenkins GM, Hannun YA, Obeid LM (1997) Identification and characterization of *Saccharomyces cerevisiae* dihydrosphingosine-1-phosphate phosphatase. J Biol Chem 272:28690–28694

Mao C, Xu R, Szulc ZM, Bielawska A, Galadari SH, Obeid LM (2001) Cloning and characterization of a novel human alkaline ceramidase. A mammalian enzyme that hydrolyzes phytoceramide. J Biol Chem 276:26577–26588

Matmati N, Kitagaki H, Montefusco D, Mohanty BK, Hannun YA (2009) Hydroxyurea sensitivity reveals a role for ISC1 in the regulation of G2/M. J Biol Chem 284:8241–8246

Matmati N, Metelli A, Tripathi K, Yan S, Mohanty BK, Hannun YA (2013) Identification of C18:1-phytoceramide as the candidate lipid mediator for hydroxyurea resistance in yeast. J Biol Chem 288:17272–17284

Meier KD, Deloche O, Kajiwara K, Funato K, Riezman H (2006) Sphingoid base is required for translation initiation during heat stress in *Saccharomyces cerevisiae*. Mol Biol Cell 17:1164–1175

Merrill AH Jr, Sereni AM, Stevens VL, Hannun YA, Bell RM, Kinkade JM Jr (1986) Inhibition of phorbol ester-dependent differentiation of human promyelocytic leukemic (HL-60) cells by sphinganine and other long-chain bases. J Biol Chem 261:12610–12615

Montefusco DJ, Chen L, Matmati N, Lu S, Newcomb B, Cooper GF, Hannun YA, Lu X (2013) Distinct signaling roles of ceramide species in yeast revealed through systematic perturbation and systems biology analyses. Sci Signal 6:rs14

Montefusco DJ, Newcomb B, Gandy JL, Brice SE, Matmati N, Cowart LA, Hannun YA (2012) Sphingoid bases and the serine catabolic enzyme CHA1 define a novel feedforward/feedback mechanism in the response to serine availability. J Biol Chem 287:9280–9289

Muir A, Ramachandran S, Roelants FM, Timmons G, Thorner J (2014) TORC2-dependent protein kinase Ypk1 phosphorylates ceramide synthase to stimulate synthesis of complex sphingolipids. eLife 3:1–34

Munn AL, Riezman H (1994) Endocytosis is required for the growth of vacuolar H(+)-ATPase-defective yeast: identification of six new END genes. J Cell Biol 127:373–386

Nagiec MM, Baltisberger JA, Wells GB, Lester RL, Dickson RC (1994) The LCB2 gene of Saccharomyces and the related LCB1 gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis. Proc Natl Acad Sci U S A 91:7899–7902

Nagiec MM, Nagiec EE, Baltisberger JA, Wells GB, Lester RL, Dickson RC (1997) Sphingolipid synthesis as a target for antifungal drugs. Complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the AUR1 gene. J Biol Chem 272:9809–9817

Nagiec MM, Skrzypek M, Nagiec EE, Lester RL, Dickson RC (1998) The LCB4 (YOR171c) and LCB5 (YLR260w) genes of Saccharomyces encode sphingoid long chain base kinases. J Biol Chem 273:19437–19442

Nickels JT, Broach JR (1996) A ceramide-activated protein phosphatase mediates ceramide-induced G1 arrest of *Saccharomyces cerevisiae*. Genes Dev 10:382–394

Niles BJ, Powers T (2012) Plasma membrane proteins Slm1 and Slm2 mediate activation of the AGC kinase Ypk1 by TORC2 and sphingolipids in *S. cerevisiae*. Cell Cycle 11:3745–3749

Niles BJ, Mogri H, Hill A, Vlahakis A, Powers T (2012) Plasma membrane recruitment and activation of the AGC kinase Ypk1 is mediated by target of rapamycin complex 2 (TORC2) and its effector proteins Slm1 and Slm2. Proc Natl Acad Sci U S A 109:1536–1541

Obeid LM, Linardic CM, Karolak LA, Hannun YA (1993) Programmed cell death induced by ceramide. Science 259:1769–1771

Oh CS, Toke DA, Mandala S, Martin CE (1997) ELO2 and ELO3, homologues of the *Saccharomyces cerevisiae* ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation. J Biol Chem 272:17376–17384

Olson DK, Frohlich F, Farese RV Jr, Walther TC (2016) Taming the sphinx: mechanisms of cellular sphingolipid homeostasis. Biochim Biophys Acta 1861:784–792

Park JH, Schuchman EH (2006) Acid ceramidase and human disease. Biochim Biophys Acta 1758:2133–2138

Patton JL, Lester RL (1991) The phosphoinositol sphingolipids of *Saccharomyces cerevisiae* are highly localized in the plasma membrane. J Bacteriol 173:3101–3108

Penno A, Reilly MM, Houlden H, Laura M, Rentsch K, Niederkofler V, Stoeckli ET, Nicholson G, Eichler F, Brown RH Jr et al (2010) Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids. J Biol Chem 285:11178–11187

Percy AK, Carson MA, Moore JF, Waechter CJ (1984) Control of phosphatidylethanolamine metabolism in yeast: diacylglycerol ethanolaminephosphotransferase and diacylglycerol cholinephosphotransferase are separate enzymes. Arch Biochem Biophys 230:69–81

Pralhada Rao R, Vaidyanathan N, Rengasamy M, Mammen Oommen A, Somaiya N, Jagannath MR (2013) Sphingolipid metabolic pathway: an overview of major roles played in human diseases. J Lipids 2013:178910

Qie L, Nagiec MM, Baltisberger JA, Lester RL, Dickson RC (1997) Identification of a *Saccharomyces* gene, LCB3, necessary for incorporation of exogenous long chain bases into sphingolipids. J Biol Chem 272:16110–16117

Roelants FM, Breslow DK, Muir A, Weissman JS, Thorner J (2011) Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 108:19222–19227

Saba JD, Nara F, Bielawska A, Garrett S, Hannun YA (1997) The BST1 gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. J Biol Chem 272:26087–26090

Sawai H, Okamoto Y, Luberto C, Mao C, Bielawska A, Domae N, Hannun YA (2000) Identification of ISC1 (YER019w) as inositol phosphosphingolipid phospholipase C in *Saccharomyces cerevisiae*. J Biol Chem 275:39793–39798

Schneiter R (1999) Brave little yeast, please guide us to thebes: sphingolipid function in *S. cerevisiae*. BioEssays: News Rev Mol, Cell Dev Biol 21:1004–1010

Schorling S, Vallee B, Barz WP, Riezman H, Oesterhelt D (2001) Lag1p and Lac1p are essential for the Acyl-CoA-dependent ceramide synthase reaction in Saccharomyces cerevisae. Mol Biol Cell 12:3417–3427

Schuchman EH (2010) Acid sphingomyelinase, cell membranes and human disease: lessons from Niemann-Pick disease. FEBS Lett 584:1895–1900

Schuldiner M, Collins SR, Thompson NJ, Denic V, Bhamidipati A, Punna T, Ihmels J, Andrews B, Boone C, Greenblatt JF et al (2005) Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile. Cell 123:507–519

Shimobayashi M, Oppliger W, Moes S, Jeno P, Hall MN (2013) TORC1-regulated protein kinase Npr1 phosphorylates Orm to stimulate complex sphingolipid synthesis. Mol Biol Cell 24:870–881

Skrzypek MS, Nagiec MM, Lester RL, Dickson RC (1998) Inhibition of amino acid transport by sphingoid long chain bases in *Saccharomyces cerevisiae*. J Biol Chem 273:2829–2834

Strub GM, Maceyka M, Hait NC, Milstien S, Spiegel S (2010) Extracellular and intracellular actions of sphingosine-1-phosphate. Adv Exp Med Biol 688:141–155

Sun Y, Miao Y, Yamane Y, Zhang C, Shokat KM, Takematsu H, Kozutsumi Y, Drubin DG (2012) Orm protein phosphoregulation mediates transient sphingolipid biosynthesis response to heat stress via the Pkh-Ypk and Cdc55-PP2A pathways. Mol Biol Cell 23:2388–2398

Sun Y, Taniguchi R, Tanoue D, Yamaji T, Takematsu H, Mori K, Fujita T, Kawasaki T, Kozutsumi Y (2000) Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. Mol Cell Biol 20:4411–4419

Tabuchi M, Audhya A, Parsons AB, Boone C, Emr SD (2006) The phosphatidylinositol 4,5-biphosphate and TORC2 binding proteins Slm1 and Slm2 function in sphingolipid regulation. Mol Cell Biol 26:5861–5875

Teixeira V, Medeiros TC, Vilaca R, Pereira AT, Chaves SR, Corte-Real M, Moradas-Ferreira P, Costa V (2015) Ceramide signalling impinges on Sit4p and Hog1p to promote mitochondrial fission and mitophagy in Isc1p-deficient cells. Cell Signal 27:1840–1849

Vaena de Avalos S, Okamoto Y, Hannun YA (2004) Activation and localization of inositol phosphosphingolipid phospholipase C, Isc1p, to the mitochondria during growth of *Saccharomyces cerevisiae*. J Biol Chem 279:11537–11545

Vallee B, Riezman H (2005) Lip1p: a novel subunit of acyl-CoA ceramide synthase. EMBO J 24:730–741

Villasmil ML, Francisco J, Gallo-Ebert C, Donigan M, Liu HY, Brower M, Nickels JT Jr (2016) Ceramide signals for initiation of yeast mating-specific cell cycle arrest. Cell Cycle 15:441–454

Watanabe R, Funato K, Venkataraman K, Futerman AH, Riezman H (2002) Sphingolipids are required for the stable membrane association of glycosylphosphatidylinositol-anchored proteins in yeast. J Biol Chem 277:49538–49544

Wilson E, Olcott MC, Bell RM, Merrill AH Jr, Lambeth JD (1986) Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. Role of protein kinase C in activation of the burst. J Biol Chem 261:12616–12623

Yi JK, Xu R, Jeong E, Mileva I, Truman JP, Lin CL, Wang K, Snider J, Wen S, Obeid LM et al (2016) Aging-related elevation of sphingoid bases shortens yeast chronological life span by compromising mitochondrial function. Oncotarget 7:21124–21144

Zanolari B, Friant S, Funato K, Sutterlin C, Stevenson BJ, Riezman H (2000) Sphingoid base synthesis requirement for endocytosis in *Saccharomyces cerevisiae*. EMBO J 19:2824–2833

Zhao C, Beeler T, Dunn T (1994) Suppressors of the Ca(2+)-sensitive yeast mutant (csg2) identify genes involved in sphingolipid biosynthesis. Cloning and characterization of SCS1, a gene required for serine palmitoyltransferase activity. J Biol Chem 269:21480–21488

Nonpolar Lipids in Yeast: Synthesis, Storage, and Degradation

19

Karin Athenstaedt

Contents

1 Introduction 364
2 Synthesis of Nonpolar Lipids 364
2.1 Synthesis of Triacylglycerols 364
2.2 Synthesis of Steryl Esters 367
3 Lipid Droplets 367
3.1 Structure of Lipid Droplets 367
3.2 Models of Lipid Droplet Biogenesis 368
4 Degradation of Triacylglycerols and Steryl Esters 370
5 Key Knowledge Gaps and Research Needs 370
References 372

Abstract

The major nonpolar lipids occurring in yeast are triacylglycerols and steryl esters. These storage lipids accumulate when cells are provided with an excess of nutrients. As substantial amounts of nonpolar lipids cannot be incorporated into biomembranes, they are sequestered from the cytosolic environment in so-called lipid droplets (lipid particles). Upon requirement storage lipids are mobilized from this compartment by triacylglycerol lipases and steryl ester hydrolases. The respective degradation products serve as energy sources and/or building blocks for membrane formation. In this chapter, the reader is introduced to different mechanisms of triacylglycerol and steryl ester synthesis, storage of these lipids in lipid droplets, and their subsequent mobilization. Finally, major gaps in our current knowledge about nonpolar lipid metabolism and research needs for a better understanding of nonpolar lipid turnover are highlighted.

K. Athenstaedt (✉)
Institute of Molecular Biosciences, University of Graz, Graz, Austria
e-mail: karin.athenstaedt@uni-graz.at

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_22

1 Introduction

Currently, there are major efforts to gain a better understanding of the role of nonpolar lipids in cell metabolism, their formation and degradation, and the regulation of these processes. From the medical point of view, a detailed knowledge about nonpolar lipid turnover is required to combat several severe diseases of modern civilization such as obesity, arteriosclerosis, and diabetes type II which are caused by detrimental accumulation of nonpolar lipids. Thus, medical studies are aimed at finding targets to prevent the excessive accumulation of nonpolar lipids and/or at increasing the mobilization of these molecules. As nonpolar lipid metabolism is well conserved from yeast to humans, many examples exist where insights in this process with the yeast system provided essential information required for a better understanding of lipid turnover in higher eukaryotes. Thus, the model organism yeast has been proven to be a highly valuable tool in biomedical research.

In contrast to the medical field, the aim of biotechnological studies related to nonpolar lipid metabolism is to find ways to increase the amount of storage lipids, because in industry microorganisms such as the oleaginous yeast *Yarrowia lipolytica* are used for, e.g., single cell oil production and production of nutrients enriched in essential fatty acids. Furthermore, in times of increasing costs for fossil fuels, the idea of producing biodiesel from triacylglycerols of microorganisms becomes more and more attractive.

2 Synthesis of Nonpolar Lipids

Triacylglycerols (TAG) and steryl esters (SE) form the main part of storage lipids in yeast. These molecules serve as "spare parts store" for sterols, diacylglycerols, and fatty acids which are used as building blocks for membrane formation and/or for energy production. However, the formation of nonpolar lipids is also important to prevent lipotoxicity which is caused by the accumulation of excessive amounts of unesterified fatty acids within the cell. Whereas in SE only one acyl chain is bound to the hydroxyl group at position C3 of the sterol moiety, TAG contain three fatty acids esterified to the glycerol backbone (Fig. 1).

2.1 Synthesis of Triacylglycerols

Due to the importance of TAG as an inert storage form for fatty acids, its synthesis seems to be well conserved among the different kingdoms of life. De novo synthesis of TAG includes the formation of phosphatidic acid (PA), a key intermediate of glycerolipid metabolism (Fig. 2). In yeast as well as in mammalian cells, PA can be synthesized by two different pathways, namely, the glycerol-3-phosphate pathway and the dihydroxyacetone phosphate pathway, both named after their respective precursor (for a review, see Athenstaedt and Daum 1999). Whereas the former pathway comprises two subsequent acylation reactions yielding PA, synthesis via

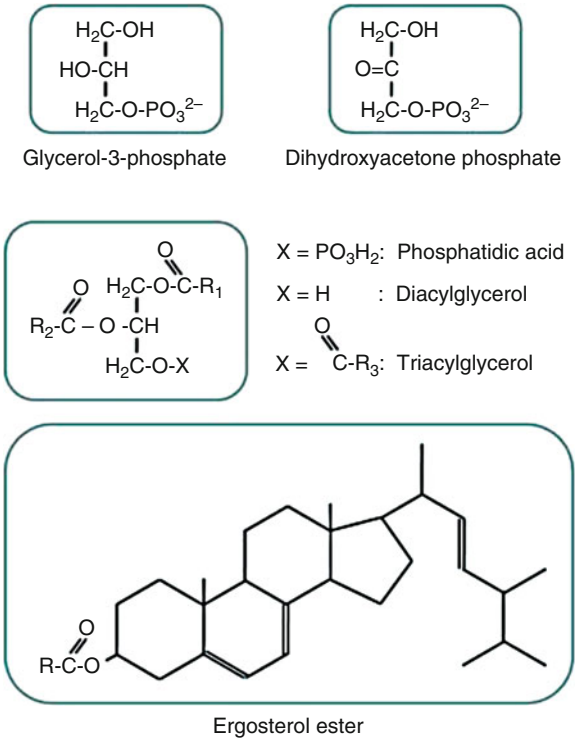


Fig. 1 Structures of triacylglycerol, some of its precursors, and ergosterol ester. R: Hydrocarbon chain

the dihydroxyacetone phosphate pathway is a three-step process – acylation of the precursor dihydroxyacetone phosphate, reduction of the intermediate 1-acyl-dihydroxyacetone phosphate to 1-acyl-glycerol-3-phosphate (lyso-PA), and a final acylation reaction yielding PA. Alternatively to de novo synthesis, PA can be formed from glycerophospholipids through the action of phospholipase D. Subsequent dephosphorylation of PA leads to diacylglycerol, which is the direct precursor for TAG synthesis. Alternative ways for the formation of diacylglycerol include the degradation of glycerophospholipids by the action of phospholipase C and the reverse reaction of TAG synthesis, namely, deacylation of TAG. In addition, diacylglycerol is formed as a by-product in the course of inositol-phosphoceramide synthesis. Noteworthy, both PA and diacylglycerol are also intermediates in de novo synthesis of glycerophospholipids. Whereas activation of PA by CTP yields CDP-diacylglycerol, which is the precursor for the synthesis of all glycerophospholipids, diacylglycerol is required for the formation of phosphatidylethanolamine and phosphatidylcholine via the Kennedy pathway (reviewed in Kent 1995; Henry et al. 2012).

The ultimate step of TAG synthesis is presented by acylation of its direct precursor diacylglycerol (Fig. 2). Mechanistically, this step occurs either by an acyl-CoA-dependent or an acyl-CoA-independent reaction. Enzymes catalyzing

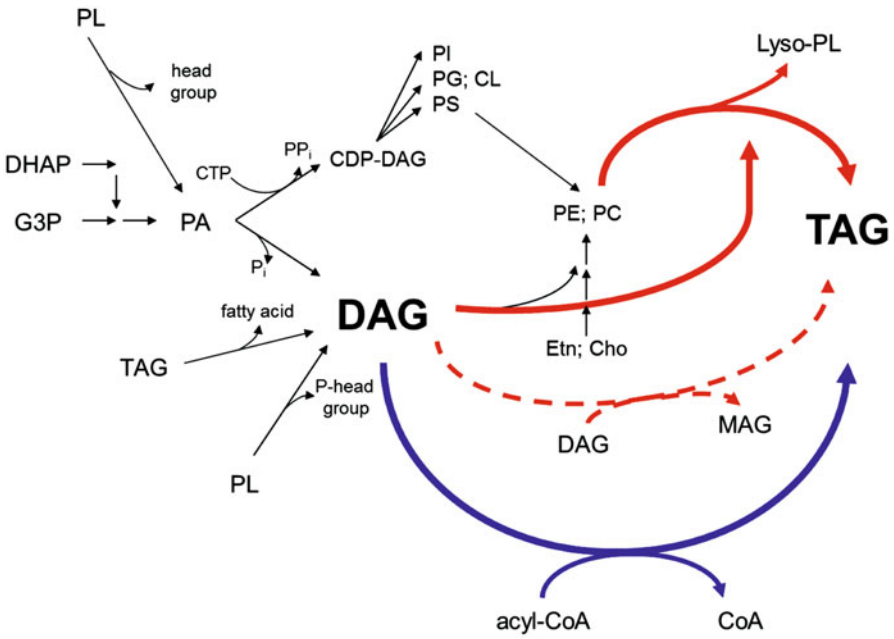


Fig. 2 Pathways leading to the formation of triacylglycerols. De novo synthesis of triacylglycerols (*TAG*) includes the formation of phosphatidic acid (*PA*). Subsequent dephosphorylation of PA yields diacylglycerol (*DAG*), which is the direct precursor for TAG formation. TAG synthesis can be accomplished independent of acyl-CoA by transferring a fatty acid either from a phospholipid (*upper arrow*) or a DAG molecule (*middle arrow*; *broken line*) to DAG. The latter mechanism has not been reported in yeast. The *lowest arrow* shows acylation of DAG via the acyl-CoA-dependent mechanism. *CDP-DAG* cytidindiphosphate-diacylglycerol, *Cho* choline, *CL* cardiolipin, *Etn* ethanolamine, *DHAP* dihydroxyacetone phosphate, *G3P* glycerol-3-phosphate, *lyso-PL* lysophospholipid, *MAG* monoacylglycerol, *P-head group* phosphorylated head group of a phospholipid, *PL* glycerophospholipid, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PI* phosphatidylinositol, *PS* phosphatidylserine

TAG synthesis via the former mechanism are present in all TAG-synthesizing organisms. Thus, the acyl-CoA-dependent acylation of diacylglycerol may be regarded as the ancestral mechanism for TAG formation. In addition, yeasts form TAG via an acyl-CoA-independent reaction using the acyl group of a glycerophospholipid as substrate. The respective enzyme catalyzes the direct transfer of a fatty acid from the *sn-2* position of a glycerophospholipid to diacylglycerol, thus combining the function of a phospholipase A_2 (B) and an acyltransferase. Another acyl-CoA-independent mechanism for TAG synthesis is represented by the transacylase reaction converting two diacylglycerol molecules to TAG and monoacylglycerol. However, enzymes catalyzing this type of reaction seem to have evolved during later periods, because TAG synthesis via this mechanism has only been described for higher eukaryotes, e.g., mammals and plants (Buhman et al. 2002; Lung and Weselake 2006), but not for yeast.

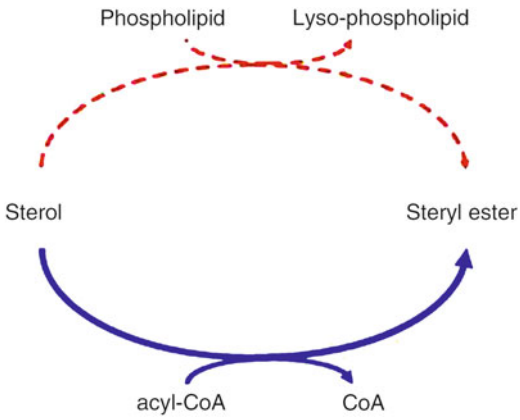


Fig. 3 Pathways leading to the formation of steryl esters. In yeast, steryl esters (*SE*) are formed by an acyl-CoA-dependent reaction (*lower arrow*). A second mechanism for SE formation which is restricted to higher eukaryotes uses a phospholipid as acyl donor (*upper arrow*, *broken line*)

2.2 Synthesis of Steryl Esters

Steryl esters are not only formed as a sterol backup required for membrane formation when nutrients are no longer provided by the environment, but also to maintain an equilibrated sterol level within the cell. Because sterols are important membrane components which modulate the physical properties of a bilayer, both excess and lack of free sterols are detrimental for the cell. In principal, similar to TAG also SE can be formed either by an acyl-CoA-dependent or an acyl-CoA-independent reaction (Fig. 3). However, the latter mechanism is restricted to higher eukaryotes (mammals and plants) and has not been described for yeast. Thus, in yeast the acylation of the hydroxyl group of ergosterol and its precursors requires the presence of acyl-CoA.

3 Lipid Droplets

3.1 Structure of Lipid Droplets

As substantial amounts of nonpolar lipids, mainly TAG and SE, cannot be incorporated into biomembranes, these lipids are sequestered in special compartments known as lipid droplets (lipid particles). These storage compartments consist of a hydrophobic core which is encompassed from the cytosolic environment by a phospholipid monolayer. Few proteins embedded into the monolayer comprise the third component of lipid droplets (Fig. 4).

The nonpolar lipid composition of the hydrophobic core of lipid droplets strongly depends on the source of this compartment. The ratio of TAG to SE differs not only between the different kingdoms of life, but also between different yeast species. In the budding yeast *Saccharomyces cerevisiae*, nearly equal amounts of TAG and SE are forming the hydrophobic core of lipid droplets (Leber et al. 1994; Grillitsch et al. 2011). In marked contrast, TAG are the main component of the respective

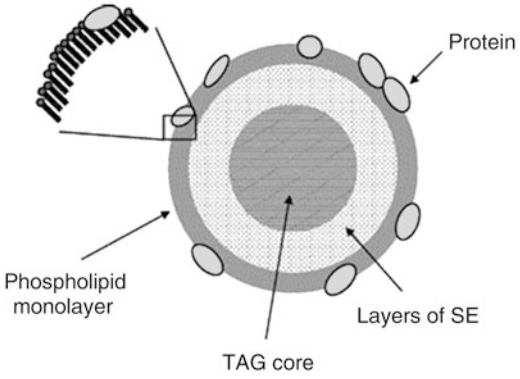


Fig. 4 Model of a lipid droplet. The inner center of the hydrophobic core of a lipid droplet is formed of triacylglycerols (*TAG*) which is circumvented by several layers of steryl esters (*SE*) and a final phospholipid monolayer. Few proteins embedded into the monolayer comprise the third component of lipid droplets

counterparts in the oleaginous yeast *Yarrowia lipolytica* (Athenstaedt et al. 2006) as well as in the methylotrophic yeast *Pichia pastoris* (Ivashov et al. 2013). Thus, lipid droplets of the latter yeast species rather resemble the respective cell compartments in adipocytes, equally storing mainly TAG, than that of the budding yeast. Structural investigations of lipid droplets from *Saccharomyces cerevisiae* revealed that TAG and SE are not randomly distributed in the hydrophobic core of the droplet but well organized (Czabany et al. 2008). Directly beneath the phospholipid monolayer, there are several layers of SE further sheltering an inner core of TAG. Similar to the ratio of TAG to SE forming the hydrophobic core, also size and number of lipid droplets vary and depend on the source. The average diameter of lipid droplets from wild-type *Saccharomyces cerevisiae* cells ranges between 0.3 and 0.4 μm, although also few larger droplets with a diameter of 1.2–1.6 μm have been observed (Leber et al. 1994). Comparison of the lipid droplet size between the oleaginous yeast *Yarrowia lipolytica* and the budding yeast grown under the same conditions reveals a twofold bigger size of the droplets in the former yeast. By shifting *Yarrowia lipolytica* cells to oleic acid-containing medium, the mean diameter of lipid droplets increases from 0.65 to 2.5 μm. In parallel to the size, also the nonpolar lipid composition and protein content changes (Athenstaedt et al. 2006). Alterations in growth conditions similarly affect the composition of lipid droplets from the budding yeast *Saccharomyces cerevisiae* (Grillitsch et al. 2011). These observations demonstrate a major influence of environmental conditions on lipid droplets. However, even though lipid droplets of different sources and formed under different conditions vary in nonpolar lipid composition and size, the total amount of lipids always constitutes more than 95% of the droplet weight with the remaining portion representing proteins.

3.2 Models of Lipid Droplet Biogenesis

It is well established that lipid droplets emerge from the endoplasmic reticulum (ER), which is the site of the ultimate reactions in nonpolar lipid synthesis. However, the exact mechanism leading from the formation of nonpolar lipids in the ER to a mature

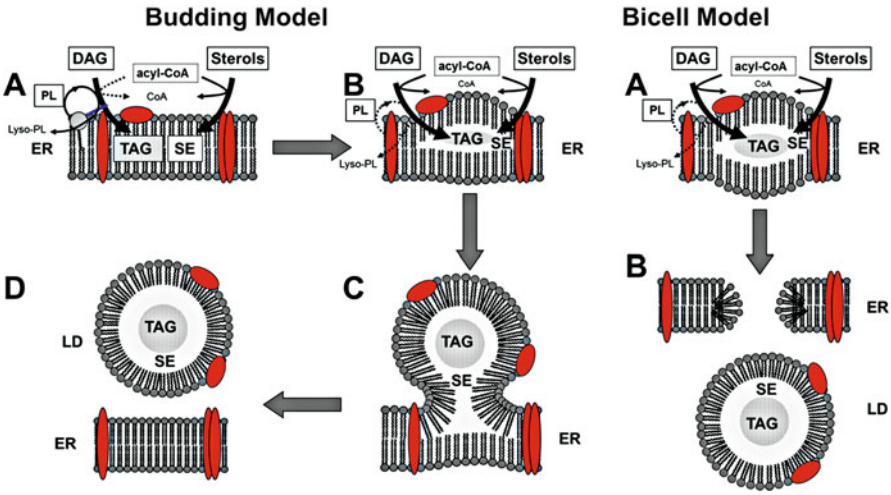


Fig. 5 Models for lipid droplet biogenesis. Budding model (*left*): (**A**) phospholipids (*PL*) or acyl-CoA serves as a fatty acid donor for diacylglycerol (*DAG*) acylation yielding triacylglycerols (*TAG*). In yeast, steryl esters (*SE*) are exclusively formed in the presence of acyl-CoA. Both nonpolar lipids do not fit into the phospholipid bilayer and are thus deposited between the two leaflets of the bilayer (**B**). Ongoing nonpolar lipid synthesis leads to the formation of a bud (**C**), which either remains associated to the ER by a phospholipid membrane bridge or buds off of the endoplasmic reticulum (*ER*) as a mature lipid droplet (*LD*) after reaching the critical size (**D**). Bicell model (*right*): (**A**) similar to the budding model, nonpolar lipids accumulate between the two leaflets of the ER and form a microdroplet. However, after reaching a certain size, the membrane region sheltering the nonpolar lipid cluster is excised forming a mature lipid droplet (**B**)

lipid droplet is still elusive. Currently, the most accepted model for lipid droplet biogenesis is the so-called budding model (Fig. 5, left).

The budding model proposes that nonpolar lipid-forming enzymes reside in the ER cluster in certain regions of this compartment and catalyze the formation of TAG and SE (Fig. 5A, left). Because substantial amounts of these nonpolar lipids cannot be incorporated into the phospholipid bilayer, they are deposited between the two leaflets of the bilayer which leads to the formation of a microdroplet (Fig. 5B, C, left). By ongoing synthesis and deposition of nonpolar lipids into the ER, the microdroplet grows in size and bulges out toward the cytosolic side of the membrane. As soon as a "critical" size is reached, this lipid-filled knob buds off as a mature lipid droplet (Fig. 5D, left) or may remain connected to the ER by a membrane bridge (Fig. 5C, left). Of note, as according to this model the droplet buds to the cytosolic side the phospholipid monolayer surrounding the hydrophobic core is exclusively formed from the outer leaflet of the ER membrane.

A related mechanism of lipid droplet biogenesis is proposed by the so-called bicell model (Ploegh 2007). Again nonpolar lipids formed by ER-resident enzymes are first deposited between the two leaflets of the ER which leads to the formation of a microdroplet (Fig. 5A, right). However, instead of forming an asymmetric knob, the microdroplet gets excised from the ER resulting in a mature lipid droplet

(Fig. 5B, right). In this case, the phospholipid monolayer circumventing the hydrophobic core of the droplet is formed from both leaflets of the ER membrane, which is in clear contrast to the budding model described above.

A third but rather controversial model of lipid droplet biogenesis is the vesicular-budding model (Walther and Farese 2008). This model suggests that nonpolar lipids are first deposited into a vesicle which is surrounded by a phospholipid bilayer. By subsequent rearrangements, the bilayer membrane of the vesicle is converted into the single membrane leaflet which finally encompasses the nonpolar lipid core of the mature lipid droplet.

4 Degradation of Triacylglycerols and Steryl Esters

It would not make sense if nonpolar lipids once formed to store the surplus of nutrients could not be mobilized upon requirement. Thus, hydrolytic enzymes catalyzing the degradation of TAG and SE exist. The breakdown products of TAG are diacylglycerol and a fatty acid. These two molecules serve either as building blocks for membrane lipid formation or as signaling molecules. In addition, fatty acids can be degraded by β-oxidation supplying the cell with energy, and complete hydrolysis of diacylglycerol yields glycerol and two more fatty acids. Sterols which are set free upon hydrolysis of SE can be directly or after further conversion incorporated into membranes. Furthermore, like fatty acids and diacylglycerols, also sterols have the capacity to act as signaling molecules. The fatty acid simultaneously yielded by SE breakdown can be again channeled either into anabolic or catabolic processes. Enzymes catalyzing the hydrolysis of SE are so-called SE hydrolases, and TAG lipases catalyze the degradation of TAG.

5 Key Knowledge Gaps and Research Needs

Although most recently several groundbreaking observations immensely contributed to our current understanding of lipid droplet biogenesis, still many aspects of this process require further investigation.

By using a mutant containing an inducible system for TAG synthesis, it was shown that indeed in the initial phase of lipid droplet biogenesis, TAG accumulate between the two leaflets of the ER membrane (Choudhary et al. 2015). Interestingly, these TAG clusters are not randomly distributed over the entire ER membrane network, but are restricted to a region of the ER contiguous to the nucleus (Wolinski et al. 2015; Choudhary et al. 2015). This observation raises the question: what distinguishes this “lipid droplet emergence region” from the rest of the ER? One major difference may be in the glycerophospholipid composition, because the phospholipid pattern of the monolayer of lipid droplets is markedly different from that of the bulk of the ER. In addition, not only the ratio of glycerophospholipid classes may be different in this region of lipid droplet biogenesis but also the glycerophospholipid species. A specific fatty acid profile in this region may be

required to allow partitioning of the two leaflets of the ER membrane to host the nonpolar lipid cluster formed in the initial phase of lipid droplet biogenesis. The second major question concerning the nature of the region of lipid droplet biogenesis is: which proteins localize in this specific region? The knowledge about the identity of the polypeptides at this site may immensely contribute to a better understanding of lipid droplet biogenesis, because most likely these polypeptides play a prominent role in this process. So far, some proteins involved in lipid droplet formation were already shown to localize to the region of lipid droplet emergence. For instance, Fld1p and Ldb16p (Wang et al. 2014; Wolinski et al. 2015; Grippa et al. 2015), two proteins required for proper lipid droplet formation, have been detected at this specific site. Similarly Nem1p, the catalytic unit of a protein phosphatase which activates the phosphatidate phosphatase Pah1p, a key enzyme in TAG synthesis, was found adjacent to lipid droplets (Adeyo et al. 2011). Which proteins involved in (nonpolar) lipid turnover are similarly present in this area of lipid droplet emergence remains to be shown.

As mentioned above, evidence is already available that lipid droplet biogenesis starts with the accumulation of nonpolar lipids between the two leaflets of the ER membrane (Choudhary et al. 2015). However, the next steps in lipid droplet biogenesis are still elusive. Is it indeed a budding process as proposed by the respective model? Does the mature lipid droplet remain physically connected to the ER by a phospholipid membrane bridge? Another open question concerns the mechanism how lipid droplets acquire their specific set of proteins. One possibility is that proteins localized to the specific region of lipid droplet emergence reach the surface of the droplet during the proposed budding process. In this case, the lipid droplet proteome would be exclusively formed of proteins which are initially present at the respective site. Alternatively, helper proteins may be required to transfer selected proteins to the surface of the droplet. This speculation is based on the observation that the retrograde transport of the lipid droplet protein Dga1p from the droplet to the ER depends on the ER protein Ice2p (Markgraf et al. 2014).

Another aspect of lipid droplet formation which remains to be elucidated is why lipid droplets of different sizes are observed within one cell. One possible explanation is that the size of the droplet is determined by the moment of budding from the ER. Alternatively, lipid droplets of similar size may emerge from the ER and subsequently increase their diameters by incorporating additional nonpolar lipids, thus leading to small “young” and huge “old” droplets. The incorporation of additional material into the hydrophobic core of lipid droplets may occur, e.g., via a potential “membrane bridge” between the droplet and the ER and/or by de novo synthesis of lipids on the droplet. The speculation that the size of a droplet increases through de novo synthesis of nonpolar lipids catalyzed by lipid droplet-resident enzymes is supported by the fact that the budding yeast contains the whole set of enzymes required for de novo synthesis of TAG except a phosphatidate phosphatase. However, a phosphatidate phosphatase is present in the cytosol and has therefore access to its substrate PA formed by enzymes localized to the lipid droplet. Furthermore, the set of TAG-synthesizing enzymes may also provide a means to compensate random TAG degradation which may occur as the lipid droplet proteome

contains also several nonpolar lipid-degrading enzymes (see also ▶ Chap. 27, "Players in the Nonpolar Lipid Game: Proteins Involved in Nonpolar Lipid Metabolism in Yeast", this volume).

In brief, even though at first sight our knowledge about different mechanisms contributing to the formation and maintenance of lipid droplets in yeast seems to be complete, several aspects remain to be elucidated. One of the biggest challenges in the future will be unraveling the enigma of lipid droplet biogenesis. Especially the identification of the key players controlling lipid droplet biogenesis will immensely contribute to a better understanding of nonpolar lipid turnover not only in yeast but also in higher eukaryotes.

For further information about nonpolar lipid metabolism, the interested reader is referred to (see also ▶ Chap. 27, "Players in the Nonpolar Lipid Game: Proteins Involved in Nonpolar Lipid Metabolism in Yeast", this volume) describing polypeptides involved in nonpolar lipid metabolism in yeast and some recent reviews covering specifically the topic of nonpolar lipid metabolism in the model organism yeast (Athenstaedt and Daum 2011; Koch et al. 2014; Wang 2015).

Acknowledgment This work was financially supported by the Austrian Science Fund (FWF) project P26308 to K. A.

References

Adeyo O, Horn PJ, Lee S, Binns DD, Chandrahas A, Chapman KD, Goodman JM (2011) The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. J Cell Biol 192:1043–1055

Athenstaedt K, Daum G (1999) Phosphatidic acid, a key intermediate in lipid metabolism. Eur J Biochem 266:1–16

Athenstaedt K, Daum G (2011) Lipid storage: yeast we can! Eur J Lipid Sci Technol 113: 1188–1197

Athenstaedt K, Jolivet P, Boulard C, Zivy M, Negroni L, Nicaud J-M, Chardot T (2006) Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. Proteomics 6:1450–1459

Buhman KK, Smith SJ, Stone SJ, Repa JJ, Wong JS, Knapp FF Jr, Burri BJ, Hamilton RL, Abumrad NA, Farese RV Jr (2002) DGAT1 is not essential for intestinal triacylglycerol absorption or chylomicron synthesis. J Biol Chem 277:25474–25479

Choudhary V, Ojha N, Golden A, Prinz WA (2015) A conserved family of proteins facilitates nascent lipid droplet budding from the ER. J Cell Biol 211:261–271

Czabany T, Wagner A, Zweytick D, Lohner K, Leitner E, Ingolic E, Daum G (2008) Structural and biochemical properties of lipid particles from the yeast *Saccharomyces cerevisiae*. J Biol Chem 283:17065–17074

Grillitsch K, Connerth M, Köfeler H, Arrey TN, Rietschel B, Wagner B, Karas M, Daum G (2011) Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim Biophys Acta 1811:1165–1176

Grippa A, Buxó L, Mora G, Funaya C, Idrissi FZ, Mancuso F, Gomez R, Muntanyà J, Sabidó E, Carvalho P (2015) The seipin complex Fld1/Ldb16 stabilizes ER-lipid droplet contact sites. J Cell Biol 211:829–844

Henry SA, Kohlwein SD, Carman GM (2012) Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. Genetics 190:317–349

Ivashov VA, Grillitsch K, Köfeler H, Leitner E, Bäumlisberger D, Karas M, Daum G (2013) Lipidome and proteome of lipid droplets from the methylotrophic yeast *Pichia pastoris*. Biochim Biophys Acta 1831:282–290

Kent C (1995) Eukaryotic phospholipid biosynthesis. Annu Rev Biochem 64:315–343

Koch B, Schmidt C, Daum G (2014) Storage lipids of yeast: a survey of nonpolar lipid metabolism in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*. FEMS Microbiol Rev 38:892–915

Leber R, Zinser E, Zellnig G, Paltauf F, Daum G (1994) Characterization of lipid particles of the yeast, *Saccharomyces cerevisiae*. Yeast 10:1421–1428

Lung SC, Weselake RJ (2006) Diacylglycerol acyltransferase: a key mediator of plant triacylglycerol synthesis. Lipids 41:1073–1088

Markgraf DF, Klemm RW, Junker M, Hannibal-Bach HK, Ejsing CS, Rapoport TA (2014) An ER protein functionally couples neutral lipid metabolism on lipid droplets to membrane lipid synthesis in the ER. Cell Rep 6:44–55

Ploegh H (2007) A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. Nature 448:435–438

Walther TC, Farese RV Jr (2008) The life of lipid droplets. Biochim Biophys Acta 1791:459–466

Wang CW (2015) Lipid droplet dynamics in budding yeast. Cell Mol Life Sci 72:2677–2695

Wang CW, Miao YH, Chang YS (2014) Control of lipid droplet size in budding yeast requires the collaboration between Fld1 and Ldb16. J Cell Sci 127:1214–1228

Wolinski H, Hofbauer HF, Hellauer K, Cristobal-Sarramian A, Kolb D, Radulovic M, Knittelfelder OL, Rechberger GN, Kohlwein SD (2015) Seipin is involved in the regulation of phosphatidic acid metabolism at a subdomain of the nuclear envelope in yeast. Biochim Biophys Acta 1851:1450–1464

Modeling Lipid Metabolism in Yeast

20

Eduard J. Kerkhoven

Contents

1 Introduction 376
2 Genome-Scale Metabolic Models 376
2.1 Generation of Genome-Scale Model 376
2.2 Computational Analysis 377
2.3 Omics Data Analysis 379
3 Models of Lipid Metabolism in Yeast 380
3.1 *Saccharomyces cerevisiae* 380
3.2 Oleaginous Yeasts 383
4 Use of Yeast Models 384
4.1 Target Prediction 384
4.2 Omics Data Analysis 385
5 Research Needs 386
References 387

Abstract

The various pathways and mechanisms behind fatty acid, lipid, and membrane biosynthesis together form a complex network. Moreover, lipid metabolism does not operate in isolation but rather functions in the context of a whole cell, surrounded by all its other metabolic pathways, a situation that results in additional connectivity and complexity. Computational models can aid to provide understanding of these complex networks and to make sense of interactions on a whole cell or genomic scale. In particular, these models have proven to be valuable to answer biotechnological questions, such as how to increase the

E. J. Kerkhoven (✉)
Systems and Synthetic Biology, Department of Biology and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden
e-mail: eduardk@chalmers.se

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_9

biosynthesis of fatty acids. This chapter discusses the development and usage of genome-scale metabolic models in the light of lipid biosynthesis. A special focus is placed on baker's yeast *Saccharomyces cerevisiae* and the oleaginous yeast *Yarrowia lipolytica* and the use of genome-scale metabolic models to answer biotechnological questions.

1 Introduction

Cellular metabolism forms a complex network, where many metabolic pathways are functioning through enzymes that perform many different catalytic functions. All metabolic pathways are interconnected, by branching off each other, producing the same products or consuming the same substrates, or by using similar cofactors such as ATP and NADH. In order to gain understanding of these complex and highly connected networks, computational models can aid as valuable tools. A recurrent approach to modeling of metabolism is constraint-based modeling (Price et al. 2004), where metabolic fluxes are predicted from the stoichiometries of the metabolic reactions. When the genome is available of an organism of interest, one can identify all enzymes that make up the metabolic network – depending on the quality of the annotation – and generate a genome-scale constraint-based model.

Also the metabolic pathways constituting lipid biosynthesis are part of the complex network of cellular metabolism, connecting to other parts of metabolism through, e.g., precursors and cofactors such as acetyl-CoA and NADPH. By investigating lipid biosynthesis in the genome-scale context, rather than studying it in isolation, we preserve the functional interactions that take place between lipid biosynthesis and the remainder of cellular metabolism.

2 Genome-Scale Metabolic Models

2.1 Generation of Genome-Scale Model

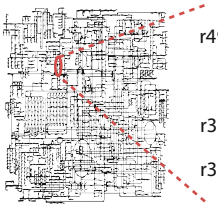
Genome-scale metabolic models (GEMs) describe the whole metabolic network that is present in a particular cell and as such represents its metabolic capabilities: what reactions it can perform, which substrates it can use, and which by-products it creates. As the majority of the metabolic reactions are catalyzed by enzymes, knowledge about all the enzymes encoded in the organism's genome is imperative to generate a new GEM. From the genome, it can be identified what proteins are present and what chemical reactions these proteins can catalyze (Fig. 1). These reactions together constitute a network, where substrates and products are nodes and reactions are edges. In addition to reactions identified from the genome sequence, unannotated and spontaneous reactions are included to generate a fully connected metabolic network. A wide range of computational tools have been developed that can assist in the construction of a GEM, such as SEED (Henry et al. 2010), RAVEN (Agren et al. 2013), and CoReCo (Pitkänen et al. 2014);

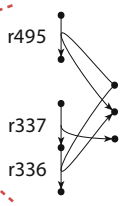
Genome sequence

Determine GPR relationships

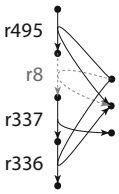
ID	Reaction equation	Gene association
r495	glycerol 3-phosphate + acyl-CoA → lyso-phosphatidic acid + CoA	GPT2 or SCT1
r8	lyso-phosphatidic acid + acyl-CoA → phosphatidic acid + CoA	ALE1 or SLC1 or TGL4 or TGL5
r337	phosphatidate + H_2O → diacylglycerol + phosphate	DPP1 or LPP1 or PAH1
r336	diacylglycerol + acyl-CoA → triacylglycerol + CoA	ARE1 or ARE2 or DGA1

Draft model





Gap-filling & manual curation



S-matrix

$$S = \begin{bmatrix} 0 & 0 & 0 & -1 \\ -1 & -1 & 0 & -1 \\ 1 & 1 & 0 & 1 \\ -1 & 0 & 0 & 1 \\ 1 & 0 & -1 & 0 \\ 0 & -1 & 1 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & -1 & 0 \end{bmatrix}$$

Fig. 1 Schematic overview of a GEM reconstruction. The genome sequence is used to infer gene-protein-reaction (GPR) relationships. Here, the triacylglycerol biosynthetic pathway is highlighted. From these relationships, a draft model can be constructed. This model is typically not a functional model. If, for whatever reason, our draft model reconstruction would have failed to annotate r8 in this example (highlighted in *gray*), this would introduce a gap in the network. Gap-filling and manual curation allow to generate a fully functional model, capable of supporting a flux through all of its reactions. This metabolic network can be represented as an S-matrix for further computational analysis

however, the generation of a high-quality genome-scale model remains an iterative process requiring intensive manual curation. The process of model construction is described in detail here (Thiele and Palsson 2010).

While the genome is used to reconstruct the metabolic network by identifying what reactions are present, also the association between genes, proteins, and reactions is stored as gene-protein-reaction (GPR) relationships. Among other things, these GPR relationships facilitate the integration of omics-level datasets with the GEM, as discussed in more detail below. The metabolic network itself can be represented in a stoichiometric matrix, where the coefficients correspond to the reaction coefficients (Fig. 1). This combination of an S-matrix and GPR relationships renders the GEM ready for further analysis.

2.2 Computational Analysis

The constraint-based modeling approach has given rise to a wide range of algorithms (Lewis et al. 2012). Underlying constraint-based modeling is the quasi-steady-state assumption (QSSA), which specifies that the system is in a state where the intracellular metabolite levels and metabolic fluxes are not changing (Fig. 2a). The QSSA can be assumed because metabolic rates are typically much faster than other cellular processes, such as cell division or depletion of nutrients from the environment.

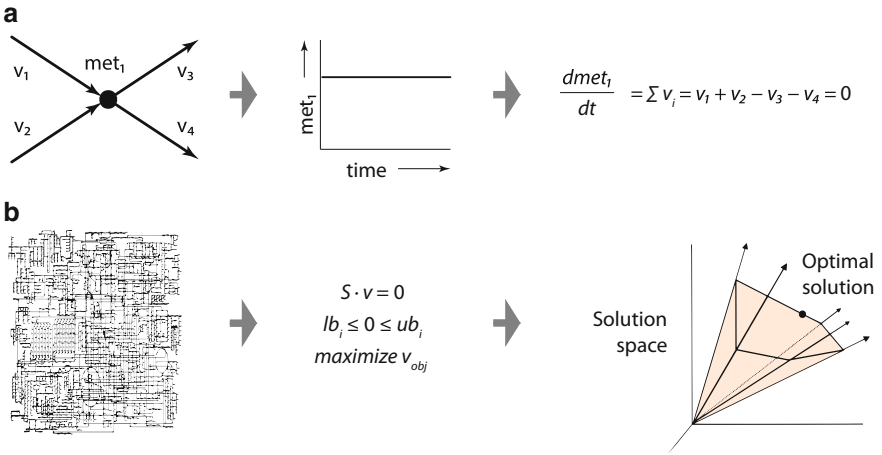


Fig. 2 Quasi-steady-state assumption and flux balance analysis. (**a**) According to the quasi-steady-state assumption (QSSA), the concentration of met_1 is constant over time, which signifies that the fluxes sum up to zero. (**b**) For flux balance analysis, the QSSA assumption is valid over the whole S-matrix. Additional constraints include lower and upper boundaries for each reaction, further restricting the solution space. Optimization for an objective function is then used to identify a possible optimal solution

Specifically, cells growing exponentially in a continuous culture can readily be presumed to be at steady state, but some caution is recommended when conditions are changing, such as during a batch fermentation. Notwithstanding, the QSSA allows balancing of metabolic fluxes in a network: if metabolite levels are constant, then the sum of the fluxes going through each metabolite has to sum up to zero (Fig. 2a). In addition to the S-matrix, upper and lower boundaries can be set for each reaction, e.g., based on experimentally determined uptake rates of nutrients, and reaction reversibility (Price et al. 2004). Methods have been developed to include additional constraints, such as the total cellular volume occupied by enzymes (Beg et al. 2007), or their kinetic efficiencies (Chakrabarti et al. 2013), but these will not be discussed here. Notwithstanding the sophistication of the applied constraints, it is now possible to determine how the flux is distributed through the metabolic network by balancing the fluxes, a method called flux balance analysis (Orth et al. 2010).

A complication with flux balance analysis (FBA) is that the metabolic network is typically large and of high complexity. This implies that instead of one unique solution, the metabolic fluxes can be balanced in numerous ways, all abiding to the same flux balancing rules and constraints that are imposed. Rather than one solution, a solution *space* can be defined (Fig. 2b). While all of the flux distributions within this *space* are feasible, not all are evenly likely. To select the *most likely* flux distribution, an objective function needs to be defined, and the most optimal flux distribution for this objective can then be calculated. Evolutionarily, it can be argued that microorganisms have evolved to grow as fast as possible in order to outcompete other microorganisms that compete for the same nutrients, and biomass production is

therefore often selected as an objective function in models of microorganisms. With growth as an objective function, it becomes important to describe the constitutive components of the organism's biomass. It is worth noting that this equation will change from species to species, but potentially also from condition to condition (Feist and Palsson 2010). Other often used objective functions are ATP generation and product formation, while a combination of different objective functions seems most suitable to describe microbial metabolism (Schuetz et al. 2012).

Even when an objective function has been selected, there are typically still a wide range of flux distributions that all abide by the same constraints and satisfy the same objective. This can be analyzed by flux variability analysis (Mahadevan and Schilling 2003) and random sampling (Bordel et al. 2010), two methods that attempt to describe all possible flux values given the constraints and objective function. From this, mean and standard deviations can be described for each reaction, providing an overview of likely metabolic fluxes in a particular condition. The importance of exploring the solution space through such methods should be stressed, identifying whether fluxes change solely based on single FBA simulations ignores the remainder of the solution space, full of equally valid flux distributions.

Now able to estimate metabolic fluxes and their uncertainty, the power of in silico modeling is that such calculations can be done extremely rapidly in a computer. This opens the way to in silico experimentation, determining the effects of changes in the metabolic network that would otherwise take days to weeks to implement in the lab. Rather, the GEM can be queried for thousands of different changes, in less than a minute. For instance, this allows the GEM to predict targets for metabolic engineering, and for this various algorithms have been developed.

A classical approach predicting metabolic engineering targets is OptKnock (Burgard and Maranas 2003), where in silico gene knockouts are performed to identify their effect on a bioengineering objective, such as increased production of a compound of interest. In silico gene knockouts can easily be implemented in the GEM by restricting the flux through the associated reaction(s) to zero. With OptKnock, solutions are found that are maximizing both the cell growth and production of target compound. From this principle, a wide array of different optimization methods has been developed to aid strain design in metabolic engineering (Lewis et al. 2012).

2.3 Omics Data Analysis

As a GEM does not only contain a description of the network stoichiometry but also GPR relationships, GEMs can readily be used as scaffolds for omics data analysis. Changes in transcript and protein levels, determined through transcriptomics and proteomics techniques, are representing regulatory changes in the cell, and the GPR relationships allow to compare these regulatory changes with changes in metabolic fluxes that are estimated from FBA. Correlation between different omics levels and metabolic fluxes, or the absence of correlation, is then indicative of whether a

reaction is regulated on a transcriptional, posttranscriptional, or metabolic level (Bordel et al. 2010).

In an alternative approach, the network stoichiometry is used to identify so-called reporter metabolites (Patil and Nielsen 2005). Given a set of differentially expressed genes, and their GPR relationship to a metabolic network, metabolites can be identified around which the most significant transcriptional changes occur. Metabolites themselves can also be used as the input for analysis in the context of the metabolic network. The Kiwi package is capable to take a list of metabolites, which, for instance, have been measured to differ between experimental conditions, and combine this list with a GEM in order to extract an interaction network between these metabolites (Väremo et al. 2014). Various other methods have been developed to integrate omics data with GEMs (Sánchez and Nielsen 2015).

3 Models of Lipid Metabolism in Yeast

3.1 *Saccharomyces cerevisiae*

The first GEM for *S. cerevisiae* was published by Förster et al. (2003). This model, iFF708, contained information from 708 genes and a total of 1,175 reactions. In the years following, this model has been updated and improved in a series of models, a process that is discussed in detail here (Heavner and Price 2015). A major improvement was the inclusion of a more detailed description of lipid metabolism in iIN800 (Nookaew et al. 2008). This improvement increased the number of genes to 800 and the number of reactions to 1,446 and was primarily done through an extensive manual curation of the lipid metabolic pathways using various online databases and research papers. Specifically, iIN800 introduced mitochondrial fatty acid synthesis, fatty acid elongation, and updated beta-oxidation, sphingolipid biosynthesis, and biosynthesis of phospholipids, triacylglyceroles, and ergosterol. As such, with iIN800 it was now possible to query yeast lipid metabolism using a genome-scale model.

Development of yeast GEMs has not remained idle after this. In an attempt to combine the various modeling efforts and to assure consistency between future models, a consensus metabolic network was created: Yeast 1 (Herrgård et al. 2008). Again, this model was improved in various updates, while the latest major expansion in lipid metabolism was obtained with the introduction of Yeast 7 (Aung et al. 2013). In previous models, fatty acids and acyl-CoAs of different lengths and saturation were pooled, according to experimentally measured ratios, implemented in various ways in different model versions (Table 1). With Yeast 7, each unique acyl group, regarding length and saturation, was modeled explicitly. This has resulted in a massive expansion of the description of lipid metabolism. As example, where diacylglycerol acyltransferase used to be modeled as one reaction in the form of diacylglycerol + acyl-CoA → triacylglycerol + CoA, these are now modeled with 32 reactions in the form of palmitoyl-CoA + diglyceride (1-16:0, 2-16:1) → triglyceride (1-16:0, 2-16:1, 3-16:0) + CoA (Table 1). Nonetheless, all 32 reactions are still

Table 1 Evolution of lipid metabolism in *S. cerevisiae* genome-scale models. For a number of models is indicated how the last step of triacylglycerol biosynthesis is described. A increased complexity is observed with newer models. ACP: acyl-carrier protein; l: lipid droplet; r: endoplasmic reticulum. Square brackets indicate localization

Model	Reaction	Equation	Gene
iFF708	U69_	Diacylglycerol + 0.017000 Decanoyl-[ACP] + 0.062000 Dodecanoyl-[ACP] + 0.100000 Myristoyl-[ACP] + 0.270000 Hexadecanoyl-[ACP] + 0.169000 Palmitoyl-[ACP] + 0.055000 Stearoyl-[ACP] + 0.235000 Oleoyl-[ACP] + 0.093000 Linolenoyl-[ACP] → Triacylglycerol + ACP	
iIN800	DGA1	Acyl-CoAs + diacylglycerol → triacylglycerol + CoA	YOR245C
	Pool_Acyl2	0.008290 Decanoyl-CoA + 0.008277 Dodecanoyl-CoA + 0.019114 Tetradecanoyl-CoA + 0.005707 Tetradecanoyl-9-ene-CoA + 0.154233 Hexadecanoyl-CoA + 0.604950 Hexadecanoyl-9-ene-CoA + 0.037531 Octadecanoyl-CoA + 0.161896 Octadecanoyl-9-ene-CoA → Acyl-CoAs	
Yeast 6	336	Acyl-CoA[l] + diglyceride[l] ↔ coenzyme A[l] + 4 H +[l] + triglyceride[l]	YOR245C
	1282	L-3-hydroxyhexacosanoyl-CoA[r] → acyl-CoA[r]	
	1283	L-3-hydroxypalmitoyl-CoA[r] → 0.78723 acyl-CoA[r]	
	1284	3-hydroxyoctadecanoyl-CoA[r] → 0.82979 acyl-CoA[r]	
	1285	L-3-hydroxytetradecanoyl-CoA[r] → 0.74468 acyl-CoA [r]	
	1288	3-oxohexacosanoyl-CoA[r] → acyl-CoA[r]	
	1289	3-oxopalmitoyl-CoA[r] → 0.78723 acyl-CoA[r]	
	1290	3-oxooctadecanoyl-CoA[r] → 0.82979 acyl-CoA[r]	
	1291	3-oxotetradecanoyl-CoA[r] → 0.74468 acyl-CoA[r]	
	1298	Hexacosanoyl-CoA[r] → acyl-CoA[r]	
	1302	Oleoyl-CoA[r] → 0.82979 acyl-CoA[r]	
	1305	Palmitoyl-CoA[r] → 0.78723 acyl-CoA[r]	
	1310	Tetracosanoyl-CoA[r] → 0.95745 acyl-CoA[r]	
	1356	Palmitoleoyl-CoA[r] → 0.78723 acyl-CoA[r]	
Yeast 7[a]	2400	Palmitoyl-CoA[l] + diglyceride (1–16:0, 2–16:1)[l] → coenzyme A[l] with [l] + triglyceride (1–16:0, 2–16:1, 3–16:0)[l]	YOR245C
	2401	Palmitoyl-CoA[l] + diglyceride (1–16:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:0, 2–18:1, 3–16:0)[l]	YOR245C
	2402	Palmitoyl-CoA[l] + diglyceride (1–16:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–16:1, 3–16:0)[l]	YOR245C
	2403	Palmitoyl-CoA[l] + diglyceride (1–16:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–18:1, 3–16:0)[l]	YOR245C
	2404	Palmitoyl-CoA[l] + diglyceride (1–18:0, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–16:1, 3–16:0)[l]	YOR245C
	2405	Palmitoyl-CoA[l] + diglyceride (1–18:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–18:1, 3–16:0)[l]	YOR245C
	2406	Palmitoyl-CoA[l] + diglyceride (1–18:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–16:1, 3–16:0)[l]	YOR245C

(*continued*)

Table 1 (continued)

Model	Reaction	Equation	Gene
	2407	Palmitoyl-CoA[l] + diglyceride (1–18:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–18:1, 3–16:0)[l]	YOR245C
	2408	Palmitoleoyl-CoA[l] + diglyceride (1–16:0, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–16:0, 2–16:1, 3–16:1)[l]	YOR245C
	2409	Palmitoleoyl-CoA[l] + diglyceride (1–16:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:0, 2–18:1, 3–16:1)[l]	YOR245C
	2410	Palmitoleoyl-CoA[l] + diglyceride (1–16:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–16:1, 3–16:1)[l]	YOR245C
	2411	Palmitoleoyl-CoA[l] + diglyceride (1–16:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–18:1, 3–16:1)[l]	YOR245C
	2412	Palmitoleoyl-CoA[l] + diglyceride (1–18:0, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–16:1, 3–16:1)[l]	YOR245C
	2413	Palmitoleoyl-CoA[l] + diglyceride (1–18:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–18:1, 3–16:1)[l]	YOR245C
	2414	Palmitoleoyl-CoA[l] + diglyceride (1–18:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–16:1, 3–16:1)[l]	YOR245C
	2415	Palmitoleoyl-CoA[l] + diglyceride (1–18:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–18:1, 3–16:1)[l]	YOR245C
	2416	Stearoyl-CoA[l] + diglyceride (1–16:0, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–16:0, 2–16:1, 3–18:0)[l]	YOR245C
	2417	Stearoyl-CoA[l] + diglyceride (1–16:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:0, 2–18:1, 3–18:0)[l]	YOR245C
	2418	Stearoyl-CoA[l] + diglyceride (1–16:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–16:1, 3–18:0)[l]	YOR245C
	2419	Stearoyl-CoA[l] + diglyceride (1–16:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–18:1, 3–18:0)[l]	YOR245C
	2420	Stearoyl-CoA[l] + diglyceride (1–18:0, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–16:1, 3–18:0)[l]	YOR245C
	2421	Stearoyl-CoA[l] + diglyceride (1–18:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–18:1, 3–18:0)[l]	YOR245C
	2422	Stearoyl-CoA[l] + diglyceride (1–18:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–16:1, 3–18:0)[l]	YOR245C
	2423	Stearoyl-CoA[l] + diglyceride (1–18:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–18:1, 3–18:0)[l]	YOR245C
	2424	Oleoyl-CoA[l] + diglyceride (1–16:0, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–16:0, 2–16:1, 3–18:1)[l]	YOR245C
	2425	Oleoyl-CoA[l] + diglyceride (1–16:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:0, 2–18:1, 3–18:1)[l]	YOR245C
	2426	Oleoyl-CoA[l] + diglyceride (1–16:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–16:1, 3–18:1)[l]	YOR245C
	2427	Oleoyl-CoA[l] + diglyceride (1–16:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–16:1, 2–18:1, 3–18:1)[l]	YOR245C
	2428	Oleoyl-CoA[l] + diglyceride (1–18:0, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–16:1, 3–18:1)[l]	YOR245C

(*continued*)

Table 1 (continued)

Model	Reaction	Equation	Gene
	2429	Oleoyl-CoA[l] + diglyceride (1–18:0, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:0, 2–18:1, 3–18:1)[l]	YOR245C
	2430	Oleoyl-CoA[l] + diglyceride (1–18:1, 2–16:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–16:1, 3–18:1)[l]	YOR245C
	2431	Oleoyl-CoA[l] + diglyceride (1–18:1, 2–18:1)[l] → coenzyme A[l] + triglyceride (1–18:1, 2–18:1, 3–18:1)[l]	YOR245C

[a]Only shown for lipid particle, same reactions exist for endoplasmic reticulum.

annotated to the same gene DGA1, as they are still all catalyzed by the same enzyme. Yeast 7 implemented this extension of lipid metabolism in combination with further manual curation and fixing localization of various pathways. The result of this expansion is that Yeast 7 includes 3,493 reactions and 2,218 metabolites (cf. 1,882 and 1,454 for Yeast 6, prelipid expansion), while the number of genes has increased only from 901 to 916, mainly due to other alterations.

While this more expansive description of lipid metabolism is a more accurate representation of what the metabolic network looks like in vivo, the expansion can be argued to have limited benefit in silico. The expansion increases the complexity of the model, and knowledge about the specific composition of each lipid is often unknown. Rather, experimental measurements typically measure lipid compositions in classes such as triacylglycerols, phosphatidylinositols, and sterol esters, and fatty acid composition is determined after hydrolyzation of the whole sample, giving an overview of all fatty acids in the cell.

Furthermore, the expansion of lipid metabolism introduced many alternative pathways within lipid metabolism, which leads to a large expansion of the solution space, resulting in more uncertainty about fluxes calculated by FBA. Interestingly, inclusion of kinetics and protein level constraints on Yeast 7 has shown to result to large decreases in flux variability in lipid metabolism (Sanchez et al. submitted). Combining the lipid expansion with such constraints is therefore preferred.

3.2 Oleaginous Yeasts

While there has always been much interest on lipid biosynthesis of *S. cerevisiae* as a model organism, from a biotechnological perspective, there are other yeasts that are arguably more suitable for the production of oleochemicals. While *S. cerevisiae* lipid content ranges from 5% to 20% in an engineered strain, oleaginous yeasts accumulate over 20% in the right conditions, while lipid contents of over 70% are also observed. The ascomycete *Yarrowia lipolytica* has been studied as model oleaginous yeast and as a promising microbial cell factory for the production of oleochemicals (Ledesma-Amaro and Nicaud 2016). To aid the study into oleaginous lipid metabolism, GEMs have also been constructed for *Y. lipolytica*. The first GEM was built using the then-existing *S. cerevisiae* GEMs as scaffolds (Loira et al. 2012), and also

the *Y. lipolytica* GEMs have gone through a number of versions where improvements have been made. The most recent version (Kerkhoven et al. 2016) used Yeast 7 as a scaffold.

In addition to *Y. lipolytica*, GEMs have been generated for other oleaginous fungi, such as *Mortierella alpina* (Ye et al. 2015) and *Mucor circinelloides* (Vongsangnak et al. 2016). While all of these models are functional, capable of simulating the production of biomass and biosynthesis of lipids, a challenge with models of nonconventional yeasts is that the quality of their genome annotation is far lower than of model organisms such as *S. cerevisiae*. The functions of most of the genes in these organisms are solely annotated based on homology to other sequences, the presence of Pfam and other protein domains, and other computational approaches. In contrast, for *S. cerevisiae*, many of the enzymes have been characterized in vitro, generating proof of their catalytic capabilities. Due to the lower level of annotation, and the higher number of genes with unknown function in nonconventional yeasts, it is to be expected that the nonconventional yeast models are poorer representatives of reality in comparison to *S. cerevisiae* models. Annotated genes might have different substrate specificities, while unannotated genes might have uncharacterized and currently unrepresented catalytic capabilities. Notwithstanding these challenges in investigating lesser-studied organisms, GEMs of nonconventional yeasts such as *Y. lipolytica* have proven to be useful for further analysis, as detailed below.

4 Use of Yeast Models

Different yeast models have been used to investigate lipid biosynthesis using the different methods detailed above. While computational target prediction for metabolic engineering, such as with OptKnock, appears like a promising method to increase the microbial production of oleochemicals, there are de facto only a few examples of successful application. Rather, GEMs have been used to integrate omics data in investigations of lipid metabolism.

4.1 Target Prediction

The first *S. cerevisiae* model iFF708 was used to investigate how to increase the production of sesquiterpene, an isoprenoid that is produced in a competing pathway branching off ergosterol biosynthesis (Asadollahi et al. 2009). Previous strategies in increasing sesquiterpene production relied on the manipulation of classical targets within the mevalonate pathway that generates the precursor for isoprenoid biosynthesis. In contrast, Asadollahi et al. used the OptGene algorithm (Patil et al. 2005), an extension of OptKnock, to identify targets anywhere in the metabolic network that would increase sesquiterpene production. The intervention suggested by OptGene entailed deletion of glutamate dehydrogenase *GDH1*, which in silico resulted in a tenfold increase in sesquiterpene production alongside a 15% reduction in growth. *GDH1* catalyzes the reaction from alpha-ketoglutarate to glutamate, fixing ammonium

while using NADPH as a cofactor. In an alternative pathway, ammonium can be fixed via the two-step GS-GOGAT pathway, mediated by glutamine synthetase (GS) and glutamate synthase (GOGAT), using NADH as a cofactor. Knocking out *GDH1* redirects flux through the GS-GOGAT pathway, making NADPH available for other processes, such as NAPDH requiring reactions in the mevalonate pathway. Experimental confirmation of this approach indeed gave an 85% increase in final sesquiterpene titer.

Predicting interventions to increase product formation does not necessarily mean genetic engineering. For *Y. lipolytica*, analysis of batch fermentation data using a GEM gave rise to a hypothesis how lipid accumulation could be increased using a different feeding strategy (Kavšček et al. 2015). It was observed that in the end of the fermentation, when nitrogen had been depleted, the major product formed was citrate rather than lipids. Initially the model was only able to replicate this behavior if the citrate excretion was explicitly constrained to the measured rate. From this, it was hypothesized that lipid biosynthesis was at its maximum rate in these experimental conditions and any additional citrate that was formed but could not be used in lipid biosynthesis was rather excreted. While fixing the lipid biosynthesis rate in the model, the glucose uptake rate was decreased until citrate was no longer excreted. In silico, citrate excretion was abolished at a glucose uptake rate of 0.152 mmol $(g \cdot h)^{-1}$, while the previous experiment had a glucose uptake rate of 0.350 mmol $(g \cdot h)^{-1}$. Therefore, a fed-batch strategy was conceived, where the glucose feed was set to match the in silico calculated optimal feeding rate. Indeed, citrate excretion was completely abolished, while the TAG biosynthesis quadrupled to 0.203 g TAG per g glucose, whereas further increasing the glucose feeding rate reinstated citrate excretion.

4.2 Omics Data Analysis

The *S. cerevisiae* GEM iIN800 has been used to study the interaction of inositol-choline and Snf1 in controlling lipid metabolism (Chumnanpuen et al. 2012). In *S. cerevisiae*, many genes that are involved in lipid biosynthesis are under transcriptional control, and this has been known to be influenced by protein kinase Snf1, inositol-choline levels, and nutrient limitation. In this investigation, a multifactorial experimental setup was used to enquire the various interactions. The first factor was the level of inositol-choline, either high or low; the second factor was the absence (*snf1Δ*) and presence (WT) of SNF1; and the third factor was the presence of either carbon or nitrogen as limiting nutrient. Chemostat fermentations exploring all eight possible combinations of the three factors were performed and sampled for transcriptomics and lipid content. The transcriptomics data was integrated with iIN800, and the reporter metabolite analysis indicated where in the metabolic network the transcriptional regulation was concentrated, while reporter transcription factor analysis revealed that Snf1 and inositol-choline exert transcriptional control on lipid metabolism through transcription factors such as Ino2, Opi1, and Mga2.

In *Y. lipolytica*, the response of lipid accumulation to nitrogen limitation has been investigated by integrating transcriptomics data with the GEM iYali4 overexpressing *Y. lipolytica* strain, known to result in high lipid accumulation (Tai and Stephanopoulos

2013), was cultivated in both nitrogen-limiting and carbon limitation chemostats and sampled for transcriptomics and lipid content (Kerkhoven et al. 2016). Interestingly, while nitrogen limitation results in an increase in lipid accumulation, this was not mediated through an increased expression of lipid biosynthetic enzymes. Contrastingly, amino acid biosynthetic pathways and beta-oxidation were transcriptionally downregulated upon nitrogen limitation. A downregulation of amino acid biosynthesis can be rationalized as the cell attempting to reduce its nitrogen usage, while a lower expression of beta-oxidation makes sense in the light of nitrogen-limiting medium being rich in carbons, such that there is no reason to mobilize storage lipids as carbon source. These results on transcription level were then correlated with metabolic flux estimated from the GEM. The random sampling framework (Bordel et al. 2010) was used to support that the downregulation of amino acid biosynthetic enzymes resulted in a decreased flux. This could then be linked to the involvement of Snf1 but also TORC1.

5 Research Needs

While the argument for studying lipid metabolism in the context of the whole metabolic network is compelling, with current genome-scale metabolic models able to simulate metabolic fluxes through the complex lipid metabolic pathways and omics data sets being ever easier to generate, there are a number of issues that would thrive this area of research further.

One of the major issues is the accurate estimation of metabolic fluxes from GEMs, which is impeded by the vast solution space in purely stoichiometric models. There have been efforts to incorporate enzyme kinetics and protein constraints in the models, and these approaches have already resulted in large reductions of the solution space. However, any additional development in this direction, further tightening the flux predictions, would make the methods more valuable.

Another issue is the annotation and characterization of lipid biosynthetic pathways in different species. Especially from biotechnological viewpoint, there is a good argument to use nonconventional organisms, but this also means the absence of high-confidence knowledge on their metabolic networks and constituent enzymes. While computational approaches can go a long way to annotate genes, there remains a need for biochemical studies where enzyme activities are measured in vitro.

Lastly, while the incorporation of kinetics and metabolic constraints add more biological information into the models, there is another layer of information that can currently only be integrated poorly with GEMs. Besides the metabolic network, there is a complex regulatory network behind much of the behaviors of (lipid) metabolism. Improvements in these three areas would enhance the applicability of genome-scale models and as such would be able to answer questions in lipid metabolism.

Acknowledgments The author would like to acknowledge support from the US Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomic Science program (DE-SC0008744), and the Novo Nordisk Foundation.

References

Agren R, Liu L, Shoaie S et al (2013) The RAVEN toolbox and its use for generating a genome-scale metabolic model for *Penicillium chrysogenum*. PLoS Comput Biol 9:e1002980. https://doi.org/10.1371/journal.pcbi.1002980

Asadollahi MA, Maury J, Patil KR et al (2009) Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through in silico driven metabolic engineering. Metab Eng 11:328–334. https://doi.org/10.1016/j.ymben.2009.07.001

Aung HW, Henry SA, Walker LP (2013) Revising the representation of fatty acid, glycerolipid, and glycerophospholipid metabolism in the consensus model of yeast metabolism. Ind Biotechnol 9:215–228. https://doi.org/10.1089/ind.2013.0013

Beg QK, Vazquez A, Ernst J et al (2007) Intracellular crowding defines the mode and sequence of substrate uptake by *Escherichia coli* and constrains its metabolic activity. Proc Natl Acad Sci U S A 104:12663–12668. https://doi.org/10.1073/pnas.0609845104

Bordel S, Agren R, Nielsen J (2010) Sampling the solution space in genome-scale metabolic networks reveals transcriptional regulation in key enzymes. PLoS Comput Biol 6:e1000859. https://doi.org/10.1371/journal.pcbi.1000859

Burgard AP, Maranas CD (2003) Optimization-based framework for inferring and testing hypothesized metabolic objective functions. Biotechnol Bioeng 82:670–677. https://doi.org/10.1002/bit.10617

Chakrabarti A, Miskovic L, Soh KC, Hatzimanikatis V (2013) Towards kinetic modeling of genome-scale metabolic networks without sacrificing stoichiometric, thermodynamic and physiological constraints. Biotech J 9:1043-1057. https://doi.org/10.1002/biot.201300091

Chumnanpuen P, Zhang J, Nookaew I, Nielsen J (2012) Integrated analysis of transcriptome and lipid profiling reveals the co-influences of inositol-choline and Snf1 in controlling lipid biosynthesis in yeast. Mol Gen Genomics 287:541–554. https://doi.org/10.1007/s00438-012-0697-5

Feist AM, Palsson BO (2010) The biomass objective function. Curr Opin Microbiol 13:344–349. https://doi.org/10.1016/j.mib.2010.03.003

Förster J, Famili I, Fu P et al (2003) Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res 13:244–253. https://doi.org/10.1101/gr.234503

Heavner BD, Price ND (2015) Comparative analysis of yeast metabolic network models highlights progress, opportunities for metabolic reconstruction. PLoS Comput Biol 11:e1004530. https://doi.org/10.1371/journal.pcbi.1004530

Henry CS, DeJongh M, Best AA et al (2010) High-throughput generation, optimization and analysis of genome-scale metabolic models. Nat Biotechnol 28:977–982. https://doi.org/10.1038/nbt.1672

Herrgård MJ, Swainston N, Dobson P et al (2008) A consensus yeast metabolic network reconstruction obtained from a community approach to systems biology. Nat Biotechnol 26:1155–1160. https://doi.org/10.1038/nbt1492

Kavšček M, Bhutada G, Madl T, Natter K (2015) Optimization of lipid production with a genome-scale model of *Yarrowia lipolytica*. BMC Syst Biol 9:72. https://doi.org/10.1186/s12918-015-0217-4

Kerkhoven EJ, Pomraning KR, Baker SE, Nielsen J (2016) Regulation of amino-acid metabolism controls flux to lipid accumulation in *Yarrowia lipolytica*. npj Syst Biol Appl 2:16005. https://doi.org/10.1038/npjsba.2016.5

Ledesma-Amaro R, Nicaud J-M (2016) *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. Prog Lipid Res 61:40–50. https://doi.org/10.1016/j.plipres.2015.12.001

Lewis NE, Nagarajan H, Palsson BO (2012) Constraining the metabolic genotype-phenotype relationship using a phylogeny of in silico methods. Nat Rev Microbiol 10:291–305. https://doi.org/10.1038/nrmicro2737

Loira N, Dulermo T, Nicaud J-M, Sherman DJ (2012) A genome-scale metabolic model of the lipid-accumulating yeast *Yarrowia lipolytica*. BMC Syst Biol 6:35. https://doi.org/10.1186/1752-0509-6-35

Mahadevan R, Schilling CH (2003) The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. Metab Eng 5:264–276. https://doi.org/10.1016/j.ymben.2003.09.002

Nookaew I, Jewett MC, Meechai A et al (2008) The genome-scale metabolic model iIN800 of *Saccharomyces cerevisiae* and its validation: a scaffold to query lipid metabolism. BMC Syst Biol 2:71. https://doi.org/10.1186/1752-0509-2-71

Orth JD, Thiele I, Palsson BØ (2010) What is flux balance analysis? Nat Biotechnol 28:245–248. https://doi.org/10.1038/nbt.1614

Patil KR, Nielsen J (2005) Uncovering transcriptional regulation of metabolism by using metabolic network topology. Proc Natl Acad Sci U S A 102:2685–2689. https://doi.org/10.1073/pnas.0406811102

Patil KR, Rocha I, Förster J, Nielsen J (2005) Evolutionary programming as a platform for in silico metabolic engineering. BMC Bioinforma 6:308. https://doi.org/10.1186/1471-2105-6-308

Pitkänen E, Jouhten P, Hou J et al (2014) Comparative genome-scale reconstruction of gapless metabolic networks for present and ancestral species. PLoS Comput Biol 10:e1003465. https://doi.org/10.1371/journal.pcbi.1003465

Price ND, Reed JL, Palsson BØ (2004) Genome-scale models of microbial cells: evaluating the consequences of constraints. Nat Rev Microbiol 2:886–897. https://doi.org/10.1038/nrmicro1023

Sánchez BJ, Nielsen J (2015) Genome scale models of yeast: towards standardized evaluation and consistent omic integration. Integr Biol 7:846–858. https://doi.org/10.1039/C5IB00083A

Sánchez BJ, Zhang C, Nilsson A et al (submitted) Improving phenotype predictions of a yeast genome-scale metabolic model by incorporating enzymatic constraints

Schuetz R, Zamboni N, Zampieri M et al (2012) Multidimensional optimality of microbial metabolism. Science 336:601–604. https://doi.org/10.1126/science.1216882

Tai M, Stephanopoulos G (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. Metab Eng 15:1–9. https://doi.org/10.1016/j.ymben.2012.08.007

Thiele I, Palsson BØ (2010) A protocol for generating a high-quality genome-scale metabolic reconstruction. Nat Protoc 5:93–121. https://doi.org/10.1038/nprot.2009.203

Vongsangnak W, Klanchui A, Tawornsamretkit I et al (2016) Genome-scale metabolic modeling of *Mucor circinelloides* and comparative analysis with other oleaginous species. Gene 583:121–129. https://doi.org/10.1016/j.gene.2016.02.028

Väremo L, Gatto F, Nielsen J (2014) Kiwi: a tool for integration and visualization of network topology and gene-set analysis. BMC Bioin 15:408. https://doi.org/10.1186/s12859-014-0408-9

Ye C, Xu N, Chen H et al (2015) Reconstruction and analysis of a genome-scale metabolic model of the oleaginous fungus *Mortierella alpina*. BMC Syst Biol 9:1. https://doi.org/10.1186/s12918-014-0137-8

Part II

Genetics and Functional Genomics of Biogenesis of Fatty Acids and Lipids

Fatty Acid Synthesis and Regulation

21

Isabel M. López-Lara and María J. Soto

Contents

1 Introduction 392
2 Overview of Fatty Acid Biosynthesis 394
3 Biochemical Regulation 396
3.1 Regulation of Enzyme Activity by Temperature 397
4 Transcriptional Regulation of Fatty Acid Biosynthesis 398
4.1 Coordination of Fatty Acid Biosynthesis and Degradation by FadR in *Escherichia coli* 398
4.2 Transcriptional Regulation of Unsaturated Fatty Acid Formation 400
4.3 FapR of *Bacillus subtilis* 402
4.4 FabT of *Streptococcus pneumoniae* 403
5 Research Needs 404
References 405

Abstract

Fatty acids are the building blocks of diverse membrane lipids and therefore are essential for the viability of bacterial cells. Fatty acids are energetically expensive to produce, and their production is highly controlled at the transcriptional and posttranscriptional level. Biosynthesis of fatty acids is catalyzed in most bacteria by a group of highly conserved proteins known as the type II fatty acid synthase (FAS II) system. This system was characterized in *Escherichia coli*, and a similar

I. M. López-Lara (✉)
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, Mexico
e-mail: isabel@ccg.unam.mx

M. J. Soto
Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain
e-mail: mariajose.soto@eez.csic.es

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_26

set of enzymes is present in different bacteria. Knowledge of biochemical regulation of fatty acid biosynthesis is inferred from studies in *E. coli*. During biosynthesis, fatty acids are esterified to the phosphopantetheine prosthetic group of the small acyl carrier protein (ACP). Long-chain acyl-ACPs, the end product of the pathway, exert feedback regulation over different key enzymes. There is a diversity of systems used for transcriptional regulation of fatty acid biosynthesis. Transcriptional regulation of fatty acid biosynthesis has been studied mainly in three model organisms, the Gram-negative *E. coli* and the Gram-positive organisms *Bacillus subtilis* and *Streptococcus pneumoniae*. The effector molecules that modulate activity of transcription factors involved in lipid biosynthesis are either substrates (malonyl-CoA) or final products (long-chain acyl-ACPs) of fatty acid biosynthesis. Long-chain acyl-CoAs, which are usually formed from exogenous fatty acids, are effectors of transcription factors that coordinate *de novo* biosynthesis with availability of fatty acids in the environment.

1 Introduction

Fatty acids are key components of the cell, and their synthesis is essential for all organisms except for Archaea, where side chains of membrane lipids are isoprenoids. The main fate of fatty acids in bacteria is formation of membrane lipids, and recent data establish fatty acid biosynthesis as a primary determinant of cell size in *Escherichia coli* and *Bacillus subtilis* (Vadia et al. 2017). Most of the energy that it takes to construct a membrane lipid is used in the biosynthesis of fatty acids. Thus, formation of palmitic acid requires 8 acetyl-CoA molecules, 14 NAD(P)H reducing equivalents, and 7 ATP molecules and is usually occurring on an acyl carrier protein (ACP). Therefore, the rate of fatty acid biosynthesis is tightly regulated in order to provide the proper amount of molecules for membrane formation during growth. Also, the type of fatty acids produced, in particular the ratio of unsaturated to saturated fatty acids, is precisely controlled in order to accommodate membrane fluidity to changing environments (Zhang and Rock 2008).

Since the fatty acid biosynthetic pathway is energetically expensive, most bacteria studied can incorporate exogenous fatty acids using different mechanisms. The three different mechanisms described for incorporation of fatty acids are (1) conversion into acyl-CoA by the *fadD* encoded long-chain acyl-CoA synthetase as in γ-proteobacteria (Fig. 1a), (2) conversion to acyl-ACP by acyl-ACP synthetase (Aas) in some Gram-negative, and (3) conversion to acyl-phosphates in Gram-positives via the fatty acid-binding protein FakB and the fatty acid kinase FakA (Fig. 1b) (reviewed in Yao and Rock 2017). Probably, the same mechanisms used for incorporation of exogenous fatty acids are used for reutilization of endogenous fatty acids as shown for cyanobacteria, *E. coli* and *Sinorhizobium meliloti* (Kaczmarzyk and Fulda 2010; Pech-Canul et al. 2011). In the presence of exogenous fatty acids, bacteria respond with a downregulation of their own fatty acid biosynthetic pathway. While *Streptococcus pneumoniae* can completely repress *de novo* fatty acid

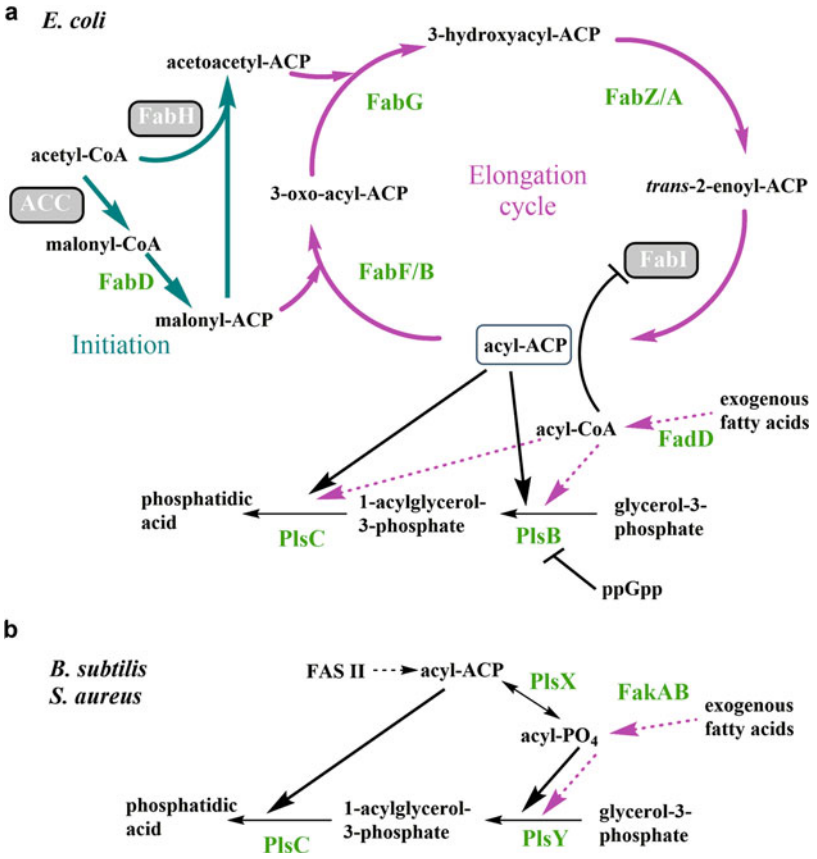


Fig. 1 Biosynthesis of fatty acids, incorporation to form phosphatidic acid and biochemical regulation in bacteria. In (**a**) the pathway of fatty acid biosynthesis for *E. coli*, which occurs through the stages of initiation and cyclic elongation, is shown. The long-chain acyl-ACP end products exert feedback regulation over three enzymes, ACC, FabH, and FabI (shown on gray squares). Acyl-ACPs are substrates of the PlsB and PlsC acyltransferases and which can also use acyl-CoAs derived from exogenous fatty acids (dotted arrows). FadD is the enzyme responsible of converting long-chain free fatty acids to acyl-CoAs. Palmitoyl-CoA is an inhibitor of FabI, and the alarmone ppGpp inhibits the acyltransferase PlsB. In (**b**) pathways for the incorporation of endogenous and exogenous fatty acids in the bacteria *B. subtilis* and *S. aureus* are shown. Acyl-ACPs are formed via the FAS II system using similar reactions to those shown in (a). PlsX converts acyl-ACP to acyl-phosphate (acyl-PO_4) which is used by the acyltransferase PlsY to form 1-acylglycerol-3-phosphate. PlsC uses acyl-ACP to form phosphatidic acid. Exogenous fatty acids are converted to acyl-phosphates by the FakAB kinase system. The acyl-phosphate formed can be used by the acyltransferase PlsY to form 1-acylglycerol-3-phosphate or can be converted to acyl-ACP by PlsX and then used by the acyltransferase PlsC

biosynthesis in the presence of exogenous fatty acids, *Staphylococcus aureus* can only reduce it to 50% (Parsons et al. 2011).

E. coli regulates fatty acid formation by biochemical regulation using the end product acyl-ACP as feedback regulator. There also exists a complex transcriptional

regulation of fatty acid biosynthesis through the transcriptional factors FadR and FabR. Different types of genetic regulation have been described in Gram-positive bacteria. In *B. subtilis*, as in *E. coli*, genes for fatty acid biosynthesis are scattered throughout the chromosome, while in *S. pneumoniae*, the 12 genes for fatty acid biosynthesis are clustered (Parsons and Rock 2013).

Although the reactions of fatty acid biosynthesis are essentially the same in all bacteria (Fig. 1a), there are different mechanisms for their regulation. In this chapter we will describe biochemical regulation of fatty acids which has been studied mainly in *E. coli* as well as the transcriptional regulation mechanisms described for three model organisms: *E. coli*, *B. subtilis*, and *S. pneumoniae*.

Fatty acid regulation has also been studied in members of the *Corynebacteriales* order, which includes the important pathogen *Mycobacterium*. *Corynebacteriales* have linear fatty acids and mycolic acid that are exceptionally long- and branched-chain fatty acids. Two fatty acid synthesis systems are required for biosynthesis of mycolic acids, and regulation of their biosynthesis is rather complex involving biochemical and transcriptional regulation (see the ▶ Chap. 11, "Mycolic Acids: From Chemistry to Biology" by Daffé et al., this volume). Recent results establish that acyl-CoA longer than C18 are sensed by the two main transcriptional regulators involved in maintaining lipid homeostasis in mycobacteria, FasR involved in regulation of the FAS I system (Mondino et al. 2013) and MabR that regulates components of the FAS II system (Tsai et al. 2017) (Table 1) (see the ▶ Chap. 22, "Components and Key Regulatory Steps of Lipid Biosynthesis in Actinomycetes" by Gago et al., this volume).

2 Overview of Fatty Acid Biosynthesis

Fatty acid biosynthesis is catalyzed in most bacteria by a group of highly conserved proteins known as the type II fatty acid synthase (FAS II) system. A key protein in this system is the ACP which acts as carrier of growing acyl chains during biosynthesis and as donor of acyl chains during transfer to target molecules. Fatty acid biosynthesis proceeds in two stages, initiation and cyclic elongation as outlined in Fig. 1. Acetyl-CoA carboxylase (ACC) catalyzes the first committed step of fatty acid biosynthesis, the ATP-consuming carboxylation of acetyl-CoA. The product of the reaction is malonyl-CoA, and the malonyl group is transferred to ACP by malonyl-CoA:ACP transacylase (FabD) to form malonyl-ACP. The condensation of malonyl-ACP with acetyl-CoA by 3-oxoacyl-ACP synthase III (FabH) with the formation of acetoacetyl-ACP and CO_2 finishes the initiation steps in fatty acid biosynthesis. Elongation of fatty acids by C2 units goes through a cycle of reactions, each cycle involving a condensation, a first reduction, a dehydration, and a second reduction step. Each new round is initiated by an elongation condensing enzyme: 3-oxoacyl-ACP-synthase II (FabF) or 3-oxoacyl-ACP-synthase I (FabB). Both catalyze a Claisen condensation using malonyl-ACP to elongate the growing acyl chain by two carbons. FabG is the 3-oxoacyl-ACP reductase, and the next step is the dehydration of the 3-hydroxyacyl-ACP to *trans*-2-enoyl-ACP by 3-hydroxyacyl-

Table 1 Transcriptional regulators of fatty acid biosynthesis in bacteria[a]

Transcription factor	Family	Signal sensed	Regulated genes	Representative organism
$FadR_{Ec}$[b,c]	GntR	LC acyl-CoA	*fabA*, *fabB*, *fabH-D-G*, *acpP-fabF*, *accA*, *accB-C*, *accD*, *fabI*	*Escherichia coli*
FabR	TetR	LC acyl-ACP LC acyl-CoA	*fabA*, *fabB*	*Escherichia coli*
DesT	TetR	LC acyl-CoA	*desC-B*, *fabA-B*	*Pseudomonas aeruginosa*
DesR	Response regulator	Membrane thickness	*des*	*Bacillus subtilis*
FapR	DeoR	malonyl-CoA malonyl-ACP	*fabHa-fabF*, *fapR-plsX-fabDfabG*, *fabI*, *fabHb*, *yhfC*, *plsC*	*Bacillus subtilis* *Staphylococcus aureus*
FabT	MarR	LC acyl-ACP (C18:1Δ11, C16:0)	*fabT-H-acpP*, *fabK-D-G-F-accB-fabZ-accC-D-A*	*Streptococcus pneumoniae*
$FasR_{Mt}$[c]	TetR	LC acyl-CoA (>C18)	*fas-acpS*	*Mycobacterium tuberculosis*
MabR	TetR	LC acyl-CoA (>C18)	*fabD-acpM-kasA-kasB*	*Mycobacterium tuberculosis*

[a]Genes forming operons are indicated by connecting with dashes, LC long-chain
[b]FadR of *E. coli* is a dual regulator of fatty acid metabolism, acting as repressor of degradation and as an activator of biosynthesis. In this table, only genes involved in fatty acid biosynthesis are indicated
[c]There are regulators in other bacteria named as FadR or FasR which are not homologues to *E. coli* FadR or *M. tuberculosis* FasR

ACP dehydratases, FabA or FabZ. Each cycle is led to completion by an enoyl-ACP reductase which reduces the double bond in *trans*-2-enoyl-ACP using the cofactor NADH (Fig. 1). The enoyl-ACP reductase FabI of *E. coli* was the first described, but, in contrast to most enzymes in the pathway, enoyl-ACP reductases show diversity among different bacteria (Parsons and Rock 2013; see the ▶ Chap. 3, "Formation of Fatty Acids" by López-Lara and Geiger, this volume). The 3-hydroxyacyl-ACP dehydratase FabA not only can remove water during fatty acid elongation but is also able to catalyze the isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP, the key step to synthesize unsaturated fatty acids. For the elongation of *cis*-3-decenoyl-ACP to palmitoleyl-ACP, FabB is essential. Biosynthesis of unsaturated fatty acids in *E. coli* requires of FabA and FabB enzymes, but genomic analyses suggest that the FabA/FabB pathway for unsaturated fatty acids might be restricted to α- and γ-proteobacteria. In a similar way as FabA, FabX introduces an

unsaturation during fatty acid biosynthesis at the level of the C10 intermediate. However, FabX uses an unprecedented combination of dehydrogenase/isomerase activities. FabX-dependent biosynthesis of unsaturated fatty acids is present in many different bacteria such as *Helicobacter*, *Clostridium*, *Campylobacter*, and *Burkholderia* where homologues to FabA/FabB are not present (Bi et al. 2016).

Acyl-ACPs of the proper chain length are substrates for acyltransferases involved in biosynthesis of different membrane lipids (López-Lara and Geiger 2017). There are two different systems for the first acylation in phospholipid biosynthesis. The PlsB system is found in *E. coli* and other γ-proteobacteria and acylates glycerol-3-phosphate using as fatty acid donors acyl-ACP or acyl-CoA (Fig. 1a). The PlsX/PlsY system is more widespread in bacteria. PlsX converts the acyl-ACP formed during fatty acid biosynthesis into acyl-phosphate, and PlsY uses the acyl-phosphate to acylate glycerol-3-phosphate (Fig. 1b). In all bacteria, PlsC transfers a second acyl group to 1-acylglycerol-3-phosphate leading to phosphatidic acid formation (Fig. 1), a central intermediate in phospholipid biosynthesis. *E. coli* PlsC can use acyl-ACP and acyl-CoA as substrates, but in many other bacteria, PlsC can only use acyl-ACP (see the ► Chap. 5, "Formation of Bacterial Glycerol-Based Membrane Lipids: Pathways, Enzymes, and Reactions" by Geiger et al., this volume).

3 Biochemical Regulation

Biochemical regulation occurs usually in the form of negative feedback, i.e., the end product of the pathway inhibits enzymes of earlier steps. The first data suggesting long-chain acyl-ACPs as negative feedback regulators of fatty acid biosynthesis in *E. coli* were obtained during overexpression of thioesterases in *E. coli*. The overexpression of thioesterases cleaves the acyl-ACP species and results in relief of the inhibition, pointing to long-chain acyl-ACPs as inhibitors of fatty acids biosynthesis (Jiang and Cronan 1994). Long-chain acyl-ACPs inhibit three key enzymes in fatty acid biosynthesis, ACC, FabH, and FabI (Fig. 1a) (Davis and Cronan 2001; Heath and Rock 1996a, b). ACC is responsible for the formation of malonyl-CoA, the first committed step in fatty acid biosynthesis, FabH condenses acetyl-CoA with malonyl-ACP starting the formation of each fatty acid, and FabI is required to complete each cycle of elongation (Fig. 1a). The inhibition of ACC by palmitoyl-ACP occurs via an allosteric mechanism, and specifically pantothenic acid is the moiety that inhibits the enzyme. ACC inhibition shows a pronounced hysteresis, meaning a delay in the response to metabolite concentrations. The hysteretic behavior mitigates dramatic changes of metabolites during fatty acid biosynthesis and controls tightly the flux through fatty acid biosynthesis (Evans et al. 2017). FabI of *E. coli* is also inhibited by palmitoyl-CoA, and this is probably important in regulating endogenous fatty acid biosynthesis when exogenous fatty acids are present (Bergler et al. 1996). Exogenous long-chain fatty acids are converted to

acyl-CoAs by the long-chain acyl-CoA synthetase FadD, and the phospholipid acyl transferases of *E. coli* can use the acyl-CoAs formed as substrates (Fig. 1a).

The alarmones guanosine penta- and tetraphosphate (ppGpp) are general stress response signals that increase after starvation and inhibit several major biosynthetic pathways including phospholipid synthesis. ppGpp inhibits PlsB activity, and consequently an accumulation of acyl-ACPs occurs that leads to feedback inhibition of fatty acid synthesis (Heath et al. 1994). SpoT can either hydrolyze or synthesize ppGpp. SpoT interacts permanently with ACP, and it is proposed that the activity of SpoT is influenced by the acyl cargo present in ACP (Battesti and Bouveret 2006). In *E. coli*, ppGpp together with the RNA polymerase cofactor DksA binds directly to RNA polymerase and inhibits transcription of many promoters. In fact, the operon *fabHDG* is inhibited directly by ppGpp/DksA (My et al. 2013), and many other genes of fatty acid metabolism might be directly or indirectly regulated by ppGpp (reviewed in Janßen and Steinbüchel 2014). Therefore, ppGpp is able to directly inhibit the PlsB enzyme and to control fatty acid biosynthesis at the level of transcription.

3.1 Regulation of Enzyme Activity by Temperature

For normal membrane functionality, lipid bilayers need to be fluid, and bacteria must regulate membrane fluidity in response to a decrease in temperature. As temperature decreases, a higher proportion of fatty acyl chains that present lower interaction among them are incorporated into the membrane. These are: i) unsaturated fatty acids with a *cis*-double bond, which introduces a kink in the chain; ii) methyl-branched fatty acids, particularly a higher proportion of *anteiso* branched-chain fatty acids; and iii) shorter fatty acids (see the ▶ Chap. 42, "Regulation of Membrane Lipid Homeostasis in Bacteria upon Temperature Change" by Mansilla and de Mendoza, this volume). Formation of unsaturated fatty acids in *E. coli* at low temperature is increased due to a higher activity of the FabF enzyme. FabF is responsible for the elongation of C16:1Δ9-ACP to C18:1Δ11-ACP and increases its activity at lower temperatures. FabF is responsible for temperature-dependent alterations in fatty acid composition, and *fabF* mutants are unable to produce *cis*-vaccenic acid (C18:1Δ11) (de Mendoza et al. 1983).

Some Gram-positive bacteria adjust membrane fluidity by increasing the proportion of *anteiso* branched-chain fatty acids at lower growth temperatures. The FabH enzyme of bacteria that form branched-chain fatty acids uses as precursors isobutyryl-CoA, isovaleryl-CoA, and 2-methylbutyryl-CoA. Only the precursor 2-methylbutyryl-CoA leads to formation of *anteiso*-fatty acids (see the ▶ Chap. 3, "Formation of Fatty Acids" by López-Lara and Geiger, this volume). At temperatures of 10 °C, the FabH enzyme of *Listeria monocytogenes* displays an increased selectivity for the 2-methylbutyryl-CoA primer, leading to a higher formation of *anteiso* branched-chain fatty acids. This selectivity seems to be an intrinsic property of the FabH enzyme (Singh et al. 2009).

4 Transcriptional Regulation of Fatty Acid Biosynthesis

There is not a single system of transcriptional regulation of fatty acid biosynthesis utilized by all bacteria. Rather, different groups of bacteria use specific types of transcription factors that control most or a subset of genes involved in fatty acid biosynthesis. The mechanism of transcriptional regulation of fatty acid biosynthesis is usually via one-component systems in which a single polypeptide contains a sensory domain and a DNA-binding domain and they function as dimers. FadR of *E. coli* is a dual transcriptional factor acting as a repressor of genes for degradation of fatty acids and as an activator of genes of fatty acid synthesis. There are transcriptional factors specifically involved in fine-tuning regulation of the ratio of unsaturated:saturated fatty acids. Transcription factors specifically involved in regulating synthesis of unsaturated fatty acids are FabR of *E. coli*, DesT in *Pseudomonas aeruginosa*, and the DesKR two-component regulator identified in *B. subtilis*. In Gram-positive bacteria, the global regulators FapR of *B. subtilis* and FabT from *S. pneumoniae* have been described in more detail.

4.1 Coordination of Fatty Acid Biosynthesis and Degradation by FadR in *Escherichia coli*

The FadR transcriptional factor was discovered in *E. coli* as a repressor of the fatty acid degradation (*fad*) regulon, which comprises genes for transport, activation, and β-oxidation of fatty acids. Later on, it was shown that FadR is an activator of the *fabA* gene that introduces the double bond of unsaturated fatty acids, as well as an activator of *iclR*. IclR acts as a repressor of the *aceBAK* operon encoding the glyoxylate shunt enzymes. This operon is responsible for the utilization of acetyl-CoA that originates from β-oxidation. FadR is also a repressor of *uspA*, coding for the universal stress response protein (reviewed in Cronan and Subrahmanyam 1998). Furthermore, FadR is a positive regulator of the *fabB* gene (Campbell and Cronan 2001), and recent results show that FadR is an activator of all genes involved in fatty acid biosynthesis except for *fabZ* (My et al. 2013, 2015). Overexpression of FadR in an engineered fatty acid-producing *E. coli* strain increases fatty acid production by 7.5-fold confirming that FadR functions as an activator of fatty acid biosynthetic genes (Zhang et al. 2012).

FadR binds to a similar 17 bp nucleotide operator sequence when acting either as repressor or activator. The distinction between these two roles depends on the location of the binding site relative to the transcriptional start site. When acting as an activator, the operator sequences are located at the −40 region, and bound FadR facilitates RNA polymerase binding resulting in increased transcription. When acting as a repressor, the operator sequences are positioned in a region downstream of the promoter sequences recognized by the RNA polymerase, and FadR binding prevents transcription (Cronan and Subrahmanyam 1998) (Fig. 2). However, the binding strength is lower for the fatty acid biosynthesis genes probably due to a low conservation of the binding consensus site (My et al. 2015). Furthermore,

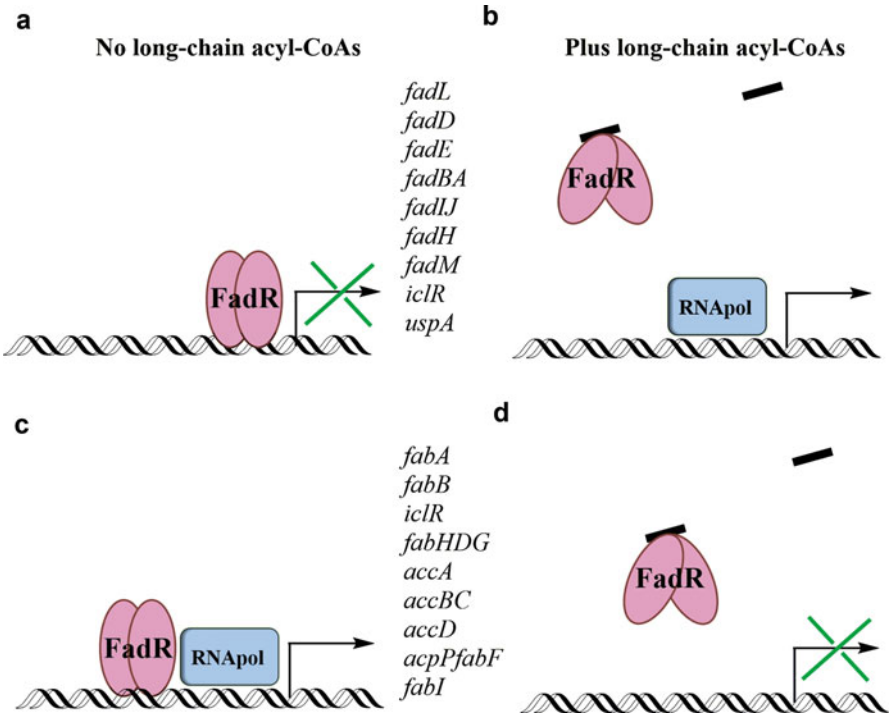


Fig. 2 Schematic representation of the *E. coli* FadR transcriptional regulatory mechanism. In the absence of long-chain acyl-CoA (**a** and **c**), FadR binds to various operators repressing transcription of the *fad* regulon (**a**) and activating transcription of fatty acid biosynthetic genes by favoring binding of RNA polymerase (RNApol) (**c**). In the presence of long-chain acyl-CoAs (**b** and **d**), FadR is released from the operator sequences resulting in the induction of *fad* expression (**b**), and transcription of fatty acid biosynthesis genes is no longer activated (**d**). Genes or operons repressed (above) or activated (below) by FadR are indicated

expression of fatty acid biosynthesis genes is driven from multiple promoters resulting in a complex genetic control of these genes. Among the *fad* genes, *fadL* (coding for a fatty acid transporter) and *fadD* (responsible for the activation of free fatty acids to acyl-CoAs) are subject to a less stringent regulation by FadR. This is logical since these enzymes are required to generate the acyl-CoA inducer.

E. coli FadR is a member of the GntR family of transcriptional regulators and functions as a dimer, with each monomer composed of an N-terminal helix-turn-helix DNA-binding domain and a C-terminal acyl-CoA-binding domain (van Aalten et al. 2000). The physiological ligands of FadR are long-chain (more than 12 C) fatty acyl-CoA thioesters. Binding of long-chain acyl-CoAs to the effector domain causes a conformational change in the dimeric protein that provokes release from the operator region. The result is activation of genes for fatty acid degradation genes,

while fatty acid biosynthesis genes are no longer induced (Fig. 2) (Cronan and Subrahmanyam 1998).

Homologues to *E. coli* FadR are found exclusively in a subset of γ-proteobacteria that colonize animals or plants. This distribution suggests that FadR regulation is involved in utilization of fatty acids present in these ecological niches (Iram and Cronan 2005). *B. subtilis* also contains a transcriptional regulator named as FadR that represses the genes of β-oxidation but does not function as an activator of genes for fatty acid biosynthesis. Furthermore, *B. subtilis* and *E. coli* FadR proteins are structurally different, *B. subtilis* FadR belongs to the TetR family, while the *E. coli* FadR is a GntR-like repressor (Fujita et al. 2007).

4.2 Transcriptional Regulation of Unsaturated Fatty Acid Formation

The proportion of unsaturated:saturated fatty acids determines the viscosity of the membrane which is essential to maintain a stable and functional membrane (Zhang and Rock 2008). In addition to enzymatic control of the formation of unsaturated fatty acids, bacteria contain specific transcriptional factors for the regulation of genes dedicated to the formation of unsaturated fatty acids. For the bacteria *E. coli*, *P. aeruginosa*, and *B. subtilis*, the regulators FabR, DesT, and DesKR, respectively, control the expression of genes involved in the formation of unsaturated fatty acids. FabR and DesT are homologous proteins, and they are both transcriptional regulators of the TetR family, while DesKR is a two-component transcriptional regulator.

The *E. coli* fatty acid biosynthesis regulator (FabR) was discovered in 2001 as a protein that bound a sequence upstream of *fabA* and *fabB*, the two essential genes for the synthesis of monounsaturated fatty acids (McCue et al. 2001). Deletion of *fabR* significantly increases the levels of unsaturated fatty acids confirming its function as repressor of *fabA* and *fabB* (Zhang et al. 2002). Homologues to FabR are found exclusively in γ-proteobacteria. FabR represses transcription of *fabA* and *fabB* by binding downstream of the FadR operator (Zhang et al. 2002). Interestingly, FabR regulates expression by sensing the composition of the cytosolic fatty acid pool. All long-chain acyl-CoAs or acyl-ACPs bind to FabR. Binding of long-chain unsaturated fatty acyl thioesters increases the affinity of FabR for the operator sequences present in the *fabB* and *fabA* promoters leading to repression of transcription. On the other hand, binding of long-chain saturated acyl-ACP or acyl-CoA to FabR does not allow binding to DNA (Fig. 3) (Zhu et al. 2009). The phospholipid acyltransferases of *E. coli* can use acyl-ACPs from *de novo* biosynthesis and acyl-CoAs which are formed from exogenous fatty acids (Fig. 1a). Therefore, FabR can adjust biosynthesis of unsaturated fatty acids in relation to the composition of fatty acids obtained from the environment (Zhu et al. 2009).

P. aeruginosa also has the FabA/FabB system responsible for the classical anaerobic pathway of unsaturated fatty acids but, in addition, has two oxygen-requiring desaturases: a Δ9-desaturase (DesA) that inserts a double bond into acyl chains of preexisting phospholipids and the DesBC system responsible for

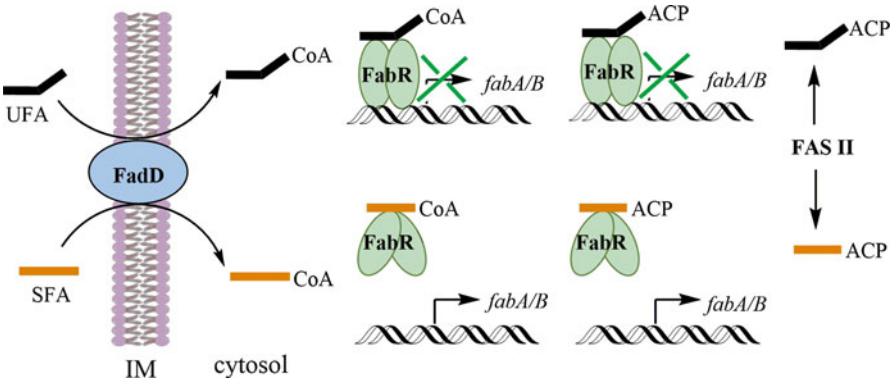


Fig. 3 Transcriptional control of unsaturated fatty acid formation by FabR. FabR binds both long-chain acyl-CoAs that arise from exogenous fatty acids and the long-chain acyl-ACPs produced by the FAS II synthase. Unsaturated fatty acid ligands induce a conformational change in FabR that increases binding to DNA, whereas binding of saturated ligands does not promote binding to DNA, and transcription of *fabA* and *fabB* is allowed. FadD, long-chain fatty acyl-CoA synthetase; UFA, unsaturated fatty acid; SFA, saturated fatty acid; IM, inner membrane

introducing double bonds at the Δ9-position into acyl-CoAs derived from exogenous fatty acids (Zhu et al. 2006). The transcriptional regulator DesT is a protein highly related to FabR of *E. coli*, and they both bind to similar DNA palindromes. DesT controls the expression of the operon *desCB* and also of *fabAB*. Similar to FabR, DesT responds to fatty acid structure, although only in the form of acyl-CoAs. Binding of unsaturated acyl-CoAs to DesT stabilizes a conformation that binds tightly to their operator sequences, and transcription of *desCB* and *fabAB* is impeded. However, binding of saturated acyl-CoAs to DesT stabilizes a conformation that cannot bind DNA, and formation of unsaturated fatty acids via the aerobic and anaerobic pathway is allowed (Zhang et al. 2007; Miller et al. 2010) (Fig. 4). The ability of DesT to respond to differences in acyl-CoA structure allows the cell to use exogenous fatty acids and, at the same time, to maintain membrane homeostasis due to the control of the degree of unsaturation of fatty acids (Zhang and Rock 2008).

The DesKR two-component system of *B. subtilis* is responsible for transcription of the Δ5-desaturase Des. DesK functions as a kinase at cold temperatures, autophosphorylating a conserved histidine within its kinase domain. Then, the phosphoryl group is transferred to the conserved aspartic acid residue of the DNA-binding response regulator DesR. Phosphorylation of DesR results in its reorganization as quaternary structure which is responsible for activating transcription of the *des* gene. A drop in temperature causes an increment in membrane thickness due to an increased order in acyl lipid chains. Experimental evidence indicates that DesK senses thickening of the membrane when temperature drops, which results in autophosphorylation (see the ▶ Chap. 42, "Regulation of Membrane Lipid Homeostasis in Bacteria upon Temperature Change" by Mansilla and de Mendoza, this volume).

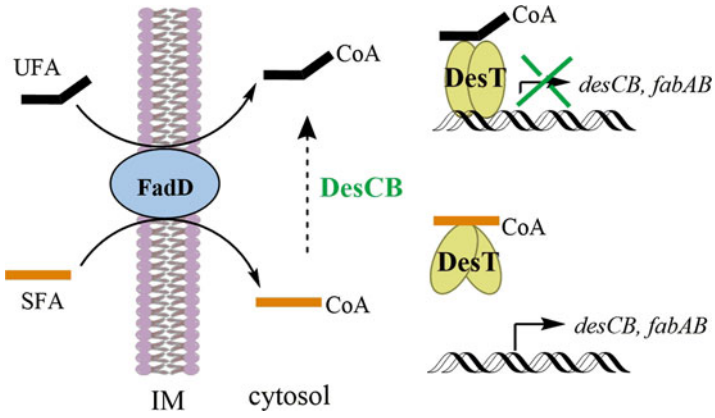


Fig. 4 Regulation of the expression of *desCB* and *fabAB* by DesT in *P. aeruginosa*. Unsaturated (UFA) and saturated (SFA) fatty acids present in the growth environment are activated to acyl-CoAs by the membrane associated acyl-CoA synthetase FadD. When cellular unsaturated acyl-CoAs are formed, the DesT·UFA-CoA complex binds tightly to the *desCB* and *fabAB* promoters leading to repression of transcription. Presence of saturated acyl-CoAs leads to formation of the DesT·SFA-CoA complex that prevents binding of DesT to the cognate promoters, and the repression of *desCB* and *fabAB* transcription is released. IM: inner membrane

4.3 FapR of *Bacillus subtilis*

FapR belongs to the DeoR family of transcriptional regulators, and it is a global regulator of lipid biosynthesis that negatively controls the expression of fatty acid and phospholipid genes, the *fap* regulon. Initially discovered in *B. subtilis* (Schujman et al. 2003), it is highly conserved in *Bacillus*, *Listeria*, and *Staphylococcus* species. The *fap* regulon consists of six operons that comprise the genes for the FAS II system, with the notable exception of the genes for ACC, and two genes involved in phospholipid synthesis (Table 1). FapR binds to a conserved 17 bp inverted repeat identified within or immediately downstream of the predicted *fap* promoters (Schujman et al. 2003). The specific binding of FapR to its DNA targets is inhibited by malonyl-CoA and malonyl-ACP, and in the presence of these effectors, FapR is unable to repress transcription of the *fap* regulon (Schujman et al. 2003, 2006; Martinez et al. 2010) (Fig. 5). Therefore, FapR activity is controlled by metabolites required at the beginning of the fatty acid biosynthetic pathway and represents a feed-forward control mechanism.

FapR is a homodimeric repressor, and each monomer consists of an N-terminal DNA-binding domain containing a typical helix-turn-helix motif and a C-terminal effector-binding domain. Interestingly, the effector domain has the hot-dog fold (Schujman et al. 2006), and a similar fold is found in many enzymes involved in fatty acid metabolism. However, the FapR transcriptional factor appears to have selected the hot-dog fold for a nonenzymatic function. Binding of malonyl-CoA induces a structural modification that propagates to the helix-turn-helix motive impairing association of FapR to its DNA target (Schujman et al. 2006; Albanesi

et al. 2013). The crystal structure of *Staphylococcus aureus* FapR in complex with malonyl-CoA shows that FapR specifically recognizes the malonyl-phosphopantetheine moiety of the effector (Albanesi et al. 2013) in agreement with the fact that either malonyl-CoA or malonyl-ACP can function as effectors (Martinez et al. 2010).

4.4 FabT of *Streptococcus pneumoniae*

FabT was identified in the human pathogen *S. pneumoniae* (Lu and Rock 2006). In this bacterium, the 12 genes for fatty acid biosynthesis are located in a cluster where the second gene is *fabT*. *fabT* encodes a helix-turn-helix DNA-binding protein belonging to the MarR superfamily of transcriptional regulators that binds to specific DNA palindromes at two promoters (Lu and Rock 2006). FabT controls the expression of all fatty acid biosynthesis genes with the exception of *fadM*, the first gene in the *fab* cluster (Lu and Rock 2006). A similar genetic organization is found in the order Lactobacillales (*Streptococcus*, *Enterococcus*, *Lactococcus*) and in *Clostridium* species. The effectors that enhance FabT binding to their DNA-binding sites are long-chain acyl-ACPs (Jerga and Rock 2009) (Fig. 5). FabT affinity for DNA increased with increasing acyl-ACP chain length with *cis*-vaccenoyl-ACP being the most effective ligand (Jerga and Rock 2009). Thus, the acyl-ACP end products of fatty acid biosynthesis act as feedback regulators of FAS II gene expression in *Streptococcus* and *Enterococcus*. The MarR regulators have a DNA winged helix-turn-helix domain and exist as dimers (Deochand and Grove 2017).

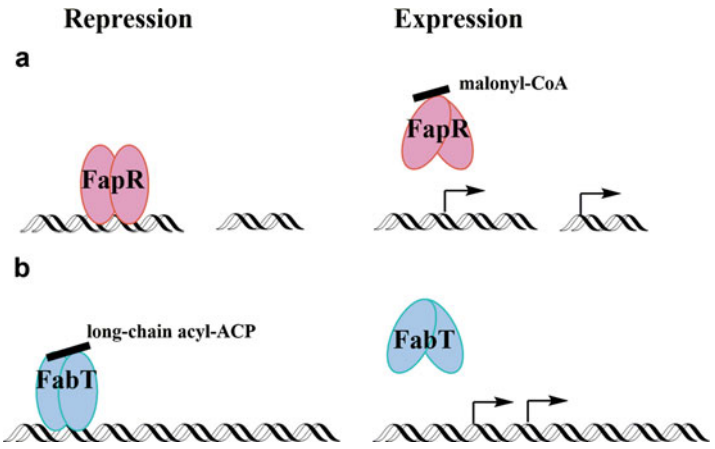


Fig. 5 Schematic comparison of transcriptional regulation of fatty acid biosynthesis through the global regulators FapR (in **a**) and FabT (in **b**). FapR represses transcription in its apo-form, while FabT represses transcription in the presence of long-chain fatty acids (left). FapR allows expression when it is bound to malonyl-CoA or malonyl-ACP, while FabT allows expression in its apo-form (right)

5 Research Needs

The steps of fatty acid biosynthesis in *E. coli* are now very well known, and many details of the regulation at the level of biochemical and transcriptional regulation are well established. Because the regulation of fatty acid biosynthesis is rather complex and subject to multiple levels even in the model bacterium *E. coli*, there are still some discoveries ahead. Recently the precise mechanism of ACC inhibition by palmitoyl-ACP has been elucidated (Evans et al. 2017). However, such a deep knowledge in the mechanisms of inhibition of FabH and FabI by acyl-ACPs or the mechanism of inhibition of PlsB by ppGpp is missing. In addition to the inhibition of the enzyme PlsB, ppGpp also exerts direct transcriptional regulation of several genes involved in fatty acid biosynthesis (My et al. 2013). ppGpp is one of the signals that coordinates fatty acid biosynthesis with formation of other macromolecules. However, there remain many open questions in connection to coordination between fatty acid synthesis and formation of other macromolecules.

The long-chain acyl-ACP end products of fatty acid biosynthesis (mainly 16- and 18-carbon acyl-ACPs) inhibit the enzymes ACC, FabH, and FabI in *E. coli* through feedback regulation. However, biochemical regulation of fatty acid biosynthesis needs to be demonstrated in other bacteria. *S. pneumoniae* deficient in FabT and *B. subtilis* lacking FapR do not show an increment of fatty acids although the genes are not regulated (Lu and Rock 2006; Schujman et al. 2003). This observation strongly argues for the presence of biochemical regulatory mechanisms in both organisms that override transcriptional control to prevent excess of fatty acid formation. Possible inhibition of ACC, FabH, or acyltransferases in different bacteria by long-chain acyl-ACPs requires further investigation.

So far, the mechanism controlling an increase in the proportion of *anteiso* branched-chain fatty acids after a downshift in temperature is little studied. Only for the case of *L. monocytogenes*, a temperature dependency of the specificity of FabH according to temperature has been described (Singh et al. 2009). It will be important to study mechanisms of transcriptional regulation that control the proportion of branched-chain fatty acids in relation to saturated fatty acids. Considering the importance of maintaining a proper fluidity of the membrane, bacteria which relay in the newly discovered mechanism of FabX for biosynthesis of unsaturated fatty acids (Bi et al. 2016) should also regulate the activity of the enzyme and/or the expression of the *fabX* gene.

In this chapter we have described transcriptional regulation of fatty acid biosynthesis in a few representative organisms. There are marked differences in the mechanisms used which reflect differences in physiology and accommodation to the niches occupied by the specific organism. Given the huge diversity of bacteria, many more different mechanisms are expected to exist. We have schematically represented in Fig. 1 two ways of incorporation of exogenous fatty acids into membrane lipids by converting them to acyl-CoAs (Fig. 1a) or to acyl-phosphates (Fig. 1b). Some bacteria convert directly exogenous fatty acids to acyl-ACPs (reviewed in Yao and Rock 2017). Acyl-CoAs and acyl-ACPs function as effectors in regulation of fatty acid biosynthesis. It will be of interest to determine if

acyl-phosphates have any regulatory role in bacteria, either modifying enzymatic activity or as effectors of transcriptional factors of lipid metabolism.

Acknowledgments Work on IML-L's lab was funded by Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México (DGAPA-UNAM; PAPIIT IN202616). Work on MJS's lab was funded by ERDF-cofinanced grant BIO2013-42801-P from the Spanish Ministry for Economy and Competitiveness. We thank Angeles Moreno-Ocampo and José Espíritu Salazar for skillful technical assistance.

References

Albanesi D, Reh G, Guerin ME, Schaeffer F, Debarbouille M, Buschiazzo A, Schujman GE, de Mendoza D, Alzari PM (2013) Structural basis for feed-forward transcriptional regulation of membrane lipid homeostasis in *Staphylococcus aureus*. PLoS Pathog 9(1):e1003108

Battesti A, Bouveret E (2006) Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. Mol Microbiol 62(4):1048–1063

Bergler H, Fuchsbichler S, Högenauer G, Turnowsky F (1996) The enoyl-[acyl-carrier-protein] reductase (FabI) of *Escherichia coli*, which catalyzes a key regulatory step in fatty acid biosynthesis, accepts NADH and NADPH as cofactors and is inhibited by palmitoyl-CoA. Eur J Biochem 242(3):689–694

Bi H, Zhu L, Jia J, Zeng L, Cronan JE (2016) Unsaturated fatty acid synthesis in the gastric pathogen *Helicobacter pylori* proceeds via a backtracking mechanism. Cell Chem Biol 23:1480–1489

Campbell JW, Cronan JE (2001) *Escherichia coli* FadR positively regulates transcription of the *fabB* fatty acid biosynthetic gene. J Bacteriol 183(20):5982–5990

Cronan JE, Subrahmanyam S (1998) FadR, transcriptional co-ordination of metabolic expediency. Mol Microbiol 29:937–943

Davis MS, Cronan JE (2001) Inhibition of *Escherichia coli* acetyl coenzyme A carboxylase by acyl-acyl carrier protein. J Bacteriol 183(4):1499–1503

de Mendoza D, Klages Ulrich A, Cronan JE (1983) Thermal regulation of membrane fluidity in *Escherichia coli*. Effects of overproduction of beta-ketoacyl-acyl carrier protein synthase I. J Biol Chem 258(4):2098–2101

Deochand DK, Grove A (2017) MarR family transcription factors: dynamic variations on a common scaffold. Crit Rev Biochem Mol Biol 52(6):595–613

Evans A, Ribble W, Schexnaydre E, Waldrop GL (2017) Acetyl-CoA carboxylase from *Escherichia coli* exhibits a pronounced hysteresis when inhibited by palmitoyl-acyl carrier protein. Arch Biochem Biophys 636:100–109

Fujita Y, Matsuoka H, Hirooka K (2007) Regulation of fatty acid metabolism in bacteria. Mol Microbiol 66(4):829–839

Heath RJ, Rock CO (1996a) Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in *Escherichia coli*. J Biol Chem 271(4):1833–1836

Heath RJ, Rock CO (1996b) Inhibition of β-ketoacyl-acyl carrier protein synthase III (FabH) by acyl-acyl carrier protein in *Escherichia coli*. J Biol Chem 271(18):10996–11000

Heath RJ, Jackowski S, Rock CO (1994) Guanosine tetraphosphate inhibition of fatty acid and phospholipid synthesis in *Escherichia coli* is relieved by overexpression of glycerol-3-phosphate acyltransferase (*plsB*). J Biol Chem 269(42):26584–26590

Iram SH, Cronan JE (2005) Unexpected functional diversity among FadR fatty acid transcriptional regulatory proteins. J Biol Chem 280(37):32148–33256

Janßen HJ, Steinbüchel A (2014) Fatty acid synthesis in *Escherichia coli* and its applications towards the production of fatty acid based biofuels. Biotechnol Biofuels 7(1):7

Jerga A, Rock CO (2009) Acyl-acyl carrier protein regulates transcription of fatty acid biosynthetic genes via the FabT repressor in *Streptococcus pneumoniae*. J Biol Chem 284(23):15364–15388

Jiang P, Cronan JE (1994) Inhibition of fatty acid synthesis in *Escherichia coli* in the absence of phospholipid synthesis and release of inhibition by thioesterase action. J Bacteriol 176(10): 2814–2821

Kaczmarzyk D, Fulda M (2010) Fatty acid activation in cyanobacteria mediated by acyl-acyl carrier protein synthetase enables fatty acid recycling. Plant Physiol 152:1598–1610

López-Lara IM, Geiger O (2017) Bacterial lipid diversity. Biochim Biophys Acta 1862:1287–1299

Lu YJ, Rock CO (2006) Transcriptional regulation of fatty acid biosynthesis in *Streptococcus pneumoniae*. Mol Microbiol 59:551–566

Martinez MA, Zaballa ME, Schaeffer F, Bellinzoni M, Albanesi D, Schujman GE, Vila AJ, Alzari PM, de Mendoza D (2010) A novel role of malonyl-ACP in lipid homeostasis. Biochemistry 49(14):3161–3167

McCue L, Thompson W, Carmack C, Ryan MP, Liu JS, Derbyshire V, Lawrence CE (2001) Phylogenetic footprinting of transcription factor binding sites in proteobacterial genomes. Nucleic Acids Res 29(3):774–782

Miller DJ, Zhang YM, Subramanian C, Rock CO, White SW (2010) Structural basis for the transcriptional regulation of membrane lipid homeostasis. Nat Struct Mol Biol 17(8):971–975

Mondino S, Gago G, Gramajo H (2013) Transcriptional regulation of fatty acid biosynthesis in mycobacteria. Mol Microbiol 89(2):372–387

My L, Rekoske B, Lemke JJ, Viala JP, Gourse RL, Bouveret E (2013) Transcription of the *Escherichia coli* fatty acid synthesis operon *fabHDG* is directly activated by FadR and inhibited by ppGpp. J Bacteriol 195:3784–3795

My L, Ghandour Achkar N, Viala JP, Bouveret E (2015) Reassessment of the genetic regulation of fatty acid synthesis in *Escherichia coli*: global positive control by the dual functional regulator FadR. J Bacteriol 197:1862–1872

Parsons JB, Rock CO (2013) Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res 52:249–276

Parsons JB, Frank MW, Subramanian C, Saenkham P, Rock CO (2011) Metabolic basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis inhibitors. Proc Natl Acad Sci U S A 108(37):15378–15383

Pech-Canul A, Nogales J, Miranda-Molina A, Álvarez L, Geiger O, Soto MJ, López-Lara IM (2011) FadD is required for utilization of endogenous fatty acids released from membrane lipids. J Bacteriol 193:6295–6304

Schujman GE, Paoletti L, Grossman AD, de Mendoza D (2003) FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis. Dev Cell 4(5):663–672

Schujman GE, Guerin M, Buschiazzo A, Schaeffer F, Llarrull LI, Reh G, Vila AJ, Alzari PM, de Mendoza D (2006) Structural basis of lipid biosynthesis regulation in Gram-positive bacteria. EMBO J 25(17):4074–4083

Singh AK, Zhang YM, Zhu K, Subramanian C, Li Z, Jayaswal RK, Gatto C, Rock CO, Wilkinson BJ (2009) FabH selectivity for *anteiso* branched-chain fatty acid precursors in low-temperature adaptation in *Listeria monocytogenes*. FEMS Microbiol Lett 301(2):188–192

Tsai YT, Salzman V, Cabruja M, Gago G, Gramajo H (2017) Role of long-chain acyl-CoAs in the regulation of mycolic acid biosynthesis in mycobacteria. Open Biol 7(7):170087

Vadia S, Tse JL, Lucena R, Yang Z, Kellogg DR, Wang JD, Levin PA (2017) Fatty acid availability sets cell envelope capacity and dictates microbial cell size. Curr Biol 27(12):1757–1767

van Aalten DM, DiRusso CC, Knudsen J, Wierenga RK (2000) Crystal structure of FadR, a fatty acid-responsive transcription factor with a novel acyl coenzyme A-binding fold. EMBO J 19(19):5167–5177

Yao J, Rock CO (2017) Exogenous fatty acid metabolism in bacteria. Biochimie 141:30–39

Zhang YM, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6(3): 222–333

Zhang YM, Marrakchi H, Rock CO (2002) The FabR (YijC) transcription factor regulates unsaturated fatty acid biosynthesis in *Escherichia coli*. J Biol Chem 277(18):15558–15565

Zhang YM, Zhu K, Frank MW, Rock CO (2007) A *Pseudomonas aeruginosa* transcription factor that senses fatty acid structure. Mol Microbiol 66(3):622–632

Zhang F, Ouellet M, Batth TS, Adams PD, Petzold CJ, Mukhopadhyay A, Keasling JD (2012) Enhancing fatty acid production by the expression of the regulatory transcription factor FadR. Metab Eng 14(6):653–660

Zhu K, Choi KH, Schweizer HP, Rock CO, Zhang YM (2006) Two aerobic pathways for the formation of unsaturated fatty acids in *Pseudomonas aeruginosa*. Mol Microbiol 60:260–273

Zhu K, Zhang YM, Rock CO (2009) Transcriptional regulation of membrane lipid homeostasis in *Escherichia coli*. J Biol Chem 284(50):34880–34888

Components and Key Regulatory Steps of Lipid Biosynthesis in Actinomycetes

22

Gabriela Gago, Ana Arabolaza, Lautaro Diacovich, and Hugo Gramajo

Contents

1 Introduction 410
2 Acyl-CoA Carboxylases: An Overview of Their Distribution and Regulation in Actinomycetes 412
2.1 Subunits Composition and Biochemical Reaction of Acyl-CoA Carboxylases 412
2.2 Acyl-CoA Carboxylases in Actinomycetes 414
2.3 Genetic and Biochemical Regulation of Acyl-CoA Carboxylases 415
3 General Principles and Structural Diversity in FA Synthesis in Actinomycetes 416
3.1 Fatty Acid Synthesis in Streptomyces 419
3.2 Fatty Acid Synthesis in Corynebacterium 421
3.3 Fatty Acid Synthesis in Mycobacterium 421
4 Architecture of Microbial FAS I Megaenzymes 423
5 Fatty Acid Biosynthesis Regulation 424
6 Research Needs 427
References 428

Gabriela Gago, Ana Arabolaza, and Lautaro Diacovich have contributed equally to this article.

G. Gago · A. Arabolaza · L. Diacovich
Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina
e-mail: gago@ibr-conicet.gov.ar; arabolaza@ibr-conicet.gov.ar; diacovich@ibr-conicet.gov.ar

H. Gramajo (✉)
Department of Microbiology, Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina
e-mail: gramajo@ibr-conicet.gov.ar

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_65

Abstract

The biochemical steps in fatty acid synthesis are highly conserved in bacteria and in most organisms. However, the data provided by the massive genomic sequencing revealed a surprising amount of diversity in the genes, enzymes, and genetic organization of the components responsible for bacterial lipid synthesis, with these differences being even more striking in the order *Actinomycetales*. Fatty acid biosynthesis is energetically very expensive for the cell; therefore, adjusting the rate of fatty acid synthesis, in order to maintain membrane lipid homeostasis, is a key factor for bacterial survival. Bacteria have evolved sophisticated and diverse mechanisms to finely control the expression of the genes responsible for the synthesis of fatty acids and, in some cases, also by regulating the activity of the pacemaker enzymes. In this chapter we summarize the main components of fatty acid biosynthesis and their regulation in different genera of actinomycetes, highlighting the main differences found between them and also with other bacteria. The main focus has been put into the acyl-CoA carboxylases, the fatty acid synthases, and on the regulatory elements that control these pathways.

1 Introduction

Actinomycetes, so-called the group of Gram-positive bacteria that belongs to the order *Actinomycetales*, are widely distributed in the environment; as a consequence, they exhibit an enormous diversity in terms of their morphology, physiology, and metabolic capabilities. Several genera of this group of bacteria have been thoroughly studied because of their implication in human health, either as important human and animal pathogens (e.g., different species of *Mycobacterium* and *Corynebacterium*) or as one of the richest sources of natural products often used in the clinic as antibiotics, anticancer agents, immunomodulators, or cholesterol-reducing drugs (e.g., *Streptomyces* spp.). More recently, other genera of actinomycetes have also been the subject of considerable research studies due to their impact in biotechnological and/or industrial processes (e.g., *Corynebacterium*, *Rhodococcus*, *Cellulomonas*).

From the early studies carried out in the composition of the cell wall of different actinomycetes genera, such as *Mycobacterium*, *Corynebacterium*, and *Rhodococcus*, it was clear that lipid metabolism in these organisms had a higher level of complexity compared with the one present in the model system *Escherichia coli*. For instance, while the outer membrane of Gram-negative bacteria is a unique asymmetric lipid bilayer composed of phospholipids (PLs) in the inner leaflet and lipopolysaccharides (LPSs) in the outer leaflet, most actinomycetes share a common feature: a unique multilaminar outer membrane composed of peptidoglycan, complex polysaccharides, and both covalently linked lipids and free lipids/lipoglycans that form the inner and outer layer of the cell wall (Hoffmann et al. 2008; Daffé et al. 2014). Thus, the complex chemical nature of the lipid-derived molecules that form part of the actinomycetes outer membrane provides a unique opportunity to illustrate the complexity and diversity of lipid metabolic pathways in bacteria.

The first committed step of fatty acid (FA) biosynthesis in bacteria, as well as in animals and plants, is catalyzed by acetyl-CoA carboxylase (ACC), an enzyme complex that carboxylates acetyl-CoA to form malonyl-CoA, the common extender unit used in FA biosynthesis (Wakil et al. 1983). In actinomycete bacteria, however, this enzyme is part of a family of enzyme complexes called acyl-CoA carboxylases (ACCases) (Haase et al. 1982; Hunaiti and Kolattukudy 1982; Diacovich et al. 2002; Gago et al. 2006). This name was adopted because of the relaxed substrate specificity evolutionary acquired by some of these enzymes; this property allows some of these enzyme complexes to synthesize, besides malonyl-CoA, other short-chain α-carboxy-acyl-CoAs (e.g., methylmalonyl-CoA). These short-chain α-carboxy-acyl-CoAs can be used in these microorganisms by polyketide synthases (PKSs) for the biosynthesis of complex lipids or for the production of a broad array of polyketides natural products (Das and Khosla 2009). To add an additional level of complexity to this enzyme system, several orthologs and paralogs of the genes encoding for the different subunits that conform ACCases are usually present in the different genera of actinomycetes (Gago et al. 2011).

Regarding the de novo FA biosynthesis pathways, actinomycetes also show interesting characteristics. The genus *Streptomyces*, for example, contains the dissociated FA synthase type II system (FAS II) present in most bacteria. However, early studies on FA synthesis showed that mycobacteria contain the two types of FAS found in nature: the so-called eukaryotic like, or FAS I, involving a large multifunctional polypeptide and also the dissociated FAS II (Kikuchi et al. 1992). More recently, biochemical and genetics studies demonstrated that those actinomycetes that synthesize mycolic acids (MA) (long-chain α-branched, ß-hydroxylated FAs) use the FAS I system for de novo FA biosynthesis, while the dissociated FAS II system has been adapted to elongate the FAS I-derived long-chain FAs to form the meromycolic chain of the MAs (for a review see Takayama et al. 2005; Quémard 2016). As an exception, *Corynebacterium* spp., that also produce MAs, only harbors a FAS I system. This enzyme synthesizes the de novo long-chain FAs that are then used for the synthesis of both, membrane phospholipids and MAs (Kalinowski et al. 2003).

While in most bacteria the FAs produced by the biosynthetic pathway are incorporated mainly as phospholipids into the membrane, in actinomycetes the products of FA biosynthesis may have other destinies besides the synthesis of phospholipids and MAs. For example, they can also be the substrate of specific PKSs for the synthesis of other complex lipids like phthiocerol dimycocerosate, sulfolipids, and mycoketides (Camacho et al. 1999; Cox et al. 1999; Astarie-Dequeker et al. 2009) or be the substrates of diacylglycerol acyltransferases to synthesize triacylglycerides (TAG) as storage compounds, another remarkable aspect of some actinomycete metabolism (Alvarez and Steinbüchel 2002). The biosynthesis of TAG has been studied in several representatives of these organisms and key enzymes of this pathway, like phosphatidic acid phosphatases (Comba et al. 2013; Hernandez et al. 2015) and diacylglycerol acyltransferases have been thoroughly characterized in different genera (for a review see Rottig and Steinbuchel 2013; Alvarez 2016).

The high competition of the de novo synthesized long-chain FAs by the different pathways mentioned above highlights the need of a tightly regulated network

in order to preserve membrane homeostasis and therefore the survival of these organisms. The high level of sophistication needed to keep lipid homeostasis in these bacteria and the mechanisms involved in this global coordination are only recently beginning to be understood (Mondino et al. 2013; Hernandez et al. 2017; Tsai et al. 2017).

This chapter attempts to capture and organize the diversity found in key steps of FA metabolism in actinomycetes and provides an overview of the emerging regulatory network that coordinates this central metabolism with other lipid related pathways.

2 Acyl-CoA Carboxylases: An Overview of Their Distribution and Regulation in Actinomycetes

The enzymatic complexes of the acyl-CoA carboxylases (ACCases) are biotin-dependent proteins extensively distributed in nature, being found in human, plants, archaea, fungi, and bacteria. This family of enzymes mostly uses coenzyme A (CoA) thioesters of short-chain organic acids as its substrates. The carboxylation occurs either in the α carbon of saturated acids, for example, acetyl- or propionyl-CoA, or at the γ carbon of the α-β unsaturated acid, such as 3-methylcrotonyl-CoA or geranyl-CoA. Here we focused on the α-ACCases, since their main role is associated with the metabolism of FAs and lipids in most organisms (Wakil et al. 1983; Tong 2013). Although some of these enzymes have relaxed substrate specificity, they are usually referred as acetyl-CoA carboxylases (ACC), propionyl-CoA carboxylases (PCC), and long-chain acyl-CoA carboxylases (LCC) (Tong 2013), according to their preferred substrate.

2.1 Subunits Composition and Biochemical Reaction of Acyl-CoA Carboxylases

In general, ACCases harbor three different components, the biotin carboxylase (BC), the biotin carboxyl carrier protein (BCCP), and the carboxyltransferase (CT). The overall reaction of these enzymes occurs in two steps; in the first half-reaction, the BC component catalyzes the phosphorylation of bicarbonate, dependent on ATP and Mg^{2+}, to form a carboxyphosphate intermediate. Then, the carboxyl group is transferred to the cofactor biotin to form carboxybiotin (Blanchard et al. 1999; Waldrop et al. 1994). Biotin is covalently attached to the BCCP component through an amide bond to a conserved lysine residue (Athappilly and Hendrickson 1995; Cronan 2001). Once the biotin is carboxylated, it is translocated to a second active site present in the CT component, where the carboxylation of the acyl-CoA occurs. The organization of the BC, BCCP, and CT components in ACCase enzymes can vary depending on the organism. Most eukaryotic ACC complexes are large multifunctional single-chain proteins containing the three active components in discrete domains (Cronan and Waldrop 2002). In contrast, in *E. coli* and in several bacterial ACC, the BC, BCCP, and CT components are organized in four separate

polypeptides, which interact to constitute the functional enzyme complex (Cronan and Waldrop 2002) (Fig. 1). Alternatively, several ACCases from actinomycetes, eukaryotic organelles, and other bacteria consist of two polypeptides, α and β. In these cases, the α subunit possesses both the BC and BCCP components with the capability to carboxylate its covalently bound biotin group, while the β subunit holds the CT activity (Diacovich et al. 2002; Gago et al. 2006). A particularity found in some actinomycetes is the presence of a third subunit, called ε (7–10 kDa), needed for maximal enzyme activity. So far the exact stoichiometry of this subunit in each complex has not been unambiguously determined

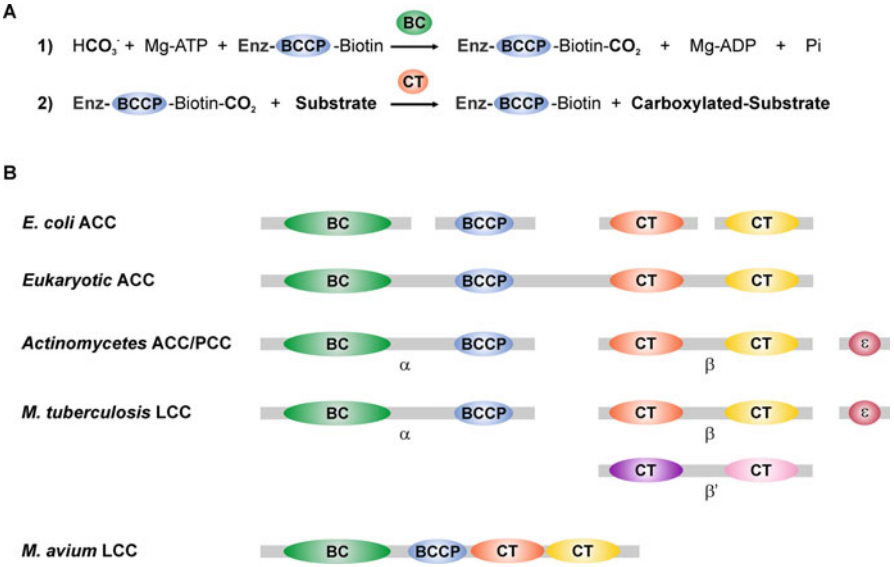


Fig. 1 Reaction and organization of ACCase complex enzymes. (**a**) The biochemical activity of biotin-dependent carboxylases occurs in two sequential reactions. First, a biotin carboxylase (BC) component catalyzes the carboxylation of the cofactor biotin in a Mg^{2+} and ATP-dependent step. Biotin is covalently linked to a conserved residue of lysine in the biotin carboxyl carrier protein (BCCP) component. In the second step, the carboxyltransferase (CT) component catalyzes the carboxyl transfer from carboxybiotin to the acyl-CoA acceptor (acetyl-, propionyl-, or long-chain acyl-CoA). (**b**) Schematic diagram of the component organization of several representative ACCases. Bacterial ACC is composed of multiple subunits; the *E. coli* ACC is formed by four proteins: BC, BCCP, and two proteins that form the CT subunit. The eukaryotic ACC is a multifunctional polypeptide chain that contains the three components (BC, BCCP, and CT). In actinomycetes, the ACC/PCC complexes are made up of two main subunits: the α subunit, which contains the BC and BCCP components, and the β subunit, which has the CT activity. In addition, some actinomycete ACCases may include a third subunit, ε, the presence of which dramatically stimulates the specific activity of the enzyme complexes. *M. tuberculosis*-LCC complex is conform by a unique α subunit, a ε subunit, and two β subunits (β and β′), and the four subunits are essential for its activity. *Mycobacterium avium* subspecies *paratuberculosis* LCC holoenzyme has an architecture that is strikingly different compared to those of related biotin-dependent carboxylases. This enzyme consists of a single-chain (120 kDa) multi-domain biotin-dependent carboxylase

(Diacovich et al. 2002; Gago et al. 2006; Bazet Lyonnet et al. 2017). Finally, two different organizations were found for the LCC complexes characterized from different actinomycetes. One consists of a single-multi-domain protein, and the other is composed of four separate polypeptides (Gande et al. 2004, 2007; Tran et al. 2015; Bazet Lyonnet et al. 2017) (Fig. 1).

2.2 Acyl-CoA Carboxylases in Actinomycetes

The complexity found in the subunit arrangement of the different actinomycete ACCases is related with their diverse physiological roles and flexibility to recognize different substrates in order to provide varied extender units for the biosynthesis of the rich diversity of complex lipids present in these bacteria. For example, *Mycobacterium tuberculosis* has six putative ACCase complexes; three of them (ACCase 5, ACCase 6, and LCC) are essential for bacterial viability and were found to be involved in lipid metabolism, as they have been characterized at the biochemical and/or genetic levels (Gago et al. 2006; Daniel et al. 2007; Kurth et al. 2009; Pawelczyk et al. 2011; Bazet Lyonnet et al. 2017). Remarkable, the three enzyme complexes mentioned above share the same α subunit (AccA3) inferring that substrate specificity, and therefore the different physiological roles of these enzymes, is given by the CT subunits. AccA3 interacts with AccD6 to form an ACC/PCC enzyme, named ACCase 6, whose main role appears to be the generation of malonyl-CoA from acetyl-CoA, thus providing the precursors for de novo FA biosynthesis by FAS I and for the elongation of FAS I products by the FAS II complex to produce meromycolic acids (Daniel et al. 2007; Kurth et al. 2009). The CT subunit AccD6 has been shown to be essential for *M. tuberculosis* viability (Pawelczyk et al. 2011). Also, AccA3 together with the CT subunit AccD5 and the small ε subunit AccE5 conforms a second enzyme complex named ACCase 5, which also has ACC and PCC activities to produce both malonyl-CoA and methylmalonyl-CoA, respectively (Gago et al. 2006; Oh et al. 2006; Lin et al. 2006). This later product is the extender unit for the biosynthesis of multimethyl-branched FAs. Finally, the LCC complex formed by AccA3, the ε subunit AccE5, and the two β subunits AccD4 and AccD5 produces long-chain α-carboxy-acyl-CoAs, one of the substrates of the polyketide synthase PKS13 that catalyzes the last condensation step of MA biosynthesis (Bazet Lyonnet et al. 2017). In *S. coelicolor*, two ACCase complexes were characterized, one containing ACC and PCC activities (AccA2-AccB-AccE), whose main role is to provide malonyl-CoA for FA and actinorhodin biosynthesis, and a second one that only has PCC activity (AccA2-PccB-PccE), being capable of producing methylmalonyl-CoA. On the other hand, *C. glutamicum* has four CT subunits (AccD1, AccD2, AccD3, AccD4), only one BC-BCCP subunit (AccBC), and one ε subunit (AccE). As in mycobacteria, AccBC α subunit interacts either with AccD1 and AccE, to build an ACC/PCC complex, or with AccD2, AccD3 (*Mtb* AccD4 ortholog), and AccE subunits to conform an LCC complex (Gande et al. 2004, 2007). These two complexes provide the precursors for the production of FAs and MAs in this organism. *C. glutamicum* AccD4 is a CT

subunit orthologous to *Mtb* AccD3; however they have not been characterized yet. In *Mycobacterium avium* subspecies *paratuberculosis*, a distinctive LCC complex has been recently characterized. This enzyme is a novel single-chain multi-domain biotin-dependent carboxylase of 120 kDa, with preference for long-chain acyl-CoAs as substrates, although it is also active with short- and medium-chain acyl-CoAs (Tran et al. 2015). The structure of this enzyme was determined by crystallography showing that the holoenzyme is a homo-hexamer with molecular weight of 720 kDa. In the same work, the authors studied at a biochemical level an LCC homologue enzyme from *Rhodopseudomonas palustris*, which showed activity with acyl-CoAs with a wide range of chain lengths (from C_2 to C_{16}); however the *K*m for palmitoyl-CoA resulted ~350-fold lower than that for acetyl-CoA (Tran et al. 2015).

2.3 Genetic and Biochemical Regulation of Acyl-CoA Carboxylases

The modulation of ACCase expression and activity was extensively studied in *E. coli* and mammals. *E. coli* ACC activity is inhibited by guanosine alarmones (Polakis et al. 1973; Cronan and Waldrop 2002) and by acyl-ACPs (C_6 to C_{20}) (Davis 2001). In mammals, there is hormonal control of ACC gene expression and a short-term modulation mediated by reversible phosphorylation or by allosteric regulation via citrate and long-chain acyl-CoAs (Munday 2002). At present none of these types of regulation has been described for any of the actinomycete ACCases.

In the actinomycete *Saccharopolyspora erythraea*, the protein PccD has been recently identified as a transcriptional repressor of the genes encoding for α, β, and ε subunits of a PCC complex in charge of providing methylmalonyl-CoA as starter units for the synthesis of erythromycin (Xu et al. 2017). In this case, methylmalonic acid is suggested to be the ligand recognized by PccD to modulate its repressor activity. In some of the actinomycete ACCases, the α and β subunits constitute the core enzyme complex with a basal activity, while the presence of the ε subunit drastically stimulates their specific enzyme activity. This small basic polypeptide sometimes acts as an accessory protein that helps the stabilization of the α-β interaction allowing maximal enzyme activity (Diacovich et al. 2002; Gago et al. 2006). The ε subunit is a distinctive feature of some of the actinomycete ACCases (Diacovich et al. 2002; Gago et al. 2006; Gande et al. 2007). We could speculate that the modulation of activity mediated by the ε subunit possesses a strictly structural nature in this intricate protein-protein interaction scenario; however, we cannot rule out the possibility of this subunit being a regulator of the enzyme activity. Finally, in *M. tuberculosis* the protein Wag31 (a DivIVA ortholog) acts as a coordinator of polar growth complexes. It was observed that Wag31 interacts with AccA3 in order to recruit the ACCase complexes to the growing pole regions, close to the subpolar space where the cell wall biosynthetic enzymes are located (Meniche et al. 2014; Xu et al. 2014). In this way Wag31 could control the positioning of AccA3 inside the cell with the purpose of localizing the precursors for the biosynthesis of lipids. However, until now, the modulation of ACC activity mediated by Wag31 has not been addressed.

3 General Principles and Structural Diversity in FA Synthesis in Actinomycetes

Fatty acids fulfill a variety of crucial functions, being the main constituents of biological membranes, serving as energy storage compounds, acting as signaling molecules, and being involved in protein modification. All organisms employ a conserved set of chemical reactions to achieve the biosynthesis of fatty acids (FA), even though, there exist variations in the structural organization of the fatty acid synthesis (FAS) machinery (Rangan and Smith 2003; White et al. 2005). In general, the process of biosynthesis of these lipid compounds is accomplished by the sequential extension of the growing carbon chain, two carbons at a time, through a series of decarboxylative condensation reactions. The first step comprises the formation of a new carbon-carbon bond by condensation of a primer substrate, usually acetyl-CoA and, the chain extender substrate, malonyl-CoA. This reaction is coupled with an energetically favorable decarboxylation, so that the carbon originating from CO_2, introduced in the reaction catalyzed by acetyl-CoA carboxylase, is recycled (Rangan and Smith 2003). In this process the chain extender substrate (malonyl-CoA) is transferred via the nucleophilic serine residue of an acyltransferase enzyme or domain (AT) to the acyl carrier protein (ACP), and the β-ketoacyl synthase (KS) catalyzes the decarboxylative condensation between the acetyl moiety and malonyl-ACP to form the new ACP-bound β-ketoacyl intermediate. The β-carbon position of this intermediate (as well as that generated in the subsequent condensation reactions) is then modified by consecutive action of the NADPH-dependent β-ketoreductase (KR), a dehydratase (DH), and the NADPH-dependent enoyl reductase (ER) to yield a saturated acyl-ACP product. This acyl-ACP functions as a starter substrate for the next round of elongation, until the growing FA chain reaches a length of 16–18 carbon atoms, being then (in the final step) released from ACP.

Regarding the organization and the architecture of the enzymatic activities needed for FA biosynthesis, FAS systems are grouped into two major classes, type I and II. In the dissociated type II FAS systems, each enzyme of the pathway is encoded by a separate gene and exists as a soluble monofunctional protein. These type II systems are found mostly in bacteria but also in specialized eukaryotic organelles such as mitochondria and plastids in plants. In contrast, type I FAS systems of fungi and animals are huge multifunctional polypeptides that integrate all steps of FA synthesis into large macromolecular assemblies, usually one or two large polypeptide chains (Rock and Jackowski 2002; White et al. 2005; Leibundgut et al. 2008).

As mentioned above, the scenario of FAS systems diversity (type I and II) in actinomycetes is unique (Figs. 2, 3, and 4). These complex genetic endowments reflect, in part, the important differences between the components of the cellular envelope in the *Actinomycetales* group. Particularly, the *Streptomyces* members that undergo a complex life cycle with distinctive developmental and morphological stages and that produce a vast array of biologically active secondary metabolites (some of them related with the FA metabolism, see below) (Manteca et al. 2008) possess a cell wall similar to that of other Gram-positive bacteria, composed of a simple peptidoglycan mesh surrounding the cytoplasmic membrane. On the other

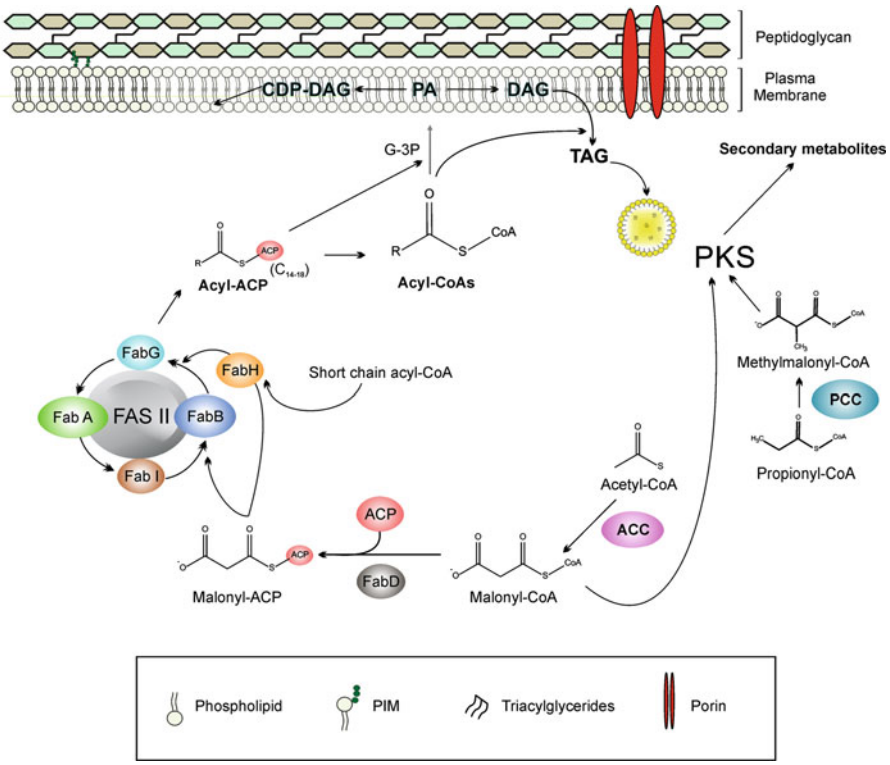


Fig. 2 Schematic representation of the individual enzyme system involved in de novo fatty acid biosynthesis in *Streptomyces* (type II FAS). The de novo synthesized fatty acids can be incorporated into membrane phospholipids or stored as neutral lipids (TAG). As in other typical Gram-positive bacteria, the cell wall consists of a peptidoglycan layer that covers the cytoplasmic membrane

hand, the cell walls of *Mycobacterium*, *Corynebacterium*, *Gordonia*, *Nocardia*, and *Rhodococcus* are composed by a thick meso-diaminopimelic acid-containing peptidoglycan covalently linked to arabinogalactan, which in turn is esterified to MA. In contrast to the linear FAs of the phospholipids, MA are α-branched β-hydroxyl FAs that requires carboxylation and a PKS-mediated condensation of two FAs for their synthesis (Portevin et al. 2004). In mycobacteria, MA are composed of very long-chain FAs of a total of 60–90 carbon atoms that may contain various oxygen functions in addition to the β-hydroxyl group (Daffe and Draper 1998). The MA found in other actinomycetes consist of a mixture of saturated and unsaturated FAs with shorter carbon chains. In corynebacteria, MA possesses around 30 carbon atoms (corynomycolates) and in rhodococci varies from 30 to 50 carbon atoms (Sutcliffe 1997; Daffe and Draper 1998; Puech et al. 2001).

MA are major and specific lipid components of the mycobacterial cell envelope and are essential for the survival and virulence of members of the genus *Mycobacterium*. Besides these properties, MA also have a central role in the

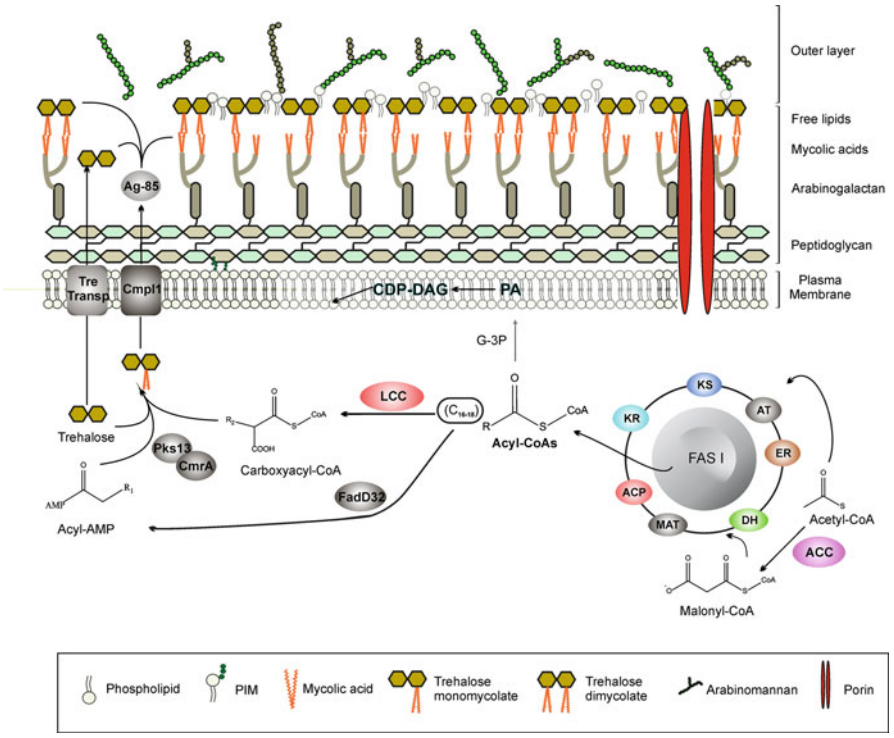


Fig. 3 Biosynthesis of fatty acids by the multifunctional type I system in *Corynebacterium*. The FAS I products are the substrates for the biosynthesis of membrane phospholipids and also for the biosynthesis of the corynomycolic acids that conform the complex cell wall envelope. FadD32 catalyzes the fatty acid's activation into their acyl-AMP derivatives. The CmrA enzyme performs the reduction of the β-keto function to yield the final corynomycolic acid motif. Cmpl1 mediates the translocation of trehalose monocorynomycolates

formation of a second permeability barrier functionally similar to the outer membrane in Gram-negative bacteria (Gebhardt et al. 2007). In mycobacteria and corynebacteria, this outer layer consists of a monolayer of mycoloyl residues covalently linked to the cell wall arabinogalactan, to form mycoloyl arabinogalactan, or acylated to trehalose units to form trehalose monomycolate (TMM) and trehalose dimycolate (TDM) (Brennan 2003; Daffe 2008). Some species of mycobacteria also contain an array of very complex lipids relevant in the infection process of the pathogenic species, such as dimycocerosate esters, sulfolipids, lipooligosaccharide, and mycoketides (Astarie-Dequeker et al. 2009). Finally, besides the MA layer, the top outer surface of these microorganisms is composed of free polysaccharides, glycolipids, and proteins (Chiaradia et al. 2017).

Therefore considering the complex, diverse, and specialized metabolism that encompassed the FA biosynthetic pathways in Actinobacteria, we briefly describe in the next three sections some distinctive features that are unique to each one of the three major genera we are focusing in the chapter.

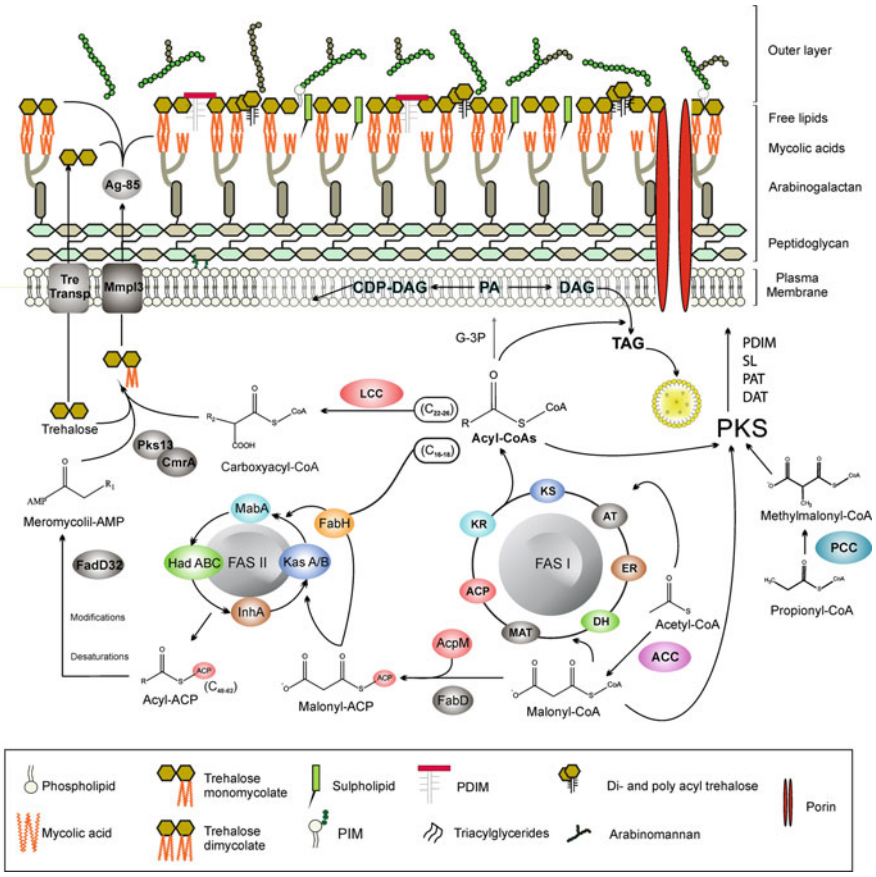


Fig. 4 Comprehensive representation of the lipid biosynthesis pathways and their interactions in *Mycobacterium*. De novo synthesis of fatty acids is operated by the mycobacterial multifunctional fatty acid synthase (FAS I). These fatty acid molecules are used for the biosynthesis of membrane phospholipids and TAGs and are also the substrates of the PKSs involved in the biosynthesis of the complex lipids present in the cell wall. Further, these molecules initiate the elongation process catalyzed by the dissociated FAS II system to yield the meromycolic acid chains, which after being activated by the enzyme FadD32 to their acyl-AMP derivatives are then condensed with the α-carboxy long-chain acyl-CoA to finally produce the mycolic acids. The α-carboxy long-chain acyl-CoA is the product of the carboxylation of the long-chain acyl-CoAs produced by FAS I by the long-chain acyl-CoA carboxylase (LCC). The mature mycolic acids under the form of trehalose mycolates are transported through the plasma membrane by MmpL3. The mycoloyltransferases Ag85 complex generate the derivative mycolate-containing compounds

3.1 Fatty Acid Synthesis in Streptomyces

Streptomyces produce primarily branched-chain FAs (80–90% of total FA content) with only a minor proportion of straight-chain FAs (Hoischen et al. 1997). The first step in the type II FAS process is carried out by FabH (β-ketoacyl synthase III),

which catalyzes a decarboxylative condensation of an acyl-CoA primer with malonyl-ACP (Fig. 2). For branched-chain FAs, the primers are typically either isobutyryl, isovaleryl, or anteisovaleryl units to give odd- and even-numbered FAs with a methyl branch at the ω-terminus. For straight-chain FAs, acetyl-CoA, butyryl-CoA, and propionyl-CoA serve as the most common primers (Kaneda 1991).

β-ketoacyl-ACP synthase enzymes are generally divided into classes: III, II, and I. Class III catalyze a Claisen condensation between acetyl-CoA and malonyl-ACP, thus being involved in initiating FA biosynthesis. β-ketoacyl-ACP synthases I and II only catalyze acyl-ACP reactions with malonyl-ACP, hence participating in the process of FA chain elongation. The enzyme responsible of the subsequent rounds of FA elongation in type II FAS is denominated FabF (β-ketoacyl synthase II). As already mentioned, the malonyl-ACP utilized in each elongation step is generated from malonyl-CoA by the action of an AT enzyme, precisely a malonyl-CoA/ACP transacylase encoded by *fabD* gene. The resulting 3-ketoacyl-ACP product is reduced by NADPH-dependent FabG (KR) to provide 3-hydroxyacyl-ACP, which is dehydrated by FabA (DH) to form enoyl-ACP. Then, the NADH-dependent FabI (ER) completes the cycle by catalyzing a reduction to provide the corresponding acyl-ACP. In streptomycetes, *fabD*, *fabH, acpP*, and *fabF* are clustered together forming an operon (Revill et al. 1995), where this genomic arrangement presents a remarkable synteny among actinomycetes (Gago et al. 2011).

Particularly in *Streptomyces*, FA biosynthesis pathway influences, overlaps, and displays a complex relationship with some secondary metabolite production. Most work on this metabolic crosstalk has focused on FabD, a well-established case of a crosstalk between FA biosynthesis and polyketide biosynthesis (Florova et al. 2002). For instance, *S. coelicolor* has a single copy of *fabD*. FabD has no significant ACP selectivity, being able to catalyze the formation of various malonyl-ACP derivatives. At least four ACP homologs are present in this microorganism; AcpP is dedicated to FAS II (Revill et al. 1996), whereas the other ACPs are required for the synthesis of the secondary metabolites produced by *S. coelicolor*: the blue-pigmented antibiotic actinorhodin, the gray pigment associated with the spore wall (Revill et al. 1996), and the red pigment undecylprodiginine (Mo et al. 2008). Thus, in this microorganism FabD generates intermediates for both the primary and the secondary metabolism. It was generally proposed that the control over these two processes or metabolism lies partially in the initial condensing enzymes (Singh et al. 2012). For example, for undecylprodiginine biosynthesis, homologues of the condensing enzymes (FabH and FabF) and the ACP (AcpP) are encoded by *redP*, *redR*, and *redQ*, respectively. These genes are located in the undecylprodiginine biosynthetic locus. RedP initiates biosynthesis of undecylprodiginine's alkyl chain by condensing an acyl-CoA with malonyl-RedQ, and then RedR would catalyze the subsequent condensation steps. Each resulting 3-ketoacyl-RedQ intermediate would presumably be reduced by the type II FAS enzymes FabG, FabA, and FabI. Specifically, RedP utilizes acetyl-CoA, whereas FabH is more efficient with branched acyl-CoA substrates, and both enzymes possess differing ACP specificities: RedP only works with RedQ, whereas FabH is more efficient with AcpP (Singh and Reynolds 2015). Therefore, possessing dedicated condensing enzymes and individual ACPs proteins,

while sharing the de novo FA processing enzymes (FabG, FabA, and FabI), appears to be the clue for differentiating FA and undecylprodiginine biosynthesis.

However, this picture varies between each *Streptomyces* species, since the number and distribution of clusters of genes involved in secondary metabolism are particular to each microorganism. Thus, competition for substrates, crosstalk, and homeostasis regulation between primary metabolism and secondary metabolism is individually achieved by each microorganism according to its own genetic background, nutritional conditions, and environmental adaptation.

3.2 Fatty Acid Synthesis in Corynebacterium

Most corynebacteria genome sequences highlight the absence of FAS II genes but the presence of two putative type I FAS genes (Fig. 3). The exceptions are the pathogenic species *C. diphtheriae* that only contains one type I FAS and *C. jeikeium* and *C. urealyticum* that contain no *fasI* gene, reflecting their strict dependence for growth on the presence of exogenous FAs (Tauch et al. 2005, 2008). Gene knockout experiments in *C. ammoniagenes* and *C. glutamicum* show that only one of the two FAS enzymes is essential for cell survival (denominated FasA and Fas IA in *C. ammoniagenes* and *C. glutamicum*, respectively), while the second (FasB and Fas IB in *C. ammoniagenes* and *C. glutamicum*, respectively) is supplementary (Radmacher et al. 2005). Duplicated versions of genes with similar or related functional activities frequently occur in Actinobacteria; however, both enzymes are necessary to produce the characteristic lipid pattern of these organisms. For example, the FAs present in *C. glutamicum* are dominated by $C_{16:0}$ and $C_{18:1}$ (Radmacher et al. 2005), and also tuberculostearic acid ($10MeC_{18:0}$) is present in minor proportion, being this branched-chain FA a characteristic feature for a number of *Corynebacterium*. In the absence of *fas IB*, the relative content of palmitate is strongly reduced, suggesting that palmitate is primarily synthesized by the Fas IB enzyme. Inactivation of *C. glutamicum fas IA* gene results in an unsaturated FA growth requirement (Radmacher et al. 2005). Indeed, in vitro, it has been shown that FAS IA synthesizes mainly $C_{18:1}$ (oleate) and $C_{18:0}$ (stearate), with only traces of the $C_{16:0}$ (palmitate), while FAS IB product is the $C_{16:0}$ (Radmacher et al. 2005; Lanéelle et al. 2013).

The consequences of these *fas* gene mutations are also manifested by an alteration in the profile of MA. The noticed direct relationship between the structure of MA and the profile of FAs present in a given corynebacterial species has led researchers to early suggest that MA result from the condensation of two FAs synthesized by the dedicated FAS I (Gastambide and Lederer 1960).

3.3 Fatty Acid Synthesis in Mycobacterium

Mycobacteria are unique in possessing both type I and type II FAS systems. The de novo FA biosynthesis is carried out by a multifunctional FAS I enzyme, catalyzing the production of long-chain acyl-CoAs from acetyl-CoA and malonyl-CoA

(extender unit). The FAS I $C_{16:0}$-CoA and $C_{18:0}$-CoA derivatives are used primarily for the synthesis of membrane phospholipids or further elongated by the same FAS I enzyme to produce $C_{24:0}$ in the fast-growing organism *M. smegmatis* and $C_{26:0}$ products in the slow-growing *M. bovis* and *M. tuberculosis* (Kikuchi et al. 1992). This is a distinguishing behavior of mycobacterial FAS I called "bimodal product distribution." Indeed, mycobacterial FAS I functional studies determined that this enzyme can either produce de novo $C_{16:0}$–$C_{18:0}$ and $C_{22:0}$–$C_{26:0}$-FAs (Kikuchi et al. 1992). Subsequently, the type II FAS enzymes mediate the elongation of the $C_{18:0}$–$C_{16:0}$ precursors to up to C_{48}–C_{62}-meromycolic acids that are then condensed with an α-carboxy $C_{22:0\text{-}26:0}$-fatty acyl-CoA to yield the C_{60}–C_{90}-MAs (Portevin et al. 2004). As described above, the α-carboxy $C_{22\text{-}26}$-fatty acyl-CoAs are generated by a dedicated LCC which uses as substrates the corresponding FAS I products (Portevin et al. 2005).

In *M. tuberculosis* the *fasI* gene has been predicted to be essential by high-density mutagenesis, and it was shown to be essential in *M. smegmatis* by the construction of a conditional *fasI* mutant (Sassetti et al. 2003; Cabruja et al. 2017). The occurrence of type I FAS in these bacteria correlates with MA biosynthesis. Specifically in this microorganism, the C_{48}-C_{62}-meromycolic acid biosynthesis is carried out by the FAS II enzymes, where the process is initiated by a unique key condensing enzyme: the β-ketoacyl-ACP synthase III or mtFabH (Fig. 4). This enzyme catalyzes a decarboxylative condensation of malonyl-ACP with the acyl-CoA ($C_{16:0}$–$C_{20:0}$) products of the type I FAS. The resulting 3-ketoacyl-AcpM derivative is then reduced to the corresponding saturated acyl-AcpM and shuffled into the FAS II cycle. Biochemical studies showed that mtFabH can utilize a wide range of $C_{12:0}$–$C_{20:0}$ acyl-CoA substrates and suggested a possible role of AcpM in modulating the substrate specificity of this enzyme (Brown et al. 2005). Structural characterization and site-directed mutagenesis studies distinguished mtFabH from *E. coli* FabH and other type II FabH enzymes that typically utilize C_2–C_6 acyl-CoA as substrates (Scarsdale et al. 2001; Brown et al. 2005). Further, general transposon mutagenesis indicated that mtFabH is not essential for this bacterial viability (Sassetti et al. 2003). However, additional studies will be needed to determine the role of mtFabH in vivo.

Next, the elongation steps that follow the FabH-dependent initial condensation are conducted by the β-ketoacyl-AcpM enzymes encoded by *kasA* and *kasB*. These two genes are part of an operon with the conserved arrange of *fabD-acpM-kasA-kasB* genes. The specific roles of KasA and KasB in FA elongation to yield the meromycolates demonstrated that KasA extended C_{16} substrates to monounsaturated FAs averaging 40 carbon units, while KasB synthesized longer multi-unsaturated acyl chains averaging 54 carbon units (Slayden and Barry 2002). KasA has an absolute requirement for ACP derivatives as substrates, catalyzing the condensation of acyl-AcpM with malonyl-AcpM, for elongating the growing meromycolate chain by a further two carbons (Schaeffer et al. 2001; Borgaro et al. 2011). *kasA* is proposed to be essential for survival, whereas *kasB* mutants of *M. marinum* (Gao et al. 2003) and of *M. tuberculosis* (Bhatt et al. 2007) gave rise to strains which produced meromycolate chains shorter than the wild-type mycolates. Also, the

M. marinum kasB mutant showed a significant reduction of the keto-mycolates, while the *M. tuberculosis kasB* mutant presented a significant reduction of the *trans*-cyclopropanated oxygenated mycolates.

The rest of the FAS II components are the β-ketoacyl-ACP reductase MabA (KR), 2-*trans*-enoyl-ACP reductase InhA (ER), and the dehydratase complex HadA–HadB–HadC (DH) (Marrakchi et al. 2002; Sacco et al. 2007). The InhA protein was demonstrated to catalyze the 2-*trans*-enoyl-ACP reduction, with a preference for long-chain substrates (Quemard et al. 1995), and it was identified as the target for the drugs isoniazid and ethionamide (Banerjee et al. 1994).

Finally, in *M. tuberculosis*, the α-carboxy $C_{26:0}$ FA generated by FAS I and the meromycolate-AcpM from FAS II are condensed by the Pks13 to form the MAs. Other dedicated PKSs that utilize as substrates the FAS I products are involved in the biosynthesis of the additional complex lipids of the cell wall envelope of certain mycobacteria. Since their distribution is more restricted and as they are not essential for in vitro growth (although they are involved in pathogenicity), we will not include their biosynthetic pathways in this chapter. For an excellent review on this topic, see Chopra and Gokhale (2009).

4 Architecture of Microbial FAS I Megaenzymes

FAS I megaenzymes can be subdivided according to their domain arrangements and oligomerization state. Mycobacterial and corynebacterial type I FAS enzymes consist of a single polypeptide organized as hexamers (α6), whereas the fungal FAS I comprises the same domain sequence as the microbial enzymes but is organized from two polypeptides forming a homohexameric or heterododecameric complex (Schweizer and Hofmann 2004; Leibundgut et al. 2007; Lomakin et al. 2007). Briefly, in the bacterial FAS I, the linear catalytic domain order is AC-ER-DH-MPT-ACP-KR-KS, while in the fungal FAS I, the domain sequence is the same: AC-ER-DH-MPT/ACP-KR-KS-PPT (as mentioned divided in two proteins), but harboring the PPT domain (phosphopantetheine transferase) as part of one of their polypeptide chains. PPT is responsible for the posttranslational modification of ACP, catalyzing the transfer of a 4′-phosphopantetheine (4′-PP) moiety from coenzyme A (CoA) to a highly conserved serine residue of the ACP protein, reaction that is initially necessary for turning the FAS into its catalytically active state. AC is a dedicated acetyltransferase in charge of loading the primer substrate, while MPT domain possesses an acyltransferase activity catalyzing a malonyl/palmitoyl-transacylase reaction.

By contrast, this organization of domains differs from that of the animal FAS megaenzyme (mFAS) that presents the following arrange: KS–AT–DH–ER–KR–ACP–TE (Schweizer and Hofmann 2004). The thioesterase (TE) domain is unique to animal FAS and determines the length of the fatty acyl chain and its ultimate release by hydrolysis. mFAS adopts an X-shaped homodimeric structure of 540 kDa (Maier et al. 2008).

As discussed below, the occurrence of acyltransferase domains depends on the particular FAS system and is exclusive of each FAS I enzyme given the catalytic

properties of these domains. Thus, regarding their acylation characteristics, the MPT domain of fungal FAS I is actually a malonyl-/palmitoyl-transacylation site and resembles the bacterial MPT domain, being involved in loading the extender substrate and in the final step of releasing the product. Sometimes in Actinobacteria this domain is denoted MAT (malonyl/acyl-transacylase) due to its substrate specificity for malonyl-CoA, as well as a more relaxed acyl-CoA releasing activity of different chain lengths, other than palmitoyl-CoA. Furthermore, a significant difference here is that mFAS utilizes the same acyltransferase domain (AT) to load the primer and the chain extender substrates, whereas as mentioned, in fungal and bacterial FAS, dedicated acyltransferase domains (denominated AC) are responsible of loading the starter primer, while the loading of the chain extender substrate (malonyl-CoA) is performed by the MPT/MAT domains. In addition, as already mentioned, mFAS releases free FAs by hydrolytic cleavage catalyzed by a thioesterase (TE) domain (Schweizer and Hofmann 2004), while the FAS I of fungi, mycobacteria, and corynebacteria use the MPT/MAT activity for transacylation of the final product from the enzyme ACP domain to CoA molecule.

Crystallographic data and electron microscopic (EM) studies showed conformational dynamics of mFAS I folds (mobile ACP within highly flexible complex) (Brignole et al. 2009; Gipson et al. 2010). A structural model of the 1.9 MDa *M. smegmatis* FAS I was derived from flexible fitting of the fungal FAS I into moderate resolution EM data, and further studies indicated this bacterial FAS I as a minimized version of fungal FAS I (Boehringer et al. 2013; Ciccarelli et al. 2013). It is proposed that compared to mammalian FAS I, fungal and bacterial FAS I represent a different structural solution for type I FA synthesis (Grininger 2014).

5 Fatty Acid Biosynthesis Regulation

The de novo FA biosynthetic pathway is a key point for the regulatory events that control lipid homeostasis. This is crucial not only because the biophysical properties of membranes are determined mostly by the composition of the FAs that are produced by the de novo biosynthesis but also for the metabolic energy that is expended in the formation of these molecules. Most of the regulatory mechanisms that control FA metabolic pathways have been elucidated in model bacteria, such as *E. coli* (DiRusso and Nystrom 1998; Cronan and Subrahmanyam 1998; Zhang and Rock 2008) and *Bacillus subtilis* (Schujman et al. 2003, 2006; Fujita et al. 2007). These investigations revealed that there is a rapid response of the integrated biochemical network, acting at the level of the activities of lipid biosynthetic enzymes and a central control at the level of gene expression involving specific transcription factors. Extensive bioinformatic analysis carried out in different actinomycete genome databases demonstrated that these microorganisms lack genes orthologous to the transcription factors described in other genera. In most bacteria, the FAs produced by the biosynthetic pathway are mainly incorporated into the membrane. Actinomycetes represent an exception because the product of FA biosynthesis can be directed to other cellular components such as complex structural lipids (e.g., MAs or

PDIM) or storage lipids (TAG). This feature adds a higher level of sophistication to the regulation of lipid homeostasis in these bacteria, and the mechanisms involved in this global coordination are only recently beginning to be understood.

A major advance in the recognition of a transcriptional control of actinobacterial lipid synthesis occurred by the identification of $FasR_{SC}$, a transcription factor involved in the activation of the expression of the main *fab* operon (*fabD-fabH-acp-fabB)* in *S. coelicolor* (Arabolaza et al. 2010). This novel regulator is widely distributed among the Actinobacteria that relies on a type II FAS system for FA biosynthesis, including *Arthrobacter*, *Frankia*, *Rhodococcus*, *Nocardia*, *Acidothermus*, *Bifidobacterium*, *Streptomyces*, and *Mycobacterium*. It is interesting to note that MA-producing Actinobacteria relies on the FAS II system for the production of the very long-chain FAs that are precursors of meromycolate chains. The first transcriptional regulator of the mycobacteria main *fasII* operon (*fabD-acpM-kasA-kasB*) that was characterized is MabR, the orthologous of $FasR_{SC}$ (Salzman et al. 2010). Genetic studies showed that *mabR* is essential for *M. smegmatis* survival, and biochemical analysis carried out in a *mabR* conditional mutant strain showed alterations in MA and in de novo FA biosynthesis, demonstrating for the first time the existence of a crosstalk between the two FAS systems and confirming MabR as one of the key modulators of lipid homeostasis in *Mycobacterium* (Salzman et al. 2010; Tsai et al. 2017). A second nonessential transcription factor, FadR, that represses the *fasII* operon expression in mycobacteria has also been described (Biswas et al. 2013). FadR is induced upon starvation, leading to reduced *fasII* expression under those conditions (Yousuf et al. 2015). The *hadABC* genes, coding for the dehydratase complexes of the FAS II system, are part of a seven-gene operon together with four genes involved in translation, and they all respond to the alarmone (p)ppGpp which leads to dramatic reprogramming of cell transcription (Jamet et al. 2015). This suggests that the coordination of the expression of the complete set of FAS II genes is really complex and indicates that an eventual interplay exists between different regulatory pathways.

An exception to this situation occurs in *Corynebacterium*, where FA and MA synthesis is significantly different and no type II FAS exists and consequently no homologs to these transcription factors. As mentioned above, *C. glutamicum* has two FAS I enzymes, with the essential FAS IA being primarily responsible of stearate and oleate synthesis and FAS IB mostly of palmitate (Radmacher et al. 2005). Nickel et al. found that *fasA* and *fasB*, as well as the genes *accD1* and *accBC* coding for the ACC complex, are under transcriptional control of the TetR-type transcriptional regulator $FasR_{CG}$ and a specific operator site, *fasO*, during growth on acetate (Nickel et al. 2010). The current model of FA regulation in *Corynebacterium* is that acyl-CoA molecules, the products of the FA synthesis pathway, interact with $FasR_{CG}$ and the resulting complex represses the transcription of *accD1* and *fasA* (Irzik et al. 2014). This also indicates that ACC and FAS activities are major determinants of lipid homeostasis in *C. glutamicum*, as is the case with the ACC in *E. coli*.

The transcription of the *fas-acpS* operon of mycobacteria (encoding for the multi-domain FAS I and the phosphopantetheinyl transferase AcpS) is regulated by FasR (Mondino et al. 2013). Analysis of a conditional mutant in *M. smegmatis* showed

that FasR is a transcriptional activator of the *fas-acpS* operon. Using in vitro and in vivo approaches, it was demonstrated that long-chain acyl-CoAs are the ligands that are sensed by FasR in order to adjust FA biosynthesis levels. FasR binds to $C_{16\text{-}20}$-CoA, and consequently it is released from its operator sites, leading to reduced levels of FA biosynthesis (Mondino et al. 2013). Although this regulatory protein also belongs to the TetR family of transcriptional regulators and is able to bind acyl-CoA molecules, it has no significant homology with FasR from *Corynebacterium* (<20%).

An important difference between *M. smegmatis* FasR and MabR with other bacteria transcriptional regulators of FA biosynthesis is that FasR and MabR are essential for bacterial survival (Salzman et al. 2010; Mondino et al. 2013), while the others have shown to be nonessential. Thus, the evolutionary divergence found for these regulatory proteins could be correlated with the different physiological roles of the FAS systems that each of them regulates.

Interestingly, the activity of most of these transcription factors (MabR, FadR, $FasR_{CG}$, and FasR) is modulated by long-chain acyl-CoAs, products of the FAS I system, highlighting a key role for these molecules in the modulation of lipid biosynthesis in MA-producing bacteria (Biswas et al. 2013; Mondino et al. 2013; Tsai et al. 2017).

The impact of having reduced levels of de novo FA biosynthesis was studied in a *fas* conditional mutant in *M. smegmatis* (Cabruja et al. 2017). As expected, *fas* was essential for survival, and in the nonpermissive conditions, the reduction of FAS I activity led to the accumulation of the FAS I substrates (acetyl-CoA and malonyl-CoA) and to a strong reduction of $C_{12\text{-}18}$ acyl-CoAs. Unexpectedly, even when de novo FA biosynthesis was impaired, the *fas* mutant was still able to synthesize MAs at the expense of TAG, suggesting that storage lipids could be an intracellular reservoir of FAs for the biosynthesis of complex lipids in mycobacteria. Understanding the interaction between FAS I and the metabolic pathways that rely on FAS I products is a key step to better understand how lipid homeostasis is regulated in *M. tuberculosis* and how this regulation could play a role during infection in pathogenic mycobacteria.

Reversible protein phosphorylation is a central mechanism for transduction of specific signals to various cellular processes, and *M. tuberculosis* is known to employ Ser/Thr and Tyr protein kinases and phosphatases to modulate essential functions such as central metabolism, gene expression, cell division, interaction with the host, and virulence (Richard-Greenblatt and Av-Gay 2017). In the last years, a model for MA synthesis regulation based on the phosphorylation of several components of the FAS II system was proposed. Remarkably, each FAS II enzyme is phosphorylated by multiple kinases (Molle et al. 2006; Veyron-Churlet et al. 2009, 2010; Khan et al. 2010; Molle and Kremer 2010; Slama et al. 2011; Vilchèze et al. 2014), and these kinases are also involved in reducing the production of the FAS II system precursors by phosphorylating malonyl-CoA-ACP transacylase (FabD) (Molle et al. 2006) and the β-ketoacyl-ACP synthase (FabH) (Veyron-Churlet et al. 2009), enabling a fine-tuning regulation of MA biosynthesis in response to variable growth environments. However, whether phosphorylation of these proteins

regulates the MA profile in vivo in response to environmental changes, and/or affects *M. tuberculosis* virulence in infected mice, has yet to be demonstrated.

On the other hand, the Ser/Thr/Tyr phosphorylation phosphoproteome in *C. glutamicum* revealed that most of the phosphorylated proteins identified are enzymes acting on central metabolic pathways such as glycolysis, tricarboxylic cycle, fatty-acid metabolism, and components of the protein synthesis machinery (Bendt et al. 2003). Particularly, an acyl-CoA synthetase (NgCl2774) and the ACCase α subunit (AccBC) were found to be phosphorylated; however, how this posttranslational modification affects their enzyme activities and how this influences lipid metabolism is not known.

6 Research Needs

In this chapter we provide an updated overview of the different biosynthetic and regulatory components of the de novo FA biosynthesis in phylogenetically distinct actinomycetes. In all of them, this central metabolic pathway has a direct interaction with other secondary biosynthesis routes that utilize the de novo FAs as substrates, for example, in the synthesis of complex lipids and/or of storage compounds. Furthermore, secondary metabolic pathways may also compete with FAS for the same substrates for the synthesis of specialized metabolites. Therefore, the crosstalk between all these biosynthesis pathways needs to be tightly controlled in order to optimize membrane homeostasis and cell viability. This implies the existence of a fine and coordinated network of regulation (transcriptional, posttranscriptional, posttranslational) from which we still know very little. Therefore, there is a clear research need in order to advance in this direction. Considering the important differences that exist in lipid metabolism, even between close species of actinomycetes, it will be significantly challenging to identify the regulatory components (proteins and effectors) that could be involved in maintaining lipid homeostasis in these organisms.

In the last decade, we have witnessed a renewed interest toward the study of lipid metabolism in several genera of actinomycetes. This particular interest was prompted not only for the importance of gaining fundamental knowledge of the still poorly understood lipid metabolism present in most actinomycete bacteria but also for the medical and industrial impacts that a better understanding of these metabolic pathways might have in the near future. For example, the fact that the *M. tuberculosis* FAS II enoyl reductase InhA has been identified as the target of isoniazid and ethionamide, two of the drugs clinically used to treat tuberculosis, suggests that other components of the complex lipid metabolism of mycobacteria must be explored as putative targets for the discovery of new antimycobacterial compounds. In this sense, the inhibition of *M. tuberculosis* growth in axenic cultures with compounds identified as inhibitors of the essential ACCases confirmed that these enzymes are interesting proteins to be explored as new targets for the identification and development of new antimycobacterial drugs. Furthermore, the demonstrated essentiality of the transcriptional regulators, FasR and MabR, for mycobacteria viability, also suggests that

these proteins could be explored as targets for the identification of new anti-mycobacterial compounds with a different mode of action.

On the other hand, some of the oleaginous members of Actinobacteria (e.g., *Rhodococcus* sp.) are between the potential microorganisms to be used as alternative sources for the production of oils and wax esters. However, to compete with plant-derived oils/waxes, it is necessary to improve the accumulation of these neutral molecules in the producer organisms using cheap and renewable carbon sources. To achieve these goals, there is still a need to focus the scientific research in the identification and characterization of all the components of the TAG biosynthetic and regulatory machinery in the native producers. More importantly understanding how the interaction of the de novo FA biosynthesis with the production of TAG biosynthesis can be modulated will certainly lead to yield improvements.

Acknowledgments This work was supported by NIH (1R01AI095183-01), ANPCyT PICT-2012-0168, PICT 2015-2022 and PID-2013-0042 to HG, PICT 2015-0796 to GG, PICT 2013-1981 to AA, and PICT 2014-1454 to LD.

References

Alvarez HM (2016) Triacylglycerol and wax ester-accumulating machinery in prokaryotes. Biochimie 120:28–39

Alvarez HM, Steinbüchel A (2002) Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol 60:367–376

Arabolaza A, D'Angelo M, Comba S, Gramajo H (2010) FasR, a novel class of transcriptional regulator, governs the activation of fatty acid biosynthesis genes in *Streptomyces coelicolor*. Mol Microbiol 78:47–63

Astarie-Dequeker C, Le Guyader L, Malaga W et al (2009) Phthiocerol dimycocerosates of *M. tuberculosis* participate in macrophage invasion by inducing changes in the organization of plasma membrane lipids. PLoS Pathog 5(2):e1000289

Athappilly FK, Hendrickson WA (1995) Structure of the biotinyl domain of acetyl-coenzyme A carboxylase determined by MAD phasing. Structure 3:1407–1419

Banerjee A, Dubnau E, Quemard A et al (1994) inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 263:227–230

Bazet Lyonnet B, Diacovich L, Gago G et al (2017) Functional reconstitution of the *Mycobacterium tuberculosis* long-chain acyl-CoA carboxylase from multiple acyl-CoA subunits. FEBS J 284:1110–1125

Bendt AK, Burkovski A, Schaffer S et al (2003) Towards a phosphoproteome map of *Corynebacterium glutamicum*. Proteomics 3:1637–1646

Bhatt A, Fujiwara N, Bhatt K et al (2007) Deletion of *kasB* in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. Proc Natl Acad Sci USA 104:5157–5162

Biswas RK, Dutta D, Tripathi A et al (2013) Identification and characterization of Rv0494: a fatty acid-responsive protein of the GntR/FadR family from *Mycobacterium tuberculosis*. Microbiology 159:913–923

Blanchard CZ, Chapman-Smith A, Wallace JC, Waldrop GL (1999) The biotin domain peptide from the biotin carboxyl carrier protein of *Escherichia coli* acetyl-CoA carboxylase causes a marked increase in the catalytic efficiency of biotin carboxylase and carboxyltransferase relative to free biotin. J Biol Chem 274:31767–31769

Boehringer D, Ban N, Leibundgut M (2013) 7.5-Å cryo-EM structure of the mycobacterial fatty acid synthase. J Mol Biol 425:841–849

Borgaro JG, Chang A, Machutta CA et al (2011) Substrate recognition by β-ketoacyl-ACP synthases. Biochemistry 50:10678–10686

Brennan PJ (2003) Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis 83:91–97

Brignole EJ, Smith S, Asturias FJ (2009) Conformational flexibility of metazoan fatty acid synthase enables catalysis. Nat Struct Mol Biol 16:190–197

Brown AK, Sridharan S, Kremer L et al (2005) Probing the mechanism of the *Mycobacterium tuberculosis* beta-ketoacyl-acyl carrier protein synthase III mt FabH: factors influencing catalysis and substrate specificity. J Biol Chem 280:32539–32547

Cabruja M, Mondino S, Tsai YT et al (2017) A conditional mutant of the fatty acid synthase unveils unexpected cross talks in mycobacterial lipid metabolism. Open Biol 7(2):160277

Camacho LR, Ensergueix D, Perez E et al (1999) Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. Mol Microbiol 34:257–267

Chiaradia L, Lefebvre C, Parra J et al (2017) Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. Sci Rep 7:12807

Chopra T, Gokhale RS (2009) Polyketide versatility in the biosynthesis of complex mycobacterial cell wall lipids. Methods Enzymol 459:259–94.

Ciccarelli L, Connell SR, Enderle M et al (2013) Structure and conformational variability of the *Mycobacterium tuberculosis* fatty acid synthase multienzyme complex. Structure 21:1251

Comba S, Menendez-Bravo S, Arabolaza A, Gramajo H (2013) Identification and physiological characterization of phosphatidic acid phosphatase enzymes involved in triacylglycerol biosynthesis in *Streptomyces coelicolor*. Microb Cell Fact 12:9

Cox JS, Chen B, McNeil M, Jacobs WR Jr (1999) Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. Nature 402:79–83

Cronan JE (2001) The biotinyl domain of *Escherichia coli* acetyl-CoA carboxylase. J Biol Chem 276:37355–37364

Cronan JE Jr, Subrahmanyam S (1998) FadR, transcriptional co-ordination of metabolic expediency. Mol Microbiol 29:937–943

Cronan JE Jr, Waldrop GL (2002) Multi-subunit acetyl-CoA carboxylases. Prog Lipid Res 41:407–435

Daffe M (2008) The global architecture of the mycobacterial cell envelope. In: Daffé M, Reyrat JM (eds) The mycobacterial cell envelope. ASM Press, Washington, DC, pp 3–11

Daffe M, Draper P (1998) The envelope layers of mycobacteria with reference to their pathogenicity. Adv Microb Physiol 39:131–203

Daffé M, Crick DC, Jackson M (2014) Genetics of capsular polysaccharides and cell envelope (glyco)lipids. Microbiol Spectr 2:MGM2-0021-2013

Daniel J, Oh TJ, Lee CM, Kolattukudy PE (2007) AccD6, a member of the Fas II locus, is a functional carboxyltransferase subunit of the acyl-coenzyme A carboxylase in *Mycobacterium tuberculosis*. J Bacteriol 189:911–917

Das A, Khosla C (2009) Biosynthesis of aromatic polyketides in bacteria. Acc Chem Res 42:631–639

Davis MS (2001) Inhibition of *Escherichia coli* acetyl coenzyme A carboxylase by acyl-acyl carrier protein. J Bacteriol 183:1499–1503

Diacovich L, Peiru S, Kurth D et al (2002) Kinetic and structural analysis of a new group of acyl-CoA carboxylases found in *Streptomyces coelicolor* A3(2). J Biol Chem 277:31228–31236

DiRusso CC, Nystrom T (1998) The fats of *Escherichia coli* during infancy and old age: regulation by global regulators, alarmones and lipid intermediates. Mol Microbiol 27:1–8

Florova G, Kazanina G, Reynolds KA (2002) Enzymes involved in fatty acid and polyketide biosynthesis in *Streptomyces glaucescens* : role of FabH and FabD and their acyl carrier protein specificity. Biochemistry 41:10462–10471

Fujita Y, Matsuoka H, Hirooka K (2007) Regulation of fatty acid metabolism in bacteria. Mol Microbiol 66:829–839

Gago G, Kurth D, Diacovich L et al (2006) Biochemical and structural characterization of an essential acyl coenzyme A carboxylase from *Mycobacterium tuberculosis*. J Bacteriol 188:477–486

Gago G, Diacovich L, Arabolaza A et al (2011) Fatty acid biosynthesis in actinomycetes. FEMS Microbiol Rev 35:475–497

Gande R, Gibson KJC, Brown AK et al (2004) Acyl-CoA carboxylases (accD2 and accD3), together with a unique polyketide synthase (Cg-pks), are key to mycolic acid biosynthesis in Corynebacterianeae such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. J Biol Chem 279:44847–44857

Gande R, Dover LG, Krumbach K et al (2007) The two carboxylases of *Corynebacterium glutamicum* essential for fatty acid and mycolic acid synthesis. J Bacteriol 189:5257–5264

Gao LY, Laval F, Lawson EH et al (2003) Requirement for *kasB* in *Mycobacterium* mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Mol Microbiol 49:1547–1563

Gastambide O, Lederer E (1960) Biosynthesis of corynomycolic acid from 2 molecules of palmitic acid. Biochem Z 333:285–295

Gebhardt H, Meniche X, Tropis M et al (2007) The key role of the mycolic acid content in the functionality of the cell wall permeability barrier in *Corynebacterineae*. Microbiology 153:1424–1434

Gipson P, Mills DJ, Wouts R et al (2010) Direct structural insight into the substrate-shuttling mechanism of yeast fatty acid synthase by electron cryomicroscopy. Proc Natl Acad Sci USA 107:9164–9169

Grininger M (2014) Perspectives on the evolution, assembly and conformational dynamics of fatty acid synthase type I (FAS I) systems. Curr Opin Struct Biol 25:49–56

Haase FC, Henrikson KP, Treble DH, Allen SHG (1982) The subunit structure and function of the propionyl coenzyme A carboxylase of *Mycobacterium smegmatis*. J Biol Chem 257:11994–11999

Hernandez MA, Comba S, Arabolaza A et al (2015) Overexpression of a phosphatidic acid phosphatase type 2 leads to an increase in triacylglycerol production in oleaginous *Rhodococcus* strains. Appl Microbiol Biotechnol 99:2191–2207

Hernandez M, Lara J, Gago G et al (2017) The pleiotropic transcriptional regulator NlpR contributes to the modulation of nitrogen metabolism, lipogenesis and triacylglycerol accumulation in oleaginous *rhodococci*. Mol Microbiol 103:366–385

Hoffmann C, Leis A, Niederweis M et al (2008) Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc Natl Acad Sci U S A 105:3963–3967

Hoischen C, Gura K, Luge C, Gumpert J (1997) Lipid and fatty acid composition of cytoplasmic membranes from *Streptomyces hygroscopicus* and its stable protoplast-type L form. J Bacteriol 179:3430–3436

Hunaiti AR, Kolattukudy PE (1982) Isolation and characterization of an acyl-coenzyme A carboxylase from an erythromycin-producing *Streptomyces erythreus*. Arch Biochem Biophys 216:362–371

Irzik K, van Ooyen J, Gätgens J et al (2014) Acyl-CoA sensing by FasR to adjust fatty acid synthesis in *Corynebacterium glutamicum*. J Biotechnol 192:96–101

Jamet S, Quentin Y, Coudray C et al (2015) Evolution of mycolic acid biosynthesis genes and their regulation during starvation in *Mycobacterium tuberculosis*. J Bacteriol 197:3797–3811

Kalinowski J, Bathe B, Bartels D et al (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J Biotechnol 104:5–25

Kaneda T (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288–302

Khan S, Nagarajan SN, Parikh A et al (2010) Phosphorylation of enoyl-acyl carrier protein reductase InhA impacts mycobacterial growth and survival. J Biol Chem 285:37860–37871

Kikuchi S, Rainwater DL, Kolattukudy PE (1992) Purification and characterization of an unusually large fatty acid synthase from *Mycobacterium tuberculosis* var. *bovis* BCG. Arch Biochem Biophys 295:318–326

Kurth DG, Gago GM, de la Iglesia A et al (2009) Accase 6 is the essential acetyl-CoA carboxylase involved in fatty acid and mycolic acid biosynthesis in mycobacteria. Microbiology 155:2664–2675

Lane MD, Moss J, Polakis SE (1974) Acetyl coenzyme A carboxylase. Curr Top Cell Regul 8:139–195

Lanéelle MA, Tropis M, Daffé M (2013) Current knowledge on mycolic acids in *Corynebacterium glutamicum* and their relevance for biotechnological processes. Appl Microbiol Biotechnol 97:9923–9930

Leibundgut M, Jenni S, Frick C, Ban N (2007) Structural basis for substrate delivery by acyl carrier protein in the yeast fatty acid synthase. Science 316:288–290

Leibundgut M, Maier T, Jenni S, Ban N (2008) The multienzyme architecture of eukaryotic fatty acid synthases. Curr Opin Struct Biol 18:714–725

Lin TW, Melgar MM, Kurth D et al (2006) Structure-based inhibitor design of AccD5, an essential acyl-CoA carboxylase carboxyltransferase domain of *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 103:3072–3077

Lomakin IB, Xiong Y, Steitz TA (2007) The crystal structure of yeast fatty acid synthase, a cellular machine with eight active sites working together. Cell 129:319–332

Maier T, Leibundgut M, Ban N (2008) The crystal structure of a mammalian fatty acid synthase. Science 321:1315–1323

Manteca A, Alvarez R, Salazar N et al (2008) Mycelium differentiation and antibiotic production in submerged cultures of *Streptomyces coelicolor*. Appl Environ Microbiol 74:3877–3886

Marrakchi H, Ducasse S, Labesse G et al (2002) MabA (FabG1), a *Mycobacterium tuberculosis* protein involved in the long-chain fatty acid elongation system FAS-II. Microbiology 148:951–960

Meniche X, Otten R, Siegrist MS et al (2014) Subpolar addition of new cell wall is directed by DivIVA in mycobacteria. Proc Natl Acad Sci USA 111:E3243–E3251

Mo S, Sydor PK, Corre C et al (2008) Elucidation of the *Streptomyces coelicolor* pathway to 2-undecylpyrrole, a key intermediate in undecylprodiginine and streptorubin B biosynthesis. Chem Biol 15:137–148

Molle V, Kremer L (2010) Division and cell envelope regulation by Ser/Thr phosphorylation: *Mycobacterium* shows the way. Mol Microbiol 75:1064–1077

Molle V, Brown AK, Besra GS et al (2006) The condensing activities of the *Mycobacterium tuberculosis* type II fatty acid synthase are differentially regulated by phosphorylation. J Biol Chem 281:30094–30103

Mondino S, Gago G, Gramajo H (2013) Transcriptional regulation of fatty acid biosynthesis in mycobacteria. Mol Microbiol 89:372–387

Munday MR (2002) Regulation of mammalian acetyl-CoA carboxylase. Biochem Soc Trans 30:1059–1064

Nickel J, Irzik K, Van Ooyen J, Eggeling L (2010) The TetR-type transcriptional regulator FasR of *Corynebacterium glutamicum* controls genes of lipid synthesis during growth on acetate. Mol Microbiol 78:253–265

Oh TJ, Daniel J, Kim HJ et al (2006) Identification and characterization of Rv3281 as a novel subunit of a biotin-dependent acyl-CoA carboxylase in *Mycobacterium tuberculosis* H37Rv. J Biol Chem 281:3899–3908

Pawelczyk J, Brzostek A, Kremer L et al (2011) Accd6, a key carboxyltransferase essential for mycolic acid synthesis in *Mycobacterium tuberculosis*, is dispensable in a nonpathogenic strain. J Bacteriol 193:6960–6972

Polakis SE, Guchhait RB, Lane MD (1973) Stringent control of fatty acid synthesis in *Escherichia*. J Biol Chem 248:7957–7967

Portevin D, De Sousa-D'Auria C, Houssin C et al (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. Proc Natl Acad Sci USA 101:314–319

Portevin D, Auria DS, Montrozier H et al (2005) The Acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth. J Biol Chem 280:8862–8874

Puech V, Chami M, Lemassu A et al (2001) Structure of the cell envelope of corynebacteria: importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane. Microbiology 147:1365–1382

Quémard A (2016) New insights into the compound biosynthesis and transport in mycobacteria. Trends Microbiol 24:725–738

Quemard A, Sacchettini C, Dessen A et al (1995) Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*. Biochemistry 34:8235–8241

Radmacher E, Alderwick LJ, Besra GS et al (2005) Two functional FAS-I type fatty acid synthases in *Corynebacterium glutamicum*. Microbiology 151:2421–2427

Rangan V, Smith S (2003) Fatty acid synthesis in eukaryotes. In: Vance D, Vance J (eds) Biochemistry of lipids, lipoproteins and membranes. Elsevier, Amsterdam, pp 151–179

Revill WP, Bibb MJ, Hopwood D (1995) Purification of a malonyltransferase from *Streptomyces coelicolor* A3(2) and analysis of its genetic determinant. J Bacteriol 177:3946–3952

Revill WP, Bibb MJ, Hopwood D (1996) Relationships between fatty acid and polyketide synthases from Streptomyces coelicolor A3(2): characterization of the fatty acid synthase acyl carrier protein. J Bacteriol 178:5660–5667

Richard-Greenblatt M, Av-Gay Y (2017) Epigenetic phosphorylation control of *Mycobacterium tuberculosis* infection and persistence. Microbiol Spectr 5(2):TBTB2-0005-2015

Rock CO, Jackowski S (2002) Forty years of bacterial fatty acid synthesis. Biochem Biophys Res Commun 292:1155–1166

Rottig A, Steinbuchel A (2013) Acyltransferases in bacteria. Microbiol Mol Biol Rev 77:277–321

Sacco E, Covarrubias AS, O'Hare HM et al (2007) The missing piece of the type II fatty acid synthase system from *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 104:14628–14633

Salzman V, Mondino S, Sala C et al (2010) Transcriptional regulation of lipid homeostasis in mycobacteria. Mol Microbiol 78:64–77

Sassetti CM, Boyd DH, Rubin EJ (2003) Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol 48:77–84

Scarsdale JN, Kazanina G, He X et al (2001) Crystal structure of the *Mycobacterium tuberculosis* beta ketoacyl-acyl carrier protein synthase III. J Biol Chem 276:20516–20522

Schaeffer ML, Agnihotri G, Volker C et al (2001) Purification and biochemical characterization of the *Mycobacterium tuberculosis* beta-ketoacyl-acyl carrier protein synthases KasA and KasB. J Biol Chem 276:47029–47037

Schujman GE, Paoletti L, Grossman AD, de Mendoza D (2003) FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis. Dev Cell 4:663–672

Schujman GE, Guerin M, Buschiazzo A et al (2006) Structural basis of lipid biosynthesis regulation in Gram-positive bacteria. EMBO J 25:4074–4083

Schweizer E, Hofmann J (2004) Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. Microbiol Mol Biol Rev 68:501–517

Singh R, Reynolds KA (2015) Characterization of FabG and FabI of the *Streptomyces coelicolor* dissociated fatty acid synthase. Chembiochem 16:631–640

Singh R, Mo S, Florova G, Reynolds KA (2012) *Streptomyces coelicolor* RedP and FabH enzymes, initiating undecylprodiginine and fatty acid biosynthesis, exhibit distinct acyl-CoA and malonyl-acyl carrier protein substrate specificities. FEMS Microbiol Lett 328:32–38

Slama N, Leiba J, Eynard N et al (2011) Negative regulation by Ser/Thr phosphorylation of HadAB and HadBC dehydratases from *Mycobacterium tuberculosis* type II fatty acid synthase system. Biochem Biophys Res Commun 412:401–406

Slayden RA, Barry CE III (2002) The role of KasA and KasB in the biosynthesis of meromycolic acids and isoniazid resistance in *Mycobacterium tuberculosis*. Tuberculosis 82:149–160

Sutcliffe IC (1997) Macroamphiphilic cell envelope components of *Rhodococcus equi* and closely related bacteria. Vet Microbiol 56:287–299

Takayama K, Wang C, Besra GS (2005) Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. Clin Microbiol Rev 18:81–101

Tauch A, Kaiser O, Hain T et al (2005) Complete genome sequence and analysis of the multi-resistant nosocomial pathogen *Corynebacterium jeikeium* K411, a lipid-requiring bacterium of the human skin flora. J Bacteriol 187:4671–4682

Tauch A, Trost E, Tilker A et al (2008) The lifestyle of *Corynebacterium urealyticum* derived from its complete genome sequence established by pyrosequencing. J Biotechnol 136:11–21

Tong L (2013) Structure and function of biotin-dependent carboxylases. Cell Mol Life Sci 70:863–891

Tran TH, Hsiao Y-S, Jo J et al (2015) Structure and function of a single-chain, multi-domain long-chain acyl-CoA carboxylase. Nature 518:120–124

Tsai YT, Salzman V, Cabruja M et al (2017) Role of long chain acyl-CoAs in the regulation of mycolic acid biosynthesis in mycobacteria. Open Biol 7:170087

Veyron-Churlet R, Molle V, Taylor RC et al (2009) The *Mycobacterium tuberculosis* β-ketoacyl-acyl carrier protein synthase III activity is inhibited by phosphorylation on a single threonine residue. J Biol Chem 284:6414–6424

Veyron-Churlet R, Zanella-Cléon I, Cohen-Gonsaud M et al (2010) Phosphorylation of the *Mycobacterium tuberculosis* β-ketoacyl-acyl carrier protein reductase MabA regulates mycolic acid biosynthesis. J Biol Chem 285:12714–12725

Vilchèze C, Molle V, Carrère-Kremer S et al (2014) Phosphorylation of KasB regulates virulence and acid-fastness in *Mycobacterium tuberculosis*. PLoS Pathog 10(5):e1004115

Wakil SJ, Stoops JK, Joshi VC (1983) Fatty acid synthesis and its regulation. Annu Rev Biochem 52:537–579

Waldrop GL, Rayment I, Holden HM (1994) Three-dimensional structure of the biotin carboxylase subunit of acetyl-CoA carboxylase. Biochemistry 33:10249–10256

White SW, Zheng J, Zhang YM, Rock (2005) The structural biology of type II fatty acid biosynthesis. Annu Rev Biochem 74:791–831

Xu WX, Zhang L, Mai JT et al (2014) The Wag31 protein interacts with AccA3 and coordinates cell wall lipid permeability and lipophilic drug resistance in *Mycobacterium smegmatis*. Biochem Biophys Res Commun 448:255–260

Xu Z, Wang M, Ye B-C (2017) TetR family transcriptional regulator PccD negatively controls propionyl coenzyme A assimilation in *Saccharopolyspora erythraea*. J Bacteriol 199:1–12

Yousuf S, Angara R, Vindal V, Ranjan A (2015) Rv0494 is a starvation-inducible, auto-regulatory FadR-like regulator from *Mycobacterium tuberculosis*. Microbiology 161:463–476

Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6:222–233

Type III Polyketide Synthases Responsible for Phenolic Lipid Synthesis

23

Akimasa Miyanaga and Yasuo Ohnishi

Contents

1 Introduction 436
2 The *ars* Gene Cluster in *A. vinelandii* 437
3 The *srs* Gene Cluster in *S. griseus* 438
4 An Alkylquinone Biosynthetic Gene Cluster in *M. smegmatis* 440
5 Phenolic Lipid Biosynthetic Machinery in Various Bacteria 440
6 Fungal and Plant Type III PKSs that Are Responsible for Phenolic Lipid Biosynthesis 441
7 Research Needs 441
References 442

Abstract

In various microorganisms, phenolic lipids, consisting of a polar aromatic ring and a hydrophobic alkyl chain, are synthesized by type III polyketide synthases (PKSs). In *Azotobacter vinelandii*, two type III PKSs, ArsB and ArsC, are responsible for the biosynthesis of alkylresorcinols and alkylpyrones, respectively, which are the major lipids in the cyst membrane. In *Streptomyces griseus*, SrsA is involved in synthesizing alkylquinones, which confer resistance to β-lactam antibiotics. In *Mycobacterium smegmatis*, PKS10 is involved in the biosynthesis of alkylquinones, which are proposed to act as electron-shuttling molecules. The phenolic lipid-producing

The authors thank the late Prof. Sueharu Horinouchi, who was a coauthor of this chapter in the first edition of this book.

A. Miyanaga (✉)
Department of Chemistry, Tokyo Institute of Technology, Tokyo, Japan
e-mail: miyanaga.a.aa@m.titech.ac.jp

Y. Ohnishi
Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
e-mail: ayasuo@mail.ecc.u-tokyo.ac.jp

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_28

type III PKSs are distributed in a wide variety of Gram-positive and Gram-negative bacteria, some fungi, and plants. Thus, phenolic lipids produced by type III PKSs play important, but so far overlooked, roles as minor components in biological membranes and, more importantly, as mobile electron carriers in the respiratory electron transport chain in some bacteria.

1 Introduction

Polyketides form a large family of natural products that are found in bacteria, fungi, and plants, and they exhibit various biological activities. Polyketides are synthesized by polyketide synthases (PKSs). PKSs possess a ketosynthase activity that catalyzes the condensation of extender units onto an acyl starter substrate or a growing polyketide chain. Type III PKSs, which consist of a homodimeric ketosynthase, are the simplest enzymes among the three types (I to III) of PKSs (Austin and Noel 2003). Formerly, it had been thought that type III PKSs were distributed exclusively in plants, and many plant type III PKSs have been discovered and characterized. For example, chalcone synthase catalyzes the formation of naringenin chalcone, an essential precursor of flavonoids and isoflavonoids, which have attracted significant attention because of their wide range of pharmacological properties. The first bacterial type III PKS to be characterized, RppA, catalyzes the condensation of five malonyl-CoA molecules to produce 1,3,6,8-tetrahydroxynaphthalene, which serves as an intermediate in the biosynthesis of hexahydroxyperylenequinone melanin in the actinomycete *Streptomyces griseus* (Ueda et al. 1995; Funa et al. 1999). PhlD, a type III PKS in *Pseudomonas fluorescens*, is responsible for the biosynthesis of 2,4-diacetylphloroglucinol, which has biocontrol activity against soilborne fungal plant pathogens (Bangera and Thomashow 1999). DpgA, a type III PKS from *Amycolatopsis mediterranei*, is involved in the biosynthesis of glycopeptide antibiotics such as vancomycin (Pfeifer et al. 2001). The discoveries of these type III PKSs have established the idea that type III PKSs are distributed widely not only in plants but also in microorganisms.

Based on bioinformatic predictions and subsequent in vivo and in vitro analyses, a number of type III PKSs have been shown to be responsible for the biosynthesis of various secondary metabolites, such as antibiotics and pigments. In addition, "steely," a type I fatty acid synthase-type III PKS fusion enzyme from the social amoeba *Dictyostelium discoideum*, synthesizes a differentiation-inducing factor (Austin et al. 2006). Furthermore, a growing number of microbial type III PKSs have been reported to be involved in the biosynthesis of phenolic lipids such as alkylresorcinols and alkylpyrones. Phenolic lipids are distributed widely in bacteria, fungi, and plants (Kozubek and Tyman 1999; Stasiuk and Kozubek 2010). They consist of a polar aromatic ring and a hydrophobic alkyl chain. The amphiphilic nature of phenolic lipids contributes to the formation of a stable monomolecular layer. Phenolic lipids also exhibit antimicrobial and antioxidation activities. In this review, we primarily describe the phenolic lipid-producing type III PKSs in three microorganisms, *Azotobacter vinelandii*, *S. griseus*, and *Mycobacterium smegmatis*.

2 The *ars* Gene Cluster in *A. vinelandii*

A. vinelandii is a Gram-negative, nitrogen-fixing soil bacterium that differentiates into metabolically dormant cysts under adverse environmental conditions (Lin and Sadoff 1968). The central body of the cyst is surrounded by a thick membrane composed of a modified capsule (the intine) and a layered outer shell (the exine). Phenolic lipids, such as alkylresorcinols and alkylpyrones, are the major lipids in the cyst membrane (Reusch and Sadoff 1983). A search of a genome database predicted the presence of a gene cluster, named the alkylresorcinol synthesis (*ars*) gene cluster, which is required for the biosynthesis of these phenolic lipids. This gene cluster consists of two type III PKS genes, *arsB* and *arsC*, and two genes, *arsA* and *arsD*, together encoding a type I fatty acid synthase (Fig. 1a). In vitro studies showed that

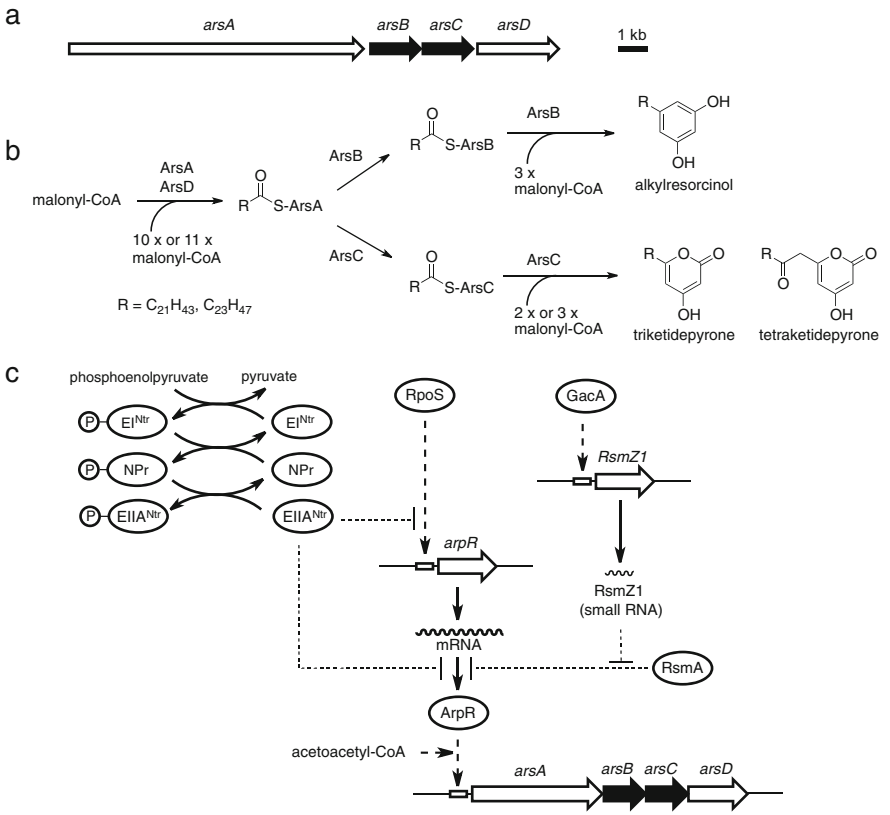


Fig. 1 Phenolic lipids produced by type III PKSs in *A. vinelandii*. (**a**) Gene organization of the *ars* gene cluster. (**b**) Pathways for the biosynthesis of alkylresorcinols and alkylpyrones in *A. vinelandii*. (**c**) Proposed model for the transcriptional regulation of the *arsABCD* operon in *A. vinelandii*. Positive and negative controls are represented by *broken arrows* and *blunt-ended dotted lines*, respectively

ArsA and ArsD synthesize an acyl starter substrate for ArsB and ArsC (Fig. 1b) (Miyanaga et al. 2008). ArsB synthesizes alkylresorcinols from an acyl starter substrate and three molecules of malonyl-CoA through a decarboxylative aldol condensation (Funa et al. 2006). In contrast, ArsC synthesizes triketide pyrones and tetraketide pyrones from an acyl starter substrate and two and three molecules of malonyl-CoA, respectively, through lactonization. An *ars* disruptant, which is unable to produce phenolic lipids, forms cysts with a disorganized exine, suggesting that phenolic lipids play a structural role in the cysts (Funa et al. 2006; Segura et al. 2009).

Biosynthesis of the phenolic lipids is elaborately controlled in *A. vinelandii* (Fig. 1c). Transcription of the *ars* genes is very low in exponentially growing vegetative cells and slightly increases (14-fold) during stationary phase, whereas it is induced 200-fold under an encystment-inducing condition (Segura et al. 2009). ArpR, a LysR-type transcriptional regulator, is the master regulator of the *arsABCD* operon; ArpR activates the transcription of the *ars* genes by binding the upstream regulatory region of *arsA* during encystment (Romero et al. 2013). Acetoacetyl-CoA, which is a precursor of poly-β-hydroxybutyrate, was shown to increase the binding affinity of ArpR to the *arsA* upstream regulatory region (Romero et al. 2013). Furthermore, the exogenous addition of acetoacetyl-CoA to the medium increased the transcription of the *ars* genes, as well as the synthesis of phenolic lipids. Taken together, acetoacetyl-CoA seems to act as a coinducer to enhance the transcriptional activation of the *ars* genes by ArpR (Romero et al. 2013). Meanwhile, the stationary-phase sigma factor RpoS is required for the transcription of *arpR*, and therefore RpoS is essential for the synthesis of phenolic lipids (Cocotl-Yañez et al. 2011; Romero et al. 2013). RpoS also controls the synthesis of other cyst components such as alginate and poly--β-hydroxybutyrate (Cocotl-Yañez et al. 2011). In addition, the nitrogen-related phosphotransferase system comprising the EI^{Ntr}, NPr, and $EIIA^{Ntr}$ regulates the synthesis of phenolic lipids (Muriel-Millán et al. 2015). The non-phosphorylated $EIIA^{Ntr}$ protein negatively affects the transcriptional activation of *arpR* by RpoS and also seems to control the expression of *arpR* at the posttranscriptional level. Very recently, another posttranscriptional regulation of *arpR* was reported in the Gac-Rsm system (Romero et al. 2016). The translational repressor protein RsmA negatively regulates the translation of *arpR* by binding its mRNA. The two-component response regulator GacA activates the transcription of the small RNA RsmZ1 that binds RsmA and counteracts its repressor activity.

3 The *srs* Gene Cluster in *S. griseus*

The genome sequence of *S. griseus* (Ohnishi et al. 2008) led to the discovery of a gene cluster, named the *Streptomyces* resorcinol synthesis (*srs*) gene cluster, which encodes another type III PKS, in addition to RppA. This gene cluster contains *srsA*, *srsB*, and *srsC*, which encode a type III PKS, a methyltransferase,

and a hydroxylase, respectively (Fig. 2a) (Funabashi et al. 2008). SrsA synthesizes alkylresorcylic acids from an acyl starter substrate, one methylmalonyl-CoA molecule, and two malonyl-CoA molecules through an aldol condensation (Nakano et al. 2012). SrsB catalyzes the decarboxylative methylation of alkylresorcylic acids to yield alkylresorcinol methyl ethers, and SrsC catalyzes the hydroxylation of alkylresorcinolmethyl ethers to yield alkylquinones (Fig. 2b). Because alkylresorcinol methyl ethers and alkylquinones are detected mainly in the cell wall/membrane fraction of *S. griseus*, these phenolic lipids seem to be associated with the cytoplasmic membrane (Funabashi et al. 2008). An *srs* disruptant, which does not produce phenolic lipids, is highly sensitive to β-lactam antibiotics, including penicillin G, which is an inhibitor of peptidoglycan synthesis, which suggests that phenolic lipids confer penicillin resistance on *S. griseus*. Phenolic lipids may affect the characteristics and rigidity of the cell wall/cytoplasmic membrane.

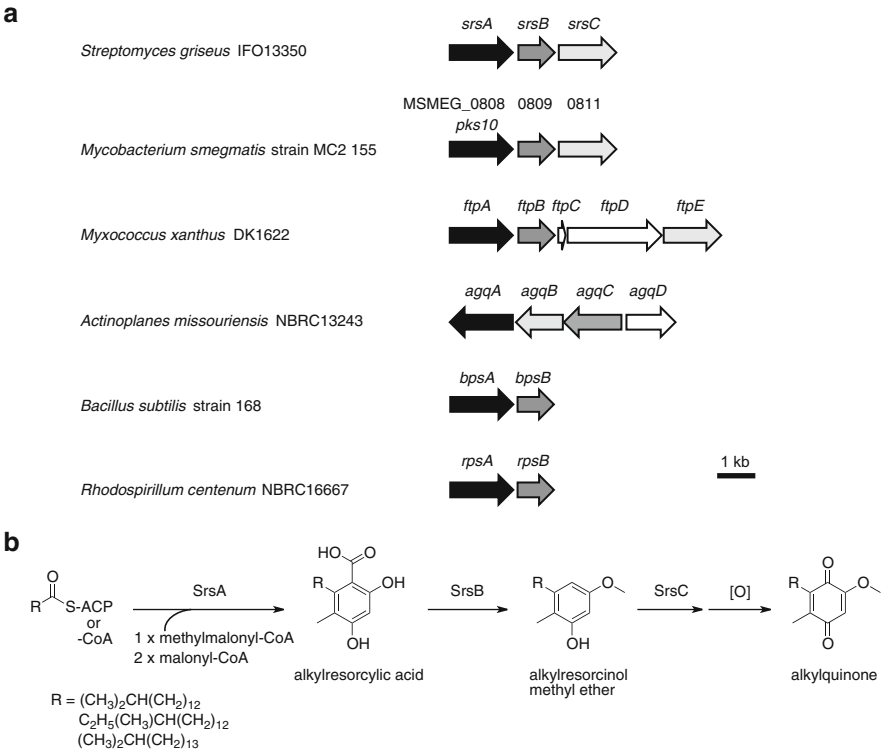


Fig. 2 Distribution of *srs*-like gene clusters among bacteria. (**a**) Gene organization of the *srs* gene cluster in *S. griseus*, and the distribution of *srs*-like gene clusters in various bacteria. *Black*, *gray*, *light gray*, and *white arrows* indicate type III PKS genes, methyltransferase genes, hydroxylase genes, and other enzyme-encoding genes, respectively. (**b**) Pathway for the biosynthesis of alkylquinones in *S. griseus*

4 An Alkylquinone Biosynthetic Gene Cluster in *M. smegmatis*

M. smegmatis has an *srs*-like gene cluster that consists of a type III PKS gene (PKS10, MSMEG_0808), a methyltransferase gene (MSMEG_0809), and a hydroxylase gene (MSMEG_0811) (Fig. 2a). Recent studies have confirmed that this gene cluster is responsible for the biosynthesis of alkylquinones (Anand et al. 2015). PKS10 synthesizes alkylresorcinols from an acyl starter substrate, methylmalonyl-CoA, and malonyl-CoA. A *pks10* knockout strain, which does not produce alkylquinones, forms fragile biofilms, and it exhibits lower levels of ATP synthesis and higher levels of NADH accumulation, which are indicative of a disruption in the respiratory chain. In addition, alkylquinones were shown to function as electron carriers in the respiratory electron transport chain. Taken together with the fact that the expression of these genes is upregulated under oxygen-depleted conditions, these observations suggest that alkylquinones are necessary for maintaining cellular respiration in biofilms. These polyketide-type alkylquinones are biosynthetically distinct from canonical respiratory quinones that are derived from the shikimate and isoprenoid pathways.

5 Phenolic Lipid Biosynthetic Machinery in Various Bacteria

srs-like gene clusters, which contain a type III PKS gene, a methyltransferase gene, and a hydroxylase gene, are found widely among Gram-positive and Gram-negative bacteria (Fig. 2a). This fact implies that alkylquinone-type phenolic lipids are produced in various bacteria. For example, *Myxococcus xanthus* has the fatty acyl-AMP ligase-dependent type III polyketide synthase (*ftp*) gene cluster for phenolic lipid biosynthesis (Hayashi et al. 2011). The *ftp* cluster consists of a type III PKS gene (*ftpA*), a methyltransferase gene (*ftpB*), a stand-alone acyl carrier protein-encoding gene (*ftpC*), an acyl-AMP ligase gene (*ftpD*), and a hydroxylase gene (*ftpE*). *M. xanthus* seems to use two additional proteins, FtpC and FtpD, to produce the FtpA starter substrate. FtpA synthesizes an alkylresorcylic acid, which is modified by FtpB and FtpE to generate an alkylquinone, as occurs in the SrsABC reaction.

Some bacterial species contain an additional prenyltransferase gene in their *srs*-like gene cluster. *Actinoplanes missouriensis* has the alkyl-*O*-dihydrogeranyl-methoxyhydroquinone (*agq*) gene cluster, which consists of a type III PKS gene (*agqA*), a hydroxylase gene (*agqB*), a methyltransferase gene (*agqC*), and a prenyltransferase gene (*agqD*) (Fig. 2a). The prenyltransferase AgqD attaches a prenyl group to the C4-hydroxy group of the alkyl-methoxyhydroquinones, which are produced by AgqABC, to yield geranylated phenolic lipids (Awakawa et al. 2011). Although the biological functions of the prenylated phenolic lipids in *A. missouriensis* remain elusive, they are assumed to have some important functions because *agq*-like gene clusters are conserved among some related actinomycetes, such as *Micromonospora aurantiaca* and *Salinispora tropica*.

In contrast, some *srs*-like gene clusters lack a hydroxylase gene (Fig. 2a). For example, the *Bacillus* pyrone synthase (*bps*) gene cluster in *Bacillus subtilis* consists of two genes: *bpsA* encoding a type III PKS and *bpsB* encoding a methyltransferase. In vivo and in vitro analyses showed that BpsA synthesizes triketide alkylpyrones from an acyl starter substrate and malonyl-CoA and that BpsB catalyzes the methylation of the alkylpyrones to yield alkylpyrone methyl ethers (Nakano et al. 2009). The *Rhodospirillum* polyketide synthase (*rps*) gene cluster in *Rhodospirillum centenum* also consists of only two genes: *rpsA* encoding a type III PKS and *rpsB* encoding a methyltransferase (Awakawa et al. 2013).

6 Fungal and Plant Type III PKSs that Are Responsible for Phenolic Lipid Biosynthesis

Filamentous fungi also contain type III PKS genes. A type III PKS from *Neurospora crassa*, 2'-oxoalkylresorcinolic acid synthase (ORAS), has been characterized (Funa et al. 2007). In vitro studies showed that ORAS has the ability to produce an alkylresorcylic acid from a long acyl starter substrate. However, *oras* gene deletions do not exhibit any apparent phenotypic changes. Moreover, production of the alkylresorcinol acid or the corresponding alkylresorcinol could not be detected in *N. crassa*, probably because only small amounts of these polyketides are produced.

In plants, phenolic lipids are also synthesized by type III PKSs. In *Sorghum bicolor*, two type III PKSs, ARS1 and ARS2, are involved in the biosynthesis of sorgoleone, which is an alkylquinone that is responsible for the allelopathic property of the plant (Cook et al. 2010). A methyltransferase (SbOMT3) and a hydroxylase (possibly a P450 monooxygenase) are also involved in sorgoleone biosynthesis (Baerson et al. 2008), as was observed for phenolic lipid biosynthesis in *S. griseus*. Rice (*Oryza sativa*) contains 31 type III PKS candidates. Among them, Os10g07040 (ARAS1) and Os10g08620 (ARAS2) were shown to catalyze the formation of alkylresorcylic acids (Matsuzawa et al. 2010). Bis-5-alkylresorcinols, which have a dihydroxybenzene ring at both terminal ends of an alkyl chain, have been isolated from some plants. Although in vitro studies suggest that bis-5-alkylresorcinols might be synthesized by type III PKSs, no genuine type III PKS has been identified to be responsible for the biosynthesis of bis-5-alkylresorcinol in any plant (Miyanaga and Horinouchi 2009).

7 Research Needs

Many type III PKSs have been discovered by genome mining. Some of the compounds that are synthesized by these type III PKSs exhibit important biological activities. Recent studies showed that some type III PKSs play important roles in the biosynthesis of biological membrane components and electron-shuttling molecules in bacteria. Further studies of other unexploited type III PKSs will identify additional novel biological components and bioactive substances.

References

Anand A, Verma P, Singh AK, Kaushik S, Pandey R, Shi C, Kaur H, Chawla M, Elechalawar CK, Kumar D, Yang Y, Bhavesh NS, Banerjee R, Dash D, Singh A, Natarajan VT, Ojha AK, Aldrich CC, Gokhale RS (2015) Polyketide quinones are alternate intermediate electron carriers during *Mycobacterial* respiration in oxygen-deficient niches. Mol Cell 60:637–650

Austin MB, Noel JP (2003) The chalcone synthase superfamily of type III polyketide synthases. Nat Prod Rep 20:79–110

Austin MB, Saito T, Bowman ME, Haydock S, Kato A, Moore BS, Kay RR, Noel JP (2006) Biosynthesis of *Dictyostelium discoideum* differentiation-inducing factor by a hybrid type I fatty acid-type III polyketide synthase. Nat Chem Biol 2:494–502

Awakawa T, Fujita N, Hayakawa M, Ohnishi Y, Horinouchi S (2011) Characterization of the biosynthesis gene cluster for alkyl-*O*-dihydrogeranyl-methoxyhydroquinones in *Actinoplanes missouriensis*. Chembiochem 12:439–448

Awakawa T, Sugai Y, Otsutomo K, Ren S, Masuda S, Katsuyama Y, Horinouchi S, Ohnishi Y (2013) 4-Hydroxy-3-methyl-6-(1-methyl-2-oxoalkyl)pyran-2-one synthesis by a type III polyketide synthase from *Rhodospirillum centenum*. Chembiochem 14:1006–1013

Baerson SR, Dayan FE, Rimando AM, Nanayakkara NP, Liu CJ, Schröder J, Fishbein M, Pan Z, Kagan IA, Pratt LH, Cordonnier-Pratt MM, Duke SO (2008) A functional genomics investigation of allelochemical biosynthesis in *Sorghum bicolor* root hairs. J Biol Chem 283:3231–3247

Bangera MG, Thomashow LS (1999) Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. J Bacteriol 181:3155–3163

Cocotl-Yañez M, Sampieri A, Moreno S, Núñez C, Castañeda M, Segura D, Espín G (2011) Roles of RpoS and PsrA in cyst formation and alkylresorcinol synthesis in *Azotobacter vinelandii*. Microbiology 157:1685–1693

Cook D, Rimando AM, Clemente TE, Schröder J, Dayan FE, Nanayakkara NP, Pan Z, Noonan BP, Fishbein M, Abe I, Duke SO, Baerson SR (2010) Alkylresorcinol synthases expressed in *Sorghum bicolor* root hairs play an essential role in the biosynthesis of the allelopathic benzoquinone sorgoleone. Plant Cell 22:867–887

Funa N, Ohnishi Y, Fujii I, Shibuya M, Ebizuka Y, Horinouchi S (1999) A new pathway for polyketide synthesis in microorganisms. Nature 400:897–899

Funa N, Ozawa H, Hirata A, Horinouchi S (2006) Phenolic lipid synthesis by type III polyketide synthases is essential for cyst formation in *Azotobacter vinelandii*. Proc Natl Acad Sci U S A 103:6356–6361

Funa N, Awakawa T, Horinouchi S (2007) Pentaketide resorcylic acid synthesis by type III polyketide synthase from *Neurospora crassa*. J Biol Chem 282:14476–14481

Funabashi M, Funa N, Horinouchi S (2008) Phenolic lipids synthesized by type III polyketide synthase confer penicillin resistance on *Streptomyces griseus*. J Biol Chem 283:13983–13991

Hayashi T, Kitamura Y, Funa N, Ohnishi Y, Horinouchi S (2011) Fatty acyl-AMP ligase involvement in the production of alkylresorcylic acid by a *Myxococcus xanthus* type III polyketide synthase. Chembiochem 12:2166–2176

Kozubek A, Tyman JH (1999) Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. Chem Rev 99:1–26

Lin LP, Sadoff HL (1968) Encystment and polymer production by *Azotobacter vinelandii* in the presence of beta-hydroxybutyrate. J Bacteriol 95:2336–2343

Matsuzawa M, Katsuyama Y, Funa N, Horinouchi S (2010) Alkylresorcylic acid synthesis by type III polyketide synthases from rice *Oryza sativa*. Phytochemistry 71:1059–1067

Miyanaga A, Horinouchi S (2009) Enzymatic synthesis of bis-5-alkylresorcinols by resorcinol-producing type III polyketide synthases. J Antibiot 62:371–376

Miyanaga A, Funa N, Awakawa T, Horinouchi S (2008) Direct transfer of starter substrates from type I fatty acid synthase to type III polyketide synthases in phenolic lipid synthesis. Proc Natl Acad Sci U S A 105:871–876

Muriel-Millán LF, Moreno S, Romero Y, Bedoya-Pérez LP, Castañeda M, Segura D, Espín G (2015) The unphosphorylated EIIA(Ntr) protein represses the synthesis of alkylresorcinols in *Azotobacter vinelandii*. PLoS One 10:e0117184

Nakano C, Ozawa H, Akanuma G, Funa N, Horinouchi S (2009) Biosynthesis of aliphatic polyketides by type III polyketide synthase and methyltransferase in *Bacillus subtilis*. J Bacteriol 191:4916–4923

Nakano C, Funa N, Ohnishi Y, Horinouchi S (2012) The *O*-methyltransferase SrsB catalyzes the decarboxylative methylation of alkylresorcyclic acid during phenolic lipid biosynthesis by *Streptomyces griseus*. J Bacteriol 194:1544–1551

Ohnishi Y, Ishikawa J, Hara H, Suzuki H, Ikenoya M, Ikeda H, Yamashita A, Hattori M, Horinouchi S (2008) Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. J Bacteriol 190:4050–4060

Pfeifer V, Nicholson GJ, Ries J, Recktenwald J, Schefer AB, Shawky RM, Schröder J, Wohlleben W, Pelzer S (2001) A polyketide synthase in glycopeptide biosynthesis: the biosynthesis of the non-proteinogenic amino acid (*S*)-3,5-dihydroxyphenylglycine. J Biol Chem 276:38370–38377

Reusch RN, Sadoff HL (1983) Novel lipid components of the *Azotobacter vinelandii* cyst membrane. Nature 302:268–270

Romero Y, Moreno S, Guzmán J, Espín G, Segura D (2013) Sigma factor RpoS controls alkylresorcinol synthesis through ArpR, a LysR-type regulatory protein, during encystment of *Azotobacter vinelandii*. J Bacteriol 195:1834–1844

Romero Y, Guzmán J, Moreno S, Cocotl-Yañez M, Vences-Guzmán MÁ, Castañeda M, Espín G, Segura D (2016) The GacS/A-RsmA signal transduction pathway controls the synthesis of alkylresorcinol lipids that replace membrane phospholipids during encystment of *Azotobacter vinelandii* SW136. PLoS One 11:e0153266

Segura D, Vite O, Romero Y, Moreno S, Castañeda M, Espín G (2009) Isolation and characterization of *Azotobacter vinelandii* mutants impaired in alkylresorcinol synthesis: alkylresorcinols are not essential for cyst desiccation resistance. J Bacteriol 191:3142–3148

Stasiuk M, Kozubek A (2010) Biological activity of phenolic lipids. Cell Mol Life Sci 67:841–860

Ueda K, Kim KM, Beppu T, Horinouchi S (1995) Overexpression of a gene cluster encoding a chalcone synthase-like protein confers redbrown pigment production in *Streptomyces griseus*. J Antibiot 48:638–646

Wax Ester and Triacylglycerol Biosynthesis in Bacteria

24

H. M. Alvarez, M. A. Hernández, O. M. Herrero, M. P. Lanfranconi, R. A. Silva, and M. S. Villalba

Contents

1 Introduction 446
2 Synthesis of TAG and WE by Bacteria 447
3 Triacylglycerol Biosynthesis in Bacteria 447
4 Wax Ester Biosynthesis in Bacteria 451
5 Metabolic Engineering in TAG Biosynthesis 451
6 Research Needs 454
References 454

Abstract

Bacteria are an extremely diverse group of organisms, some of which possess the ability to synthesize and accumulate neutral lipids, such as triacylglycerols (TAG) and wax esters (WE). Among these microorganisms, Actinobacteria are specialized in the accumulation of TAG, whereas Gram-negative Proteobacteria, such as *Acinetobacter* and *Marinobacter*, produce predominantly WE. The capability for accumulating large amounts of TAG seems to be restricted to some members of Actinobacteria, such as those belonging to *Rhodococcus*, *Gordonia*, and *Streptomyces* genera, and to the Gram-negative *Alcanivorax borkumensis*. The biosynthesis

H. M. Alvarez (✉) · M. A. Hernández · M. P. Lanfranconi · R. A. Silva
Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina
e-mail: halvarez@unpata.edu.ar; mahernandez@unpata.edu.ar; mlanfranconi@unpata.edu.ar; rsilva@unpata.edu.ar

O. M. Herrero · M. S. Villalba
Faculty of Natural Science, Bioscience Institute Patagonia (INBIOP), University of Patagonia San Juan Bosco, CONICET, Comodoro Rivadavia, Chubut, Argentina

Oil m&s, Comodoro Rivadavia, Chubut, Argentina
e-mail: mherrero@grupoindalo.com.ar; msol_villalba@yahoo.com.ar

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_30

and accumulation of TAG and/or WE require the occurrence of a set of genes/proteins working in a coordinated metabolic and regulatory context in the cell. Some components of the lipid-accumulating machinery in native producers have been identified and characterized. They include genes coding for: (1) enzymes catalyzing the last reactions of TAG and/or WE synthesis; (2) enzymes involved in the reduction of fatty acids to the respective fatty alcohols for the synthesis of WE; (3) enzymes of central metabolism which generate NADPH for fatty acid synthesis; (4) a structural protein involved in the assembly and stabilization of lipid inclusion bodies; and (5) a lipid transporter protein involved in the balance and homeostasis of cellular lipids. Some of these genes identified in native producers have been used for engineering bacterial hosts, which are naturally unable to produce these lipids, in order to produce TAG/WE with bacterial strains of biotechnological relevance.

1 Introduction

Bacteria have developed diverse strategies for surviving under the fluctuating conditions of the environment. The synthesis and accumulation of storage lipids, such as polyhydroxyalkanoates (PHA), wax esters (WE), and triacylglycerols (TAG), is one of the mechanisms evolved by some prokaryotes to colonize and thrive in natural environments. Interestingly, the production of TAG or WE might represent an ancestral biosynthetic innovation and a conservative strategy that facilitated bacterial colonization of harsh, early terrestrial environments (Finkelstein et al. 2010). The ability to produce TAG (and WE to minor extent) allowed cells to adapting and tolerating the main stresses found in soil environments, such as desiccation, oxidative stress, and osmotic stress, among others. Neutral lipids seem to play multiple roles in the physiology of lipid-accumulating bacteria. TAG are excellent reserve materials, since their oxidation produces higher yields of energy compared to other storage compounds, such as carbohydrates or PHA. The energy obtained by the mobilization of stored lipids may provide bacterial cells of energetic autonomy and a temporal independence from the environment and contributes to cell survival when they do not have access to energy resources in the environment. In addition, their extremely hydrophobic properties allow their accumulation in large amounts in cells without changing the osmolarity of the cytoplasm. On the other hand, TAG may also provide a source of metabolic water from their oxidation under desiccation conditions. Bacterial TAG or WE may play additional roles in cells, such as: (1) a source of lipid precursors for membranes and cell envelopes; (2) a source of metabolic precursors for the synthesis of secondary metabolites, such as antibiotics; (3) a form to balance central metabolism and the redox state of the cell, and (4) a form to detoxify unusual fatty acids and the excess of free fatty acids, among others.

The biosynthesis and accumulation of neutral lipids by bacteria is a process related to the stationary growth phase or as a response to stress. The limitation of the nitrogen source in the presence of an excess of the carbon source usually

stimulates lipid accumulation in diverse bacteria; thus, the nutritional stress seems to be the main condition that influences lipid accumulation in prokaryotes. The total amount of TAG or WE accumulated by bacteria depends on both the strain (taxonomic affiliation) and the carbon source used for growth. The stored lipids are usually mobilized by bacterial cells in the absence of any carbon source.

Beyond the physiological relevance of the stored TAG and WE in prokaryotes, these lipids have recently gained the interest of the industry because of its potential biotechnological applications. Neutral lipids produced by bacteria may serve as a source of precursors and components for the production of a variety of biofuels, biolubricants, and oleochemicals, among other commercial products. This situation driven recent advances in basic research in this field and the identification of different genes coding for proteins involved in TAG and WE metabolism in Gram-positive as well as in Gram-negative bacteria.

2 Synthesis of TAG and WE by Bacteria

The ability to synthesize TAG or WE is found in a diversity of bacteria, although there are some qualitative and quantitative differences in their accumulation profiles. In general, Gram-positive Actinobacteria are specialized in the accumulation of TAG, whereas WE are synthesized and accumulated predominantly by Gram-negative bacteria, as is shown in Fig. 1. Interestingly, some members of Proteobacteria, such as *Alcanivorax borkumensis* and *Alcanivorax jadensis*, produce TAG as main storage lipids.

On the other hand, some of the lipid-accumulating bacteria are able to produce high amounts of TAG (higher than 20% of cellular dry weight, CDW); thus, they can be considered as oleaginous microorganisms. Among them, Actinobacteria belonging to *Rhodococcus*, *Gordonia*, and *Streptomyces*, and Proteobacteria belonging to *Alcanivorax*, exhibit "oleaginous phenotypes" during growth on different carbon sources (Fig. 1). Diverse substrates support growth and lipid accumulation in bacteria, including sugars, organic acids, hydrocarbons, as well as agro-industrial wastes, such as whey, sugar cane molasses, carob, and orange wastes, among others.

In summary, the combination of the carbon source used for cultivation of cells and the metabolic characteristics of the bacterial strain influence the type and the amount of neutral lipid accumulated by microorganisms.

3 Triacylglycerol Biosynthesis in Bacteria

TAG are composed of three fatty acyl groups esterified to a glycerol backbone at the *sn*-1, *sn*-2, and *sn*-3 positions. Bacterial TAG are predominantly synthesized via the acyl-CoA-dependent acylation of the glycerol backbone derived from *sn*-glycerol-3-phosphate (G3P), referred to as the "Kennedy pathway" (Fig. 2). G3P is generated from dihydroxyacetone phosphate derived from the glycolysis through the catalytic action of *sn*-glycerol-3-phosphate dehydrogenase. The first acylation step of G3P is

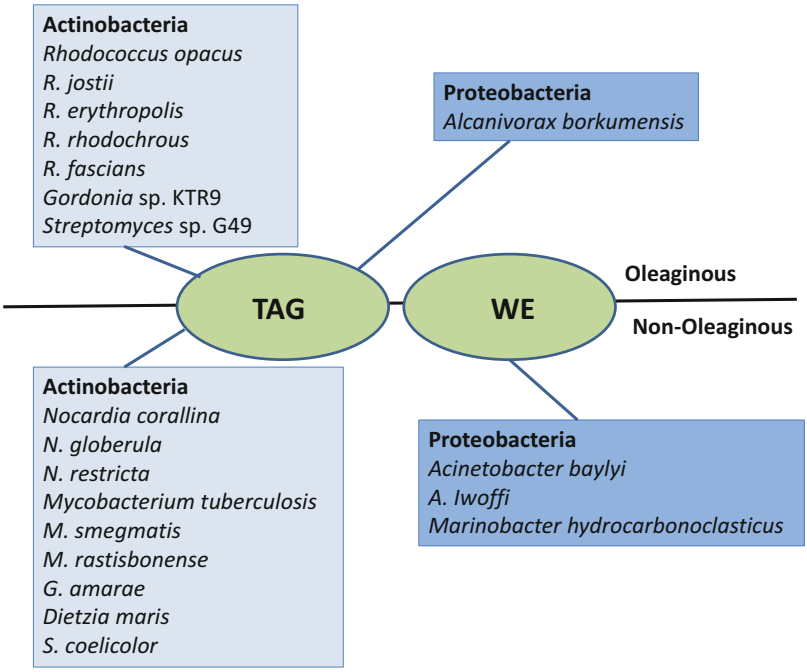


Fig. 1 Bacteria able to synthesize TAG and/or WE. The figure shows the relation between microorganisms and the predominant lipid species which they produce. It is noteworthy that some TAG-accumulating Actinobacteria are able to produce traces of WE and some Gram-negative Proteobacteria that produce WE are also able to synthesize minor amounts of TAG

catalyzed by an *sn*-glycerol-3-phosphate-*O*-acyltransferase (GPAT), whereas the second acylation by an acylglycerol-3-phosphate-*O*-acyltransferase (AGPAT). A subsequent removal of the phosphate group by a phosphatidic acid phosphatase enzyme to generate diacylglycerol (DAG) occurs prior to the final acylation. The final acyl-CoA-dependent acylation of the resulting DAG is catalyzed by a diacylglycerol-acyltransferase enzyme (DGAT) (Fig. 2). Genes and enzymes involved in Kennedy pathway have been intensively investigated during the last years in diverse TAG-synthesizing bacteria. Several DGAT enzymes have been characterized in Actinobacteria as well as in Proteobacteria, since the presence of these enzymes seems to be a key feature that differentiates bacteria capable for synthesizing TAG or WE to those unable to produce these lipids. Kalscheuer and Steinbüchel (2003) reported the first WS/DGAT in *Acinetobacter baylyi* ADP1, which is able to produce WE as main storage lipids plus minor amounts of TAG. This enzyme exhibited simultaneously both acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WS) and diacylglycerol acyltransferase (DGAT) activities. Later, other DGATs were identified and characterized in several TAG-synthesizing bacteria, such as those belonging to *Alcanivorax*, *Streptomyces*, *Mycobacterium*, *Gordonia, Marinobacter*, and *Rhodococcus* genera (Daniel et al. 2004; Holtzapple

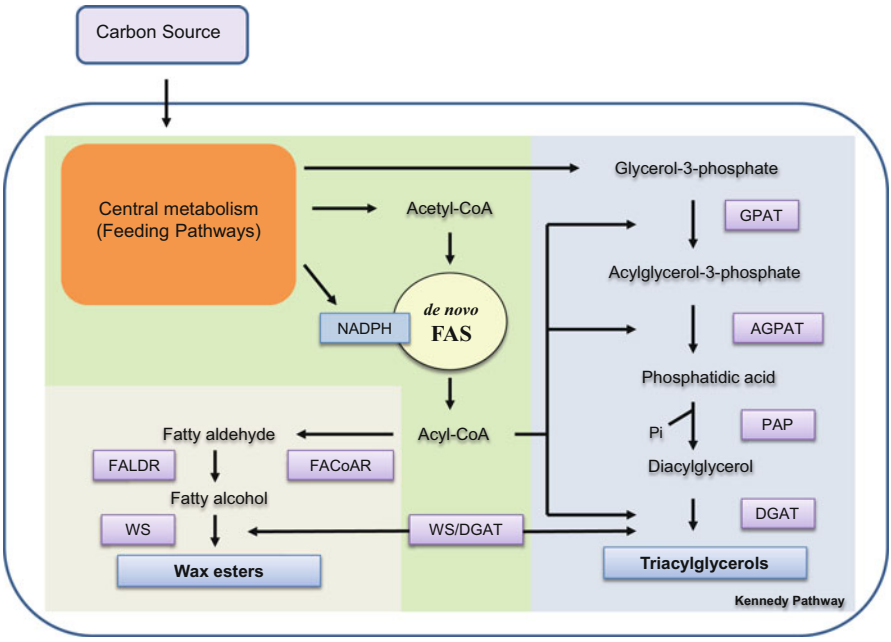


Fig. 2 Schematic of metabolic pathways involved in the synthesis of TAG and WE in bacteria. Major metabolic pathways are shown with different colored backgrounds according to their occurrence in Gram-positive Actinobacteria (*light blue*), Gram-negative Proteobacteria (*violet*), or in both bacteria (*green*). *Abbreviations*: *FAS* fatty acid synthesis, *WS* wax ester synthase, *DGAT* diacylglycerol acyltransferase, *GPAT* glycerol-3-phosphate *O*-acyltransferase, *AGPAT* acyl-glycerol-3-phosphate acyltransferase, *PAP* phosphatidic acid phosphatase, *FALDR* fatty aldehyde reductase, *FACoAR* fatty acyl-CoA reductase

and Schmidt-Dannert 2007; Kalscheuer et al. 2007; Alvarez et al. 2008; Arabolaza et al. 2008; Indest et al. 2015). Interestingly, bacterial DGATs belong to a new class of TAG-synthesizing enzymes, exhibiting no extended sequence similarities to any known eukaryotic acyltransferase. Genes coding for DGAT enzymes are particularly abundant in TAG-accumulating Actinobacteria, although the number of isoenzymes occurring in cells seems to be a strain-/species-dependent feature. Some members of *Mycobacterium* and *Rhodococcus* genera possess up to 15–17 DGAT isoenzymes in their genomes. This feature may represent an ecological advantage, providing more physiological and regulatory flexibility to lipid metabolism in these microorganisms. The abundance of DGAT enzymes and the high promiscuity of their activities may confer to Actinobacteria the ability to produce TAG and/or WE, using a diversity of intermediates from the cellular metabolism, and under a wide range of environmental conditions.

Other studies led to the identification and characterization of genes encoding additional enzymes of the Kennedy pathway, such as AGPAT in *Mycobacterium bovis* BCG and phosphatidic acid phosphatase (PAP2) in *Streptomyces coelicolor*

(Low et al. 2010; Comba et al. 2013) and *Rhodococcus jostii* RHA1 (Hernández et al. 2015; Fig. 2). In addition to the Kennedy pathway, other routes may be involved in the synthesis of TAG in prokaryotes, as occur in yeasts and plants. The existence of phospholipid:diacylglycerol acyltransferase-like enzymes (PDAT) in TAG-producing bacteria is expected. PDAT catalyzes the transfer of fatty acids at the *sn*-2 position of phospholipid species to DAG to generate TAG. The occurrence of PDAT-like enzymes in prokaryotes remains to be investigated. On the other hand, the key intermediate for TAG synthesis (DAG) can be produced by different metabolic reactions in bacteria. Phospholipase C produces DAG by cleavage of phospholipids, whereas the headgroup transfer from phospholipids to membrane-derived oligosaccharides or periplasmic cyclic glucans is also a source of DAG (Sohlenkamp and Geiger 2015).

Holder et al. (2011) proposed that 261 genes are implicated in TAG metabolism in the oleaginous *R. opacus* strain PD630. Thus, the ability to synthesize and accumulate TAG (principally in oleaginous bacteria) depends not only on the presence of DGAT enzymes but also on the existence of a special metabolic and regulatory network, which includes several genes/enzymes involved in the central metabolism, the fatty acid synthesis, and the production of reducing equivalents, as well as several genes/enzymes involved in the assembly, stabilization, and structure of lipid inclusion bodies, lipid transport required for maintaining a balanced distribution of lipids between lipid fractions in the cell, and regulation of the lipid accumulation process. Some of the components of the TAG-accumulating machinery have been identified and characterized in detail, such as TadA protein that plays a structural role in the formation of lipid bodies by *R. opacus* PD630 (MacEachran et al. 2010), Ltp1 protein which is an ATP-binding cassette transporter that functions as an importer of long-chain fatty acids in *R. jostii* RHA1 (Villalba and Alvarez 2014), and an acyl-CoA synthetase which participates in the activation of fatty acids for TAG synthesis in *Mycobacterium tuberculosis* H37Rv (Daniel et al. 2014).

The de novo fatty acid biosynthesis, which is a key pathway for the subsequent TAG formation, demands the generation of high levels of NADPH and ATP by cells, since the synthesis of one molecule of palmitic acid ($C_{16:0}$) requires eight molecules of acetyl-CoA, 14 molecules of NADPH, and seven molecules of ATP. In this context, previous studies identified and characterized $NADP^+$-dependent enzymes in TAG-producing Actinobacteria, which provide reducing equivalents and participate in the carbon flux homeostasis during fatty acid and TAG synthesis. Rodriguez et al. (2012) reported the occurrence of two genes coding for malic enzymes in *Streptomyces coelicolor* A3(2), whereas MacEachran and Sinskey (2013) identified and characterized a gene encoding a non-phosphorylative glyceraldehyde dehydrogenase enzyme in *R. opacus* PD630, which is specifically activated during TAG accumulation.

According to the current knowledge on TAG-producing prokaryotes, the ability of bacterial cells to synthesize and accumulate those lipids is not only supported by the presence of key specific enzymes involved in their synthesis, such as WS/DGAT's; but also by the occurrence of an integrated metabolic and regulatory network in lipid-accumulating bacteria.

4 Wax Ester Biosynthesis in Bacteria

Waxes are esters of a long-chain alcohol and a long-chain fatty acid (WE). The key step for WE synthesis is catalyzed by a WS, or by the bifunctional WS/DGAT enzyme, which transfers the acyl group from an acyl-CoA to a fatty acyl alcohol (Fig. 2). In general, WS/DGATs accept acyl groups with carbon chain length of C_{16} to C_{18} and *n*-alcohols with carbon chain length between C_{12} and C_{20} (Shi et al. 2012). The WS/DGAT from *Acinetobacter baylyi* ADP1 (AtfA) exhibits a preference for C_{14} and C_{16} acyl-CoA substrates with C_{14} to C_{18} fatty alcohols (Kalscheuer and Steinbüchel 2003). Interestingly, *Marinobacter hydrocarbonoclasticus* DSM 8798 was able to synthesize isoprenoid WE after growth on phytol as sole carbon source (Rontani et al. 1999). Two WS/DGAT enzymes (WS1 and WS2) were involved in the biosynthesis of isoprenoid WE by strain DSM 8798 (Holtzapple and Schmidt-Dannert 2007).

The ability to produce WE from unrelated carbon sources, such as acetate or glucose, depends not only on the occurrence of a WS/DGAT enzyme but also on the capability of the microorganism for reducing fatty acids to the respective fatty alcohols. Fatty alcohols can be produced by a two-step reduction of either acyl-ACP or acyl-CoA to the respective fatty alcohol via an intermediate fatty aldehyde (Fig. 2). *A. baylyi* ADP1 possesses this route for the sequential reduction of fatty acids to fatty alcohols for WE synthesis (Reiser and Somerville 1997). On the other hand, *Marinobacter aquaeolei* VT8 possesses two fatty acyl-CoA reductases (FarA and AcrB) that able to reduce not only fatty acids to fatty aldehydes but also fatty acids to fatty alcohols directly with consumption of NADPH (Hofvander et al. 2011; Lenneman et al. 2013). Fatty acyl-CoA reductases also occur in Actinobacteria, as has been reported for *M. tuberculosis* (Sirakova et al. 2012). These enzymes produce WE that are accumulated in the cell wall of mycobacteria as a permeability barrier limiting replication by inhibition of nutrient uptake under dormancy state.

In summary, the ability to synthesize WE in bacteria depends on the occurrence of, at least, two types of enzymes: the fatty acid reductases for the de novo production of key alcohol precursors and WS (or WS/DGAT enzymes) for the esterification of fatty acids and fatty alcohols to produce WE. The accumulation of WE in intracellular inclusion bodies is a property only restricted to a few prokaryotes, predominantly Gram-negative Proteobacteria. Other bacteria, such as mycobacteria, produce WE for their accumulation in the cell wall in order to modify its permeability for controlling the uptake of nutrients. Thus, only few bacteria can accumulate large amounts of WE, and most of them produce just trace of these lipids.

5 Metabolic Engineering in TAG Biosynthesis

During the last years, the increasing availability of genomic databases, omic studies, and molecular tools led to a diversity of interesting approaches to enhance lipid production in native oil-accumulating bacteria and enlarge their application

spectrum, or to establish lipid production in the canonical bacterial hosts, which are naturally not able to produce TAG and/or WE, through genetic engineering. In the first line of study, bacteria belonging to *Rhodococcus* and *Acinetobacter* genera have been predominantly used as lipid production platforms via genetic engineering. The overexpression of native genes has been the preferred strategy to improve TAG production in rhodococci. A variable increase of TAG accumulation has been obtained after overexpression of the following genes in rhodococci: (1) *atf1* and *atf2* genes coding for DGAT enzymes in *R. opacus* PD630 (Alvarez et al. 2008; Hernández et al. 2013), and *atf8* in *R. jostii* RHA1 (Amara et al. 2016); (2) a gene coding for a phosphatidic acid phosphatase (PAP2) enzyme in *R. jostii* RHA1 (Hernández et al. 2015); (3) a gene encoding a non-phosphorylative glyceraldehyde dehydrogenase enzyme in *R. opacus* PD630 for enhancing NADPH formation (MacEachran and Sinskey 2013); (4) a gene encoding a thioesterase enzyme in *R. opacus* PD630 (Huang et al. 2016); (5) a gene coding for an ATP-binding cassette transporter protein for the uptake of long chain fatty acids (Villalba and Alvarez 2014); and (6) genes encoding enzymes involved in the catabolism of glycerol from *R. fascians* transferred to *R. opacus* during growth on glycerol as sole carbon source (Herrero et al. 2016). On the other hand, the overexpression of genes encoding $NADP^+$-dependent malic enzymes and PAP2 enzymes also improved TAG accumulation by *Streptomyces coelicolor* (Rodríguez et al. 2012; Comba et al. 2013).

Rhodococci have been also engineered to utilize lignocellulosic raw materials, which are important agro-industrial wastes, as substrates for oil production. In this context, some genes/enzymes from *Streptomyces* involved in the xylose metabolism pathway were used to construct *R. jostii* RHA1 and *R. opacus* PD630 xylose-fermenting strains (Xiong et al. 2012; Kurosawa and Sinskey 2013). The successful engineering of xylose (Xiong et al. 2012; Kurosawa and Sinskey 2013) and cellobiose (Hetzler and Steinbüchel 2013) catabolic pathways in *Rhodococcus* strains suggested that lignocellulosic biomass can be utilized by rhodococci to produce TAG.

Other studies considered the Gram-negative *Acinetobacter baylyi* as a convenient model organism for studying the potentiality and modifiability of WE in a natural host by means of synthetic biology. Santala et al. (2014) successfully expressed a fatty acid reductase complex LuxCDE with an inducible arabinose promoter in *A. baylyi* ADP1 for the production of WE. The introduction of the synthetic pathway also led to a modification of fatty acid and alcohol species in the produced WE, demonstrating the potentiality of recircuiting a biosynthesis pathway in a natural producer, for a regulated production of a customized bioproduct (Santala et al. 2014).

The second line of study involves the possibility to transfer the capability for producing TAG and/or WE by host strains that are naturally not able to synthesize those lipids, such as *Escherichia coli*. Different molecular approaches have been successfully used to produce TAG and/or WE by recombinant *E. coli* strains. Rucker et al. (2013) engineered an *E. coli* strain to synthesize and accumulate TAG from cell wall phospholipid precursors through heterologous expression of two enzymes, phosphatidic acid phosphatase (PAP) and diacylglycerol acyltransferase (DGAT) (Fig. 2). Other study investigated the production of TAG in cells of the wild type of

E. coli and of a strain with a deleted diacylglycerol kinase gene (*dgkA*). The overexpression of *atfA* from *A. baylyi* ADP1 and *fadD* from *E. coli* in the *dgkA* deletion mutant led to a production of approximately 4.9% (w/w) of TAG in batch cultivation (Janβen and Steinbüchel 2014). On the other hand, Comba et al. (2014) used an *E. coli* BL21 derivative strain containing a deletion in the *dgkA* gene as platform for TAG production and the heterologous expression of some TAG biosynthesis genes from *Streptomyces coelicolor*, such as the acyl-CoA: diacylglycerol acyltransferase (DGAT) Sco0958 and the phosphatidic acid phosphatase (PAP) Lppβ. Moreover, the authors performed a series of stepwise optimizations of the chassis: (1) fine-tuning the expression of the heterologous *Sco0958* and *lppβ* genes, (2) overexpression of the *S. coelicolor* acetyl-CoA carboxylase complex, and (3) mutation of *fadE*, the gene encoding for the acyl-CoA dehydrogenase that catalyzes the first step of the β-oxidation cycle in *E. coli*. They obtained a recombinant strain able to produce up to 4.85% cell dry weight (CDW) TAG in batch cultivation (Comba et al. 2014). In other study, Röttig et al. (2015) engineered an *E. coli* strain by expressing *fadD* (acyl-CoA synthetase), *fadR* (transcriptional activator of fatty acid biosynthesis genes), *pgpB* (phosphatidic acid phosphatase), *plsB* (glycerol-3-phosphate acyltransferase), and *tesA* (thioesterase A) in combination with *atfA* (WS/DGAT) from *Acinetobacter baylyi*, which led to a total fatty acid contents of up to 16% (w/w) on complex media, corresponding to approximately 9% (w/w) of TAG. *E. coli* has been also used for the biosynthesis of WE upon metabolic engineering. Kalscheuer et al. (2006) co-expressed in a recombinant *E. coli* strain a fatty alcohol-producing bifunctional acyl-coenzyme A reductase from the jojoba plant and the bacterial WS from *A. baylyi* strain ADP1 for the synthesis of WE. In the presence of oleate, jojoba oil-like WEs such as palmityl oleate, palmityl palmitoleate, and oleyl oleate were produced, amounting to up to ca. 1% of CDW.

Recently, Plassmeier et al. (2016) reported the use of *Corynebacterium glutamicum* as platform for TAG biosynthesis through metabolic engineering. Firstly, they performed the heterologous expression of four genes involved in TAG biosynthesis and accumulation in rhodococci, such as *atf1* and *atf2*, encoding DGAT enzymes; *pgpB*, encoding the phosphatidic acid phosphatase enzyme to complete the TAG biosynthesis pathway; and *tadA* coding for a structural protein for lipid body assembly. On the other hand, the authors applied four metabolic strategies to increase TAG accumulation: (i) boosting precursor supply by heterologous expression of *tesA* (encoding a thioesterase to form free fatty acid to reduce the feedback inhibition by acyl-ACP) and *fadD* (encoding acyl-CoA synthetase to enhance acyl-CoA supply), (ii) reduction of TAG degradation and precursor consumption by deleting four cellular lipases (*cg0109*, *cg0110*, *cg1676* and *cg1320*) and the diacylglycerol kinase (*cg2849*), (iii) enhancement of fatty acid biosynthesis by deletion of *fasR* (*cg2737*, TetR-type transcriptional regulator of genes for the fatty acid biosynthesis), and (iv) elimination of the observed by-product formation of organic acids by blocking the acetic acid (*pqo*) and lactic acid production (*ldh*) pathways. The recombinant *C. glutamicum* strain was able to produce TAG, and the intracellular fatty acid content amounted approximately 17% of the CDW.

All these studies demonstrated that the synthesis and accumulation of TAG and/or WE can be engineered using the native oil producers or other nonlipid-accumulating bacterial hosts as cellular production platforms.

6 Research Needs

Oleagenicity demands not only the occurrence of key enzymes for the esterification of alcohols (glycerol or long-chain fatty alcohols) with fatty acids, such as WS and DGAT enzymes, but also the integration of these reactions with the metabolic and regulatory network of cell. Oleaginous microorganisms must be able to generate a high carbon flux through metabolic pathways that provide key intermediates for lipogenesis; they need a set of reactions which generate the necessary NADPH pool for supporting high levels of fatty acid biosynthesis, an efficient mechanism that coordinate and balance phospholipid and TAG pathways which share common reactions: a set of proteins involved in the assembly, stabilization and structure of lipid inclusion bodies, and efficient regulatory circuits for the fine regulation of the lipid accumulation process. This could be one of the main reasons why bacterial host such as *E. coli*, which is naturally unable to produce TAG and/or WE, is not able to accumulate large amounts of lipids after expression of heterologous genes from lipid-accumulating bacteria. Although promising advances on basic aspects of lipid accumulation in native bacterial producers and the metabolic engineering of diverse bacterial hosts were done, further efforts are necessary for understanding the ecological role of these lipids and for their application in the industry. It is necessary to focus research on the identification and characterization of new components of the lipid-accumulating machinery in native bacteria. The combination of these results obtained in model microorganisms with the increasing availability of a wide variety of new genetic tools and genome sequences and the application of high-throughput techniques and synthetic biology strategies will led to promising advances in the basic knowledge of lipid-accumulating process and in the metabolic engineering of diverse bacterial hosts for the design of new oil-derived bioproducts.

References

Alvarez AF, Alvarez HM, Kalscheuer R, Wältermann M, Steinbüchel A (2008) Cloning and characterization of a gene involved in triacylglycerol biosynthesis and identification of additional homologous genes in the oleaginous bacterium *Rhodococcus opacus* PD630. Microbiology 154:2327–2335

Amara S, Seghezzi N, Otani H, Diaz-Salazar C, Liu J, Eltis LD (2016) Characterization of key triacylglycerol biosynthesis processes in rhodococci. Sci Report 6:24985

Arabolaza A, Rodriguez E, Altabe S, Alvarez H, Gramajo H (2008) Multiple pathways for triacylglycerol biosynthesis in *Streptomyces coelicolor*. Appl Environ Microbiol 74:2573–2582

Comba S, Menendez-Bravo S, Arabolaza A, Gramajo H (2013) Identification and physiological characterization of phosphatidic acid phosphatase enzymes involved in triacylglycerol biosynthesis in *Streptomyces coelicolor*. Microb Cell Factories 12:9

Comba S, Sabatini M, Menendez-Bravo S, Arabolaza A, Gramajo H (2014) Engineering a *Streptomyces coelicolor* biosynthesis pathway into *Escherichia coli* for high yield triglyceride production. Biotechnol Biofuels 7:172

Daniel J, Deb C, Dubey VS, Sirakova T, Abomoelak MHR, Kolattukudy PE (2004) Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. J Bacteriol 186:5017–5030

Daniel J, Sirakova T, Kolattukudy P (2014) An acyl-CoA synthetase in *Mycobacterium tuberculosis* involved in triacylglycerol accumulation during dormancy. PLoS One 9:e114877

Finkelstein DB, Brassell SC, Pratt LM (2010) Microbial biosynthesis of wax esters during desiccation: adaptation for colonization of the earliest terrestrial environments? Geology 38:247–250

Hernández MA, Arabolaza A, Rodríguez E, Gramajo H, Alvarez HM (2013) The *atf*2 gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous *Rhodococcus opacus* PD630. Appl Microbiol Biotechnol 97:2119

Hernández MA, Comba S, Arabolaza A, Gramajo H, Alvarez HM (2015) Overexpression of a phosphatidic acid phosphatase type 2 leads to an increase in triacylglycerol production in oleaginous *Rhodococcus* strains. Appl Microbiol Biotechnol 99:2191

Herrero OM, Moncalián G, Alvarez HM (2016) Physiological and genetic differences amongst *Rhodococcus* species for using glycerol as a source for growth and triacylglycerol production. Microbiology 162(2):384–397

Hetzler S, Steinbüchel A (2013) Establishment of cellobiose utilization for lipid production in *Rhodococcus opacus* PD630. Appl Environ Microbiol 79(9):3122–3312

Hofvander P, Doan TT, Hamberg M (2011) A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. FEBS Lett 585:3538–3543

Holder JW, Ulrich JC, DeBono AC, Godfrey PA, Desjardins CA, Zucker J, Zeng Q, Leach ALB, Ghiviriga I, Dancel C, Abeel T, Gevers D, Kodira CD, Desany B, Affourtit JP, Birren BW, Sinskey AJ (2011) Comparative and functional genomics of *Rhodococcus opacus* PD630 for biofuels development. PLoS Genet 7(9):e1002219

Holtzapple E, Schmidt-Dannert C (2007) Biosynthesis of isoprenoid wax ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: identification and characterization of isoprenoid coenzyme A synthetase and wax ester synthases. J Bacteriol 189:3804–3812

Huang L, Zhao L, Zan X, Song Y, Ratledge C (2016) Boosting fatty acid synthesis in *Rhodococcus opacus* PD630 by overexpression of autologous thioesterases. Biotechnol Lett 38(6):999–1008

Indest KJ, Eberly JO, Ringelberg DB, Hancock DE (2015) The effects of putative lipase and wax ester synthase/acyl-CoA: diacylglycerol acyltransferase gene knockouts on triacylglycerol accumulation in Gordonia sp. KTR9. J Ind Microbiol Biotechnol 42(2):219–227

Janßen HJ, Steinbüchel A (2014) Production of triacylglycerols in *Escherichia coli* by deletion of the diacylglycerol kinase gene and heterologous overexpression of atfA from *Acinetobacter baylyi* ADP1. Appl Microbiol Biotechnol 98(4):1913–1924

Kalscheuer R, Steinbüchel A (2003) A novel bifunctional wax ester synthase/acyl-CoA: diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. J Biol Chem 278:8075–8082

Kalscheuer R, Stöveken T, Luftmann H, Malkus U, Reichelt R, Steinbüchel A (2006) Neutral lipid biosynthesis in engineered *Escherichia coli*: jojoba oil-like wax esters and fatty acid butyl esters. Appl Environ Microbiol 72(2):1373–1379

Kalscheuer R, Stöveken T, Malkus U, Reichelt R, Golyshin PN, Sabirova JS, Ferrer M, Timmis KN, Steinbüchel A (2007) Analysis of storage lipid accumulation in *Alcanivorax borkumensis*: evidence for alternative triacylglycerol biosynthesis routes in bacteria. J Bacteriol 189:918–928

Kurosawa K, Sinskey AJ (2013) Engineering xylose metabolism in triacylglycerol producing *Rhodococcus opacus* for lignocellulosic fuel production. Biotechnol Biofuels 6:134–147

Lenneman EM, Ohlert JM, Palani NP, Barney BM (2013) Fatty alcohols for wax esters in *Marinobacter aquaeolei* VT8: two optional routes in the wax biosynthesis pathway. Appl Environ Microbiol 79:7055–7062

Low KL, Shui G, Natter K, Yeo WK, Kohlwein SD, Dick T, Wenk MR (2010) Lipid droplet-associated proteins are involved in the biosynthesis and hydrolysis of triacylglycerol in *Mycobacterium bovis* bacillus Calmette-Guerin. J Biol Chem 285(28):21662–21670

MacEachran DP, Sinskey AJ (2013) The *Rhodococcus opacus* TadD protein mediates triacylglycerol metabolism by regulating intracellular NAD(P)H pools. Microb Cell Factories 12:104

MacEachran DP, Prophete ME, Sinskey AJ (2010) The *Rhodococcus opacus* PD630 heparin-binding hemagglutinin homolog TadA mediates lipid body formation. Appl Environ Microbiol 76:7217–7225

Plassmeier J, Li Y, Rueckert C, Sinskey AJ (2016) Metabolic engineering *Corynebacterium glutamicum* to produce triacylglycerols. Metab Eng 33:86–97

Reiser S, Somerville C (1997) Isolation of mutants of *Acinetobacter calcoaceticus* deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme A reductase. J Bacteriol 179:2969–2975

Rodriguez E, Navone L, Casati P, Gramajo H (2012) Impact of malic enzymes on antibiotic and triacylglycerol production in *Streptomyces coelicolor*. Appl Environ Microbiol 78 (13):4571–4579

Rontani JF, Bonin PC, Volkman JK (1999) Production of wax esters during aerobic growth of marine bacteria on isoprenoid compounds. Appl Environ Microbiol 65:221–230

Röttig A, Zurek PJ, Steinbüchel A (2015) Assessment of bacterial acyltransferases for an efficient lipid production in metabolically engineered strains of *E. coli*. Metab Eng 32:195–206

Rucker J, Paul J, Pfeifer BA, Lee K (2013) Engineering *E. coli* for triglyceride accumulation through native and heterologous metabolic reactions. Appl Microbiol Biotechnol 97:2753–2759

Santala S, Efimova E, Koskinen P, Karp MT, Santala V (2014) Rewiring the wax ester production pathway of *Acinetobacter baylyi* ADP1. ACS Synth Biol 3(3):145–151

Shi S, Valle-Rodríguez JO, Khoomrung S, Siewers V, Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. Biotechnol Biofuels 5:7

Sirakova TD, Deb C, Daniel J, Singh HD, Maamar H, Dubey VS, Kolattukudy PE (2012) Wax ester synthesis is required for *Mycobacterium tuberculosis* to enter in vitro dormancy. PLoS One 7 (12):e51641

Sohlenkamp C, Geiger O (2015) Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev 40:133–159

Villalba MS, Alvarez HM (2014) Identification of a novel ATP-binding cassette transporter involved in long-chain fatty acid import and its role in triacylglycerol accumulation in *Rhodococcus jostii* RHA1. Microbiology 160:1523–1532

Xiong XC, Wang X, Chen SL (2012) Engineering of a xylose metabolic pathway in *Rhodococcus* strains. Appl Environ Microbiol 78(16):5483–5491

Biogenesis of Medium-Chain-Length Polyhydroxyalkanoates

25

Ryan Kniewel, Olga Revelles Lopez, and M. Auxiliadora Prieto

Contents

1 Introduction 458
2 mcl-PHA Biogenesis 458
2.1 PHA Synthases 460
2.2 The Carbonosome Is the Subcellular Structure for mcl-PHA Production 463
2.3 PHAs and Central Metabolism 466
3 Bioengineering mcl-PHA Production 467
3.1 Cultivation 467
3.2 Metabolic Engineering 468
3.3 Improving PHA Synthesis 470
3.4 PHA Recovery 471
4 Research Needs and Synthetic Biology Tools 471
References 474

Abstract

Medium-chain-length polyhydroxyalkanoates (mcl-PHA) are biotechnologically useful natural products found in many bacteria. This biopolymer functions as a carbon and energy storage reservoir in cells but has physical and mechanical properties that make it a promising bioplastic with applications ranging from adhesives to medical implants. Therefore, there is much interest in understanding the biology of mcl-PHA synthesis and metabolism. Increased knowledge of PHA biology serves as a foundation for the bioengineering of PHA and its eventual use as a biologically derived product. This chapter covers the state of knowledge on

R. Kniewel · M. A. Prieto (✉)
Department of Environmental Biology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain
e-mail: rkniewel@cib.csic.es; auxi@cib.csic.es

O. Revelles Lopez
Bioprocess Laboratory D-BSSE, ETH Zürich, Basel, Switzerland
e-mail: olga.revelles@bsse.ethz.ch

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_29

mcl-PHA, including its synthesis and its central role in cellular metabolism. Moreover, this chapter discusses methods for bioengineering mcl-PHA production in bacteria as well as synthetic biology methods for its study and production in the natural mcl-PHA producer, *Pseudomonas putida*.

1 Introduction

Polyhydroxyalkanoate (PHA) production is widespread in bacteria, being found in many Gram-negative and Gram-positive genera, as well as some archaea (Han et al. 2010; for a complete list of bacterial genera with references, see Lu et al. 2009). PHAs are hydrophobic polymers that accumulate in intracellular inclusions termed PHA granules or carbonosomes. The traditional view is that PHA serves an energy storage function with favorable conditions for its production commonly being a nutrient imbalance, including the availability of a suitable carbon source coupled with an imbalance in at least one other nutrient. These other lacking nutrients vary, with one commonly being nitrogen, but can include phosphate, oxygen, sulphate, or various elements (Kim and Lenz 2001). Recently, a more nuanced view presents PHA as not solely having an energy storage function but as a key component in the cycle of carbon storage and energy expenditure by the cell (Escapa et al. 2012).

While numerous organisms have been studied for their natural or recombinant production of short-chain-length PHA (scl-PHA), i.e., poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxyvalerate) (PHV), fewer organisms are natural producers of medium-chain-length PHA (mcl-PHA), with *Pseudomonas* spp. being the best studied. Mcl-PHAs have elastomeric and thermal properties that generally make them superior bioplastics for rubber and viscoelastic applications when compared to scl-PHA (Sudesh et al. 2000). The following sections will focus on mcl-PHA-producing *Pseudomonas* species with descriptions from the scl-PHA-producing *Cupriavidus necator* (formerly named *Ralstonia eutropha*) or other scl-PHA producers presented for comparison.

2 mcl-PHA Biogenesis

mcl-PHA is synthesized by the enzymatic polymerization of *(R)*-3-hydroxyacyl-CoA monomers containing between six and 14 carbons (3-hydroxyhexanoate and 3-hydroxytetradecanoate, respectively). PHA molecules are highly variable in regard to their number of monomeric repeats and the length and composition of their side chains. For example, a PHA homopolymer made up of solely 3-hydroxyhexanoate (poly(3-hydroxyhexanoate)) has a side chain of three carbon atoms, whereas a copolymer or heteropolymer consisting of 3-hydroxybutyrate and 3-hydroxyoctanoate (poly(3-hydroxybutyrate-co-3-hydroxyoctanoate)) has side chains of one carbon and five carbon atoms, respectively. Several factors determine whether a copolymer is random or contains blocks of monomers, including the metabolic state of the bacteria and the

time of exposure to various carbon sources, both of which influence what precursor substrates are available to the cell for PHA production. PHA polymer composition varies widely both among bacterial species and within individual bacteria as it depends on the carbon source(s), the metabolic pathway utilized, and the enzymes used to supply monomers. Additional variety in PHAs stems from the ability of some PHA synthases to naturally incorporate 4-, 5-, and 6-hydroxyacids or unsaturated hydroxyacids into the polymer, in addition to 3-hydroxyacids (Lageveen et al. 1988; Saito et al. 1996; Madison and Huisman 1999).

Three metabolic pathways generate precursors for PHA synthesis (Fig. 1) (reviewed in Prieto et al. 2016). Typically, scl-PHAs such as PHB are synthesized through the glycolysis of sugars to produce acetyl-CoA (Fig. 1, Pathway I). Two acetyl-CoA molecules are then combined by β-ketothiolase (PhaA) creating acetoacetyl-CoA, which is then reduced into 3-hydroxybuturyl-CoA (3HB) by acetoacetyl-CoA reductase (PhaB). The 3HB monomers are then polymerized by

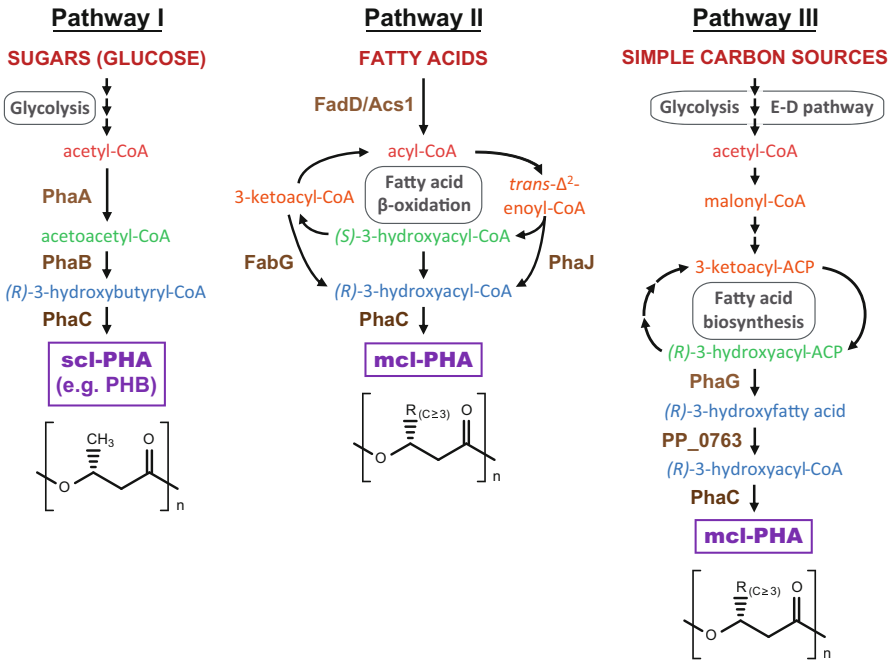


Fig. 1 Three metabolic pathways involved in the synthesis of PHA Three metabolic pathways used by bacteria to create PHA precursors. Pathway I is predominantly used in PHB-producing organisms, such as *C. necator*, while Pathways II and III are present in mcl-PHA producing pseudomonads. Enzymes are indicated in *bold* adjacent to arrows; processes involving many steps are indicated by rounded *rectangles*: glycolysis, the Entner–Doudoroff (E-D) pathway (used in pseudomonads), as well as the cycles for β-oxidation and biosynthesis of fatty acids. PHAs produced are indicated within rectangles with the chemical structure of the resulting PHA monomer given below. Note that Pathways II and III have the ability to produce mcl-PHAs from 3-hydroxyhexanoate and longer chain hydroxyacids resulting in polymers containing side chains (R-groups) with three or more carbons

PHA synthase (PhaC). This is the typical scl-PHA synthesis pathway utilized in bacteria such as *C. necator*.

In contrast, Pathways II and III are commonly used for mcl-PHA synthesis in *Pseudomonas* spp. Longer carbon chain monomers required for mcl-PHA synthesis are generated through Pathway II by the degradation of fatty acids by the β-oxidation cycle (Fig. 1, Pathway II). β-oxidation oxidizes fatty acids into *trans*-Δ^2-enoyl-CoA, *(S)*-3-hydroxyacyl-CoA, and 3-ketoacyl-CoA. These intermediates are then converted into *(R)*-3-hydroxyacyl-CoA (3HA-CoA) monomers, respectively, by a stereospecific enoyl-CoA hydratase (PhaJ), an epimerase, and a 3-ketoacyl-CoA reductase (FabG) (Tortajada et al. 2013). As with Pathway I, these mcl-3HA-CoA monomers are incorporated into PHA polymers by PhaC.

mcl-PHA monomers can also be synthesized by de novo fatty acid biosynthesis (Fig. 1, Pathway III). The de novo biosynthesis of fatty acids has special role in PHA biosynthesis as this pathway can provide various types of 3-hydroxyacyl (3HA) precursors from simple carbon sources such as glucose, fructose, gluconate, glycerol, ethanol, and acetate (Tortajada et al. 2013). These precursors are oxidized into acetyl-CoA, followed by their conversion by a series of reactions into malonyl-CoA and culminating in (*R*)-3HA-acyl carrier protein (ACP). This forms the first unit that is further elongated by successive two-carbon units. (*R*)-3HA-ACP intermediates were thought to be subsequently transformed into (*R*)-3HA-CoA thioesters by a specific CoA transferase (PhaG) found in most pseudomonads (Rehm et al. 1998). Recently, Wang et al. reported that PhaG functions as a (*R*)-3HA-ACP thioesterase that produces free (*R*)-3-hydroxyfatty acids rather than (*R*)-3HA-CoA (Wang et al. 2012). Evidence was presented showing that the PP_0763 gene product in *P. putida* functions as the medium-chain fatty acid-CoA ligase necessary to incorporate (*R*)-3-hydroxyfatty acids derived from fatty acid biosynthesis into the PHA polymer chain.

PHA production and catabolism are connected through a continuous cycle in which PHA synthesis and depolymerization are simultaneously active, resulting in constant turnover of PHA (Fig. 2a) (Ren et al. 2009a; de Eugenio et al. 2010a; Arias et al. 2013). PHA depolymerases (PhaZ) are continuously releasing 3-hydroxyfatty acids from PHA granules. These released 3-hydroxyfatty acids are then converted into 3HA-CoAs by the FadD/Acs1 acyl-CoA synthetase and can then be either catabolized via β-oxidation (Fig. 1, Pathway II) or directly reincorporated to the granule by PHA synthase.

2.1 PHA Synthases

PHA synthases are α-/β-hydrolase family enzymes that catalyze the polymerization of *(R)*-3HA-CoA thioester monomers. PHA synthases are grouped into four classes. Class I and class II synthases require a single subunit, PhaC, for activity, while classes III and IV require an additional subunit, PhaE or PhaR, respectively (Table 1). The substrate specificities vary among the classes: classes I, III, and IV synthases have high affinities for 3–5 carbon scl-3HA-CoA precursors to form scl-PHA, with class I and III synthases generally having additional low affinities

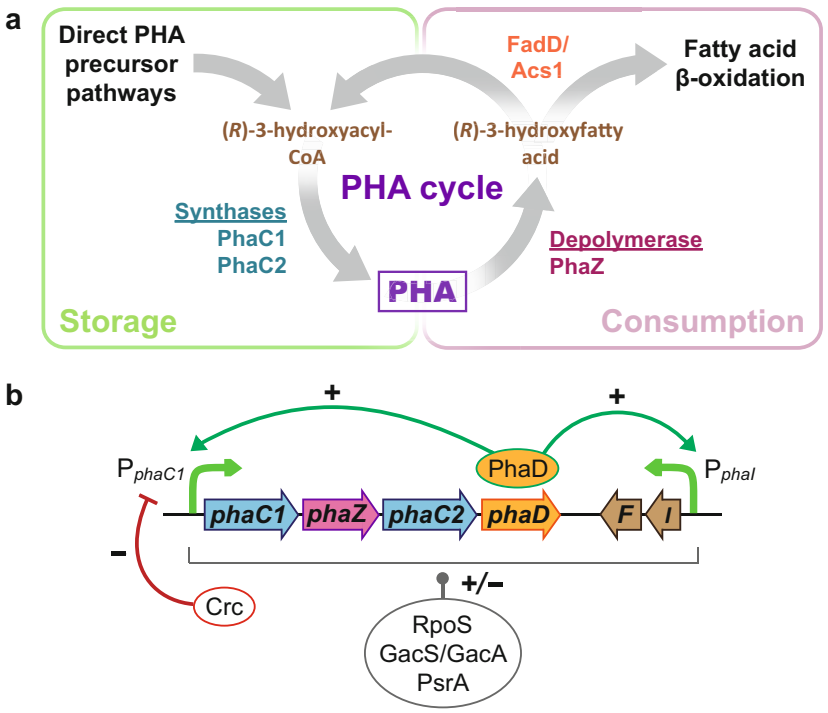


Fig. 2 The PHA cycle and regulation of *pha* gene expression in pseudomonads. (**a**) PHA production and utilization proceeds in a continuous cycle that is altered depending upon the energy and carbon needs of the cell. In response to an imbalance of specific nutrients, PHA is synthesized by the PHA synthases, PhaC1, and PhaC2 as a means to store carbon and reducing equivalents from precursors obtained from pathways II and III. Conversely, when consumed, PHA is depolymerized by the PHA depolymerase, PhaZ. The released 3-hydroxyfatty acids can then be recycled into 3-hydroxyacyl-CoAs by the acyl-CoA synthetase, FadD/Acs1, thus completing the PHA cycle, or can be used in the fatty acid β-oxidation pathway. (**b**) Schematic of the primary *pha* gene cluster in *P. putida* KT2440 and its regulatory elements. The two operons, *phaC1ZC2D* and *phaIF*, are under the partial control of the regulator, PhaD, which enhances transcription across the cluster. The global transcriptional regulator Crc has an inhibitory effect on *phaC1* transcription, and additional transcriptional regulators have been implicated to modulate *pha* gene transcription, yet their specific effects are to be determined

for ≥6 carbon mcl-3HA-CoA precursors, yet this varies by species. Notably, the class I synthases from *Aeromonas caviae* and *Rhodospirillum rubrum* or the class III synthase from *Thiocapsa pfennigii* are capable of producing mcl-PHAs containing 3-hydroxyhexanoate or 3-hydroxyoctanoate when provided with carbon sources of these lengths (Brandl et al. 1989; Fukui and Doi 1997; Liebergesell et al. 2000). However, the class II PHA synthases found in *Pseudomonas* species are clearly different than the other classes in regard to their substrate specificity as these synthases readily generate mcl-PHAs from mcl-3HA-CoA precursors (Huisman et al. 1989; Matsusaki et al. 1998).

Table 1 PHA synthase classes and representative PHA-producing species

PHA synthase class	Subunits	Representative species	Characteristics	Preferred substrate[a]	PHAs produced[b]		References[c]
					scl-PHA 3-5C	mcl-PHA ≥6C	
I	PhaC	*Cupriavidus necator*, *C. necator* H16		3HB-CoA, mcl-3HA-CoA (low affinity)	P(3HB), P(3HB-co-3HV)		(Antonio et al. 2000; Zhang et al. 2001; Reinecke and Steinbüchel 2009)
		Aeromonas caviae	Uncommon class I PHA synthase	scl- and shorter mcl-3HA-CoA	P(3HB)	P(3HB-co-3HHx) P(3HB-co-3HA)	(Doi et al. 1995; Fukui and Doi 1997)
		Rhodospirillum rubrum		scl- and shorter mcl-3HA-CoA	P(3HB), P(3HV)	P(3HHx)	(Brandl et al. 1989; Jin and Nikolau 2012)
II	PhaC	*Pseudomonas putida*, *P. aeruginosa*		mcl-3HA-CoA	P(3HB), P(3HV)	P(3HA)	(Huisman et al. 1989, 1992; Timm and Steinbüchel 1990)
		Pseudomonas sp. 61-3		3HB-CoA, mcl-3HA-CoA	P(3HB)	P(3HB-co-3HA)	(Matsusaki et al. 1998)
III	PhaC PhaE	*Allochromatium vinosum*		3HB-CoA, mcl-3-HA-CoA (low affinity)	P(3HB), P(3HB-co-3HV)		(Liebergesell and Steinbüchel 1992; Yuan et al. 2001)
		Thiocapsa pfennigii	Uncommon class III PHA synthase	scl- and shorter mcl-3HA-CoA	P(3HB), P(3HV), P(4HV)	low P(3HHx), P(3HO)	(Liebergesell et al. 2000; Gorenflo et al. 2001)
IV	PhaC PhaR	*Bacillus* spp.		3HB-CoA	P(3HB), P(3HB-co-3HV)		(McCool and Cannon 2001; Shamala et al. 2003; Singh et al. 2009)

[a]As determined by enzymatic analysis of purified PHA synthase
[b]Examples of PHAs produced, products vary depending on carbon source. P(3HB), poly(3-hydroxybutyrate); P(3HV), poly(3-hydroxyvalerate); P(4HV), poly(4-hydroxyvalerate); P(3HHx), poly(3-hydroxyhexanoate); P(3HO), poly(3-hydroxyoctanoate); P(3HA), poly(3-hydroxalkanoates) ≥ 6 carbons
[c]References are relevant examples and are not exhaustive

Pseudomonas species commonly have two genes encoding PhaC synthase, *phaC1*, and *phaC2*. When expressed separately in recombinant *E. coli*, the two enzymes had similar substrate specificities with decanoate or dodecanoate being the carbon sources that produced the highest yield of PHA when *phaC1* or p*haC2* were expressed, respectively (Langenbach et al. 1997; Qi et al. 1997). Likewise, the composition of the PHA produced by PhaC1 or PhaC2 were similar with the formation of 3-hydroxydecanoate being predominant (50–75 mol %) when cells were provided with decanoate or dodecanoate.

Much interest is centered on engineering the single subunit class I and class II PHA synthases to improve their activities, substrate specificities, product polymer characteristics, and stabilities (for review, see Nomura and Taguchi 2007). This and other bioengineering synthetic biology approaches are further discussed below in Sect. 3.

PHA synthase genes, along with the other genes involved in PHA metabolism, are often organized in operons, though their organization varies among species (reviewed in Taguchi et al. 2005). Several common patterns exist. One pattern has the *phaC* gene contained within the same locus as the *phaA* and *phaB* genes, as found in the *phaCAB* operon of *C. necator*. A second pattern is found in many pseudomonads wherein the two synthase genes, *phaC1* and *phaC2,* flank the *phaZ* depolymerase gene, forming the *phaC1ZC2* operon (Fig. 2b). Some pseudomonads, such as *Pseudomonas* sp. 61-3, contain a hybrid of these two patterns, with one locus containing the *phaC1ZC2* operon and another containing the *phaBAC* operon (Matsusaki et al. 1998).

2.2 The Carbonosome Is the Subcellular Structure for mcl-PHA Production

Bacteria producing PHA form intracellular, roughly spherical inclusions (carbonosomes) that contain densely packed hydrophobic PHA (Fig. 3) (Jendrossek and Pfeiffer 2014). The size, number, and cellular distribution of carbonosomes vary considerably among bacterial species. In pseudomonads, carbonosomes can account for nearly 90% of cell dry weight and fill the majority of the intracellular space (Jendrossek 2009; Galán et al. 2011). Carbonosomes are generally concentrated around the center of the longitudinal axis of the cell with a 500 nm median diameter and distributions ranging from one to approximately ten inclusions per cell with a mean of 5–6 (Beeby et al. 2012). Little is known regarding the precise steps leading to the development of carbonosomes, including whether fission or fusion of carbonosomes occurs. Several models have been proposed to describe the intracellular distribution of carbonosomes (Jendrossek and Pfeiffer 2014). Among these, the scaffold model is currently the best supported and posits that carbonosomes are arranged around a yet to be confirmed scaffold that determines their localization and mediates their segregation during cell division. The nucleoid is suspected to comprise this scaffold, as supported by both the observation that carbonosomes are concentrated around the cell center in most species as well as the ability of granule-associated proteins (phasins) to interact with DNA (see below) (Galán et al. 2011; Pfeiffer et al. 2011). The scaffold-mediated

arrangement of carbonosomes around the nucleoid could provide a motive underlying the observation that carbonosomes are faithfully segregated into both daughter cells during cell division, as occurs with replicated nucleoid.

2.2.1 PHA Granule Composition

The first determined chemical composition of isolated PHB carbonosomes was from *Bacillus megaterium*, which contained 97.7% PHB, 1.87% protein, and 0.46% lipids (Griebel et al. 1968). However, the exact fraction and composition of the lipid component remain elusive (Steinbuchel et al. 1995; Bresan et al. 2016). Electron and atomic force microscopy studies revealed that carbonosomes are composed of a thin, approximately 4 nm lattice-like surface layer that overlies a denser core of packed hydrophobic PHA (Fig. 3c) (Mayer and Hoppert 1997; Dennis et al. 2003, 2008). Carbonosomes appear to function to segregate nonpolar PHA molecules aggregated by the hydrophobic effect away from the aqueous environment of the cytoplasm. This oil-in-water model of carbonosomes posits that the surface coat functions as an amphipathic interface, yet its exact composition remains to be fully determined. A recent microscopic study to detect phospholipids on carbonosomes in *P. putida* and *C. necator* failed to show their presence in situ, casting doubt on whether carbonosomes contain both protein and phospholipids on their surface (Bresan et al. 2016).

Carbonosomes isolated from a variety of bacterial species were found to contain a number of surface-bound proteins when separated by electrophoresis (Fuller et al. 1992; Steinbuchel et al. 1995; Stuart et al. 1998) and/or detected by mass spectrometry (Jendrossek and Pfeiffer 2014). Further studies identified specific carbonosome

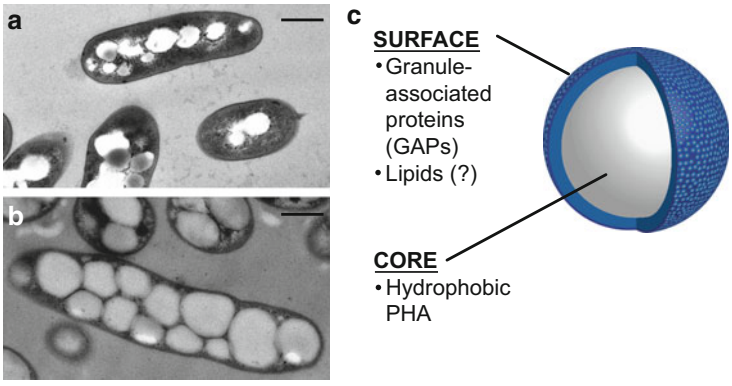


Fig. 3 Carbonosomes in bacteria producing PHA. Electron micrographs of Gram-negative bacteria producing PHA with white carbonosome spheroids visible in (**a**) *P. putida* KT2440 and (**b**) *C. necator* H16 (scale bar = 0.5 μm). (**c**) Depiction of the proposed carbonosome structure. In *Pseudomonas* spp., the granule-associated proteins (GAPs) include PHA synthase, PhaC1; PHA depolymerase, PhaZ; acyl-CoA synthetase, Acs1; and the phasins, PhaF and PhaI. The precise composition of the lipid component remains to be resolved

granule-associated proteins (GAPs). In *Pseudomonas* spp., these include PHA synthase, PhaC1; PHA depolymerase, PhaZ; acyl-CoA synthetase, Acs1; and phasins (see below) (Stuart et al. 1996; Ruth et al. 2008; Ren et al. 2009a, b). This compliment of enzymes belies the metabolic cycle of PHA synthesis for carbon and energy storage and depolymerization to release stored PHA resources. The presence of the acyl-CoA synthetase Acs1 on the surface of carbonosomes likely functions to CoA-activate 3-hydroxyfatty acids released by the action of the PHA depolymerase PhaZ thereby making them available for degradation by β-oxidation for use as the PHA-derived energy source (Fig. 2a) (Ruth et al. 2008). Additionally, a unique class of proteins, the phasins, is found on the surface of carbonosomes, which in pseudomonads are PhaF and PhaI (Prieto et al. 1999; Sandoval et al. 2007; Ren et al. 2009b). These small proteins are the predominant protein component of carbonosomes and comprise a unique class of intrinsically disordered proteins (Pötter et al. 2004; Neumann et al. 2008; Maestro et al. 2013). Phasins have been most thoroughly investigated in *P. putida*, with two identified phasins, and in *C. necator* H16, which has seven identified phasins (Kuchta et al. 2007; Pfeiffer and Jendrossek 2011, 2012).

A representative and well-studied phasin is PhaF from *P. putida*. Biophysical analyses of purified PhaF and its structural modeling revealed that it is likely a tetramer in solution with each monomer adopting a secondary structure of predominantly alpha-helices that are interspersed by regions of random coil (Maestro et al. 2013). Structural modeling predicted an extended amino-terminal alpha-helical region as well as a separate central region. The central region was predicted to contain amphipathic alpha-helices and leucine residues contained within heptad repeats with the potential to form a leucine zipper dimerization domain. The carboxy-terminal region contains AAKP repeats that are found in DNA-binding proteins, such as eukaryotic histone H1 and AlgP from *Pseudomonas aeruginosa* (Timm and Steinbüchel 1992). Further structure-function studies of PhaF revealed that the amino-terminal region forms a stable structure that is necessary for carbonosomes interaction, whereas the carboxyl-terminal region was capable of binding DNA in a sequence-independent manner (Moldes et al. 2004; Galán et al. 2011). The smaller phasin protein in *P. putida*, PhaI, is homologous to the amino-terminal region of PhaF but lacks the carboxy-terminal DNA-binding region (Prieto et al. 1999).

The function of phasins was revealed through genetic studies. The deletion of *P. putida phaF* resulted in a twofold reduction in PHA production, a decrease in the number and size of PHA granules, as well as their aberrant localization and mis-segregation during cell division (Prieto et al. 1999; Galán et al. 2011; Dinjaski and Prieto 2013). The phenotype of the Δ*phaF* mutant was mitigated to some extent by the presence of *phaI,* which likely has some redundant functions. The deletion of *phaI* alone had a similar reduction in PHA accumulation as seen with Δ*phaF*, yet the deletion of both *phaF* and *phaI* led to an eightfold reduction in PHA accumulation compared to wild type. This suggests that both phasins are required for proper PHA accumulation. These results of *P. putida* phasins are mirrored by studies of phasin-like proteins from other PHA-producing bacteria (Pfeiffer et al. 2011).

2.3 PHAs and Central Metabolism

2.3.1 Regulatory Network of the mcl-PHA Cycle

Due to the tight connection between PHA biosynthesis and cellular metabolism, its regulation is quite complex and dependent upon several factors, including the carbon source(s), the specificity of the PHA synthase, and the metabolic pathways involved. PHA accumulation is under systemic regulation that involves indirect PHA precursor routes that link the catabolism of carbon sources to PHA metabolism, fatty acid β-oxidation and de novo fatty acid synthesis, and specific PHA metabolism (the PHA cycle) (Fig. 2a). Furthermore, the regulation of PHA metabolism takes place at different levels, at the transcriptional and posttranscriptional levels by specific and global regulatory factors and through regulation of the carbon flow of different pathways that provide PHA precursors.

Transcriptional regulation is one means for regulating PHA synthesis. In *P. putida* and other pseudomonads, the *pha* cluster is organized into two main operons, *phaC1ZC2D* and *phaIF,* that are under the regulation of the TetR-like transcriptional activator, PhaD, in response to β-oxidation metabolites (Fig. 2b) (Klinke et al. 2000; de Eugenio et al. 2010b). Additionally, the global regulator Crc negatively regulates the expression of the *pha* genes under balanced C/N conditions (La Rosa et al. 2014). It was demonstrated that Crc activity is influenced not only by the carbon source but also by the C/N ratio of the culture medium. Other global transcriptional regulators, including RpoS, PsrA, and the GacS/GacA system, also affect PHA accumulation (Ruiz et al. 2004; de Eugenio et al. 2010b; Fonseca et al. 2014).

As mentioned above, active turnover in PHA accumulation and degradation is linked to central carbon metabolism. Intracellular ratios of acetyl-CoA/free CoA and $NADH/NAD^+$ orchestrate the carbon flux of 3HA-CoA with cellular demand by regulating the PHA cycle (Ruth et al. 2008; de Eugenio et al. 2010a; Zinn et al. 2011). Therefore, the control of carbon flux influences PHA metabolism by regulating the availability of PHA precursors.

2.3.2 Impact of the mcl-PHA Cycle on Central Carbon Metabolism

PHA allows cells to cope with carbon starvation by acting as a reservoir of carbon and energy. The direct link between PHA and carbon metabolism permits bacteria to use PHA as a carbon store that can be directed to central carbon metabolism under situations of carbon stress. PHA metabolism represents a bidirectional continuous process whereby PHA synthase and depolymerase are simultaneously active, ensuring PHA turnover (Fig. 2a) (de Eugenio et al. 2010a). The PHA cycle is thus linked to the metabolic network of the cell by adapting the carbon flow to cellular demand. Furthermore, PHA acts as a sink for reducing equivalents. PHA production is controlled by intracellular levels of NADH, NADPH, and acetyl-CoA and thus is under the regulation of the redox state of the cell (Schlegel and Gottschalk 1962; Schubert et al. 1988; Budde et al. 2010).

The PHA cycle has a strong impact on cell physiology as it balances global biomass (including PHA carbon/energy storage), cell division, and energy spillage (Escapa et al. 2012). It is important to stress that the PHA cycle also links carbon and

nitrogen metabolism. *P. putida* unable to accumulate PHA grown under a high C/N ratio produced the same PHA-free biomass as wild-type cells (de Eugenio et al. 2010a). However, the number of cells was tenfold more than the wild type. Thus, the number and size of cells were related to the ability to accumulate PHA. Furthermore, Martinez and coworkers showed that the cell size of a *P. putida pha* mutant was inversely related to the C/N ratio of the culture medium (Martinez et al. 2013).

Finally, in *Pseudomonas* spp. and *C. necator*, PHB has been proposed as a potential metabolic strategy to cope with cold environments, since the PHA cycle enhances the resistance of bacterial cells to low temperature and freezing conditions (Ayub et al. 2009; Obruca et al. 2016).

3 Bioengineering mcl-PHA Production

Much effort is being extended to optimize PHA production and recovery from bacteria. Many of the potential points for optimization are listed in Table 2, which focuses mainly on optimizations carried out in pseudomonads or optimizations that could be adapted from other microbiological systems for use in pseudomonads. The following subsections highlight in brief some of the strategies in use or conceived to rationally design mcl-PHA production for exploitation with an eye toward producing a defined and consistent product. The final subsection is an overview of research needs and synthetic biology tools that are available for bioengineering the mcl-PHA producer, *P. putida*.

3.1 Cultivation

Work is under way to understand the precise growth conditions needed to optimize mcl-PHA production while maximizing economy. Likely the most important consideration during cultivation is the carbon source provided. *Pseudomonas* spp. provided with solely glucose as the carbon source produce low amounts of mcl-PHAs and generally require fatty acids to generate longer-chain PHAs (Huijberts et al. 1992). Thus, various co-feeding strategies have been developed to produce mcl-PHAs of higher quality through the inclusion of two carbon sources in the media with one being a fatty acid (Sun et al. 2007; Kenny et al. 2012; Jiang et al. 2012). This allows for the employment of rational co-feeding to produce mcl-PHAs of defined composition. Moreover, there are many studies testing the feasibility of growth on a variety of inexpensive waste materials to produce mcl-PHAs. These growths generally yield varied results depending on the composition of substrates contained within the feedstock (Nikodinovic-Runic et al. 2013; Muhr et al. 2013). Furthermore, combining genetic manipulations of metabolic pathways along with cofeeding provides a promising strategy that was demonstrated in one study to increase PHA production by twofold (Escapa et al. 2013). It is likely that the future of pseudomonal mcl-PHA production will require similar genetic metabolic

Table 2 Points of technological optimization for mcl-PHA production

	Point or idea for optimization	Details	Example references
Cultivation	Media components/ carbon source	Many parameters, e.g., cofeeding strategies	(Sun et al. 2007; Kenny et al. 2012; Jiang et al. 2012)
Cellular metabolic engineering	Elimination of bystander metabolic pathways	Manipulation of a metabolic pathway	(Escapa et al. 2013)
	Carbon source utilization	Glucose as sole carbon source	(Poblete-Castro et al. 2013)
Improving PHA synthesis	PHA synthase mutagenesis	Altering substrate specificity, e.g., 2-hydroxyacids, lactyl-CoA; improving enzyme activity	(Taguchi and Doi 2004; Takase et al. 2004; Taguchi et al. 2008; Yang et al. 2010)
	Elimination of depolymerase activity	Mutations of *phaZ* depolymerase, recombinant production in non-PHA producing species	(de Eugenio et al. 2010a; Eggers and Steinbüchel 2014)
	Custom-tailored PHAs	Consistent composition, unsaturations, modifications	(Olivera et al. 2001; Liu et al. 2011; Escapa et al. 2011; Wang et al. 2011; Tortajada et al. 2013)
	Cell volume	Larger cells could accommodate more or larger carbonosomes	(Jiang et al. 2015; Kadoya et al. 2015)
	In vitro PHA synthesis	Cell-free system	(Qi et al. 2000; Tajima et al. 2016)
PHA recovery	Autolysis	Inducible or automatic expression of a lytic gene	(Hori et al. 2002; Martínez et al. 2011)
	Fragile cells	Cells engineered for easy rupture	N/A
	Lysis by predator	Use of *Bdellovibrio* sp.	(Martínez et al. 2013, 2016)

Gray shading indicates that optimization likely requires genetic engineering of the PHA-producing bacteria. *N/A* theoretical and lacks support by published results

manipulation and/or a combination of strategies to create high-quality product from inexpensive and readily available carbon sources.

3.2 Metabolic Engineering

PHA diversity – reflected in monomers, homopolymers, random copolymers, block copolymers, functional polymers, graft polymers, as well as the ranges of molecular weights and combinations of the above – is usually achieved by different bacterial

species having PHA synthases with different specificities, different substrate preferences, and possibly different metabolic pathways. It has become increasingly possible to generate PHA diversity in a single bacterial species as embodied by the PHAome concept (Chen and Hajnal 2015). For example, *P. putida* can be engineered to produce various types of PHA homopolymers, including various scl- and mcl-PHA random copolymers, mcl-PHA random copolymers containing adjustable monomer ratios, PHA block copolymers, as well as functionalized PHAs (Tortajada et al. 2013; Chen et al. 2015).

The emerging field of systems metabolic engineering combines systems biology and metabolic engineering approaches in order to identify metabolic pathway bottlenecks and provide solutions to optimize and reconstruct metabolic pathways. This requires the use of omics datasets from transcriptomic, proteomic, and metabolic studies for in silico genome-scale metabolic reconstruction. Once created in silico, the metabolic model allows one to study the cell as global network (Nogales et al. 2008; Puchałka et al. 2008; Peplinski et al. 2010). This strategy is contributing to the design of new strains and fermentation strategies aimed to increase PHA production, permitting the use of alternative carbon sources and an augmentation in PHA synthesis.

The in silico metabolic reconstruction of *P. putida* (*i*JN746) expanded the pool of potential substrates for use as carbon sources for the production of PHA precursors to include a large set of non-glycolytic substrates, such as aromatic compounds (Nogales et al. 2008). A further use of computational metabolic modeling was carried out by Poblete-Castro et al. who reengineered the metabolic network of *P. putida* toward more efficient PHA production when glucose was the carbon source (Poblete-Castro et al. 2013, 2014).

Thanks to the increasing number of omics datasets, combined with statistical tools, metabolic reconstruction is boosting our knowledge about cell metabolism and physiology. For example, Sohn and coworkers (Sohn et al. 2010) implemented in silico flux analysis in *P. putida* metabolic models (*i*JN746 and *i*JP815) and showed that both the de novo fatty acid biosynthesis and β-oxidation pathways contributed simultaneously to the synthesis of different monomers of PHA. The authors also reported that varying ratios of $NADH/NAD^+$ affected not only the accumulation of PHA but also the rate of substrate consumption. Thus, computational pathway prediction algorithms will provide a systematic framework to redesign metabolic pathways and to predict novel engineering strategies oriented to boost PHA yields. Although natural PHA producers such as *C. necator* or pseudomonads can accumulate high concentrations of PHA polymer (up to 60–90% of cell dry weight), the ability to engineer non-PHA-producing bacteria to produce PHA could be an advantage over natural producers due to their lack of PHA depolymerases and absence of tight regulation over PHA synthesis (Jambunathan and Zhang 2016). In this sense, much effort has been carried out to engineer *E. coli* to synthesize PHB by transferring the PHB biosynthetic pathway from other organisms such as *C. necator* (Slater et al. 1988). In these transgenic strains, PHB synthesis depends on the amount of free acetyl-CoA and does not require any nutrient limitations. A broad range of studies has been carried out directed toward increasing the availability of PHB

precursors such as acetyl-CoA and/or NADPH in recombinant *E. coli* (Wang et al. 2013).

Pioneering studies in strain engineering for PHA production were reports that *E. coli* strains blocked in the 3-ketoacyl thiolase (FadA) or 3-HA-CoA dehydratase (FadB) enzyme activity of the fatty acid β-oxidation pathway were able to accumulate mcl-PHA with solely the *phaC1* or *phaC2* synthase gene from *P. putida* GPo1 heterologously expressed (Klinke et al. 1999; Ren et al. 2000). Since then, the production of various copolymers of scl-PHA and mcl-PHA has been achieved in recombinant *E. coli* (Park and Lee 2004; Phithakrotchanakoon et al. 2013; Tappel et al. 2014). Additional examples include the production of poly(3HB-co-3HA) copolymers containing mcl-3HA units in *E. coli* with glucose as the carbon source (Tappel et al. 2014), or by coexpression of PhaG and the PP_0763 gene product coding for a fatty acid-CoA ligase (Wang et al. 2012), or expressing engineered PHA synthase from *Pseudomonas* sp. 61-3 (Taguchi and Doi 2004). These are examples of metabolic engineering strategies in non-PHA-producing bacteria to control PHA precursor production by redirecting central metabolism toward PHA accumulation.

3.3 Improving PHA Synthesis

An idea for improving PHA synthesis is the direct mutagenic engineering of PHA synthases. Targeted mutation of active site residues was carried out in order to alter substrate specificity sufficiently to produce new-to-nature product chemistries, an example of which is the incorporation of 2-hydroxyacids into the PHA polymer. Additionally, active site residues of a pseudomonal PhaC1 were mutated to permit the use of lactyl-CoA as a substrate to create poly(lactate-co-3HB) copolymer (Taguchi et al. 2008; Yang et al. 2010). Further mutagenic strategies of pseudomonal PhaC enzymes have resulted in increased production of PHA when compared to the wild-type enzyme, specifically as a means to produce poly(3HB-co-3HA) (Takase et al. 2004).

A further technological optimization is the development of consistent homopolymeric or functionalized PHAs containing unsaturations, modifications, and novel chemistries. Pseudomonads provided with fatty acids produce PHA precursors via the β-oxidation pathway (Fig. 1, Pathway II). Because β-oxidation shortens the fatty acid chain by two carbon atoms with each cycle, this results in PHAs containing an ensemble of precursors shorter than the provided fatty acid by multiples of two carbons. A solution to this problem was the creation of strains mutated in the β-oxidation pathway that resulted in the synthesis of PHAs with the same chain length as the provided fatty acid (Olivera et al. 2001; Liu et al. 2011; Escapa et al. 2011; Wang et al. 2011). This also allowed for the synthesis of block PHA copolymers of defined chain lengths by including specific fatty acids in the culture. An extension of this approach is the ability to make customized PHAs that contain any number of functional groups including double and triple bonds and/or phenyl, carbonyl, epoxy, etc. modifications. These specialized PHAs have unique and

novel physical properties making them tailorable to specific needs, including medical applications, packaging, and adhesives. The problem is to find a way to synthesize these functionalized PHAs in a consistent manner as current methods for providing precursors for PHA functionalization to cells result in the formation of diverse polymers with generally low yields (Tortajada et al. 2013).

Addressing the problem of augmenting PHA synthesis from another angle would be increasing the total cellular volume, thus providing increased space for PHA carbonosomes. For example, an increase in PHB content was demonstrated in recombinant *E. coli* by deleting genes involved in peptidoglycan synthesis or an actin-like protein, which resulted in larger cells (Jiang et al. 2015; Kadoya et al. 2015). Another solution would be to eliminate the need to use cells as factories through the development of an in vitro synthesis system for mcl-PHA production. Currently, the metabolic pathways required for the production of PHA precursors from carbon source substrates are far too complex to carry out extensive in vitro metabolism using existing technology, yet some progress has been made. A chemoenzymatic system using purified recombinant enzymes was shown to be capable of synthesizing PHB and poly(lactate-co-3HB) copolymer from 3-hydroxybutyrate and lactic acid substrates (Tajima et al. 2016).

3.4 PHA Recovery

An obstacle to scaling up PHA production is finding methods to simplify the recovery of PHA from cells. Current methods generally use organic solvents and/or physical disruption strategies to extract PHA. The use of solvents is especially damaging as many solvents can hydrolyze PHA, decreasing both the yield and consistency of the product. Thus streamlining PHA recovery through improved techniques is a priority. Several innovative ideas exist to extract PHA from cell factories. One strategy is cell autolysis, wherein a lytic enzyme is inducibly or automatically expressed to lyse the cells and simplify PHA harvesting (Hori et al. 2002; Martínez et al. 2011). Another lytic strategy is the use of a predatory bacterium that can be added to a cell culture and cause lysis, allowing for PHA to be more easily recovered from the media (Martínez et al. 2016). A final theoretical possibility is to produce PHA in cells modified to be fragile and rupture as the carbonosomes expand in size during PHA accumulation. Cells could be modified to have inherent structural fragility in the cell envelope through chemical exposure or mutations.

4 Research Needs and Synthetic Biology Tools

P. putida can produce a large variety of mcl-PHAs under highly controlled laboratory conditions. However, manipulating *P. putida* to create mcl-PHAs of consistently high yield and quality and to be able to harvest the product in a sustainable manner remains elusive. Maximizing cell growth as well as PHA yield in PHA-producing

media conditions requires the channeling of metabolic resources into PHA production while not sacrificing the resources required to increase biomass. Solving this puzzle remains a key obstacle for scaling up microbial PHA yields. Promise comes via the use of strain designs that optimize entire metabolic pathways that are derived from computational metabolic models based on omics data. However, constructing these strains requires the targeted editing of a number of genes to create multiple mutations. Fortunately, much effort is being directed toward developing *P. putida* into a general chassis suitable for a wide variety of synthetic biology applications due to its metabolic flexibility, tolerance to various toxins, and it being generally regarded as safe (GRAS certified) (Timmis 2002; Nikel et al. 2014). The recent development of a genome-minimalized strain of *P. putida* KT2440 is progress toward streamlining the use of this bacteria to produce PHA on a large scale (Martínez-García et al. 2014b). This strain lacks 4.3% of its genome and displays superior growth characteristics and improved expression of heterologous genes. An extension on this theme is the work currently being carried out by the EmPowerPutida international consortium of researchers toward the development of a *P. putida* chassis for production of value-added products, which could be used as a new platform for PHA production (http://www.empowerputida.eu/).

The choice of using *P. putida* as a PHA production chassis is made easier by its ability to maintain plasmids containing common broad-host-range multicopy replicons, for example, pBBR1, pRO1600/ColE1, and RK2. However, due to the high level of natural tolerance to antibiotics found in pseudomonads, care must be taken in selecting the bacterial resistance markers present on these vectors (Martínez-García and de Lorenzo 2011). Numerous chemical inducer-mediated expression systems function in *P. putida*, including those that rely upon a native activator such as P*m*/XylS induced by 3-methylbenzoate or a heterologous activator, e.g., P_{lac}/LacI with IPTG (Loeschcke and Thies 2015). More recently, a set of genome-integrated promoters was developed that allow the fine tuning of the level of constitutive expression of a gene (Zobel et al. 2015). Taken together, these tools allow for the easy manipulation of expression of genes and for rapid screening of potential genetic constructs involved in PHA production.

In order to carry out large-scale genomic engineering to optimize cellular metabolism and/or PHA synthesis, the ability to rapidly create multiple genomic changes needs to be developed in *P. putida*. This bacterium has a low rate of homologous recombination, thus the use of genome-integrating, mini-transposon-containing vectors is heavily relied upon in order to make insertions. Both Tn*5*- or Tn*10*-based random knock-in genome editing (de Lorenzo and Timmis 1994; Martínez-García et al. 2014a) as well as site-directed Tn*7*-mediated insertions are possible (Lambertsen et al. 2004; Choi and Schweizer 2006; de las Heras et al. 2008). Owing to the creativity of the scientists in this field, several other genome engineering technologies are being developed that could be scalable to carry out medium- to high-throughput editing. These include homing nuclease-based knockout technology (Martínez-García and de Lorenzo 2011), λ/Red-based deletions (Luo et al. 2016), deletions via custom mini-Tn*5* transposons combined with the Flp-*FRT* recombination system (Leprince et al. 2012), as well as significant steps toward

oligonucleotide-based recombineering (Aparicio et al. 2016). Moreover, two systems using counterselectable markers function in pseudomonads for creating markerless genome edits (Schweizer 1992; Wang et al. 2015). Finally, gene expression knockdowns through the use of CRISPRi to engineer PHA composition in recombinant *E. coli* have been described and are expected to function similarly in *P. putida*, yet the deployment of CRISPRi in *P. putida* remains to be reported (Lv et al. 2015).

Despite the numerous techniques mentioned above that have been established to carry out genome editing in *P. putida*, a pressing need remains for the development of high-throughput genome engineering strategies to carry out large-scale manipulation of metabolic pathways for tuning them toward PHA production. One possibility is the adaptation of multiplex automated genomic engineering (MAGE) technology for use in *P. putida*, which has been demonstrated in *E. coli* to simultaneously mutate dozens of loci (Carr et al. 2012). A recent development in MAGE involves the transient suppression of the highly conserved DNA mismatch repair system, which makes MAGE possible in many bacterial species (Nyerges et al. 2016). Other methods for rationally designing metabolic pathways include adaptive laboratory evolution to directionally evolve a strain toward a desired phenotype, for example, to enhance growth on a particular carbon source (Ibarra et al. 2002). Additional mutagenic approaches exist to reprogram gene expression dynamics either at the transcriptional level with global transcription machinery engineering (gTME) or through altering global translation using ribosome engineering (Hosokawa et al. 2002; Alper and Stephanopoulos 2007). These technologies could be used to implement PHA yield-biased metabolic pathway optimizations based on computational models, as well as the ability to simultaneously create a panel of orthogonal engineered strains for head-to-head comparisons of PHA production.

Related to the need to metabolically tune bacteria in order to improve mcl-PHA production is the desire to ascertain the most inexpensive and readily available carbon source that allows for the production of the desired PHA. It is a tantalizing prospect to be able to produce mcl-PHAs through bioremediation by growing on waste or hazardous materials. This objective requires metabolic engineering techniques in order to adapt bacteria to a particular waste carbon source. Interesting progress has been made toward the upcycling of petrochemical-derived plastic waste for use as a feedstock for PHA production. Examples include the conversion of pyrolyzed polystyrene into mcl-PHA by *P. putida* CA-3, the production of predominantly poly(3-hydroxydecanoate) and poly(3-hydroxydodecanoate) from several *Pseudomonas* isolates fed with terephthalic acid from waste polyethylene terephthalate (PET) plastic, and the production of mcl-PHAs from pyrolyzed polyethylene (PE) waste (Ward et al. 2006; Kenny et al. 2008; Guzik et al. 2014). However, advances in these plastic waste-utilizing *Pseudomonas* strains need to be made in order to develop strains fully capable of upcycling plastic waste – a goal currently under development by the European research consortium, P4SB: From Plastic Waste to Plastic Value Using *P. putida* Synthetic Biology (http://www.p4sb.eu/).

Acknowledgments Research on polymer biotechnology in the laboratory of M. Auxiliadora Prieto is supported by funding from the European Union's Horizon 2020 research and innovation program under grant agreements number 633962 and 679050. We also acknowledge support from the Community of Madrid (P2013/MIT2807) and the Spanish Ministry of Economy (BIO201344878R, BIO2014-61515-EXP).

References

Alper H, Stephanopoulos G (2007) Global transcription machinery engineering: a new approach for improving cellular phenotype. Metab Eng 9:258–267. https://doi.org/10.1016/j.ymben.2006.12.002

Antonio RV, Steinbüchel A, Rehm BH (2000) Analysis of in vivo substrate specificity of the PHA synthase from Ralstonia eutropha: formation of novel copolyesters in recombinant Escherichia coli. FEMS Microbiol Lett 182:111–117

Aparicio T, Jensen SI, Nielsen AT et al (2016) The Ssr protein (T1E_1405) from Pseudomonas putida DOT-T1E enables oligonucleotide-based recombineering in platform strain P. putida EM42. Biotechnol J 11(10):1309–1319. https://doi.org/10.1002/biot.201600317

Arias S, Bassas-Galia M, Molinari G, Timmis KN (2013) Tight coupling of polymerization and depolymerization of polyhydroxyalkanoates ensures efficient management of carbon resources in Pseudomonas putida. Microb Biotechnol 6:551–563. https://doi.org/10.1111/1751-7915.12040

Ayub ND, Tribelli PM, López NI (2009) Polyhydroxyalkanoates are essential for maintenance of redox state in the Antarctic bacterium Pseudomonas sp. 14-3 during low temperature adaptation. Extrem Life Extreme Cond 13:59–66. https://doi.org/10.1007/s00792-008-0197-z

Beeby M, Cho M, Stubbe J, Jensen GJ (2012) Growth and localization of polyhydroxybutyrate granules in Ralstonia eutropha. J Bacteriol 194:1092–1099. https://doi.org/10.1128/JB.06125-11

Brandl H, Knee EJ, Fuller RC et al (1989) Ability of the phototrophic bacterium Rhodospirillum rubrum to produce various poly (beta-hydroxyalkanoates): potential sources for biodegradable polyesters. Int J Biol Macromol 11:49–55

Bresan S, Sznajder A, Hauf W et al (2016) Polyhydroxyalkanoate (PHA) granules have no phospholipids. Sci Rep 6:26612. https://doi.org/10.1038/srep26612

Budde CF, Mahan AE, Lu J et al (2010) Roles of multiple acetoacetyl coenzyme A reductases in polyhydroxybutyrate Biosynthesis in Ralstonia eutropha H16. J Bacteriol 192:5319–5328. https://doi.org/10.1128/JB.00207-10

Carr PA, Wang HH, Sterling B et al (2012) Enhanced multiplex genome engineering through co-operative oligonucleotide co-selection. Nucleic Acids Res 40:e132. https://doi.org/10.1093/nar/gks455

Chen G-Q, Hajnal I (2015) The "PHAome". Trends Biotechnol 33:559–564. https://doi.org/10.1016/j.tibtech.2015.07.006

Chen G-Q, Hajnal I, Wu H et al (2015) Engineering biosynthesis mechanisms for diversifying polyhydroxyalkanoates. Trends Biotechnol 33:565–574. https://doi.org/10.1016/j.tibtech.2015.07.007

Choi K-H, Schweizer HP (2006) mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. Nat Protoc 1:153–161. https://doi.org/10.1038/nprot.2006.24

de Eugenio LI, Escapa IF, Morales V et al (2010a) The turnover of medium-chain-length polyhydroxyalkanoates in Pseudomonas putida KT2442 and the fundamental role of PhaZ depolymerase for the metabolic balance. Environ Microbiol 12:207–221. https://doi.org/10.1111/j.1462-2920.2009.02061.x

de Eugenio LI, Galán B, Escapa IF et al (2010b) The PhaD regulator controls the simultaneous expression of the pha genes involved in polyhydroxyalkanoate metabolism and turnover in Pseudomonas putida KT2442. Environ Microbiol 12:1591–1603. https://doi.org/10.1111/j.1462-2920.2010.02199.x

de las Heras A, Carreño CA, de Lorenzo V (2008) Stable implantation of orthogonal sensor circuits in Gram-negative bacteria for environmental release. Environ Microbiol 10:3305–3316. https://doi.org/10.1111/j.1462-2920.2008.01722.x

de Lorenzo V, Timmis KN (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol 235: 386–405

Dennis D, Liebig C, Holley T et al (2003) Preliminary analysis of polyhydroxyalkanoate inclusions using atomic force microscopy. FEMS Microbiol Lett 226:113–119

Dennis D, Sein V, Martinez E, Augustine B (2008) PhaP is involved in the formation of a network on the surface of polyhydroxyalkanoate inclusions in Cupriavidus necator H16. J Bacteriol 190:555–563. https://doi.org/10.1128/JB.01668-07

Dinjaski N, Prieto MA (2013) Swapping of phasin modules to optimize the in vivo immobilization of proteins to medium-chain-length polyhydroxyalkanoate granules in Pseudomonas putida. Biomacromolecules 14:3285–3293. https://doi.org/10.1021/bm4008937

Doi Y, Kitamura S, Abe H (1995) Microbial synthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate). Macromolecules 28:4822–4828. https://doi.org/10.1021/ma00118a007

Eggers J, Steinbüchel A (2014) Impact of Ralstonia eutropha's poly(3-Hydroxybutyrate) (PHB) depolymerases and phasins on PHB storage in recombinant Escherichia coli. Appl Environ Microbiol 80:7702–7709. https://doi.org/10.1128/AEM.02666-14

Escapa IF, Morales V, Martino VP et al (2011) Disruption of β-oxidation pathway in Pseudomonas putida KT2442 to produce new functionalized PHAs with thioester groups. Appl Microbiol Biotechnol 89:1583–1598. https://doi.org/10.1007/s00253-011-3099-4

Escapa IF, García JL, Bühler B et al (2012) The polyhydroxyalkanoate metabolism controls carbon and energy spillage in Pseudomonas putida. Environ Microbiol 14:1049–1063. https://doi.org/10.1111/j.1462-2920.2011.02684.x

Escapa IF, del Cerro C, García JL, Prieto MA (2013) The role of GlpR repressor in Pseudomonas putida KT2440 growth and PHA production from glycerol. Environ Microbiol 15:93–110. https://doi.org/10.1111/j.1462-2920.2012.02790.x

Fonseca P, de la Peña F, Prieto MA (2014) A role for the regulator PsrA in the polyhydroxyalkanoate metabolism of Pseudomonas putida KT2440. Int J Biol Macromol 71:14–20. https://doi.org/10.1016/j.ijbiomac.2014.04.014

Fukui T, Doi Y (1997) Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of Aeromonas caviae. J Bacteriol 179:4821–4830

Fuller RC, O'Donnell JP, Saulnier J et al (1992) The supramolecular architecture of the polyhydroxyalkanoate inclusions in Pseudomonas oleovorans. FEMS Microbiol Rev 103:279–288. https://doi.org/10.1111/j.1574-6968.1992.tb05849.x

Galán B, Dinjaski N, Maestro B et al (2011) Nucleoid-associated PhaF phasin drives intracellular location and segregation of polyhydroxyalkanoate granules in Pseudomonas putida KT2442. Mol Microbiol 79:402–418. https://doi.org/10.1111/j.1365-2958.2010.07450.x

Gorenflo V, Schmack G, Vogel R, Steinbüchel A (2001) Development of a process for the biotechnological large-scale production of 4-hydroxyvalerate-containing polyesters and characterization of their physical and mechanical properties. Biomacromolecules 2:45–57

Griebel R, Smith Z, Merrick JM (1968) Metabolism of poly-beta-hydroxybutyrate. I. Purification, composition, and properties of native poly-beta-hydroxybutyrate granules from Bacillus megaterium. Biochemistry (Mosc) 7:3676–3681

Guzik MW, Kenny ST, Duane GF et al (2014) Conversion of post consumer polyethylene to the biodegradable polymer polyhydroxyalkanoate. Appl Microbiol Biotechnol 98:4223–4232. https://doi.org/10.1007/s00253-013-5489-2

Han J, Hou J, Liu H et al (2010) Wide distribution among halophilic archaea of a novel polyhydroxyalkanoate synthase subtype with homology to bacterial type III synthases. Appl Environ Microbiol 76:7811–7819. https://doi.org/10.1128/AEM.01117-10

Hori K, Kaneko M, Tanji Y et al (2002) Construction of self-disruptive Bacillus megaterium in response to substrate exhaustion for polyhydroxybutyrate production. Appl Microbiol Biotechnol 59:211–216. https://doi.org/10.1007/s00253-002-0986-8

Hosokawa K, Park N-H, Inaoka T et al (2002) Streptomycin-resistant (rpsL) or rifampicin-resistant (rpoB) mutation in Pseudomonas putida KH146-2 confers enhanced tolerance to organic chemicals. Environ Microbiol 4:703–712

Huijberts GN, Eggink G, de Waard P et al (1992) Pseudomonas putida KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. Appl Environ Microbiol 58:536–544

Huisman GW, de Leeuw O, Eggink G, Witholt B (1989) Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl Environ Microbiol 55:1949–1954

Huisman GW, Wonink E, de Koning G et al (1992) Synthesis of poly(3-hydroxyalkanoates) by mutant and recombinant Pseudomonas. Appl Microbiol Biotechnol 38:1–5. https://doi.org/10.1007/BF00169409

Ibarra RU, Edwards JS, Palsson BO (2002) Escherichia coli K-12 undergoes adaptive evolution to achieve in silico predicted optimal growth. Nature 420:186–189. https://doi.org/10.1038/nature01149

Jambunathan P, Zhang K (2016) Engineered biosynthesis of biodegradable polymers. J Ind Microbiol Biotechnol 43:1037–1058. https://doi.org/10.1007/s10295-016-1785-z

Jendrossek D (2009) Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). J Bacteriol 191:3195–3202. https://doi.org/10.1128/JB.01723-08

Jendrossek D, Pfeiffer D (2014) New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly(3-hydroxybutyrate). Environ Microbiol 16:2357–2373. https://doi.org/10.1111/1462-2920.12356

Jiang X, Sun Z, Marchessault RH et al (2012) Biosynthesis and properties of medium-chain-length polyhydroxyalkanoates with enriched content of the dominant monomer. Biomacromolecules 13:2926–2932. https://doi.org/10.1021/bm3009507

Jiang X-R, Wang H, Shen R, Chen G-Q (2015) Engineering the bacterial shapes for enhanced inclusion bodies accumulation. Metab Eng 29:227–237. https://doi.org/10.1016/j.ymben.2015.03.017

Jin H, Nikolau BJ (2012) Role of genetic redundancy in polyhydroxyalkanoate (PHA) polymerases in PHA biosynthesis in Rhodospirillum rubrum. J Bacteriol 194:5522–5529. https://doi.org/10.1128/JB.01111-12

Kadoya R, Matsumoto K, Ooi T, Taguchi S (2015) MtgA deletion-triggered cell enlargement of Escherichia coli for enhanced intracellular polyester accumulation. PLoS One 10(6):e0125163. https://doi.org/10.1371/journal.pone.0125163

Kenny ST, Runic JN, Kaminsky W et al (2008) Up-cycling of PET (polyethylene terephthalate) to the biodegradable plastic PHA (polyhydroxyalkanoate). Environ Sci Technol 42:7696–7701

Kenny ST, Runic JN, Kaminsky W et al (2012) Development of a bioprocess to convert PET derived terephthalic acid and biodiesel derived glycerol to medium chain length polyhydroxyalkanoate. Appl Microbiol Biotechnol 95:623–633. https://doi.org/10.1007/s00253-012-4058-4

Kim YB, Lenz RW (2001) Polyesters from microorganisms. Adv Biochem Eng Biotechnol 71:51–79

Klinke S, Ren Q, Witholt B, Kessler B (1999) Production of medium-chain-length poly(3-hydroxyalkanoates) from gluconate by recombinant Escherichia coli. Appl Environ Microbiol 65:540–548

Klinke S, de Roo G, Witholt B, Kessler B (2000) Role of phaD in accumulation of medium-chain-length poly(3-Hydroxyalkanoates) in Pseudomonas oleovorans. Appl Environ Microbiol 66:3705–3710

Kuchta K, Chi L, Fuchs H et al (2007) Studies on the influence of phasins on accumulation and degradation of PHB and nanostructure of PHB granules in ralstonia eutropha H16. Biomacromolecules 8:657–662. https://doi.org/10.1021/bm060912e

La Rosa R, de la Peña F, Prieto MA, Rojo F (2014) The Crc protein inhibits the production of polyhydroxyalkanoates in Pseudomonas putida under balanced carbon/nitrogen growth conditions. Environ Microbiol 16:278–290. https://doi.org/10.1111/1462-2920.12303

Lageveen RG, Huisman GW, Preusting H et al (1988) Formation of polyesters by Pseudomonas oleovorans: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Appl Environ Microbiol 54:2924–2932

Lambertsen L, Sternberg C, Molin S (2004) Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. Environ Microbiol 6:726–732. https://doi.org/10.1111/j.1462-2920.2004.00605.x

Langenbach S, Rehm BH, Steinbüchel A (1997) Functional expression of the PHA synthase gene phaC1 from Pseudomonas aeruginosa in Escherichia coli results in poly(3-hydroxyalkanoate) synthesis. FEMS Microbiol Lett 150:303–309

Leprince A, de Lorenzo V, Völler P et al (2012) Random and cyclical deletion of large DNA segments in the genome of Pseudomonas putida. Environ Microbiol 14:1444–1453. https://doi.org/10.1111/j.1462-2920.2012.02730.x

Liebergesell M, Steinbüchel A (1992) Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in Chromatium vinosum strain D. Eur J Biochem FEBS 209:135–150

Liebergesell M, Rahalkar S, Steinbüchel A (2000) Analysis of the Thiocapsa pfennigii polyhydroxyalkanoate synthase: subcloning, molecular characterization and generation of hybrid synthases with the corresponding Chromatium vinosum enzyme. Appl Microbiol Biotechnol 54:186–194

Liu Q, Luo G, Zhou XR, Chen G-Q (2011) Biosynthesis of poly(3-hydroxydecanoate) and 3-hydroxydodecanoate dominating polyhydroxyalkanoates by β-oxidation pathway inhibited Pseudomonas putida. Metab Eng 13:11–17. https://doi.org/10.1016/j.ymben.2010.10.004

Loeschcke A, Thies S (2015) Pseudomonas putida—a versatile host for the production of natural products. Appl Microbiol Biotechnol 99:6197–6214. https://doi.org/10.1007/s00253-015-6745-4

Lu J, Tappel RC, Nomura CT (2009) Mini-review: biosynthesis of poly(hydroxyalkanoates). Polym Rev 49:226–248. https://doi.org/10.1080/15583720903048243

Luo X, Yang Y, Ling W et al (2016) Pseudomonas putida KT2440 markerless gene deletion using a combination of λ Red recombineering and Cre/loxP site-specific recombination. FEMS Microbiol Lett. https://doi.org/10.1093/femsle/fnw014

Lv L, Ren Y-L, Chen J-C et al (2015) Application of CRISPRi for prokaryotic metabolic engineering involving multiple genes, a case study: Controllable P(3HB-co-4HB) biosynthesis. Metab Eng 29:160–168. https://doi.org/10.1016/j.ymben.2015.03.013

Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Maestro B, Galán B, Alfonso C et al (2013) A new family of intrinsically disordered proteins: structural characterization of the major phasin PhaF from Pseudomonas putida KT2440. PLoS One 8:e56904. https://doi.org/10.1371/journal.pone.0056904

Martínez V, García P, García JL, Prieto MA (2011) Controlled autolysis facilitates the polyhydroxyalkanoate recovery in Pseudomonas putida KT2440. Microb Biotechnol 4:533–547. https://doi.org/10.1111/j.1751-7915.2011.00257.x

Martínez V, Jurkevitch E, García JL, Prieto MA (2013) Reward for Bdellovibrio bacteriovorus for preying on a polyhydroxyalkanoate producer. Environ Microbiol 15:1204–1215. https://doi.org/10.1111/1462-2920.12047

Martínez V, Herencias C, Jurkevitch E, Prieto MA (2016) Engineering a predatory bacterium as a proficient killer agent for intracellular bio-products recovery: the case of the polyhydroxyalkanoates. Sci Rep 6:24381. https://doi.org/10.1038/srep24381

Martínez-García E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of Pseudomonas putida KT2440. Environ Microbiol 13:2702–2716. https://doi.org/10.1111/j.1462-2920.2011.02538.x

Martínez-García E, Aparicio T, de Lorenzo V, Nikel PI (2014a) New transposon tools tailored for metabolic engineering of Gram-negative microbial cell factories. Front Bioeng Biotechnol. https://doi.org/10.3389/fbioe.2014.00046

Martínez-García E, Nikel PI, Aparicio T, de Lorenzo V (2014b) Pseudomonas 2.0: genetic upgrading of P. putida KT2440 as an enhanced host for heterologous gene expression. Microb Cell Factories 13:159. https://doi.org/10.1186/s12934–014–0159-3

Matsusaki H, Manji S, Taguchi K et al (1998) Cloning and molecular analysis of the poly (3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyalkanoate) biosynthesis genes in Pseudomonas sp. strain 61-3. J Bacteriol 180:6459–6467

Mayer F, Hoppert M (1997) Determination of the thickness of the boundary layer surrounding bacterial PHA inclusion bodies, and implications for models describing the molecular architecture of this layer. J Basic Microbiol 37:45–52. https://doi.org/10.1002/jobm.3620370108

McCool GJ, Cannon MC (2001) PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in Bacillus megaterium. J Bacteriol 183:4235–4243. https://doi.org/10.1128/JB.183.14.4235-4243.2001

Moldes C, García P, García JL, Prieto MA (2004) In vivo immobilization of fusion proteins on bioplastics by the novel tag BioF. Appl Environ Microbiol 70:3205–3212. https://doi.org/10.1128/AEM.70.6.3205-3212.2004

Muhr A, Rechberger EM, Salerno A et al (2013) Novel description of mcl-PHA biosynthesis by Pseudomonas chlororaphis from animal-derived waste. J Biotechnol 165:45–51. https://doi.org/10.1016/j.jbiotec.2013.02.003

Neumann L, Spinozzi F, Sinibaldi R et al (2008) Binding of the major phasin, PhaP1, from Ralstonia eutropha H16 to poly(3-hydroxybutyrate) granules. J Bacteriol 190:2911–2919. https://doi.org/10.1128/JB.01486-07

Nikel PI, Martínez-García E, de Lorenzo V (2014) Biotechnological domestication of pseudomonads using synthetic biology. Nat Rev Microbiol 12:368–379. https://doi.org/10.1038/nrmicro3253

Nikodinovic-Runic J, Guzik M, Kenny ST et al (2013) Carbon-rich wastes as feedstocks for biodegradable polymer (polyhydroxyalkanoate) production using bacteria. Adv Appl Microbiol 84:139–200. https://doi.org/10.1016/B978-0-12-407673-0.00004-7

Nogales J, Palsson BØ, Thiele I (2008) A genome-scale metabolic reconstruction of Pseudomonas putida KT2440: iJN746 as a cell factory. BMC Syst Biol 2:79. https://doi.org/10.1186/1752-0509-2-79

Nomura CT, Taguchi S (2007) PHA synthase engineering toward superbiocatalysts for custom-made biopolymers. Appl Microbiol Biotechnol 73:969–979. https://doi.org/10.1007/s00253-006-0566-4

Nyerges Á, Csörgő B, Nagy I et al (2016) A highly precise and portable genome engineering method allows comparison of mutational effects across bacterial species. Proc Natl Acad Sci U S A 113:2502–2507. https://doi.org/10.1073/pnas.1520040113

Obruca S, Sedlacek P, Mravec F et al (2016) Evaluation of 3-hydroxybutyrate as an enzyme-protective agent against heating and oxidative damage and its potential role in stress response of poly(3-hydroxybutyrate) accumulating cells. Appl Microbiol Biotechnol 100:1365–1376. https://doi.org/10.1007/s00253-015-7162-4

Olivera ER, Carnicero D, Jodra R et al (2001) Genetically engineered Pseudomonas: a factory of new bioplastics with broad applications. Environ Microbiol 3:612–618

Park SJ, Lee SY (2004) Biosynthesis of poly(3-hydroxybutyrate- co-3-hydroxyalkanoates) by metabolically engineered Escherichia coli strains. Appl Biochem Biotechnol 113–116:335–346

Peplinski K, Ehrenreich A, Döring C et al (2010) Genome-wide transcriptome analyses of the "Knallgas" bacterium Ralstonia eutropha H16 with regard to polyhydroxyalkanoate metabolism. Microbiol Read Engl 156:2136–2152. https://doi.org/10.1099/mic.0.038380-0

Pfeiffer D, Jendrossek D (2011) Interaction between poly(3-hydroxybutyrate) granule-associated proteins as revealed by two-hybrid analysis and identification of a new phasin in Ralstonia eutropha H16. Microbiol Read Engl 157:2795–2807. https://doi.org/10.1099/mic.0.051508-0

Pfeiffer D, Jendrossek D (2012) Localization of poly(3-hydroxybutyrate) (PHB) granule-associated proteins during PHB granule formation and identification of two new phasins, PhaP6 and PhaP7, in Ralstonia eutropha H16. J Bacteriol 194:5909–5921. https://doi.org/10.1128/JB.00779-12

Pfeiffer D, Wahl A, Jendrossek D (2011) Identification of a multifunctional protein, PhaM, that determines number, surface to volume ratio, subcellular localization and distribution to daughter cells of poly(3-hydroxybutyrate), PHB, granules in Ralstonia eutropha H16. Mol Microbiol 82:936–951. https://doi.org/10.1111/j.1365-2958.2011.07869.x

Phithakrotchanakoon C, Champreda V, Aiba S et al (2013) Engineered Escherichia coli for short-chain-length medium-chain-length polyhydroxyalkanoate copolymer biosynthesis from glycerol and dodecanoate. Biosci Biotechnol Biochem 77:1262–1268. https://doi.org/10.1271/bbb.130073

Poblete-Castro I, Binger D, Rodrigues A et al (2013) In-silico-driven metabolic engineering of Pseudomonas putida for enhanced production of poly-hydroxyalkanoates. Metab Eng 15:113–123. https://doi.org/10.1016/j.ymben.2012.10.004

Poblete-Castro I, Rodriguez AL, Lam CMC, Kessler W (2014) Improved production of medium-chain-length polyhydroxyalkanoates in glucose-based fed-batch cultivations of metabolically engineered Pseudomonas putida strains. J Microbiol Biotechnol 24:59–69

Pötter M, Müller H, Reinecke F et al (2004) The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in Ralstonia eutropha. Microbiol Read Engl 150:2301–2311. https://doi.org/10.1099/mic.0.26970-0

Prieto MA, Bühler B, Jung K et al (1999) PhaF, a polyhydroxyalkanoate-granule-associated protein of Pseudomonas oleovorans GPo1 involved in the regulatory expression system for pha genes. J Bacteriol 181:858–868

Prieto A, Escapa IF, Martínez V et al (2016) A holistic view of polyhydroxyalkanoate metabolism in *Pseudomonas putida*: polyhydroxyalkanoate metabolism in *Pseudomonas putida*. Environ Microbiol 18:341–357. https://doi.org/10.1111/1462-2920.12760

Puchałka J, Oberhardt MA, Godinho M et al (2008) Genome-scale reconstruction and analysis of the Pseudomonas putida KT2440 metabolic network facilitates applications in biotechnology. PLoS Comput Biol 4(10):e1000210. https://doi.org/10.1371/journal.pcbi.1000210

Qi Q, Rehm BH, Steinbüchel A (1997) Synthesis of poly(3-hydroxyalkanoates) in Escherichia coli expressing the PHA synthase gene phaC2 from Pseudomonas aeruginosa: comparison of PhaC1 and PhaC2. FEMS Microbiol Lett 157:155–162. https://doi.org/10.1111/j.1574-6968.1997.tb12767.x

Qi Q, Steinbüchel A, Rehm BH (2000) In vitro synthesis of poly(3-hydroxydecanoate): purification and enzymatic characterization of type II polyhydroxyalkanoate synthases PhaC1 and PhaC2 from Pseudomonas aeruginosa. Appl Microbiol Biotechnol 54:37–43

Rehm BH, Krüger N, Steinbüchel A (1998) A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. The PHAG gene from Pseudomonas putida KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme a transferase. J Biol Chem 273:24044–24051

Reinecke F, Steinbüchel A (2009) Ralstonia eutropha strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. J Mol Microbiol Biotechnol 16:91–108. https://doi.org/10.1159/000142897

Ren Q, Sierro N, Kellerhals M et al (2000) Properties of engineered poly-3-hydroxyalkanoates produced in recombinant Escherichia coli strains. Appl Environ Microbiol 66:1311–1320

Ren Q, de Roo G, Ruth K et al (2009a) Simultaneous accumulation and degradation of polyhydroxyalkanoates: futile cycle or clever regulation? Biomacromolecules 10:916–922. https://doi.org/10.1021/bm801431c

Ren Q, de Roo G, Witholt B et al (2009b) Overexpression and characterization of medium-chain-length polyhydroxyalkanoate granule bound polymerases from Pseudomonas putida GPo1. Microb Cell Factories 8:60. https://doi.org/10.1186/1475-2859-8-60

Ruiz JA, López NI, Méndez BS (2004) rpoS gene expression in carbon-starved cultures of the Polyhydroxyalkanoate-accumulating species Pseudomonas oleovorans. Curr Microbiol 48:396–400. https://doi.org/10.1007/s00284-003-4183-5

Ruth K, de Roo G, Egli T, Ren Q (2008) Identification of two acyl-CoA synthetases from Pseudomonas putida GPo1: one is located at the surface of polyhydroxyalkanoates granules. Biomacromolecules 9:1652–1659. https://doi.org/10.1021/bm8001655

Saito Y, Nakamura S, Hiramitsu M, Doi Y (1996) Microbial synthesis and properties of poly (3-hydroxybutyrate-co-4-hydroxybutyrate). Polym Int 39:169–174. https://doi.org/10.1002/(SICI)1097-0126(199603)39:3<169::AID-PI453>3.0.CO;2-Z

Sandoval A, Arias-Barrau E, Arcos M et al (2007) Genetic and ultrastructural analysis of different mutants of Pseudomonas putida affected in the poly-3-hydroxy-n-alkanoate gene cluster. Environ Microbiol 9:737–751. https://doi.org/10.1111/j.1462-2920.2006.01196.x

Schlegel HG, Gottschalk G (1962) Poly-β-hydroxybuttersäure, ihre verbreitung, funktion und biosynthese. Angew Chem 74:342–347. https://doi.org/10.1002/ange.19620741003

Schubert P, Steinbüchel A, Schlegel HG (1988) Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol 170:5837–5847

Schweizer HP (1992) Allelic exchange in Pseudomonas aeruginosa using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counter-selectable Bacillus subtilis sacB marker. Mol Microbiol 6:1195–1204

Shamala TR, Chandrashekar A, Vijayendra SVN, Kshama L (2003) Identification of polyhydroxyalkanoate (PHA)-producing Bacillus spp. using the polymerase chain reaction (PCR). J Appl Microbiol 94:369–374

Singh M, Patel SK, Kalia VC (2009) Bacillus subtilis as potential producer for polyhydroxyalkanoates. Microb Cell Factories 8:38. https://doi.org/10.1186/1475-2859-8-38

Slater SC, Voige WH, Dennis DE (1988) Cloning and expression in Escherichia coli of the Alcaligenes eutrophus H16 poly-beta-hydroxybutyrate biosynthetic pathway. J Bacteriol 170:4431–4436

Sohn SB, Kim TY, Park JM, Lee SY (2010) In silico genome-scale metabolic analysis of Pseudomonas putida KT2440 for polyhydroxyalkanoate synthesis, degradation of aromatics and anaerobic survival. Biotechnol J 5:739–750. https://doi.org/10.1002/biot.201000124

Steinbuchel A, Aerts K, Babel W et al (1995) Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions. Can J Microbiol 41(Suppl 1):94–105

Stuart ES, Foster LJ, Lenz RW, Fuller RC (1996) Intracellular depolymerase functionality and location in Pseudomonas oleovorans inclusions containing polyhydroxyoctanoate. Int J Biol Macromol 19:171–176

Stuart ES, Tehrani A, Valentin HE et al (1998) Protein organization on the PHA inclusion cytoplasmic boundary. J Biotechnol 64:137–144

Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 25:1503–1555. https://doi.org/10.1016/S0079-6700(00)00035-6

Sun Z, Ramsay JA, Guay M, Ramsay B (2007) Increasing the yield of MCL-PHA from nonanoic acid by co-feeding glucose during the PHA accumulation stage in two-stage fed-batch fermentations of Pseudomonas putida KT2440. J Biotechnol 132:280–282. https://doi.org/10.1016/j.jbiotec.2007.02.023

Taguchi S, Doi Y (2004) Evolution of polyhydroxyalkanoate (PHA) production system by "enzyme evolution": successful case studies of directed evolution. Macromol Biosci 4:146–156. https://doi.org/10.1002/mabi.200300111

Taguchi K, Taguchi S, Sudesh K, et al. (2005) Metabolic pathways and engineering of polyhydroxyalkanoate biosynthesis. In: Biopolymers online. Wiley-VCH Verlag GmbH & Co. KGaA,

Taguchi S, Yamada M, K'ichiro M et al (2008) A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. Proc Natl Acad Sci U S A 105:17323–17327. https://doi.org/10.1073/pnas.0805653105

Tajima K, Han X, Hashimoto Y et al (2016) In vitro synthesis of polyhydroxyalkanoates using thermostable acetyl-CoA synthetase, CoA transferase, and PHA synthase from thermotorelant bacteria. J Biosci Bioeng. https://doi.org/10.1016/j.jbiosc.2016.06.001

Takase K, K'ichiro M, Taguchi S, Doi Y (2004) Alteration of substrate chain-length specificity of type II synthase for polyhydroxyalkanoate biosynthesis by in vitro evolution: in vivo and in vitro enzyme assays. Biomacromolecules 5:480–485. https://doi.org/10.1021/bm034323+

Tappel RC, Pan W, Bergey NS et al (2014) Engineering Escherichia coli for improved production of short-chain-length-co-medium-chain-length poly[(R)-3-hydroxyalkanoate] (SCL-co-MCL PHA)

copolymers from renewable nonfatty acid feedstocks. ACS Sustain Chem Eng 2:1879–1887. https://doi.org/10.1021/sc500217p

Timm A, Steinbüchel A (1990) Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by Pseudomonas aeruginosa and other fluorescent pseudomonads. Appl Environ Microbiol 56:3360–3367

Timm A, Steinbüchel A (1992) Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of Pseudomonas aeruginosa PAO1. Eur J Biochem FEBS 209:15–30

Timmis KN (2002) Pseudomonas putida: a cosmopolitan opportunist par excellence. Environ Microbiol 4:779–781. https://doi.org/10.1046/j.1462-2920.2002.00365.x

Tortajada M, da Silva LF, Prieto MA (2013) Second-generation functionalized medium-chain-length polyhydroxyalkanoates: the gateway to high-value bioplastic applications. Int Microbiol Off J Span Soc Microbiol 16:1–15. https://doi.org/10.2436/20.1501.01.175

Wang H, Zhou X, Liu Q, Chen G-Q (2011) Biosynthesis of polyhydroxyalkanoate homopolymers by Pseudomonas putida. Appl Microbiol Biotechnol 89:1497–1507. https://doi.org/10.1007/s00253-010-2964-x

Wang Q, Tappel RC, Zhu C, Nomura CT (2012) Development of a new strategy for production of medium-chain-length polyhydroxyalkanoates by recombinant Escherichia coli via inexpensive non-fatty acid feedstocks. Appl Environ Microbiol 78:519–527. https://doi.org/10.1128/AEM.07020-11

Wang Q, Zhuang Q, Liang Q, Qi Q (2013) Polyhydroxyalkanoic acids from structurally-unrelated carbon sources in Escherichia coli. Appl Microbiol Biotechnol 97:3301–3307. https://doi.org/10.1007/s00253-013-4809-x

Wang Y, Zhang C, Gong T et al (2015) An upp-based markerless gene replacement method for genome reduction and metabolic pathway engineering in Pseudomonas mendocina NK-01 and Pseudomonas putida KT2440. J Microbiol Methods 113:27–33. https://doi.org/10.1016/j.mimet.2015.03.022

Ward PG, Goff M, Donner M et al (2006) A two step chemo-biotechnological conversion of polystyrene to a biodegradable thermoplastic. Environ Sci Technol 40:2433–2437

Yang TH, Kim TW, Kang HO et al (2010) Biosynthesis of polylactic acid and its copolymers using evolved propionate CoA transferase and PHA synthase. Biotechnol Bioeng 105:150–160. https://doi.org/10.1002/bit.22547

Yuan W, Jia Y, Tian J et al (2001) Class I and III polyhydroxyalkanoate synthases from Ralstonia eutropha and Allochromatium vinosum: characterization and substrate specificity studies. Arch Biochem Biophys 394:87–98. https://doi.org/10.1006/abbi.2001.2522

Zhang S, Kamachi M, Takagi Y et al (2001) Comparative study of the relationship between monomer structure and reactivity for two polyhydroxyalkanoate synthases. Appl Microbiol Biotechnol 56:131–136

Zinn M, Durner R, Zinn H et al (2011) Growth and accumulation dynamics of poly (3-hydroxyalkanoate) (PHA) in Pseudomonas putida GPo1 cultivated in continuous culture under transient feed conditions. Biotechnol J 6:1240–1252. https://doi.org/10.1002/biot.201100219

Zobel S, Benedetti I, Eisenbach L et al (2015) Tn7-based device for calibrated heterologous gene expression in Pseudomonas putida. ACS Synth Biol 4:1341–1351. https://doi.org/10.1021/acssynbio.5b00058

Storage of Hydrophobic Polymers in Bacteria

26

Luísa S. Serafim, Ana M. R. B. Xavier, and Paulo C. Lemos

Contents

1 Introduction 484
2 Polyhydroxyalkanoates (PHAs) 486
2.1 Metabolism of PHAs Production 488
2.2 Function of PHAs 491
2.3 Occurrence of PHAs Producing Bacteria 491
2.4 Mixed Microbial Cultures 492
2.5 Industrial Production of PHAs 494
3 Triacylglycerols (TAGs) 497
4 Wax Esters (WEs) 500
5 Research Needs 502
References 503

Abstract

The accumulation of storage reserves is broadly spread in nature, and among the different compounds stored, carbohydrates and lipids are the most common and important. The accumulation of storage compounds in inclusion bodies is a strategy that allows the survival of microorganisms in different environments since most of these compounds act as element and/or energy sources. A variety of storage reserves is known and among lipids, polyhydroxyalkanoates (PHAs), triacylglycerols (TAGs), and wax esters (WEs) are the most important. These

L. S. Serafim (✉) · A. M. R. B. Xavier
CICECO – Aveiro Institute of Materials, Departamento de Química, Universidade de Aveiro, Aveiro, Portugal
e-mail: luisa.serafim@ua.pt; abx@ua.pt

P. C. Lemos
LAQV-REQUIMTE, Departmento de Química, Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, Lisboa, Portugal
e-mail: paulo.lemos@fct.unl.pt

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_33

carbon-based internal reserves gained importance in the last years due to the possibility of using them as substitutes of materials and fuels usually obtained from mineral oil. For this reason, the knowledge about the microorganisms that store them, the metabolic routes involved on their formation, and the process conditions that allow their efficient production were subject of many scientific works and constitute the main topic of the present chapter.

1 Introduction

Intracellular storage of hydrophobic compounds in bacteria has been widely known for decades. The accumulation of storage reserves is widely spread in nature and found in numerous species of Bacteria. Among the different compounds stored, the most common and important are carbohydrates and lipids. Until some decades ago, those compounds together with polyphosphate, cyanophycins in cyanobacteria, and sulfur granules in sulfurbacteria were the only known storage compounds in bacteria (Shively 1974). Currently, a variety of storage reserves is well-known and among lipids the number of possibilities increased considerably. If 40 years ago, only polyhydroxyalkanoates (PHAs), more specifically poly(3-hydroxybutyrate), was the only group of compounds considered (Shively 1974), now, a wide variety of PHAs is recognized and this class also includes triacylglycerols (TAGs), and wax esters (WEs), all of them being esters of fatty acids (Shively et al. 2011). While the firsts are long polymers of hydroxyl-fatty acids, TAGs are made out of three fatty acids and a glycerol molecule, and WE are oxoesters of long-chain alcohols and fatty acids.

Microorganisms have developed a variety of strategies allowing their survival in different environments, and the accumulation of storage compounds in inclusion bodies is only one of them. These compounds usually act as carbon and energy sources to be used in periods of lack of their external availability but other functions can be attributed. Mostly, the storage of intracellular reserves represents a competitive advantage for microorganisms since it implies a survival strategy in adverse environments. Storage compounds can occur as intracellular inclusions in Bacteria (Shively 1974). Those inclusions are defined as discrete structures seen within the confines of prokaryotic cells, usually intracytoplasmic, but in some instances also in the periplasmic region of the cell. The inclusion may exist without a boundary layer or may be enclosed in either a mono- or a bilayer membrane (Shively et al. 2011).

Bacterial inclusions are the visual expression of metabolic aspects of the bacterial cell. They not only exist in a variety of shapes and sizes but are also involved in an assortment of functions. Most commonly, the inclusions act as reserve or storage depots of an element and/or energy (metabolic reserves). Also, some inclusions can function as simple organelles that contribute directly to the metabolic capabilities of the cell, whereas others are involved in the ability to cope with changing environmental conditions, as cell positioners (Murphy 1993; Wältermann and Steinbüchel 2005). Other inclusions potentially contribute to both of these functions. Glycogen, PHAs, WEs, TAGs, cyanophycins, polyphosphate, and sulfur globules are considered metabolic reserves, which are commonly accumulated in response to

a nutrient imbalance, for example, under conditions of an excess of carbon/energy. Magnetosomes and gas vesicles contribute to cell mobility thereby assisting the cells in attaining nutrient and/or redox needs. Carboxysomes, containing the enzymes carbonic anhydrase and ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo), and chlorosomes, sacs of self-aggregated bacteriochlorophyll are carbon fixing and light-harvesting organelles, respectively (Shively et al. 2011; Rae et al. 2013).

In many habitats, bacteria are exposed to unsteady conditions and imbalanced nutrition supply. Thus, the ability to store carbon compounds intracellularly conveys an advantage over competitors in the same habitat when growth substrates become scarce. Consequently, nearly all prokaryotes known so far are able to accumulate at least one type of storage compounds. Among the storage compounds, lipids represent ideal reserve materials, as they are highly calorific, water-insoluble, and osmotically inert (Murphy 1993; Wältermann and Steinbüchel 2005). Moreover, in nature, some bacteria are able to store more than one type of storage reserves. Polyphosphate-accumulating organisms, the target organisms of enhanced biological phosphorus removal in wastewater treatment plants by playing an important role on phosphorus natural cycle, are a good example of versatility in intracellular reserves. Despite not being yet isolated, these bacteria are known to accumulate PHAs, glycogen, and polyphosphate that play different roles during the process and are consumed and restored in different periods (Oehmen et al. 2007).

Due to the limitation of using raw materials such as petroleum, natural gas, or those belonging to the human food chain, compounds produced and accumulated by bacteria became of high relevance. Not only bacteria can use different substrates from many raw materials, but also these processes can signify a cost reduction or more environmental-friendly processes. Carbon-based internal reserves, especially the hydrophobic ones gained importance in the last years due to the possibility of using them as substitutes of materials and fuels usually obtained from mineral oil. PHAs are polymeric materials with similar properties to many synthetic plastics with the additional advantage of being biodegradable. TAGs can be used as components of biodiesel. WEs can be utilized by the cosmetic, lubricants, or coatings industries (Hauschild et al. 2017).

Lipids are important storage compounds in many living organisms. Stored lipids from both plants and microalgae have been used in industrial applications such as the production of renewable fuels. In recent years, bacterial lipids have drawn the same attention as replacements for oil fuels and plastics from petrochemical origin. Bacterial lipids present several advantages when compared with those found in plants and algae. While for plants, mainly, the oleaginous seeds are used, with bacteria less space is needed for their cultivation and they present a better yield of oil content per unit of biomass having a high flexibility of using different carbon sources. Also, there is no competition for food as with bio-energy crops, no seasonality, and obtaining different lipidic compositions by changing the type of carbon substrates and other nutrients is easier in Bacteria. As for microalgae, they are most suited for production of low amounts of compounds for example specialty lipids and pigments, as carotenoids, presenting a slower growth and lower cell densities. Bacterial storage lipids have further benefits as the

reduction of greenhouse gas emissions, the potential to use agricultural residues, and other waste sources as substrates contributing to the environmental sustainability (Qadeer et al. 2017).

Compared with carbohydrates and proteins, bacterial lipids are better reserve materials in several aspects. In their function as energy reserves, they present a two times higher energy density, 9.3 kcal/g, than the two other counterparts, 4.1 kcal/g (Hauschild et al. 2017). Bacterial lipids can also act as source of metabolic water, of particular importance in environments with water scarcity. Upon lipid degradation out of 100 g of fat, 107.1 g of metabolic water can be recovered while for the same amount of carbohydrates and proteins only 55.5 g and 41.3 g of metabolic water can be gained, respectively (Hauschild et al. 2017). Bacterial lipids also act upon the redox balance in cells, either by consuming redox equivalents during their production or being a source of those same reducing equivalents upon degradation. Another important feature of lipid reserves is their involvement in cell membrane integrity upon temperature stress by controlling the proportion of saturated and unsaturated fatty acids in membrane phospholipids. Lipid reserves also function as a sink for potential toxic metabolites that are physiologically active (Alvarez and Steinbüchel 2010).

Lipids are preferably stored as neutral lipids, so they do not interfere with cell stability. Both TAG and WE degradation produce the highest energetic yield when compared with PHA or glycogen, due to the nature of their acyl moieties that are in their most reductive form (Alvarez 2016). The principal constraint for lipids accumulation is a high carbon to nitrogen (C/N) ratio.

This chapter is dedicated to the hydrophobic storage compounds produced by bacteria, with special emphasis on those of lipidic origin, including PHAs, TAGs, and WEs. PHAs, TAGs, and WEs result from the esterification of (hydroxyl-) fatty acids. PHAs, polymers of hydroxyalkanoic acids, are the most common lipophilic storage reserves being ubiquitous in nature, since more than 300 genera were identified as being able of their production (Serafim et al. 2016). Still, a large number of PHAs-producing bacteria remain unknown in nature. TAGs and WEs are non-polymeric neutral lipids and accumulated by fewer genera of bacteria than PHAs. TAGs and WEs are mostly known to be accumulated by eukaryotic organisms but some bacteria, as those belonging to the taxonomic group of Actinomycetales, were found to be able to store them (Röttig and Steinbüchel 2013).

2 Polyhydroxyalkanoates (PHAs)

Poly(3-hydroxybutyrate) (P(3HB)), the homopolyester of 3-hydroxybuyric acid (3HB) (Fig. 1), was the first PHA to be observed in 1926, by Lemoigne, inside *Bacillus megaterium* cells. Since then, several polyesters of various hydroxyacids belonging to PHAs group were reported to be produced by more than 300 genera of prokaryotes (Steinbüchel and Hein 2001). The general molecular structure of P(3HB) is shown in Fig. 1.

Fig. 1 Chemical structure of several lipids (P(3HB), poly-3-hydroxybutyrate; FA, Fatty acid; WE, Wax ester; TAG, Tri-acyl-glycerol)

According to Steinbüchel and Valentin (1995), around 100 different monomers of PHAs were already identified. As revised by Volova (2004), the majority of monomers of PHAs are 3-alkanoic acids (3HAs), linear and saturated molecules covering a range from 3 to 16 carbon atoms. However, other structures were already detected in nature or in laboratory, resulting from in vivo or in vitro production. Those include unsaturated monomers like 3-hydroxy-4-pentenoic acid, branched monomers like 3-hydroxy-4-methyvaleric acid, 3HAs containing sulfur (3-hydroxypropylthiobutyric acid) or halogens (3-hydroxy-6-chlorohexanoic acid) or non-3HAs as 4-hydroxybutyric acid (Volova 2004).

PHAs are accumulated intracellularly as complex inclusion bodies or granules. As a result of their multiple functions, these granules were recently designated as carbonosomes by Jendrossek and Pfeiffer (2014). PHAs granules are partitioned from the cell content by a layer of proteins that include enzymes involved in PHAs synthesis (e.g., PHA synthase) and in their depolymerization, regulatory proteins, and structural proteins, the so-called phasins (Rehm 2006). Usually, microorganisms accumulate PHAs when facing unbalanced conditions in their living environment, e.g., when a carbon substrate is present in excess but other nutrients required to grow, as oxygen or nitrogen, are limited or absent. In such conditions, the storage of the external carbon substrate as PHAs will allow them to use it as carbon and energy sources when the growth conditions are restored. This ability to store PHAs represents a competitive advantage for microorganisms (Serafim et al. 2016).

PHAs are thermoplastics or elastomers with unique properties such as biodegradability and biocompatibility, being very interesting from the commercial point of view since they result in environmental friendly materials that can be applied in medical devices (Laycock et al. 2013). It is possible to control the formation of the different monomers by choosing the suitable carbon sources, organisms and reactor operational conditions (Reis et al. 2011). Consequently, tailoring thermal and mechanical properties (Li et al. 2014), or the size of PHAs chains, usually in the range from 10^4 to 10^6 (Holdren 2011), became possible, which is something that is not easily achieved by chemical synthesis (Chen and Hajnal 2015).

Despite the diversity of their monomers, PHAs are usually classified based on the number of carbons in three different groups. PHAs containing only monomeric units with 3 to 5 carbon atoms are usually designated short-chain length PHAs (SCL-PHAs). The presence of monomers with 6 to 14 carbon atoms corresponds to medium-chain length PHAs (MCL-PHAs). The formation of monomers with more than 14 carbon atoms is not very often but those polymers constitute the long-chain length PHAs (LCL-PHAs) group. Besides being the most frequent PHA produced in nature, the homopolymer P(3HB) is the most studied and well-characterized PHAs. Microorganisms are able to include different types of monomers in the PHAs chains resulting in co- or terpolymers. MCL-PHAs are elastomers with lower melting points and crystallinities than SCL-PHAs (Bugnicourt et al. 2014).

PHAs can be also classified based on the way the different monomers are incorporated in the polymer chains. It was previously shown that by manipulating the feeding of substrates can result in different polymer structures and random-, block-, or random-block copolymers can be obtained. These different monomeric distributions result in different properties (Laycock et al. 2013). As summarized by Bugnicourt et al. (2014), in general, PHAs are water insoluble and relatively resistant to hydrolytic degradation; offer good resistance to moisture and aromas; show good ultra-violet resistance but poor resistance to acids and bases; are soluble in few organic solvents, mostly halogenated hydrocarbons; are more dense than water which facilitates their biodegradation in aquatic environments and sediments; are less "sticky" than traditional polymers when melted. However, some PHAs tend to recrystallize with aging and loose flexibility and elongation capacity. This tendency can be inverted by blending PHAs with plasticizers or nucleating agents. PHAs can be also blended or grafted with other polymers resulting in new materials with improved characteristics (Bugnicourt et al. 2014).

2.1 Metabolism of PHAs Production

Since poly-3-hydroxyalkanoates are the most frequently produced PHAs, the metabolic pathways related to their production are well studied (Fig. 1). The production of P(3HB) by *Cupriavidus necator* starts with the conversion of acetate molecules to acetyl-CoA units, two of these units condense forming acetoacetyl-CoA by the action of ß-ketothiolase. This compound is further transformed to the monomer R-3-hydroxybutyryl-CoA (3HB) by acetoacetyl-CoA reductase that is added to the growing polymeric chain by PHA synthase. Acetic acid is not the only short-chain organic acid (SCOA) easily converted to PHAs, other SCOAs can also originate 3HAs. Butyric or valeric acids can derive their respective 3-hydroxyacyl-CoA, the former, 3HB, and the later 3-hydroxyvaleryl-CoA (3 HV). From propionic acid, several compounds can be obtained, 3-HV or 3-hydroxy-2-methylbutyrate when one -propionyl-CoA unit combines with one acetyl-CoA, while 3-hydroxy-2-methylvalerate results from combining two units of propionyl-CoA (Serafim et al. 2016).

Other substrates like sugars and long-chain organic acids (LCOAs) can be converted into PHAs via different metabolic pathways that both produce SCL and

MCL monomers. Sugars require a previous conversion via glycolysis to pyruvate and further into acetyl or propionyl moieties that can be combined as described above or introduced in the de novo fatty acids biosynthesis (Fig. 2). In the de novo biosynthetic pathway, acetyl-CoA will be converted to R-3-hydroxyacyl-ACP intermediates that are processed by a transacylase followed by a reductase to the corresponding R-3-hydroxyacyl-CoAs. LCOAs can also be converted to monomers in the de novo pathway but usually are processed in the ß-oxidation pathway. In this pathway, LCOAs can be drifted for R-3-hydroxyacyl-CoA production by reductases, hydratases, and epimerases (Serafim et al. 2016).

It is widely accepted that the type of PHAs produced depends on the substrate but mostly on the microorganism, since different types of PHA synthase were detected in different bacteria and this enzyme defines their ability to produce SCL-PHAs or MCL-PHAs. Based on the substrate specificity and on the subunit structures, four different classes of PHA-synthases were defined after sequencing the nucleotide sequences of 59 PHA synthase genes obtained from 44 different bacteria (Rehm 2003). Each class corresponds to a model bacterium: Class I to *C. necator*, II to *Pseudomonas aeruginosa*, III to *Allochromatium vinosum*, and IV to *Bacillus megaterium*. The different classes are also related to the type of PHAs formed. PHA synthases of classes I and III incorporate only SCL hydroxyacyl monomers, while PHA synthases of class II, MCL hydroxyacyl monomers. Some diversity seems to exist among those belonging to Class IV since the majority of enzymes described are able to polymerize SCL units. However, the production of MCL- and LCL-PHAs was reported for some strains of *B. megaterium* and *Bacillus* sp. US163, while *Bacillus* sp. US177 produces MCL- or LCL-PHAs (Tsuge et al. 2015).

From a commercial point of view, the production of a copolymer is much more important than a homopolymer, since the incorporation of different monomers in the polymer chains often signify improved physical and mechanical characteristics (Bugnicourt et al. 2014). Concerning copolymers, the best studied is the copolymer of 3-HB and 3-HV (P(3HB-co-3 HV)). The search for molecules that provide the propionyl moiety present in 3-HV has been the focus of many research works. One example is the use of other carbon compounds investigated as possible substrates for PHA production, as the aminoacids valine and isoleucine. These aminoacids were fed to *Alcaligenes eutrophus* (presently *C. necator*) as co-substrates and resulted in the production of P(3HB-co-3 HV), which indicates the involvement of the propionyl moiety in the biosynthetic process (Steinbüchel and Pieper 1992).

Carbon dioxide, methane, and methanol were also tested as substrates due to their low cost and the need to tackle their effect as greenhouse gases. Autotrophic strains *C. necator* and *Ideonella* sp. were able to grow and produce PHAs from CO_2 by using H_2 as electron donor under aerobic conditions (Tanaka et al. 1995, 2011). The enzyme RuBisCo from Calvin cycle allows drifting 3-phosphoglycerate for central metabolism via pyruvate, and the necessary acetyl-CoA moieties can result from the reverse tricarboxylic acid cycle. Methane and methanol can be utilized by Methylothophs as *Methylocystis*, *Methylosinus*, *Methylococcus*, and *Methylomonas* spp. through the serine pathway for PHAs production or the ribulose monophosphate pathway (Khosravi-Darani et al. 2013).

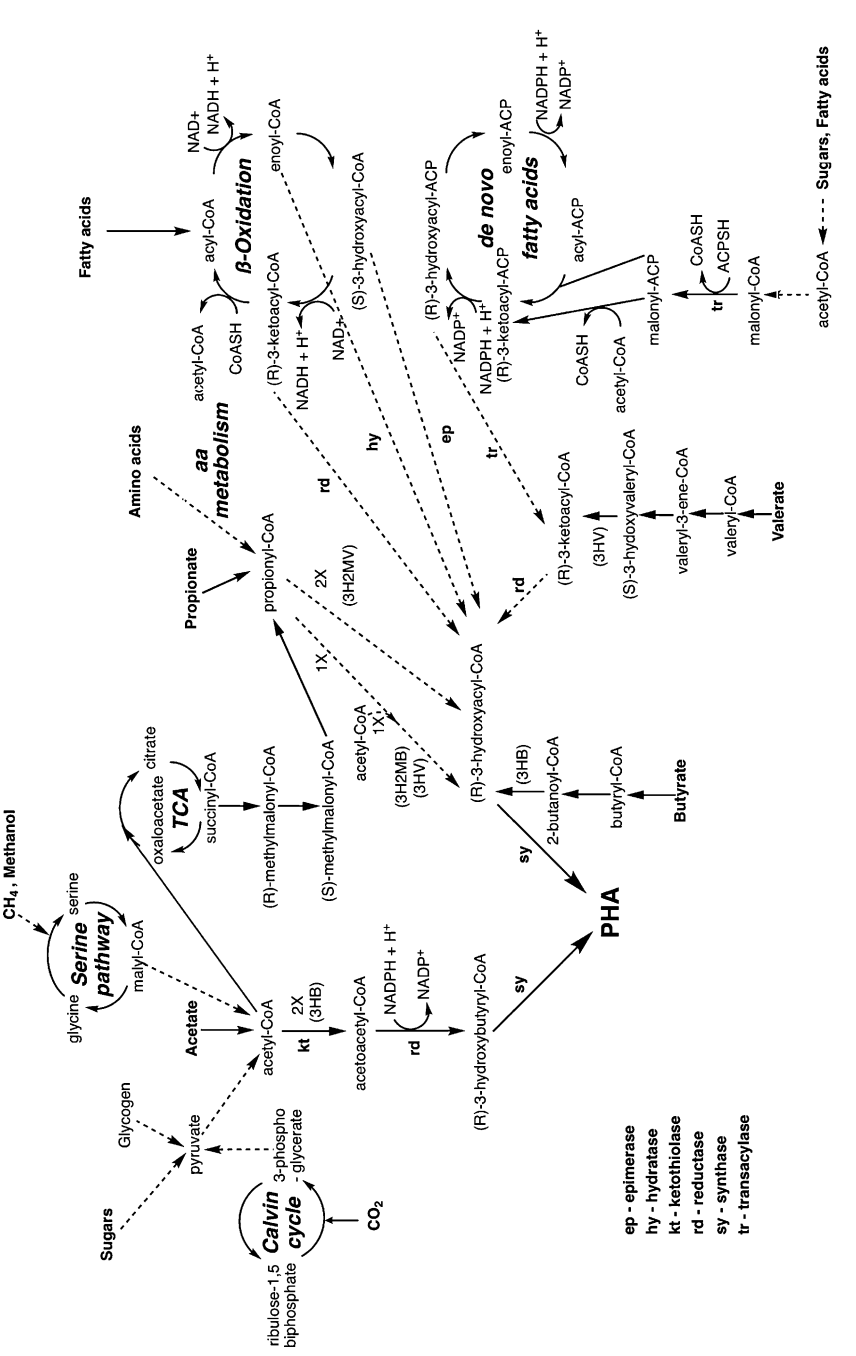


Fig. 2 Metabolic pathways for poly-3-hydroxyalkanoates production. (Adapted from Serafim et al. 2008a; Tan et al. 2014)

2.2 Function of PHAs

Besides the aforementioned storage function, ubiquitous among bacteria as a way to provide nutrition, recent studies showed that PHAs accumulation is involved in additional cellular functions, especially under adverse conditions by enhancing the resistance to stress. The mechanisms by which PHAs provide a protective role and other physiological functions are not well clarified but some examples of those functionalities can be found in the literature as reviewed by López et al. (2015) and Obruca et al. (2018).

PHAs were found to maintain anoxic photosynthesis and the sulfur cycle in microbial mats dependent on photosynthetic carbon fixation (Rothermich et al. 2000; Urmeneta et al. 1995), triggering sporulation in *Bacilli* (Slepecky and Law 1961), supporting the extension of nitrogen fixation by diazotrophs in the dark (Bergersen et al. 1991), or maintaining energy production and NADH oxidation in nitrogen-fixing bacteria (Encarnación et al. 2002). Also PHAs-storing cells were found to be more resistant to UV irradiation, low temperatures, desiccation, and osmotic pressure than mutants without storage ability. As reviewed by Obruca et al. (2018), this protective role of PHAs was observed in cells of *Azospirilum brasilense*, *Aeromonas hydrophila,* or recombinant *Escherichia coli*. PHAs were also found in Bacteria living in extreme environments of cold, showing a cryoprotective function, and in those living in high saline media, as most of the halophiles, behaving as compatible solutes to prevent the effect of high osmotic pressure (Obruca et al. 2018).

2.3 Occurrence of PHAs Producing Bacteria

As mentioned, PHAs are produced by a wide range of bacterial genera in nature and are usually produced under unbalanced growth conditions. The majority of the studied genera were isolated under such conditions. The bacterium which is considered a model organism for PHAs production, *Cupriavidus necator* (formerly known as *Alcaligenes eutrophus*, *Ralstonia eutropha*, and *Wautersia eutropha*) was isolated from freshwater sludge (Koller et al. 2011). Freshwaters, as well as most of the aquatic environments, are subject to fluctuations of nutrient availability. *Pseudomonas* strains with the same ability were isolated from lake water (Mukai et al. 1994). Also, microbial mats are a good source of PHAs-producing organisms. Not only these organisms are able to store the organic carbon produced by photosynthetic organisms that dominate those habitats (Koller et al. 2011) but also PHAs production was found to play a role on anoxic photosynthesis as an electron donor (Urmeneta et al. 1995; Rothermich et al. 2000). Several strains of the *Halomonas* genera, moderate halophilic bacteria are considered very promising for industrial PHAs production, were isolated from hypersaline microbial mats (Villanueva et al. 2010; Berlanga et al. 2015; Quillaguamán et al. 2010).

Soil is another example of habitats with nutritional fluctuations and is a well-known source of microorganisms of biotechnological significance (Koller et al. 2011). *Pseudomonas* strains, usually MCL-PHAs producers, were often isolated

from soil samples including rice field soil (Song et al. 2008). Quillaguamán et al. (2005) isolated *Halomonas boliviensis* from saline soil. This moderate halophile is able to use residues as substrates for PHA production, which is advantageous at an industrial level since this ability could contribute to the decrease of producing costs (Quillaguamán et al. 2005). Several *Bacillus* strains were also isolated from different types of soil with diverse locations as reviewed by Koller et al. (2011). PHAs-producing bacteria can be often found in rhizosphere soil since it is a habitat with nutritional fluctuations controlled by plant roots necessities (Koller et al. 2011). *Burkholderia sacchari* is a very efficient PHA-storing bacteria isolated from sugar cane soil and currently used at industrial level (Brämer et al. 2001). Other terrestrial habitats where PHA-storing bacteria can be found include gas field soils, industrial waste drainage sites, or contaminated soils (Koller et al. 2011).

2.4 Mixed Microbial Cultures

In nature, microorganisms live in communities with different relationships among them and the isolation of bacteria with specific characteristics is quite difficult. It is believed that more than 99% of microorganisms have not been successfully cultivated yet (Wintermute and Silver 2010) and, surely, among this number, many PHAs-producing strains can be found (Serafim et al. 2016). The majority of natural mixed microbial communities includes activated sludge populations, the best known and studied mixed microbial culture (MMC) and usually composed by bacteria with the ability to store intracellular reserves. Many of these complex consortia of microorganisms have an undefined composition, which depends on feeding and process conditions, often experiencing variations on the external carbon availability, being PHAs-producing organisms quite frequent. MMCs can be found in open biological systems that develop in natural environments, as soil, seawater, sediments, rumen, N_2-fixing bacteria, or as activated sludge, in human-created environments under nonsterile conditions, namely, ponds, waste storage facilities, etc.

PHAs granules were firstly observed inside cells belonging to an activated sludge community (Wallen and Rohwedder 1974). Some years later, PHAs were found to play a key role in enhanced biological phosphorus removal (EBPR). PHAs are stored when the MMC faces a sudden increase in the availability of the external carbon source when there is lack of electron sink. Under the impossibility of growing, bacteria store the external carbon source as PHAs that will be used as carbon and energy sources when the external substrate is exhausted and/or an electron sink is available again (Pereira et al. 1996). The first processes to be developed for PHAs production by MMC were based on EBPR conditions.

In aerobic activated sludge processes, bulking is a frequent problem but, by alternating the periods of excess of an external carbon source with depletion, floc-forming bacteria with enhanced PHA storage capacity are favored (Majone et al. 1996). The absence of external carbon source results on the dropping of levels of enzymes and RNA required for growth. If a sudden increase on external substrate occurs, it can trigger two types of response. Cells use carbon to grow and start to

synthesize a large variety of enzymes and high amounts of RNA, which requires a complex physiological adaptation and signifies a slow response. Instead of growing, PHAs-storing bacteria can produce internal reserves, which require lower amounts of enzymes and RNA, resulting in a faster response (Daigger and Grady 1982). This mechanism was the basis for the concept of aerobic "feast and famine," a process later known as aerobic dynamic feeding (ADF), first proposed by Majone et al. (1996) and widely used in the subsequent years (Serafim et al. 2008a). The ADF process became the most popular and developed process using MMC to produce PHAs but others were being developed (Serafim et al. 2016).

The development of processes using MMC is believed to contribute to the decrease of PHAs production costs. In 2004, Dionisi et al. proposed a three-step process based on the ADF process for using wastes or surplus-based feedstock as carbon source. This process starts with the acidification of organic components of the feedstock, mostly sugars, in order to obtain readily biodegradable carbon sources, followed by the enrichment of the MMC in PHAs-storing microorganisms. Finally, the obtained MMC is used in PHAs accumulation assays (Dionisi et al. 2004).

The use of inexpensive substrates as agro-forestry residues, industrial by-products, and industrial and urban wastes could also contribute to decrease costs. Several complex substrates, namely, olive mill wastewaters (Dionisi et al. 2005), paper mill wastewaters (Bengtsson et al. 2008; Jiang et al. 2012), pulp and paper mill by-products (Queirós et al. 2014), palm oil mill effluents (Mohd et al. 2012), pyrolysis by-products, crude glycerol (Moita and Lemos 2012; Moita et al. 2014; Freches and Lemos 2017), and sugar cane molasses (Albuquerque et al. 2007) were successfully tested in PHAs production. Jiang et al. (2012), using fermented paper mill wastewaters, obtained a MMC able to accumulate the highest PHA content so far reported with a complex substrate, 77% of cell dry weight.

MMC processes became competitive with pure and recombinant cultures and represent a clear alternative with reduced PHAs production costs. While pure and recombinant culture processes deeply depend on tight sterile conditions in order to avoid the contamination by non PHAs-producing bacteria, this is not a concern in processes using MMCs. In these processes, the dominance of PHA-storing organisms result from the operational conditions imposed. The establishment of true feast and famine conditions usually only allows for the survival of PHAs-storing organisms. The non-storing bacteria will be eliminated during the famine period, while PHAs-producing organisms will use their internal reserves as carbon and energy source.

Despite the wide variety of bacteria already identified in MMC processes, as reviewed by Queirós et al. (2015), many of them are traditional bacteria related with pure culture systems, including genus such as *Pseudomonas*, *Alcaligenes*, *Paracoccus,* or *Bacillus*. The diversity of populations, instead of being a drawback of the process, since it could signify too much variability on the polymer produced, is usually related with the robustness of the systems to cope with a feedstock that suffers periodic variations in its composition (Coats et al. 2007; Carvalho et al. 2014). Moreover, several researchers already showed that the diversity of genera in MMC do not result in variability of PHAs, which was confirmed after analyzing

molecular weights, polydispersities, melting and glass transition temperatures, melting enthalpies, or crystallinity (Serafim et al. 2008b; Laycock et al. 2013). Also, the monomeric composition of PHAs can be tailored by feeding the MMC with the right mixture of SCOAs (Serafim et al. 2008b; Albuquerque et al. 2011). Nevertheless, pure and recombinant cultures still allow for a better control of PHAs characteristics. However, the variety of monomers that can be obtained by MMC is limited when compared with pure cultures. Only in a few works with MMC, monomers with a number of carbons higher than six were obtained (Pisco et al. 2009).

Low biomass concentration is another weakness that can be assigned to MMC processes, due to its strong impact on the amount of polymer produced and on the downstream processing. The downstream processing itself can be another drawback of PHAs production by MMC, as cell wall and floc structure seem to be much more complex and harder to disrupt than pure and recombinant cultures that become fragile when attaining high PHAs storage contents (Samori et al. 2015).

2.5 Industrial Production of PHAs

Due to their biodegradability, biocompatibility, and similar characteristics to common plastics, PHAs are being produced industrially. Their widespread utilization is not as high as would be desirable, based on PHAs potential, as several drawbacks do not allow lower production costs.

In the recent years, numerous approaches were studied for the production of PHAs, making use of a variety of organisms such as bacteria, plants, and fungi. Also, with the advent of genetic engineering, the use of several recombinant organisms became possible, like the well-known recombinant *Escherichia coli* or new transgenic plants (Koller et al. 2011). With the aim of achieving industrial production, several considerations should be taken into account: cheap and suitable substrates in accordance with the metabolism of the producing organism; its storage capacity; the need for sterile conditions; type of reactor, feeding pattern; oxygen demand; behavior; and evolution of the culture.

More than 300 bacterial species were reported as PHAs producers but only 75 were tested for large-scale production. Currently, industrial production processes rely on pure cultures of microorganisms in their wild form, or genetically modified, namely, *Cupriavidus necator*, *Alcaligenes latus*, *Comamonas acidovorans*, *Pseudomonas putida*, *Chromobacterium* sp., *Hydrogenophaga pseudoflava*, *Rhodococcus ruber*, *Azotobacter vinelandii*, *Pseudomonas oleovorans*, *Paracoccus denitrificans*, *Protomonas extorquens,* and recombinant *Escherichia coli* (Magdouli et al. 2015). Genetic engineering allowed for the construction of recombinant strains for a more cost-effective PHA production.

As reviewed by Mozejko-Ciesielska and Kiewisz (2016) and Kourmentza et al. (2017), several industrial and pilot processes were already implemented using either wild-type or recombinant bacteria (Table 1). Earlier, Wang et al. (2014) discussed extensively the challenges that researchers on this field need to tackle in order to make the industrial production of PHAs economically competitive with the well-

Table 1 PHAs production at industrial and pilot scale. (Adapted from Mozejko-Ciesielska and Kiewisz 2016; Kourmentza et al. 2017)

Manufacturer	Trade mark	Strain	DNA recomb.	Carbon source	PHAs type	Cell cont. (% CDW)	Cell DW (g/L)	Production capacity
Biomatera, Canada	Biomatera	Non-pathogenic bacteria isolated from soil	No	Renewable raw materials	PHAs	–	–	–
Biomer, Germany	Biomer®	*Alcaligenes latus*	No	Sucrose	P(3HB)	>75%	>60	–
Bio-On Srl., Italy	Minerv®PHA	*Cupriavidus necator*	–	Sugar beets and cane processing waste materials	P(3HB), P(3HB-3 HV)	–	–	10,000 t/a
BluePHA, China	-	Microbial strains via synthetic biology	Yes	–	P(HB-HV-HHx), P (3 HV) P(3HP-3HB), P (3HB-4HB), P(3HP), P (4HB)	–	–	–
CJ CheilJedang Corp, South Korea	Mirel	*Cupriavidus necator*	No	Glucose, corn, sugarcane, vegetable oils	P(3HB), P(3HB-4HB)	>75%	>100	10,000 t/a
Danimer Scientific, USA	Nodax®PHA	Soil bacteria	Yes	Cold press canola oil	mcl-PHAs	–	–	30,000 t/a
EcoMann Biotechnology, China	AmBio®	Genetic engineered bacteria	Yes	Starch, sugar, or glucose	P(3HB-3 HV)	–	–	5000 t/a
ETH, Switzerland	PHA	*Pseudomonas putida*	No	Fatty acids	mcl-PHAs	≈60%	≈45	–
Jiangsu Nantian, China	Jiangsu Nantian	*Escherichia coli*	Yes	Glucose	P(3HB)	>80%	>150	1000 t/a

(*continued*)

Table 1 (continued)

Manufacturer	Trade mark	Strain	DNA recomb.	Carbon source	PHAs type	Cell cont. (% CDW)	Cell DW (g/L)	Production capacity
Kaneka Corporation, Japan	AONILEX®	*Cupriavidus necator*	Yes	Plant oils (fatty acids)	P(3HB-HHx)	>80%	>100	3500 t/a
Newlight Technologies LLc, USA	AirCarbon™	Newlight's 9X biocatalyst	–	Air oxygen and carbon from captured methane emissions	PHAs resins	–	–	–
PHB Industrial S.A., Brazil	Biocycle®	*Alcaligenes* sp.	No	Sugarcane	P(3HB)	>90%	>90	3000 t/a
PolyFerm, Canada	VersaMer™PHA	Naturally selected microorganisms	No	Sugars, vegetable oils	mcl-PHAs	–	–	–
SIRIM Bioplastics Pilot plant, Malaysia	–	–	–	Palm oil mill effluent (POME), crude palm, kernel oil	PHAs	–	–	2000 t/a
Tianan Biologic Material Co., Ltd. Ningbo, China	ENMAT™	*Cupriavidus necator*	No	Glucose +propionate, dextrose derived from corn or cassava	P(3HB), P(3HB-3 HV)	>75%	>160	10,000 t/a, 50,000 t/a by 2020
Tianjin GreenBio Materials Co, China	SoGreen®	*Escherichia coli*	Yes	Glucose+1,4-butanodiol, starch, cellulose	P(3HB-4HB)	>75%	>100	10,000 t/a

Abbreviations: *Cell cont.* cell content, *Cell DW* Cell dry weight, *DNA recomb.* DNA recombinant, *HB* hydroxybutyrate, *HHx* hydroxyhexanoate, *HP* hydroxypropionate, *HV* hydroxyvalerate, *mcl* medium chain length, *PHAs* polyhydroxyalkanoates

established industry of conventional plastics. These challenges include high cell density processes, the use of cheap and fast metabolized substrates, fast-growing strains, low sterility processes, development of oxygen limited processes, cell contents over 95%, and easier downstream processing. Comparing these findings with the processes described in Table 1, most of them are still far from fulfilling the portrayal of a competitive process.

3 Triacylglycerols (TAGs)

Triacylglycerols (TAGs) are fatty acid (FA) polyesters of glycerol being neutral and nonpolar lipids. Figure 1 shows a TAG molecule with the glycerol moiety linked to three molecules of an unsaturated FA. The biosynthesis of TAGs is determined by FA availability and often different and/or unsaturated FAs are also incorporated. Contrarily to animals and plants, Bacteria present FA chains composed not only by even carbon chains coming from acetyl-CoA metabolic insertion but also by odd and branched carbon chains. TAGs physicochemical properties are determined by FAs constitution, namely, their chain length and saturation degree (Brigham et al. 2013). In nature, TAGs are often found in eukaryotic organisms. Plants commonly store TAGs in seeds, while in animals, the production and storage of TAGs occur in adipocytes. Fungi and yeasts are also described as TAGs producers, mainly under metabolic stress conditions (Alvarez and Steinbuchel 2002).

The prokaryotic ability, which only certain bacterial strains possess, to synthesize and accumulate neutral lipids, is related to the expression of genetic and enzymatic complex mechanisms for surveillance against stress environmental conditions. Also, as previously shown, TAGs and waxes correspond to the highest energetic content, among bacterial storage reserves, since the acyl moieties are in the most reduced form. Only a few genera have the capacity of changing the usual cell growth metabolism to lipid accumulation by maintaining the balance of precursors and reducing equivalents (Alvarez 2016). Gram-negative bacteria from the phylae *Actinobacteria* (genera *Mycobacterium* and *Streptomyces)* and *Proteobacteria* (genera *Acinetobacter)* when submitted to specific experimental culture conditions triggering for TAGs biosynthesis accumulate high contents of TAGs. The accumulated TAGs bear the physiological function of carbon and energy reserve compounds (Qadeer et al. 2017).

Studies related to TAGs biosynthesis process by microbial cultures are quite recent. One of the first ones reported the ability of *Rhodococcus opacus*, an *Actinobacteria* isolated from soil, to store more than 70% of its dry biomass as TAGs (Alvarez and Steinbuchel 2002). TAGs were identified as intracellular inclusion bodies which allowed reaching high cell contents. Furthermore, TAGs were also associated with bacterial regulation of membrane fluidity, namely, the composition of the phospholipidic bilayer. TAGs could act as a source of the right FAs for phospholipid biosynthesis and allows keeping unusual FAs away from the cellular membrane (Dahlqvist et al. 2000). Consequently, TAGs play an important role on the adaptation of the cell membrane to the environmental changes without disturbing

its rheological properties of fluidity, metabolite permeability, and transportation dynamism of protein complexes (Alvarez and Steinbuchel 2002). TAGs have also an intracellular thermodynamic role since they can dissipate reducing equivalents released by other metabolisms. They seem to function as a bacterial self-defensive protection, especially in very aggressive environments such as deserts, where they can work as a source of intracellular water. Additionally, TAGs accumulation was also associated to pathogeny since it was reported that *Mycobacterium tuberculosis* can accumulate TAGs even when submitted to conditions of dormancy and virulence (Wältermann et al. 2007). This specific TAGs metabolic role together with the role described during antibiotic biosynthesis by *Streptomyces coelicolor* were determinant for the development of subsequent research studies. These studies could provide TAGs characterization and analytical determination of the respective components in order to understand and describe biosynthesis pathways and mechanisms of TAGs accumulation in prokaryotes. The possibility of performing genetic modification for increasing the production and accumulation of high-value TAGs in bioreactor cultures resulted in an increasing interest in this research field and motivated the development of different biochemical approaches related to the genes, enzymes, and regulatory mechanisms involved.

TAG accumulation has been identified and studied in the Gram-negative bacteria of genera *Acinetobacter*, *Marinobacter,* and *Alcanivorax* sp. Gram-positive *Actinobacteria* genera *Streptomyces*, *Gordonia*, *Mycobacterium*, *Rhodococcus*, and *Nocardia* were also reported as accumulating TAGs. Alvarez (2016) presented and compared metabolic pathways of biosynthesis, respective enzymes, and genes highlighting the similarities and differences between Gram-positive and Gram-negative bacteria. Figure 3 (right) shows TAGs biosynthesis in Prokaryotes through the Kennedy pathway: acyl-CoA reacts with glycerol-3-phosphate (G3P) to produce lyso-phosphatidic acid, the precursor of phosphatidic acid. This reaction is catalyzed by the enzyme wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT), which is also involved in WEs biosynthesis (Alvarez et al. 2012). Then, phosphatidic acid can be converted into diacylglycerol, a substrate for producing TAG by acylation. Alternatively, phosphatidic acid can also be utilized to phospholipid biosynthesis suggesting a competition between these two metabolic routes for the same substrate. Metabolic regulation is the key for growth or for attaining TAG accumulation in Prokaryotes. Some authors associated TAGs accumulation to nitrogen limitation situations or, at least, to high carbon to nitrogen ratios (Amara et al. 2016). Metabolic regulation and functions of enzymes involved in TAGs metabolism (Fig. 3, right) and proteins associated with TAGs granules are still the subject of diverse on-going research (Alvarez et al. 2012).

Genes codifying for WS/DGAT in each species and the substrates fed to microbial cultures determine the chemical composition of TAGs. Genetic information and improvement of knowledge about metabolic routes of TAGs biosynthesis gave rise to metabolic engineered or genetically engineered strains to provide microbial cultures that could produce oils (Yan and Liao 2009). Such strains were assayed with different carbon sources in order to perspective TAGs cell factories from low-value carbon sources such as lignocellulosic materials and industrial by-products. An

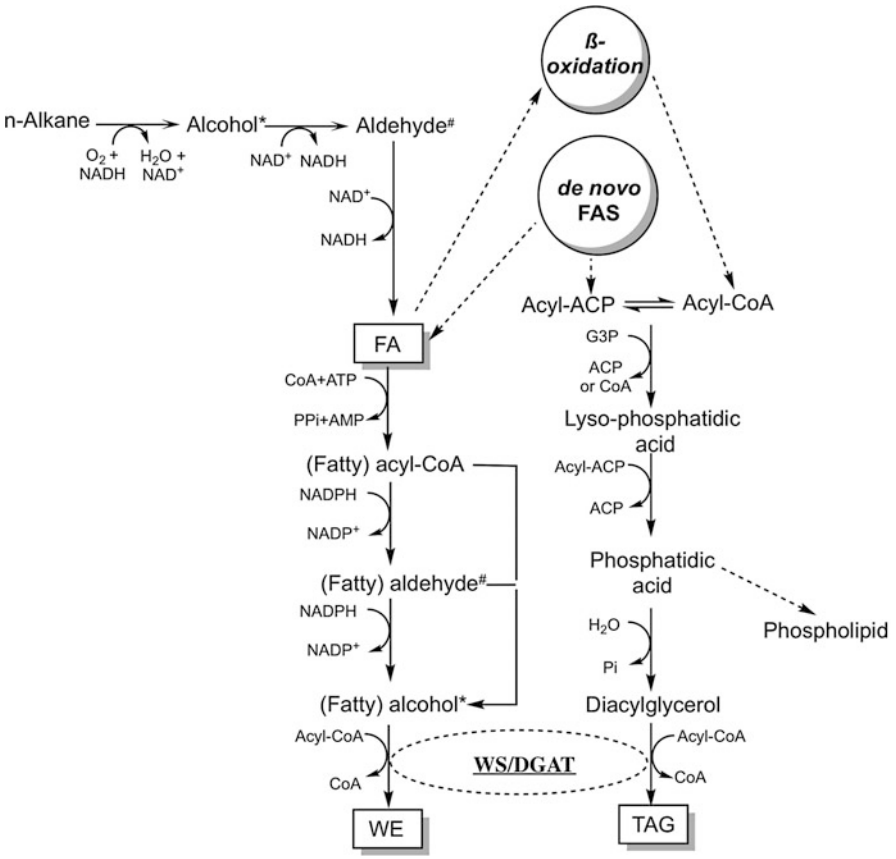


Fig. 3 Pathways for the production of some storage neutral lipids. (Adapted from Muller et al. 2014; Rontani 2010; Acyl-CoA, Acyl-coenzyme A; Acyl-ACP, acyl-acyl carrier protein; FA, Fatty acid; FAS, Fatty acid synthesis; G3P, Glycerol-3-phosphate; TAG, Tri-acyl-glycerol; WE, Wax ester; WS/DGAT, Wax ester synthase/acyl-CoA:diacylglycerol acyltransferase; #, * the same compound)

example is the genetically modified *Acinetobacter baylyi* M4 that showed an improved production of TAGs in gluconate and glycerol (Santala et al. 2011). However, the majority of studies were done using *Rhodococcus* species. Promising results were obtained by phosphatidic acid phosphatase overexpression, involved in the production of diacylglycerol from phosphatidic acid, in *R. jostii* and in *R. fasciens* allowing increased TAGs accumulation using glucose and fructose, respectively (Hernández et al. 2015). *R. opacus* the most tested species was modified for xylose utilization (Kurosawa et al. 2014) in order to use lignocellulosic raw materials. Two of the modified strains were effective in xylose/glucose mixtures as well as in Kraft hardwood hydrolysates (Kurosawa et al. 2013). The most successful strain, *R. opacus* MITXM-61, was able to use glucose and acetic acid, accumulating a maximum of 54% of TAGs from high concentrated corn stover hydrolysate (Kurosawa et al. 2014).

In order to obtain cultures for TAGs production with high cell contents, different *E. coli* genetic modifications were attained in strains tested for glucose or lactose consumption but with TAGs contents lower than 9% (Röttig et al. 2015).

In the future, optimized cultures and bioprocesses could provide significant TAGs contents with diverse potential applications, since TAGs can be a source of pharmaceuticals, cosmetics, and nutraceuticals. TAGs production from lignocellulosic residual materials is also being searched in order to be used as biofuel and to replace fossil fuels.

Waste streams rich in lipids were studied for TAGs production by MMCs (Tamis et al. 2015). This bioprocess needs a specific strategy for TAGs accumulation with the advantage of lowering the costs associated with their production when compared with pure or recombinant cultures. Also, activated sludge from existing wastewater treatment facilities were subject of study for perspective potential TAGs production (Revellame et al. 2013). The approach would provide a double benefit since it would decrease the need for waste stream post-treatment together with the bacterial TAGs production that could be a solution for biofuel availability.

4 Wax Esters (WEs)

Wax esters are oxoesters of long-chain fatty acids esterified with long-chain alcohols (Fig. 1). WEs from biological sources contribute to the formulation of several industrial products such as lubricants, printing inks, coating of materials, candles, and cosmetics (Ishige et al. 2003).

The most generic metabolism for WEs production (Fig. 3) involves the activation of FAs and its subsequent conversion into the fatty alcohol by the activity of several reductases. The final steps of WEs and TAGs production share the enzyme WS/DGAT, a CoA-dependent acyltransferase with a broad substrate range that transfers an acyl-CoA either onto a fatty alcohol (WE) or a diacylglycerol (TAG). The formation of the lipid bodies enclosing WEs/TAGs also involves the same enzyme. The enzyme attaches to the cytoplasmic membrane producing lipid microdroplets towards the cytoplasmic side that will be released, aggregated, and surrounded by a phospholipid monolayer infused with proteins resulting in insoluble intracytoplasmic lipid-bodies (Wältermann et al. 2005; Muller et al. 2014). These proteins are important in the accumulation of lipids and in the determination of the size of lipid-bodies (Qadeer et al. 2017). The organization of WEs lipid-bodies can be diverse from spherical, flat, disk-like to rectangular structures (Rontani 2010).

WEs production can result from the bioremediation of land and water bodies contaminated with petrochemical hydrocarbons. Gram-negative hydrocarbon degrading bacteria such as *Acinetobacter* sp., *Marinobacter* sp., *Thalassolituus* sp., *Alcanivorax* sp., and Gram-positive Actinobacteria as *Mycobacterium* sp., *Streptomyces* sp., *Rhodococcus* sp., *Nocardia* sp., *Microthrix* sp. are able to accumulate TAGs and/or WEs (Muller et al. 2014; Alvarez 2016). The accumulation occurs under external carbon source availability with nitrogen limitation, usually in

low amounts, but some strains can accumulate up to 10–20% of their dry weight (Alvarez 2016). The type and amount of stored lipid are strain-dependent with *Acinetobacter baylyi* SDP1 and *Marinobacter hydrocarbonoclasticus* SP17 and DSM 8798 accumulating preferably WEs. Actinobacteria are more specialized in accumulating TAGs being able to reach much higher lipid storage than their Gram-negative counterparts. Lipid storage is quite demanding for bacteria since they must drift carbon, reducing equivalents and energy from their normal growth and division processes. To achieve that, a complex metabolic balance has to be reached at any given moment, involving many different metabolic pathways (glycolysis, pentose-phosphate, ß-oxidation, *de-novo* fatty acids, amino acids catabolism, etc.)

Most of WEs-producing organisms use related carbon sources but some, as *Marinobacter aqualeolei* VT8, can use unrelated substrates to produce precursors as medium and long-chain fatty acyl and fatty alcohols, for its synthesis (Willis et al. 2011). Microorganisms can use n-alkanes both to grow and to store WEs as reserves. Several strains of *Acinetobacter sp.* are able to store high amounts of WEs from n-alkenes or long-chain length alkanols usually under growth-limitation conditions (nitrogen). *Acinetobacter sp.* strain ADP1 utilizes n-alkenes from C_{12}–C_{18}, but strains as *Acinetobacter sp.* strain M1 can use much longer chain-length alkenes (C_{20}–C_{44}, till C_{60}). This last strain is versatile in terms of alkane carbon number utilization. However, with increasing alkane chain-length, the amount of stored WEs decreases, and in terms of composition alkanoates with a shorter-chain length are becoming more important in WEs while alkanol moiety remains the same as the alkane fed. This fact suggests that alkanes will be processed through ß-oxidation in order to produce the shorter acyl-CoAs used for WE synthesis (Ishige et al. 2003). Several *Acinetobacter* sp. can also produce WEs from alkyl-alcohols. The final step in WEs production involves the reaction of an alcohol with acyl-CoA. In *Acinetobacter sp.* strain M1, the alcohol moiety for WEs production can be derived from two possible pathways. One involves the direct utilization of the n-alkanol or the n-alkanal in the n-alkane degradation pathway, while the other comprises the conversion of acyl-CoA into the corresponding alcohol via aldehyde. *Acidovorax borkumensis* SK2 possesses WS/DGAT enzymes with affinity to a diverse range of substrates being able to produce storage lipids from related and unrelated sources. Contrary to most Gram-negative lipid storage bacteria, SK2 strain produces preferably TAGs (about 20%CDW) together with small amounts of WEs (Kalscheuer et al. 2007). Recently, Da Silva et al. (2016) observed the production of WEs using an industrial wastewater composed mostly of a mixture of linear alkanes (C10–C34) associated with the growth phase of *Alcanivorax borkumensis* SK2.

Actinobacteria store preferably TAGs using different carbon sources, but in the presence of n-alkanes and n-alcohols, they are able to produce WEs. *Streptomyces avermitilis* MA-4680 produces both TAGs and WEs (minor fraction) when grown in the presence of both glucose and hexadecanol (Kaddor et al. 2009). *Mycobacterium tuberculosis* was able to produce WE, both saturated and unsaturated ones, under iron limitation (Bacon et al. 2007). Another *M. tuberculosis* (Rv3391) under dormancy conditions produced WEs from acyl-CoA derived from fatty alcohols (Sirakova et al. 2012). *Rhodococcus* sp. produces TAGs from unrelated carbon

sources, namely, sugars, but with related ones, as hexadecanol and hexadecane, the fatty acid and the derived acyl alcohol are incorporated into WEs.

5 Research Needs

The neutral lipid accumulation in Bacteria is an intricate process with a strict regulation for fine tuning of many different reactions in several metabolic pathways. The metabolic intermediates need an adequate distribution in order to allow for the proper cell metabolism and, at the same time, allowing responses to different stress conditions. PHAs still require more research in order to become competitive to conventional plastics and increase their market share. The possibility of using MMCs processes seems to be a viable hypothesis but the process needs more pilot studies and overcomes some of their bottlenecks previously enumerated: low biomass densities and difficult downstream processing.

A deeper understanding of the metabolic regulation of TAGs accumulation in Bacteria is needed, namely, for the clarification of its biological trigger. Both nitrogen limitation and the ratio of carbon to nitrogen were reported as potential triggers. The gained scientific knowledge would support strains optimization. New experimental strategies for process optimization and results that could provide the implementation of cell factories for TAGs production are needed. TAGs from low-price feedstock, such as lignocellulosic residues or industrial by-products, by pure cultures or waste streams by mixed microbial cultures are also an important focus of research. TAGs extraction is a determinant issue to be deeply studied. Besides cell recovery of enriched intracellular TAG cultures, cell disruption and also granule disruption steps have to be well studied. This is a drawback that ought to be overcome in order to enable the recovery of TAGs for their possible multiple applications.

Concerning Wes, they share most of the problems related with TAG production, namely, because of the common WS/DGAT enzyme indispensable for lipid storage. WEs accumulation is not as effective as TAGs, indicating that an effort has to be made in order to increase their accumulation. An advantage of the WEs is their tailor-made production by controlling the substrate, the growth conditions, and the strains used. Concerning costs for lipids production half came from the nutrient medium needed for cell growth while a quarter came from cost of solvent for lipid extraction. To circumvent such problems, a low-to-zero value substrate could be used, namely, considering the strategies for remediation and lipid's waste valorization. New and less toxic solvents that could be reused have to be applied, as non-halogenated solvents or ionic liquids, but also other strategies, as cell disruption, in order to increase extraction efficiency. When comparing lab-scale processes with pilot-scale studies, the low efficiency of the bacterial strains in the later reinforces the need for further research in this area.

Acknowledgments This work was financed by Fundação para a Ciência e a Tecnologia through IF/01054/2014. This work was also developed within the scope of the project CICECO-Aveiro

Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID/CTM/50011/2013), financed by national funds through the FCT/MEC and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement. This work was also supported by the Associate Laboratory for Green Chemistry- LAQV which is financed by national funds from FCT/MCTES (UID/QUI/50006/2013) and co-financed by the ERDF under the PT2020 Partnership Agreement (POCI-01-0145-FEDER – 007265).

References

Albuquerque MGE, Eiroa M, Torres C, Nunes BR, Reis MAM (2007) Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. J Biotechnol 130:411–421

Albuquerque MGE, Martino V, Pollet E, Avérous L, Reis MAM (2011) Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties. J Biotech 151:66–76

Alvarez HM, Steinbuchel A (2002) Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol 60:367–376

Alvarez HM, Steinbüchel A (2010) Physiology biochemistry and molecular biology of triacylglycerol accumulation by *Rhodococcus*. In: Alvarez HM (ed) Biology of *Rhodococcus*, Microbiology monographs series. Springer, Heidelberg, pp 263–290

Alvarez HM, Silva RA, Herrero M, Hernández MA, Villalba MS (2012) Metabolism of triacylglycerols in *Rhodococcus* species: insights from physiology and molecular genetics. J Mol Biochem 2:69–78

Alvarez HM (2016) Triacylglycerol and wax ester-accumulating machinery in prokaryotes. Biochimie 120:28–39

Amara S, Seghezzi N, Otani H, Diaz-Salazar C, Liu J, Eltis LD (2016) Characterization of key triacylglycerol biosynthesis processes in rhodococci. Sci Rep 6:24985

Bacon J, Dover LG, Hatch KA, Zhang Y, Gomes JM, Kendall S, Wernisch L, Stoker NG, Butcher PD, Besra GS, Marsh PD (2007) Lipid composition and transcriptional response of *Mycobacterium tuberculosis* grown under iron-limitation in continuous culture: identification of a novel wax ester. Microbiology 153:1435–1444

Bengtsson S, Werker A, Christensson M, Welander T (2008) Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. Bioresour Technol 99:509–516

Bergersen FJ, Peoples MB, Turner GL (1991) A role for poly-beta-hydroxybutyrate in bacteroids of soybean root nodules. Proc R Soc Lond 245:59–64

Berlanga M, Miñana-Galbis D, Domènech O, Guerrero R (2015) Enhanced polyhydroxyalkanoates accumulation by *Halomonas* spp. in artificial biofilms of alginate beads. Int Microbiol 15:191–199

Brämer CO, Vandamme P, da Silva LF, Gomez JGC, Steinbüchel (2001) *Burkholderia sacchari* sp. nov., a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. Int J Syst Evol Microbiol 51:1709–1713

Brigham CJ, Kurosawa K, Rha C, Sinskey AJ (2013) Bacterial carbon storage to value added products. J Microbial Biochem Technol S3:002:2–13

Bugnicourt E, Cinelli P, Lazzeri P, Alvarez V (2014) Polyhydroxyalkanoate (PHA): review of synthesis, characteristics, processing and potential applications in packaging. Express Polym Lett 8:791–808

Carvalho G, Oehmen A, Albuquerque MGE, Reis MAM (2014) The relationship between mixed microbial culture composition and PHA production performance from fermented molasses. New Biotechnol 31:257–263

Chen G-Q, Hajnal I (2015) The 'PHAome'. Trends Biotechnol 33:559–564

Coats ER, Loge FJ, Smith W, Thompson DN, Wolcott MP (2007) Functional stability of a mixed microbial consortium producing PHA from waste carbon sources. Appl Biochem Biotechnol 137:909–925

Da Silva DMP, Lima F, Alves MM, Bijmans MFM, Pereira MA (2016) Valorization of lubricant-based wastewater for bacterial neutral lipids production: growth-linked biosynthesis. Water Res 101:17–24

Dahlqvist A, Stähl U, Lanman M, Banas A, Lee M, Sandager L, Ronne H, Stymne S (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci U S A 12:6487–6492

Daigger GT, Grady CPL (1982) An assessment of the role of physiological adaptation in the transient-response of bacterial cultures. Biotechnol Bioeng 24:1427–1444

Dionisi D, Majone M, Papa V, Beccari M (2004) Biodegradable polymers from organic acids by using activated sludge enriched by aerobic periodic feeding. Biotechnol Bioeng 85:569–579

Dionisi D, Carucci G, Petrangeli Papini M, Riccardi C, Majone M, Carrasco F (2005) Olive oil mill effluents as a feedstock for production of biodegradable polymers. Water Res 39:2076–2084

Revellame ED, Hernandez R, French WT, Holmes WE, Forks A, Callahan R II (2013) Lipid-enhancement of activated sludges obtained from conventional activated sludge and oxidation ditch processes. Bioresour Technol 148:487–493

Encarnación S, Vargas MC, Dunn MF, Davalos A, Mendoza G, Mora Y, Mora J (2002) AniA regulates reserve polymer accumulation and global protein expression in *Rhizobium etli*. J Bacteriol 184:2287–2295

Freches A, Lemos PC (2017) Microbial selection strategies for polyhydroxyalkanoates production from crude glycerol: effect of OLR and cycle length. New Biotechnol 39:22–28

Hauschild P, Röttig A, Madkour MH, Al-Ansari AM, Almakishah NH, Steinbüchel A (2017) Lipid accumulation in prokaryotic microorganisms from arid habitats. Appl Microbiol Biotechnol 101:2203–2216

Hernández MA, Comba S, Arabolaza A, Gramajo H, Alvarez HM (2015) Overexpression of a phosphatidic acid phosphatase type 2 leads to an increase in triacylglycerol production in oleaginous *Rhodococcus* strains. Appl Microbiol Biotechnol 99:2191–2207

Holdren JP (2011) Materials genome initiative for global competitiveness. National Science and Technology Council OSTP, Washington, DC

Ishige T, Tani A, Sakai Y, Kato N (2003) Wax ester production by bacteria. Curr Opin Microbiol 6:244–250

Jendrossek D, Pfeiffer D (2014) New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly(3-hydroxybutyrate). Environ Microbiol 16: 2357–2373

Jiang Y, Marang L, Tamis J, van Loosdrecht MCM, Dijkman H, Kleerebezem R (2012) Waste to resource: converting paper mill wastewater to bioplastic. Water Res 46:5517–5530

Kaddor C, Biermann K, Kalscheuer R, Steinbüchel A (2009) Analysis of neutral lipid biosynthesis in *Streptomyces avermitilis* MA-4680 and characterization of an acyltransferase involved herein. Appl Microbiol Biotechnol 84:143–155

Kalscheuer R, Stöveken T, Malkus U, Reichelt R, Golyshin PN, Sabirova JS, Ferrer M, Timmis KN, Steinbüchel A (2007) Analysis of storage lipid accumulation in *Alcanivorax borkumensis*: evidence for alternative triacylglycerol biosynthesis routes in bacteria. J Bacteriol 189:918–928

Khosravi-Darani K, Mokhtari Z-B, Amai T, Tanaka K (2013) Microbial production of poly (hydroxybutyrate) from C1 carbon sources. Appl Microbiol Biotechnol 97:1407–1424

Koller M, Gasser I, Schimd F, Berg G (2011) Linking ecology with economy: insights into polyhydroxyalkanoate-producing microorganisms. Eng Life Sci 11:222–237

Kourmentza C, Plácido J, Venetsaneas N, Burniol-Figols A, Varrone C, Gavala HN, Reis MAM (2017) Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering 4:55

Kurosawa K, Wewetzer SJ, Sinskey AJ (2013) Engineering xylose metabolism in triacylglycerol-producing *Rhodococcus opacus* for lignocellulosic fuel production. Biotechnol Biofuels 6:134

Kurosawa K, Wewetzer SJ, Sinskey AJ (2014) Triacylglycerol production from corn Stover using a xylose-fermenting *Rhodococcus opacus* strain for lignocellulosic biofuels. J Microbial Biochem Technol 6:254–259

Laycock B, Halley P, Pratt S, Werker A, Lant P (2013) The chemomechanical properties of microbial polyhydroxyalkanoates. Prog Polym Sci 38:536–583

Li S, Cai L, Wu L, Zeng G, Chen J, Wu Q, Chen C-Q (2014) Microbial synthesis of functional homo-, random, and block polyhydroxyalkanoates by β-oxidation deleted *Pseudomonas entomophila*. Biomacromolecules 15:2310–2319

López NI, Pettinari MJ, Nikel PI, Méndez BS (2015) Polyhydroxyalkanoates: much more than biodegradable plastics. Adv Appl Biotechnol 93:73–106

Magdouli S, Brar SK, Blais JF, Tyagi RD (2015) How to direct the fatty acid biosynthesis towards polyhydroxyalkanoates production? Biomass Bioenergy 74:268–279

Majone M, Massanisso P, Carucci A, Lindrea K, Tandoi V (1996) Influence of storage on kinetic selection to control aerobic filamentous bulking. Water Sci Technol 34(223–2):32

Mohd MF, Mohanadoss P, Ujang Z, van Loosdrecht M, Yunus SM, Chelliapan S, Zambare V, Olsson G (2012) Development of Bio-PORec system for polyhydroxyalkanoates (PHA) production and its storage in mixed cultures of palm oil mill effluent (POME). Bioresour Technol 124:208–216

Moita R, Lemos PC (2012) Biopolymers production from mixed cultures and pyrolysis by-products. J Biotechnol 157:578–583

Moita R, Freches A, Lemos PC (2014) Crude glycerol as feedstock for polyhydroxyalkanoates production by mixed microbial cultures. Water Res 58:9–20

Mozejko-Ciesielska J, Kiewisz R (2016) Bacterial polyhydroxyalkanoates: still fabulous? Microbiol Res 192:271–282

Mukai K, Yamada K, Doi Y (1994) Efficient hydrolysis of polyhydroxyalkanoates by *Pseudomonas stutzeri* YM1414 isolated from lake water. Polym Degrad Stab 43:319–327

Muller EEL, Sheik AR, Wilmes P (2014) Lipid-based biofuel production from wastewater. Curr Opin Biotechnol 30:9–16

Murphy DJ (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. Prog Lipid Res 32:247–280

Obruca S, Sedlacek P, Koller M, Kucera D, Pernicova I (2018) Involvement of polyhydroxyalkanoates in stress resistance of microbial cells: biotechnological consequences and applications. Biotechnol Adv 36:856 (in press)

Oehmen A, Lemos PC, Carvalho G, Yuan Z, Keller J, Blackall LL, Reis MAM (2007) Advances in enhanced biological phosphorus removal: from micro to macro scale. Water Res 41:2271–2300

Pereira H, Lemos PC, Reis MAM, Crespo JPSG, Carrondo MJT, Santos H (1996) Model for carbon metabolism in biological phosphorus removal processes based on in vivo ^{13}C-NMR labelling experiments. Water Res 30:2128–2138

Pisco AR, Bengtsson S, Werker A, Reis MAM, Lemos PC (2009) Community structure evolution and enrichment of glycogen-accumulating organisms producing polyhydroxyalkanoates from fermented molasses. Appl Environ Microbiol 75:4676–4686

Qadeer S, Khalid A, Mahmood S, Anjum M, Ahmad Z (2017) Utilizing oleaginous bacteria and fungi for cleaner energy production. J Clean Prod 168:917–928

Queirós D, Rossetti S, Serafim LS (2014) PHA production by mixed cultures: a way to valorize wastes from pulp industry. Bioresour Technol 157:197–205

Queirós D, Lemos PC, Rossetti S, Serafim LS (2015) Unveiling PHA-storing populations using molecular methods. Appl Microbiol Biotechnol 99:10433–10446

Quillaguamán J, Hashim S, Bento F, Mattiasson B, Hatti-Kaul R (2005) Poly(b-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as substrate. J Appl Microbiol 99:151–157

Quillaguamán J, Guzmán H, Doan Van T, Hatti-Kaul R (2010) Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects. Appl Microbiol Biotechnol 85:1687–1696

Rae BD, Long BM, Murray RB, Price GD (2013) Functions, compositions, and evolution of the two types of carboxysomes: polyhedral microcompartments that facilitate CO_2 fixation in cyanobacteria and some proteobacteria. Microbiol Mol Biol Rev 77:357–379

Rehm BHA (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33

Rehm B (2006) Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: the key role of polyester synthases. Biotechnol Lett 28:207–213

Reis M, Albuquerque M, Villano M, Majone M (2011) Mixed culture processes for polyhydroxyalkanoate production from agro-industrial surplus/wastes as feedstocks. In: Moo-Young M (ed) Comprehensive biotechnology, 2nd edn. Academic, Burlington, pp 669–683

Rontani J-F (2010) Production of wax esters by bacteria. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin/Heidelberg, pp 459–470

Rothermich MM, Guerrero R, Lenz RW, Goodwin S (2000) Characterization, seasonal occurrence, and diel fluctuation of poly(hydroxyalkanoate) in photosynthetic microbial mats. Appl Environ Microbiol 66:4279–4291

Röttig A, Steinbüchel A (2013) Acyltransferases in bacteria. Microbiol Mol Biol Rev 77:277–321

Röttig A, Zurek PJ, Steinbüchel A (2015) Assessment of bacterial acyltransferases for an efficient lipid production in metabolically engineered strains of E. coli. Metab Eng 32:195–206

Samori C, Abbondanzi F, Galletti P, Giorgini L, Mazzocchetti L, Torri C, Tagliavini E (2015) Extraction of polyhydroxyalkanoates from mixed microbial cultures: impact on polymer quality and recovery. Bioresour Technol 189:195–202

Santala S, Efimova E, Kivinen V, Larjo A, Aho T, Karp M, Santala V (2011) Improved triacylglycerol production in *Acinetobacter baylyi* ADP1 by metabolic engineering. Microb Cell Factories 10:36

Serafim LS, Lemos PC, Albuquerque MGE, Reis MAM (2008a) Strategies for PHA production by mixed cultures and renewable waste materials. Appl Microbiol Biotechnol 81:615–628

Serafim LS, Lemos PC, Torres C, Reis MAM, Ramos AM (2008b) The influence of process parameters on the characteristics of polyhydroxyalkanoates produced by mixed cultures. Macromol Biosci 8:355–366

Serafim LS, Queirós D, Rossetti S, Lemos PC (2016) Biopolymer production by mixed-microbial cultures: integrating remediation with valorization. In: Koller M (ed) Recent advances in biotechnology – volume 1 – microbial polyester production, performance and processing – microbiology, feedstocks, and metabolism, 1st edn. Bentham Science Publishers, Sharjah, pp 226–264

Shively JM (1974) Inclusion bodies of prokaryotes. Annu Rev Microbiol 28:167–188

Shively JM, Cannon GC, Heinhorst S, Bryant DA, DasSarma S, Bazylinski D, Preiss J, Steinbüchel A, Docampo R, Dahl C (2011) Bacterial and archaeal inclusions. In: eLS. Wiley, Chichester, pp 1–14

Sirakova TD, Deb C, Daniel J, Singh HD, Maamar H, Dubey VS, Kolattukudy PE (2012) Wax ester synthesis is required for *Mycobacterium tuberculosis* to enter in vitro dormancy. PLoS One 7: e51641

Slepecky RA, Law JH (1961) Synthesis and degradation of poly-beta-hydroxybutyric acid in connection with sporulation of *Bacillus megaterium*. J Bacteriol 82:37–42

Song JH, Jeon CO, Choi MH, Yoon SC, Park W (2008) Polyhydroxyalkanoate (PHA) production using waste vegetable oil by *Pseudomonas* sp. strain DR2. J Microbiol Biotechnol 18:1408–1415

Steinbüchel A, Pieper U (1992) Production of a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources by a mutant of *Alcaligenes eutrophus*. Appl Microbiol Biotechnol 37:1–6

Steinbüchel A, Valentin HE (1995) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 128:219–228

Steinbüchel A, Hein S (2001) Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. Adv Biochem Eng Biotechnol 71:81–123

Tamis J, Sorokin DY, Jiang Y, van Loosdrecht MCM, Kleerebezem R (2015) Lipid recovery from a vegetable oil emulsion using microbial enrichment cultures. Biotechnol Biofuels 8:39

Tan G-Y, Chen C-L, Li L, Ge L, Wang L, Razaad I, Li Y, Zhao L, Mo Y, Wang J-Y (2014) Start a research on biopolymer Polyhydroxyalkanoate (PHA): a review. Polymers (Basel) 6:706–754

Tanaka K, Ishizaki A, Kanamaru T, Kawano T (1995) Production of poly(D-3-hydroxybutyrate) from CO_2, H_2, and CO by high cell density autotrophic cultivation of *Alcaligenes eutrophus*. Biotechnol Bioeng 45:268–275

Tanaka K, Miyawaki K, Yamaguchi A, Khosravi-Darani K, Matsusaki H (2011) Cell growth and P (3HB) accumulation from CO_2 of a carbon monoxide-tolerant hydrogen-oxidizing bacterium, *Ideonella* sp. O-1. Appl Microbiol Biotechnol 92:1161–1169

Tsuge T, Hyakutake M, Mizuno K (2015) Class IV polyhydroxyalkanoate (PHA) synthases and PHA-producing *Bacillus*. Appl Microbiol Biotechnol 99:6231–6240

Urmeneta J, Mas-Castella J, Guerrero R (1995) Biodegradation of poly-b-hydroxyalkanoates in a lake sediment sample increases bacterial sulfate reduction. Appl Environ Microbiol 61:2046–2048

Villanueva L, Del Campo J, Guerrero R (2010) Diversity and physiology of polyhydroxyalkanoate-producing and -degrading strains in microbial mats. FEMS Microbiol Ecol 74:42–54

Volova TG (2004) Polyhydroxyalkanoates – plastic materials of the 21st century: production, properties, applications. Nova Science Publishers, Inc., New York

Wallen LL, Rohwedder WK (1974) Poly-b-hydroxyalkanoate from activated sludge. Environ Sci Technol 8:576–579

Wältermann M, Hinz A, Robenek H, Troyer D, Reichelt R, Malkus U, Galla H-J, Kalscheuer R, Stöveken T, von Landenberg P, Steinbüchel A (2005) Mechanism of lipid-body formation in prokaryotes: how bacteria fatten up. Mol Microbiol 55(3):750–763

Wältermann M, Steinbüchel A (2005) Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. J Bacteriol 187:3607–3619

Wältermann M, Stoveken T, Steinbuchel A (2007) Enzymes for biosynthesis of neutral lipid storage compounds in prokaryotes: properties, function and occurrence of wax ester synthases/acyl-CoA:diacylglycerol acyltransferases. Biochimie 89:230–242

Wang Y, Yin J, Chen CQ (2014) Polyhydroxyalkanoates, challenges and opportunities. Curr Opin Biotechnol 30:59–65

Willis RM, Wahlen BD, Seefeldt LC, Barney BM (2011) Characterization of a fatty acyl-CoA reductase from *Marinobacter aquaeolei* VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol. Biochemistry 50:10550–10558

Wintermute EH, Silver PA (2010) Dynamics in the mixed microbial concourse. Genes Dev 24:2603–2614

Yan Y, Liao JC (2009) Engineering metabolic systems for production of advanced fuels. J Ind Microbiol Biotechnol 36:471–479

Players in the Nonpolar Lipid Game: Proteins Involved in Nonpolar Lipid Metabolism in Yeast

27

Karin Athenstaedt

Contents

1 Introduction 510
2 Enzymes Catalyzing Nonpolar Lipid Synthesis 511
2.1 Triacylglycerol Synthesis 511
2.2 Steryl Ester Synthesis 512
3 Mutations Affecting Lipid Droplet Formation 513
4 Nonpolar Lipid Degradation 514
4.1 Triacylglycerol Lipases 514
4.2 Degradation of Steryl Esters 516
5 Key Knowledge Gaps and Research Needs 517
References 520

Abstract

Synthesis, storage, and degradation of nonpolar lipids enable cells to continue cell metabolism when nutrients are no longer provided by the environment. Major nonpolar lipids occurring in yeast are triacylglycerols and steryl esters. These hydrophobic molecules are sequestered from the cytosolic environment in the core of special organelles termed lipid droplets (lipid particles). When nutrients are no longer provided by the environment, hydrolytic enzymes catalyze the degradation of triacylglycerols and steryl esters. The respective breakdown products serve as energy source and/or building blocks for membrane formation. Here, enzymes catalyzing nonpolar lipid synthesis and degradation in the budding yeast *Saccharomyces cerevisiae* are described with special emphasis to their localization and regulation. Furthermore, examples are presented showing that the formation of lipid droplets is not only disturbed in cells defective in polypeptides directly involved in nonpolar lipid synthesis and degradation, but also in

K. Athenstaedt (✉)
Institute of Molecular Biosciences, University of Graz, Graz, Austria
e-mail: karin.athenstaedt@uni-graz.at

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_31

a number of mutants defective in polypeptides and pathways which are not obviously linked to nonpolar lipid turnover. Although research over the past decade provided major insights into nonpolar lipid metabolism, many aspects of nonpolar lipid synthesis, storage, and degradation remain to be elucidated. Research needs for a better understanding of nonpolar lipid turnover are outlined at the end of this chapter.

1 Introduction

A detailed knowledge about enzymes catalyzing the formation and degradation of nonpolar lipids, the regulation of these enzymes, as well as the correlation of different pathways with nonpolar lipid metabolism is required for biomedical purposes as well as studies in the field of biotechnology. Besides a general understanding of nonpolar lipid metabolism, investigations in nonpolar lipid turnover are aimed at finding targets for altering the accumulation of storage molecules. In humans excessive accumulation of nonpolar lipids is related to several severe diseases such as arteriosclerosis, obesity, and diabetes type II. Thus, scientists are seeking for means how to avoid detrimental accumulation of storage molecules. In contrast, genetic manipulations resulting in high amounts of triacylglycerols (TAG) are in focus of biotechnological research, because in industry microorganisms such as the oleaginous yeast *Yarrowia lipolytica* are used for, e.g., single cell oil production and production of nutrients enriched in essential fatty acids. In the budding yeast *Saccharomyces cerevisiae* as well as in other eukaryotes, TAG and steryl esters (SE) constitute the major portion of nonpolar lipids. Investigations with the model organism yeast identified numerous polypeptides involved in nonpolar lipid metabolism. For many of these proteins, counterparts in other microorganisms as well as in higher eukaryotes have been identified, thus indicating that lipid metabolism is well conserved across the different kingdoms of life.

In *Saccharomyces cerevisiae* four enzymes catalyze the formation of SE and TAG. As substantial amounts of nonpolar lipids cannot be incorporated into the phospholipid bilayer, these hydrophobic molecules are sequestered in so-called lipid droplets, cell compartments destined for storage of these compounds. The formation of lipid droplets is not only disturbed in mutants defective in enzymes catalyzing nonpolar lipid synthesis and degradation, but also in strains blocked in other pathways which are not obviously connected to lipid metabolism. In yeast six hydrolytic enzymes have been identified which catalyze the degradation of TAG and SE. The breakdown products of nonpolar lipid degradation are sterols, diacylglycerol, and fatty acids which serve as building blocks for membrane formation and/or for energy production. For general information about nonpolar lipid synthesis, storage, and degradation, the reader is referred to ▶ Chap. 19, "Nonpolar Lipids in Yeast: Synthesis, Storage, and Degradation" of this volume and some recent reviews covering specifically nonpolar lipid metabolism in yeast (Athenstaedt and Daum 2011; Koch et al. 2014a; Wang 2015).

2 Enzymes Catalyzing Nonpolar Lipid Synthesis

2.1 Triacylglycerol Synthesis

Yeast synthesizes TAG via an acyl-CoA-dependent as well as an acyl-CoA-independent mechanism (▶ Chap. 19, "Nonpolar Lipids in Yeast: Synthesis, Storage, and Degradation"). The first TAG synthase identified in *Saccharomyces cerevisiae* has been Lro1p, which catalyzes TAG synthesis independent of acyl-CoA (Dahlqvist et al. 2000; Oelkers et al. 2000). Lro1p has been identified due to its homology to the mammalian lecithin:cholesterol acyltransferase (LCAT). Like its mammalian homolog, Lro1p uses glycerophospholipids as acyl donors, but transfers the acyl chain to the *sn*-3 position of diacylglycerol instead. Analysis of the substrate specificity showed that Lro1p exhibits a preference for unsaturated fatty acids which are bound to the *sn*-2 position of phosphatidylethanolamine and phosphatidylcholine (Ghosal et al. 2007). The transfer of the fatty acid from the glycerophospholipid to diacylglycerol occurs at the endoplasmic reticulum (ER), the site where Lro1p is located (Dahlqvist et al. 2000; Oelkers et al. 2000). A detailed study of the topology of Lro1p revealed the presence of a transmembrane domain at the N-terminus (Choudhary et al. 2011). This domain is most probably responsible for the localization of Lro1p to the ER, because a truncated version of Lro1p lacking the first 98 amino acids (including the transmembrane domain) fused to alpha-factor secretion signal is efficiently secreted into the medium upon heterologous expression in *Pichia pastoris* (Ghosal et al. 2007). The small part of Lro1p which is N-terminal from the transmembrane domain faces the cytosol, whereas the bulk of this enzyme including its active site residues is oriented toward the luminal side of the ER (Choudhary et al. 2011). This latter part contains also several glycosylation sites. Interestingly, deglycosylation significantly reduces the enzymatic activity of Lro1p, but Lro1p lacking its N-terminus is highly active (Ghosal et al. 2007), showing that the N-terminus of Lro1p is only required for membrane localization, but not for catalysis. Lro1p is the only acyl-CoA-independent TAG synthase of *Saccharomyces cerevisiae*, because in a strain lacking *LRO1* TAG are exclusively formed via the acyl-CoA-dependent pathway.

The major acyl-CoA-dependent TAG synthase of *Saccharomyces cerevisiae* is Dga1p. Enzymatic measurements revealed a 70- to 90-fold enrichment of acyl-CoA-dependent TAG synthase activity in lipid droplets over homogenate which is ascribed to Dga1p (Sorger and Daum 2002). However, Dga1p is also present in the ER (Oelkers et al. 2002; Sorger and Daum 2002). Similar to Lro1p, a transmembrane domain is predicted at the N-terminus of Dga1p. However, whereas the active site of Lro1p is oriented to the luminal side of the ER, that of Dga1p is most likely exposed to the cytosol (Ståhl et al. 2004). These differences in orientation may lead to different TAG pools being physically separated and having different physiological functions. Moreover, Lro1p and Dga1p are active at different growth stages of the cell. Whereas Lro1p is the main contributor to TAG synthesis in cells of the exponential growth phase, the latter enzyme is highly active in cells of the stationary phase (Oelkers et al. 2002).

Overexpression of *DGA1* and enhanced leucine biosynthesis in a *snf2*Δ mutant background, but not in wild type, significantly increases TAG accumulation (Kamisaka et al. 2007). As the protein levels of Dga1p are similar in both respective mutants, it is hypothesized that posttranslational modifications of Dga1p account for the higher amount of TAG in the *snf2*Δ mutant overexpressing *DGA1*. By contrast, overexpression of *LRO1* in *snf2*Δ cells reduces TAG accumulation. Snf2p is a transcription factor forming a part of the SWI/SNF (switching/sucrose non-fermenting) chromatin remodeling complex. Because *SNF2* disruption markedly decreases the expression of genes encoding the phospholipid-biosynthetic enzymes regulated by inositol and choline (Kodaki et al. 1995), a defect in Snf2p may significantly reduce the amount of glycerophospholipids which serve as a substrate for Lro1p. Alternatively, Lro1p may compensate the defect in phospholipid synthesis by catalyzing the reverse reaction, namely, transferring an acyl chain from TAG to a lyso-phospholipid. A similar link between Lro1p activity, glycerophospholipid synthesis, and the cellular TAG level was observed in *eki1*Δ*cki1*Δ*dpl1*Δ (Horvath et al. 2011). In this mutant the formation of phosphatidylethanolamine via the Kennedy pathway is blocked, which markedly reduces the relative amount of this lipid in the ER. As Lro1p resides in the ER and uses preferentially phosphatidylethanolamine as a substrate, it seems that an insufficient supply of Lro1p with phosphatidylethanolamine reduces its enzymatic activity and consequently decreases the TAG level in the *eki1*Δ*cki1*Δ*dpl1*Δ mutant.

In wild-type cells, Lro1p and Dga1p together account for 90% of TAG synthase activity (Oelkers et al. 2002; Sandager et al. 2002). Are1p and Are2p, two acyl-CoA: cholesterol acyltransferase (ACAT)-related enzymes (see below), have been shown to be responsible for the residual TAG synthase activity in *lro1*Δ*dga1*Δ cells. Like Dga1p, both Are1p and Are2p are catalyzing TAG synthesis via the acyl-CoA-dependent mechanism. However, the preferred substrates of Are1p and Are2p are sterols; thus these two enzymes are referred as acyl-CoA-dependent SE synthases.

2.2 Steryl Ester Synthesis

In contrast to higher eukaryotic organisms, *Saccharomyces cerevisiae* catalyzes the esterification of sterols only in the presence of acyl-CoA. In the budding yeast, Are1p and Are2p, two acyl-CoA:cholesterol acyltransferase (ACAT)-related enzymes, catalyze the formation of SE (Yang et al. 1996; Yu et al. 1996). As SE cannot be detected in a mutant deleted of both *ARE1* and *ARE2*, the respective gene products are the only SE synthases of yeast. Both Are1p and Are2p are localized to the ER (Zweytick et al. 2000). Different substrate specificities may account for the redundancy of SE synthesizing enzymes in the ER. Whereas Are1p has a pronounced preference for sterol precursors, ergosterol is the main substrate of Are2p. A defect in *ARE2* is reflected by the reduction of the total amount of SE to 26% of control (Yang et al. 1996; Yu et al. 1996). In contrast, in an *are1*Δ mutant, the cellular SE level remains nearly unaffected. This is not surprising having in mind that ergosterol is the most abundant sterol in wild-type cells. The higher contribution

of Are2p to SE formation is paralleled by a significantly higher transcription activity through the *ARE2* promoter as well as a 12 times higher half-life of the respective mRNA compared to *ARE1* (Jensen-Pergakes et al. 2001). Under anaerobic conditions, however, the major portion of SE is formed by Are1p. This observation can be explained by the fact that ergosterol biosynthesis is an oxygen-consuming process, and limitation of oxygen leads to the accumulation of sterol precursors which are the preferred substrate of Are1p.

3 Mutations Affecting Lipid Droplet Formation

As outlined above, in the budding yeast *Saccharomyces cerevisiae*, four enzymes, namely, Lro1p, Dga1p, Are1p, and Are2p, contribute to the formation of TAG and SE. Nonpolar lipids synthesized by these polypeptides are sequestered from the cytosolic environment forming the hydrophobic core of cell compartments known as lipid droplets (lipid particles; ▶ Chap. 19, "Nonpolar Lipids in Yeast: Synthesis, Storage, and Degradation"). Under standard growth conditions, a quadruple mutant deleted of *LRO1*, *DGA1*, *ARE1*, and *ARE2* is viable but lacks lipid droplets. By contrast, triple mutant cells expressing only one of these four genes form lipid droplets, albeit with a significantly broader size distribution than wild type (Czabany et al. 2008). Interestingly, when oleic acid serves as a carbon source, the triple mutant *dga1*Δ*lro1*Δ*are1*Δ (Are2p active), but not the other triple mutants, is unable to form lipid droplets similar to the quadruple mutant (Connerth et al. 2010). This phenomenon is explained by competitive enzymatic inhibition of Are2p by unesterified oleate, which thus strongly compromises SE formation.

A defect in TAG catabolism which affects the appearance of lipid droplets is the lack of the major TAG lipase Tgl3p (see below). Deletion of *TGL3* augments the total amount of TAG threefold compared with control (Athenstaedt and Daum 2003). Upon staining these cells with the lipophilic fluorescent dye Nile red, increased fluorescence of lipid droplets can be observed (Fei et al. 2008).

However, not only defects in processes obviously linked to nonpolar lipid turnover cause alterations in lipid droplet formation, but also defects in several other metabolic pathways. For instance, Henry and coworkers (Gaspar et al. 2008) provided evidence that a block in the secretory pathway leads to lipid droplet proliferation due to increased TAG formation. The link between the secretory pathway and TAG synthesis is phosphatidic acid (PA), the key intermediate for the formation of all glycerolipids. Dephosphorylation of PA leads to diacylglycerol, the direct precursor of TAG synthesis. Activation of PA by CTP, on the other hand, yields CDP-diacylglycerol, the precursor for the formation of all glycerophospholipids. In cells defective in secretion, membrane material (glycerophospholipids) in the form of vesicles cannot exit the ER and may thus cause secretory stress to the ER. By favoring dephosphorylation of PA over activation with CTP, further conversion of PA is rerouted from glycerophospholipid synthesis toward TAG formation, thus providing a degree of protection to cells during secretory stress.

Another mutation which strongly affects the formation of lipid droplets is the lack of a protein termed Fld1p (few lipid droplets; Fei et al. 2008). This polypeptide is the yeast homolog of the human lipodystrophy protein seipin (Wang et al. 2014). In cells lacking *FLD1*, the appearance of lipid droplets is quite diverse. Some cells form supersized lipid droplets, whereas in other cells small lipid droplet clusters are observed which are entangled with the ER (Szymanski et al. 2007; Fei et al. 2008). In line with a function in lipid droplet biogenesis, Fld1p localizes to the ER/lipid droplet interface where it occurs in a complex with Ldb16p (Wang et al. 2014; Wolinski et al. 2015). Because of this latter fact, it is not surprising that cells lacking *LDB16* similarly form lipid droplets with aberrant morphology. In addition, both *fld1*Δ and *ldb16*Δ contain not only lipid droplets in the cytosol, but also in the nucleus (Wolinski et al. 2015). This observation points to major disturbance in the lipid droplet budding process, which normally occurs exclusively toward the cytosol.

Two additional proteins which are similarly important for proper budding of lipid droplets from the ER are Scs3p and Yft2p (Choudhary et al. 2015). These two polypeptides are homologs of mammalian fat storage-inducing transmembrane (FIT) proteins. A defect in Scs3p and/or Yft2p affects the morphology of lipid droplets in that several lipid droplets are wrapped by an additional membrane which is part of the ER (Choudhary et al. 2015).

These few examples already demonstrate that proper formation of lipid droplets is a complex process influenced by numerous factors which are often not obviously linked to nonpolar lipid turnover.

4 Nonpolar Lipid Degradation

Degradation of TAG and SE is catalyzed by hydrolytic enzymes, namely, TAG lipases and SE hydrolases. The breakdown products formed by these catabolic processes are sterols, diacylglycerol, and unesterified fatty acids. Whereas sterols can be directly integrated into membrane bilayers, the latter two molecules serve as building blocks for membrane lipid formation. Alternatively, β-oxidation of fatty acids supplies the cell with energy.

4.1 Triacylglycerol Lipases

In *Saccharomyces cerevisiae* TAG degradation is accomplished by three TAG lipases, namely, Tgl3p, Tgl4p, and Tgl5p (Athenstaedt and Daum 2003, 2005; Kurat et al. 2006). All three TAG lipases are located to lipid droplets, thus close to their substrate. Whereas in comparison to wild-type cells a defect in *TGL3* is reflected by a threefold increased TAG level, TAG accumulation is only moderately increased in *tgl4*Δ cells and not changed at all in *tgl5*Δ cells. This can be explained by different substrate preferences of Tgl3p, Tgl4p, and Tgl5p. Tgl3p is a rather unspecific TAG lipase regarding the fatty acids cleaved off of TAG. Tgl4p, on the

other hand, prefers myristic (C14:0) and palmitic (C16:0) acid, and Tgl5p exhibits a preference for hexacosanoic (C26:0) acid. Tgl3p has been the first TAG lipase identified of a lipase protein family which contains polypeptides with the GXSXG motif characteristic for lipolytic enzymes but lacking additional homologies to other previously known lipases. By now this protein family not only comprises Tgl3p, Tgl4p, and Tgl5p of yeast, but also TAG lipases of mammals, insects, and plants (reviewed in Athenstaedt and Daum 2006). However, the catabolic activity of Tgl3p and Tgl4p is not restricted to TAG degradation. To a minor extent Tgl3p catalyzes similarly degradation of diacylglycerol (Kurat et al. 2006), and Tgl4p exhibits lipolytic activity toward diacylglycerol, SE, and phospholipids (Rajakumari and Daum 2010a; Kurat et al. 2006). Interestingly, all three TAG lipases contain besides the GXSXG motif also a HX_4D motif which is characteristic for acyltransferases (Rajakumari and Daum 2010a, b). In vitro studies revealed that indeed Tgl3p, Tgl4p, and Tgl5p act also as lyso-phospholipid acyltransferases. Whereas Tgl3p prefers lyso-phosphatidylethanolamine as a substrate, Tgl4p and Tgl5p catalyze preferentially the conversion of lysophosphatidic acid to phosphatidic acid.

As mentioned above all three TAG lipases localize to lipid droplets (Athenstaedt and Daum 2003, 2005; Kurat et al. 2006). However, minor amounts of Tgl3p and Tgl5p were also detected in the ER (Schmidt et al. 2013; Koch et al. 2014b; Klein et al. 2016). In a mutant lacking lipid droplets, i.e., the quadruple mutant *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ, all three TAG lipases are retained in the ER which reflects the close relationship of these two compartments (Schmidt et al. 2013; Klein et al. 2016). Interestingly, Tgl3p and Tgl5p are very stable in lipid droplets, but when located to the ER, their protein stability is strongly compromised. In contrast, Tgl4p associated with wild-type lipid droplets is highly instable, but in the absence of nonpolar lipids when the TAG lipase is retained in the ER, Tgl4p gains stability (Klein et al. 2016). The reason for the different stability of the TAG lipases located to either compartment seems to be a difference in the protein topology, at least for Tgl3p. Koch et al. (2014b) unveiled that the orientation of the C-terminus of Tgl3p is critical for protein stability. In Tgl3p located to a lipid droplet, this part of the TAG lipase protrudes into the interior of the droplet, which protects the polypeptide from degradation. In contrast, in Tgl3p residing at the ER, the C-terminal part is exposed to the cytosol, and the protein becomes readily degraded. Besides the protein stability, differences in the intracellular localization affect also the enzymatic activities of the Tgl proteins. In the absence of nonpolar lipids (lipid droplets), Tgl3p loses both its lipolytic activity and its activity as a lyso-phospholipid acyltransferase (Schmidt et al. 2013). Similarly, Tgl4p and Tgl5p exhibit no TAG lipase activity in cells lacking TAG and SE (Klein et al. 2016). However, in marked contrast to Tgl3p, Tgl4p and Tgl5p retain still their side activity as lyso-phospholipid acyltransferases. Analysis for a potential cross talk between the Tgl proteins revealed that the absence of two Tgl proteins does not affect the behavior of the remaining TAG lipase (Klein et al. 2016). Thus, it seems that all three enzymes act as separate entities.

As TAG lipases localize to lipid droplets, which is close to their substrate, a mechanism for regulating the activity of TAG lipases is required to avoid unspecific TAG degradation. Kurat et al. (2009) demonstrated that Tgl4p is a substrate of

cyclin-dependent kinase Cdk1p, thus linking lipolysis to cell cycle progression. Most interestingly, the phosphatidate phosphatase Pah1p which catalyzes the degradation of PA yielding diacylglycerol, the direct precursor of TAG, is also controlled by Cdk1p, however, in the opposite way (O'Hara et al. 2006). Whereas phosphorylation of Tgl4p through Cdk1p activates the lipase, phosphorylation of Pah1p renders this protein inactive resulting in a higher amount of PA which is channeled toward glycerophospholipid synthesis. Thus, simultaneous phosphorylation of Tgl4p and Pah1p contributes to cell proliferation.

Although under standard growth conditions TAG degradation is accomplished by Tgl3p, Tgl4p, and Tgl5p, additional polypeptides contribute to TAG hydrolysis in cells grown on oleic acid-containing media. These additional TAG lipases comprise Ayr1p and Ldh1p, which are both located to lipid droplets, as well as Lpx1p, a peroxisomal protein (Ploier et al. 2014). Furthermore, in vitro also the SE hydrolase Tgl1p (see below) and mitochondrial Tgl2p showed minor lipolytic activity with TAG as a substrate (Jandrositz et al. 2005; Ham et al. 2010).

The primary products of TAG degradation are diacylglycerol and a fatty acid which can be either channeled into anabolic pathways or further degraded. The only enzymes identified so far which exhibit at least minor lipolytic activity toward diacylglycerol are Tgl3p and Tgl4p (see above; Kurat et al. 2006). Monoacylglycerol, the degradation product of the former reaction, is finally hydrolyzed to glycerol and a fatty acid. This latter reaction is catalyzed either by the monoacylglycerol lipase Yju3p, which is localized to lipid droplets (Heier et al. 2010), or Mgl2p (Selvaraju et al. 2016).

4.2 Degradation of Steryl Esters

Similar to TAG degradation, three enzymes catalyze the deacylation of SE, namely, Tgl1p, Yeh1p, and Yeh2p (Jandrositz et al. 2005; Köffel et al. 2005; Müllner et al. 2005). Whereas Tgl1p and Yeh1p are located to lipid droplets, Yeh2p has been detected in the plasma membrane. Analysis of SE in a *yeh2*Δ mutant revealed a slightly higher level of zymosterol, thus indicating a preference of Yeh2p for this sterol precursor (Müllner et al. 2005). In yeast, anaerobic conditions can be mimicked by heme deficiency. Investigations of mutants deleted of two out of the three genes encoding Tgl1p, Yeh1p, and Yeh2p and the triple deletion mutant *tgl1*Δ*yeh1*Δ*yeh2*Δ in a heme-deficient background indicated that Yeh1p is the major enzyme catalyzing SE degradation under anaerobic conditions (Köffel and Schneiter 2006).

A SE hydrolase which exclusively hydrolyzes acetylated sterols is Say1p (Tiwari et al. 2007). This enzyme is part of a quality control cycle which allows the secretion of unusual sterols which would otherwise exhibit toxic effects. In this cycle the acetyltransferase Atf2p conducts the formation of acetylated sterols which are subsequently cleaved by Say1p if accepted as substrate. Those sterols which get deacetylated are retained in the cell, whereas acetylated sterols which are not

substrate of Say1p are secreted. Like Tgl1p and Yeh1p, Say1p localizes to lipid droplets, but is also found in the ER (Table 1).

5 Key Knowledge Gaps and Research Needs

Although to date major players in nonpolar lipid turnover in yeast are identified, the answers to several questions related to nonpolar lipid synthesis, storage, and degradation are still missing. As outlined above, enzymes catalyzing nonpolar lipid synthesis and degradation occur in redundancy. This leads to the question why at least two enzymes exist which are catalyzing essentially the same reaction. One simple explanation is that isoenzymes allow ongoing turnover even if one of these polypeptides is defective. However, this seems to be only half of the truth. It is hypothesized that each isoenzyme has also a specific function which makes it unique. As an example, whereas the TAG mobilization defect of *tgl3*Δ cells can at least to some extent be compensated by the additional TAG lipases Tgl4p and Tgl5p, the homologs of Tgl3p are unable to reverse the sporulation defect of a homozygous diploid *tgl3*Δ/*tgl3*Δ mutant (Athenstaedt and Daum 2003). This demonstrates the indispensable role of Tgl3p in spore formation. For several isoenzymes involved in nonpolar lipid turnover, a unique function remains to be elucidated. This will not only significantly contribute to our current understanding of nonpolar lipid metabolism, but furthermore reveal some "novel" relationships of nonpolar lipid synthesis, storage, and degradation with other cell metabolic functions.

One approach which has a high potential to answer the question concerning the existence of enzymes with overlapping function is studying regulatory aspects of nonpolar lipid metabolism. Furthermore, by investigating regulation at the gene level as well as the protein level, some unexpected links between nonpolar lipid turnover and other cell metabolic processes may be indicated. As an example, a link between nonpolar lipid degradation, glycerophospholipid synthesis, and cell cycle progression has been observed by studying the regulation of the TAG lipase Tgl4p (see above). As currently information about regulatory aspects is rather limited, one major aim of future investigations has to be the elucidation of these aspects.

As outlined in detail above, all enzymes catalyzing nonpolar lipid synthesis are localized to the ER. The major acyl-CoA-dependent TAG synthase Dga1p, however, is also present on lipid droplets. The dual localization of Dga1p leads to several questions. One question concerns the mechanism by which Dga1p reaches the lipid droplet surface. Is it by a budding process as suggested by the budding model for lipid droplet biogenesis (▶ Chap. 19, "Nonpolar Lipids in Yeast: Synthesis, Storage, and Degradation")? Why is it Dga1p, which is dually localized, and not one of the other nonpolar lipid synthesizing enzymes? In vitro measurements revealed that Dga1p is active in both ER and lipid droplets. However, to date we can only speculate whether this is also true in vivo. An active Dga1p on lipid droplets may be required to compensate random degradation of TAG which may occur because all TAG lipases are equally localized to the droplet. In a mutant deleted of the major TAG lipase Tgl3p, the amount of TAG is significantly increased suggesting that

Table 1 Polypeptides involved in nonpolar lipid metabolism in the budding yeast *Saccharomyces cerevisiae*

Involved in	Gene product	Main molecular function	Substrate	Product	Regulation/ modification	Localization
Synthesis	Lro1p	Phospholipid:diacylglycerol acyltransferase	Phospholipids, diacylglycerol	TAG	Glycosylation	ER
	Dga1p	Acyl-CoA:diacylglycerol acyltransferase	Acyl-CoA, diacylglycerol	TAG		ER, LD
	Are1p	Acyl-CoA:sterol acyltransferase	Acyl-CoA, sterols (diacylglycerol)	SE (TAG)	Induced upon anaerobic conditions	ER
	Are2p	Acyl-CoA:sterol acyltransferase	Acyl-CoA, sterols (diacylglycerol)	SE (TAG)		ER
Degradation	Tgl3p	TAG lipase (lyso-phospholipid acyltransferase, diacylglycerol lipase)	TAG (diacylglycerol)	Diacylglycerol, fatty acid		LD, ER
	Tgl4p	TAG lipase (SE hydrolase, phospholipase; lyso-phospholipid acyltransferase, diacylglycerol lipase)	TAG (SE, phospholipids, diacylglycerol)	Diacylglycerol, fatty acid (MAG)	Phosphorylation	LD
	Tgl5p	TAG lipase (lyso-phospholipid acyltransferase)	TAG	Diacylglycerol, fatty acid (sterol, lyso-phospholipid, MAG)		LD, ER
	Tgl2p	TAG lipase	TAG	Diacylglycerol, fatty acid		MT

	Ayr1p	TAG lipase; 1-acyl-dihydroxyacetone phosphate reductase	TAG	Diacylglycerol, fatty acid	Oleic acid medium	LD
	Ldh1p	Esterase (lipase)	TAG	Diacylglycerol, fatty acid	Oleic acid medium	LD, MT, PX
	Lpx1p	Esterase (lipase)	Esters	Alcohol, fatty acid	Oleic acid medium	PX
	Yju3p	MAG lipase	MAG	Glycerol, fatty acid		LD
	Mgl2p	MAG lipase	MAG	Glycerol, fatty acid		n.d.
	Tgl1p	SE hydrolase	SE (TAG)	Sterol, fatty acid (diacylglycerol)		LD
	Yeh1p	SE hydrolase	SE	Sterol, fatty acid	Induced upon anaerobic conditions	LD
	Yeh2p	SE hydrolase	SE	Sterol, fatty acid		PM
	Say1p	SE hydrolase	SE	Sterol, acetate		LD, ER

ER endoplasmic reticulum, *LD* lipid droplet, *MAG* monoacylglycerol, *MT* mitochondrium, *PM* plasma membrane, *PX* peroxisome, *SE* steryl ester, *TAG* triacylglycerol, *n.d.* not determined

Dga1p localized to lipid droplets is indeed active in vivo. This observation leads to several additional questions, e.g., is TAG formed by Dga1p the preferred substrate of Tgl3p? How is Tgl3p prevented from hydrolyzing just synthesized TAG? Or is there a concomitant synthesis and degradation of TAG? What would be the sense of such a permanent turnover?

Already those few questions/hypotheses mentioned above reveal that many aspects of nonpolar lipid metabolism are currently far from being understood and require further investigation. However, with our detailed knowledge about the features of single polypeptides governing nonpolar lipid metabolism, we are already well equipped to set the next steps: to elucidate the interplay of enzymes and organelles during nonpolar lipid turnover and to unravel the significance of nonpolar lipid synthesis, storage, and degradation for other cell metabolic processes.

Acknowledgment This work was financially supported by the Austrian Science Fund (FWF) project P26308 to K.A.

References

Athenstaedt K, Daum G (2003) *YMR313c/TGL3* encodes a novel triacylglycerol lipase located in lipid particles of *Saccharomyces cerevisiae*. J Biol Chem 278:23317–23323

Athenstaedt K, Daum G (2005) Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast *Saccharomyces cerevisiae* are localized to lipid particles. J Biol Chem 280:37301–37309

Athenstaedt K, Daum G (2006) The life cycle of neutral lipids: synthesis, storage and degradation. Cell Mol Life Sci 63:1355–1369

Athenstaedt K, Daum G (2011) Lipid storage: yeast we can! Eur J Lipid Sci Technol 113:1188–1197

Choudhary V, Jacquier N, Schneiter R (2011) The topology of the triacylglycerol synthesizing enzyme Lro1 indicates that neutral lipids can be produced within the luminal compartment of the endoplasmatic reticulum: implications for the biogenesis of lipid droplets. Commun Integr Biol 4:781–784

Choudhary V, Ojha N, Golden A, Prinz WA (2015) A conserved family of proteins facilitates nascent lipid droplet budding from the ER. J Cell Biol 211:261–271

Connerth M, Czabany T, Wagner A, Zellnig G, Leitner E, Steyrer E, Daum G (2010) Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast. J Biol Chem 285:26832–26841

Czabany T, Wagner A, Zweytick D, Lohner K, Leitner E, Ingolic E, Daum G (2008) Structural and biochemical properties of lipid particles from the yeast *Saccharomyces cerevisiae*. J Biol Chem 283:17065–17074

Dahlqvist A, Ståhl U, Lenman M, Banaś A, Lee M, Sandager L, Ronne H, Stymne S (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci U S A 97:6487–6492

Fei W, Alfaro G, Muthusamy B-P, Klaasen Z, Graham TR, Yang H, Beh CT (2008) Genome-wide analysis of sterol-lipid storage and trafficking in *Saccharomyces cerevisiae*. Eukaryot Cell 7:401–414

Gaspar ML, Jesch SA, Viswanatha R, Antosh AL, Brown WJ, Kohlwein SD, Henry SA (2008) A block in ER-to-Golgi trafficking inhibits phospholipid synthesis and induces neutral lipid accumulation. J Biol Chem 283:25735–25751

Ghosal A, Banaś A, Ståhl U, Dahlqvist A, Lindqvist Y, Stymne S (2007) *Saccharomyces cerevisiae* phospholipid:diacylglycerol acyl transferase (PDAT) devoid of its membrane anchor region is a

soluble and active enzyme retaining its substrate specificities. Biochim Biophys Acta 1771:1457–1463

Ham HJ, Rho HJ, Shin SK, Yoon HJ (2010) The *TGL2* gene of *Saccharomyces cerevisiae* encodes an active acylglycerol lipase located in the mitochondria. J Biol Chem 285:3005–3013

Heier C, Taschler U, Rengachari S, Oberer M, Wolinski H, Natter K, Kohlwein SD, Leber R, Zimmermann R (2010) Identification of Yju3p as functional orthologue of mammalian monoglyceride lipase in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1801:1063–1071

Horvath SE, Wagner A, Steyrer E, Daum G (2011) Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1811:1030–1037

Jandrositz A, Petschnigg J, Zimmermann R, Natter K, Scholze H, Hermetter A, Kohlwein SD, Leber R (2005) The lipid droplet enzyme Tgl1p hydrolyzes both steryl esters and triglycerides in the yeast, *Saccharomyces cerevisiae*. Biochim Biophys Acta 1735:50–58

Jensen-Pergakes K, Guo Z, Giattina M, Sturley SL, Bard M (2001) Transcriptional regulation of the two sterol esterification genes in the yeast *Saccharomyces cerevisiae*. J Bacteriol 183: 4950–4957

Kamisaka Y, Tomita N, Kimura K, Kainou K, Uemura H (2007) *DGA1* (diacylglycerol acyltransferase gene) overexpression and leucine biosynthesis significantly increase lipid accumulation in the Δ*snf2* disruptant of *Saccharomyces cerevisiae*. Biochem J 408:61–68

Klein I, Klug L, Schmidt C, Zandl M, Korber M, Daum G, Athenstaedt K (2016) Regulation of the yeast triacylglycerol lipases Tgl4p and Tgl5p by the presence/absence of nonpolar lipids. Mol Biol Cell 27:2014–2024

Koch B, Schmidt C, Daum G (2014a) Storage lipids of yeast: a survey of nonpolar lipid metabolism in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*. FEMS Microbiol Rev 38:892–915

Koch B, Schmidt C, Ploier B, Daum G (2014b) Modifications of the C terminus affect functionality and stability of yeast triacylglycerol lipase Tgl3p. J Biol Chem 289:19306–19316

Kodaki T, Hosaka K, Nikiwa J, Yamashita S (1995) The *SNF2*/*SWI2*/*GAM1*/*TYE3*/*RIC1* gene is involved in the coordinate regulation of phospholipid synthesis in *Saccharomyces cerevisiae*. J Biochem (Tokyo) 117:362–368

Köffel R, Schneiter R (2006) Yeh1 constitutes the major steryl ester hydrolase under heme-deficient conditions in *Saccharomyces cerevisiae*. Eukaryot Cell 5:1018–1025

Köffel R, Tiwari R, Falquet L, Schneiter R (2005) The *Saccharomyces cerevisiae YLL012/YEH1*, *YLR020/YEH2*, and *TGL1* genes encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis. Mol Cell Biol 25:1655–1668

Kurat CF, Natter K, Petschnigg J, Wolinski H, Scheuringer K, Scholz H, Zimmermann R, Leber R, Zechner R, Kohlwein SD (2006) Obese yeast: triglyceride lipolysis is functionally conserved from mammals to yeast. J Biol Chem 281:491–500

Kurat CF, Wolinski H, Petschnigg J, Kaluarachchi S, Andrews B, Natter K, Kohlwein SD (2009) Cdk1/Cdc28-dependent activation of the major triacylglycerol lipase Tgl4 in yeast links lipolysis to cell-cycle progression. Mol Cell 33:53–63

Müllner H, Deutsch G, Leitner E, Ingolic E, Daum G (2005) *YEH2/YLR020c* encodes a novel steryl ester hydrolase of the yeast *Saccharomyces cerevisiae*. J Biol Chem 280:13321–13328

O'Hara L, Han GS, Peak-Chew S, Grimsey N, Carman GM, Siniossoglou S (2006) Control of phospholipid synthesis by phosphorylation of the yeast lipin Pah1p/Smp2p Mg^{2+}-dependent phosphatidate phosphatase. J Biol Chem 281:34537–34548

Oelkers P, Tinkelenberg A, Erdeniz N, Cromley D, Billheimer J, Sturley SL (2000) A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. J Biol Chem 275:15609–15612

Oelkers P, Cromley D, Padamsee M, Billheimer JT, Sturley SL (2002) The *DGA1* gene determines a second triglyceride synthetic pathway in yeast. J Biol Chem 277:8877–8881

Ploier B, Scharwey M, Koch B, Schmidt C, Schatte J, Rechberger G, Kollroser M, Hermetter A, Daum G (2014) Screening for hydrolytic enzymes reveals Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. J Biol Chem 288:36061–36072

Rajakumari S, Daum G (2010a) Multiple functions as lipase, steryl ester hydrolase, phospholipase, and acyltransferase of Tgl4p from the yeast *Saccharomyces cerevisiae*. J Biol Chem 285: 15769–15776

Rajakumari S, Daum G (2010b) Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Mol Biol Cell 21:501–510

Sandager L, Gustavsson MH, Ståhl U, Dahlqvist A, Wiberg E, Banaś A, Lenman M, Ronne H, Stymne S (2002) Storage lipid synthesis is non-essential in yeast. J Biol Chem 277:6478–6482

Schmidt C, Athenstaedt K, Koch B, Ploier B, Daum G (2013) Regulation of the yeast triacylglycerol lipase Tgl3p by formation of nonpolar lipids. J Biol Chem 288:19939–19948

Selvaraju K, Gowsalya R, Vijayakumar R, Nachiappan V (2016) *MGL2*/YMR210w encodes a monoacylglycerol lipase in *Saccharomyces cerevisiae*. FEBS Lett 590:1174–1186

Sorger D, Daum G (2002) Synthesis of triacylglycerols by the acyl-coenzyme A:diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. J Biol Chem 184:519–524

Ståhl U, Carlsson AS, Lenman M, Dahlqvist A, Wiberg E, Banaś W, Banaś A, Stymne S (2004) Cloning and characterization of a phospholipid:diacylglycerol acyltransferase from *Arabidopsis*. Plant Physiol 135:1324–1335

Szymanski KM, Binns D, Bartz R, Grishin NV, Li WP, Agarwal AK, Garg A, Anderson RG, Goodman JM (2007) The lipodystrophy protein seipin is found at endoplasmic reticulum lipid droplet junctions and is important for droplet morphology. Proc Natl Acad Sci U S A 104: 20890–20895

Tiwari R, Köffel R, Schneiter R (2007) An acetylation/deacetylation cycle controls the export of sterols and steroids from *S. cerevisiae*. EMBO J 26:5109–5119

Wang CW (2015) Lipid droplet dynamics in budding yeast. Cell Mol Life Sci 72:2677–2695

Wang CW, Miao YH, Chang YS (2014) Control of lipid droplet size in budding yeast requires the collaboration between Fld1 and Ldb16. J Cell Sci 127:1214–1228

Wolinski H, Hofbauer HF, Hellauer K, Cristobal-Sarramian A, Kolb D, Radulovic M, Knittelfelder OL, Rechberger GN, Kohlwein SD (2015) Seipin is involved in the regulation of phosphatidic acid metabolism at a subdomain of the nuclear envelope in yeast. Biochim Biophys Acta 1851:1450–1464

Yang H, Bard M, Bruner DA, Gleeson A, Deckelbaum RJ, Aljinovic G, Pohl TM, Rothstein R, Sturley SL (1996) Sterol esterification in yeast: a two-gene process. Science 272:1353–1356

Yu C, Kennedy NJ, Chang CCY, Rothblatt J (1996) Molecular cloning and characterization of two isoforms of *Saccharomyces cerevisiae* acyl-CoA:sterol acyltransferases. J Biol Chem 271:24157–24163

Zweytick D, Leitner E, Kohlwein SD, Yu C, Rothblatt J, Daum G (2000) Contribution of Are1p and Are2p to steryl ester synthesis in the yeast *Saccharomyces cerevisiae*. Eur J Biochem 267:1075–1082

Part III

Biochemistry of Biogenesis of Membranes

Membrane Lipid Biogenesis

28

Howard Goldfine

Contents

1 Introduction 526
2 Lipid Bilayer Formation 526
2.1 Requirements 526
2.2 The Liquid Crystalline Phase 528
2.3 The Formation of Non-bilayer Phases: Effects of Hydrocarbons, Solvents, and Other Small Molecules 528
3 Regulation of Membrane Lipid Composition 531
3.1 Hydrocarbon Chains 531
3.2 Polar Headgroups 532
4 Lipid Domains 534
5 Knowledge Gaps/Research Needs 535
References 535

Abstract

In order to maintain a fluid lipid bilayer in the cell membrane, microorganisms must adjust to environmental conditions including the ambient temperature, pressure, and the presence of solutes that affect the physical state of the membrane. Although the types of amphipathic lipids present in the cell membrane can vary widely between species, the variety of adjustments made, including changes in the compositions of the hydrocarbon chains and the polar headgroups, appear to obey certain rules. The regulation of lipid biosynthesis to adapt to the cellular environment will be discussed in this chapter.

H. Goldfine (✉)
Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA
e-mail: goldfinh@upenn.edu; goldfinh@mail.med.upenn.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_34

1 Introduction

Biological membranes are self-assembled structures largely composed of lipids and proteins. The cell membrane forms a semipermeable barrier and contains proteins involved in solute transport, the formation of chemical energy, the synthesis and translocation of macromolecules, and the synthesis of the membrane lipids. Microbes are no exception, and they show considerable diversity with respect to the types of lipids they contain. Of paramount importance for the formation of a stable, functional membrane is the ability of the ensemble of lipids to form a fluid bilayer within the growth temperature boundaries of the organism. In most cases the predominant polar lipids are phospholipids, but in some organisms, glycosyldiacylglycerols represent a significant fraction of the total membrane lipids (Fig. 1). In this chapter the general rules for fluid bilayer formation and maintenance will be considered as will the special requirements of organisms that produce apolar compounds.

2 Lipid Bilayer Formation

2.1 Requirements

Most bacterial membrane lipids are amphipathic or bipolar. They have relatively long hydrocarbon chains, predominantly from 14 to 20 carbons long, which are usually, but not always, linked to a glycerol backbone by means of an ester, ether, or alk-1-enyl ether bond. The hydrocarbon chains are hydrophobic, point toward the center of the bilayer, and form stable associations with adjacent chains through

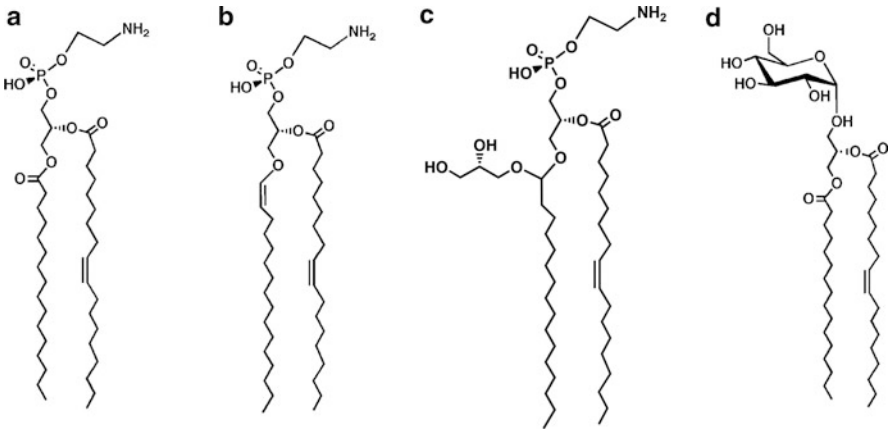


Fig. 1 Structures of some bacterial lipids. (**a**) phosphatidylethanolamine; (**b**) plasmalogen form of phosphatidylethanolamine; (**c**) glycerol acetal of the plasmalogen form of phosphatidylethanolamine; (**d**) glucosyldiacylglycerol an example of a monoglycosyldiacylglycerol (Courtesy of C.R. Raetz)

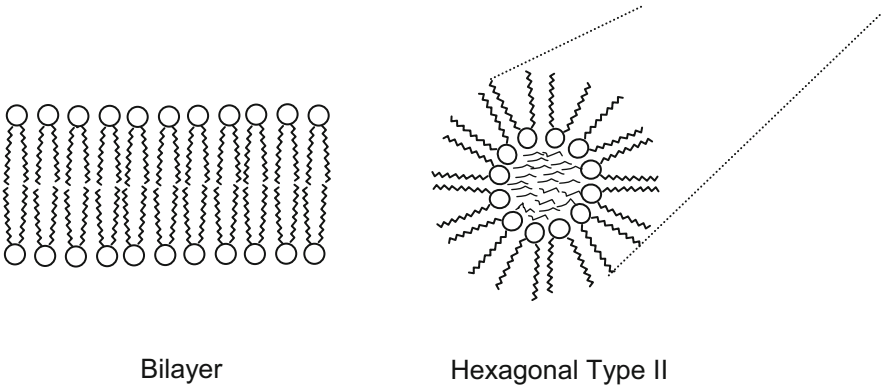


Fig. 2 A typical bilayer and an illustration of the tubelike structure of the hexagonal II phase in which the center is aqueous. These tubes are packed into hexagonal arrays in which the tips of the hydrocarbon chains are touching

hydrophobic, non-covalent interactions. At the *sn*-3 position of the glycerol backbone in *Bacteria* or the *sn*-1 position in *Archaea*, a polar, hydrophilic group is attached, and in the case of phospholipids, this group will consist of a phosphodiester to a short-chain primary alcohol such as ethanolamine, glycerol, or serine and in a subgroup of bacteria choline (Goldfine 1982; Geiger et al. 2013). In the bacteria that have glycolipids, these are mostly of the glycosyl diradyl[1] glycerol type (Fig. 1).

In order to form a bilayer, a significant proportion of the lipid molecules must be cylindrical in shape (Israelachvili et al. 1980). These lipids will spontaneously form bilayers with minimum curvature on the scale of a cell membrane. Among the polar lipids in bacteria that tend to form bilayers are phosphatidylglycerol (PG)[2], phosphatidylserine, and diglycosyl diradylglycerol (DGDG). In most bacterial membranes, there is a significant proportion of polar lipids that do not tend to form bilayers, but rather produce the inverted hexagonal (H_{II}) or other non-lamellar phases (Fig. 2). These lipids are conical in shape with relatively small polar headgroups and acyl chains that sweep out a wider conical volume than that occupied by the headgroup. Among these are phosphatidylethanolamine (PE), monoglycosyl diradylglycerols (MGDG), and cardiolipin in the presence of certain divalent metal ions (Cullis and De Kruijff 1979). These non-bilayer-preferring lipids are normally present with sufficient bilayer-forming lipids to maintain the bilayer arrangement.

[1]Diradyl refers to chains that may be linked through acyl ester bonds, alk-1-enyl ether, or saturated ether bonds.

[2]Abbreviations: DGDG, diglycosyldiacylglycerol; DGluDG, diglycosyldiacylglycerol; GAPlaE, glycerol acetal of the plasmalogen form of phosphatidylethanolamine; MGDG, monoglycosyldiacylglycerol; MGluDG, monoglucosyldiacylglycerol; MV, membrane vesicles; PE, phosphatidylethanolamine; PlaE, plasmalogen form of phosphatidylethanolamine; PG, phosphatidylglycerol

2.2 The Liquid Crystalline Phase

At their normal growth temperatures, biological membranes are said to be fluid or to be more precise, to exist in the lamellar liquid crystalline phase, termed *Lα*. In this state the hydrocarbon chains are disordered with rapid flexion all along the chains. The intact lipid molecules rotate in the plane of the bilayer and diffuse laterally. They rarely flip from one side of the bilayer to the other unless aided by proteins called flippases or phospholipid translocators. This state is essential for the normal function of membrane proteins embedded within the bilayer. If the ambient temperature cools, lipids in the bilayer will eventually undergo a transition to a gel phase (*Lβ*) in which the chains are packed in a stiff, near crystalline arrangement. Nature ensures the fluidity of bacterial membranes by incorporating a significant proportion of either unsaturated or branched hydrocarbon chains in the lipids. In general the former is true for gram-negative bacteria, while the latter is the case in gram-positive bacteria, but these rules do not hold universally. Unsaturation in hydrocarbon chains introduces a kink which decreases lateral close packing, thus lowering the temperature of transition to a gel state. The presence of methyl branches usually at the penultimate (*anteiso*) or the terminal carbon (*iso*) also deters close packing with a similar result. When bacteria are grown at lower temperatures, they usually increase either the content of unsaturated chains or the ratio of *anteiso* to *iso* chains.

2.3 The Formation of Non-bilayer Phases: Effects of Hydrocarbons, Solvents, and Other Small Molecules

As the ambient temperature is increased above the melting point, bilayers consisting of mixtures of polar lipids will eventually transition to other liquid crystalline states including the cubic phase *Qα* and the inverted hexagonal H_{II} phase. The temperature at which this occurs depends on the lipid polar headgroups and the compositions of the hydrocarbon chains. As noted above, smaller headgroups promote the formation of non-lamellar phases. Hydrogen bonding of the polar headgroups can also cause them to come close together, favoring the formation of a non-lamellar phase. In addition, for example, in the case of PE or MGDG, the presence of unsaturated hydrocarbon chains also increases the tendency to form non-lamellar phases (Lindblom and Rilfors 1989; Cullis and De Kruijff 1979). The lateral pressure exerted by these lipids in the bilayer leads to curvature stress. Near the lamellar/non-lamellar transition, “The bilayer leaflets are literally straining to bend” (Gruner and Shyamsunder 1991). Bacteria have adopted different strategies to avoid the disruption of the bilayer caused by formation of non-bilayer arrangements. For example, when the degree of unsaturation of the membrane lipids in *Acholeplasma laidlawii* is increased artificially, the ratio of diglucosyldiacylglycerol (DGlcDAG) to monoglucosyldiacylglycerol (MGlcDAG) increases. The ratios of other polar lipids are also changed, and the overall result is to decrease the tendency to form non-lamellar structures (Rilfors and Lindblom 2002; Wieslander et al. 1980). In

Clostridium butyricum a similar degree of enrichment with unsaturated hydrocarbon chains leads to an increase in the amount of a glycerol acetal of the plasmalogen form of PE (GAPlaE) and a decrease in diradyl PE (Fig. 1), again leading to stabilization of the bilayer (Goldfine et al. 1987). Studies with PE-deficient mutants of *E. coli* lead to a similar conclusion: these bacteria maintain an optimal ratio between lipids that prefer the bilayer structure and those that prefer the H_{II} phase (Rietveld et al. 1993). Furthermore, it has been shown with both *A. laidlawii* and *E. coli* that these cells appear to adjust the composition of their membrane lipids in order to maintain lamellar liquid crystalline phases, avoiding the formation of a gel phase and non-lamellar liquid crystalline phases. The cells are said to grow in a "window" between the boundaries of the gel and non-lamellar phases (Rilfors and Lindblom 2002). *C. butyricum* appears to regulate its lipid composition according to a similar plan (Goldfine et al. 1987).

Production of solvents or growth in the presence of solvents presents special problems for microorganisms that have these lifestyles. In *A. laidlawii* (Wieslander et al. 1986) and *C. butyricum* (MacDonald and Goldfine 1991), growth in the presence of solvents such as cyclohexane increases the proportions of the bilayer-preferring membrane lipids DGlcDAG and GAPlaE, respectively. Shorter-chain alcohols such as butanol have little effect. Shorter-chain alcohols are expected to expand the headgroup region thus stabilizing the lamellar phase (Weber and De Bont 1996; Janes 1996). Longer-chain alcohols, i.e., n-hexanol, will expand the hydrocarbon region in addition to the headgroup region and thus will stabilize the H_{II} over the lamellar state. This is counterbalanced by increasing the proportions of bilayer-preferring lipids in both organisms. In experimental systems in which alcohols are added to PE, the crossover point is between butanol and longer-chain alcohols (Weber and De Bont 1996), and similar crossover points were seen with *A. laidlawii* and *C. butyricum* (see references above).

Other adaptive changes are seen in microorganisms in response to the presence of alcohols and other solvents. These compounds increase the permeability of artificial lipid vesicles. Addition of ethanol to the growth medium of *E. coli* leads to an increase in the ratio of unsaturated to saturated acyl chains in the membrane lipids, whereas C6–C10 alcohols have the opposite effect. *Pseudomonas putida* grown in the presence of toluene undergoes an interesting adaptation in which the normal *cis*-unsaturated fatty acids are converted to the corresponding *trans*-fatty acids. This would have the effect of increasing lipid ordering and raising the temperature of the lamellar to H_{II} transition. Another effect seen was a decrease in the proportion of PE relative to the sum of PG and cardiolipin (CL), again resulting in an increase in the lamellar to H_{II} transition temperature (Weber and De Bont 1996).

There are several solvent-producing clostridia. Among these is *Clostridium beijerinckii* which has a lipid composition similar to that of *C. butyricum* with the exception of having phosphatidyl-*N*-methylethanolamine in addition to PE. It also has plasmalogens and glycerol acetals of the plasmalogen forms of these lipids (Goldfine and Johnston 2005) (Fig. 1). Its response to artificially increased unsaturated fatty acids in the membrane lipids is similar to that described above for

C. butyricum, i.e., an increase in the relative amounts of the glycerol acetals of the plasmalogens (Goldfine 1984).

The lipids of *C. beijerinckii* ATCC 39057, previously identified as *Clostridium acetobutylicum*, were studied during acetone/butanol production. As solvents accumulated in the culture fluid, the ratio of GAPlaE + GAPlaME/diradyl (PE + PME) increased and the saturated/unsaturated (SU) acyl chain ratio also increased. Similar changes were seen in the acyl chains along with a shortening of these chains upon addition of *n*-hexanol or *n*-octanol. Ethanol or butanol produced smaller changes in the composition of the acyl chains (Lepage et al. 1987). These changes in polar headgroups and acyl chains counteract the effects of longer-chain alcohols that would perturb the lamellar/non-lamellar equilibrium as described above. *C. acetobutylicum* has a high proportion of glycosyl diradylglycerols in addition to phosphoglycerolipids. It responds to increased membrane lipid unsaturated chains similarly to *C. beijerinckii* and *C. butyricum* by an increase in GAPlaE, but only at the highest level of unsaturated chains. Glycerol acetals of plasmalogens have been found in all of the solvent-producing clostridia examined to date, but not in other clostridia (Goldfine and Johnston 2005). All the solvent-producing clostridia have PlaE, which is more prone to undergo a lamellar to H_{II} transition than PE. The studies cited above suggest that glycerol acetals evolved to improve the ability of these organisms to withstand the stress of solvent formation.

Increased unsaturation of the lipid hydrocarbon chains of *C. acetobutylicum* produced the greatest effects on the glycolipids. The glycolipid composition undergoes changes similar to those seen in *A. laidlawii*, i.e., an increase in DGDRG relative to MGDRG. Another glycolipid modification in response to increased unsaturation of the membrane lipids or addition of a medium-chain alcohol is the addition of ethanolamine-P to the MGDRG (Tian et al. 2013). Although not proven, it is likely that this addition would decrease the non-bilayer tendency of MGDRG by increasing the size of the polar headgroup. It would also provide a charge in the polar headgroup region rather than an additional uncharged sugar.

Studies on the effects of butanol challenge and growth temperature on the membrane lipids and fluidity of *C. acetobutylicum* ATCC 824 and a butanol-tolerant mutant, SA-2, showed that addition of butanol to the growth medium also led to an increase in the ratio of saturated to unsaturated (plus cyclopropane) acyl chains in the membrane lipids (Baer et al. 1987; Vollherbst-Schneck et al. 1984). Shorter-chain alcohols in model membranes cause a shift to lower temperatures in the transition from the gel to the liquid crystalline phase. This is often referred to as an increase in membrane fluidity, which can be measured with various probes (Weber and De Bont 1996). The increase in the SU ratio seen in both *C. beijerinckii* and *C. acetobutylicum* upon butanol challenge would tend to counteract the effects of butanol on membrane fluidity. In summary, changes observed can be viewed as a response to the addition of solvents to the growth medium or to the production of solvents by bacteria that serves to maintain the cell membrane in an essential window ($L\alpha$) between the gel and non-lamellar phases.

3 Regulation of Membrane Lipid Composition

3.1 Hydrocarbon Chains

The two dominant mechanisms for adjustment of membrane fluidity involve the formation of *cis*-unsaturated fatty acids or the formation of terminally branched fatty acids. The latter mechanism mainly occurs in gram-positive bacteria. The central double bond in unsaturated fatty acids introduces a kink in the chain which reduces packing of the chains in the bilayer. As unsaturation of a given lipid increases, the temperature of transition from the gel ($L\beta$) phase to the lamellar liquid crystalline phase, termed $L\alpha$, decreases. Polyunsaturated fatty acids are not commonly found in bacteria. *Anteiso*-branched fatty acids with the branch on the penultimate carbon provide lower temperatures of transition to the $L\alpha$ liquid crystalline phase, than *iso*-branched fatty acids with the branch at the terminal carbon.

In contrast to higher organisms in which *cis*-unsaturated fatty acids are produced by direct desaturation of a long-chain acyl-CoA by an oxygen-dependent desaturase, bacteria mostly introduce the double bond during elongation of the fatty acid chain which does not require molecular oxygen (Goldfine and Bloch 1961; Scheuerbrandt et al. 1961). The branching of the saturated and unsaturated fatty acid synthesis pathways usually occurs at the dehydration of β-hydroxydecanoyl-ACP to form *trans*-2-decenoyl-ACP, which is in equilibrium with *cis*-3-decenoyl-ACP. Elongation of the latter produces the common bacterial unsaturated fatty acids, *cis*-9,10–16:1 and *cis*-11,12–18:1 (*cis*-vaccenic acid) (Fig. 3). FabF is temperature sensitive and its activation leads to increased synthesis of *cis*-vaccenic acid at lower growth temperatures (Cronan and Thomas 2009; Zhang and Rock 2008).

FabH from bacteria that produce mostly branched-chain fatty acids prefers branched-chain acyl-CoAs rather than acetyl-CoA in initiating the process of fatty acid synthesis (Zhang and Rock 2008). In *Listeria monocytogenes*, which is capable of growth at low temperatures, selectivity of FabH for precursors of *anteiso*-branched-chain fatty acids such as 2-methylbutyryl-CoA at lower growth temperatures (10 °C) determines the ratio of *anteiso*- to *iso*-branched fatty acids, thereby lowering the temperature of transition to the liquid crystalline phase (Singh et al. 2009; Zhu et al. 2005).

An unusual response of bacteria to solvents that destabilize the cell membrane is *cis-trans* isomerization of unsaturated fatty acids in *Pseudomonas putida*. An increase in the *trans*-unsaturated fatty acid content would lead to an increase in the gel to liquid crystalline phase transition temperature (Cronan and Gelmann 1975; Weber et al. 1994). The finding of a strain of *P. putida* that tolerated high concentrations of toluene (Inoue and Horikoshi 1989) led to the understanding of *cis-trans* isomerization as a mechanism for this adaptation (Weber and De Bont 1996; Weber et al. 1994; Holtwick et al. 1997; Halverson and Firestone 2000). The activity of a *cis-trans* isomerase, Cti, which is constitutively expressed, increases in response to temperature stress and the presence of solvents in the medium of *Pseudomonas syringae* (Kiran et al. 2005; Zhang and Rock 2008). The mechanism for increased activity is not known.

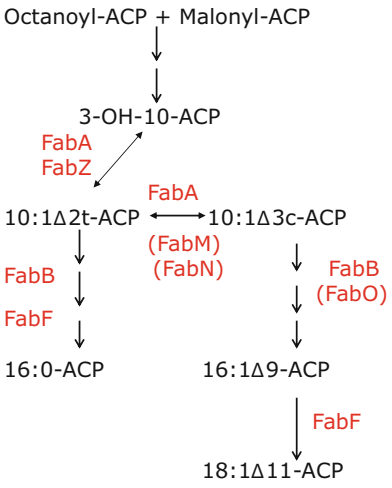


Fig. 3 Biosynthesis of unsaturated fatty acids in *E. coli*. The branched point at the level of 3-hydroxydecanoyl-ACP leads to the production of *trans*-2-decenoyl-ACP, which is isomerized to *cis*-3-decenoyl-ACP by FabA. This reaction is selective for the ten-carbon intermediate. FabB, but not FabF, catalyzes the elongation of *cis*-3-decenoyl-ACP. The elongation to 18:1-ACP is controlled by FabF, which is naturally temperature sensitive. The parentheses show enzymes from other bacteria that catalyze the same reactions shown (Adapted from Cronan and Thomas (2009))

3.2 Polar Headgroups

The biosynthesis of polar lipids in most bacteria begins with the acylation of *sn*-glycerol-3-P by acyl-P formed by the transfer of an acyl group from acyl-ACP to inorganic phosphate, which is catalyzed by PlsX. PlsY then catalyzes the acylation of glycerol-3-P to form lysophosphatidic acid (LPA). Phosphatidic acid (PA) is formed by the transfer of an acyl chain from acyl-ACP to LPA catalyzed by PlsC. With few exceptions, e.g., *E. coli*, bacteria do not use acyl-CoAs in the acylation of glycerol-3-P or LPA (Goldfine et al. 1967; Goldfine and Ailhaud 1971; Zhang and Rock 2008; Lueking and Goldfine 1975). In *E. coli,* PlsB catalyzes the transfer of an acyl group from either acyl-CoA or acyl-ACP to glycerol-3-P (Ailhaud and Vagelos 1966; Zhang and Rock 2008; Green et al. 1981). CDP-diacylglycerol (CDP-DAG) is formed from PA and CTP catalyzed by CDP- diacylglycerol synthase (CdsA) (Fig. 4). The next steps form a critical branch point in which either serine or glycerol-3-P is exchanged with CMP to form phosphatidylserine (PS) or phosphatidylglycerol-P (PG-P), respectively. The final steps leading to the formation of phosphatidylethanolamine (PE) or phosphatidylglycerol (PG) involve a decarboxylation of PS and removal of the terminal phosphate of PG-P, respectively. PG in turn can be converted to cardiolipin (CL) by one of three different mechanisms (Walton and Goldfine 1987; Tan et al. 2012).

In *E. coli,* the ratio of PE to PG plus CL is tightly regulated, which preserves the balance between PE, a zwitterionic non-bilayer lipid, and PG plus CL, negatively

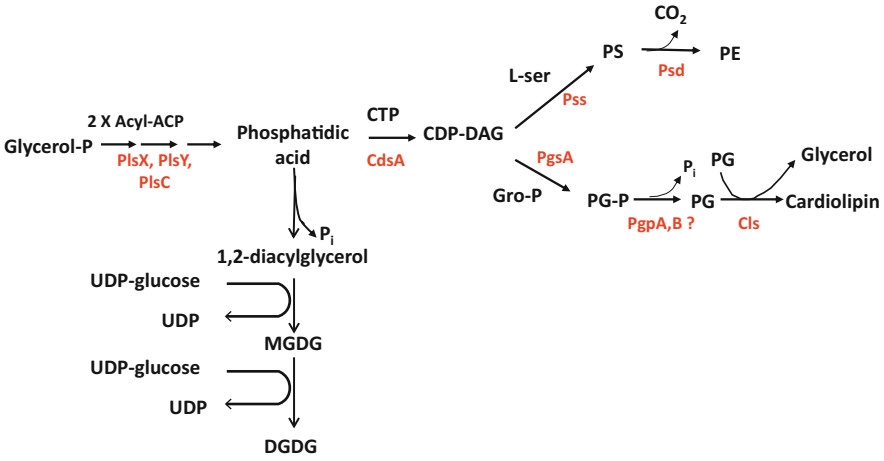


Fig. 4 Biosynthesis of polar lipids in bacteria. The pathway for phospholipid biosynthesis has been found in many bacteria as elucidated by examination of their genomes and by biochemical studies (Yao and Rock 2013; Zhang and Rock 2008; Dowhan 2013). The pathway for formation of the common glycolipids, MGluDG, and DGluDg has been found in many gram-positive bacteria (Edman et al. 2003)

charged bilayer lipids. As noted above, CL may tend toward formation of a non-lamellar phase in the presence of divalent cations. The genes involved appear to be overexpressed and further enhancement does not result in changes in the ratios of the major lipids. On the other hand, reduction in the production of PS synthase through dilution of the gene *pss* resulted in decreased PE production when the enzyme level was at approximately 25% of the wild-type level. Cellular PG could be similarly reduced by decreased expression of *pgsA* (Dowhan 2013).

It appears that the enzymes of phospholipid synthesis in *E. coli* are responsive to the relative amounts of zwitterionic and negatively charged lipids in the cell membrane (Rilfors et al. 1999; Linde et al. 2004). The activity of reconstituted PS synthase from *E. coli* in lipid vesicles containing the detergent octyl glucoside showed a strong dependence on the fraction and type of anionic phospholipid in the vesicles. PG, CL, and phosphatidic acid provided the strongest stimulation, but phosphatidylinositol, a lipid not found in *E. coli*, gave a lower activity (Rilfors et al. 1999). Thus, the synthesis of PE is expected to be enhanced when the relative amounts of anionic phospholipids exceeds the normal ratios.

Acholeplasma laidlawii is the best-studied example of a bacterium that regulates the content of its glycolipids in order to balance the relative amounts of bilayer and non-lipids in its cell membrane. The seminal work of Lindblom, Rilfors, Wieslander, and their colleagues proposed and solidified the concept that there is a required balance of these two types of lipids in biological membranes (Wieslander et al. 1980, 1981a, b, 1986). In this organism a major bilayer-forming lipid is diglucosyldiacylglycerol (DGluDG) and a major non-bilayer-forming lipid is monoglucosyldiacylglycerol (MGluDG). These are formed by successive additions of glucose from UDP-glucose,

initially to diacylglycerol (Fig. 4). As the growth temperature and degree of unsaturation of the cellular lipids were increased, which would favor the non-bilayer arrangement of MGluDG, the ratio of DGluDG to MGluDG increased.

Studies on the regulation of the responsible glycosyltransferases have shown that they sense the state of the membrane with regard to lipid curvature and charge. DGDG synthase, purified from *A. laidlawii*, is activated by both anionic and non-bilayer lipids. Among the latter are dioleyoylphosphatidylethanolamine, 1,3-dioleoylglycerol, and cholestenone in mixed micelles with the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). When both MGluDG and DGluDG synthases were reconstituted in mixed micelles, the relative amounts of MGluDG and DGluDG formed were strongly influenced by addition of the potent non-bilayer lipid cholestenone. As the mol % of cholestenone was increased, the ratio of DGluDG to MGluDG formed also increased, in accordance with the changes observed in response to membrane curvature stress in growing cells (Vikstrom et al. 1999). The general conclusion is that regulation of the ratios of bilayer vs. non-bilayer lipids occurs at the level of the lipid biosynthetic enzymes in the membrane, which respond to membrane curvature stress induced by variation in the relative amounts of these two classes of lipids. A mathematical treatment of this model has been presented in which the binding of the regulated enzyme through an amphipathic α-helix is dependent on the stored elastic energy in the membrane, thus providing a biophysical feedback mechanism (Alley et al. 2008).

An alternative mechanism for balancing the non-bilayer tendency of PE is to form phosphatidylcholine (PC), which by virtue of the larger size of its headgroup readily forms bilayers. In bacteria this can be done in two different ways, the phospholipid *N*-methylation pathway or the PC synthase pathway. In the former PE is methylated sequentially by an *N*-methyltransferase which uses *S*-adenosylmethionine as methyl donor. Some bacteria have a single *N*-methyltransferase, which catalyzes all three methylations, but other bacteria have a second *N*-methyltransferase, which catalyzes the second and third methylation. In the PC synthase pathway, choline is condensed with CDP-diacylglycerol to form PC and CMP (Geiger et al. 2013).

PC is found in many bacteria that have high contents of unsaturated fatty acids, and it was proposed that the formation of PC would compensate for the non-bilayer property of unsaturated PE (Goldfine 1984). In mutants lacking PC, there is necessarily an increase in the relative amounts of PE. These PC-deficient mutants are often severely affected in their ability to grow at elevated temperatures, presumably as a result of the increased tendency of PE to form the non-lamellar H_{II} phase at higher temperatures (Geiger et al. 2013).

4 Lipid Domains

Lipid rafts or lipid microdomains are thought to exist in eukaryotic cells where they result from the segregation of cholesterol and sphingomyelin into discrete patches of membrane. Bacteria do not have cholesterol and most do not have sphingomyelin, though sterol-like, pentacyclic triterpenoids based on the hopane structure are found

in many bacteria. Evidence for cardiolipin domains at the septal regions and at the poles in both *Bacillus subtilis* and *E. coli* based on specific staining with the fluorescent dye 10-N-nonyl-acridine orange (NAO) has been presented (Kawai et al. 2004; Mileykovskaya and Dowhan 2000). However, the specificity of NAO for CL has been questioned since it may also recognize other anionic phospholipids such as PG (Oliver et al. 2016). PE has also been found to segregate at the septal membranes of *B. subtilis*, but not in *E. coli*, in which it is the major lipid (Nishibori et al. 2005). Based on green fluorescent protein fusions to the enzymes needed for phospholipid synthesis in *B. subtilis*, these authors have suggested that lipid synthesis is mostly localized in the septal membranes. Some localization of anionic phospholipids results from specific interactions with proteins. Lin and Weibel have recently summarized bacterial processes regulated by interactions of membrane proteins with anionic phospholipids. These include ATP synthesis, DNA replication, DNA repair, protein translocation, and others (Lin and Weibel 2016). Some macromolecules may sort to regions of either negative (i.e. concave) or positive (i.e. convex) membrane curvature (Huang and Ramamurthi, 2010) resulting from the presence of specific lipids.

5 Knowledge Gaps/Research Needs

Although core knowledge on the membranes of hydrocarbon-producing bacteria is evolving, much remains to be learned about the mechanisms by which the cells respond to hydrocarbons.

Since lipid composition is affected by the presence of hydrocarbons and other solvents, more information is needed concerning the effects of genetic manipulation of lipid compositions on resistance to and the production of solvents.

What changes in the expression of membrane proteins occur in response to hydrocarbon production or hydrocarbons in the environment?

What is the mechanism for translocation of hydrocarbons in the bacterial cell?

References

Ailhaud GP, Vagelos PR (1966) Palmitoyl-acyl carrier protein as acyl donor for complex lipid biosynthesis in *Escherichia coli*. J Biol Chem 241:3866–3868

Alley SH, Ces O, Templer RH, Barahona M (2008) Biophysical regulation of lipid biosynthesis in the plasma membrane. Biophys J 94:2938–2954

Baer SH, Blaschek HP, Smith TL (1987) Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl Environ Microbiol 53:2854–2861

Cronan JE Jr, Gelmann EP (1975) Physical properties of membrane lipids: biological relevance and regulation. Bacteriol Rev 39:232–256

Cronan JE, Thomas J (2009) Bacterial fatty acid synthesis and its relationships with polyketide synthetic pathways. Methods Enzymol 459:395–433

Cullis PR, De Kruijff B (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. Biochim Biophys Acta 559:399–420

Dowhan W (2013) A retrospective: use of *Escherichia coli* as a vehicle to study phospholipid synthesis and function. Biochim Biophys Acta 1831:471–494

Edman M, Berg S, Storm P, Wikstrom M, Vikstrom S, Ohman A et al (2003) Structural features of glycosyltransferases synthesizing major bilayer and nonbilayer-prone membrane lipids in *Acholeplasma laidlawii* and *Streptococcus pneumoniae*. J Biol Chem 278:8420–8428

Geiger O, Lopez-Lara IM, Sohlenkamp C (2013) Phosphatidylcholine biosynthesis and function in bacteria. Biochim Biophys Acta 1831:503–513

Goldfine H (1982) Lipids of procaryotes-structure and distribution. Curr Top Membr Transport 17:1–43

Goldfine H (1984) Bacterial membranes and lipid packing theory. J Lipid Res 25:1501–1507

Goldfine H, Ailhaud GP (1971) Fatty acyl-acyl carrier protein and fatty acyl-CoA in the biosynthesis of phosphatidic acid in *Clostridium butyricum*. Biochem Biophys Res Commun 45:1127–1133

Goldfine H, Bloch K (1961) On the origin of unsaturated fatty acids in clostridia. J Biol Chem 236:2596–2601

Goldfine H, Johnston NC (2005) Membrane lipids of clostridia. In: Dürre P (ed) Handbook on clostridia. Taylor & Francis, Boca Raton, pp 297–310

Goldfine H, Ailhaud GP, Vagelos PR (1967) Involvement of acyl carrier protein in acylation of glycerol 3-phosphate in *Clostridium butyricum.* II Evidence for the participation of acyl thioesters of acyl carrier protein. J Biol Chem 242:4466–4475

Goldfine H, Johnston NC, Mattai J, Shipley GG (1987) The regulation of bilayer stability in *Clostridium butyricum*: studies on the polymorphic phase behavior of the ether lipids. Biochemistry 26:2814–2822

Green PR, Merrill AH Jr, Bell RM (1981) Membrane phospholipid synthesis in *Escherichia coli*. Purification, reconstitution, and characterization of *sn*-glycerol-3-phosphate acyltransferase. J Biol Chem 256:11151–11159

Gruner SM, Shyamsunder E (1991) Is the mechanism of general anesthesia related to lipid membrane spontaneous curvature? Ann N Y Acad Sci 625:685–697

Halverson LJ, Firestone MK (2000) Differential effects of permeating and nonpermeating solutes on the fatty acid composition of *Pseudomonas putida*. Appl Environ Microbiol 66:2414–2421

Holtwick R, Meinhardt F, Keweloh H (1997) cis-trans isomerization of unsaturated fatty acids: cloning and sequencing of the cti gene from *Pseudomonas putida* P8. Appl Environ Microbiol 63:4292–4297

Huang KC, Ramamurthi KS (2010) Macromolecules that prefer their membranes curvy. Mol Microbiol 76:822–832

Inoue A, Horikoshi K (1989) A *Pseudomonas* thrives in high-concentrations of toluene. Nature (London) 338:264–266

Israelachvili JN, Marcelja S, Horn RG (1980) Physical principles of membrane organization. Q Rev Biophys 13:121–200

Janes N (1996) Curvature stress and polymorphism in membranes. Chem Phys Lipids 81:133–150

Kawai F, Shoda M, Harashima R, Sadaie Y, Hara H, Matsumoto K (2004) Cardiolipin domains in *Bacillus subtilis* Marburg membranes. J Bacteriol 86:1475–1483

Kiran MD, Annapoorni S, Suzuki I, Murata N, Shivaji S (2005) Cis-trans isomerase gene in psychrophilic *Pseudomonas syringae* is constitutively expressed during growth and under conditions of temperature and solvent stress. Extremophiles 9:117–125

Lepage C, Fayolle F, Hermann M, Vandecasteele J-P (1987) Changes in membrane lipid composition of *Clostridium acetobutylicum* during acetone-butanol fermentation: effects of solvents, growth temperature and pH. J Gen Microbiol 133:103–110

Lin TY, Weibel DB (2016) Organization and function of anionic phospholipids in bacteria. Appl Microbiol Biotechnol 100:4255–4267

Lindblom G, Rilfors L (1989) Cubic phases and isotropic structures formed by membrane lipids -possible biological relevance. Biochim Biophys Acta 988:221–256

Linde K, Grobner G, Rilfors L (2004) Lipid dependence and activity control of phosphatidylserine synthase from *Escherichia coli*. FEBS Lett 575:77–80

Lueking DR, Goldfine H (1975) *sn*-Glycerol-3-phosphate acyltransferase activity in particulate preparations from anaerobic, light-grown cell of *Rhodopseudomonas sphaeroides*. The involvement of acyl thioester derivatives of acyl carrier protein in complex lipid synthesis. J Biol Chem 250:530–8535

MacDonald DL, Goldfine H (1991) Effects of solvents and alcohols on the polar lipid composition of *Clostridium butyricum* under conditions of controlled lipid chain composition. Appl Environ Microbiol 57:3517–3521

Mileykovskaya E, Dowhan W (2000) Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange. J Bacteriol 182:1172–1175

Nishibori A, Kusaka J, Hara H, Umeda M, Matsumoto K (2005) Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes. J Bacteriol 187:2163–2174

Oliver PM, Crooks JA, Leidl M, Yoon EJ, Saghatelian A, Weibel DB (2016) Localization of anionic phospholipids in *Escherichia coli* cells. J Bacteriol 196:3386–3398

Rietveld AG, Killian JA, Dowhan W, De Kruijff B (1993) Polymorphic regulation of membrane phospholipid composition in *Escherichia coli*. J Biol Chem 268:12427–12433

Rilfors L, Lindblom G (2002) Regulation of lipid composition in biological membranes – biophysical studies of lipids and lipid synthesizing enzymes. Colloids Surf B Biointerfaces 26:112–124

Rilfors L, Niemi A, Haraldsson S, Edwards K, Andersson AS, Dowhan W (1999) Reconstituted phosphatidylserine synthase from *Escherichia coli* is activated by anionic phospholipids and micelle-forming amphiphiles. Biochim Biophys Acta 1438:281–294

Scheuerbrandt G, Goldfine H, Baronowsky P, Bloch K (1961) A novel mechanism for the biosynthesis of unsaturated fatty acids. J Biol Chem 236:PC70–PC71

Singh AK, Zhang YM, Zhu K, Subramanian C, Li Z, Jayaswal RK et al (2009) FabH selectivity for anteiso branched-chain fatty acid precursors in low-temperature adaptation in *Listeria monocytogenes*. FEMS Microbiol Lett 301:188–192

Tan BK, Bogdanov M, Zhao JS, Dowhan W, Raetz CRH, Guan Z (2012) Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci U S A 109:16504–16509

Tian B, Guan Z, Goldfine H (2013) An ethanolamine-phosphate modified glycolipid in *Clostridium acetobutylicum* that responds to membrane stress. Biochim Biophys Acta 1831:1185–1190

Vikstrom S, Li L, Karlsson OP, Wieslander A (1999) Key role of the diglucosyldiacylglycerol synthase for the nonbilayer-bilayer lipid balance of *Acholeplasma laidlawii* membranes. Biochemistry 38:5511–5520

Vollherbst-Schneck K, Sands JA, Montenecourt BS (1984) Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 47:193–194

Walton PA, Goldfine H (1987) Transphosphatidylation activity in *Clostridium butyricum* Evidence for a secondary pathway by which membrane phospholipids may be synthesized and modified. J Biol Chem 262:10355–10361

Weber FJ, De Bont JAM (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim Biophys Acta 1286:225–245

Weber FJ, Isken S, deBont JAM (1994) Cis/trans isomerization of fatty-acids as a defense-mechanism of *Pseudomonas-Putida* strains to toxic concentrations of toluene. Microbiology-UK 140:2013–2017

Wieslander Å, Christiansson A, Rilfors L, Lindblom G (1980) Lipid bilayer stability in membranes. Regulation of lipid composition in *Acholeplasma laidlawii* as governed by molecular shape. Biochemistry 19:3650–3655

Wieslander Å, Christiansson A, Rilfors L, Khan A, Johansson LBÅ, Lindblom G (1981a) Lipid phase structure governs the regulation of lipid composition in membranes of *Acholeplasma laidlawii*. FEBS Lett 124:273–278

Wieslander Å, Rilfors L, Johansson LBÅ, Lindblom G (1981b) Reversed cubic phase with membrane glucolipids from *Acholeplasma laidlawii*. ^{1}H, ^{2}H, and diffusion nuclear magnetic resonance measurements. Biochemistry 20:730–735

Wieslander Å, Rilfors L, Lindblom G (1986) Metabolic changes of membrane lipid composition in *Acholeplasma laidlawii* by hydrocarbons, alcohols, and detergents: arguments for effects on lipid packing. Biochemistry 25:7511–7517

Yao J, Rock CO (2013) Phosphatidic acid synthesis in bacteria. Biochim Biophys Acta 1831:495–502

Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6:222–233

Zhu K, Bayles DO, Xiong AM, Jayaswal RK, Wilkinson BJ (2005) Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain alpha-keto acid dehydrogenase. Microbiology-SGM 151:615–623

Biosynthesis and Evolution of Archaeal Membranes and Ether Phospholipids

29

Yosuke Koga

Contents

1 Introduction 541
2 Biosynthesis of Archaeal Phospholipids: Similarity Between the Biosynthetic Pathways in Archaea and Bacteria 541
2.1 G1P Formation 542
2.2 Isoprenoid-PP Formation and Modified Mevalonate (MVA) Pathway 544
2.3 Ether Bond Formation 544
2.4 CTP Activation of Archaetidic Acid (DGGGP) 546
3 Inference of Membrane Lipid Evolution by Suggestive Biochemical Events 546
3.1 Commonality of Major Polar Head Groups and the Enzyme Superfamily 547
3.2 Polar Head Groups and Phospholipid Backbones Were Derived from Gluconeogenesis 547
3.3 Broad Substrate Specificity of Polar Head Group-Attaching Enzymes 547
4 Do Archaea and Bacteria Have Different Ancestors: Are They Completely Different? 548
4.1 Wächtershäuser's "Surface Metabolism" 548
4.2 An Innumerable Number of Compounds Were Synthesized by Catalysts with Broad Substrate Specificity 549
4.3 Lipid-Driven Speciation of Archaea and Bacteria 549
4.4 An Extra Anionic Charge on the Polar Head Group and Phospholipid Intermediates 550
5 Conclusions and Research Needs: A Scenario of Membrane Lipid Evolution Based on the Biochemical Events 550
References 551

Y. Koga (✉)
University of Occupational and Environmental Health, Kitakyushu City, Japan

Munakata City, Fukuoka, Japan
e-mail: yokoga@ams.odn.ne.jp

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_47

Abstract

The fundamental biosynthetic pathway of archaeal phospholipids has been elucidated by in vitro studies based on comparison with the bacterial phospholipid biosynthesis pathway in bacteria. The glycerophosphate (*sn*-glycerol-1-phosphate; G1P) backbone of archaeal phospholipids (an enantiomer of bacterial *sn*-glycerol-3-phosphate; G3P) is formed from dihydroxyacetonephosphate (DHAP) catalyzed by a novel enzyme, G1P dehydrogenase. Isoprenoid chains are synthesized either by one of the classical or modified mevalonate pathways. The first ether bond is formed at the *sn*-3 position of the G1P backbone by a soluble enzyme geranylgeranylglycerophosphate (GGGP) synthase. G1P dehydrogenase is specific for G1P. GGGP synthase involves the step where three major characteristics of archaeal phospholipid structure are fixed into one molecule. Therefore, this enzyme can be regarded as "a quality control mechanism" in the biosynthesis of archaeal membrane lipid. Digeranylgeranylglycerophosphate (DGGGP) synthase catalyzes the formation of a second ether bond between the G1P backbone and an isoprenoid chain. The DGGGP synthase belongs to the hydrophobic ring-forming prenyltransferase enzyme family. The core lipid is activated by CTP catalyzed by CDP-archaeol synthase. Subsequently, CMP is replaced in CDP-archaeol by L-serine or other polar head groups. The entire pathway of phospholipid biosynthesis (starting from DHAP to the synthesis of one of amphiphilic phospholipids) in Archaea is quite similar to the bacterial pathway. It is especially remarkable that serine phospholipid and inositol phospholipid synthases from archaea and bacteria show unusually broad substrate specificities. These enzymes display nearly the same level of specific activities with all the substrates, CDP-activated core lipids of the archaeal or bacterial types (*sn*-2,3-diphytanyl glycerol-1-phosphate, Ai or *sn*-1,2-diacyl glycerol-3-phosphate, Bf) or with unnatural types (*sn*-2,3-diacyl glycerol-1-phosphate, Af or *sn*-1,2-diphytanyl glycerol-3-phosphate, Bi). In particular, the entire archaeal lipid biosynthesis pathway and the broad substrate specificity displayed by the enzymes involved suggest that in the ancient times, one enzyme reacted with various compounds including lipid-like compounds that had been formed abiotically. In the primitive lipid biosynthetic pathway, a variety of similar lipids was produced with the catalysis by a small number of catalysts with a broad substrate specificity. Although innumerous lipid components were abiotically synthesized, most of these lipids did not persist. The abundant lipid compounds included Ai, Bf, Af, and Bi. Presently, we can observe a large variety of amphiphilic phospholipids which constitute cellular membranes. The extremely broad substrate specificity of polar head group-attaching enzymes suggests that the differentiation of one pathway into two pathways, a bacterial and an archaeal, occurred.

These phenomena lead to the hypothesis that postulates the existence of a promiscuous stage, a historical stage during which a complex mixture of the isomers of the lipid components coexisted. Nowadays, archaeal and bacterial membrane lipids consist exclusively of the Ai core and Bf core, respectively. This fact implies that biological differentiation was driven by membrane segregation.

Abbreviations

Ai *sn*-2,3-Diphytanyl Glycerol-1-phosphate
Bf *sn*-1,2-Diacyl Glycerol-3-phosphate
Af *sn*-2,3-Diacyl Glycerol-1-phosphate
Bi *sn*-1,2-Diphytanyl Glycerol-3-phosphate

1 Introduction

Generally, archaeal polar lipids are composed of a core lipid, an isoprenoid glycerol ether (archaeol or caldarchaeol; for the nomenclature of archaeal lipids, see Nishihara et al. 1987; Koga et al. 1993). The isoprenoid chains are bound to the *sn*-2 and *sn*-3 positions of the G1P backbone, which is the most remarkable feature of archaeal phospholipids. Since 1990, when the first in vitro experimental results on the biosynthesis of archaeal phospholipids were published (Zhang et al. 1990), by now the basic pathway for archaeal phospholipid biosynthesis has been almost completely elucidated. Here the outline of the discoveries on the archaeal phospholipid biosynthesis is briefly reviewed. Most researchers studying archaeal lipids have focused their studies on the three remarkable differences (Koga et al. 1993), also known as the "lipid divide," whereas the similarities in biochemical processes of the bacterial and the archaeal pathway have received little attention. In the latter half of this chapter, the inference based on the past history of prebiotic events and biochemistry is presented. A consistent scenario is illustrated with supporting biochemical data, which have been summarized in Table 1. As the distinctness of archaeal and bacterial core lipids is well known, there might be a tendency to overestimate the distinctness (Koga et al. 1998). There is a real possibility that Archaea and Bacteria were evolutionarily far apart from each other and that they originated from different ancestors. However, the similarity and commonality found among them in the field of biochemistry would be challenging to explain. Initially, it is difficult to imagine how lipid materials might be abiotically synthesized. Therefore, Wächtershäuser's "Surface Metabolism" hypothesis (Wächtershäuser 1988) was adopted, based on scientific logical reasoning. Wächtershäuser proposed that the "last universal common ancestor (LUCA) possessed various kinds of phospholipids.

2 Biosynthesis of Archaeal Phospholipids: Similarity Between the Biosynthetic Pathways in Archaea and Bacteria

In this section, the biosynthesis pathways of archaeal and bacterial phospholipids are compared, and their remarkable similarities with regard to structure and biosynthesis are highlighted. For example, (A) in contrast to the distinctness of phospholipid

Table 1 The ancient evolutionary events of membrane lipids (B) were inferred from the biochemical studies (A)

Stage	Observed real events (A)	Inferred evolutionary events (B) from (A)
I	[First prebiotic organic compounds were from SM]	*SM on pyrite was hypothesized
II	*Intermediates of PL synthesis have anionic groups *Membrane lipids are composed of multifarious parts organisms *are found on the precursors of PHGs	*Multifarious LCP were synthesized by the SM *SM would be involved in the early evolution
III	*PHG compounds are all present since ancient times the same as glycolysis	*PHG compounds are all present since ancient times the same as glycolysis
IV	*Archaeal and bacterial phospholipid biosynthetic pathways are quite similar *CDP-OH PTFase can react with artificially synthesized core lipids as well as natural core lipids as substrates *CDP-OH PTFase from all organisms are universal and homologous	*A few or only one set of enzymes or inorganic catalysts with broad substrate specificity had worked for synthesis of multifarious lipid components, which were metabolized by only few catalysts
V	*Major PHGs are bound to membrane lipids but present in cytoplasmic compartments	Since cytoplasmic constituents were passively divided into two daughter cells, the PHG and their synthesizing enzymes were passed down to the later generations of Archaea and Bacteria
VI	The core lipids Ai or Bf exist exclusively in Archaea or Bacteria, respectively, without exception	Membrane segregation drove differentiation of Archaea and Bacteria to aim toward membranes with the purer core lipid composition

SM surface metabolism, *PL* phospholipid, *LCP* lipid component parts, *PHG* polar head group, *CDP-OH PTFase* cytidine diphosphate-alcohol phosphatidyltransferase

structures with racemic GP backbones and lipid component parts, GP backbones are formed by the direct reduction of DHAP by NADH. The only difference observed is the stereoisomeric structure. (B) The core lipids are activated by CTP in order to accept a polar head group. (C) The CMP moiety in CDP-archaeol is replaced by a polar head group. (D) The enzymes that link a polar head group to a core lipid are members of the CDP-alcohol phosphatidyltransferase family and are homologous. (E) The sequences of the reactions in the archaeal and bacterial pathways are essentially the same (Fig. 1). These structural and biochemical aspects suggest that the various component parts of phospholipids were combined with the aid of inorganic or proteinacious catalysts with broad substrate specificity (see Sect. 3.2), and during the course of the reactions they reacted promiscuously.

2.1 G1P Formation

Characteristic formation of G1P is one of the primary interests in the investigation of archaeal phospholipid biosynthesis. After several in vivo experiments, Nishihara and

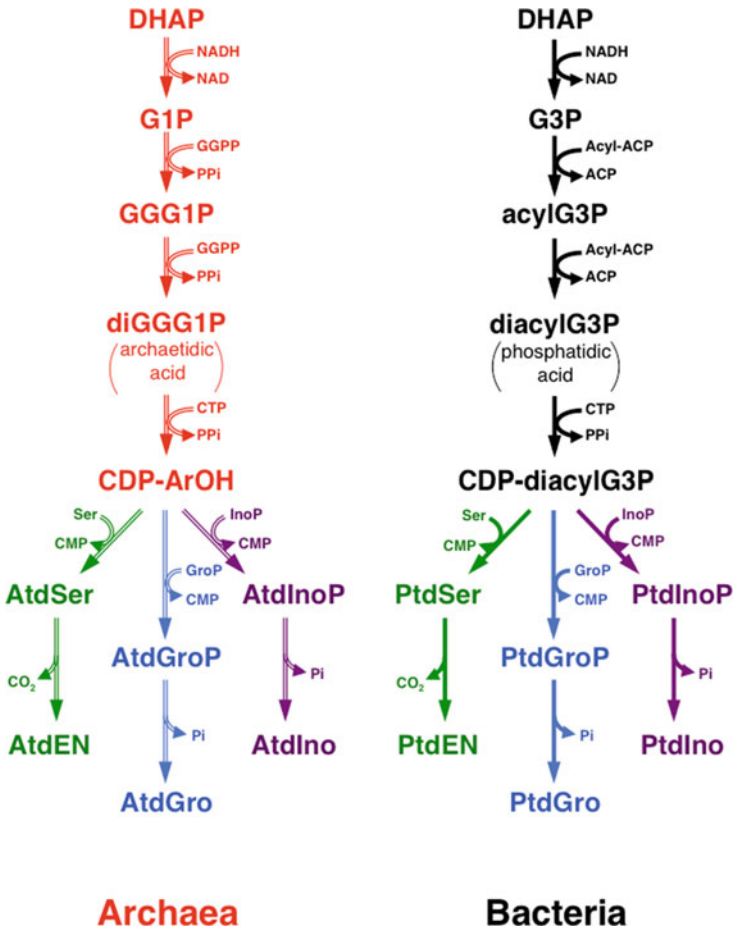


Fig. 1 Similarity between phospholipid synthesis pathways in Archaea and Bacteria. *DHAP* dihydroxyacetonephosphate, *G1P* *sn*-glycerol-1-phosphate, *G3P* *sn*-glycerol-3-phosphate, *GGG1P* geranylgeranyl-*sn*-glycerol-1-phosphate, *acylG3P* 1-acyl-*sn*-glycerol-3-phosphate, *diGGG1P* 2,3-digeranylgeranyl-*sn*-glycerol-1- phosphate, *diacyl-G3P* 1,2-diacyl-*sn*-glycerol-1-phosphate, *CDP-ArOH* CDP-2,3-digeranylgeranyl-*sn*-glycerol, *CDP-1,2-diacylG3P* CDP-1,2-*sn*-diacylglycerol, *AtdSer* archaetidylserine, *ArtGroP* archaetidylglycerolphosphate, *ArtInoP* archaetidyl-*myo*inositolphosphate, *PtdIno* phosphatidyl-*myo*inositolphosphate, *AtdEN* archaetidylethanolamine, *AtdGro* archaetidylglycerol, *ArtIno* archaetidyl-*myo*inositol, *PtdEN* phosphatidylethanolamine, *PtdIno* phosphatidyl-*myo*inositol

Koga (1995) found that DHAP is reduced to G1P by NAD(P)H by means of a new enzyme, G1P dehydrogenase. DHAP is supplied from the glycolysis or gluconeogenesis pathways. This enzyme activity has been ubiquitously detected in all Archaea surveyed thus far. G1P dehydrogenase and the encoding gene *egs*A are responsible for the formation of the enantiomeric GP backbone of polar lipids specific to Achaea (Nishihara et al. 1999). Although archaeal G1P dehydrogenase and bacterial G3P dehydrogenase are both dehydrogenases, they show no homology

and do not share common ancestral enzymes (Daiyasu et al. 2002). Recently, it was observed that, although archaeal G1P dehydrogenase and bacterial G3P dehydrogenase catalyze the same reaction mode (reduction of DHAP by NAD(P)H), G3P dehydrogenase transfers the hydride (H^-) ion of NAD(P)H from the opposite side as the archaeal G1P dehydrogenase (Daiyasu et al. 2002; Koga et al. 2003). However, a G1P dehydrogenase has also been found in *Bacillus subtilis* and many other bacterial species (Guldan et al. 2008). Additionally, an ether linkage-forming activity (like GGGP synthase, see below) on the *sn*-3 position of the G1P with a C35 isoprenoid was found. This latter feature looks like an exception from the "lipid divide" (Koga 2011). However, since no Ai type phospholipids have been detected in Bacteria, it is hard to deny the existence of the "lipid divide." This divide is a hallmark of the membrane lipid composition (Ai or Bf) but not of the existence of the related enzymes.

2.2 Isoprenoid-PP Formation and Modified Mevalonate (MVA) Pathway

Although in vivo incorporation experiments produced results, consistent with a MVA pathway in archaea (Ekiel et al. 1983), some of the expected genes are not necessarily detected in the respective genomes. In most archaeal species, except *Sulfolobus*, the orthologous genes for phosphomevalonate kinase (PMK) or MVA-5PP decarboxylase (DMD) are not encountered. After the discovery of isopentenylphosphate kinase (IPK) (Grochowski et al. 2006), the missing link was detected. The finding that the orthologous gene of DMD in the extremely halophilic archaeon *Haloferax* was not DMD but mevalonate-5-decarboxylase established a new, modified MVA pathway. In addition, a new pathway, which takes the route of phosphorylation at the 3- and 5-positions of MVA has been proposed. The missing links of the archaeal MVA pathway have been almost completely elucidated (Azami et al. 2014) (Fig. 2). The significance of this diversification of the MVA pathway is not yet understood. Isopentenyl-PP isomerase is also unique in its primary structure. In either case, isopentenyl-PP (C5) and its isomer, dimethylallyl-PP (C5), are condensed to geranyl-PP (C10). This type of condensation reaction is repeated two more times to obtain geranylgeranyl-PP (GGPP; C20).

2.3 Ether Bond Formation

G1P is sequentially etherified to form, at first, geranylgeranyl-G1P (GGGP), and subsequently digeranylgeranyl-G1P (DGGGP or unsaturated archaetidic acid). The first ether bond is formed at the *sn*-3-hydroxyl group of G1P by a soluble enzyme (GGGP synthase). This enzyme was characterized by Soderberg et al. (2001) as the first ether bond-forming enzyme, and its 3D crystal structure was analyzed by Payandeh et al. (2006). The enzyme prefers G1P to G3P as the acceptor of a geranylgeranyl chain and was not active with DHAP or glycerol. It is involved in

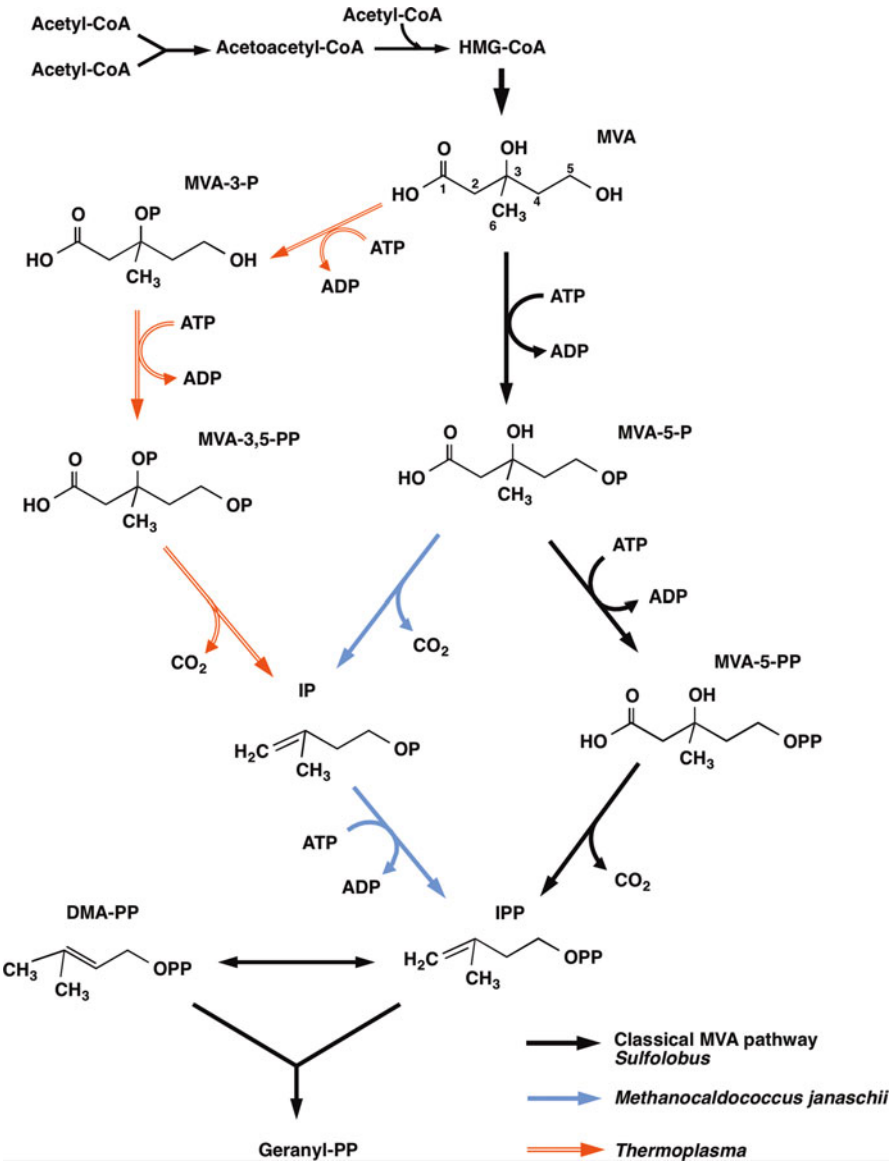


Fig. 2 Modified mevalonate pathways for isoprenoid synthesis in Archaea. *HMG-CoA* hydroxy methyl glutaryl-CoA, *MVA* mevalonate, *MVA-3-P* mevalonate-3-phosphate, *MVA-5-P* mevalonate-5-phosphate, *MVA-3,5-PP* mevalonate-3,5-pyrophosphate, *MVA-5-PP* mevalonate-5-pyrophaspate, *IP* isoprenyl phosphate, *IPP* isoprenyl pyrophosphate, *DMA-PP* dimethylallyl pyrophosphate

the step at which three major characteristics of archaeal polar lipid structure (stereochemistry of the GP backbone, isoprenoid chain, and ether bond) are assembled into one molecule (Payandeh et al. 2006). Therefore, this enzyme may be regarded as having the most important role in the differentiation of the two domains of life.

Furthermore, the catalytic mechanism of GGGP synthase has been reported on the basis of its crystal structure (Payandeh et al. 2006). Crucially, this enzyme is regarded as a keeper of the three distinct features of archaeal membrane lipids. Many archaeal cells produce not only G1P but also G3P (Nishihara et al. 1999), whereas their backbone for membrane lipids should be limited to G1P only. Accordingly, candidate compounds other than G1P must be excluded and should not enter the biosynthetic pathway. The GGGP synthase-catalyzed reaction is considered as the "quality control mechanism" in archaeal phospholipid biosynthesis. The second ether bond-forming enzyme is a prenyl transferase that transfers prenyl groups to large hydrophobic rings such as hemes. This enzyme is bound to membranes and belongs to the UbiA prenyl lipid-related transferase family (Hemmi et al. 2004). The nascent ether lipids are allyl ether compounds, which are quite labile to acid. Therefore, the conventional view that all ether bonds are connected to the thermophilicity of lipids is not necessarily true as some kind of ether bonds are as labile as ester bonds (Koga 2012).

2.4 CTP Activation of Archaetidic Acid (DGGGP)

After completing the core lipid formation, the next step involves activation of the core lipid by CTP. The activated form is CDP-archaeol, the archaeal analogue of CDP-diacylglycerol (CDP-DAG). The reaction involves the same mode of activation as phosphatidic acid by CTP. The enzyme involved in this reaction is CDP-unsaturated archaeol synthase (Morii et al. 2000). Recently Jain et al. (2014) purified and crystallized the enzyme. It exhibits similarity to bacterial CDP-DAG synthase, which does not recognize the characteristic structure of archaeal phospholipids, but is specifically active with substrates possessing geranylgeranyl chains, irrespective of the backbone stereoisomer (G1P or G3P) of the CDP-DAG, CDP-diprenylglycerol, or to the ether or ester bonds. Amphiphilic phospholipids are absolutely required as constituents for membranes, which, in turn, spontaneously generate vesicles. Accordingly, these enzymes are one of the most significant enzymes for cell formation. In other words, these enzymes are the "membrane-forming enzymes." Comparison of the archaeal and bacterial enzymes indicates several historical events in primordial times.

3 Inference of Membrane Lipid Evolution by Suggestive Biochemical Events

The properties of enzymes for biosynthesis or comparison of archaeal phospholipid synthesizing enzymes with bacterial ones may indicate the historical events that ancient ancestor organisms experienced. Here, this will be reviewed in depth.

3.1 Commonality of Major Polar Head Groups and the Enzyme Superfamily

Four major polar head groups (L-serine, ethanolamine, glycerol, and myoinositol) are shared by organisms of the three domains. In addition, the polar head group-attaching enzymes are homologous and belong to the enzyme superfamily of CDP-alcohol phosphatidyltransferases. The enzymes that synthesize archaetidylserine (AS), archaetidyl-*myo*inositolphosphate (AIP), archaetidylglycerol (AG), phosphatidylserine (PS), phosphatidyl-*myo*inositolphosphate (PIP), and phosphatidylglycerolphosphate (PGP) were identified in archaea or bacteria, biochemically or phylogenetically (Koga and Morii 2007; Daiyasu et al. 2005).

3.2 Polar Head Groups and Phospholipid Backbones Were Derived from Gluconeogenesis

The intermediates of lipid biosynthesis and polar head groups were originally generated at a time when glycolysis or gluconeogenesis appeared first on Earth. Intermediates or polar head groups were derived from these pathways. The fact that CDP-alcohol phosphatidyltransferases show broad specificities toward natural or artificial substrates indicates that multifarious compounds were formed on pyrite surfaces and could be combined at random with nonspecific catalysts.

3.3 Broad Substrate Specificity of Polar Head Group-Attaching Enzymes

Surprisingly, the enzymes of the CDP-alcohol phosphatidyltransferase family exhibit broad substrate specificity: the enzymes do not discriminate distinct CDP-activated core lipids (stereoisomers of GP, ester or ether bonds, fatty acyl or isoprenoid as hydrocarbon chains). This suggests that various lipid component parts were combined by a small number of enzymes or by inorganic catalysts based on a broad substrate specificity (Morii and Koga 2003) saturated or unsaturated chemically synthesized hydrocarbon analogs, such as Ai-sat-ether, Bi-unsat-ether, Bf-ester, and Af-ester. Moreover, bacterial type II PS synthase exhibited a similar activity. In addition to serine phospholipid-synthesizing (Bacillus subtilis) enzymes, *myo*-inositol phospholipid-synthesizing enzymes also exhibited a similar phenomenon (Morii et al. 2009). In the phospholipid biosynthetic pathway, the core lipid requires hydrocarbons but neglects the stereochemistry of the core lipids. The broad substrate specificity and their analogous locations in the pathways support the abovementioned inference that various lipid component parts were combined to distinct lipids by a small number of enzymes or inorganic catalysts with a broad substrate specificity. It is unlikely that the broad substrate specificity was acquired after the establishment of the "lipid divide," since a broader substrate specificity

would not be required after the "lipid divide" had been established. The enzymes forming AS, AIP, AG, PS, PIP, or PGP belong to the CDP-alcohol phosphatidyltransferase superfamily and the similarity of lipid-biosynthetic pathways, and the enzymes with quite broad substrate specificities suggest that these primordial catalyst groups compose a primordial and early pathway that synthesizes varieties of phospholipids. Of course, lipid composition of LUCA membranes included all kinds of core lipids and PHG. In the context of these promiscuous lipid compositions in membranes, the question arises how the "lipid divide" emerged.

4 Do Archaea and Bacteria Have Different Ancestors: Are They Completely Different?

Pyrite surfaces can exhibit highly active surface metabolism, and in a recent study a large number of lipid-related compounds were synthesized, among the principal compounds Ai, Bf, Af, and Bi. Ai and Bf survived and became the membrane-forming phospholipids of Archaea and Bacteria, respectively. The mechanism of this differentiation is not known. However, from a number of studies, it is clear that to some extent the archaeal and bacterial lipids are different. There are two propensities on how to interpret the significance of the lipid distinctness. The first suggests that the lipids of both organisms are considerably different from each other and that they came from different ancestors. In contrast, the second concept postulates that the well-known differences can be seen in the background of similarities and commonalities of the lipid structures and biosynthesis mechanisms. According to the second view, careful comparison of archaeal and bacterial lipid membranes could provide some evidence for the evolution of membrane lipids. Since the initial origin of organic compounds could not be explained with experimental biochemical data, Wächtershäuser's "Surface Metabolism" hypothesis was be adopted, because this hypothesis provides some major advantages (Koga 2014). This reference also provides arguments why Ai and Bf core lipids might have survived from an initial pool of at least Ai, Bf, Af, and Bi (Koga 2014).

4.1 Wächtershäuser's "Surface Metabolism"

The second concept that archaeal and bacterial lipids have a common ancestor was inferred from sharing DNA, RNA, and protein as the most important informational polymers. The theory of "Surface Metabolism" proposed by Wächtershäuser (1988) seems to be the most appropriate explanation for the prebiotic origin of organic compounds, since (A) the Gibbs standard free energy change for formic acid formation from CO_2 coupled with pyrite (FeS_2) formation from H_2S + FeS is negative (which means reactions proceed spontaneously); (B) as pyrite has a positive charge, compounds with negative charges are bound electrically; (C) an electrically positive or neutral compounds cannot stay on the surface and therefore cannot grow by fixation of more CO_2; and (D) the origin of a cell can be explained by the growth

of lipid membranes. The postulation of an autotrophic origin of life seems the most appropriate, since it is reasonable to assume that biological evolution took over abiotic evolution. After encapsulation of Surface Metabolism by lipid membranes, catalysis was taken over by protein catalysts.

4.2 An Innumerable Number of Compounds Were Synthesized by Catalysts with Broad Substrate Specificity

The similarity of lipid biosynthetic pathways and the quite broad specificity membrane-synthesizing enzymes lead me to infer that these compounds for membrane lipid formation were composed of a lot of varieties of structural and stereoisomers. A great variety of membrane lipid structures provide the minimal requirement for membrane formation and that lipids are the main constituents of biomembranes. Amphiphilic membrane lipids must have structures, which are composed of two long-chain hydrocarbons and one small hydrophilic compound bound to a three-carbon backbone. Generally, amphiphilic membrane lipids must have a structure that is composed of two long-chain hydrocarbons and one small hydrophilic compound bound on a three-carbon backbone. The linkage between the hydrocarbons and the three-carbon backbone may consist of ester, ether, alkenyl-ether, amide, or even C-C bonds (Koga and Morii 2007). Hydrocarbons may be straight chains, or highly methyl-branched isoprenoid chains, which may be saturated or unsaturated. Amphiphilic lipids by random combinations of the ancient lipid components would compose LUCA membranes. As G1P and G3P cores were mixed in the LUCA membranes, these membranes were heterochiral. However, phase separation, growth, collision, fusion, or division could lead to separation into more homogeneous lipid vesides and more homochiral membranes. Based mainly on phylogenetic data, Lombard et al. (2012) proposed that LUCA membranes were composed of innumerable components of amphiphilic polar lipids present in the cenancestor (LUCA). The individual polar head group compound was incorporated into each phospholipid by replacing the CMP moiety of CDP-unsaturated archaeol. These reaction modes were probably similar to each other, and it would be inferred that a small number of catalysts with broad substrate specificity could catalyze in the LUCA stage. Lombard et al. (2012) concluded, similarly as the present author, that there were many ancient abiotic compounds. However, on the basis of the broad specificity, only a small number of abiotic catalysts could be conceivable.

4.3 Lipid-Driven Speciation of Archaea and Bacteria

As mentioned earlier, various lipid and lipid-like compounds were accumulated in the LUCA membranes. The major components of the membranes were lipids with the core lipids (Ai, Bf, Af, or Bi) and one of the four kinds of major polar head groups. The core portion was buried in the hydrocarbon layers of the membrane, while polar head groups came into contact with the aqueous environment due to their

hydrophilic property. The fact that there is no exception in the distinctness of archaeal and bacterial lipids suggests that the distinctness of lipids was the cause of the "lipid divide," i.e., the distinctness drove the differentiation of the first speciation of organisms. The next question is how the two types presently existing core lipids (Ai and Bf) have survived from an initial pool of at least four types core lipids (Ai, Bf, Af, and Bi). The archaeal core lipid (Ai) has seven chiral centers (1 *S* in GP and 6 *R* in isoprenoids), and bacterial core lipid (Bf) has only one chiral center (1*R* in GP). These Ai and Bf are diastereomers of each other and provide the largest possible difference between each other. The stereochemical difference between Ai and Bf may give rise to phase separation faster than the other combinations of different core lipids. This may be a lipid-driven differentiation.

4.4 An Extra Anionic Charge on the Polar Head Group and Phospholipid Intermediates

Archaetidylinositol (AI) had been thought to be synthesized from CDP-archaeol plus *myo*inositol in an analogous way as it had been reported for either bacteria or eukarya. However, archaeal AI and bacterial PI are formed from either CDP-DAG or CDP-archaeol and *myo*inositolphosphate (*myo*InoP) (Morii and Koga 2003, 2009, 2010). The extra phosphate group in the polar head group is removed just after completion of polar head group attachment reaction. These polar head group compounds were generated in the ancient times because they had extra anionic groups which seems to be a sign of "Surface Metabolism" (see above). Once the extra anionic group was incorporated in the lipid bilayer, the membranes would become unstable due to electric repulsion by uncountable numbers of anionic charges. Therefore, the anionic groups have to be removed as soon as possible after incorporation into the lipid bilayer. If the archaeal inositol phospholipid precursor would not have been found to be *myo*inositolphosphate, the bacterial case might also not have been revised, and the inferences addressed here would be delayed. Clearly, studies on archaea turn out to be useful in unexpected turns.

5 Conclusions and Research Needs: A Scenario of Membrane Lipid Evolution Based on the Biochemical Events

The biosynthetic pathway of archaeal membrane lipids has been almost completely illuminated, starting with G1P formation to its completion upon the formation of amphiphilic phospholipids, on the enzymatic, genetic, and genomic levels. Although three crucial, structural features of archaeal membrane phospholipids are different from bacterial phospholipids, pathways and enzymes for their biosynthesis are quite similar. In addition, the broad substrate specificity of phospholipid synthesis enzymes is quite an unexpected result. Lipid membranes separate an inside cytoplasmic compartment of a cell from the outside environment. Therefore, membranes must have a barrier function for compounds that migrate to and from the outside and

the inside of the cell. At the same time, the membrane must have the ability to incorporate and eliminate materials and energy out of and into the cell. To comprehend the intrinsic qualities of life, the understanding of a cell which has such functions, is essential to the basic sciences and applied technologies.

The abovementioned scenario of early evolution of membrane lipids includes still many hypothesis or speculations. These ideas should be verified by suitable experiments or observations.

Acknowledgment The author greatly indebted to Prof. Otto Geiger for his excellent help in the last stage of preparing of this manuscript, and wishes to thank to Prof. Hiroyuki Morii for his help in preparing figures and to Dr. Yuichi Koga for his help in rearrangement of the manuscript.

References

Azami Y, Hattori A, Nishimura H, Kawaide H, Yoshimura T, Hemmi H (2014) (*R*)-Mevalonate 3-phosphate is an intermediate of the mevalonate pathway in *Thermoplasma acidophilum*. J Biol Chem 289:15957–15967

Daiyasu H, Hiroike T, Koga Y, Toh H (2002) The analysis of membrane stereochemistry with the homology modeling *sn*-glycerol-1-phosphate dehydrogenase. Protein Eng 15:987–995

Daiyasu H, Kuma K, Yokoi T, Morii H, Koga Y, Toh H (2005) A study of archaeal enzymes involved in polar lipid synthesis linking amino acid sequence information, genomic contexts and lipid composition. Archaea 1:399–410

Ekiel I, Smith IC, Sprott GD (1983) Biosynthetic pathways in *Methanospirillum hungatei* as determined by 13C nuclear magnetic resonance. J Bacteriol 56:316–326

Grochowski LL, Xu HM, White RH (2006) *Methanocaldococcus jannaschii* uses a modified mevalonate pathway for biosynthesis of isopentenyl diphosphate. J Bacteriol 188:3192–3198

Guldan H, Sterner R, Babinger P (2008) Identification and characterization of a bacterial glycerol-1-phosphate dehydrogenase: Ni^{2+}-dependent *Ara*M from *Bacillus subtilis*. Biochemistry 47:7376–7384

Hemmi H, Shibuya K, Takahashi Y, Nakayama T, Nishino T (2004) (S)-2,3-Di-O-geranylgeranylglyceryl phosphate synthase from the thermoacidophilic archaeon *Sulfolobus solfataricus*. Molecular cloning and characterization of a membrane-intrinsic prenyltransferase involved in the biosynthesis of archaeal ether-linked membrane lipids. J Biol Chem 279:50197–50203

Jain S, Caforio A, Fodran P, Lolkema JS, Minnaard AJ, Driessen AJ (2014) Identification of CDP-archaeol synthase, a missing link of ether lipid biosynthesis in archaea. Chem Biol 21:1392–1401

Koga Y (2011) Early evolution of membrane lipids: how did the lipid divide occur? J Mol Evol 72:274–282

Koga Y (2012) Thermal adaptation of the archaeal and bacterial lipid membranes. Archaea 2012: 789652, 6 pages. https://doi.org/10.1155/2012/789652

Koga Y (2014) From promiscuity to the lipid divide: on the evolution of distinct membranes in archaea and bacteria. J Mol Evol 78:234–242

Koga Y, Morii H (2007) Biosynthesis of ether-type polar lipids in archaea and evolutionary considerations. Microbiol Mol Biol Rev 71:97–120

Koga Y, Nishihara M, Morii H, Akagawa-Matsushita M (1993) Ether polar lipids of methanogenic bacteria: structures, comparative aspects, and biosyntheses. Microbiol Rev 57:164–182

Koga Y, Kyuragi T, Nishihara M, Sone N (1998) Did archaeal and bacterial ells arise independently from noncellular precursors? A hypothesis stating that the advent of membrane phospholipid with enantiomeric glycerophosphate backbones caused the separation of the two lines of descent. J Mol Evol 46:54–63

Koga Y, Sone N, Noguchi S, Morii H (2003) Transfer of pro-*R* hydrogen from NADH to dihydroxyacetonephosphate by *sn*-glycerol-1-phosphate dehydrogenase from the archaeon *Methanothermobacter thermautotrophicus*. Biosci Biotechnol Biochem 67:1605–1608

Lombard C, Lopez-Garcia P, Moreira D (2012) The early evolution of lipid membranes and the three domains of life. Nat Rev Microbiol 10:507–515

Morii H, Koga Y (2003) CDP-2,3-Di-*O*-geranylgeranyl-*sn*-glycerol:L-serine *O*-archaetidyltransferase (archaetidylserine synthase) in the methanogenic archaeon *Methanothermobacter thermautotrophicus*. J Bacteriol 185:1181–1189

Morii H, Nishihara M, Koga Y (2000) CTP:2,3-di-O-geranylgeranyl-*sn*-glycero-1-phosphate cytidyltransferase in the methanogenic archaeon *Methanothermobacter thermoautotrophicus*. J Biol Chem 275:36568–36574

Morii H, Kiyonari S, Ishino Y, Koga Y (2009) A novel biosynthetic pathway of archaetidyl-*myo*-inositol via archaetidyl-*myo*-inositol-1-phosphate from CDP-archaeol and glucose 6-phosphate in the methanoarchaeon *Methanothermobacter thermautotrophicus* cells. J Biol Chem 284:30766–30774

Morii H, Ogawa M, Fukuda K, Taniguchi H, Koga Y (2010) A revised biosynthetic pathway for phosphatidylinositol in mycobacteria. J Biochem 148:593–602

Nishihara M, Koga Y (1987) Structure determination of a quartet of novel tetraether lipids from *Methanobacterium thermoautotrophicum*. J Biochem 101:1007–1015

Nishihara M, Koga Y (1995) *sn*-glycerol-1-phosphate dehydrogenase in *Methanobacterium thermoautotrophicum*: key enzyme in biosynthesis of the enantiomeric glycerophosphate backbone of ether phospholipids of archaebacteria. J Biochem 117:933–935

Nishihara M, Yamazaki T, Oshima T, Koga Y (1999) *sn*-glycerol-1-phosphate-forming activities in archaea: separation of archaeal phospholipid biosynthesis and glycerol catabolism by glycerophosphate enantiomers. J Bacteriol 181:1330–1333

Payandeh J, Fujihashi M, Gillon W, Pai EF (2006) The crystal structure of (*S*)-3-*O*-geranylgeranylglyceryl phosphate synthase reveals an ancient fold for an ancient enzyme. J Biol Chem 281:6070–6078

Soderberg T, Chen AJ, Poulter CD (2001) Geranylgeranylglyceryl phosphate synthase: characterization of the recombinant enzyme from *Methanobacterium thermoautotrophicum*. Biochemistry 40:14847–14854

Wächtershäuser G (1988) Before enzymes and templates: theory of surface metabolism. Microbiol Rev 52:452–584

Zhang DL, Daniels L, Poulter CD (1990) Biosynthesis of archaebacterial membranes. Formation of isoprene ethers by a prenyl transfer reaction. J Amer Chem Soc 112:1264–1265

Functional Roles of Individual Membrane Phospholipids in *Escherichia coli* and *Saccharomyces cerevisiae*

30

William Dowhan, Mikhail Bogdanov, Eugenia Mileykovskaya, and Heidi Vitrac

Contents

1 Introduction 554
2 Phospholipid Synthesis and Genetics in *E. coli* 555
3 Incorporation of Foreign Lipids into *E. coli* 558
4 Lipids as Determinants of Membrane Protein Organization 559
5 Membrane Domains Enriched in Anionic Phospholipids 561
6 Are Non-bilayer-Prone Lipids Essential for Cell Viability? 562
7 Functions of Anionic Lipids and Lipid Domains 563
8 Lipid Metabolism and Genetics in *S. cerevisiae* 564
9 Anionic Phospholipid Functions in Mitochondria 566
10 Cardiolipin and Organization of the Respiratory Chain 567
11 Phosphatidylethanolamine and Mitochondrial Function 569
12 Research Needs 569
References 569

Abstract

The physical and chemical properties of membrane lipids containing both a hydrophobic and hydrophilic domain result in organization into the lipid bilayer that provides the barrier function and defines the limits of cells and organelles. The lipid bilayer provides the solvent for integral membrane proteins and the scaffold with which peripheral membrane proteins associate. The properties of the lipid bilayer are defined by the collective properties of the diverse mixture of resident lipids. The lipidome is composed of glycerol-based phospholipids and glycolipids found in bacteria and the additional steroids and sphingolipids found in eukaryotic cells. The diversity of the lipidome far exceeds that of the proteome

W. Dowhan (✉) · M. Bogdanov · E. Mileykovskaya · H. Vitrac
Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, McGovern Medical School, Houston, TX, USA
e-mail: william.dowhan@uth.tmc.edu; mikhail.v.bogdanov@uth.tmc.edu; eugenia.mileykovskaya@uth.tmc.edu; heidi.vitrac@uth.tmc.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_36

when the composition of both the hydrophilic and hydrophilic domains is considered. Therefore, it is not surprising that the diverse physical, chemical, and collective properties of the membrane bilayer have a profound effect on the structure and function of membrane-associated proteins. Lipids affect membrane proteins through the collective properties of the lipid bilayer and through direct specific lipid-protein interactions. Given the vast complexity and diversity of the influence of lipids on membrane proteins throughout nature, this review will focus on selected roles of lipids in *Escherichia coli* and *Saccharomyces cerevisiae*. The lipid genetics of each organism will be reviewed in relation to the phenotypes resulting from mutations in lipid biosynthetic pathways. The combined use of genetic manipulation and biochemical characterization will be outlined as a means to establish an understanding at the molecular level of the importance of lipids in cellular processes.

1 Introduction

Membrane lipids in bacteria and eukaryotic microbes are composed of a complex mixture of molecular species with largely amphipathic properties, i.e., containing both hydrophilic and hydrophobic properties (Dowhan 1997). The major components of bacterial membranes are charged phospholipids and neutral glycolipids. Membranes of eukaryotic microbes also contain charged sphingolipids and highly hydrophobic sterols. The amphipathic character of these major lipids results into their organization into a lipid bilayer that defines the permeability barrier to hydrophilic molecules for cell and organelle membranes. Synergistic interactions between these hydrophobic and hydrophilic domains of lipids also influence the collective properties of the membrane, which affect the organization and function of proteins spanning or surface associated with membranes. Besides the properties of the membrane as a solvent or scaffold for proteins, lipid-protein interactions regulate structure and function of membrane-associated proteins. Many large multisubunit membrane protein (MP) complexes contain essential lipids integrated into their structure. Thus, the effects of lipids on MP structure and function can be attributed either to collective bilayer properties (thickness, fluidity, lateral pressure, spontaneous curvature, and surface tension) or to the binding of specific lipids to the protein surface. Individual lipids are not covalently bound in membranes but rather interact dynamically to form transient arrangements with asymmetry both perpendicular and parallel to the plane of the lipid bilayer. Individual lipids display different relative propensities for spontaneous curvature and formation of non-bilayer phases, which are favored by deviation from an overall cylindrical shape for a lipid molecule. Increasing numbers of acyl chains and degrees of unsaturation increase the size of the hydrophobic domain, while molecular size and charge nature determine the effective space occupied by the head group. Lipid composition varies drastically among different prokaryotes and eukaryotes, single-cell and multicellular organisms, and among different subcellular organelles and intracellular compartments. The chemical nature and physical heterogeneity of individual lipids and their uneven

distribution laterally, vertically and temporally multiply their functional diversity and allow a large number of arrangements to modulate the properties of the cell membrane and residing MPs in response to harsh changes in environment and different protein needs. The collective chemical and physical properties of lipid molecules define the properties of any given membrane.

Determining the collective and individual properties of membranes and the role individual lipids play has been very challenging. Lipids have no inherent catalytic activity so that function must be defined by their effect on proteins and cell functions. Historically many functions were defined through in vitro studies using model lipids of narrow physical and chemical properties. Such studies provided initial clues to lipid function but are subject to artifacts due to the differences in physical properties between defined lipid mixtures and natural-occurring membrane lipid mixtures. Genetic approaches, which have been very successful in defining protein functions, are complicated by the fact that genes do not directly encode lipids. In order to alter lipid composition in vivo, mutations must be made in enzymes along lipid biosynthetic pathways. Such mutations are highly pleiotropic especially in eukaryotic microorganisms that contain multiple organelles. The elimination of major lipids can result in lethality, which may support a requirement for membrane integrity without providing clues to other important functions. Also, the accumulation of normally traced amounts of upstream intermediates complicates the interpretation of results. However, combining genetic studies with biochemical characterization of in vivo phenotypes and studies in defined reconstituted systems in vitro have uncovered specific and general roles for membrane lipids in cell function (Dowhan 2009).

This review will cover genetic and biochemical approaches that have been employed to define membrane lipid function in primarily *Escherichia coli* and *Saccharomyces cerevisiae*. Results from such well-defined and detailed studies can be extrapolated to more complex organisms.

2 Phospholipid Synthesis and Genetics in *E. coli*

Studies in *E. coli* (Dowhan 2013) represent the most extensive source of information on the biosynthesis, genetics, and function of bacterial phospholipids (Fig. 1). The major membrane lipids of the *E. coli* inner membrane and the inner leaflet of the outer membrane are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). The relative amounts of these phospholipids are maintained roughly in the molar ratio of 70–75:20–25:5–10, respectively. Since the inner leaflet of the outer membrane is made up of about 90% PE, the inner membrane must contain a different ratio than the overall ratio. The outer leaflet of the outer membrane is composed of lipopolysaccharide reviewed extensively elsewhere (Whitfield and Trent 2014). A major consequence of systemic infection by Gram-negative organisms is toxic shock due to the host organism's response to lipopolysaccharides. The bacterial lipidome contains a much richer population of phospholipids including phosphatidylcholine (PC) and phosphatidylinositol (PI) historically thought to be restricted to eukaryotic cells. Nonphosphate containing derivatives of diacylglycerol

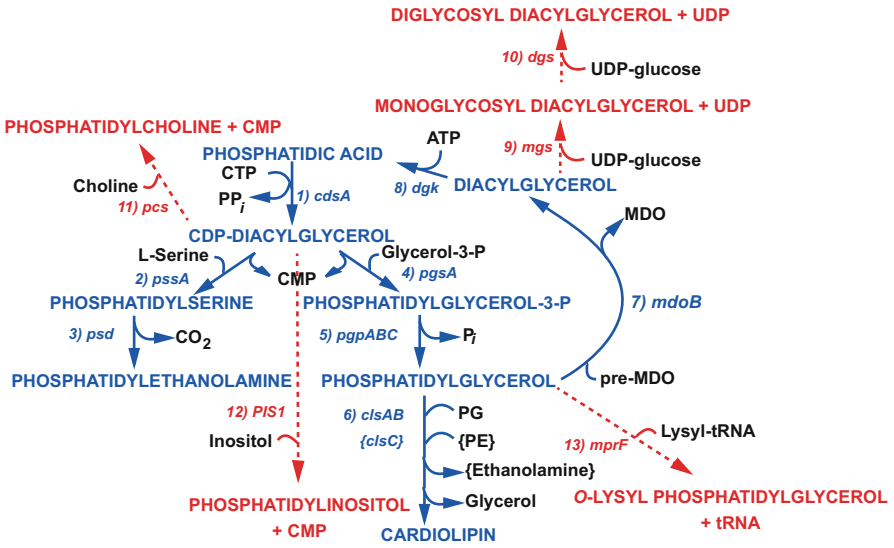


Fig. 1 Synthesis of native and foreign phospholipids in *E. coli*. Pathways native to *E. coli* are noted with *solid arrows*, and pathways resulting from foreign genes introduced into *E. coli* are noted with *dashed arrows*. The genes encoding the following enzymes and associated with each biosynthetic step are listed next to the arrows: (*1*) CDP-diacylglycerol synthase, (*2*) PS synthase, (*3*) PS decarboxylase, (*4*) PGP synthase, (*5*) PGP phosphatases, (*6*) CL synthases, (*7*) PG:MDO *sn*-glycerol-1-P transferase, (*8*) diacylglycerol kinase, (*9*) glucosyl diacylglycerol synthase (*Acholeplasma laidlawii*), (*10*) diglucosyl diacylglycerol synthase (*A. laidlawii*), (*11*) PC synthase (*Legionella pneumophila*), (*12*) PI synthase (*S. cerevisiae*), and (*13*) *O*-Lysyl PG synthase (*Staphylococcus aureus*)

include sulfolipids, glycolipids, and ornithine-containing lipids. A few bacteria also contain sphingolipids. This diversity is found in both Gram-negative and Gram-positive bacteria. The analysis of the function of this diverse collection of lipids over many bacteria is well beyond the scope of this review, which will focus on *E. coli* with occasional reference to other lipids not found in *E. coli*. *Archaea* phospholipids contain the *sn*-glycerol-1-phosphate rather than the *sn*-glycerol-3-phosphate backbone and ether-linked branched chain polyisoprenes rather than ester-linked fatty acids as found in all other organisms. Details on the structure, synthesis, and distribution of these additional lipids can be found elsewhere (Caforio and Driessen 2016).

Surprisingly, genetic manipulation of *E. coli* leading to or replacing the three major phospholipid classes is not lethal under defined laboratory conditions. However, changes in membrane lipid composition have dramatic effects on cell physiology, which would compromise viability outside of a controlled laboratory setting. Such genetic manipulation does result in compromised growth, which provided the initial clues to important lipid functions (see Dowhan (2013) for an extensive list of references). Null mutants in the *pssA* gene lack all amino-containing and zwitterionic phospholipids (i.e., normally traced amounts of phosphatidylserine (PS) and *N*-acyl-PE and the major lipid PE). Such mutants require millimolar amounts of divalent

cations (Ca^{2+}, Sr^{2+}, or Mg^{2+} but not Ba^{2+}) in the growth medium for viability (DeChavigny et al. 1991). Removal of the divalent ions from the medium results in rapid cell lysis. Cells lacking PE also display a defect in a late step in cell division resulting in elongated cells with multiple genomes separated by assembled divisomes that are arrested prior to cell envelope constriction and cell division (Mileykovskaya et al. 1998). These mutants show extensive defects in the active uptake of many sugars and amino acids dependent on secondary transporters energetically linked to the membrane potential. However, membrane potential is normal in these mutants (Bogdanov and Dowhan 1995). Mutants in the *psd* gene, which also have similar growth properties to cells lacking PE, accumulate PS at the expense of PE indicating that the growth phenotypes are due to the lack of PE and/or accumulation of PG and CL.

Null mutants in the *pgsA* gene are lethal, but lethality can be suppressed by a secondary mutation in the *lpp* gene, which encodes the major outer membrane lipoprotein (Shiba et al. 2004). The C-terminus of a subset of this protein is covalently attached to the peptidoglycan structure that resides in the periplasm of Gram-negative bacteria and provides the structural rigidity and shape of the cell envelope. The N-terminal cysteine is in sulfoether linkage to a diacylglycerol derived from PG. Phospholipids are also general donors of a fatty acid in aminoacyl linkage to the N-terminus. This lipid structure is embedded in the inner leaflet of the outer membrane. Failure to lipidate the N-terminus of the lipoprotein results in its lethal accumulation in the inner membrane. The *pgsA lpp* double mutant is also temperature sensitive for growth, which further complicated sorting out the function of PG beyond a membrane bilayer building block. The outer membrane of Gram-negative bacterial contains numerous lipoproteins with the same lipidated N-terminal structure derived from PG. Therefore, none of these proteins become properly lipidated in *pgsA* null mutants. Unlike the *lpp* gene product, most of these lipoproteins are expressed at low levels and appear not to be essential for viability. The temperature sensitivity of *pgsA* mutants appears to be due to the unlipidated *nlpE* gene product trapped in the inner membrane (Nagahama et al. 2006; Konovalova et al. 2016). This protein is normally a sensory of outer membrane mis-assembly, which activates the Rcs stress response system. Temperature sensitivity appears to be due to up regulation of this stress response system at elevated temperatures. Whether anionic phospholipids are absolutely required for cell viability has not been established since the precursors (phosphatidic acid (PA) and CDP-diacylglycerol) to PG and CL are all anionic phospholipids that accumulate to about 10% of total phospholipid content in mutants lacking PG and CL. Such mutants also have elevated levels of *N*-acyl-PE, which is also anionic (Mileykovskaya et al. 2009). Any mutation prior to the formation of CDP-diacylglycerol is lethal most likely due to the failure to form a suitable lipid bilayer.

Mutants in phospholipid metabolism uncovered an important role for membrane lipids as the source of components of other cell envelope constituents. Ethanolamine phosphate that decorates the core of lipopolysaccharide (Reynolds et al. 2005) as well as the membrane-derived oligosaccharide (MDO) of the inner membrane space (an osmolarity regulator) comes from PE (Miller and Kennedy 1987). The

source of glycerophosphate modification of MDO is PG. All these functions of PE and PG are important in the bigger picture of *E. coli* survival over time but are not absolutely essential for cell viability when growth medium is appropriately adjusted.

Cells lacking CL show very weak phenotypes most likely because PG can substitute for most functions. Initially, *E. coli* was thought to have only one gene encoding a CL synthase; the *clsA* gene product catalyzes the condensation of two PG molecules to form CL and glycerol. More detailed analysis of the *clsA* null mutant uncovered two additional synthase genes (*clsB* and *clsC*) (Tan et al. 2012). The *clsC* gene product condenses PE and PG to make CL plus ethanolamine. The *clsB* gene product condenses two PGs to make CL and also can convert PE to PG by a head group exchange reaction. This latter result explains the presence of low amounts of PG in a *pgsA* null strain (Li et al. 2016). The latter two CL synthases contribute significant CL in late log to stationary phase, while the majority of CL during exponential growth is dependent on *clsA*. Interestingly *clsABC* or *pssA* null mutants are viable, but cells unable to make both PE and CL are not viable (Bogdanov and Dowhan, unpublished). This suggests a common unknown interchangeable function for these two phospholipids. Why *E. coli* and now many other bacteria possess multiple CL synthases is not known. In *Bacillus* species one of the multiple CL synthases is necessary for sporulation (Kawai et al. 2004). *Staphylococcus aureus* requires CL for survival during prolonged exposure to high salt conditions (Tsai et al. 2011).

3 Incorporation of Foreign Lipids into *E. coli*

The surprising resilience of *E. coli* to major changes in its normal lipid composition extends to the incorporation of foreign lipids into the membrane (Dowhan and Bogdanov 2012). Depending on the physical and chemical properties of these latter lipids, partial correction of mutant phenotypes is observed. Such complementation provides clues as to which properties of lipids are important for a particular cellular function. The expression of the *PIS* gene of *S. cerevisiae* (see Fig. 1) in *E. coli* with supplementation of inositol results in about 10–15% PI in cell membranes (Xia and Dowhan 1995b). A series of net neutral or positively charged phospholipids can be introduced into *E. coli* wild-type and PE-lacking strains. The neutral glycolipids mono- and diglucosyl diacylglycerol reach 30–40% of total membrane lipids by the expression of either the *mgs* or *mgs* plus *dgs* gene, respectively, of *Acholeplasma laidlawii* (Wikstrom et al. 2009). The expression of the *pcs* gene product of *Legionella pneumophila* in *E. coli* supplemented with choline results in about 30–40% PC in the wild-type cells and up to 70% PC in cells lacking PE (Bogdanov et al. 2010). The expression of the *mprF* gene product of *Staphylococcus aureus* results in 30–40% of the total lipid being positively charged *O*-lysyl-PG (Bogdanov and Dowhan, unpublished). The above modified *E. coli* strains are useful in defining the function of lipids unique to other bacterial systems that are not easily manipulated genetically.

4 Lipids as Determinants of Membrane Protein Organization

A common architectural feature of polytopic α-helical MPs is their topological organization, i.e., the number of transmembrane domains (TMDs) and extramembrane domains (EMDs) and their orientation with respect to the plane of the membrane lipid bilayer. A fundamental question in the generation of MP topology is how the final structure is attained in a given lipid environment. Dogma dictates that MP topology once formed is fixed and is determined solely at the time of initial assembly by interplay among the ribosome, topogenic signals residing in MP sequences, MP insertion machinery, and interactions within a MP. The properties of the lipid bilayer, other than a hydrophobic solvent for TMDs, have not been generally considered as important. The ability to genetically manipulate *E. coli* membrane composition at steady state and dynamically during the cell growth uncovered a specific role for lipids in determining MP organization (Bogdanov et al. 2014). Early studies of purified lactose permease of *E. coli* (LacY, *lacY* gene protein responsible for energy-dependent update of lactose) indicated an absolute requirement for PE to support energy-dependent uphill transport of substrate after reconstitution into proteoliposomes (Chen and Wilson 1984). Cells lacking PE display the same transport phenotype (Bogdanov and Dowhan 1995), which was extended to several other secondary transport systems coupled to the proton electrochemical potential.

Further investigation of this partial loss of function in the absence of PE revealed an inversion of the N-terminal six TMD helical bundle of LacY with respect to the remaining TMDs and the plane of the lipid bilayer (Fig. 2). Similar inversions were observed for other secondary substrate transporters of *E. coli*. By placing the *pssA* gene under control of the anhydrotetracylcine dose-dependent *tet* promoter, the level of PE in cells can be controlled at variable steady-state levels or changed in a dynamic manner during cell growth. The ratio of native to inverted LacY topological conformer was found to be directly proportional to the level of PE in membranes. More surprising is that a change in PE content post-assembly of LacY resulted in a change in the ratio of native to inverted conformer (Bogdanov et al. 2008; Bogdanov and Dowhan 2012). Therefore, MP structural organization is highly dynamic and responsive to the lipid environment during initial membrane insertion as well as after final folding.

The dependence of LacY topological organization can be fully replicated in a reconstituted proteoliposome system solely dependent on protein sequence and lipid environment (Vitrac et al. 2013a, 2015). The rate of topological inversion in response to a change in lipid environment in vitro occurs on a time scale of seconds. Thus, MP folding and dynamic rearrangement post-assembly are thermodynamically driven processes dependent on inherent lipid-MP interactions that do not necessarily require other cellular factors including the MP assembly machinery or metabolic energy. MP topological organization is therefore potentially responsive to changes in the lipid environment at any time and in any membrane, not only in bacteria but throughout nature.

What is the molecular basis for the effects of lipid composition on MP topological organization? Statistical data, supported by experimental results, show that 85% of

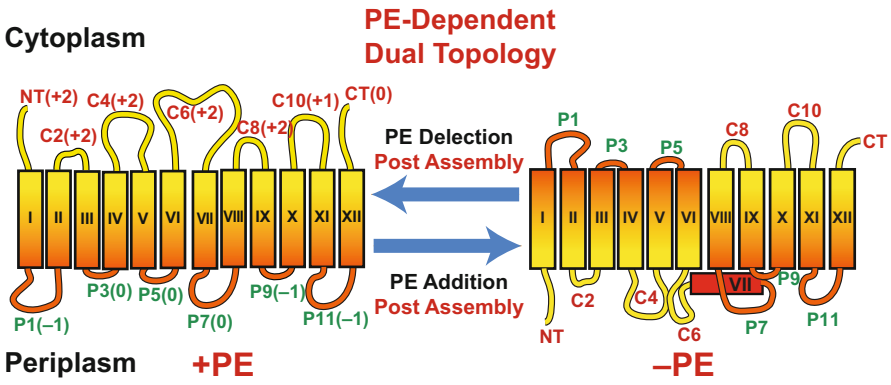


Fig. 2 Topological organization of LacY as a function of membrane lipid composition. TMDs (*Roman numerals*) and EMDs (*Arabic numerals*) are sequentially numbered from the N-terminus to C-terminus with EMDs exposed to the periplasm (*P*) or cytoplasm (*C*) as in wild-type cells. Net charge of EMDs is shown. Topology of LacY is shown after initial assembly in PE-containing cells (+PE) or after initial assembly in PE lacking (−PE). The interconversion of topological conformers and the ratio of native to inverted conformer are reversible in both directions depending on the dynamic level of PE in membranes

EMDs exposed to the cytoplasm have a net positive charge, while those exposed to the trans side of the membrane are either net negative or neutral. These observations are summarized in the Positive-Inside Rule that governs the orientation of TMDs neighboring EMDs (von Heijne 2006). The Charge Balance Rule, as an extension of the Positive-Inside Rule, incorporates the effect of membrane lipid composition on MP topology (Bogdanov et al. 2014). Several neutral glycerol-based glycolipids (Xie et al. 2006; Wikstrom et al. 2009), the zwitterionic-lipid PC (Bogdanov et al. 2010) or the net-positive lipid *O*-lysyl-PG (Bogdanov and Dowhan, unpublished) fully substitute for PE in supporting LacY native topology when introduced into *E. coli* cells or included in proteoliposome reconstitution systems (Vitrac et al. 2013a, b). The common physicochemical feature of these lipids is their ability to dilute the high negative charge density of the membrane surface contributed by PG and CL rather than whether they are bilayer- or non-bilayer-prone lipids. Cytoplasmically oriented EMDs containing a mixture of negative and positive residues appear to be most sensitive to negative charge density on the membrane surface. In the case of several secondary transporters, the effect of interfacial protein and lipid charges is cooperative and cumulative since a progressive increase in the negative charge density of the membrane surface or a progressive increase in the negative charge of EMDs normally exposed to the cytoplasm favors the inverted MP topology in cells (Bogdanov et al. 2008; Vitrac et al. 2011) and proteoliposomes (Vitrac et al. 2013a, b). Native topology is observed with increasing positive charges in EMDs or decreasing negative charge density of the membrane surface. Coexistence of native and inverted forms of a MP is observed at intermediate EMD and membrane surface charge character (Bogdanov and Dowhan 2012). Long-range intramolecular interactions also play a role in the sensitivity to lipid environment. In the case of LacY,

TMD VII of low hydrophobicity becomes exposed to the periplasm in the inverted conformer. Increasing the hydrophobicity of TMD VII prevents its exposure to the solvent in PE-lacking cells (Bogdanov et al. 2008), while the high hydrophobicity of TMD VII appears to desensitize the sucrose permease topology to changes in membrane lipid composition (Vitrac et al. 2011). This complex interplay between charges of EMDs, lipid environment, and long-range interactions within a MP probably explains why only a subset of MPs are topologically responsive to changes in their lipid environment.

A change in the membrane surface charge properties can clearly induce a rapid change in MP topological organization. According to the Charge Balance Rule, a change in the charge nature of EMDs should also result in similar post-assembly topological changes. Post-assembly phosphorylation of proteins resulting in changes in or modulation of function is widespread throughout nature. Phosphorylation of an EMD of a MP has been assumed to stabilize topological organization due to the high activation energy associated with moving a charged residue through the lipid bilayer. However, phosphorylation of an EMD domain of LacY in a proteoliposome with a native lipid composition was shown to result in a rapid topological inversion at a rate comparable to lipid-induced inversion (Vitrac et al. 2017). Such topological inversions could change on which side of a membrane an active site is exposed as was postulated for CD38 of mammalian cells (Zhao et al. 2014) or even change the function of a MP. Interestingly, membrane lipid composition and physical properties of the lipid bilayer affected the rate and extent of phosphorylation-induced topological changes for LacY.

Such TMD rearrangements constitute an entirely novel lipid-dependent biological switch for the generation of functional and structural heterogeneity of a single polypeptide chain. An increasing number of examples is being reported of proteins that have dual topologies or appear to change their topological orientation depending on their location in cells or during cell differentiation (Dunlop et al. 1995; Levy 1996; Swameye and Schaller 1997; Moise et al. 2004; Moser et al. 2013; Kainulainen and Korhonen 2014; Zhao et al. 2014; Herate et al. 2016). The above results provide a molecular basis for the existence of proteins with multiple topological organizations and of "moonlighting proteins" (i.e., proteins with multiple functions, many of which have multiple locations within cells), which increase gene pleiotropy. In eukaryotic cells, MPs could potentially undergo TMD flipping in response to changes in the local lipid environment, which occurs during cell division, membrane fission and fusion, and vesicular trafficking. Cell membranes throughout nature contain domains of different lipid composition, which could affect MP organization within the same membrane.

5 Membrane Domains Enriched in Anionic Phospholipids

As observed in eukaryotic cells, bacterial membranes display a heterogeneous distribution of proteins and lipids (Matsumoto et al. 2015). Domains enriched in specific lipids provide a means to localize and segregate cellular process directed by

integral and peripheral MPs. Lipophilic fluorescent probes were used to show heterogeneous distribution of PG and PE in *E. coli* (Vanounou et al. 2003) and phospholipid domains in mycobacteria (Christensen et al. 1999). The fluorescent dye 10-*N*-nonyl-3,6-bis(dimethylamino)acridine (NAO), when used in low and limiting amounts, specifically binds to anionic phospholipids generating a green fluorescence. Binding to CL elicits both a green and red fluorescence (Mileykovskaya and Dowhan 2009). Using fluorescent microscopy, NAO was shown to bind to nascent septal division sites, and with time these sites matured into polar domains after cell division (Mileykovskaya and Dowhan 2009; Matsumoto et al. 2015). Polar localization of CL was verified by CL enrichment of minicells (Koppelman et al. 2001), which are released from the polar region of Δ*minCDE* cells. Similar localization of CL was observed in *Bacillus subtilis* during exponential growth and sporulation (Kawai et al. 2004). Viable *pgsA* null mutants, which lack both PG and CL, showed only septal and polar green fluorescence when treated with low levels of NAO indicating an enrichment of anionic lipids. Minicells isolated from a Δ*pgsA* Δ*minCDE* cell showed an enrichment in PA and *N*-acyl-PE over whole cells (Mileykovskaya et al. 2009). These lipids are elevated in cells lacking PG and CL. PA and *N*-acyl-PE like CL (in the presence of divalent cations, see below) are considered anionic non-bilayer-prone lipids due to their cone shape in contrast to PG, which occupies a cylindrical space. When present in a bilayer such lipids induce membrane curvature, which has been postulated as either the driving force for membrane curvature or their favorable location at sites of membrane curvature. Therefore, *E. coli* appears to have a mechanism for localization of anionic non-bilayer-prone lipids to the polar/septal highly curved regions.

6 Are Non-bilayer-Prone Lipids Essential for Cell Viability?

Non-bilayer-prone lipids undergo a transition in aqueous solution from a bilayer organization to various non-bilayer arrangements as the temperature is increased. Although total non-bilayer organization would compromise the barrier function of membranes, the presence of non-bilayer-prone lipids within a lipid bilayer results in lateral stress, which can affect the properties of integral MPs (see (Dowhan 1997) for a summary). The major non-bilayer-prone lipids in *E. coli* are PE and CL. However, the majority of PE molecular species contain one saturated and one unsaturated fatty, which places the beginning of their transition from bilayer to non-bilayer above 55 °C (Killian et al. 1992). The CL transition to the non-bilayer phase occurs at a similar temperature only in the presence of divalent cations such Ca^{2+}, Sr^{2+}, and Mg^{2+} but not Ba^{2+} (Killian et al. 1994). However, *E. coli* total lipid extracts begin to undergo this transition at about 42 °C (Killian et al. 1992). The following results with *E. coli* cells lacking PE suggest that the presence of lipids whose beginning transition temperature is above but near the optimal growth temperature for *E. coli* of 37 °C is of biological significance.

Viability and growth of cells lacking PE are supported by millimolar levels of Ca^{2+}, Sr^{2+}, and Mg^{2+} but not Ba^{2+} in the growth medium (DeChavigny et al. 1991;

Rietveld et al. 1993, 1994). Ca^{2+} is a more effective inducer of the non-bilayer phase of CL than is Mg^{2+}. Ca^{2+} is required at significantly lower levels than Mg^{2+} to support growth of Δ*pssA* cells, which also contain with lower levels of CL during exponential growth in the former versus the latter cation. Phospholipids extracts resuspended in the same divalent cation in which the cells were grown displayed a bilayer to non-bilayer transition pattern similar to phospholipids extracted from wild-type cells determined in the absence of divalent cations, i.e., beginning near 42 °C with a midpoint transition temperature (T_m) at 55 °C. However, lipids from Mg^{2+}-grown cells when analyzed in the presence of Ca^{2+} displayed a T_m at 40 °C. Lipids from Ca^{2+}-grown cells when analyzed in the presence of Mg^{2+} displayed a T_m above 75 °C, which is consistent with the respective differences in CL levels and strength of non-bilayer induction by these two cations. Ba^{2+} did not induce a phase transition at any temperature or support growth of cells lacking PE. Thus, there is a close correlation between the ability of divalent cations to support growth of PE-lacking cells and the T_m of the cation-dependent induction of the non-bilayer phase.

7 Functions of Anionic Lipids and Lipid Domains

Requirements for PG and CL have been more difficult to establish since the anionic character of these lipids is more important in bacteria than the specific structure of the head group. As noted earlier, total elimination of net negatively charged lipids is not possible in vivo. Therefore, much of the evidence comes from in vitro experiments complemented by partially evident in vivo phenotypes observed for *B. subtilis* and *E. coli*. Early studies in cells with reduced levels of PG and CL indicated a slower rate of SecA-dependent export of proteins across the inner membrane to the periplasm (de Vrije et al. 1988). Follow-up studies in vitro confirmed that SecA specifically binds to and is activated by anionic lipids in membranes (Lill et al. 1990; Hendrick and Wickner 1991). CL is tightly associated with the SecYEG protein membrane translocation machine (Gold et al. 2010). It stabilized the dimer and provides a binding site for SecA. CL and subsequently PG was shown in vitro and indirectly in vivo to rejuvenate DnaA-dependent initiation of new rounds of DNA replication in order for cells to progress along the cell cycle (Xia and Dowhan 1995a). These studies indicated that anionic lipids form binding sites for peripheral MPs followed by their activation.

Although PE-lacking inverted membrane vesicles of *E. coli* are highly enriched in PG and CL, they were found not to support SecA/SecY-dependent import of proteins unless divalent cations were added to the lumen of the vesicles (Rietveld et al. 1995); as noted above, SecA function is dependent on anionic phospholipids. The supplementation of these vesicles with non-bilayer prone but not bilayer-prone PE species restored import function. These results support a requirement for the presence of non-bilayer-prone lipids for proper function of the protein translocation machinery. These results are also consistent with the effectiveness of Ca^{2+}, which is excluded from the cytoplasm, in supporting viability of PE-lacking cells. Ba^{2+}, which does not

induce non-bilayer properties of CL, does not support viability of PE-lacking cells or protein import in PE-lacking membrane vesicles.

A role for anionic lipid domains in various cell functions comes from the localization of proteins within the septal and distal regions of cells, which are enriched in anionic phospholipids. A recent review provides extensive details on the function of discontinuous lipid domains in mostly *B. subtilis* and *E. coli* (Matsumoto et al. 2015), so only a few examples are summarized here. In the former organism, both PE and CL appear to be enriched at the septum where essentially all the core enzymes responsible for phospholipid synthesis have been co-localized (Nishibori et al. 2005). No similar concentration of PE has been seen in *E. coli*. However, several proteins have been localized to the poles of *E. coli* cells with varied dependence on cellular CL levels (Romantsov et al. 2010).

A common mode of interaction of peripheral proteins with anionic lipid-enriched domains is through an amphipathic positively charged helix at either the amino or carboxyl terminus of a protein that serves as a membrane targeting sequence. The hydrophobic face of the helix appears to insert into the bilayer and is stabilized by the interaction of the positively charged face with anionic phospholipid head groups. MinD of *E. coli* is an example of such a protein (reviewed in Matsumoto et al. (2015)). MinD, in association with MinC and ATP, binds to the cell poles enriched in anionic phospholipids through its amphipathic helix and prevents organization of the FtsZ-based divisome at the cell poles. The interaction of MinCD with polar-localized MinE activates MinD ATPase activity resulting in dissociation of MinCD polymers from the cell poles. Fluorescently tagged MinD can be visualized cycling between the cell poles. In vitro studies support the affinity of MinD-ATP for anionic phospholipid domains. Cells lacking PE and enriched in CL and PG display aberrant MinD cycling with an increased membrane-associated dwell time associated presumably due to increased anionic lipid content (Mileykovskaya et al. 2003). Cells lacking PG and CL display normal division properties but are now enriched in PA and *N*-acyl-PE at the poles (Mileykovskaya et al. 2009).

8 Lipid Metabolism and Genetics in *S. cerevisiae*

The pathways for synthesis of the major phospholipids in *S. cerevisiae* (Fig. 3) are very similar, with some exceptions, to those in bacteria (Henry et al. 2012). All the proteins are encoded by nuclear genes. Yeast has two genes encoding CDP-diacylglycerol synthase. The *CDS1* gene product is located in the endoplasmic reticulum and supports PI synthesis (Shen and Dowhan 1996; Shen et al. 1996) in the endoplasmic reticulum. The *TAM41* gene product is in the inner mitochondrial membrane (Tamura et al. 2013) and provides CDP-diacylglycerol for PG and CL synthesis. *PSD1*-encoded phosphatidylserine decarboxylase (Clancey et al. 1993; Trotter et al. 1993) is also localized to the mitochondria and is the primary source of mitochondrial PE derived from PS imported from the endoplasmic reticulum (Kannan et al. 2015). There is a second *PSD2* gene product localized to the Golgi/endosome system (Kannan et al. 2015). Yeast has only one PGP phosphatase (encoded by *GEP3*) localized to the mitochondria. There is only

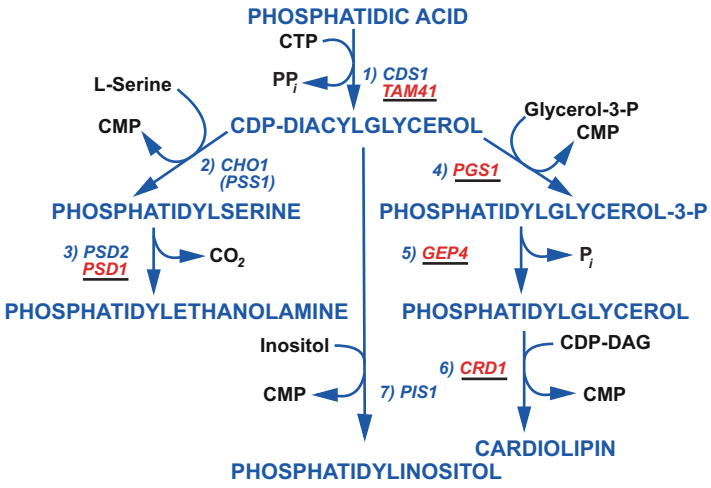


Fig. 3 Pathways for phospholipid synthesis in *S. cerevisiae*. Genes encoding the enzymes responsible for each step are indicated. Those nuclear genes encoding enzymes localized to the mitochondria are underlined. The remaining gene products are localized to the endoplasmic reticulum

one CL synthase (encoded by *CRD1*) in yeast, and it adds a phosphatidic acid moiety derived from CDP-diacylglycerol to PG rather than condensing two PG molecules. In addition to the PS synthase (encoded by *PSS1* formally denoted as *CHO1*) route to PE synthesis, yeast utilizes the "Kennedy Pathway" for synthesis of PE and PC outside of the mitochondria starting with ethanolamine and choline. Analogous to the mammalian pathway, the two amines are first phosphorylated and converted to CDP-alcohol substrates, which are the donors of the phosphorylated alcohol amines to diacylglycerol. The latter is derived from PA by a specific phosphatase (encoded by the *PAH1* gene). In addition, PE can be methylated using *S*-adenosyl-methionine to form PC by sequential action of the *CHO2/PEM1* and *OPI3/PEM2* gene products.

There is only limited sequence homology at the protein level with the bacterial enzymes even though the substrates and products are very similar. The *E. coli pssA*, *pgsA*, and *psd* gene products show variable levels of homology with the *PGS1*, *CRD1*, and *PSD1* gene products of yeast. The yeast *CHO1/PSS* gene product (PS synthase) has homology with the *B. subtilis pss* gene product. The latter two enzymes, which require Mg^{2+} for activity, are integral MPs and proceed by a different mechanism than the *E. coli* peripheral membrane *pssA* gene product (Raetz et al. 1987), which has no divalent cation requirement.

Null mutants in the *PIS1* gene are lethal (Nikawa et al. 1987) presumably due to the importance of PI polyphosphorylated derivatives as signaling molecules and as a precursor to yeast sphingolipids (Megyeri et al. 2016). PS appears not to be essential as long as the medium of a *PSS1* null mutant is supplemented with ethanolamine or choline, which can be used by the Kennedy Pathways for PE or PC synthesis, respectively. Although PC makes up about 60% of the total membrane phospholipid of yeast, neither mono-, di-, nor trimethylated (PC) PE appears to be required

(Choi et al. 2004). Null mutants of *PEM1* and *PEM2* can only make PC via the Kennedy Pathway dependent on choline-supplemented media. Propanolamine (Prn) can be incorporated in place of choline into membrane phospholipids. The resulting phosphatidyl propanolamine (PPrn) has the chemical and physical properties of PE (i.e., a primary amine, zwitterionic, and non-bilayer-prone lipid). Yeast in which PPrn completely replaced PC grew slower but was otherwise normal for growth on fermentable and non-fermentable carbon sources, which also indicated normal mitochondrial function. However, also blocking the majority of PE synthesis by adding a null mutation in the *PSD1* gene resulted in the lack of growth on a non-fermentable carbon source. This is consistent with a very specific requirement for PE in mitochondrial function (discussed below), while PPrn can substitute in functions other than those of the mitochondria.

Complete depletion of yeast PE requires multiple mutations. The elimination of the *PSD1* and *PSD2* gene products reduces PE levels significantly, but the cells still contain low levels of PE and remain viable. The remaining source of PE comes from the release of ethanolamine by the *DPL1* gene product that is a sphingosine lyase. Mutants null in all three genes require ethanolamine for viability on any carbon source indicating a strict requirement for PE (rather than PC) even outside of the mitochondrial requirement (Storey et al. 2001). The requirement for PE during cell division extends beyond the initial observation in *E. coli,* where PE-deficient cells are compromised in the final stages of cell division (DeChavigny et al. 1991; Mileykovskaya et al. 1998). PE is exposed to the surface of yeast (Iwamoto et al. 2004) and mammalian (Emoto et al. 1996) cells at the cleavage furrow during cell division. If the PE is immobilized on the outer surface of cells, cell division is blocked until the PE is free to move to the inner leaflet of the cell membrane.

9 Anionic Phospholipid Functions in Mitochondria

In all eukaryotes CL is found only in the mitochondria. PG is also restricted to the mitochondria except for its presence in the complex mixture of proteins and lipids making up surfactant exported from type II alveolar cells of the lung (Kandasamy et al. 2016). PG makes up only about 1% of mitochondrial phospholipid, while CL is about 20%. The difference in this ratio from bacteria is due to the final step of eukaryotic CL biosynthesis being energetically irreversible in contrast to the step in bacteria, which is reversible. Null mutants in the *PGS1* gene lack PG and CL, grow normally on a fermentable carbon source, are not viable on a non-fermentable carbon source, and contain morphologically and functionally impaired mitochondria (Chang et al. 1998a). The severe defects in mitochondria appear to be due to the lack of translation of mitochondrial encoded mRNAs and one nuclear-encoded mRNA that produce essential subunits for Complexes III (cytochrome bc_1) and IV (cytochrome *c* oxidase) of the mitochondrial respiratory chain (Ostrander et al. 2001). Although the molecular basis is not known, *PGS1* null mutants display a defect in cell wall synthesis resulting in the loss of cell integrity at elevated temperatures suggesting an indirect role for mitochondrial anionic phospholipids

in cell wall synthesis (Zhong et al. 2005). Null mutants in the *CRD1* gene are viable, accumulate PG to the levels normally seen for CL, display a reduced inner mitochondrial membrane potential, and grow normally on a fermentable carbon source and at a reduced rate and to a lower cell density on a non-fermentable carbon source (Jiang et al. 1997, 2000; Chang et al. 1998b). Therefore, loss of CL compromises but does not eliminate energy produced by mitochondrial oxidative phosphorylation.

The fatty acids of CL in yeast, as in mammalian cells, are remodeled from that reflecting the overall complex phospholipid fatty acid composition of the cell to one of a much narrower composition highly enriched in unsaturated fatty acids (Ye et al. 2016; Schlame and Greenberg 2017). This remodeling event requires a CL-specific phospholipase encoded by the *CLD1* gene that generates monolyso-CL (MLCL) and the *TAZ* gene product that reacylates MLCL by transferring fatty acids donated by phospholipids. *TAZ* null mutants in yeast display many of the phenotypes of humans with deficiency in the homologous *TAZ1* gene product found in the male-inherited disease Barth Syndrome. The phenotypes are mostly related to mitochondrial oxidative phosphorylation compromised energy production. In both cases CL fails to be remodeled to the normal highly unsaturated species, which was thought to be the molecular basis for the disease. However, *TAZ* mutants also show a significant reduction in the ratio of CL to MLCL suggesting the molecular basis lies in severely reduced CL levels that cannot be compensated for by the increase in MLCL. The latter basis appears to be the case. A *TAZ CLD1* double null of yeast displays normal CL levels (although not remodeled) and CL/MLCL ratios as well as a wild-type mitochondrial phenotype with respect to energy metabolism (Baile et al. 2014; Ye et al. 2014). In spite of the lack of phenotype for *CLD1* null strains, the remodeling of CL initiated by this gene product may be important as a protection against the accumulation of oxidized derivatives of highly unsaturated CL species (Ye et al. 2016).

10 Cardiolipin and Organization of the Respiratory Chain

The respiratory chain is organized within the inner membrane of mitochondria (Mileykovskaya and Dowhan 2015). *S. cerevisiae* lacks the mammalian Complex I (NADH:ubiquinone oxidoreductase), which is replaced by three membrane-associated NADH dehydrogenases, that transfer electrons from NADH to ubiquinone. In contrast to Complex I, these latter dehydrogenases are not proton pumps. In both cases ubiquinone shuttles electrons to Complex III followed by the transfer of electrons to Complex IV via cytochrome *c*. Complexes III and IV are proton pumps that are responsible for generating the inner mitochondrial proton electrochemical gradient. Although these individual complexes were originally postulated to be organized into higher-order supercomplexes (Chance and Williams 1955), it was not until methods such as blue native polyacrylamide gel electrophoresis (BN-PAGE) were developed that these complexes solubilized by gentle detergents such as digitonin were visualized as organized respirasomes (Schagger and Pfeiffer 2000). The stoichiometry in yeast is III_2IV_2 and in mammalian cells is $I_1III_2IV_1$. There is very high homology between the yeast and mammalian Complexes III and

IV even though the yeast Complex III contains only ten instead of 11 subunits, and the yeast Complex IV contains only 11 instead of 13 subunits. Both complexes contain several phospholipids tightly integrated within their respective subunit structure. The yeast Complex III homodimer contains four CLs, two PIs, six PEs, and two PCs (Hunte 2005). The mammalian Complex IV monomer contains two CL, three PEs, one PC, and four PGs (Shinzawa-Itoh et al. 2007). In yeast *CRD1* null strains Complexes III and IV remain intact and fully functional suggesting that the elevated levels of PG functionally and structurally substitute for CL. Such a conclusion is also consistent in the absence of PG and CL resulting in the lack of Complexes III and IV in the mitochondria (Ostrander et al. 2001).

If Complexes III and IV are functional in the absence of CL, what then is the basis for the reduced efficiency of energy production in the mitochondria? The answer appears to be in the reduced ability to form the respirasome higher-order supercomplexes of Complexes III and IV. Indeed, there is a CL dose-dependent formation of the III_2IV_2 supercomplex as the level of CL is increased from zero to full levels in yeast mitochondria (Zhang et al. 2002). Furthermore, a kinetic investigation of electron transfer from ubiquinone to oxygen in whole mitochondria demonstrated cytochrome c-mediated channeling of electrons between Complexes III and IV in wild-type mitochondria and the lack of channeling in the absence of CL (Zhang et al. 2005). This is consistent with the lack of supercomplex formation in the absence of CL in situ. Although such higher-order organization appears not to be essential, it contributes to the efficiency derived from close contact of related processes. The isolated III_2IV_2 supercomplex contains about 50 CL molecules, which is in large excess of the eight molecules integrated into the individual complexes (Mileykovskaya et al. 2012). Imaging of the supercomplex by cryo-electron microscopy (Mileykovskaya et al. 2012) revealed that the Complex III homodimer is flanked on each side by a Complex IV monomer (IV-III_2-IV). The interfaces between Complexes III and IV, which lie within the lipid bilayer, are separated by significant "empty spaces" in the density map that are postulated to be filled with phospholipid including CL. Therefore, an important role for CL over and above PG is in "gluing" together individual respiratory complexes into the supermolecular organization of the respirasome.

This conclusion is further supported by the absolute requirement for CL in the reconstitution of the individual purified Complexes III and IV (stripped of their excess CL) into the tetrameric supercomplex in vitro (Bazán et al. 2013). This reconstitution is independent of the fatty acid composition of CL employed, which further supports reduced total CL levels rather than lack of CL remodeling in Barth Syndrome and yeast *TAZ* null mutants as the molecular basis for observed phenotypes. A mostly unrecognized difference between the resulting phenotypes of *TAZ* and *CRD1* null mutants, both of which affect CL levels, is that *CRD1* mutants in contrast of *TAZ* mutants contain high levels of PG that partially compensates for reduced CL levels.

CL is also required for other mitochondrial processes, which are compromised in *CRD1* null strains. The ADP/ATP carrier (*ACC2* gene product) of the inner mitochondrial membrane has a near absolute requirement in vitro for CL for which PG can compensate (Claypool 2009). However, the association of the carrier with the

III_2IV_2 supercomplex is CL dependent. The lack of CL also results in compromised acetyl-CoA synthesis in yeast mitochondria due to a CL requirement by pyruvate dehydrogenase (Raja et al. 2017). The ability of CL to organize higher-order structures in the mitochondria most likely extends to additional mitochondrial, as well as bacterial systems.

11 Phosphatidylethanolamine and Mitochondrial Function

As noted earlier, the lack of mitochondrial PE or CL compromised mitochondrial ability to grow on a non-fermentable carbon source, but cells still remained viable if grown on glucose. However, a null mutation in *PSD1* was found to be synthetically lethal with a null *CRD1* mutation (Baker et al. 2016). Thus, lacking the ability to make PE and CL in the mitochondria not only compromises mitochondria function but also cell viability. Interestingly similar synthetic lethality was observed in *E. coli* unable to make PE and CL. Since PE is also integral to the structure of mitochondrial respiratory complexes, its absence is understandably detrimental to normal mitochondrial function. Depletion of mitochondrial PE via a *PSD1* null mutation results in the loss of growth on a non-fermentable carbon source, defects in assembly of mitochondrial protein complexes, and loss of mitochondrial DNA (Horvath and Daum 2013). Supplementation with ethanolamine restores limited growth on a non-fermentable carbon source. Outer mitochondrial MP import and assembly are also compromised. Although the lack of PE, especially in mitochondria, results in multiple defects, there have been few studies that illuminate the precise molecular details of these PE-dependent phenotypes.

12 Research Needs

The diverse ways in which lipids modulate and support cellular processes was only partially addressed. Emphasis was placed on systems in which detailed genetic and biochemical studies have been possible to address molecular detail. The examples reviewed should provide a framework to extend such studies to less well-characterized systems. Similar motifs for lipid involvement in mammalian systems, plants and pathogenic bacteria should exist. The unique lipid composition of each system must be taken into account, and new studies will illuminate unrecognized roles for lipids. The systems reviewed illustrate the importance of involving studies on the role lipid environment plays in any membrane-related process.

References

Baile MG, Sathappa M, Lu YW, Pryce E, Whited K, McCaffery JM, Han X, Alder NN, Claypool SM (2014) Unremodeled and remodeled cardiolipin are functionally indistinguishable in yeast. J Biol Chem 289:1768–1778

Baker CD, Basu Ball W, Pryce EN, Gohil VM (2016) Specific requirements of nonbilayer phospholipids in mitochondrial respiratory chain function and formation. Mol Biol Cell 27:2161–2171

Bazán S, Mileykovskaya E, Mallampalli VK, Heacock P, Sparagna GC, Dowhan W (2013) Cardiolipin-dependent reconstitution of respiratory supercomplexes from purified *Saccharomyces cerevisiae* complexes III and IV. J Biol Chem 288:401–411

Bogdanov M, Dowhan W (1995) Phosphatidylethanolamine is required for *in vivo* function of the membrane-associated lactose permease of *Escherichia coli*. J Biol Chem 270:732–739

Bogdanov M, Dowhan W (2012) Lipid-dependent generation of dual topology for a membrane protein. J Biol Chem 287:37939–37948

Bogdanov M, Xie J, Heacock P, Dowhan W (2008) To flip or not to flip: lipid-protein charge interactions are a determinant of final membrane protein topology. J Cell Biol 182:925–935

Bogdanov M, Heacock P, Guan Z, Dowhan W (2010) Plasticity of lipid-protein interactions in the function and topogenesis of the membrane protein lactose permease from *Escherichia coli*. Proc Natl Acad Sci U S A 107:15057–15062

Bogdanov M, Dowhan W, Vitrac H (2014) Lipids and topological rules governing membrane protein assembly. Biochim Biophys Acta 1843:1475–1488

Caforio A, Driessen AJ (2016) *Archaeal* phospholipids: Structural properties and biosynthesis. Biochim Biophys Acta. https://doi.org/10.1016/j.bbalip.2016.1012.1006

Chance B, Williams GR (1955) A method for the localization of sites for oxidative phosphorylation. Nature 176:250–254

Chang SC, Heacock PN, Clancey CJ, Dowhan W (1998a) The *PEL1* gene (renamed *PGS1*) encodes the phosphatidylglycerophosphate synthase of *Saccharomyces cerevisiae*. J Biol Chem 273:9829–9836

Chang SC, Heacock PN, Mileykovskaya E, Voelker DR, Dowhan W (1998b) Isolation and characterization of the gene (*CLS1*) encoding cardiolipin synthase in *Saccharomyces cerevisiae*. J Biol Chem 273:14933–14941

Chen CC, Wilson TH (1984) The phospholipid requirement for activity of the lactose carrier of *Escherichia coli*. J Biol Chem 259:10150–10158

Choi JY, Martin WE, Murphy RC, Voelker DR (2004) Phosphatidylcholine and *N*-methylated phospholipids are nonessential in *Saccharomyces cerevisiae*. J Biol Chem 279:42321–42330

Christensen H, Garton NJ, Horobin RW, Minnikin DE, Barer MR (1999) Lipid domains of mycobacteria studied with fluorescent molecular probes. Mol Microbiol 31:1561–1572

Clancey CJ, Chang SC, Dowhan W (1993) Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from *Saccharomyces cerevisiae* by complementation of an *Escherichia coli* mutant. J Biol Chem 268:24580–24590

Claypool SM (2009) Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function. Biochim Biophys Acta 1788:2059–2068

de Vrije T, de Swart RL, Dowhan W, Tommassen J, de Kruijff B (1988) Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes. Nature 334:173–175

DeChavigny A, Heacock PN, Dowhan W (1991) Phosphatidylethanolamine may not be essential for the viability of *Escherichia coli*. J Biol Chem 266:5323–5332

Dowhan W (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids? Annu Rev Biochem 66:199–232

Dowhan W (2009) Molecular genetic approaches to defining lipid function. J Lipid Res 50(Suppl): S305–S310

Dowhan W (2013) A retrospective: use of *Escherichia coli* as a vehicle to study phospholipid synthesis and function. Biochim Biophys Acta 1831:471–494

Dowhan W, Bogdanov M (2012) Molecular genetic and biochemical approaches for defining lipid-dependent membrane protein folding. Biochim Biophys Acta 1818:1097–1107

Dunlop J, Jones PC, Finbow ME (1995) Membrane insertion and assembly of ductin: a polytopic channel with dual orientations. EMBO J 14:3609–3616

Emoto K, Kobayashi T, Yamaji A, Aizawa H, Yahara I, Inoue K, Umeda M (1996) Redistribution of phosphatidylethanolamine at the cleavage furrow of dividing cells during cytokinesis. Proc Natl Acad Sci U S A 93:12867–12872

Gold VA, Robson A, Bao H, Romantsov T, Duong F, Collinson I (2010) The action of cardiolipin on the bacterial translocon. Proc Natl Acad Sci U S A 107:10044–10049

von Heijne G (2006) Membrane-protein topology. Nat Rev Mol Cell Biol 7:909–918

Hendrick JP, Wickner W (1991) SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. J Biol Chem 266:24596–24600

Henry SA, Kohlwein SD, Carman GM (2012) Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. Genetics 190:317–349

Herate C, Ramdani G, Grant NJ, Marion S, Gasman S, Niedergang F, Benichou S, Bouchet J (2016) Phospholipid Scramblase 1 Modulates FcR-Mediated Phagocytosis in Differentiated Macrophages. PLoS One 11:e0145617

Horvath SE, Daum G (2013) Lipids of mitochondria. Prog Lipid Res 52:590–614

Hunte C (2005) Specific protein-lipid interactions in membrane proteins. Biochem Soc Trans 33:938–942

Iwamoto K, Kobayashi S, Fukuda R, Umeda M, Kobayashi T, Ohta A (2004) Local exposure of phosphatidylethanolamine on the yeast plasma membrane is implicated in cell polarity. Genes Cells 9:891–903

Jiang F, Rizavi HS, Greenberg ML (1997) Cardiolipin is not essential for the growth of *Saccharomyces cerevisiae* on fermentable or non-fermentable carbon sources. Mol Microbiol 26:481–491

Jiang F, Ryan MT, Schlame M, Zhao M, Gu Z, Klingenberg M, Pfanner N, Greenberg ML (2000) Absence of cardiolipin in the crd1 null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. J Biol Chem 275:22387–22394

Kainulainen V, Korhonen TK (2014) Dancing to another tune-adhesive moonlighting proteins in bacteria. Biology (Basel) 3:178–204

Kandasamy P, Numata M, Berry KZ, Fickes R, Leslie CC, Murphy RC, Voelker DR (2016) Structural analogs of pulmonary surfactant phosphatidylglycerol inhibit toll-like receptor 2 and 4 signaling. J Lipid Res 57:993–1005

Kannan M, Riekhof WR, Voelker DR (2015) Transport of phosphatidylserine from the endoplasmic reticulum to the site of phosphatidylserine decarboxylase2 in yeast. Traffic 16:123–134

Kawai F, Shoda M, Harashima R, Sadaie Y, Hara H, Matsumoto K (2004) Cardiolipin domains in *Bacillus subtilis* marburg membranes. J Bacteriol 186:1475–1483

Killian JA, Fabrie CH, Baart W, Morein S, de Kruijff B (1992) Effects of temperature variation and phenethyl alcohol addition on acyl chain order and lipid organization in *Escherichia coli* derived membrane systems. A 2H- and 31P-NMR study. Biochim Biophys Acta 1105:253–262

Killian JA, Koorengevel MC, Bouwstra JA, Gooris G, Dowhan W, de Kruijff B (1994) Effect of divalent cations on lipid organization of cardiolipin isolated from *Escherichia coli* strain AH930. Biochim Biophys Acta 1189:225–232

Konovalova A, Mitchell AM, Silhavy TJ (2016) A lipoprotein/beta-barrel complex monitors lipopolysaccharide integrity transducing information across the outer membrane. Elife 5 (e15276):1–17

Koppelman CM, Den Blaauwen T, Duursma MC, Heeren RM, Nanninga N (2001) *Escherichia coli* minicell membranes are enriched in cardiolipin. J Bacteriol 183:6144–6147

Levy D (1996) Membrane proteins which exhibit multiple topological orientations. Essays Biochem 31:49–60

Li C, Tan BK, Zhao J, Guan Z (2016) *In vivo* and *in vitro* Synthesis of Phosphatidylglycerol by an *Escherichia coli* Cardiolipin Synthase. J Biol Chem 291:25144–25153

Lill R, Dowhan W, Wickner W (1990) The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell 60:271–280

Matsumoto K, Hara H, Fishov I, Mileykovskaya E, Norris V (2015) The membrane: transertion as an organizing principle in membrane heterogeneity. Front Microbiol 6:572

Megyeri M, Riezman H, Schuldiner M, Futerman AH (2016) Making Sense of the Yeast Sphingolipid Pathway. J Mol Biol 428:4765–4775

Mileykovskaya E, Dowhan W (2009) Cardiolipin domains in prokaryotes and eukaryotes. Biochim Biophys Acta 1778:2084–2091

Mileykovskaya E, Dowhan W (2015) The role of cardiolipin in mitochondrial supercomplex assembly. In: Louro RO, Diaz-Moreno I (eds) Redox proteins in supercomplexes and signalosomes. Lumina Datamatics, Puducherry, pp 81–105

Mileykovskaya E, Sun Q, Margolin W, Dowhan W (1998) Localization and function of early cell division proteins in filamentous *Escherichia coli* cells lacking phosphatidylethanolamine. J Bacteriol 180:4252–4257

Mileykovskaya E, Fishov I, Fu X, Corbin BD, Margolin W, Dowhan W (2003) Effects of phospholipid composition on MinD-membrane interactions *in vitro* and *in vivo*. J Biol Chem 278:22193–22198

Mileykovskaya E, Ryan AC, Mo X, Lin CC, Khalaf KI, Dowhan W, Garrett TA (2009) Phosphatidic acid and *N*-acyl phosphatidylethanolamine form membrane domains in *Escherichia coli* mutant lacking cardiolipin and phosphatidylglycerol. J Biol Chem 284:2990–3000

Mileykovskaya E, Penczek PA, Fang J, Mallampalli VK, Sparagna GC, Dowhan W (2012) Arrangement of the respiratory chain complexes in *Saccharomyces cerevisiae* supercomplex III2IV2 revealed by single particle cryo-electron microscopy. J Biol Chem 287:23095–23103

Miller KJ, Kennedy EP (1987) Transfer of phosphoethanolamine residues from phosphatidylethanolamine to the membrane-derived oligosaccharides of *Escherichia coli*. J Bacteriol 169:682–686

Moise AR, Grant JR, Lippe R, Gabathuler R, Jefferies WA (2004) The adenovirus E3-6.7K protein adopts diverse membrane topologies following posttranslational translocation. J Virol 78:454–463

Moser M, Nagamori S, Huber M, Tokuda H, Nishiyama K (2013) Glycolipozyme MPIase is essential for topology inversion of SecG during preprotein translocation. Proc Natl Acad Sci U S A 110:9734–9739

Nagahama H, Sakamoto Y, Matsumoto K, Hara H (2006) *RcsA*-dependent and -independent growth defects caused by the activated Rcs phosphorelay system in the *Escherichia coli pgsA* null mutant. J Gen Appl Microbiol 52:91–98

Nikawa J, Kodaki T, Yamashita S (1987) Primary structure and disruption of the phosphatidylinositol synthase gene of *Saccharomyces cerevisiae*. J Biol Chem 262:4876–4881

Nishibori A, Kusaka J, Hara H, Umeda M, Matsumoto K (2005) Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes. J Bacteriol 187:2163–2174

Ostrander DB, Zhang M, Mileykovskaya E, Rho M, Dowhan W (2001) Lack of mitochondrial anionic phospholipids causes an inhibition of translation of protein components of the electron transport chain. A yeast genetic model system for the study of anionic phospholipid function in mitochondria. J Biol Chem 276:25262–25272

Raetz CR, Carman GM, Dowhan W, Jiang RT, Waszkuc W, Loffredo W, Tsai MD (1987) Phospholipids chiral at phosphorus. Steric course of the reactions catalyzed by phosphatidylserine synthase from *Escherichia coli* and yeast. Biochemistry 26:4022–4027

Raja V, Joshi AS, Li G, Maddipati KR, Greenberg ML (2017) Loss of cardiolipin leads to perturbation of Acetyl-CoA synthesis. J Biol Chem 292:1092–1102

Reynolds CM, Kalb SR, Cotter RJ, Raetz CR (2005) A phosphoethanolamine transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of *Escherichia coli* lipopolysaccharide. Identification of the *eptB* gene and Ca^{2+} hypersensitivity of an *eptB* deletion mutant. J Biol Chem 280:21202–21211

Rietveld AG, Killian JA, Dowhan W, de Kruijff B (1993) Polymorphic regulation of membrane phospholipid composition in *Escherichia coli*. J Biol Chem 268:12427–12433

Rietveld AG, Chupin VV, Koorengevel MC, Wienk HL, Dowhan W, de Kruijff B (1994) Regulation of lipid polymorphism is essential for the viability of phosphatidylethanolamine-deficient *Escherichia coli* cells. J Biol Chem 269:28670–28675

Rietveld AG, Koorengevel MC, de Kruijff B (1995) Non-bilayer lipids are required for efficient protein transport across the plasma membrane of *Escherichia coli*. EMBO J 14:5506–5513

Romantsov T, Battle AR, Hendel JL, Martinac B, Wood JM (2010) Protein localization in *Escherichia coli* cells: comparison of the cytoplasmic membrane proteins ProP, LacY, ProW, AqpZ, MscS, and MscL. J Bacteriol 192:912–924

Schagger H, Pfeiffer K (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J 19:1777–1783

Schlame M, Greenberg ML (2017) Biosynthesis, remodeling and turnover of mitochondrial cardiolipin. Biochim Biophys Acta 1862:3–7

Shen H, Dowhan W (1996) Reduction of CDP-diacylglycerol synthase activity results in the excretion of inositol by *Saccharomyces cerevisiae*. J Biol Chem 271:29043–29048

Shen H, Heacock PN, Clancey CJ, Dowhan W (1996) The CDS1 gene encoding CDP-diacylglycerol synthase in *Saccharomyces cerevisiae* is essential for cell growth. J Biol Chem 271:789–795

Shiba Y, Yokoyama Y, Aono Y, Kiuchi T, Kusaka J, Matsumoto K, Hara H (2004) Activation of the Rcs signal transduction system is responsible for the thermosensitive growth defect of an *Escherichia coli* mutant lacking phosphatidylglycerol and cardiolipin. J Bacteriol 186:6526–6535

Shinzawa-Itoh K, Aoyama H, Muramoto K, Terada H, Kurauchi T, Tadehara Y, Yamasaki A, Sugimura T, Kurono S, Tsujimoto K, Mizushima T, Yamashita E, Tsukihara T, Yoshikawa S (2007) Structures and physiological roles of 13 integral lipids of bovine heart cytochrome c oxidase. EMBO J 26:1713–1725

Storey MK, Clay KL, Kutateladze T, Murphy RC, Overduin M, Voelker DR (2001) Phosphatidylethanolamine has an essential role in *Saccharomyces cerevisiae* that is independent of its ability to form hexagonal phase structures. J Biol Chem 276:48539–48548

Swameye I, Schaller H (1997) Dual topology of the large envelope protein of duck hepatitis B virus: determinants preventing pre-S translocation and glycosylation. J Virol 71:9434–9441

Tamura Y, Harada Y, Nishikawa S, Yamano K, Kamiya M, Shiota T, Kuroda T, Kuge O, Sesaki H, Imai K, Tomii K, Endo T (2013) Tam41 is a CDP-diacylglycerol synthase required for cardiolipin biosynthesis in mitochondria. Cell Metab 17:709–718

Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CR, Guan Z (2012) Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci U S A 109:16504–16509

Trotter PJ, Pedretti J, Voelker DR (1993) Phosphatidylserine decarboxylase from *Saccharomyces cerevisiae*. Isolation of mutants, cloning of the gene, and creation of a null allele. J Biol Chem 268:21416–21424

Tsai M, Ohniwa RL, Kato Y, Takeshita SL, Ohta T, Saito S, Hayashi H, Morikawa K (2011) *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. BMC Microbiol 11:13

Vanounou S, Parola AH, Fishov I (2003) Phosphatidylethanolamine and phosphatidylglycerol are segregated into different domains in bacterial membrane. A study with pyrene-labelled phospholipids. Mol Microbiol 49:1067–1079

Vitrac H, Bogdanov M, Heacock P, Dowhan W (2011) Lipids and topological rules of membrane protein assembly: balance between long- and short-range lipid-protein interactions. J Biol Chem 286:15182–15194

Vitrac H, Bogdanov M, Dowhan W (2013a) *In vitro* reconstitution of lipid-dependent dual topology and postassembly topological switching of a membrane protein. Proc Natl Acad Sci U S A 110:9338–9343

Vitrac H, Bogdanov M, Dowhan W (2013b) Proper fatty acid composition rather than an ionizable lipid amine is required for full transport function of lactose permease from *Escherichia coli*. J Biol Chem 288:5873–5885

Vitrac H, MacLean DM, Jayaraman V, Bogdanov M, Dowhan W (2015) Dynamic membrane protein topological switching upon changes in phospholipid environment. Proc Natl Acad Sci U S A 112:13874–13879

Vitrac H, MacLean DM, Karlstaedt A, Taegtmeyer H, Jayaraman V, Bogdanov M, Dowhan W (2017) Dynamic lipid-dependent modulation of protein topology by post-translational phosphorylation. J Biol Chem 292:1613–1624

Whitfield C, Trent MS (2014) Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem 83:99–128

Wikstrom M, Kelly AA, Georgiev A, Eriksson HM, Klement MR, Bogdanov M, Dowhan W, Wieslander A (2009) Lipid-engineered *Escherichia coli* membranes reveal critical lipid headgroup size for protein function. J Biol Chem 284:954–965

Xia W, Dowhan W (1995a) *In vivo* evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*. Proc Natl Acad Sci U S A 92:783–787

Xia W, Dowhan W (1995b) Phosphatidylinositol cannot substitute for phosphatidylglycerol in supporting cell growth of *Escherichia coli*. J Bacteriol 177:2926–2928

Xie J, Bogdanov M, Heacock P, Dowhan W (2006) Phosphatidylethanolamine and monoglucosyldiacylglycerol are interchangeable in supporting topogenesis and function of the polytopic membrane protein lactose permease. J Biol Chem 281:19172–19178

Ye C, Lou W, Li Y, Chatzispyrou IA, Huttemann M, Lee I, Houtkooper RH, Vaz FM, Chen S, Greenberg ML (2014) Deletion of the cardiolipin-specific phospholipase Cld1 rescues growth and life span defects in the tafazzin mutant: implications for Barth syndrome. J Biol Chem 289:3114–3125

Ye C, Shen Z, Greenberg ML (2016) Cardiolipin remodeling: a regulatory hub for modulating cardiolipin metabolism and function. J Bioenerg Biomembr 48:113–123

Zhang M, Mileykovskaya E, Dowhan W (2002) Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. J Biol Chem 277:43553–43556

Zhang M, Mileykovskaya E, Dowhan W (2005) Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. J Biol Chem 280:29403–29408

Zhao YJ, Zhu WJ, Wang XW, Zhang LH, Lee HC (2014) Determinants of the membrane orientation of a calcium signaling enzyme CD38. Biochim Biophys Acta 1853:2095–2103

Zhong Q, Gvozdenovic-Jeremic J, Webster P, Zhou J, Greenberg ML (2005) Loss of function of KRE5 suppresses temperature sensitivity of mutants lacking mitochondrial anionic lipids. Mol Biol Cell 16:665–675

Bacterial Lipid Domains and Their Role in Cell Processes

31

Adrián F. Alvarez and Dimitris Georgellis

Contents

1 Introduction 576
2 Membrane Composition 576
3 Bacterial Membrane Heterogeneities 577
3.1 Bacterial Lipid Bilayers and Transversal Asymmetry 577
3.2 Uneven Distribution of Anionic Phospholipids 579
3.3 Physiological Roles of Anionic PL Domains 580
4 The New Paradigm: Proteins Organize Membrane Domains with Different Fluidity in Bacteria 583
4.1 Membrane Domains with Increased Fluidity 583
4.2 Lipid Rafts 585
5 Conclusions and Research Needs 588
References 589

Abstract

Bacterial plasma membranes, mainly composed of phospholipids and proteins, separate the interior of a cell from their environment and maintain cell homeostasis. Early notions of membrane organization gave rise to a model in which a homogeneous lipid bilayer permits free protein diffusion within the membrane. However, proteins and phospholipids are distributed unevenly in bacterial membranes, and specific membrane localization is often crucial for protein activity or function. Bacterial membrane domains with different lipid compositions and with differential physical properties in comparison with the surrounding membrane have now been described. These membrane domains appear to influence

A. F. Alvarez · D. Georgellis (✉)
Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad de Mexico, Mexico
e-mail: aalvarez@ifc.unam.mx; dimitris@ifc.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_39

localization, diffusion, and function of membrane proteins, and thereby seem to be involved in many cellular processes. Here, we describe the different types of bacterial membrane domains and discuss their involvement in various bacterial cellular processes.

1 Introduction

Cellular plasma membranes serve to delimit the interior of a cell from the exterior environment. For decades these plasma membranes were thought to be a two-dimensional homogeneous and dynamic assortment of phospholipids (PL) and proteins, as proposed by the Singer and Nicolson fluid mosaic model (Singer and Nicolson 1972). According to this model the lipid bilayer functions as a neutral two-dimensional solvent, having no influence on membrane protein function. During the late 1980s and 1990s, however, the fluid mosaic model was challenged by numerous studies that provided evidence for specialized membrane structures, with differential composition and fluidity from the rest of the membrane, within the lipid bilayer of eukaryotic membranes (Simons and Van Meer 1988; Brown and Rose 1992; Simons and Ikonen 1997). These membrane platforms, coined lipid rafts, were shown to have the ability to spatially organize proteins, by including or excluding specific proteins to variable extents, thereby promoting kinetically favorable interactions (Allen et al. 2007) and regulating a variety of cellular functions. The fact that the composition of these eukaryotic membrane platforms contained invariably glycosphingolipid and cholesterol that are absent in almost all bacterial membranes, diverted attention on possible bacterial membrane domains. Nevertheless the development of new lipid staining techniques and imaging technologies, during the last decade, allowed the observation of specific membrane regions with different characteristics. These observations were initially interpreted as heterogeneous distribution of different lipid species in bacterial membranes, thus giving rise to the notion of bacterial membrane domains (Fishov and Woldringh 1999; Mileykovskaya and Dowhan 2000; Barák et al. 2008). Moreover, it was observed that bacterial membranes have heterogeneous protein distribution, as various proteins were found to localize to specific sites on the plasma membrane. Finally, several reports in the few last years, provided evidence that bacterial membrane domains are crucial for many physiological processes, such as cell division, cell wall biosynthesis, signal transduction, protein secretion, among others (Barák and Muchová 2013). Here, the different types of bacterial membrane domains will be described, and their importance for various cellular processes will be discussed.

2 Membrane Composition

The most abundant lipid components of biological membranes are phospholipids, which are amphipathic molecules that tend to spontaneously form a bilayer in an aqueous environment. The major lipid class defining this bilayer in practically all membranes is the glycerophospholipid. This lipid is based on an esterified glycerol moiety with two

acyl-chains (fatty acids) and with a phosphate group. The phosphate group is bound to a specific alcohol head group, resulting in the different types of glycerophospholipids, i.e., phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidic acid (PA), etc. (Fig. 1) (Sohlenkamp and Geiger 2016). In addition to glycerophospholipids, other lipids, such as polyisoprenoids and hopanoids in bacteria as well as sterols and the ceramide-based sphingolipids in eukaryotic membranes, but exceptionally also in some bacterial membranes, are usually found to form part of biological membranes (Fig. 1) (van Meer et al. 2008; Sohlenkamp and Geiger 2016).

Lipids account for almost half the mass of a membrane, whereas proteins account for the other half. Many of these proteins have one or more α-helices inserted into the lipid bilayer and can only be separated from it by detergent treatments. These integral proteins include transporters, electron carriers, signaling and structural proteins, among others.

Curiously, many proteins, known as peripheral proteins, that have been shown to co-localize with PG or CL are apparently soluble proteins, lacking any obvious transmembrane region. A common characteristic of such proteins is that all of them have at least one amphipathic α-helix, which can be located either at the C- or N-terminus, and mediates interactions with the lipid bilayer (Orgel 2006). These amphipathic α-helices, known also as membrane-targeting sequences (MTS), are necessary and sufficient to achieve membrane association of proteins. In MTSs, one face of the helix is highly hydrophobic whereas the other one is strongly polar, usually containing positive charged residues. This characteristic explains the anionic-PL affinity to MTSs, depending on hydrophobic interactions between one face of the helix and the acyl-chains of PL, and on electrostatic interactions due to the net positive charge of the polar face of the helix and the negative charge of the head group of the PG or CL domain. MTSs are found in a variety of proteins such as MinD (Szeto et al. 2002), the actin homologues MreB (Salje et al. 2011) and FtsA (Pichoff and Lutkenhaus 2005), the CL synthase ClsA (Kusaka et al. 2016), the exoribonuclease RNaseII (Lu and Taghbalout 2013), among others. The physiological role of some of them and their possible involvement in the coordination of lipid domain formation in *Escherichia coli* and *Bacillus subtilis* will be discussed below.

3 Bacterial Membrane Heterogeneities

3.1 Bacterial Lipid Bilayers and Transversal Asymmetry

Specific staining techniques and microscopic observation constituted the first approaches to study bacterial envelopes. In 1884, the Danish bacteriologist Hans Christian Gram developed a staining technique that is used until the present day. Depending on their capability to retain the Gram stain (the crystal violet dye), most bacteria fall into one of two main groups: Gram-negative or Gram-positive bacteria. This differential feature relies on essential structural differences in the cell envelope. Gram-negative bacteria, such as *E. coli,* are surrounded by a lipopolysaccharide containing membrane (outer membrane, OM), a thin peptidoglycan cell wall, and a

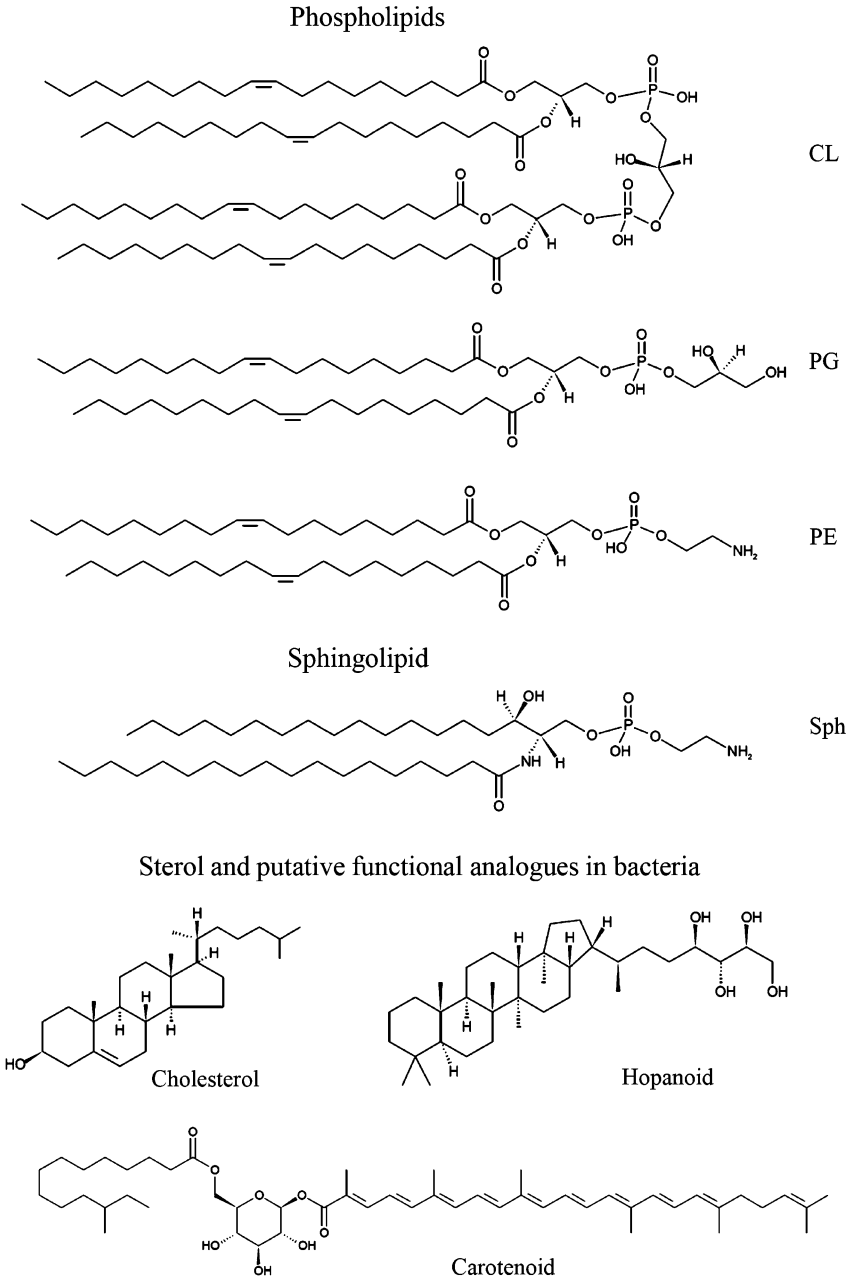


Fig. 1 Chemical structures of the major bacterial phospholipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and representative structures of sphingolipid (Sph), cholesterol, hopanoid and carotenoid are presented

cytoplasmic phospholipid bilayer or inner membrane (IM). In contrast, Gram-positive bacteria have a single membrane that is surrounded by a thick peptidoglycan wall. The OM of *E. coli* and many other Gram-negative bacteria is a highly asymmetric bilayer, with phospholipids predominantly on the inner leaflet and lipopolysaccharides (LPS) on the outer one. This composition confers to OM and the proteins embedded therein, natural resistance to solubilization by detergents, such as bile salts, a property that enables bacteria to survive in the mammalian gut. Asymmetry has also been observed in bacterial cytoplasmic membranes, with PE being preferentially situated in the inner leaflet and PG in the outer face of the lipid bilayer (Rothman and Kennedy 1977). In addition, it was proposed that CL is predominantly distributed on the inner leaflet in bacterial cytoplasmic membranes (McAuley et al. 1999; Huang and Ramamurthi 2010). Taken into account that CL accumulates in polar regions of rod-shaped bacteria (see below), this asymmetry in CL distribution, with preference to the concave cytoplasmic leaflet of the bilayer, is consistent with the predicted conical shape of a CL molecule, composed by two phosphatidyl moieties, four acyl chains and a relatively small polar head group.

Interestingly, asymmetry in the distribution of phospholipids in both leaflets of eukaryotic membranes has been shown to be actively maintained by the activity of lipid translocases and to promote the formation of ordered micro-domains (lipid rafts) (Cheng et al. 2009). However, no data exists from bacterial cytoplasmic membranes relating transversal asymmetry to membrane micro-domain assembly.

3.2 Uneven Distribution of Anionic Phospholipids

The *E. coli* cytoplasmic membrane is composed mainly of three glycerophospholipid species, the zwitterionic PE, representing the 75% of the total membrane phospholipids, and the anionic PG and CL, accounting for 20% and 5%, respectively (Raetz and Dowhan 1990). However, the membrane composition not only differs strongly from one taxonomic group to another and between genera from the same group, but also in the same strain depending on the physiological conditions. Consequently, the question of whether the different phospholipid species are homogeneously distributed across the membrane or whether determined lipids are enriched in specific regions of the membrane was raised. Indeed, by using dyes with different lipid specificities, PG and CL enriched domains were observed to be located in specific membrane regions. One of the first agents used to study the organization of bacterial membranes was FM 4–64, which is a positively charged molecule and it was therefore believed to bind anionic lipids, such as PG and CL. Fishov and Woldringh (1999) observed a pattern with dark bands between bright regions in FM 4–64 stained membranes of living *E. coli* cells. It was therefore assumed that the dark bands represented PE enriched regions, whereas the brightest areas, which were close to the cell poles, represented PG and CL enriched regions (Fishov and Woldringh 1999). Later on, by the use of the membrane dye 10-*N*-Nonyl acridine orange (NAO), which was suggested to have a selective fluorescent emission in the presence of CL, membrane regions enriched in CL were observed at cell poles and

septal regions of *E. coli* (Mileykovskaya and Dowhan 2000, 2009), *Pseudomonas putida* (Bernal et al. 2007), *B. subtilis* (Kawai et al. 2004) and *Mycobacterium tuberculosis* (Maloney et al. 2011). The preferential localization of CL at cell poles in *E. coli* was confirmed by later studies and with different techniques. On the one hand, lipid analysis of minicells, in contrast to vegetative cells, showed that they are enriched in CL (Koppelman et al. 2001). Minicells are spherical and anucleated membrane vesicles that are produced by inactivation of any of the *minC*, *minD* or *minE* genes. These mutations cause a shift in the division site from mid-cell to the vicinity of the cell poles, and therefore the membrane of polar regions of normal cells are well represented in minicell membranes. On the other hand, in vitro experiments demonstrated that changes in membrane curvature cause a change in CL localization, displaying a significant preference to localize at negative curvatures encountered in the inner leaflet of the IM of the *E. coli* cell poles (Renner and Weibel 2011). Nevertheless, bacteria devoid of CL are essentially healthy cells under lab growth conditions, and their content of PG appears to increase in a compensatory manner. Curiously, in these cells, NAO-stained enriched regions were still observed, casting doubt on the use of NAO as a specific CL-staining agent. In fact, NAO was found to bind promiscuously to other anionic PLs, such as PG and PA, producing nearly the same spectroscopic changes and rendering it impossible to differentiate between these lipid species (Oliver et al. 2014). In addition, *E. coli* cells devoid of both CL and PG are still viable, and compensate this deficiency by accumulating between 5% and 10% of other anionic PLs such us phosphatidic acid (PA) and *N*-acylphosphatidylethanolamine (*N*-acyl-PE), which are also located principally at cell poles and septal regions (Mileykovskaya et al. 2009). Thus, it seems most likely that bacterial cells need anionic PLs to carry out essential physiological processes, as anionic PL domains may play a vital role providing the required environment for the function of many proteins.

3.3 Physiological Roles of Anionic PL Domains

Several studies, in the last two decades, provided evidence for the involvement of CL or PG in several cellular functions. Many of these evidences came from studies in which CL or PG were found to co-purify or were found to be physically associated with a variety of membrane proteins. Moreover, anionic PLs were shown to modulate enzymatic activities or to be required for the reconstitution of active proteins in vitro (Arias-Cartin et al. 2012). For instance, the Mg^{2+} transporter MgtA of *E. coli* co-localizes with CL domains at the cell poles and requires CL or PG to be reconstituted as an active ATPase in vitro (Subramani et al. 2016). Another example is provided by the association of anionic PLs with the protein translocation machinery, consisting of the SecYEG complex and the ATPase SecA. CL, and to a lesser degree PG, were shown to associate with the SecYEG translocon, thereby stabilizing its active dimeric form, and to stimulate the in vitro ATPase activity of SecA (Gold et al. 2010). Curiously, in *B. subtilis* the PG pool, instead of CL, was found to improve SecA activity and to control its localization, thereby affecting the whole translocon behavior (Campo et al. 2004). A third example for a physiological role is the association of CL with the

osmotic stress response in *E. coli*. Under increasing osmotic pressure bacteria respond by adjusting the distribution of solutes (osmolytes) across the membrane. In *E. coli*, this is achieved by ProP, which acts as an osmosensory transporter that allows the cytosolic accumulation of osmolytes. Under such osmotic stress conditions it was noted that the expression of the *cls* genes, encoding for CL synthases, is activated, leading to a significant increase in CL content. Curiously, ProP was found to localize in the cell poles, a CL-rich membrane region, on a CL-dependent manner, as no polar localization of ProP was observed in *clsA* mutant cells (Romantsov et al. 2007). However, the activity of ProP appeared to not be affected. This was explained by the fact that inactivation of CL synthesis is compensated by an increase in the amount of PG, another anionic PL, as ProP activity was completely lost in a CL- and PG- deficient strain. Therefore, it was concluded that an anionic PL enriched membrane environment, rather than localization at the cell poles, is required for proper function of ProP (Romantsov et al. 2007, 2009). Thus, anionic PL domains appear to serve as an anchor site for several proteins, leading to their localization to specific sites in the cell, and they seem to be required for their activity.

3.3.1 PG Domains and Cell Division: Lipid Spirals in *B. subtilis*

A last example worth describing in more detail is the process of bacterial cell division, in which MTS containing proteins and anionic lipid domains have been shown to be essential. Cytokinesis is finely coordinated with replication and segregation of the chromosome, ensuring that each daughter cell can receive a copy of the chromosome after division. The earliest step of bacterial cytokinesis is the formation of the membrane-tethered Z-ring at the division site, by the polymerization of the tubulin homologue protein FtsZ, a cytosolic protein. In *B. subtilis*, membrane tethering of the FtsZ filament depends on FtsA, a MTS containing peripheral protein. Subsequently, the Z-ring acts as a scaffold that recruits more than 10 essential proteins to produce a mature division-machinery that constricts the cell and completes cytokinesis (Aj De Boer 2010).

The nucleoid occlusion (NOC) system and Min system provide the main mechanisms that control the above process. On one hand the Noc protein associates to the membrane by a N-terminal MTS, and at the same time binds to specific sites on the DNA, enabling membrane-chromosome association and physically impeding Z-ring formation in the vicinity of the nucleoid (Adams et al. 2015). On the other hand, the Min system, composed by MinC and MinD, inhibits cell division at the poles while allowing it to occur at mid-cell. MinD, an ATPase that is associated to the inner face of the membrane by a C-terminal MTS (Szeto et al. 2002), forms extended filaments on the membrane surface. These filaments recruit MinC from the cytosol and activate it as a cell division inhibitor. MinC, which is the effector of the Min system, directly interacts with FtsZ, thus impeding functional associations with FtsA and with itself, thereby impeding Z-ring formation (Dajkovic et al. 2008; Shen and Lutkenhaus 2009). A question that comes to mind is how does this system select the lateral positioning of the Z-ring, restricted to be at mid-cell. The DivIVA protein gives the answer to this question. DivIVA associates to the inner face of the plasma membrane, by an N-terminal amphiphatic α-helix, which possesses an intrinsic affinity

to negative curvatures, and therefore it accumulates at the cell poles (Lenarcic et al. 2009). Consequently, MinCD recruitment by DivIVA generates a concentration gradient of the first, ensuing a minimum concentration at the cell center where Z-ring formation can take place. It is noteworthy that in *E. coli*, which lacks DivIVA, a dynamic mechanism in which MinE-stimulated oscillatory movements of MinCD produce a concentration gradient of the MinCD complex between cell poles, thereby confining Z-ring formation at mid-cell (Meinhardt and de Boer 2001), [see more references in (Lutkenhaus 2007; Vats et al. 2009; Aj De Boer 2010)].

The MTS-containing protein MinD of *B. subtilis*, which is distributed on the inner face of the membrane, was observed to form spiral-like structures (Barák et al. 2008). Interestingly, by using the positive charged lipid dye FM 4–64 and confocal microscopy, Barak and colleagues described the same spiral-like pattern in *B. subtilis* cells, presumably composed of PG, since NAO-stained cells showed that CL were preferentially distributed in the septal regions and at the poles of cells. Moreover, the authors observed that the MinD spirals co-localized with the lipid helices on the membrane surface. Because depletion of anionic lipids produced a MinD delocalization it was proposed that PG-enriched membrane domains, determine the localization of MinD, and therefore are essential for the functioning of the Min system (Fig. 2)

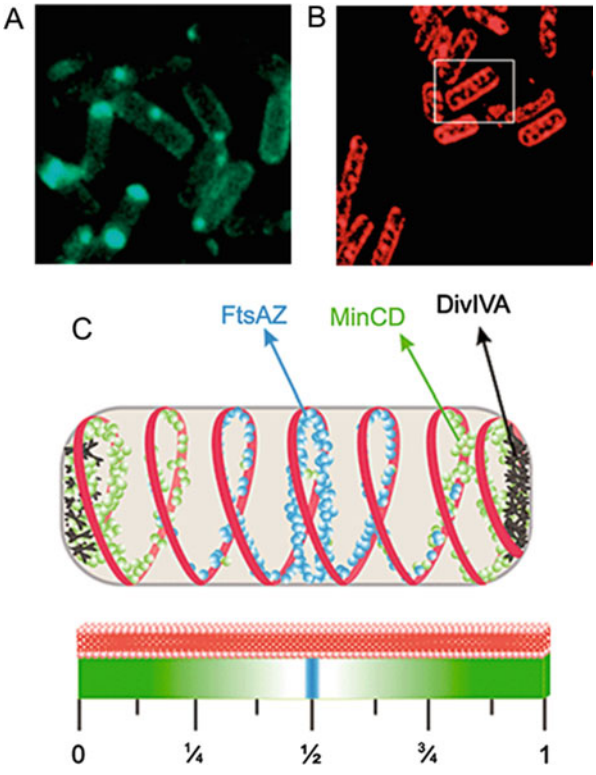


Fig. 2 Staining of lipid species and PG spirals in *B. subtilis*. A. Visualization of polar- and septum-localized cardiolipin domains by NAO staining of wild-type *B. subtilis*. B. PG helices in *B. subtilis* revealed by FM 4–64 staining of live cells. C. Model of lipid spirals in *B. subtilis*: localization of the MTS-containing proteins (MinD and FtsA) in PG-enriched domains (red stripes) drives septum formation at mid-cell (Reproduced from reference (Barák et al. 2008) with permission of John Wiley and Sons)

(Barák et al. 2008). Thus, PG domains appear to control the process of cell division by determining the specific localization of the cell division-machinery.

It is worth mentioning that spiral-like distribution on the plasma membrane is not exclusive for MinD, as other MTS-containing proteins such as MreB and SecA exhibit spiral-like patterns (Jones et al. 2001; Campo et al. 2004), highlighting the importance of MTS motifs in the localization of proteins in PG domains.

Considering the above observations, a model providing a physiological link between the heterogeneous distributions of lipid species on the cytoplasmic membrane and essential cellular processes was put forward. In this model, CL-enriched domains seemed to be needed by a variety of proteins to be active at the cell poles and PG-enriched helical domains appeared to localize other proteins that partake in different processes.

Nevertheless, the above model may not apply for Gram-negative bacteria. This is because PG-enriched helical domains could not be observed in *E. coli* cells, in which bands or dots around the membrane were observed after staining with the positive charged lipid dye FM 4–64. This was despite that MinD showed a spiral-like localization (Shih et al. 2003).

Moreover, different MTS-containing proteins were shown to have differential localization patterns. For instance, the CL synthase A, ClsA, of *E. coli* contain a C-terminal MTS but has been shown to be preferentially at cell poles and septum, presumably associated with CL-enriched domains (Kusaka et al. 2016). In another case, the actin-like protein MreB of *E. coli* was shown to be organized forming long helical structures that extend along the length of the cell, similarly to MinD, but curiously these structures did not colocalize, suggesting that both MTS-containing proteins are not part of the same lipid domain (Shih et al. 2005). Hence, in addition to a PG and CL enriched environments other factors may contribute to the proper localization of membrane-associated proteins.

4 The New Paradigm: Proteins Organize Membrane Domains with Different Fluidity in Bacteria

Until recently, membrane domains in bacteria have been thought to be limited to discrete regions enriched in PLs with a specific hydrophilic head. However, recent findings have shifted the focus in the analysis of membrane heterogeneity in bacterial cytoplasmic membranes. That is, physiologically relevant membrane regions with increased fluidity (RIFs) with respect to adjacent areas, and dynamic membrane assemblies with lower fluidity, similar to eukaryotic lipid rafts, were found to exist in bacterial membranes (Fig. 3).

4.1 Membrane Domains with Increased Fluidity

The discovery of membrane regions with increased fluidity came from the study of the bacterial MreB cytoskeleton. MreB, a peripheral protein, is a bacterial actin

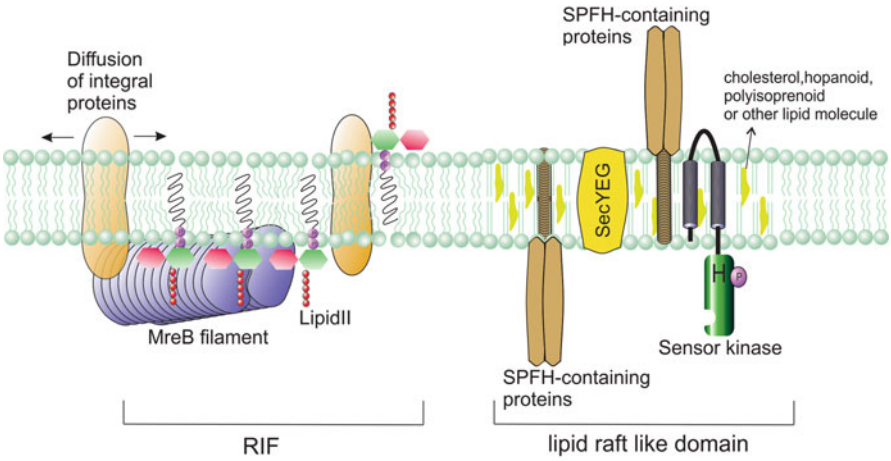


Fig. 3 Scheme of the organization of bacterial membrane regions with increased fluidity (RIF) and lipid raft-like domains. (Left) MreB-induced RIF affects both the localization and the diffusion of certain membrane proteins. The isoprenoid complex molecule Lipid II, which is a cell wall precursor carrier, has been proposed to be involved in the increase of membrane fluidity. (Right) Lipid raft like domains serve as organizing centers that compartmentalize cellular processes, such as signaling and protein translocation and diffuse laterally as a whole in the lipid bilayer. It has been proposed that hopanoids, carotenoids or other unknown lipid species are necessary for the decreased fluidity of these membrane domains, and that the *S*tomatin, *P*rohibitin, *F*lotillin and *H*flK/C (SPFH)-containing proteins help to nucleate proteins/protein complexes into them

homologue that is widely distributed in rod-shaped bacteria and has been shown to be involved in cell shape maintenance. Like other MTS-containing proteins, MreB associates reversibly with the inner face of the plasma membrane by hydrophobic and electrostatic interactions. Subsequently, it interacts with the integral membrane proteins MreC and MreD, and recruits multiple peptidoglycan cell-wall biosynthetic proteins. Intriguingly MreB was observed to form spiral-like filaments in *B. subtilis* (Jones et al. 2001), and since then various models have been put forward for its function and localization [(Errington 2015) and refs therein]. In an actual model, MreB is believed to form extended filaments that move around the membrane in form of coils. MreB filament motion is driven by the peptidoglycan cell wall biosynthetic machinery, and in a direction dictated by the MreB filament (Garner et al. 2011; van Teeffelen et al. 2011). Therefore, the MreB cytoskeleton differs from the cortical actin cytoskeleton of eukaryotic cells. The latter one determines the cell shape by the organization of a network of actin filaments attached to the inner face of the plasma membrane, whereas MreB determines bacterial cell shape by enabling and coordinating cell wall formation.

Recently, Strahl and colleagues observed that delocalization of MreB in *B. subtilis* produces changes in the Nile Red and FM 4–64 staining patterns. Nile red is an uncharged lipid dye that appears to stain uniformly the cell membranes, whereas FM 4–64 was observed to have a helical distribution that was interpreted as

PG enriched domains. However, after delocalization of MreB by membrane depolarization, staining with both dyes resulted in fluorescent foci in diverse regions of the cell membrane. These foci were independent of the CL, PG or PE content but were dependent of the presence of MreB (and its paralogs in *B. subtilis*, Mbl and MreBH), with which they were co-localized. Because the same punctuated staining pattern was observed with both dyes, the authors discarded the possibility that the bright foci represent domains enriched in lipids with specific head groups. Instead, by using dyes with high specificity to fluid membrane areas such as Laurdan and DiI-C12, it was noted that the MreB associated foci represented membrane regions with increased fluidity (RIFs). Importantly, using these dyes, RIFs were observed to colocalize with MreB even without membrane depolarization of *B. subtilis* cells (Strahl et al. 2014). Thus, MreB appears to be able to organize membrane domains by increasing local membrane fluidity. This can be achieved either by reorganizing the distribution of fatty acids with more insaturations or with branched chains, or by the association with specialized lipid species. In this respect, the peptidoglycan biosynthesis precursor lipid II, which is a complex isoprenoid lipid linked to a sugar moiety and a pentapeptide, was proposed to be involved in the increase of membrane fluidity associated to MreB filaments (Scheffers et al. 2015; Strahl and Errington 2017). More recently, the role of MreB in membrane organization was also observed in *E. coli* cells, in which RIFs were found to be associated with polymerized MreB (Oswald et al. 2016). Most importantly, RIFs could have crucial physiological roles, since the MreB-induced domains were shown to alter the localization of certain membrane proteins, and to affect the diffusion of lipid species and membrane proteins (Strahl et al. 2014; Oswald et al. 2016).

It, therefore, seems likely that differential fluidity of membrane regions, given by fatty acid composition, specialized lipid species and/or membrane-protein associations, organize physiological processes by controlling spatio-temporal localization of proteins. Moreover, this novel model of bacterial membrane organization begins to resemble the eukaryotic membrane compartmentalization, where the cortical actin cytoskeleton organizes membrane compartments. This is achieved by creating a physical barrier for phospholipid and membrane protein diffusion (Kusumi et al. 2005; Andrade et al. 2015), and by affecting directly or indirectly the dynamics of lipid rafts (Viola and Gupta 2007). However, the bacterial MreB cytoskeleton is more permissive in terms of protein or lipid diffusion and organizes fluid membrane domains by a thus far unknown mechanism.

4.2 Lipid Rafts

In eukaryotic cells, signal transduction and membrane trafficking depend on the integrity of membrane assemblies that have differential lipid and protein composition and thereby physical properties, in comparison with the surrounding membrane (Simons and Toomre 2000; Pike 2003). These membrane domains, known as lipid rafts, are defined as sterol- and sphingolipid-enriched liquid-ordered lipid clusters with decreased fluidity. Sterols, such as cholesterol and ergosterol, favor a close

packing of the acyl chains within the phospholipid bilayer, increasing membrane thickness and reducing its fluidity. In addition, the high affinity of cholesterol to the longer and saturated fatty acid chains of sphingolipids promotes their segregation from glycerophospho lipids, forming more rigid structures in artificial bilayers that are suggested to resemble lipid rafts. Thus, the presence of sterols allows the phase separation within liquid-ordered domains, which have lesser fluidity. Also, an invariant component of lipid rafts is a group of proteins characterized by the *S*tomatin, *P*rohibitin, *F*lotillin and *H*flK/C (SPFH) domain (Browman et al. 2007). SPFH-containing proteins are thought to be involved in protein recruitment to the rafts and in the anchoring of lipid rafts to the cortical actin cytoskeleton (Langhorst et al. 2005, 2007; Browman et al. 2007). Since SPFH-containing proteins are essential for eukaryotic lipid raft formation and they are found exclusively in these domains, they are commonly used as lipid raft markers.

4.2.1 Lipid Raft-like Domains in Bacteria

The fact that SPFH-containing proteins are widely distributed in prokaryotes, being present in more than 90% of bacterial genomes (Hinderhofer et al. 2009), raises the question of whether these proteins are able to drive the formation of lipid raft-like domains in bacterial membranes. And then, if such membrane structures exist in bacteria, how do they achieve the local increase of stickiness and decreased fluidity, characteristic of lipid rafts, in the absence of sterols and sphingolipids? Indeed, lipid raft-like domains, coined functional membrane microdomains (FMMs), have been identified in the cytoplasmic membrane of *B. subtilis* (López and Kolter 2010). In this study, Lopez and Kolter demonstrated that two SPFH-containing proteins, FloT (YuaG) and FloA (YqfA), which localize in discrete foci along the cell membrane, are spatially and functionally associated with a signaling pathway, which is involved in regulation of biofilm formation. Interestingly, this association was shown to depend on the activity of YisP, which catalyzes the formation of farnesol from farnesyl diphosphate (Feng et al. 2014), suggesting that farnesol or farnesol-derived polyisoprenoid lipids might be needed for lipid raft-like domain formation in *B. subtilis*. Thus, the participation of specialized lipids that mimic the properties of sterols and sphingolipids and/or the involvement of proteins that affect the local fluidity of membranes may provide an answer to the above questions.

The tight packing of lipids and the phase separation characteristic of lipid rafts, render them resistant to the solubilization by nonionic detergents at low temperatures. Therefore, the biochemical characterization of lipid rafts has been largely pursued by the analysis of detergent-resistant membranes (DRM) in both eukaryotic and prokaryotic organisms. In *B. subtilis*, analysis of the DRM protein content revealed the presence of signaling proteins (histidine kinases and chemoreceptors), proteins of the cell wall biosynthesis machinery, transporters, and secretion proteins, along with SPFH-containing proteins (López and Kolter 2010; Bach and Bramkamp 2013). Some of these proteins have been shown to colocalize and directly interact with FloT and/or FloA. Moreover, functional dependence on flotillins was found for the histidine kinase KinC and for the Sec protein translocation apparatus (López and Kolter 2010; Bach and Bramkamp 2013; Schneider et al. 2015). Thus, similarly to

lipid rafts in eukaryotic cells, bacterial FMMs appear to be involved in signaling and protein secretion.

SFPH containing proteins, or flotillins, seem to organize in whole the formation of bacterial FMMs. Indeed, flotillins were found to act as the scaffold for proteins that find the appropriate environment in FMMs to carry out kinetically favorable interactions and regulate their activities. In addition, analysis of Laurdan staining of *B. subtilis* cells showed that FloT and FloA form dynamic assemblies that can locally influence membrane fluidity, allowing liquid-ordered phase formation (Bach and Bramkamp 2013). This change in local fluidity was found to be dependent of YisP, indicating that polyisoprenoid lipids are required.

This body of recent data about FFMs in the model bacterium *B. subtilis*, advocates for the occurrence of bacterial lipid raft-like domains that are involved in many membrane-associated cellular processes. In analogy with eukaryotic lipid rafts, specialized lipid species, such as farnesol or its derived polyisoprenoid lipids, appear to be essential for FMM formation. By various approaches and to different extents of detail, lipid raft-like domains have been identified in other bacteria, such as *E. coli* (López and Kolter 2010; Guzmán-Flores et al. 2017), *Staphylococcus aureus* (López and Kolter 2010), *B. anthracis* (Somani et al. 2016), *Bacteroides fragilis* (An et al. 2011), *Borrelia burgdorferi* (LaRocca et al. 2013), and *Helicobacter pylori* (Hutton et al. 2017). *B. burgdorferi* and *H. pylori* are two of the few bacteria which possess cholesterol as a membrane component, despite that they do not carry cholesterol biosynthetic genes and therefore cannot carry out de novo sterol biosynthesis. Instead, both bacteria are able to obtain cholesterol from the host epithelial cells to generate glyco-cholesterol derivatives, which are incorporated into bacterial membranes. Interestingly, both bacterial species seem to form flotillin-dependent cholesterol-containing lipid raft microdomains that are assembled into the outer membrane (LaRocca et al. 2013; Hutton et al. 2017). *Bacteroides* species, which are common residents of the mammalian gut, form part of a small group of bacteria that possess sphingolipids in their outer membranes. Remarkably, they can also use the host-provided cholesterol to form sphingolipid- and cholesterol-containing membrane domains, which have been shown to control stress response pathways. On the other hand, in *Methylobacterium extorquens* it was observed that, comparable to cholesterol in lipid rafts, hopanoids, lipid molecules present in many bacterial species, favor high order in the outer membrane.

The case for the model bacterium *E. coli* may be quite different. The *E. coli* genome encodes for 4 SPFH-containing proteins, which are all inner membrane integral proteins. These proteins are distributed in foci in different cell locations and are enriched in DRM fractions obtained from the inner membrane (López and Kolter 2010; Guzmán-Flores et al. 2017). Until now, *E. coli* is the unique Gram-negative bacterium in which inner membrane located lipid raft-like domains have been identified. Furthermore, *E. coli* lacks sterol, carotenoid and hopanoid lipids. Interestingly, the genome of some pathogenic *E. coli* strains, such as the enterotoxigenic *E. coli* B7A, carry a gene codifying for a putative serine palmitoyltransferase (Spt). Spt is responsible for the first step of sphingolipid biosynthesis in both bacteria and eukaryotic cells, suggesting that sphingolipids might exist in the membranes of these

bacterial strains (Geiger et al. 2010). However, a functional or structural connection between sphingolipids and membrane domains in the above *E. coli* strains has not been reported thus far. Therefore, further work will be needed to determine the lipid composition of lipid raft-like domains and their protein cargo.

5 Conclusions and Research Needs

Lateral heterogeneity clearly occurs in bacterial membranes and it is essential for the coordination of a multitude of membrane-associated processes that occur simultaneously in a bacterial cell. Such cellular processes include signal transduction, transport, energy metabolism, cell division, cell wall metabolism, among others. The first notion of membrane domains in bacteria came from the observation of membrane regions enriched in anionic lipids, such as CL or PG. The occurrence and physiological importance of these domains has been largely discussed, and the irregular distribution of lipid dyes had diverse interpretations. However, membrane zones enriched in lipids with a determined head group could be related to the more recent view in which membrane heterogeneity is determined by differences in fluidity rather than by individual lipid species. In this respect, two different membrane domains have been described: membrane regions with increased fluidity and lipid raft-like domains, characterized by being less fluid (Fig. 3). Both types of domains coincide in bacterial membranes and seem to be organized by specific proteins, namely, the actin homologues MreB and the SPFH-containing proteins. In an early study, it was suggested that two types of membrane domains coexist in plasma membranes of *E. coli* and *B. subtilis*, one mainly lipid enriched and the other being a proteolipid domain (Vanounou et al. 2002). Interestingly, the authors interpreted their observations in a predictive manner, since it was not until 2010 that the coexistence of two types of membrane domains was demonstrated. However, both types of membrane domains have important protein components and may require a complex lipid composition. This is exemplified by the fact that increase of local membrane fluidity has been proposed to require MreB in association with the cell wall precursor lipid II, whereas lipid raft segregation needs a liquid-ordered phase formation, which has only been observed in the presence of sterols, carotenoids, polyisoprenoids or hopanoids. Because these lipid species are absent in many bacteria further investigation is needed to determine the essential lipid components in lipid raft-like domains of these bacteria. In fact, CL has been shown to be associated to FFMs, since CL enriched regions often colocalize with flotillin foci and CL molecules have been shown to copurify with this lipid raft markers (Donovan and Bramkamp 2009). However, CL depletion does not appear to affect FMMs formation, in contrast to eukaryotic cells and *B. burgdorferi* where no lipid rafts were formed after cholesterol depletion.

Efforts for the identification of specialized lipid species essential for membrane rafting in *E. coli* and other γ-proteobacteria are of uppermost importance. Taken into account that the integrity of FMMs has been shown to be essential for proper signal transduction by two component systems (TCSs) (Lopez and Kolter 2010, and own

unpublished observations), and that many TCSs usually regulate the expression of virulence factors, the possibility to impede FMMs formation, e.g., by blocking a lipid biosynthetic pathway, becomes of special interest.

Acknowledgments Research in our laboratory is funded by grants from the Consejo Nacional de Ciencia y Tecnología (CONACyT, 178033), and from Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México (DGAPA-UNAM; PAPIIT IN209215 and IA203216). We thank Claudia Rodriguez for her technical assistance.

References

Adams DW, LJ W, Errington J (2015) Nucleoid occlusion protein Noc recruits DNA to the bacterial cell membrane. EMBO J 34:491–501

Aj De Boer P (2010) Advances in understanding *E. coli* cell fission. Curr Opin Microbiol 13:730–737

Allen JA, Halverson-Tamboli RA, Rasenick MM (2007) Lipid raft microdomains and neurotransmitter signalling. Nat Rev Neurosci 8:128–140

An D, Na C, Bielawski J, Hannun YA, Kasper DL (2011) Membrane sphingolipids as essential molecular signals for Bacteroides survival in the intestine. Proc Natl Acad Sci U S A 108:4666–4671

Andrade DM, Clausen MP, Keller J, Mueller V, Wu C, Bear JE, Hell SW, Christoffer Lagerholm B, Eggeling C (2015) Cortical actin networks induce spatio-temporal confinement of phospholipids in the plasma membrane – a minimally invasive investigation by STED-FCS. Sci Rep 5:11454

Arias-Cartin R, Grimaldi S, Arnoux P, Guigliarelli B, Magalon A (2012) Cardiolipin binding in bacterial respiratory complexes: structural and functional implications. Biochim Biophys Acta 1817:1937–1949

Bach JN, Bramkamp M (2013) Flotillins functionally organize the bacterial membrane. Mol Microbiol 88:1205–1217

Barák I, Muchová K (2013) The role of lipid domains in bacterial cell processes. Int J Mol Sci 14:4050–4065

Barák I, Muchová K, Wilkinson AJ, O'Toole PJ, Pavlendová N (2008) Lipid spirals in *Bacillus subtilis* and their role in cell division. Mol Microbiol 68:1315–1327

Bernal P, Muñoz-Rojas J, Hurtado A, Ramos JL, Segura A (2007) A *Pseudomonas putida* cardiolipin synthesis mutant exhibits increased sensitivity to drugs related to transport functionality. Environ Microbiol 9:1135–1145

Browman DT, Hoegg MB, Robbins SM (2007) The SPFH domain-containing proteins: more than lipid raft markers. Trends Cell Biol 17:394–402

Brown DA, Rose JK (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68:533–544

Campo N, Tjalsma H, Buist G, Stepniak D, Meijer M, Veenhuis M, Westermann M, Müller JP, Bron S, Kok J, Kuipers OP, Jongbloed JDH (2004) Subcellular sites for bacterial protein export. Mol Microbiol 53:1583–1599

Cheng H-T, Megha LE (2009) Preparation and properties of asymmetric vesicles that mimic cell membranes: effect upon lipid raft formation and transmembrane helix orientation. J Biol Chem 284:6079–6092

Dajkovic A, Lan G, Sun SX, Wirtz D, Lutkenhaus J (2008) MinC spatially controls bacterial cytokinesis by antagonizing the scaffolding function of FtsZ. Curr Biol 18:235–244

Donovan C, Bramkamp M (2009) Characterization and subcellular localization of a bacterial flotillin homologue. Microbiology 155:1786–1799

Errington J (2015) Bacterial morphogenesis and the enigmatic MreB helix. Nat Rev Microbiol 13:241–248

Feng X, Hu Y, Zheng Y, Zhu W, Li K, Huang C-H, Ko T-P, Ren F, Chan H-C, Nega M, Bogue S, López D, Kolter R, Götz F, Guo R-T, Oldfield E (2014) Structural and functional analysis of *Bacillus subtilis* YisP reveals a role of its product in biofilm production. Chem Biol 21:1557–1563

Fishov I, Woldringh CL (1999) Visualization of membrane domains in *Escherichia coli*. Mol Microbiol 32:1166–1172

Garner EC, Bernard R, Wang W, Zhuang X, Rudner DZ, Mitchison T (2011) Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis*. Science 333:222–225

Geiger O, González-Silva N, López-Lara IM, Sohlenkamp C (2010) Amino acid-containing membrane lipids in bacteria. Prog Lipid Res 49:46–60

Gold VAM, Robson A, Bao H, Romantsov T, Duong F, Collinson I (2010) The action of cardiolipin on the bacterial translocon. Proc Natl Acad Sci U S A 107:10044–10049

Guzmán-Flores JE, Alvarez AF, Poggio S, Gavilanes-Ruiz M, Georgellis D (2017) Isolation of detergent-resistant membranes (DRMs) from *Escherichia coli*. Anal Biochem 518:1–8

Hinderhofer M, Walker CA, Friemel A, Stuermer CA, Möller HM, Reuter A (2009) Evolution of prokaryotic SPFH proteins. BMC Evol Biol 9:10

Huang KC, Ramamurthi KS (2010) Macromolecules that prefer their membranes curvy. Mol Microbiol 76:822–832

Hutton ML, D'Costa K, Rossiter AE, Wang L, Turner L, Steer DL, Masters SL, Croker BA, Kaparakis-Liaskos M, Ferrero RL (2017) A *Helicobacter pylori* homolog of eukaryotic flotillin is involved in cholesterol accumulation, epithelial cell responses and host colonization. Front Cell Infect Microbiol 7:219

Jones LJF, Carballido-Ló Pez R, Errington J (2001) Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. Cell 104:913–922

Kawai F, Shoda M, Harashima R, Sadaie Y, Hara H, Matsumoto K (2004) Cardiolipin domains in *Bacillus subtilis* marburg membranes. J Bacteriol 186:1475–1483

Koppelman CM, Den Blaauwen T, Duursma MC, Heeren RM, Nanninga N (2001) *Escherichia coli* minicell membranes are enriched in cardiolipin. J Bacteriol 183:6144–6147

Kusaka J, Shuto S, Imai Y, Ishikawa K, Saito T, Natori K, Matsuoka S, Hara H, Matsumoto K (2016) Septal localization by membrane targeting sequences and a conserved sequence essential for activity at the COOH-terminus of *Bacillus subtilis* cardiolipin synthase. Res Microbiol 167:202–214

Kusumi A, Nakada C, Ritchie K, Murase K, Suzuki K, Murakoshi H, Kasai RS, Kondo J, Fujiwara T (2005) Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. Annu Rev Biophys Biomol Struct 34:351–378

Langhorst MF, Reuter A, Stuermer CAO (2005) Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. Cell Mol Life Sci 62:2228–2240

Langhorst MF, Solis GP, Hannbeck S, Plattner H, Stuermer CAO (2007) Linking membrane microdomains to the cytoskeleton: regulation of the lateral mobility of reggie-1/flotillin-2 by interaction with actin. FEBS Lett 581:4697–4703

LaRocca TJ, Pathak P, Chiantia S, Toledo A, Silvius JR, Benach JL, London E (2013) Proving lipid rafts exist: membrane domains in the prokaryote *Borrelia burgdorferi* have the same properties as eukaryotic lipid rafts. PLoS Pathog 9:e1003353

Lenarcic R, Halbedel S, Visser L, Shaw M, LJ W, Errington J, Marenduzzo D, Hamoen LW (2009) Localisation of DivIVA by targeting to negatively curved membranes. EMBO J 28:2272–2282

López D, Kolter R (2010) Functional microdomains in bacterial membranes. Genes Dev 24:1893–1902

Lu F, Taghbalout A (2013) Membrane association via an amino-terminal amphipathic helix is required for the cellular organization and function of RNase II. J Biol Chem 288:7241–7251

Lutkenhaus J (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring cytoskeletal: protein filaments that provide for intracellular organization. Annu Rev Biochem 76:539–562

Maloney E, Lun S, Stankowska D, Guo H, Rajagoapalan M, Bishai WR, Madiraju MV (2011) Alterations in phospholipid catabolism in *Mycobacterium tuberculosis lysX* mutant. Front Microbiol 2:19

McAuley KE, Fyfe PK, Ridge JP, Isaacs NW, Cogdell RJ, Jones MR (1999) Structural details of an interaction between cardiolipin and an integral membrane protein. Proc Natl Acad Sci U S A 96:14706–14711

Meinhardt H, de Boer PA (2001) Pattern formation in *Escherichia coli*: a model for the pole-to-pole oscillations of Min proteins and the localization of the division site. Proc Natl Acad Sci U S A 98:14202–14207

Mileykovskaya E, Dowhan W (2000) Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-*N*-nonyl acridine orange. J Bacteriol 182:1172–1175

Mileykovskaya E, Dowhan W (2009) Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochim Biophys Acta 1788:2084–2091

Mileykovskaya E, Ryan AC, Mo X, Lin C-C, Khalaf KI, Dowhan W, Garrett TA (2009) Phosphatidic acid and N-acylphosphatidylethanolamine form membrane domains in *Escherichia coli* mutant lacking cardiolipin and phosphatidylglycerol. J Biol Chem 284:2990–3000

Oliver PM, Crooks JA, Leidl M, Yoon EJ, Saghatelian A, Weibel DB (2014) Localization of anionic phospholipids in *Escherichia coli* cells. J Bacteriol 196:3386–3398

Orgel JP (2006) Surface-active helices in transmembrane proteins. Curr Protein Pept Sci 7:553–560

Oswald F, Varadarajan A, Lill H, Peterman EJG, Bollen YJM (2016) MreB-dependent organization of the *E. coli* cytoplasmic membrane controls membrane protein diffusion. Biophys J 110:1139–1149

Pichoff S, Lutkenhaus J (2005) Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA. Mol Microbiol 55:1722–1734

Pike LJ (2003) Lipid rafts: bringing order to chaos. J Lipid Res 44:655–667

Raetz CR, Dowhan W (1990) Biosynthesis and function of phospholipids in Escherichia coli. J Biol Chem 265:1235–1238

Renner LD, Weibel DB (2011) Cardiolipin microdomains localize to negatively curved regions of *Escherichia coli* membranes. Proc Natl Acad Sci U S A 108:6264–6269

Romantsov T, Helbig S, Culham DE, Gill C, Stalker L, Wood JM (2007) Cardiolipin promotes polar localization of osmosensory transporter ProP in *Escherichia coli*. Mol Microbiol 64:1455–1465

Romantsov T, Guan Z, Wood JM (2009) Cardiolipin and the osmotic stress responses of bacteria. Biochim Biophys Acta 1788:2092–2100

Rothman JE, Kennedy EP (1977) Asymmetrical distribution of phospholipids in the membrane of *Bacillus megaterium*. J Mol Biol 110:603–618

Salje J, Van Den Ent F, De Boer P, Lö We J (2011) Direct membrane binding by bacterial actin MreB. Mol Cell 43:478–487

Scheffers D-J, Tol MB (2015) LipidII: just another brick in the wall? PLoS Pathog 11:e1005213

Schneider J, Mielich-Süss B, Böhme R, Lopez D (2015) *In vivo* characterization of the scaffold activity of flotillin on the membrane kinase KinC of *Bacillus subtilis*. Microbiology 161:1871–1887

Shen B, Lutkenhaus J (2009) The conserved C-terminal tail of FtsZ is required for the septal localization and division inhibitory activity of MinC/MinD. Mol Microbiol 72:410–424

Shih Y-L, Le T, Rothfield L (2003) Division site selection in *Escherichia coli* involves dynamic redistribution of Min proteins within coiled structures that extend between the two cell poles. Proc Natl Acad Sci U S A 100:7865–7870

Shih Y-L, Kawagishi I, Rothfield L (2005) The MreB and Min cytoskeletal-like systems play independent roles in prokaryotic polar differentiation. Mol Microbiol 58:917–928

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569–572

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31–39
Simons K, Van Meer G (1988) Lipid sorting in epithelial cells. Biochemistry 27:6197–6202
Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175:720–731
Sohlenkamp C, Geiger O (2016) Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev 40:133–159
Somani VK, Aggarwal S, Singh D, Prasad T, Bhatnagar R (2016) Identification of novel raft marker protein, FlotP in *Bacillus anthracis*. Front Microbiol 7:169
Strahl H, Errington J (2017) Bacterial membranes: structure, domains, and function. Annu Rev Microbiol 71:519–538
Strahl H, Bürmann F, Hamoen LW (2014) The actin homologue MreB organizes the bacterial cell membrane. Nat Commun 5:3442
Subramani S, Perdreau-Dahl H, Morth JP (2016) The magnesium transporter A is activated by cardiolipin and is highly sensitive to free magnesium *in vitro*. elife 5:e11407
Szeto TH, Rowland SL, Rothfield LI, King GF (2002) Membrane localization of MinD is mediated by a C-terminal motif that is conserved across eubacteria, archaea, and chloroplasts. Proc Natl Acad Sci U S A 99:15693–15698
van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9:112–124
van Teeffelen S, Wang S, Furchtgott L, Huang KC, Wingreen NS, Shaevitz JW, Gitai Z (2011) The bacterial actin MreB rotates, and rotation depends on cell-wall assembly. Proc Natl Acad Sci U S A 108:15822–15827
Vanounou S, Pines D, Pines E, Parola AH, Fishov I (2002) Coexistence of domains with distinct order and polarity in fluid bacterial membranes. Photochem Photobiol 76:1–11
Vats P, Yu J, Rothfield L (2009) The dynamic nature of the bacterial cytoskeleton. Cell Mol Life Sci 66:3353–3362
Viola A, Gupta N (2007) Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins. Nat Rev Immunol 7:889–896

Outer Membrane Vesicles of Bacteria: Structure, Biogenesis, and Function

32

Armaity Nasarabadi, James E. Berleman, and Manfred Auer

Contents

1 Introduction 594
2 EMVs Across the Tree of Life 598
3 OMV Structures, Composition, and Function 599
4 Mechanism(s) of OMV Biogenesis 600
5 Regulation of OMV Biogenesis 601
6 OMV Functions 602
6.1 Biofilm Formation 602
6.2 Pathogenesis 603
6.3 OMV Associated with Predatory Behavior 604
7 Future Research Need 604
References 605

Abstract

Extracellular membrane vesicles (*EMVs*), a characteristic present across each domain of life, are subcellular shuttles of biologically active cargo that have a variety of functions ranging from cell-to-cell communication to predatory behavior. Mechanism(s) governing EMV biogenesis remain elusive; however, several initiators have been determined such as stress stimuli, sensing a potential prey or intruder, and signaling molecules. Regardless of function, increased membrane curvature and bulging is a key characteristic that leads to budding and release.

A. Nasarabadi (✉) · M. Auer
Lawrence Berkeley National Laboratory, Berkeley, CA, USA
e-mail: anasarabadi@lbl.gov; mauer@lbl.gov

J. E. Berleman
Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Saint Mary's College of California, Moraga, CA, USA
e-mail: jeb8@stmarys-ca.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_44

This chapter highlights the differences between biogenesis processes of the bacteria, archaea and eukarya. We then focus on the outer membrane vesicles (*OMVs*) specific to Gram-negative bacteria, including several mechanism(s) that potentially explain how the loss of crucial OM-peptidoglycan (*PGN*) and OM-PGN-inner membrane (*IM*) interactions can destabilize the OM to result in OMV biogenesis. Despite gaps present in the current understanding of these novel organelles, OMVs are one mechanism that allow microbial cells to function as multicellular organisms, as pathogens, and act as key predators in their environment. We discuss the importance in better understanding OMV biogenesis for greater insight into how this form of membrane architecture can be utilized for vaccines and targeted/specific treatments for infections.

1 Introduction

After initial controversy about their in vivo existence and functional relevance for microbial physiology, outer membrane vesicles (*OMVs*) in recent years have risen to high prominence as they mediate remote cell-to-cell interactions in biofilms, allowing for the exchange of macromolecules (lipids and proteins) and small molecules including quorum signals (*QS*) and toxins (Mashburn-Warren et al. 2008b; reviewed in Kulp and Kuehn 2010; Berleman et al. 2014; Rivera et al. 2010; Remis et al. 2014). The triggers for OMV formation and fusion are still being elucidated, as are the (macro-)molecular mechanisms underlying these mechanisms. Once formed, OMVs reside in the extracellular space, where they may immediately promote extracellular activities or later fuse to another cell (Kulp and Kuehn 2010; Mashburn and Whiteley 2005; Kadurugamuwa and Beveridge 1996; Li et al. 1996, 1998). Because of their hydrophobic nature, the molecules packed within OMVs are not diffused throughout an aqueous environment, but instead serve as a discrete package of goods that another cell, perhaps much later, may receive. It is not clear if different messages packaged in OMVs are targeted for intra- or interspecies delivery, but the complex array of cell surface proteins and lipopolysaccharide modifications may constitute a specific "bar code" or constrain the distribution of uptake. There are intriguing examples of OMV potential, from the motility proteins shared as a community resource through OMV transfer between cells of *Myxococcus xanthus* to the QS-induced OMVs in *Pseudomonas aeruginosa* biofilms (reviewed in Berleman and Auer 2013; Remis et al. 2014; Schaber et al. 2007; Mashburn and Whiteley 2005; Palsdottir et al. 2009). OMVs may also contain a lethal cargo, killing the receiving cell upon vesicle fusion and corresponding uptake of an undiluted chemical dose (Mashburn-Warren et al. 2008a). This mode of a contact-only delivery system clearly has stood the test of time, and we review here the current state of the field in understanding the biogenesis of these structures, their roles in nature and the applications that OMVs may have.

Gram-negative bacteria have a dual membrane envelope, with distinctive inner membrane (*IM*) and outer membrane (*OM*) structures (Fig. 1a). But Gram-negative bacteria are not monophyletic, and it is not clear whether there exists one OMV biogenesis mechanism for all Gram-negative bacteria or a variety of mechanisms

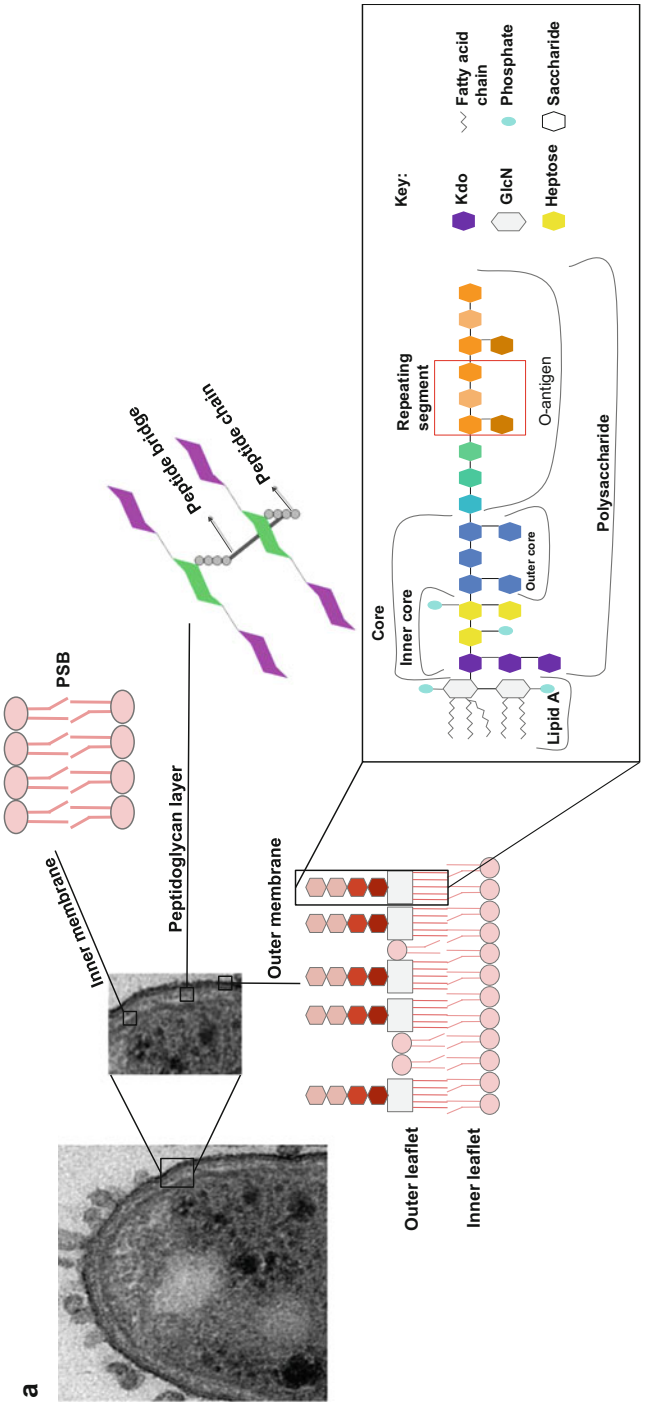


Fig. 1 (continued)

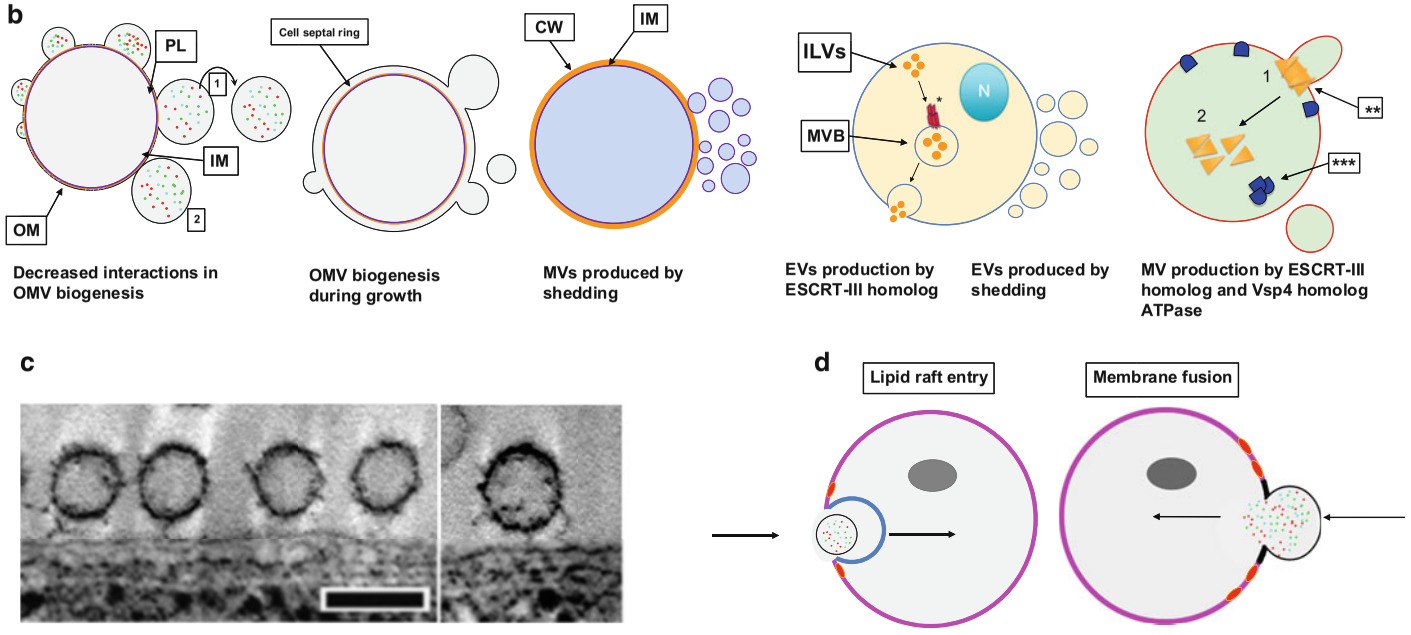


Fig. 1 Gram-negative bacteria membrane structure, MV biogenesis and OMV acceptance in target cell: Gram-negative bacteria make OMVs from the OM, which either stay connected or bud off (**a**, *far left panel*) (Images adopted from Berleman and Auer 2013). The inner membrane is composed of phospholipids, but the OM inner leaflet is composed of phospholipids and the outer leaflet is mainly LPS. LPS is composed of a polysacchride chain and lipid body, with the *O*-antigen being presented to the extracellular matrix (Key: Kdo, 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid). The *O*-antigen is responsible for inducing the endotoxic response in the host's immune system. The peptidoglycan layer makes up the bacterial cell wall and is composed of strands of alternating *N*-acetylmuramic acid (*green*) and *N*-acetylglucosamine (*purple*) which are connected by peptide bridges at the peptide chains that extends from *N*-acetylmuramic acid.

that vary significantly from clade to clade. The OM is a unique envelope structure, with an inner leaflet of phospholipid and an outer leaflet composed of lipopolysaccharides (*LPS*), and is far more complex in its composition (Fig. 1a). While membrane vesicle formation is best documented and understood in Gram-negative bacteria, species with other cell architectures also release extracellular membrane vesicles (*MVs*) (Fig. 1b). Gram-positive bacteria and archaea have also been shown to produce extracellular MVs that serve similar functions as OMVs in Gram-negative bacteria (reviewed in Deatherage and Cooksona 2012; Rivera et al. 2010). Despite having only a single membrane and their extensive envelope with a thick cell wall (peptidoglycan or S-layer protein coat/pseudopeptidoglycan, respectively), MVs are still observed. For Gram-negative bacteria, with their clearly defined inner membrane (*IM*) and outer membrane (*OM*) systems, OMVs bud off the OM with the IM typically held intact. But in the case of Gram-positive bacteria, MVs must originate from the cytoplasmic membrane. These MVs lack LPS and consist predominantly of a phospholipid bilayer. Similarly, archaeal MVs reflect the unique biochemistry of archaeal cytoplasmic membranes, containing ether linkages, isoprenoid chains, and transmembrane phospholipids. Extracellular secretion of vesicles also exists in eukaryotes, where they are known as exosome vesicles (*EVs*). Such EVs have been shown to play a vital role in red blood cell maturation, including the sequestration of certain membrane proteins and nucleic acids breakdown fragments of the cell nuclear

Fig. 1 (continued) EMV formation occurs in all three domains of life (**b**). Gram-negative (*two light grey cells to the left*) bacteria are observed making OMVs by two mechanisms: reducing the number of OM-PL (*light blue lines*) and OM-PL-IM (*purple lines*) interactions in areas around the cell in response to stimuli and at points called nanoterritories or reducing the interactions previously mentioned through cell growth since the cell wall grows at a slower rate than the OM at the septum of the dividing cell. OMVs can either be released from the cell (*1*) or remain attached (*2*). Once removed, OMVs are found to have a variety of contents, including proteins, secondary metabolites and nucleic acids. Gram-positive bacteria (*center left blue cell*) have been observed producing MVs, which is suspected to be done by shedding, similar to eukaryotic microbes, at areas of weakened or absent cell wall. Eukaryotic microbe cells (*two center right beige cells*) are also observed producing EVs by the gathering of ILVs in MVBs by ESCRT-III homolog mediation which then fuse to the cell membrane and release its contents into the extracellular matrix. Archaea (*light green cell far right*) utilize a ESCRT-III homolog to make the membrane bulge to produce the MV, which is released through ESCRT-III membrane complex disassembly by Vsp4 homolog ATPase. Note that the archaeal cell membrane is shown above, but the S-layer and cell wall would be present during MV genesis, as well. Electron density increases from the center of the vesicles to its edge and are observed aligned on the surface of a *Myxococcus xanthus* cell (imaged by cryo-tomography) with cargo or an underlying protein organization (**c**). Scale bar, 100 nm (Palsdottir et al. 2009) (Images adopted from Berleman and Auer 2013; Palsdottir et al. 2009). OMVs enter host cells by two methods: lipid rafts or membrane fusion (**d**). Lipid rafts are areas that are rich in sphingolipids and cholesterol, causing the membrane to invaginate (*blue*) at areas of dense lipid packing and allow OMVs to enter the cell whole. OMV fusion to the target cell's membrane also leads to OMV contents entering the cell, despite variations in membrane structure, at colocalized lipid raft rich areas. Note abbreviations and symbols: *PL* peptidoglycan layer, *IM* inner membrane, *OM* outer membrane, *PSB* phospholipid bilayer, *CW* cell wall, * (ESCRT-III homolog), ** (Vsp4 homolog ATPase assembled)

chromatin. Exosomes also have been implemented in cancer development, as EVs are capable to remove unwanted chemicals from the cancer cell.

We will focus here mostly on OMVs, where a host of functions have evolved for optimal survival, ranging from indirect cell–cell communication, acquisition of nutrients, evasion of the host's immune system and functioning as pathogens. For examples, soil bacteria live in a dynamic and complex environment that constantly changes with respect to nutrient availability and competition from other species. Communication is key in such environments, and in order to maintain their territory and avoid annihilation, a concerted response mediated by OMVs is needed, for example, coordinating a targeted and lethal attack and alerting other biofilm members to the threat.

2 EMVs Across the Tree of Life

Secreted vesicles are found in Gram-negative and Gram-positive bacteria, archaea, as well as eukaryotic cell systems. The lumen or membrane envelope of OMV often contains toxins, small signaling molecules and biologically active proteins, including virulence factors (Fig. 1c). Gram-negative bacterial OMVs have been extensively studied, whereas the ability of Gram-positive bacteria to produce MVs, that are similar in size to OMVs (20–100 nm), has been more recently reported (reviewed in Deatherage and Cooksona 2012; Lee et al. 2008, 2009; Rivera et al. 2010). *Bacillus* MVs can be double-layered (Lee et al. 2008, 2009; Rivera et al. 2010) and contain deadly cargo, such as the anthrax toxin (Rivera et al. 2010). Plausible mechanisms that govern Gram-positive bacteria membrane vesicle formation are largely unknown. However, there is experimental evidence that suggest vesicle shedding as a mechanism (Lee et al. 2009).

Archaeal and eukaryotic microbes produce vesicles that are of somewhat similar size to bacteria, 90–230 nm and 40–100 nm, respectively (Prangishvili et al. 2000; Ellen et al. 2008; reviewed in Deatherage and Cooksona 2012). Archaea related MV production appears to be created by pinching off the cell membrane and outer S-layer of the cell wall, whereas eukaryotic microbes utilize exocytosis of intraluminal vesicles (*ILVs*) for EV formation, a process more similar to the production of OMVs by bacteria and the shedding microvesicles (*SMVs*) of eukaryotes (Deatherage and Cooksona 2012) (Fig. 1b). Both archaeal and eukaryotic microbes are suspected to utilize *e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport (*ESCRT-III*) and *v*acuolar *s*orting *p*rotein (*Vps4*) homologues in the biogenesis of their membrane vesicles (Deatherage and Cooksona 2012; Ellen et al. 2008), since this class of machinery is conserved between both domains (reviewed in Deatherage and Cooksona 2012). In eukaryotic cells, ILVs are packaged in larger membrane vesicle congregates called *m*ulti *v*esicular *b*odies (*MVBs*) by ESCRT-III homologues, which can be thought of as a larger vesicle holding smaller vesicles (Keller et al. 2006; Deatherage and Cooksona 2012) (Fig. 1b). MVBs fuse to the plasma membrane and release of ILVs as exosomes (Deatherage and Cooksona 2012). Eukaryotic microbes utilize similar machinery which allows the release of ILVs in the form of exosomes. Production of exosomes for eukaryotic microbes is the result of shedding, similar to bacteria, although the exact mechanisms are still unknown.

Archaea utilize ESCRT-III homologue proteins to form membrane vesicles and appears to require Vsp4 homologs for vesicle release (Ellen et al. 2008; Deatherage and Cooksona 2012). ESCRT-III homolog proteins are thought to congregate at the plasma membrane surface to create a bulge, with pinching off of the vesicle occurring when Vps4 homolog ATPases leads to archaeal ESCRT-III homolog disassembly (Ellen et al. 2008; Deatherage and Cooksona 2012). ESCRT-III homolog and Vps4 homolog ATPase proteins are membrane associated, which upon vesicle release mostly stay with the cell (Deatherage and Cooksona 2012; Ellen et al. 2008). While the detailed mechanisms between bacterial and archaea/eukaryotes appear to be different, it would seem that neither process is random or passive but highly orchestrated and controlled.

3 OMV Structures, Composition, and Function

OMVs were first observed with *Escherichia coli* when it was placed in growth limiting nutritional conditions resulting in OMVs that appeared as protrusions on the outer surface of cells, still attached in many cases (Bishop and Work 1965; Knox et al. 1966). As the nutrient concentration decreased, vesicle growth seemed to increase (Knox et al. 1966; Work et al. 1966). Analysis initially done by Bishop and Work (1965) showed that LPS was incorporated into the vesicles. The origin of the vesicles was identified using time lapse imaging and initially it was hypothesized that an excess of membranes due to stalled growth of *E. coli* was the reason for vesicle formation (Work et al. 1966). Biochemical analysis of vesicles and outer membrane revealed an identical LPS signature, which supported an early hypothesis, that the vesicles solely originated from the outer membrane (Katsui et al. 1982; Hoekstra et al. 1976).

The cytotoxic protein ClyA in an *E. coli* K-12 derivative strain accumulates initially as periplasm-folded ClyA monomers underneath OM bulges (Wai et al. 2003; reviewed in De Geyter et al. 2016) in regions of reduced peptidoglycan linkages. Oligomerization of ClyA occurs in OMVs forming pores which contribute to the infection of erythrocytes and other mammalian cells (Hunt et al. 2010; Wai et al. 2003). When observed by TEM imaging, vesicles appear hollow, and devoid of major macromolecular proteins and complexes, in accordance with the hypothesis of their origins (Katsui et al. 1982). These structures have been observed being relatively spherical in shape, and the size reported was typically between 30 and 80 nm or larger in diameter (Lee et al. 2008, 2009; Rivera et al. 2010; Kadurugamuwa and Beveridge 1996; Palsdottir et al. 2009).

Recent proteomics studies support the idea that OMVs share compositional characteristics with the outer membrane, but are not an exact replica (Kulp and Kuehn 2010; Berleman et al. 2014; Loeb and Kilner 1979). Certain proteins are only found in OMVs but not in the OM, whereas others are shared between OMVs and the OM; supporting the notion that there are possible organization or selection mechanisms that determine outer membrane vesicle composition (reviewed in Kulp and Kuehn 2010; Berleman et al. 2014). In addition to OMV-OM-shared and OMV-unique proteins, periplasmic proteins, inner membrane proteins and cytoplasmic components have also

been found in OMVs as well (Berleman et al. 2014; Roier et al. 2016; Deatherage and Cooksona 2012; Kulkarni and Jagannadham 2014; Deatherage et al. 2009; Schwechheimer and Kuehn 2015), and they do not appear to represent mere contamination issues as certain other high abundance proteins were not found.

OMVs have been found to enter mammalian cells by cholesterol and sphingolipids-rich areas that form lipid rafts and fusion to a target membrane at colocalized areas of lipid rafts (O'Donoghue and Krachler 2016) which result in the content to be released into its cytoplasms (Fig. 1d). However as a word of caution, LPS serves as an endotoxin that elicits an immune response in humans. Though, upon entry into eukaryotic cells, the content of OMVs is eventually released and may contain secondary metabolites and proteins that maybe have a broad cytotoxic effect and can induce cell apoptosis.

4 Mechanism(s) of OMV Biogenesis

While the biological processes governing the formation of OMVs have yet to be fully understood, differences in OMV and OM composition suggest that OMV formation is not a passive process, but is somehow regulated (Berleman et al. 2014; Wensink and Witholt 1981; Kato et al. 2001; Hoekstra et al. 1976; Deatherage and Cooksona 2012). Given the different roles OMV play in bacterial physiology, ranging from mediating benign and supportive interactions such as the sharing and exchange of OM proteins, to hostile and detrimental interactions, such as the delivery of toxins, one would expect that both the cargo-load as well as the targeting mechanisms are well controlled and adjusted to the respective situation. However, none of the proteins in this sorting machinery have been identified.

In order for the vesicles to exhibit any kind of function they first have to be secreted. Three mechanisms for OMVs formation has been proposed (reviewed in Berleman and Auer 2013): (I) in the first mechanism small molecules or membrane proteins associate with the outer membrane to induce curvature that then subsequently leads to budding of the vesicle; (II) in the second mechanism, the accumulation of periplasmic proteins pushes the outer membrane out to form a vesicle; and (III) in the third mechanism the contact between the OM and peptidoglycan layer is lost, leading to the buildup of lipids, and subsequent vesicle formation. In this scenario links between the OM and the cell wall are severed (Schwechheimer and Kuehn 2015). Either such links are actively broken, or there may exist as nanoterritories, or areas in the membrane, with reduced interactions (e.g., near the poles) (Schwechheimer and Kuehn 2015). Recent studies of OMV formation in *Haemophilus influenza*, suggests the involvement of an ABC transporter protein for the accumulation of lipids in the outer leaflet of the outer membrane (Roier et al. 2016). These proposed mechanisms are not mutually exclusive and may all in some part contribute to OMV biogenesis. Proteomics analysis has revealed that more than half of the proteins found in *Myxococcus* OMVs have no known function (Berleman et al. 2014). Comparison to other Gram-negative OMV, proteomics studies (Kulp and Kuehn 2010; Deatherage and Cooksona 2012) have not yielded a clearly defined set of proteins that is always present and thus evolutionary conserved.

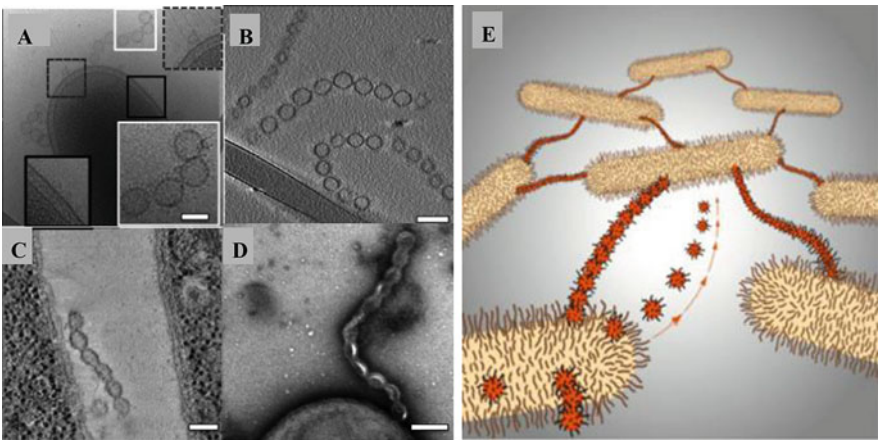


Fig. 2 Structural analysis of *M. xanthus* vesicle chains and model of vesicle facilitated interactions in biofilm formation. *M. xanthus* forms both single vesicles and vesicle chains. Cryo-fixed *M. xanthus* cells were imaged by TEM to view OMVs that have a single membrane (**a**, *white bordered square*) when compared to the parent cell double-membrane structure (**a**, *black line border square*). Vesicles were discernible from pili (**a**, *black dotted square*). Vesicle chains prepared by fast-frozen methods (**b**) were observed being of similar size in a single chain, with size variation observed between chains. Continuous lumen was observed in constricted vesicle chains of a slice through a 3D tomogram of a HPF/FS, resin-embedded sample and negatively stained whole mount sample (**c**, **d**, respectively). (**e**) Remis et al. propose that connections between individual cells within a biofilm are maintained by extracellular appendages including free diffusing vesicles and vesicle chains. These appendages are coated in carbohydrates (i.e., LPS and OM lipids) utilized for cell-cell recognition and communication through the transfer of intercellular matter. This model shows individual cells being organized into a highly integrated multicellular community through free vesicles diffusion and direct intercellular connections by vesicle chains. Scale bars represent: 50 nm (**a**) and 100 nm (**b**, **c**, **d**) (Images adopted from Remis et al. 2014)

From an ultrastructural point of view, the center of the vesicles is found to be relatively hollow with a single membrane bilayer surrounding the luminal content (Knox et al. 1966; Work et al. 1966; Kadurugamuwa and Beveridge 1996). Electron tomographic analysis (Palsdottir et al. 2009) of resin-embedded samples confirmed a relatively sparse occupancy of the lumen (Fig. 1c). OMVs were often found to be tethered to the membrane, and even tethered to one another, with OMVs often forming a tight linear like pearl array on a string (Fig. 2). At this point it remains uncertain, whether there exists a cage-like structure just underneath the membrane, not unlike clathrin in eukaryotes, but experimental validation, for example, by electron tomographic imaging, remains to be carried out.

5 Regulation of OMV Biogenesis

Outer membrane vesicle biogenesis occurs under normal growth conditions but is upregulated upon encountering environmental stress (Kulp and Kuehn 2010; Berleman and Auer 2013; Baumgarten et al. 2012; Deatherage and Cooksona 2012;

Roier et al. 2016). These stressors include salinity, acidity, temperature, and nonrelated microbial communities occupying the same space (Kulp and Kuehn 2010; Baumgarten et al. 2012; Katsui et al. 1982; Deatherage and Cooksona 2012; Roier et al. 2016). Such external stressors can even alter the bacterial envelope physiology, for example, *Pseudomonas putida* DOT-T1E increases OMV formation and renders the hydrophobicity of the cell surface to facilitate biofilm formation (Baumgarten et al. 2012). *P. aeruginosa* increases production of membrane vesicles after exposure to the antibiotic gentamicin with an altered contents and integrated membrane composition when compared to normal OMV biogenesis (Mashburn-Warren et al. 2008a; Kadurugamuwa and Beveridge 1995). OMV production is also altered by the exogenous introduction of a quorum signaling molecule (such as PQS), which induces curvature and perturbation of the OM (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008b; Schertzer and Whiteley 2012). It is thought that the interaction of PQS with the outer LPS leaflet of the OM drives the biogenesis of vesicles (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008b; Schertzer and Whiteley 2012). When confronted with another species, *M. xanthus* will form OMVs that are filled with a lethal load resulting in the lysis of the opposing colony and the uptake of nutrients (Berleman et al. 2014; Evans et al. 2012; reviewed in Keane and Berleman 2016).

6 OMV Functions

Outer membranes vesicles are present during basal activity and often upregulated in times of stress, suggesting that they carry a variety of functions. The formation of OMVs was observed by Knox et al. (1966) when imaging *E. coli* under stress due to the lack of lysine in the growth medium. Since then, OMVs have been observed in pathogenic bacteria, as a hunting and defense mechanism and appear to play a role in biofilm formation. The exact mechanism of delivery remains to be determined; one can envision autolysis in the vicinity of the target cell (Kulp and Kuehn 2010; Mashburn-Warren et al. 2008a; Kadurugamuwa and Beveridge 1996; Li et al. 1998) or the fusion of the vesicle with its target cell, and thus release of the content into its periplasm (Kulp and Kuehn 2010; Mashburn and Whiteley 2005; Kadurugamuwa and Beveridge 1995; Li et al. 1996, 1998), possibly disrupting the peptidoglycan structure and leaving the bacterium vulnerable. Results from our and other labs support the fusion model.

6.1 Biofilm Formation

Biofilms are constructs of large microbial colonies that emerge in order for cells to communicate and serve as protection against harsh environments. *P. aeruginosa* biofilms have also been observed in chronic infections and in burn wounds, with vesicles found in the extracellular matrix surrounding cells (reviewed in Berleman and Auer 2013; Schaber et al. 2007). PQS facilitates the formation of OMVs, and

while in itself it is not sufficient for biofilm formation, it affects group dynamics and triggers PQS-related group behavior (Mashburn and Whiteley 2005). Presence of PQS leads to the formation of vesicles that contain quinolone/quinolines that have active antimicrobial properties (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008b). Within the biofilm borders of *M. xanthus*, it was found that OMVs fill available space that cell bodies do not occupy (Palsdottir et al. 2009), providing ultrastructural evidence that OMVs are closely interacting with the OM of cells via proteinaceous tethers. Thus, OMVs are not only utilized for the initial formation of biofilms but are prominently found in mature biofilms. The upregulation of OMV secretion in *M. xanthus* biofilms compared to its planktonic state further emphasizes the key role they play in intraspecies, intercellular communication.

Among the more surprising findings of the last few years is that OMVs are not only secreted as individual vesicles, but they often form more complex structures, such a membrane vesicles chains, regular-interval constricted membranes tubes, as well as membrane tubes with a uniform circular or oval-shaped diameter (Remis et al. 2014). Dubbed as "bacterial social networks," they can be viewed as a stealth intercellular communication and molecule-sharing system, where large numbers of cells are physically connected at the level of their respective periplasmic space (Fig. 2). Such interconnection of cells in a biofilm would allow *Myxococcus* biofilms to behave like a complex multicellular organism with a spatially organized division of labor, not unlike one that is found in plants or animals. This type of network would also allow regions of the biofilms that are far away from a competing species to be alerted in time and thus prepare for such threats or opportunities.

6.2 Pathogenesis

Biofilms have been found in infected wounds or vulnerable tissue (lungs of individuals with cystic fibrosis), with pathogenesis apparently correlating with OMV presence. In order for the pathogenicity to occur, a microbial community/biofilm has to be established, that conducts an attack while shielding itself. OMVs may have two functions, communication between colonies of microbes and attacking cells. The packaging of virulence and pathogenic factors into OMVs presents different advantages: first, concentrating virulence factors to the target cell for more efficient delivery, and second, virulence factors would be protected against degradation and recognition by the host and other microorganisms (Mashburn-Warren et al. 2008a). When harmful antimicrobial agents (including gentamicin) are presented to *P. aeruginosa* OMV formation is induced, leading to the packaging of *P. aeruginosa* antimicrobial agents (Kadurugamuwa and Beveridge 1996; Mashburn-Warren et al. 2008a), including autolysins, hydrolytic enzymes, phospholipase C, proteases, alkaline phosphatases and hemolysins (Mashburn-Warren et al. 2008a; Kadurugamuwa and Beveridge 1995).

OMV mediated toxin attack is facilitated by endocytosis into eukaryotic cells or fusion with the plasma membrane (Kulp and Kuehn 2010; Furuta et al. 2009). OMVs by *Acinetobacter baumannii* cause host cells to undergo apoptosis through

an OmpA channel protein-mediated process that disrupts the host membrane (Jin et al. 2011). *Aggregatibacter actinomycetemcomitans* uses OMVs to deliver the cytolethal toxins to both HeLa cells and human gingival fibroblasts (Rompikuntal et al. 2012). While the mode of producing a cytotoxic effect on these two eukaryotic target cells differs, in both cases OMVs utilized to evade the host's immune response and deliver the toxic agent.

6.3 OMV Associated with Predatory Behavior

In addition to biofilm formation and pathogenesis, OMV also play a role in predation, as "predatory [O]MVs" (Mashburn-Warren et al. 2008a; Mashburn and Whiteley 2005; Kadurugamuwa and Beveridge 1996), which may fuse with both Gram-negative bacteria and eukaryotic cells (Kadurugamuwa and Beveridge 1996; Evans et al. 2012). Predatory [O]MVs contain enzymes that disrupt the OM and peptidoglycan walls, thus killing a wide spectrum of bacteria (Li et al. 1998). Salt bridges forming stable interactions between the OMV and the Gram-positive cell wall have been proposed, which may deform OMVs resulting in OMV bursting in close proximity to the target cell (Kadurugamuwa and Beveridge 1996). Likewise scavenging and predatory soil bacteria take advantage of this long-range, but targeted mode of attack and/or defense.

M. xanthus, a Gram-negative bacterium, is a key predator species in the soil that utilizes OM vesicles not only to maintain its territories, but also to acquire essential nutrients. *M. xanthus* requires close contact with prey cell to induce prey death, without invasion or engulfment, and OMVs have been found to facilitate predation (Keane and Berleman 2016; Berleman et al. 2014; Evans et al. 2012). OMV content analysis revealed the presence of 16 molecules with antibiotic activities and several homologs of hydrolytic enzymes. Proteomics analysis revealed 234 OMV-enriched proteins, with 46 specific to OMVs and 188 shared with the OM (Berleman et al. 2014). Enrichment suggests a selection mechanism for the packing of the OMV, which may suggest that OMV formation may not be a rather passive process, but entails a high level of control. Furthermore, it is possible that OMVs contain a type of "zip-code" (suggested by Evans et al. 2012), that ensures proper targeting of the OMV content, which could be protein- or more interestingly LPS-based.

Whatever the mode of targeting, delivering an undiluted lethal cargo to a targeted cell is an effective way to avoid resistance that may otherwise occur since dilution of the antimicrobial molecules would reduce their efficacy, which in by itself may justify the energy cost of OMV biogenesis.

7 Future Research Need

Many advances have been made in understanding the biological processes governing the formation of vesicles in the extracellular environment. We still lack an understanding of the exact mechanisms of OMV formation and their molecular

triggers. We also have little insight about the control of vesicle content, but discrepancies in OM and OMV macromolecular composition suggest that OMV formation is not a passive but instead a highly controlled active process. The question of a possible ZIP code that may be protein and/or polysaccharide based is a particularly intriguing one, as it would allow control over specificity of messaging. Given the success of OMVs in communication and predation, and the fact that targeted species have not developed resistance despite millions to billions of years of coevolution, OMVs may be an intriguing novel approach for antibiotics development. Packaging of one or several antibiotic/antimicrobial (macro-)molecules into vesicles offers protection and stability.

Given their ubiquitous presence in extracellular fluids, their apparent biostability, and their small size, OMVs may serve as noninfectious vehicles to deliver small molecules or macromolecules to different human cells and tissues and to initiate or suppress a host immune response (Ellis and Kuehn 2010). Such "designer OMVs" are likely to be taken up by microbes and human cells in ways similar to pathogens (Kulp and Kuehn 2010; Furuta et al. 2009; Ellis and Kuehn 2010). An OMV-based vaccine for *Neisseria meningitidis* is the most successful case of OMVs initiating an immune response with moderately high efficacy (Acevedo et al. 2014). OMV vaccines for *N. meningitidis* were proposed as an alternate source due to a lower risk of potentially developing autoimmunity (reviewed in Acevedo et al. 2014).

OMV formation could also be an important drug target, as interference in this process may stall biofilm formation and pathogenesis altogether. Clearly, it is early in the days of OMV research, as further research is needed to better understand the various biological processes involved in OMV formation and molecular packing, so that ultimately we may control this process and design OMVs with specific characteristics, content and ZIP-code for the control and treatment of infectious diseases or for other cell-specific cargo-delivery (e.g., lung diseases, cancer, or immune response abnormalities).

References

Acevedo R, Fernández S, Zayas C et al (2014) Bacterial outer membrane vesicles and vaccine applications. Front Immunol 5:121. https://doi.org/10.3389/fimmu.2014.00121

Baumgarten T, Sperling S, Seifert J et al (2012) Membrane vesicle formation as a multiple-stress response mechanism enhances *Pseudomonas putida* DOT-T1E cell surface hydrophobicity and biofilm formation. Appl Environ Microbiol 78(17):6217–6224. https://doi.org/10.1128/AEM.01525-12

Berleman J, Auer M (2013) The role of bacterial outer membrane vesicles for intra- and interspecies delivery. Environ Microbiol 15(2):347–354. https://doi.org/10.1111/1462-2920.12048

Berleman JE, Allen S, Danielewicz MA et al (2014) The lethal cargo of *Myxococcus xanthus* outer membrane vesicles. Front Microbiol 5:1–11. https://doi.org/10.3389/fmicb.2014.00474

Bishop DG, Work E (1965) An extracellular glycolipid produced by *Escherichia coli* grown under lysine-limiting conditions. Biochem J 96(2):567–576. https://doi.org/10.1042/bj0960567

Deatherage BL, Cooksona BT (2012) Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect Immun 80(6):1948–1957. https://doi.org/10.1128/IAI.06014-11

Deatherage BL, Lara JC, Bergsbaken T, Barrett SLR, Lara S, Cookson BT (2009) Biogenesis of bacterial membrane vesicles. Mol Microbiol 72(6):1395–1407. https://doi.org/10.1111/j.1365-2958.2009.06731.x.Biogenesis

De Geyter J, Tsirigotaki A, Orfanoudaki G, Zorzini V, Economou A, Karamanou S (2016) Protein folding in the cell envelope of *Escherichia coli*. Nat Microbiol 1:16107. https://doi.org/10.1038/nmicrobiol.2016.107

Ellen AF, Albers SV, Huibers W et al (2008) Proteomic analysis of secreted membrane vesicles of archaeal *Sulfolobus* species reveals the presence of endosome sorting complex components. Extremophiles 13(1):67–79. https://doi.org/10.1007/s00792-008-0199-x

Ellis TN, Kuehn MJ (2010) Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiol Mol Biol Rev 74(1):81–94. https://doi.org/10.1128/mmbr.00031-09

Evans A, Davey H, Cookson A, Currinn H, Cooke-Fox G, Stanczyk P, Whitworth D (2012) Predatory activity of *Myxococcus xanthus* outer-membrane vesicles and properties of their hydrolase cargo. Microbiology 158(11):2742–2752. https://doi.org/10.1099/mic.0.060343-0

Furuta N, Takeuchi H, Amano A (2009) Entry of *Porphyromonas gingivalis* outer membrane vesicles into epithelial cells causes cellular functional impairment. Infect Immun 77(11):4761–4770. https://doi.org/10.1128/IAI.00841-09

Hoekstra D, van der Laan JW, de Leij L, Witholt B (1976) Release of outer membrane fragments from normally growing *Escherichia coli*. Biochim Biophys Acta 455:889–899

Hunt S, Green J, Artymiuk P (2010) Hemolysin E (HlyE, ClyA, SheA) and related toxins. Adv Exp Med Biol 677:116–126

Jin JS, Kwon S-O, Moon DC et al (2011) *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via outer membrane vesicles. PLoS One 6(2):e17027. https://doi.org/10.1371/journal.pone.0017027

Kadurugamuwa JL, Beveridge TJ (1995) Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. J Bacteriol 177(14):3998–4008

Kadurugamuwa JL, Beveridge TJ (1996) Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. J Bacteriol 178(10):2767–2774

Kato S, Kowashi Y, Demuth DR (2001) Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. Microb Pathog 32(1):1–13. https://doi.org/10.1006/mpat.2001.0474

Katsui N, Tsuchido T, Hiramatsu R, Fujikawa S, Takano M, Shibasaki I (1982) Heat-induced blebbing and vesiculation of the outer membrane of *Escherichia coli*. J Bacteriol 151(3):1523–1531

Keane R, Berleman J (2016) The predatory life cycle of *Myxococcus xanthus*. Microbiology 162(1):1–11. https://doi.org/10.1099/mic.0.000208

Keller S, Sanderson MP, Stoeck A, Altevogt P (2006) Exosomes: from biogenesis and secretion to biological function. Immunol Lett 107(2):102–108. https://doi.org/10.1016/j.imlet.2006.09.005

Knox KW, Vesk M, Work E (1966) Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*. J Bacteriol 92(4):1206–1217

Kulkarni HM, Jagannadham MV (2014) Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. Microbiology 160(2014):2109–2121. https://doi.org/10.1099/mic.0.079400-0

Kulp A, Kuehn MJ (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol:163–184

Lee E, Choi D, Kim K, Cho Y (2008) Proteomics in Gram-negative bacterial outer membrane vesicles. Mass Spectrom Rev 27:535–555

Lee E-Y, Choi D-Y, Kim D-K et al (2009) Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. Proteomics 9(24):5425–5436. https://doi.org/10.1002/pmic.200900338

Li Z, Clarke AJ, Beveridge TJ (1996) A major autolysin of *Pseudomonas aeruginosa*: subcellular distribution, potential role in cell growth and division and secretion in surface membrane vesicles. J Bacteriol 178(9):2479–2488. http://www.ncbi.nlm.nih.gov/pubmed/8626312

Li Z, Clarke AJ, Beveridge TJ (1998) Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. J Bacteriol 180(20):5478–5483

Loeb MR, Kilner J (1979) Effect of growth medium on the relative polypeptide composition of cellular outer membrane and released outer membrane material in *Escherichia coli*. J Bacteriol 137(2):1031–1034

Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437(7057):422–425. https://doi.org/10.1038/nature03925

Mashburn-Warren L, McLean RJC, Whiteley M (2008a) Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology 6(3):214–219. https://doi.org/10.1111/j.1472-4669.2008.00157.x

Mashburn-Warren L, Howe J, Garidel P et al (2008b) Interaction of quorum signals with outer membrane lipids: insights into prokaryotic membrane vesicle formation. Mol Microbiol 69(2):491–502

O'Donoghue EJ, Krachler AM (2016) Mechanisms of outer membrane vesicle entry into host cells. Cell Microbiol:1508–1517. https://doi.org/10.1111/cmi.12655

Palsdottir H, Remis JP, Schaudinn C et al (2009) Three-dimensional macromolecular organization of cryofixed *Myxococcus xanthus* biofilms as revealed by electron microscopic tomography. J Bacteriol 191(7):2077–2082. https://doi.org/10.1128/JB.01333-08

Prangishvili D, Holz I, Stieger E, Nickell S, Kristjansson JK, Zillig W (2000) Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus *Sulfolobus*. J Bacteriol 182(10):2985–2988. https://doi.org/10.1128/JB.182.10.2985-2988.2000

Remis JP, Wei D, Gorur A et al (2014) Bacterial social networks: structure and composition of *Myxococcus xanthus* outer membrane vesicle chains. Environ Microbiol 16(2):598–610. https://doi.org/10.1111/1462-2920.12187

Rivera J, Cordero RJB, Nakouzi AS, Frases S, Nicola A, Casadevall A (2010) *Bacillus anthracis* produces membrane-derived vesicles containing biologically active toxins. Proc Natl Acad Sci U S A 107(44):19002–19007. https://doi.org/10.1073/pnas.1008843107

Roier S, Zingl FG, Cakar F et al (2016) A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. Nat Commun 7:10515. https://doi.org/10.1038/ncomms10515

Rompikuntal PK, Thay B, Khan M et al (2012) Perinuclear localization of internalized outer membrane vesicles carrying active cytolethal distending toxin from *Aggregatibacter actinomycetemcomitans*. Infect Immun 80(1):31–42. https://doi.org/10.1128/IAI.06069-11

Schaber JA, Triffo WJ, Sang JS et al (2007) *Pseudomonas aeruginosa* forms biofilms in acute infection independent of cell-to-cell signaling. Infect Immun 75(8):3715–3721. https://doi.org/10.1128/IAI.00586-07.1

Schertzer J, Whiteley M (2012) A bilayer-couple model of bacterial outer membrane vesicle. MBio 3(2):e00297–e00211. https://doi.org/10.1128/mBio.00297-11.Editor

Schwechheimer C, Kuehn MJ (2015) Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol 13(10):605–619. https://doi.org/10.1038/nrmicro3525

Wai SN, Lindmark B, Söderblom T et al (2003) Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell 115(1):25–35. https://doi.org/10.1016/S0092-8674(03)00754-2

Wensink J, Witholt B (1981) Outer-membrane vesicles released by normally growing *Escherichia coli* contain very little lipoprotein. Eur J Biochem 116:331–335

Work E, Knox KW, Vesk M (1966) The chemistry and electron microscopy of an extracellular lipopolysaccharide from *Escherichia coli*. Ann N Y Acad Sci 133:438–449. https://doi.org/10.1111/j.1749-6632.1966.tb52382.x

Flip-Flopping Membrane Proteins: How the Charge Balance Rule Governs Dynamic Membrane Protein Topology

33

Mikhail Bogdanov, Heidi Vitrac, and William Dowhan

Contents

1 Introduction 610
2 TMD Protein Structure: Static or Dynamic? 611
3 Justifying SCAM™ as a Method of Choice 611
4 SCAM™ to Probe TMD Protein Topology Dynamics In Vivo 614
5 Charge Balance Rule of Membrane Protein Assembly and Topogenesis 621
6 Membrane Protein Topological Duality and Lipid–Dependent Reversibility of Topological Interconversion 624
7 From Liposomes to Fliposomes: Monitoring TMD Topology Dynamics and Interconversions In Vitro 627
8 Lipid and Protein Dynamics in Real Time: Monitoring Membrane Protein and Lipid Flip-Flops 628
9 On the Biological Relevance of TMD Reorientations 628
10 Can Pathogenic Mutations or Other Genetically Induced Changes Affect Topologies? 632
11 Research Needs 632
References 633

Abstract

Transmembrane and lateral phospholipid asymmetries are not absolute as is the case for integral membrane proteins where asymmetry does not have to be actively maintained due to the enormous energy required to flip across the hydrophobic barrier of the membrane. Although the lipid bilayer is widely considered as a non-flipping zone for most proteins, some integral membrane

M. Bogdanov (✉) · H. Vitrac · W. Dowhan
Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, McGovern Medical School, Houston, TX, USA
e-mail: mikhail.v.bogdanov@uth.tmc.edu; heidi.vitrac@uth.tmc.edu; william.dowhan@uth.tmc.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_62

proteins possess the capacity to reversibly reorient themselves during or after insertion if membrane phospholipid composition is changed, the membrane is depolarized or components of the translocon interact with each other during ATP-driven protein substrate translocation. Membrane proteins can be also engineered to flip after assembly if a strong topological retention signal is introduced at the very end of the polypeptide and then removed post-insertionally. Phosphorylation of an extramembrane domain, which alters its charge nature, could also induce post-insertional topological changes. A structural approach for dynamic membrane protein organization is not achievable by X-ray crystallography. Therefore, a set of unique in vivo and in vitro approaches should be used to establish a detailed mechanistic understanding for how lipid-protein interactions govern dynamic membrane protein structure and function. Novel approaches and concepts have been developed to analyze dynamic lipid-protein interactions and mechanisms of membrane protein folding and topogenesis. Such methods have the advantage of probing the dynamics of biological membrane organization, membrane protein structure, and lipid-protein interactions both in vitro and in vivo.

1 Introduction

Lipids are not covalently bound in membranes but rather interact dynamically to form transient arrangements, either laterally or across the membrane. Lipid composition varies drastically among different prokaryotes and eukaryotes, between different intracellular compartments, and can be influenced by disease and changes in cell environment. Membrane lipids display a diverse array of hydrophilic and hydrophobic surfaces, positive and negative charges, ionizable and nonionizable moieties, and chemically and structurally different groups. These lipid properties together or separately provide both hydrophobic and topological constraints for nascent and inserted integral membrane proteins containing hydrophobic transmembrane domains (TMD) and hydrophilic extramembrane domains (EMD) with various charges, lengths, and different abilities to fold.

Membrane proteins represent at least 30% of the encoded genes of all currently sequenced genomes and almost 60% of drug targets. Effective drug design is dependent on understanding membrane protein structure and the rules that govern the folding and assembly of native membrane proteins. Rapid progress in this important field is unfortunately hindered by the complexity of the protein folding mechanism since membrane proteins are folded and inserted into a lipid bilayer. The multiple assembly rules, topogenic sequences, and topological determinants (SecYEG/Sec61 translocon, phospholipids, membrane potential) should not only ensure proper steady-state membrane protein topogenesis but allow also for post-assembly dynamic changes in topological organization in response to internal and external changes (Bogdanov et al. 2009, 2014; Dowhan and Bogdanov 2009; von Heijne 2006).

2 TMD Protein Structure: Static or Dynamic?

The simplest way to achieve the correct orientation of a polytopic membrane protein is to orient the first TMD followed by alternating orientation of EMDs to the *cis* and *trans* side of the membrane (von Heijne 2006). However, a membrane protein is very often not committed to a final topology after insertion of the first TMD, and TMDs may reorient themselves either co- or posttranslationally. It is widely accepted that TMDs may reorient themselves before protein synthesis is completed (Lu et al. 2000) or until further reorientation is blocked by glycosylation (Goder et al. 1999). Viral hepatitis C protein NS4B (Lundin et al. 2003) and human aquaporin 1 (Lu et al. 2000) are synthesized in an initial orientation and then flipped following synthesis of the remainder of the protein during posttranslational maturation. Topological analyses have demonstrated that the initial topology of the hepatitis B virus large (L) envelope protein at the endoplasmic reticulum (ER) membrane contains its entire N-terminal pre-S domain oriented to the cytosolic side, whereas for the mature protein this domain faces the luminal side. This protein undergoes a TMD topological rearrangement via posttranslational translocation of the pre-S initially exposed on the cytosolic surface of the ER. This post-insertional topological reorientation is orchestrated by molecular chaperones Hsp70-BiP in the lumen of the ER (Awe et al. 2008) and cytoplasmic Hsc70 /Hsp40, which simultaneously may keep the pre-S domain competent for post-insertional translocation (Lambert and Prange 2003).

A topological reorientation of internal TMDs and adjacent EMDs has been observed to occur during the maturation of aquaporin-1 (Lu et al. 2000). Aquaporin-1 initially adopts a 4 TMD-spanning topology with the second EMD mis-oriented to the cytosolic face of the ER membrane. To acquire its mature 6 TMD-spanning topology, TMDs 2, 3, and 4 are reoriented during late stages of biogenesis. TMD 3 undergoes a 180° rotation accompanied by translocation of the N-terminal flanking loop from the lumen to the cytosol and C-terminal flanking loop from its initial cytosolic location into ER lumen.

The structural basis for these dynamic TMD reorientation events remains unapproachable by X-ray crystallography since membrane protein crystal structures are static. Therefore, the development of new in vivo and in vitro approaches is required to establish a detailed mechanistic understanding for how co-translational, posttranslational, and post-insertional lipid-protein interactions govern dynamic protein organization, re-organization, and function.

3 Justifying SCAM™ as a Method of Choice

Non-crystallographic approaches have been developed and employed to determine steady-state and dynamic changes in topological arrangement of TMDs of full length membrane proteins in order to understand the mechanism for establishing membrane protein topology and its relationship to biological function (Bogdanov et al. 2005;

Bochud et al. 2013). Such biochemical topological analyses can be invaluable for building reliable mechanistic models even in the absence of highly resolved protein structures (Fleishman et al. 2006; Islam and Lam 2013; Liapakis 2014). These tools document EMD accessibility to the surrounding solvent and therefore topological orientation of neighboring TMDs. The Substituted Cysteine Accessibility Method (Karlin and Akabas 1998) was adapted to measure transmembrane (TM) orientation (SCAM™) (Bogdanov et al. 2005, 2010a; Bogdanov 2017) and has emerged as the method of choice due to its relative simplicity, reliability, and feasibility. This approach is based on replacement of noncritical residues with cysteine residues one at a time into putative EMDs or TMDs in an otherwise cysteine-less protein. Chemical modification with membrane impermeable thiol-specific probes is then used either before or after compromising membrane integrity to determine membrane sidedness of EMDs or lack of reactivity of cysteines within TMDs. Aside from simplicity, the advantage of this approach is that topology is documented in the context of the full-length membrane protein molecule. The chemical modification can be done using whole cells, thereby avoiding problems related to the conversion of cells into membrane vesicles with a uniform orientation. SCAM™ was also further developed to map either uniform, dual, mixed, or unusual membrane protein topology in either intact cells, isolated membranes vesicles, or proteoliposomes by using a two-step labeling protocol (Wang et al. 2002; Bogdanov et al. 2002, 2005, 2008; Vitrac et al. 2011, 2017a; Bogdanov and Dowhan 2012; Woodall et al. 2015, 2017; Bogdanov 2017).

The commonly used thiol-specific reagents and general design of labeling experiments is outlined in Figs. 1 and 2, respectively. Intact cells or lysates (sonication or detergent treatment) of cells expressing the target protein with cysteine replacements are exposed to membrane impermeable 3-(*N*-maleimidylpropionyl) biocytin (MPB) (Fig. 1b) resulting in biotinylation of sulfhydryl groups exposed on the exterior of cells or on both sides of the membrane, respectively. The target protein is then isolated by immunoprecipitation after dissolving cells in SDS followed by SDS polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting to detect the biotinylated protein. It is important to note that the extent of biotinylation should be the same before and after sonication for an extracellular cysteine. Although single cysteine replacements could affect protein expression, conclusions are based on comparison of the extent of labeling in whole cells and disrupted cells for the same protein. This approach simplifies interpretation of data obtained with a series of protein derivatives that may express at different levels since conclusions about topology are based on relative reactivity of cysteines in the same sample before and after cell disruption.

Confirmation of labeling of external water-exposed cysteines by MPB can be achieved by first blocking putative external cysteines in intact *Escherichia coli* cells with a thiol-specific reagent that is transparent in the detection phase of the procedure. A set of impermeable blocking reagents that effectively react with thiols exposed to solvent but are transparent in the detection phase is available for SCAM™ (Bogdanov et al. 2005) One such reagent is 4-acetamido-4`-maleimidyl-stilbene-2,2`-disulfonic acid (AMS) (Fig. 1c).

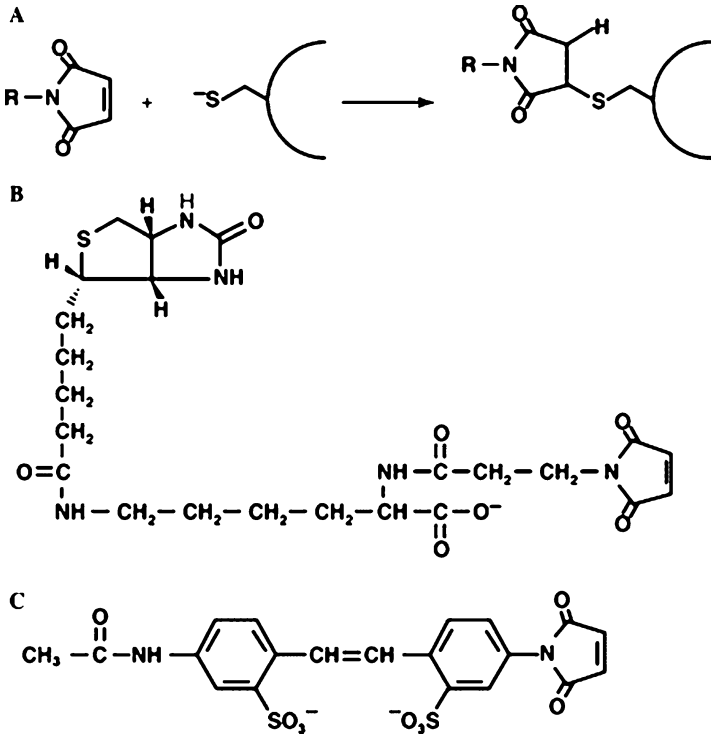


Fig. 1 Chemical structure of thiol-modifying reagents and their reaction with a thiolate anion. (**a**) Reaction of a maleimide moiety with the thiolate of a protein cysteine to form a covalent adduct via nucleophilic addition to the double bond of the maleimide ring. Maleimides are virtually unreactive until they encounter an available ionized thiol group. For most water-exposed cysteine residues in proteins, the pKa of the thiol of cysteine lies in the range of 8–9 and formation of cysteinyl thiolate anions is optimum in aqueous rather in a nonpolar environment where the pKa of the thiol of cysteine is around 14. Therefore, the reaction rate of different sulfhydryls is controlled primarily by their water exposure making the residues that reside in regions of TMDs unfavorable for the generation of thiolate anions. Thus, the labeling characteristics of intramembrane (unreactive) and extramembrane (reactive) cysteines should be consistent with their localization either in a nonpolar or polar environment, respectively (Bogdanov et al. 2005, 2010a; Bogdanov 2017). (**b**) Structure of biotin-containing labeling reagent MPB. (**c**) Structure of blocking non-detectable reagent AMS. (Figure is reproduced from Bogdanov (2017) with permission from Elsevier)

The strategic use of SCAM™ for mapping membrane protein topology has circumvented many limitations of alternative methods used to map the topology of integral membrane proteins and has the following advantages:

1. Analysis can be done on the full-length protein.
2. Monocysteine chemistry has the highest resolution in that water accessibility of individual cysteine residues can be determined.
3. Cysteine replacements are often well tolerated with retention of membrane protein topology and function.

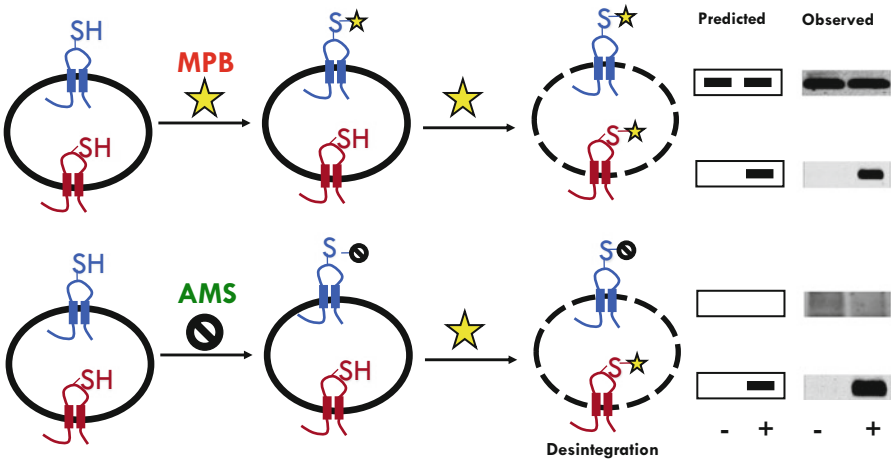


Fig. 2 General strategy for SCAM™ using impermeable MPB and non-permeant transparent AMS to probe sidedness of EMDs. A target membrane protein (only one TMD hairpin is shown) containing a single cysteine replacement exposed either to the extracellular (blue, periplasmic) or intracellular (red, cytoplasmic) side of the membrane is expressed in host cells. Half of the cells is treated with the detectable thiol reagent MPB to specifically label the externally exposed cysteine (top panel) and the other half is treated with the non-detectable thiol reagent AMS (bottom panel) to protect external cysteines in subsequent labeling steps. Both halves of cells either kept intact or desintegrated by sonication to expose the interior cysteine are treated with MPB to specifically label previously inaccessible cytoplasmic cysteine residues (top panel). Labeling by MPB that can be blocked completely by pretreatment with AMS is an independent verification of an outside facing residue (bottom panel). Labeling by MPB that cannot be blocked by such AMS treatment is independent verification of a residue that is facing the cytoplasm. The target protein is immunoprecipitated and resolved by SDS-PAGE and biotinylated protein is detected using avidin-HRP and chemiluminescence (right panel). (Figure is reproduced from Bogdanov (2017) with permission from Elsevier)

4. Detection of engineered cysteine modifications is simple, and analysis is done by chemical modification using a broad range of commercially available reagents that differ in charge, size, mass, and hydrophilicity.
5. Detection of protein SH groups is highly sensitive (femtomole range).
6. Chemical modification can be done using intact cells.
7. Capable of distinguishing accessibility of residues separated by only three or four residues and is useful in establishing the locations of the membrane interface in the protein structure and precise fine-structure mapping of the ends of TMDs in polytopic membrane proteins.
8. Can be adapted to any membrane protein and expression system.

4 SCAM™ to Probe TMD Protein Topology Dynamics In Vivo

Common dogma dictates a high thermodynamic barrier to topological flipping of TMDs after insertion. Therefore, flip-flop of integral proteins should not occur and all EMDs of membrane proteins are assumed to be static and permanently fixed at the

time of initial membrane insertion. In most cases, SCAM™ provides topological information after orientation of proteins within membranes (Islam and Lam 2013; Zhu and Casey 2007; Lee and Kim 2014). Application of this approach allowed either detailed mapping or significant refinement of the topology of a variety of integral membrane proteins including a more accurate mapping of the ends of TMDs of proteins, which had been established by other methods (Bogdanov et al. 2005). However, SCAM™ is not only an alternative approach to low-resolution determination of membrane protein structure but also constitutes an attractive independent approach to dynamic studies of membrane proteins. Dynamic aspects of protein structure as a function of the physiological state of the cell are best probed in whole cells or membranes. Although the labeling patterns derived from SCAM™ assays usually reflect steady-state topology of a membrane protein, semi-quantitative analysis of the surface accessibility of individual cysteines introduced into extramembrane loops can be carried out at various stages of protein assembly. In this case SCAM™ can be used to provide topological information during membrane insertion, folding, and assembly of proteins. For example, cysteine accessibility during bacteriorhodopsin translation was monitored by pulse-chase radiolabeling and modification by AMS to determine the order and timing of insertion of TMD segments into the membrane of *Halobacterium salinarum* (Dale et al. 2000). In this in vivo translocation assay, the rate of insertion of TMDs into the *H. salinarum* cytoplasmic membrane was monitored by rapid modification of unique cysteines in extracellular EMDs of the protein with AMS resulting in a shift in mobility of the protein in SDS PAGE. Thus, SCAM™ can provide topological information at the time TMD orientation is established co-translationally during the insertion of protein into the membrane.

SCAM™ was also used to monitor dynamic topological changes accompanied with substrate binding and release during membrane machinery function. SecG was originally shown to undergo membrane topology inversion, since SecA-dependent protein translocation renders the membrane-protected region of SecG sensitive to external proteases (Nishiyama et al. 1996). SCAM™ was successfully utilized later to examine this topology inversion in detail without protease treatment (Nagamori et al. 2002). SecG derivatives with a single cysteine residue at various positions were labeled in the presence and absence of protein translocation with an AMS (Fig. 3a). Labeling of single cysteine replacements of a major component (SecG) of the SecYEG translocon with this maleimide either in the resting state or during ATP-dependent preprotein translocation clearly demonstrated that a periplasmic and cytoplasmic region of SecG undergoes topology inversion (Nagamori et al. 2002). Treatment of spheroplasts with AMS revealed that Cys39 in the cytoplasmic region of SecG could be labeled from the periplasmic side only in the presence of ATP and during protein translocation, whereas a cytoplasmic protein elongation factor Tu remained unlabeled. Accordingly, treatment of inverted inner membrane vesicles with AMS also revealed that Cys88 and Cys111 residues in the periplasmic region were labeled exclusively from the cytoplasmic side of membranes only during protein translocation as demonstrated by the amount of SecG that is gel-shifted after alkylation (Fig. 3a). The extent of cytoplasmically exposed Cys39 derivatized by AMS appeared to decrease in the presence of ATP while Cys60, located within membrane-spanning region, was not labeled by AMS irrespective of

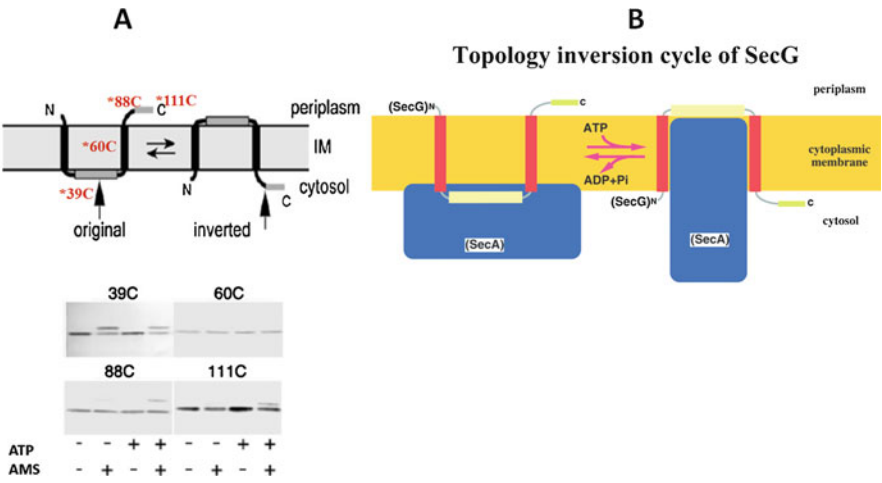


Fig. 3 **Visualization of SecG topology inversion cycle by SCAM™.** (**a**) Topological inversion of SecG was detected by labeling with membrane-impermeable AMS of a periplasmic Cys from the cytoplasmic side of membranes. Inside out oriented inner membrane vesicles containing the specified single cysteine SecG derivative were subjected to proOmpA translocation at 37 °C in the presence and absence of ATP**.** (**b**) A model for the dynamic reorientation of SecG coupled with insertion–deinsertion cycle of SecA. SecG, a subunit of the SecYEG channel, undergoes topology inversion coupled with SecA-ATPase dependent translocation of protein precursors across membrane. (Figure is kindly provided by Drs. Ken-ichi Nishiyama and Hajime Tokuda and partially reproduced from Nagamori et al. (2002) with permission Oxford University Press on behalf of the Japanese Biochemical Society)

the presence or absence of ATP (Fig. 3a). Therefore, SecG displays an unusual property of inverting its orientation in the membrane, which is tightly coupled to its function and linked with the insertion-deinsertion cycle of SecA (Fig. 3b). A translocation ATPase provides direct driving force for preprotein translocation through ATP binding and hydrolysis. Remarkably, the interaction between SecG and SecA in membranes markedly increases when SecA and SecG undergo membrane-insertion and topology inversion, respectively. Thus, the two most dynamic components of the translocation machinery were found, for the first time, to interact with each other when both undergo conformational changes.

In the absence of translocation, SecG is an α-helical hairpin with the N- and C-termini exposed to the periplasmic space. The N-terminal domain of SecG is net negative and does not possess any positive charge, which is generally found in the cytoplasmic EMDs of membrane proteins. Positively and negatively charged residues coexist close to each other and are present in equal amounts among the interhelical loop and the long C-terminal domain of this protein. The membrane sidedness of the C-terminal region is changed when just one negatively charged glutamic acid residue within N-terminal segment was mutated to an arginine (Nishiyama et al. 1996) anticipating a mechanism driven by Charge Balance Rule as will be explained in the following sections. The inverted topology of this mutant was irreversibly fixed, which appears to render the protein nonfunctional.

SCAM™ was similarly successfully utilized to establish the packing geometry of the pilin VirB2 subunit and its ATP-dependent in- and out- membrane dynamic organization within the T pilus and T4SS secretion channel (Kerr and Christie 2010). Propilin (pVirB2) is processed by signal sequence cleavage followed by covalent linkage of the N- and C-termini. The cyclized pilin integrates into the inner membrane as a pool that is the source for assembly of the secretion channel and T pilus. pVirB2 exhibited dramatic differences in MPB accessibility of engineered monocysteines when synthesized in the absence (ΔVirB) versus presence of two VirB proteins (VirB+), VirB4, and VirB11. In the absence of VirB4 and VirB11, a strategically engineered Cys94 in pVirB2 was inaccessible to biotinylation and labeled only after disintegration of cells indicating that this residue is located in the cytoplasm (Fig. 4a). Cys51 and Cys64 displayed opposite labeling patterns: Cys51 was labeled only in a strain lacking VirB4 and VirB11 proteins, whereas Cys64 was labeled by MPB only in the VirB4 and VirB11 producing strain. Normally cytoplasmic Cys94 became accessible from the periplasm only in the presence of the VirB4 ATPase. The synthesis of both VirB4 and VirB11 was necessary and sufficient for labeling of the Cys64 periplasmic residue. Biotinylation

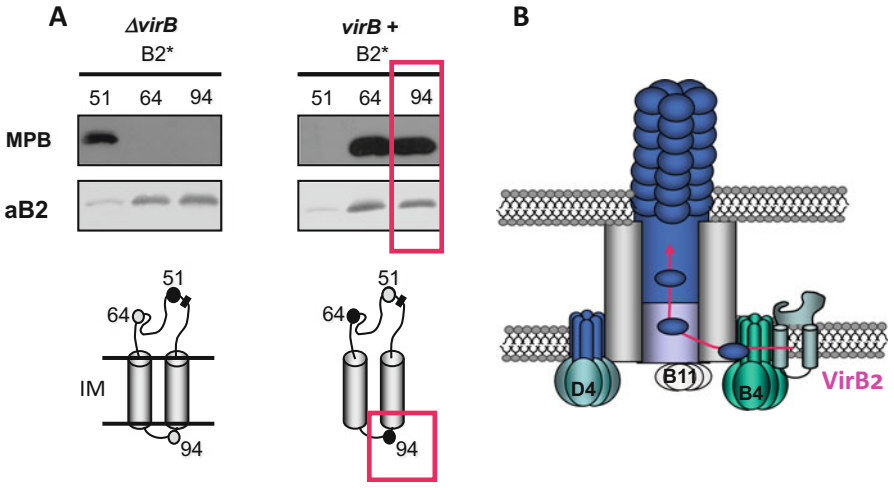


Fig. 4 Utilization of SCAM™ to establish a packing geometry and dynamic organization of the pilin VirB2 subunit. (**a**) VirB2 single cysteine derivatives were synthesized in *Escherichia coli* cells in the absence (ΔVirB) or presence (+VirB) of VirB4 and VirB11 proteins followed by SCAM™ analysis. (**b**) Dynamic model in which (i) VirB4 catalyzes dislocation of VirB2 from the inner membrane (rendering Cys94 accessibility), (ii) VirB11 coordinates with VirB4 (most likely prior to pilin dislocation) to induce a structural change in the periplasmic domain of the pilin (rendering Cys64 accessibility to MPB), and (iii) VirB4 and VirB11 coordinate to mediate VirB2 pilin binding with a VirB partner protein(s) (rendering Cys51 inaccessibility to MPB) required for further channel and T pilus assembly. VirB2 protein abundance was assessed by immunoblot analysis with anti-VirB2 antibodies (aB2). (Figure is kindly provided by Dr. Peter Christie and partially reproduced from Kerr and Christie (2010) with permission from American Society of Microbiology)

profiles for a collection of Cys-substituted pilins defined interacting partners and the dynamics of the T4SS assembly pathway (Fig. 4b).

The applicability of SCAM™ was successfully extended to the study of TMD topology of membrane proteins assembled in different lipid environments (Bogdanov et al. 2002, 2005, 2008; Xie et al. 2006; Vitrac et al. 2011, 2017a; Bogdanov and Dowhan 2012; Zhang et al. 2003; Bogdanov 2017). This approach was essential to test TMD protein orientation sensitive to the lipid composition and directly monitor changes in topological organization of membrane proteins associated with changes of phospholipid composition in vivo (Bogdanov et al. 2002, 2008; Zhang et al. 2003; Vitrac et al. 2011; Bogdanov and Dowhan 2012). The use of cysteine specific probes in combination with "lipid" mutants makes this approach a powerful means of achieving a molecular understanding of highly dynamic topogenesis using static endpoints experiments (Bogdanov et al. 2008; Bogdanov and Dowhan 2012; Vitrac et al. 2013, 2015).

By taking advantage of *Escherichia coli* strains in which lipid composition can be controlled temporally during membrane protein synthesis and assembly as well as post-assembly, it was possible for first time to observe dynamic changes in protein topology as a function of membrane lipid composition (Bogdanov et al. 2002, 2008; Zhang et al. 2003; Bogdanov and Dowhan 2012). When assembled in membranes of phosphatidylserine synthase gene (*pssA)* null mutants of *Escherichia coli* lacking phosphatidylethanolamine (PE), the major phospholipid of this organism, the N-terminal six-TMD helical bundle of lactose permease (LacY) adopts an inverted topology where periplasmic domains become cytoplasmic and vice versa. TMD VII with low hydrophobicity exits the membrane and the remaining C-terminal five TMDs remain in their native topology (Bogdanov et al. 2002).

The *pssA* gene (Fig. 5a) was initially placed under control of the *araB* promoter (Bogdanov et al. 2002), which was later replaced by the more tightly regulated *tet* promoter (Bogdanov et al. 2008). The latter is regulated by the positive inducer anhydrotetracycline (aTC) to turn on or turn off the synthesis PE. In these experiments cells were grown first in the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) without aTc to allow membrane insertion and misfolding of LacY in the absence of PE. Then cells were switched to growth without IPTG in the presence of aTc to permit biosynthesis of PE in the absence of newly synthesized LacY. The orientation of EMDs of LacY relative to the membrane bilayer was determined before (-PE) or after (+PE) growth in the absence or presence, respectively, of aTc using SCAM™. As shown in Fig. 5b, diagnostic cysteines strategically engineered residing within normally (+PE) cytoplasmic domains (NT-C6), when analyzed in cells grown in the absence of aTc (<3% PE), were labeled whether or not cells were disrupted, indicating periplasmic exposure. Diagnostic cysteines residing in normally periplasmic domain P1, P3, or P5 were labeled only after cell disruption, indicating cytoplasmic exposure. Then topology of LacY initially assembled in -PE cells was examined after induction of PE synthesis. Cysteines residing in EMDs C2, C4, or C6 initially exposed to the periplasm in -PE cells were only biotinylated after cell disruption consistent with a return to normal topology (Fig. 5b).

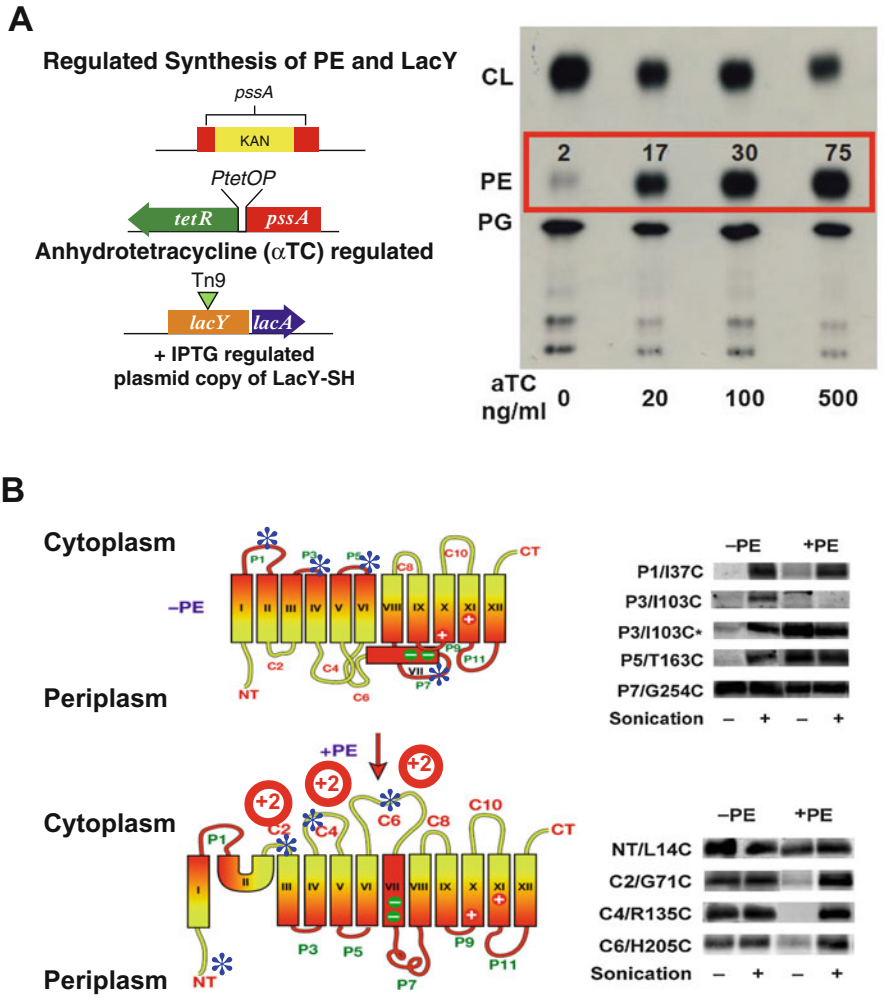


Fig. 5 Building a dynamic picture of transmembrane large scale rearrangement of LacY from end-point topology assays. (**a**) Experimental rationale. A plasmid copy of OP_{tac}-*lacY* controlled expression of monocysteine LacY (IPTG initiates expression of LacY) and a chromosomal copy of OP_{tet}-*pssA* controlled expression of phosphatidylserine synthase (aTc initiates biosynthesis of PE) were combined in the same *lacY pssA null* cells (strain AT2033). Phospholipid composition of *E. coli* cells as a function of dose-dependent homogenous *pssA* gene induction by various amount of aTC is shown (panel A). (**b**) SCAM™ was performed on intact cells either after initial assembly of LacY in PE-deficient cells, with IPTG induction but before addition of aTc or after complete removal of IPTG and induction of PE synthesis (+PE) by aTc for 3 h (maximum PE level) during logarithmic growth. The monocysteines residing within normally (+PE) cytoplasmic domains (NT-C6) were examined by SCAM™ in AT2033 grown in the absence of aTc (< 3% PE) or examined after induction of PE synthesis (PE is reaching physiologically normal level, 75% as shown on panel A) in the absence of new LacY synthesis. (Figure is reproduced from Bogdanov et al. (2008) with permission from Rockefeller Press and American Society of Cell Biology)

The same strain has been employed to determine the effects of reversible changes in lipid composition on preexisting TMD protein structure and function in a bi-directional manner (Bogdanov and Dowhan 2012). In this case, cells were grown first in the presence of IPTG and aTc to allow membrane assembly of LacY containing a diagnostic L14C (NT) or H205C (C6) replacement in EMDs flanking the N-terminal six-TMD bundle. It appears that at intermediate PE levels (30%), LacY displays a dual topology. More than 50% of cysteines for LacY L14C (NT) or H205C (C6) were protected from biotinylation during sonication by pretreatment of intact cells with AMS indicating that more than half of the LacY molecules inserted in the wild type orientation, while the remainder adopted an inverted topology. Then cells were switched to growth without IPTG in the absence of aTc to stop biosynthesis of PE in the absence of newly synthesized LacY. Reduction of PE levels to 5% due to continued cell growth resulted in misorientation of nearly all of the LacY.

Therefore, a mixture of topologically oriented forms is dependent on both protein sequence (Gafvelin and von Heijne 1994; Vitrac et al. 2011) and membrane lipid composition, and topological inversions of TMDs are reversible in both directions. The ability to change lipid composition in vivo post-assembly of a membrane protein (by either re-supplying or diluting a desired lipid metabolite) demonstrated the potential for polytopic membrane proteins to change their topological organization after insertion and assembly in the membrane. Therefore, SCAM™ can be used in vivo to observe how lipid-protein interactions (Bogdanov et al. 2002, 2008; Zhang et al. 2003; Bogdanov and Dowhan 2012) and interactions within a protein itself (Vitrac et al. 2011) contribute to overall TMD topogenesis (Bogdanov et al. 2009, 2014; Dowhan and Bogdanov 2009). Thus, SCAM™ is a powerful, well-tested technique for examining static and dynamic membrane protein topologies. Although such posttranslational reorientation of eukaryotic, bacterial, and viral membrane proteins is not unprecedented, *E. coli* LacY and phenylalanine permease (PheP) are so far the only native polytopic membrane proteins for which post-insertional TMD domain flipping after folding into a compact structure has been definitively demonstrated in vivo (Bogdanov et al. 2002, 2008; Zhang et al. 2003).

Post-insertional flipping of a membrane protein was further confirmed in in vivo experiments (Woodall et al. 2017). Complete inversion of the multi-drug resistant transporter EmrE protein can occur after the protein is fully inserted into membrane. To demonstrate that topology is not irreversibly fixed in the membrane, a strong cytoplasmic retention topological signal (positively charged KKKHHHHHH) was engineered at the C-terminus of the protein downstream from a TEV protease cleavage site. The protease site allowed removal of the retention signal after EmrE insertion following induction of TEV expression (Fig. 6). In cells expressing EmrE alone, a near complete biotinylation with MPB was achieved in AMS pre-blocked cells, consistent with cytoplasmic location of the C-terminus. After cleavage of the topological retention signal at the C-terminus, almost half of the EmrE population reoriented to move the diagnostic T108C from cytoplasm to the periplasm (Fig. 6).

Voltage-dependent gating of the monomeric colicin Ia channel is capable of moving a substantial fraction of its pore-forming domain across the lipid bilayer in association with opening and closing (Fig. 7a and b) (Kienker et al. 1997). This

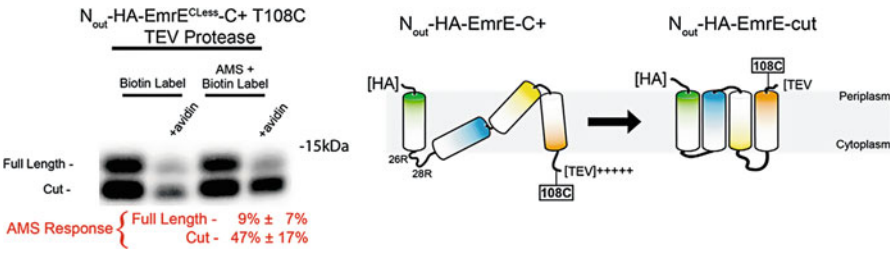


Fig. 6 Post-insertional topology inversion of the entire EmrE protein. SCAM™ established the transmembrane topology dynamics of EmrE constructs before and after cleavage by TEV protease by detecting whether a diagnostic monocysteine reside (T108C) faces either the cytoplasm or the periplasm. The engineered C-terminal tag with a cluster of the positive charges provides a strong topological retention signal that localizes the C-termini into the cytoplasm. Pre-blocking of intact cells with the membrane-impermeable AMS quantitatively prevents biotinylation of the engineered monocysteine with MPB after solubilization of protein from cells with SDS. Otherwise, added avidin gel-shifts a biotinylated EmrE. Biotinylation yield was quantified by the amount of EmrE that is gel-shifted upon the addition of avidin while the yield of protection was quantified as the AMS response. A plasmid copy with OP_{araB}–controlled expression of EmrE *(*arabinose initiates synthesis of EmrE construct) and a plasmid copy with OP_{tet}–controlled expression of TEV protease (aTC initiates expression of protease post-assembly of EmrE) were combined in the same cells. Protection from biotinylation of the monocysteine (T108C) residing within normally cytoplasmically located C-terminus of full-length protein was examined by SCAM™ before or after induction of TEV protease expression. (Figure is reproduced from Woodall et al. (2017) with permission from the Protein Society)

flipping charged stretch (containing 15 positively and 8 negatively charged residues) may fully or partially unfold to cross the membrane. If so, it evidently refolds correctly on the *trans* side. The opening and closing of colicin Ia channel is accompanied by the translocation 68 residues (474–541) back and forth across the lipid bilayer in close association with channel opening and closing driven by membrane depolarization events (Jakes et al. 1998). Moreover a 134-residue hydrophilic amino acid sequence inserted into this flipping stretch between TMD 3 and TMD4 of colicin A is still capable of translocation to the *trans* side of the bilayer (Slatin et al. 2002). Large-scale conformational reversible transmembrane reorientations of the colicin Ia channel pore-forming TMDs with adjacent EMD across the lipid bilayer upon voltage activation are drastically affected by the membrane surface charge density and strictly dependent on anionic lipid (phosphatidylglycerol) content (Yao and Hong 2006) anticipating a mechanism driven by Charge Balance Rule as will be explained in followed section.

5 Charge Balance Rule of Membrane Protein Assembly and Topogenesis

According to a central dogma in membrane biology the TMD orientation (i.e. topology) of polytopic membrane proteins is dictated primarily by the encoding sequence which folds and orients in a lipid bilayer in accordance with the Positive

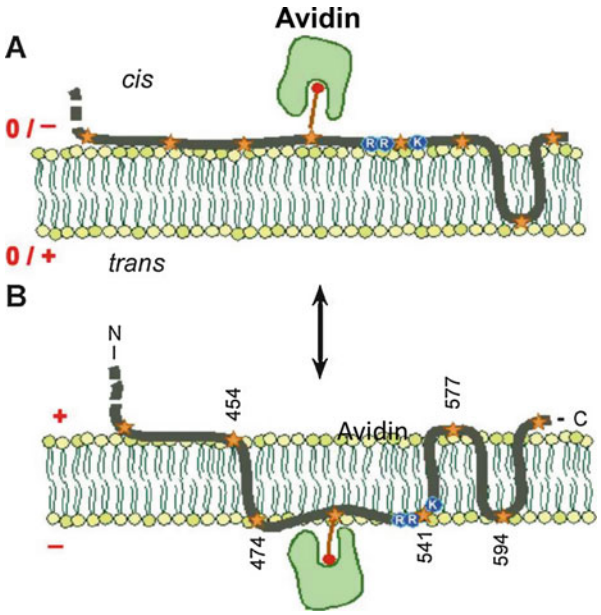


Fig. 7 Colicin Ia is capable of moving its pore-forming domain across the lipid bilayer in association with channel opening and closing driven by membrane depolarization events (A and B). A monocysteine was engineered in the flipping stretch to probe the reversible large-scale TMD rearrangement by biotin-avidin technology, which allowed trapping a pore-forming domain at either side of the membrane. Avidin was added either to the *trans* or *cis* compartments of planar bilayer experimental set up. The N-terminus is translocated to the *trans* side when the channel opened at positive voltages and is translocated back to the *cis* side when the channel closed at negative voltages. Its binding to *trans* avidin would prevent channel closure by hindering the movement of the amino terminus back to the *cis* side. (The figure is reproduced from Zakharov et al. (2004) with permission from Elsevier)

Inside Rule with net positively charged EMDs facing the cytoplasm (von Heijne 2006). Positively charged residues appear to be stronger determinants of topology than negatively charged residues due to a dampening of the translocation potential of negatively charged residues by a still unknown mechanism. Moreover, the presence of conserved oppositely charged amino acid residues within the same EMD raises more questions about the mechanism of membrane protein topogenesis. If negatively charged residues can be topologically active when present in high numbers (Nilsson and von Heijne 1990) or lie within close vicinity to TMD end (Rutz et al. 1999), why is their potency attenuated in most cases? Where is the dampening effect coming from? Moreover, the Positive Inside Rule cannot explain (i) posttranslational dynamic changes in topology (ii) coexistence of membrane proteins with dual and multiple topologies, and (iii) post-insertional changes in topological organization of already inserted membrane proteins.

SCAM™ revealed that the N-terminal six TMD bundle of LacY assembled in PE-lacking cells is inverted with respect to the plane of the membrane bilayer and the

C-terminal five TMD helical bundle (Fig. 5b). Thus, LacY assembled in the absence of PE and the presence of 100% of negatively charged anionic lipids violates the Positive Inside Rule since net positively charged EMDs are now facing the periplasm instead of the cytoplasm. This observation raises the question of the relative topological power of charged residues in the context of different lipid compositions. To determine whether membrane lipid composition has an effect on the EMD net charge-dependence for TMD orientation, the distribution and nature of charged residues within the cytoplasmic EMDs of the N- terminal six TMD of LacY was varied along with membrane lipid composition. Changing the net charge of cytoplasmically exposed EMD surface of the N-terminal bundle (i.e., C2, C4, and C6 with a cumulative +6 charge) by +1 through either neutralization of one negative residue or adding one positive residue in a position independent manner prevented topological inversion in PE-lacking cells. A neutral change of adding both a plus and minus charge had no effect. This result suggested that PE dampens the translocation potential of negatively charged residues in favor of the cytoplasmic retention potential of positively charged residues (Bogdanov et al. 2008; Vitrac et al. 2011). It appears that in the absence of PE, negative residues become much stronger translocation signals. The weak translocation signal provided by negative residues in the presence of PE was further demonstrated by the need to convert the +6 net charge of LacY to −6 in order to effect topological inversion of the N-terminal bundle in PE-containing cells (Bogdanov et al. 2008). To integrate the contribution of lipid-protein interfacial transient interactions and to extend this finding to other polytopic membrane proteins, the Charge Balance Rule was formulated to explain why increasing the content of anionic phospholipids does not favor retention of positively charged EMDs (as one might expect) (van Klompenburg et al. 1997) and appears to increase the translocation potential of domains containing acidic residues. According to this Rule, the charge density of the membrane surface and the charge content of EMDs act in concert to determine TMD orientation (Bogdanov et al. 2008, 2009, 2014; Dowhan and Bogdanov 2009; Vitrac et al. 2013). These effects are due to synergistic lipid-protein charge interactions and are not structure specific as illustrated by the spectrum of lipids (phosphatidylcholine, neutral glycolipids, and positively charged lysyl-phosphatidylglycerol resulting from an expression of foreign "lipid" genes introduced into *E. coli*) that support native topology (Bogdanov et al. 2010b, 2014; Wikstrom et al. 2004, 2009; Xie et al. 2006) and the lack of amino acid specificity and position within EMDs in determining final topology (Bogdanov et al. 2008; Vitrac et al. 2011, 2017a). Whatever the precise mechanism, lipids that dilute the high negative charge on the membrane surface by phosphatidylglycerol (PG) and cardiolipin (CL) clearly attenuate the translocation potential of negatively charged residues in favor of the cytoplasmic retention potential of positively charged residue (Fig. 8).

The initial topological decision of how to orient a protein according to the Positive-Inside Rule could be made by the translocon, which provides the permissive hydrophilic environment required for concurrent TMD translocation of the flanking regions with different net charges to the opposite side of the membrane. An alternative view is that upon incomplete or interfacial insertion of growing polypeptide into

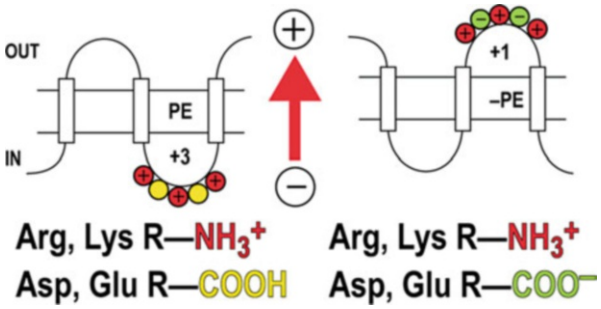


Fig. 8 Charge Balance Rule for membrane protein assembly. According this Rule the translocation potential of negatively charged residues is normally suppressed by PE and other net zero charge lipids in cytoplasmic EMDs containing both negative and positive residues. The presence of these lipids thus increases the positive amino acid retention potential of EMDs, while the absence of net zero charge lipids would increase the negative amino acid translocation potential. The pKa upshift caused by PE would selectively protonate negatively charged aspartate and glutamate residues, therefore neutralizing them and increasing the net positive charge of the corresponding domain (+3) and thereby favoring their retention on cytoplasmic side of the membrane. In the absence of PE, negative residues exert their full translocation potential and result in translocation of a domain that exhibits a lower effective net positive charge. Responses to net charge of domains or charge difference across domain may be mediated by electrochemical potential (positive outward) or alternatively the net negatively charged EMD can be electrorepelled by negatively charged membrane leaflet. (Figure is reproduced from Bogdanov et al. (2008) with permission from Elsevier)

the translocon channel, nascent sequences can be precisely positioned with respect to not only the protein interior of the channel but also to the lipid bilayer (Bogdanov et al. 2014; Cymer et al. 2015). In this process, specific lipids associated with the lateral opening of translocon channel could co-determine or directly determine protein topology within the membrane. Although water-soluble EMDs can still temporally enter the cytoplasmic half of the "hour-glass" translocon pore to escape contact with the hydrophobic part of the bilayer, hydrophobic TMDs sliding along a lateral gate of translocon can never be fully inserted and engaged with translocon (Cymer et al. 2015).

6 Membrane Protein Topological Duality and Lipid–Dependent Reversibility of Topological Interconversion

Common dogma states that all copies of a given type of integral protein have to have the same orientation relative to the plane of the lipid bilayer. Yet a number of membrane proteins have been shown to exist in more than one topological organization with respect to their TMD segments, either in the same membrane (Kim and Hegde 2002; Woodall et al. 2015; Rapp et al. 2006) or in different membranes (Levy 1996).

An *E. coli* strain with the *pssA* gene placed under *tet* promoter regulation (Fig. 5a) has been employed to determine the effects of reversible changes in lipid composition on preexisting TMD protein structure. In this strain, the level of PE can be regulated in a dose responsive manner between near 0% and 75% of total phospholipid uniformly throughout a cell culture (Fig. 5a) (Bogdanov and Dowhan 2012). In vivo *E. coli* LacY (Bogdanov and Dowhan 2012) and a charge modified sucrose permease (CscB) (Vitrac et al. 2017a) display a mixture of topological conformations ranging from complete inversion of the N-terminal helical bundle to mixed topology to completely native topology as PE is increased from near 0% to 75% of membrane phospholipid composition. At intermediate levels of PE (about 30%), LacY and CscB coexist in two topologically distinct and stable conformations in about equal amounts. There was no threshold level of PE determining a sharp transition from one topological isoform to the other, suggesting that coexisting conformers are thermodynamically locked and are not in rapid equilibrium at a static given lipid composition. CscB also displays coexisting dual topologies dependent on changes in the charge balance within EMDs (Vitrac et al. 2017a). Therefore, mixtures of topological forms in the same membrane are dependent not only on the protein sequence, as previously demonstrated (Gafvelin and von Heijne 1994; Vitrac et al. 2011), but are also regulated by membrane lipid composition in a dose-dependent manner.

Remarkably a purified LacY reconstituted into liposomes of increasing PE content also displayed inverted topology at low PE (<10%) and then a mixture of inverted and proper topologies with the latter increasing with increasing PE until all LacY adopted its native topology (Vitrac et al. 2015). Therefore, LacY is inserted into the liposomal membrane during reconstitution into a lipid bilayer in two opposite orientations at different ratios, which are proportional to the mol% of PE, closely mimicking in vivo results (Bogdanov and Dowhan 2012). Thus, dual topological states of LacY at a steady state lipid composition are not rapidly interconverting with each other and their coexistence is not maintained by other cellular factors, providing a thermodynamic basis for membrane protein heterogeneity. An intriguing question is how LacY flips between multiple topological forms and is inserted into the membrane in two opposite orientations at different mol% of PE. The topological heterogeneity can arise simply through perturbations of this thermodynamic equilibria between multiple topologies that present themselves as folding intermediates during initial membrane insertion. Rapid reversible interconversion between multiple subpopulations of a membrane protein with different TMD orientations is limited by the activation energy barrier in a given static lipid environment, but a change in lipid environment could result in partial unfolding of the protein to the point where multiple folding intermediates are in rapid equilibrium. Most likely the N-terminal bundle lies close to an equilibrium point between two alternative orientations and therefore able to switch conformations in response to changes in the lipid environment. Thus the simplest interpretation of the bi-directional reorganization process and formation of dual topologies is that the change in lipid composition would destabilize the folded state of LacY and remove the protein structure from equilibrium resulting in reorientation of TMD bundle in

order for the protein to assume its new minimum energy state in spontaneous response to a change in the lipid environment (Bogdanov and Dowhan 2012; Bogdanov et al. 2014; Vitrac et al. 2013).

These results can be also explained mechanistically by the Charge Balance Rule. Progressive depletion of PE levels increases the translocation potential of negatively charged amino acids resulting in an increasing amount of LacY with inverted topology. Progressive increase in the amount of PE simply raises the probability of the N-terminal half of LacY to adopt a correct orientation by decreasing the translocation potential of negatively charged amino acids that act in opposition to the Positive Inside Rule. Therefore, topology of a membrane protein is strongly dependent on a given ratio of anionic and net neutral lipids. This result provides a potential molecular basis for the existence in nature of proteins that exhibit topological and functional duality. This novel mechanism opens an intriguing possibility that multiple membrane protein topologies can be interchanged post-insertionally either during lateral movement of protein in and out of raft/lipid microdomains or between different intracellular membranes (Bogdanov et al. 2014).

Several lines of experimental evidence extend the Charge Balance Rule to additional lipids, which establishes a more general principle. Changes in cholesterol (also net neutral) levels in the endoplasmic reticulum membrane may affect topogenesis and result in either aberrant topology or topological heterogeneity of membrane proteins. The percentage of opposing topologies (based on pre-S domain orientation across the ER membrane) of the L envelope protein of hepatitis B virus was varied depending on the cholesterol level, which was manipulated either by growing virus-producing HepG2 cells in lipoprotein-depleted serum (cholesterol was reduced by 40%) or exposing cells to chlorpromazine (reduced cholesterol levels by ~80%) (Dorobantu et al. 2011). Therefore, this protein can exist in two functionally distinct topological isoforms whose topological distribution varies with the endoplasmic reticulum cholesterol content. There are several possibilities for cholesterol effects on membrane protein topogenesis: (i) because cholesterol does not possess any charge it can act simply as a negatively charged surface diluent supporting the Charge Balance Rule; (ii) membrane stiffness could restrict the freedom of the lateral gate of the translocon pore and thus affect the contact or timing of interaction of charged and/or hydrophobic segments with the membrane lipids.

The existence of dual and multiple topologies within the same membrane raises provocative and intriguing questions regarding the mechanism of membrane protein assembly. The generation of equal or different amounts of oppositely oriented proteins within the same membrane is well beyond the control of the translocon (Bowie 2006; Schuldiner 2007). The mechanism by which membrane proteins may adopt dual TMD folds remains unknown. The Charge Balance Rule explains how the balance of interfacial protein and lipid charges allows nascent or inserted protein to adopt exclusively uniform or dual topology since topogenesis is simply viewed as a probabilistic process. Moreover, dual topologies were not only observed but also converted into a single population of uniformly oriented protein either by increasing the negatively charged bias (CscB) or positive inside charge bias (PheP) in a lipid-

dependent manner (Vitrac et al. 2011). Hydrophilic EMDs are different in the number and positions of charged basic and acidic amino acids acting as retention and translocation signals, respectively. The different degree of sequence-specific response for interconversions between two topological isoforms of CscB and PheP to the presence or absence of PE further confirms that biogenesis of proteins is probabilistic by nature and the balance of forces near equilibrium is an important decision-making step in thermodynamically driven membrane protein topogenesis.

7 From Liposomes to Fliposomes: Monitoring TMD Topology Dynamics and Interconversions In Vitro

The environment experienced by EMDs and TMDs of membrane proteins during insertion is very complex ranging, respectively, from an aqueous permissive folding on either side of membrane and in the translocon to the hydrophobic lipid bilayer flanked by different head group charges. The fact that post-insertional correction of topology of the N-terminal bundle of LacY presumably occurs independent of the translocon following restoration of wild type lipid composition favors thermodynamic, rather than kinetic control of initial folding directed by the membrane insertion and translocation machineries. Since two oppositely oriented topological conformers are fully interconvertible by post-assembly synthesis or dilution of PE, in vivo TMD switching may require no cellular factors and therefore is determined solely by lipid-protein interactions.

To investigate the molecular determinants required for post-assembly topological reorganization, LacY was analyzed in an in vitro proteoliposome system in which lipid composition can be systematically controlled before (liposomes) and after (fliposomes) membrane protein reconstitution using a methyl-β-cyclodextrin (MβCD)-mediated lipid exchange technique (Vitrac et al. 2013, 2015). MβCD is able to facilitate the exchange of phospholipids between the outer monolayers of donor multilamellar lipid vesicles (MLVs) and recipient small unilamellar vesicles (liposomes) without bilayer fusion or disruption of bilayer integrity (Cheng and London 2009). This new "fliposome" technology provides a means to determine the minimum and sufficient requirements for a protein to flip between topologically distinct states and determine whether this interconversion is thermodynamically determined by the properties of the protein interacting with its lipid environment independent of other cellular factors.

Purified LacY was first reconstituted in vitro in the absence or presence of PE followed by a post-reconstitution supply of PE to almost wild type levels (up to 60%) or a reduction of PE levels from 70% to <20%, respectively. LacY unilamellar proteoliposomes were mixed with MβCD-treated MLVs containing only PE in the first case to incorporate PE or with MβCD-treated MLVs containing only PG plus CL in the second case to dilute PE content of liposomes after reconstitution. Remarkably, in vitro results fully mirror in vivo observations. Interconversion between topological conformers of LacY was observed in a PE dose-dependent manner by either increasing or decreasing PE levels in proteoliposomes after

reconstitution of LacY. Switching between the two opposing TMD topologies can occur in either direction not only in vivo but also in proteoliposomes and therefore requires no additional cellular factors. Therefore, such post-insertional topological changes are thermodynamically driven and thus probabilistic processes that can occur at any time and in any cell membrane depending on changes in the lipid environment.

8 Lipid and Protein Dynamics in Real Time: Monitoring Membrane Protein and Lipid Flip-Flops

A recently developed fliposome system in which lipid composition can be controlled before and after membrane protein reconstitution was successfully utilized further to assess the kinetics of changes in TMD orientation and phospholipid flipping within the lipid bilayer triggered by a change in lipid composition (Vitrac et al. 2015). Either bona fide periplasmic LacY residue or "flipping" cytoplasmic residues were strategically mutated to create Förster resonance energy transfer (FRET) pairs in order to monitor the TMD rearrangement of this inherently dynamic membrane protein (Fig. 9a). Additionally, fluorescently labeled phospholipids were used to monitor the kinetics of lipid exchange by FRET (using Rhodamine- and NBD-derivatives), as well as lipid flipping (using NBD-derivatives and dithionite quenching) in proteoliposomes. The combination of these three fluorescence-based assays was used to follow, in real time, the kinetics of EMD and lipid flipping induced by either resupply or dilution of PE or phosphatidylcholine (PC). FRET was successfully used to follow transfer of lipids from MβCD-loaded MLVs to LacY containing proteoliposomes, topological changes in LacY, and individual lipid flip-flop in real time. LacY flipping lags behind lipid transfer as expected but occurs on a time scale of seconds in both directions at room temperature (Fig. 9b) (Vitrac et al. 2015).

Altogether, these measurements established that (i) lipid exchange occurs rapidly on time scale of second, (ii) TMD reorientation, which occurs on a second to minute time scale, follows the incorporation/dilution of "enough" zwitterionic/net neutral PE/PC in the proteoliposome membrane, (iii) lipid flipping from the outer leaflet to the inner leaflet of the membrane is the slowest of the three processes, and (iv) the rate of lipid flipping depends on the presence of a protein in the membrane and the ability of this protein to reorient during lipid exchange.

9 On the Biological Relevance of TMD Reorientations

Is the functioning of membrane proteins controlled by their dynamic reorientation across membranes?

The ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase CD38 is an ectoenzyme made up of a short N-terminal domain, a single TMD, and a large C-terminal domain containing its catalytic site, as well as multiple glycosylation sites. CD38 is predicted to be a type-II transmembrane protein with its catalytic C-terminal domain located

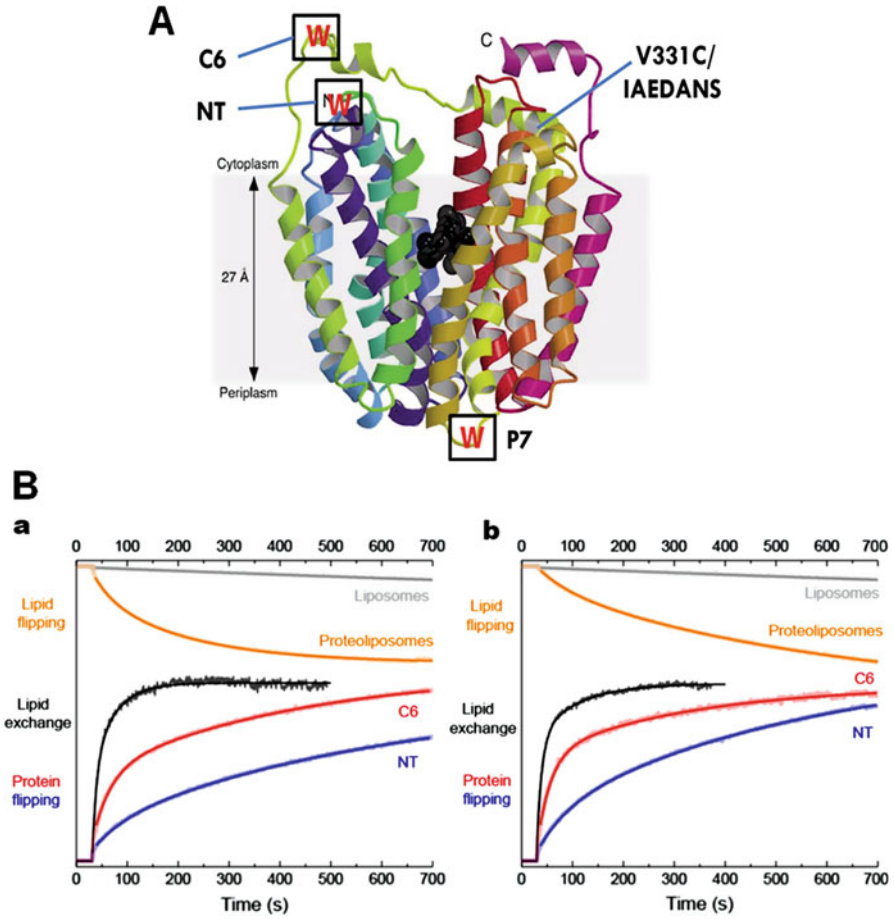


Fig. 9 Monitoring membrane protein and lipid flipping. (**a**) FRET between Trp replacements introduced one at the time in EMDs NT, C6 and P7 and1,5-((((2-Iodoacetyl)amino)ethyl)amino) Naphthalene-1-Sulfonic Acid (IAEDANS) was utilized to follow topological changes in LacY in real-time. A Cys replacement at position 331 (near the cytoplasmic end of TMD X) was labeled by IAEDANS as the acceptor of the FRET pair. Since the second half of LacY (from TMD VIII to EMD CT) does not undergo any lipid-dependent topological switch, the labeled Cys331 will always remain on the outside of fliposomes. (**b**) Rate of lipid exchange and protein flipping in fliposomes determined by real-time FRET observed either with MβCD/NBD-PE loaded donor MLVs and rhodamine-PE containing recipient LacY proteoliposomes or with IAEDANS-labeled H205W/V331C and L14 W/V331C LacY mutants, reconstituted into recipient liposomes and submitted to lipid exchange to trigger addition (**a**) or dilution of PE (**b**). Lipid flipping was monitored in the same conditions using NBD-PE quenching by dithionite encapsulated in the lumen of proteoliposomes. (Figure is reproduced from Vitrac et al. (2015) with permission from the National Academy of Sciences)

outside of the cell. This scenario presents a "topological paradox" for the cADPR/Ca^{2+}-signaling cascade due to the localization of the substrate in a different compartment. Two hypotheses were formulated to explain how CD38 can exert its biological functions: (i) CD38 indeed adopts a type-II topology and its substrates

are transported in and out of the cell by transporters/channels (such as connexins or nucleotide transporters) or (ii) CD38 adopts a type-III topology, which results in the exposure of its catalytic domain to the cytoplasm where it can access its substrates. The ability of CD38 to coexist in two different orientations (type-II and type-III) in various cell types was recently demonstrated using a combination of in vivo topological assays (Zhao et al. 2012). The presence of several charged residues in the N-terminal domain of CD38, along with potential phosphorylation sites, led the investigators to propose that modulation of the net charge of the N-terminal domain dictates the topological orientation of CD38 in the membrane (Zhao et al. 2012). Increase in the negative charge content of the N-terminal domain using site-directed mutagenesis led to an increase in the amount of type-III topology in vivo. Based on these observations, the authors proposed that, in accordance to the Charge Balance Rule, phosphorylation of the N-terminal domain may trigger the topological reorientation of CD38 from its type-II to its type-III topology, which constitutes an attractive mechanism for regulating the signaling activity of this protein (Zhao et al. 2015).

Thus, we hypothesized that alterations of EMD charge induced by such post-translational modification may trigger membrane protein topological reorientation, with the flipping proceeding at a physiologically significant rate. Therefore, naturally occurring phosphorylation within EMDs of a membrane protein could serve as a physiologically active electronegative topogenic signal, which would force a protein or its EMD to adopt a new TMD topology. To test this idea, phosphoinositide-dependent protein kinase 1 (PDK1) and liver kinase B1 (LKB1) phosphorylation sites were strategically engineered in LacY and sidedness of a single diagnostic cysteine located in EMD C6 was analyzed by SCAM™ in proteoliposomes before and after kinase(s)-mediated EMD phosphorylation (Vitrac et al. 2017b). Net charge of the N-terminal EMDs of LacY were modified from +2/+2/+2 to intrinsically metastable −2/+2/+2 or −2/0/−2 both of which, however, still display native topology in PE-containing cells and proteoliposomes composed of wild-type lipid composition. Addition of two phosphate groups to LacY after assembly in liposomes resulted in mixed topology for the −2/+2/+2 template and full inversion for the −2/0/−2 template demonstrating the dependence of EMD orientation on net charge. The double phosphorylation simply reduces further the net positive charge of flanking EMDs and promotes reorientation of adjacent TMDs. Using LacY containing FRET pairs, phosphorylation-induced protein flipping was found to occur at the same rate as lipid-induced flipping. Therefore, phosphorylation-induced TMD flipping can be explained and predicted by the Charge Balance Rule.

The demonstrated ability of phosphorylated residues to act as efficient translocation signals in a model system suggests that posttranslational modification of membrane proteins could have important physiological implications for the generation of post-insertional TMD switches in biological membranes. The change in the charge nature of EMDs during phosphorylation/dephosphorylation cycles of proteins could induce topological changes in membrane proteins resulting in changes of function. Membrane protein topogenesis within biogenic or between different

nonbiogenic cellular membranes is not necessarily a dead-end folding pathway. New TMD organization could occur through interaction not only with molecular chaperones but also by posttranslational (phosphorylation) modifications of proteins or by exposure to different lipid compositions along the protein secretory pathway. Since many proteins are subjected to multiple phosphorylation events, the degree of phosphorylation could regulate the ratio of inverted to non-inverted EMDs and result in multiple topologies for membrane protein.

Phospholipid scramblase 1 (PLSC1), which is also phosphorylated, undergoes topological changes during monocyte-to-macrophage cell differentiation. It is interesting that although constitutive phosphatidylserine (PS) exposure to the exterior of cells is not affected by phosphorylation of PLSC1, the reorientation of scramblase domains coincides with this negatively charged lipid externalization and therefore could be the cumulative consequence of disrupted phospholipid asymmetry (Herate et al. 2016). Scramblase PLSCR1 is not only phosphorylated but also palmitoylated and colocalized with raft markers anticipating a potential involvement of mechanism driven by Charge Balance Rule.

Nuclear factor-erythroid 2 (NF-E2)-related factor (Nrf1) regulates critical antioxidant responsive element sequence-driven genes and developmental target genes transactivated by a vast variety of stresses (e.g., oxidative, nutrient (glucose) dependent, etc.) and pathophysiological stimuli (e.g., inflammation and aging). Nrf1 protein exists as two topological isoforms in the ER membrane. Transactivation activity of Nrf1 is controlled through its membrane-topology and post-synthetic glycosylation state. In the presence of glucose, the DNA-binding Transactivation Domain (TAD) consisting of acidic domain (AD1) (DIDLID/DLG) and cholesterol recognition amino acid consensus motif (CRAC3) is transiently flipped into the ER lumen, where it is N-glycosylated to yield a transcriptionally inactive 120-kDa glycoprotein. Upon glucose deprivation, TAD is flopped to the cytoplasm where this domain is deglycosylated to yield an active 95-kDa transcription factor (Zhang et al. 2014). Remarkably, TAD can flip back to the lumen again in the presence of glucose. Thus, transcriptional activity of this membrane protein can be controlled topologically within ER membrane and physiologically via dynamic TMD reorientation and movement of its subdomain into and out of the ER lumen. It appears that glycosylation does not impose a barrier to flipping from the lumen to the cytoplasm monitored by conversion of the 120-kDa glycosylated isoform into the 95-kDa deglycosylated isoform (Zhang et al. 2014). It is interesting that TMD flipping of Nrf1 relies on acidic residues within AD1 and interaction of adjacent CRAC3 domain with cholesterol, which is potentially required for TMD rearrangement of Nfr1 in stressed ER or Nrf1 molecules sorted out of ER into extra-ER cyto/nucleoplasmic compartments anticipating a mechanism driven by Charge Balance Rule (named as Charge Difference Rule by authors).

The experiments with CD38, PLSCR1, and Nrf1 demonstrate that transmembrane topology switching between opposing orientations of an integral membrane protein could be physiologically important.

10 Can Pathogenic Mutations or Other Genetically Induced Changes Affect Topologies?

Aberrant protein folding and assembly of nascent membrane proteins has been implicated as the molecular basis for a growing number of diseases. The EMDs and TMDs of membrane proteins are a frequent target for disease-causing mutations. Surveying the effects of 470 known pathogenic missense mutations occurring within or near TMDs and comparing predictions for wild-type and mutant sequences established that roughly 10% of the mutations influence the efficiency with which these variants acquire their correct membrane topology (Schlebach and Sanders 2015).

The members of the astroglial glutamate transporter type 1 (GLT) and high affinity, Na^+-dependent glutamate/aspartate transporter GLAST family are predominantly expressed in the plasma membrane and play an important role in maintaining the extracellular glutamate concentrations below neurotoxic levels. The first three TMDs of these transporters are encoded by exons 2, 3, and 4. It was reported that the loss of exon 3 converts three TMDs into two and results in transmembrane inversion of the second half of protein expressed in rat cerebellum as the unglycosylated form (Huggett et al. 2000). The inverted protein fails to clear glutamate, which is the predominant excitatory neurotransmitter, from the extracellular space. Such inversion has been attributed to the development of neurological disorders. All members of the glutamate transporter family share similar membrane topology and transport mechanism. Remarkably both the rate of aspartate transport and the transmembrane topology of Na^+-dependent prokaryotic homolog of eukaryotic glutamate transporters from *Pyrococcus horikoshii* (Glt_{Ph}) required PE to display native topology in proteoliposomes (McIlwain et al. 2015). Although neurotoxic release of glutamate due to anoxia was found to be largely due to reversed operation of glutamate transporters, the role of PE and/or its asymmetry, which could be lost under these conditions, was never addressed.

11 Research Needs

Overall, membrane protein folding and topogenesis appears to be intimately dependent on a native lipid profile since lipids and proteins have coevolved to adopt a topological constrains of lipid bilayer and follow a set of rules governing the final topological organization of membrane proteins. By taking advantage of SCAM™, the simple yet well-characterized bacterial system and in vitro membrane protein reconstitution techniques, we can easily manipulate the lipid composition in genetically altered strains or in proteoliposomes (fliposomes), both in a dose-dependent manner and temporally, to identify the function of individual lipids involved in complex and dynamic processes like topogenesis of membrane proteins. Since the dependence on lipids is fully replicated in vitro, the role of lipid composition in determining protein topological organization can be extrapolated to the folding and assembly rules in more complex eukaryotic systems.

The Charge Balance Rule as an extension of the Positive Inside Rule (which is "static") explains topology dynamics and provides an attractive mechanism for generation of membrane protein structural heterogeneity within the same biological membrane. The Charge Balance Rule provides (i) a molecular basis for why positive residues are dominant topological determinants over negative residues in native membranes, (ii) a mechanistic understanding of how lipid-protein interactions determine membrane protein organization at the time of initial assembly and dynamically after assembly, and (iii) an explanation of how membrane proteins may adopt multiple topologies within either the same or different cellular membranes. According to this topology switch paradigm, it is possible that specific regions of a preexisting membrane protein can undergo reversible TMD reorganization in vivo, dictated not only by membrane lipid composition but also by components of the translocation machinery or other cellular events like membrane depolarization. Clearly membrane protein structure is metastable and dynamic rather than static and can respond to changes in membrane lipid composition in the local environment, which occurs during cell division, membrane fission and fusion, movement of proteins in and out of lipid rafts, and metabolic changes in polyphosphorylated inositol phosphatidylglyceride pools. The changes in membrane lipid composition either locally or during intracellular movement of proteins along the organelle-based secretory pathway can lead to misinterpretation of existing topological signals or different interpretation of new ones. Current dogma assumes that the initial topology of a protein in the ER membrane accurately reflects the topology of the protein elsewhere in the cell. Is topology fixed during co-translationally membrane insertion in the ER or does further remodeling of topology occur with changes in lipid composition as proteins move laterally or through different organelles to their final destination? Are the "rules" for membrane protein assembly different between different organisms and organelles? More studies are required to address and resolve a topological paradox in formation and maintenance of dual topologies in all types of cells. We have little information about the functions of lipid distribution and transbilayer phospholipid movement in bacterial, plasma, and intracellular membranes and their role in membrane protein topogenesis, which remains an experimental challenge to be tackled.

References

Awe K, Lambert C, Prange R (2008) Mammalian BiP controls posttranslational ER translocation of the hepatitis B virus large envelope protein. FEBS Lett 582(21–22):3179–3184

Bochud A, Ramachandra N, Conzelmann A (2013) Adaptation of low-resolution methods for the study of yeast microsomal polytopic membrane proteins: a methodological review. Biochem Soc Trans 41(1):35–42

Bogdanov M (2017) Mapping of membrane protein topology by substituted cysteine accessibility method (SCAM™). In: Bacterial protein secretion systems: methods and protocols, pp 105–128

Bogdanov M, Dowhan W (2012) Lipid-dependent generation of a dual topology for a membrane protein. J Biol Chem 287:37939–37948

Bogdanov M, Heacock PN, Dowhan W (2002) A polytopic membrane protein displays a reversible topology dependent on membrane lipid composition. EMBO J 21(9):2107–2116

Bogdanov M, Zhang W, Xie J, Dowhan W (2005) Transmembrane protein topology mapping by the substituted cysteine accessibility method (SCAM™): application to lipid-specific membrane protein topogenesis. Methods 36(2):148–171

Bogdanov M, Xie J, Heacock P, Dowhan W (2008) To flip or not to flip: lipid-protein charge interactions are a determinant of final membrane protein topology. J Cell Biol 182(5):925–935

Bogdanov M, Xie J, Dowhan W (2009) Lipid-protein interactions drive membrane protein topogenesis in accordance with the positive inside rule. J Biol Chem 284(15):9637–9641

Bogdanov M, Heacock PN, Dowhan W (2010a) Study of polytopic membrane protein topological organization as a function of membrane lipid composition. Methods Mol Biol 619:79–101

Bogdanov M, Heacock P, Guan Z, Dowhan W (2010b) Plasticity of lipid-protein interactions in the function and topogenesis of the membrane protein lactose permease from *Escherichia coli*. Proc Natl Acad Sci U S A 107(34):15057–15062

Bogdanov M, Dowhan W, Vitrac H (2014) Lipids and topological rules governing membrane protein assembly. Biochim Biophys Acta 1843(8):1475–1488

Bowie JU (2006) Flip-flopping membrane proteins. Nat Struct Mol Biol 13(2):94–96

Cheng H-T, London E (2009) Preparation and properties of asymmetric vesicles that mimic cell membranes effect upon lipid raft formation and transmembrane helix orientation. J Biol Chem 284(10):6079–6092

Cymer F, von Heijne G, White SH (2015) Mechanisms of integral membrane protein insertion and folding. J Mol Biol 427(5):999–1022

Dale H, Angevine CM, Krebs MP (2000) Ordered membrane insertion of an archaeal opsin in vivo. Proc Natl Acad Sci USA 97(14):7847–7852

Dorobantu C, Macovei A, Lazar C, Dwek RA, Zitzmann N, Branza-Nichita N (2011) Cholesterol depletion of hepatoma cells impairs hepatitis B virus envelopment by altering the topology of the large envelope protein. J Virol 85(24):13373–13383

Dowhan W, Bogdanov M (2009) Lipid-dependent membrane protein topogenesis. Annu Rev Biochem 78:515–540

Fleishman SJ, Unger VM, Ben-Tal N (2006) Transmembrane protein structures without X-rays. Trends Biochem Sci 31(2):106–113

Gafvelin G, von Heijne G (1994) Topological "frustration" in multispanning *E. coli* inner membrane proteins. Cell 77(3):401–412

Goder V, Bieri C, Spiess M (1999) Glycosylation can influence topogenesis of membrane proteins and reveals dynamic reorientation of nascent polypeptides within the translocon. J Cell Biol 147(2):257–266

Herate C, Ramdani G, Grant NJ, Marion S, Gasman S, Niedergang F, Benichou S, Bouchet J (2016) Phospholipid scramblase 1 modulates FcR-mediated phagocytosis in differentiated macrophages. PLoS One 11(1):e0145617

Huggett J, Vaughan-Thomas A, Mason D (2000) The open reading frame of the Na+-dependent glutamate transporter GLAST-1 is expressed in bone and a splice variant of this molecule is expressed in bone and brain. FEBS Lett 485(1):13–18

Islam ST, Lam JS (2013) Topological mapping methods for alpha-helical bacterial membrane proteins–an update and a guide. Microbiology 2(2):350–364

Jakes KS, Kienker PK, Slatin SL, Finkelstein A (1998) Translocation of inserted foreign epitopes by a channelforming protein. Proc Natl Acad Sci USA 95(8):4321–4326

Karlin A, Akabas MH (1998) Substituted-cysteine accessibility method. Methods Enzymol 293:123–145

Kerr JE, Christie PJ (2010) Evidence for VirB4-mediated dislocation of membrane-integrated VirB2 pilin during biogenesis of the agrobacterium VirB/VirD4 type IV secretion system. J Bacteriol 192(19):4923–4934

Kienker P, Qiu X-Q, Slatin S, Finkelstein A, Jakes K (1997) Transmembrane insertion of the colicin Ia hydrophobic hairpin. J Membrane Biol 157(1):27–37

Kim SJ, Hegde RS (2002) Cotranslational partitioning of nascent prion protein into multiple populations at the translocation channel. Mol Biol Cell 13(11):3775–3786

Lambert C, Prange R (2003) Chaperone action in the posttranslational topological reorientation of the hepatitis B virus large envelope protein: implications for translocational regulation. Proc Natl Acad Sci 100(9):5199–5204

Lee H, Kim H (2014) Membrane topology of transmembrane proteins: determinants and experimental tools. Biochem Biophys Res Commun 453(2):268–276

Levy D (1996) Membrane proteins which exhibit multiple topological orientations. Essays Biochem 31:49–60

Liapakis G (2014) Obtaining structural and functional information for GPCRs using the substituted-cysteine accessibility method (SCAM). Curr Pharm Biotechnol 15(10):980–986

Lu Y, Turnbull IR, Bragin A, Carveth K, Verkman AS, Skach WR (2000) Reorientation of aquaporin-1 topology during maturation in the endoplasmic reticulum. Mol Biol Cell 11(9):2973–2985

Lundin M, Monne M, Widell A, Von Heijne G, Persson MA (2003) Topology of the membrane-associated hepatitis C virus protein NS4B. J Virol 77(9):5428–5438

McIlwain BC, Vandenberg RJ, Ryan RM (2015) Transport rates of a glutamate transporter homologue are influenced by the lipid bilayer. J Biol Chem 290(15):9780–9788

Nagamori S, Nishiyama K, Tokuda H (2002) Membrane topology inversion of SecG detected by labeling with a membrane-impermeable sulfhydryl reagent that causes a close association of SecG with SecA. J Biochem 132(4):629–634

Nilsson I, von Heijne G (1990) Fine-tuning the topology of a polytopic membrane protein: role of positively and negatively charged amino acids. Cell 62(6):1135–1141

Nishiyama K-i, Suzuki T, Tokuda H (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. Cell 85(1):71–81

Rapp M, Granseth E, Seppälä S, Von Heijne G (2006) Identification and evolution of dual-topology membrane proteins. Nat Struct Mol Biol 13(2):112

Rutz C, Rosenthal W, Schulein R (1999) A single negatively charged residue affects the orientation of a membrane protein in the inner membrane of *Escherichia coli* only when it is located adjacent to a transmembrane domain. J Biol Chem 274(47):33757–33763

Schlebach JP, Sanders CR (2015) Influence of pathogenic mutations on the energetics of translocon-mediated bilayer integration of transmembrane helices. J Membr Biol 248(3):371–381

Schuldiner S (2007) Controversy over EmrE structure. Science 317(5839):748–751

Slatin SL, Nardi A, Jakes KS, Baty D, Duché D (2002) Translocation of a functional protein by a voltage-dependent ion channel. Proc Natl Acad Sci 99(3):1286–1291

van Klompenburg W, Nilsson I, von Heijne G, de Kruijff B (1997) Anionic phospholipids are determinants of membrane protein topology. EMBO J 16(14):4261–4266

Vitrac H, Bogdanov M, Heacock P, Dowhan W (2011) Lipids and topological rules of membrane protein assembly balance between long- and short-range lipid-protein interactions. J Biol Chem 286:15182–15194

Vitrac H, Bogdanov M, Dowhan W (2013) In vitro reconstitution of lipid-dependent dual topology and postassembly topological switching of a membrane protein. Proc Natl Acad Sci USA 110(23):9338–9343

Vitrac H, MacLean DM, Jayaraman V, Bogdanov M, Dowhan W (2015) Dynamic membrane protein topological switching upon changes in phospholipid environment. Proc Natl Acad Sci 112(45):13874–13879

Vitrac H, Dowhan W, Bogdanov M (2017a) Effects of mixed proximal and distal topogenic signals on the topological sensitivity of a membrane protein to the lipid environment. Biochim Biophys Acta 1859(7):1291–1300

Vitrac H, MacLean DM, Karlstaedt A, Taegtmeyer H, Jayaraman V, Bogdanov M, Dowhan W (2017b) Dynamic lipid-dependent modulation of protein topology by post-translational phosphorylation. J Biol Chem 292(5):1613–1624

von Heijne G (2006) Membrane-protein topology. Nat Rev Mol Cell Biol 7(12):909–918

Wang X, Bogdanov M, Dowhan W (2002) Topology of polytopic membrane protein subdomains is dictated by membrane phospholipid composition. EMBO J 21(21):5673–5681

Wikstrom M, Xie J, Bogdanov M, Mileykovskaya E, Heacock P, Wieslander A, Dowhan W (2004) Monoglucosyldiacylglycerol, a foreign lipid, can substitute for phosphatidylethanolamine in essential membrane-associated functions in *Escherichia coli*. J Biol Chem 279(11):10484–10493

Wikstrom M, Kelly AA, Georgiev A, Eriksson HM, Klement MR, Bogdanov M, Dowhan W, Wieslander A (2009) Lipid-engineered *Escherichia coli* membranes reveal critical lipid headgroup size for protein function. J Biol Chem 284(2):954–965

Woodall NB, Yin Y, Bowie JU (2015) Dual-topology insertion of a dual-topology membrane protein. Nat Commun 6:8099

Woodall NB, Hadley S, Yin Y, Bowie JU (2017) Complete topology inversion can be part of normal membrane protein biogenesis. Protein Sci 26(4):824–833

Xie J, Bogdanov M, Heacock P, Dowhan W (2006) Phosphatidylethanolamine and monoglucosyldiacylglycerol are interchangeable in supporting topogenesis and function of the polytopic membrane protein lactose permease. J Biol Chem 281(28):19172–19178

Yao X, Hong M (2006) Effects of anionic lipid and ion concentrations on the topology and segmental mobility of colicin Ia channel domain from solid-state NMR. Biochemistry 45(1):289–295

Zakharov SD, Kotova EA, Antonenko YN, Cramer WA (2004) On the role of lipid in colicin pore formation. Biochim Biophys Acta 1666(1):239–249

Zhang W, Bogdanov M, Pi J, Pittard AJ, Dowhan W (2003) Reversible topological organization within a polytopic membrane protein is governed by a change in membrane phospholipid composition. J Biol Chem 278(50):50128–50135

Zhang Y, Ren Y, Li S, Hayes JD (2014) Transcription factor Nrf1 is topologically repartitioned across membranes to enable target gene transactivation through its acidic glucose-responsive domains. PLoS One 9(4):e93458

Zhao YJ, Lam CMC, Lee HC (2012) The membrane-bound enzyme CD38 exists in two opposing orientations. Sci Signal 5(241):ra67

Zhao YJ, Zhu WJ, Wang XW, Zhang L-H, Lee HC (2015) Determinants of the membrane orientation of a calcium signaling enzyme CD38. Biochim Biophys Acta Mol Cell Res 1853(9):2095–2103

Zhu Q, Casey JR (2007) Topology of transmembrane proteins by scanning cysteine accessibility mutagenesis methodology. Methods 41(4):439–450

Lactose Permease: From Membrane to Molecule to Mechanism

34

Lan Guan and H. Ronald Kaback

Contents

1 Introduction 638
2 Active Transport, Efflux, Exchange, and Counterflow 639
3 Three-Dimensional Crystal Structure of LacY 640
3.1 Overall Fold and Helix Packing 640
3.2 Sugar Binding and Specificity 641
3.3 The H^+-Binding Residue 643
4 The Alternating-Access Process 644
5 The H^+/Lactose Symport Mechanism 646
5.1 Lactose Active Transport 646
5.2 Lactose Influx or Efflux down a Concentration Gradient 647
5.3 Lactose Equilibrium Exchange and Counterflow 647
6 Future Research 648
References 648

Abstract

Lactose permease of *Escherichia coli* (LacY), a galactoside/H^+ symporter, is a paradigm for cation-coupled membrane-transport proteins. This integral membrane protein is composed of two pseudo-symmetrical six-helix bundles surrounding an internal hydrophilic cavity with binding sites for sugar and H^+ at the apex of the molecule in the approximate middle of the membrane. These

L. Guan (✉)
Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX, USA
e-mail: Lan.Guan@ttuhsc.edu

H. R. Kaback
Departments of Physiology, Microbiology, Immunology and Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, CA, USA
e-mail: RKaback@mednet.ucla.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_48

structural features allow LacY to utilize an alternating-access mechanism to catalyze sugar/H^+ symport in either direction across the cytoplasmic membrane. The H^+-binding site is occupied under most physiological conditions because of a markedly perturbed pK_a, and galactoside binding causes transition of the ternary complex to an occluded intermediate that then opens to alternative sides of the membrane. Binding and alternating access of the binding sites to either side of the membrane occur independently of the electrochemical H^+ gradient $(\Delta\tilde{\mu}_{H^+})$. However, in the absence of $\Delta\tilde{\mu}_{H^+}$, deprotonation is rate limiting, while in the presence of $\Delta\tilde{\mu}_{H^+}$ (interior negative and/or alkaline), deprotonation is no longer rate limiting because there is a driving force on the H^+. Although the dissociation constant (K_d) value for galactoside remains constant on either side of the membrane, K_m decreases 50- to 100-fold, and by this means accumulation against a sugar concentration gradient is achieved.

1 Introduction

Membrane proteins are major structural and functional components for cell membranes, which not only function as barriers but also selectively allow permeation of specific solutes. The function of cell membranes relies on both lipids and membrane proteins, as well as their interactions. In this chapter, the *E. coli* lactose permease (LacY) is used as a paradigm for understanding the transport processes catalyzed by major facilitator superfamily (MFS).

LacY is the product of the *lacY* gene, the second structural gene in the *lac* operon, and the *lacY* gene was the first gene encoding a membrane transport protein to be cloned into a recombinant plasmid and sequenced (Büchel et al. 1980). It was solubilized from the membrane, purified to homogeneity, functionally reconstituted into proteoliposomes, and shown to catalyze all the translocation reactions typical of the transport system *in vivo* with comparable turnover numbers (Guan and Kaback 2006). Thus, the product of the *lacY* gene is solely responsible for all translocation reactions catalyzed by LacY.

LacY is a member of the oligosaccharide/H^+ symporter subfamily (TCDB 2.A.1.5) of the MFS, and the natural substrate is lactose (Fig. 1). However, LacY is also selective for other disaccharides containing a D-galactopyranosyl ring, as well as D-galactose, but has no affinity for D-glucose or D-glucopyranosides. LacY is the first symporter for which a high-resolution 3-D crystal structure was determined by X-ray crystallography (Abramson et al. 2003). The hydrophobic polypeptide is composed of 417 amino-acid residues with 65–70% unequivocally hydrophobic side chains, and has a mass of 46,517 Da. Notably, a library of mutants containing a single-Cys residue at virtually every position of this protein (Cys-Scanning Mutagenesis) (Frillingos et al. 1998) has been constructed and subjected to a variety of biochemical and biophysical characterizations. As a particularly well-studied sugar/H^+ symporter, LacY is a paradigm for exploring the molecular mechanism(s) of symport catalyzed by secondary active transporters, including the MFS that now contains >40,000 members and which are found ubiquitously in all living organisms.

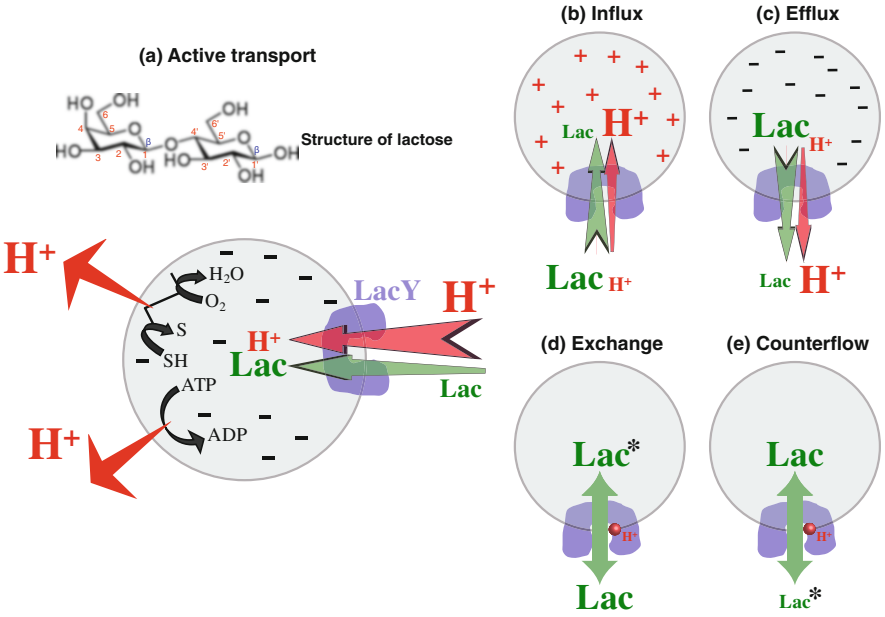


Fig. 1 **Lactose/H$^+$ symport**. (**a**) **Active transport**. The electrochemical H$^+$ gradient ($\Delta\tilde{\mu}_{H^+}$) across the cytoplasmic membrane of *E. coli* is generated by pumping out of H$^+$ via the respiratory chain or through the hydrolytic activity of F_1F_o ATPase. Free energy released from the energetically favored downhill movement of H$^+$ catalyzed by LacY (*purple color*) is converted to the uphill accumulation of lactose as indicated by the direction of the *arrows* and *font size*. The structure of native substrate lactose (β-D-galactopyranosyl-(1 → 4)-D-glucose) is illustrated. The carbon position is labeled in *red*, and the anomeric form is labeled in *blue*. (**b**) **Influx** and (**c**) **Efflux**. Energetically downhill lactose transport generates $\Delta\tilde{\mu}_{H^+}$, the polarity of which depends upon the direction of lactose concentration gradient (influx generates a $\Delta\tilde{\mu}_{H^+}$ that is interior positive and acid; efflux generates a $\Delta\tilde{\mu}_{H^+}$ that is interior negative and alkaline). (**d**) **Equilibrium exchange**. At equal intra- and extracellular lactose concentrations, lactose exchange across the membrane is catalyzed by protonated LacY. (**e**) **Counterflow**. At a high intracellular and low extracellular lactose concentrations, transient influx of labeled external lactose or "counterflow" across the membrane is observed. Both of the exchange and counterflow reactions are catalyzed by protonated LacY. *, radio-labeled lactose

2 Active Transport, Efflux, Exchange, and Counterflow

LacY is an effective chemiosmotic machine that catalyzes the coupled translocation of a galactopyranoside with an H$^+$ in the same direction (i.e., galactoside/H$^+$ symport), and coupling is obligatory (Kaback et al. 2001). Sugar accumulation against a concentration gradient (i.e., active transport) is achieved by transduction of the free energy released from the downhill movement of H$^+$ energetically generated by an electrochemical H$^+$ gradient ($\Delta\tilde{\mu}_{H^+}$; interior negative and/or alkaline) (Fig. 1a). Conversely, because coupling is obligatory, downhill sugar translocation

catalyzed by LacY drives uphill translocation of H^+ with the generation of $\Delta\tilde{\mu}_{H^+}$, the polarity of which depends upon the direction of the sugar concentration gradient, either influx (inwardly directed flux) or efflux (outwardly directed flux) (Fig. 1b, c). Notably, LacY also catalyzes equilibrium exchange or counterflow of internal galactoside for external LacY substrates in a manner that is independent of $\Delta\tilde{\mu}_{H^+}$ (Fig. 1d, e). The primary driving force for the global conformational change that drives alternating access of galactoside- and H^+-binding sites to alternative sides of the membrane (an alternating access mechanism) is association/dissociation of galactoside by protonated LacY. Without bound galactoside, LacY does not translocate H^+ in the absence or presence of $\Delta\tilde{\mu}_{H^+}$, and unprotonated LacY does not effectively bind a galactoside, so binding of both substrates is required for efficient symport.

3 Three-Dimensional Crystal Structure of LacY

3.1 Overall Fold and Helix Packing

LacY is ~86% α-helix and contains 12 mostly irregular α-helices traversing the membrane in zig–zag fashion connected by hydrophilic loops with both N- and C-termini on the cytoplasmic side of the membrane. Initial X-ray crystallography (Abramson et al. 2003; Guan et al. 2007) reveals that LacY is heart-shaped with a cavernous water-filled cleft open on the cytoplasmic side only, an inward-open conformation (Fig. 2a). The largest dimensions of the molecule are 60 × 60 Å.

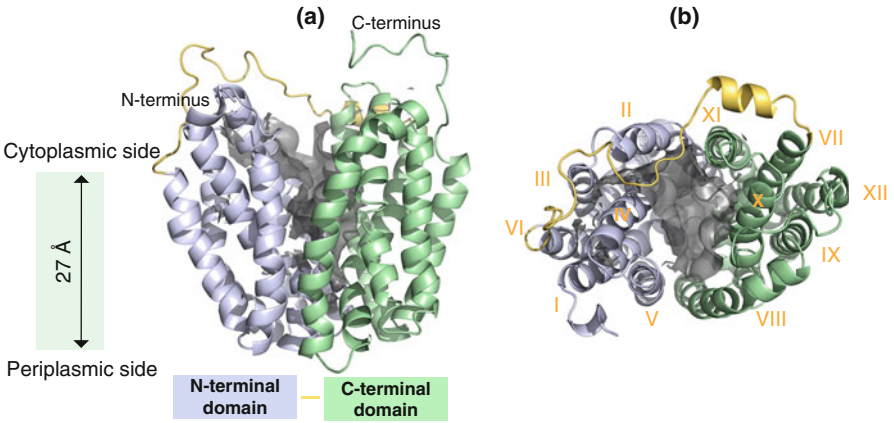


Fig. 2 X-ray crystal structure of the wild-type LacY (PDB Access ID, 2V8N). The LacY molecule exhibits an inward-facing conformation. In the ribbon representations of LacY (**a**, **b**), the N- and C-terminal six transmembrane helices are colored in *blue* and *green*, and the cytoplasmic loop is colored in *yellow*. The inward-facing cavity is illustrated by gray-colored area. (**a**) Viewed parallel to the membrane with helices V and VIII in front. The sidedness of membrane is labeled. (**b**) Cytoplasmic view normal to the membrane. Helices are labeled with Roman numerals

Viewed normal to the membrane from the cytoplasmic side (Fig. 2b), the molecule has a distorted oval shape with dimensions of 30 × 60 Å. Electrostatic surface potentials calculated from the crystal structure reveal a positively charged belt around the periphery of the cytoplasmic opening (Yousef and Guan 2009) with negatively charged residues distributed preferentially on the periplasmic side of the molecule. Thus, LacY follows the positive-inside rule (von Heijne 1992).

LacY is organized into two 6-helix bundles, or two domains, connected by a 33-residue long cytoplasmic loop between helices VI and VII (Fig. 2a, b), and most helices are heavily distorted with kinks. Helices III, VI, IX, and XII are largely embedded in the membrane. The inward-open conformation shows that the cytoplasmic regions of helices I, II, IV, and V of the N-terminal domain and helices VII, VIII, X, and XI of the C-terminal domain line a water-filled cytoplasmic cavity. The inward-open conformation represents the resting state of LacY (Nie and Kaback 2010; Smirnova et al. 2011; Kaback 2015).

LacY in an outward-open conformation (Fig. 3) has been also determined by X-ray crystallography of a LacY mutant (Kumar et al. 2014; Kumar et al. 2015). This mutant was constructed with two bulky Trp residues in place of conserved Gly residues at positions 46 (helix I) and 262 (helix VIII) on the periplasmic side that alters the resting state of LacY from inward-facing to outward-facing (Smirnova et al. 2013). In the outward-open conformation, the periplasmic region exhibits a narrow opening and a closed cytoplasmic side that prevents sugar from escaping (Kumar et al. 2014; Kumar et al. 2015).

The N- and C-terminal six-helix domains have a similar topology with the helical packing in a twofold pseudo-symmetry (Fig. 2b). Within each domain, two 3-helix repeats also exhibit an inverted topology with respect to helix packing. It has also been suggested that interconversion between the conformations of the neighboring triplets is associated with the inward- and outward-facing structural transitions (Radestock and Forrest 2011). These structural features are observed in both inward- and outward-conformations.

3.2 Sugar Binding and Specificity

LacY binds and transports one molecule of monosaccharide galactose or disaccharides containing a galactosyl moiety. Two lactose analogues, 4-nitrophenyl-α D-galactopyranoside (α-NPG) and β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), bind LacY with a dissociation constant (K_d) value of approximate 30–50 μM. The native substrate lactose has a relatively poor affinity, and galactose is the most specific substrate for LacY with the lowest affinity. Various adducts (particularly if they are hydrophobic) at the anomeric carbon C_1 of the galactopyranosyl ring can greatly increase affinity with little or no effect on specificity (Fig. 1). Glucose is an epimer of galactose since the two sugars differ only in the configuration at C_4, but neither glucose nor glucosides is recognized by LacY. The C_4OH is unequivocally the most important determinant for the specificity. While

C_2OH group is not so important, the C_4, C_3, and C_6OH groups play important roles in determining binding affinity (Sahin-Toth et al. 2000; Sahin-Toth et al. 2001).

Sugar-bound LacY in either an inward-facing or outward-facing conformation have been resolved by crystallography (Chaptal et al. 2011; Kumar et al. 2014; Kumar et al. 2015). One sugar molecule is lined by the similar residues regardless of the conformation of LacY, thereby confirming a conclusion from biochemical studies that there is only one sugar-binding site in LacY (Guan and Kaback 2004). The sugar site is located at the apex of the two inverted cavities, which enables the reversible transport process. The sugar-bound inward-facing structure is from single-Cys122 LacY with covalently bound methanethiosulfonyl-galactoside (Chaptal et al. 2011), a suicide inactivator for this mutant (Guan et al. 2003b). The positioning of galactosyl moiety in these sugar-bound structures are similar, which provides strong evidence to support the conclusion that the galactosyl moiety determines the specificity. The sugar molecule in the occluded partially outward-open conformation has tight interactions with LacY (Fig. 3), and the sugar binding in LacY may involve an induced-fit mechanism. His322 in helix X, which also contains the H^+-binding residue Glu325 one-helical turn away, forms direct contact with the sugar molecule, so helix X hosts the H^+-binding site and also participates in sugar binding.

Cys-scanning and site-directed mutagenesis have been extensively applied to study the sugar-binding site with LacY (Frillingos et al. 1998), and the biochemical results were largely confirmed crystallographically (Chaptal et al. 2011; Kumar et al. 2014; Kumar et al. 2015). A clear structure/function relationship in this transporter has been established. The structures show that the galactopyranosyl ring of α-NPG or TDG stacks hydrophobically with Trp151 on helix V (Fig. 3b, c), which has been suggested to play a role in orienting the galactopyranosyl ring and allowing specific hydrogen-bonding interactions to be realized, in addition to increasing the binding affinity (Guan et al. 2003a). Replacement of Trp151 with Tyr largely decreases binding affinity for galactosides but has no effect on the K_m value of lactose transport (Guan et al. 2003a; Vazquez-Ibar et al. 2003; Smirnova et al. 2009). Glu269 on helix VIII forms two hydrogen-bonding interactions with C_4 and C_3OH groups of the galactopyranosyl ring (Fig. 3b, c), which can explain why mutations lead to a marked decrease in the sugar-binding affinity, and Glu269 is also likely the primary determinant for the sugar specificity (Weinglass et al. 2003). These important interactions are further stabilized by the hydrogen bonds between C_4 and C_3OH groups with Asn272 on helix VIII and His322 on helix X, respectively. Asn272 can only be replaced with Gln without significant loss in the sugar-binding affinity, and Glu269 and His322 are irreplaceable residues in terms of sugar binding and lactose transport. Two other irreplaceable residues, Arg144 and Glu126, in the N-terminal domain form a salt-bridge interaction; and Arg144 directly serves as hydrogen bond donors to C_5 and C_6OH groups of the galactopyranosyl ring of TDG or α-NPG, as well as the $C_{2'}$OH group in another galactopyranosyl ring. As described above, this second ring can be replaced by various adducts that only affects the binding affinity. Glu126, in addition to stabilizing the positioning of Arg144, also forms hydrogen-bonding interactions with $C_{2'}$ and $C_{3'}$OH groups of TDG. Arg144 cannot even be replaced with positively charged Lys, and Asp replacement for Glu126 also

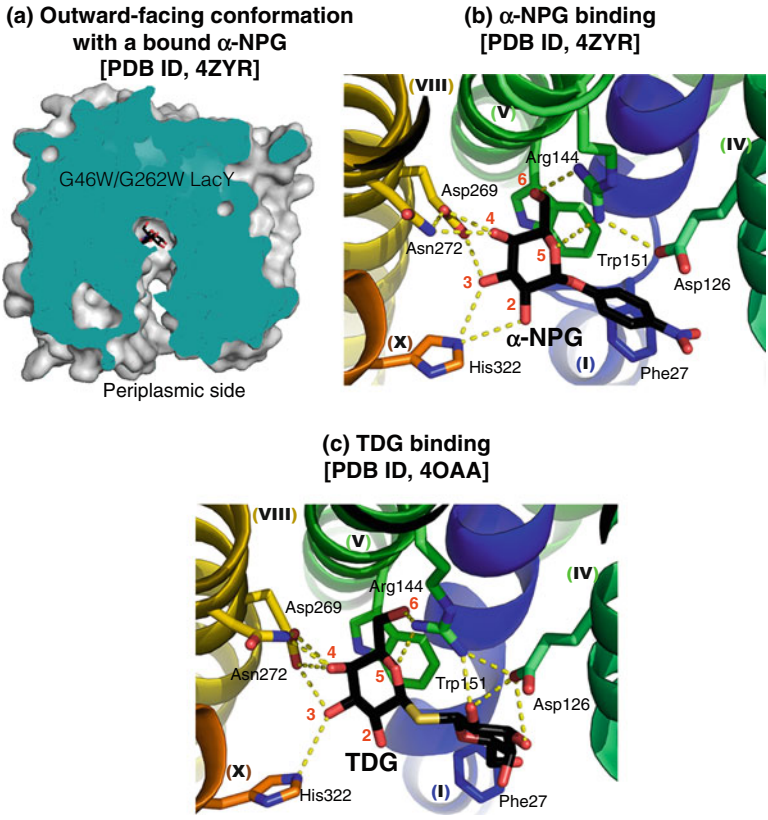


Fig. 3 Sugar binding in LacY. Crystal structures of the LacY G46W/G262W mutant in an outward-facing partially occluded conformation have bound α-NPG (PDB Access ID, 4ZYR) or TDG (PDB Access ID, 4OAA) molecule. Both lactose analogues are shown in *black*. (**a**) Slab view of a surface representation of LacY G46W/G262W molecule revealed by crystallography (PDB Access ID, 4ZYR). An occluded α-NPG molecule is shown in sticks. (**b**) Binding interactions of α-NPG with LacY. (**c**) Binding interactions of TDG with LacY. The helices of LacY are colored in *rainbow* and labeled with *Roman numerals*. Side chains that directly contact with the sugar are shown in *sticks*. *Dotted lines* indicate salt bridge/hydrogen-bonding interactions between sugar and protein, as well as between these polar/charged residues

decreases the affinity for galactosides significantly with little or no effect on apparent affinity for H^+ (Smirnova et al. 2009).

3.3 The H^+-Binding Residue

LacY affinity for galactosides is pH-dependent and exhibits a remarkably high pK_a of ~10.5 for substrate binding (Fig. 4). This pH dependence is abolished when Glu325 is replaced with a neutral side chain (Smirnova et al. 2008), although these Glu325

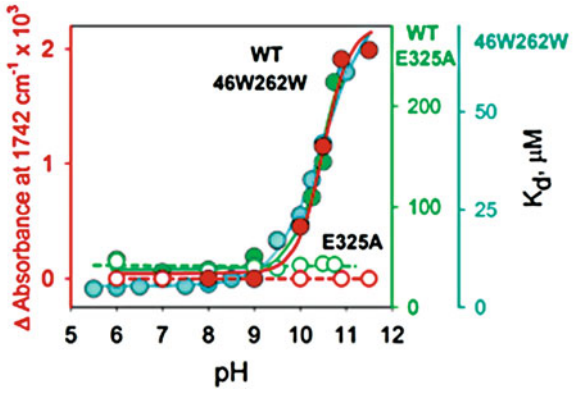


Fig. 4 pK_a of Glu325 of LacY. The pH dependence of Δ-IR intensity change at 1742 cm^{-1} was measured with the LacY G46 W/G262 W mutant in the absence or presence of α-NPG (*filled red circles*) or E325A LacY (*open red circles*). The K_d values for α-NPG binding to WT LacY (*filled green circles*), E325A LacY (*open green circles*), or LacY G46 W/G262 W mutant (*filled cyan circles*) were calculated as the ratio of rate constants (k_{off}/k_{on}) measured by stopped-flow fluorescence

mutants bind sugar with an affinity similar to the WT. The pK_a value of the side chain of Glu325 in the inward-facing WT or outward-facing LacY G46W/G262W mutant is remarkably high, also ~10.5 (Fig. 4), as determined by surface enhanced infrared absorption spectroscopy (Grytsyk et al. 2017). Glu325 likely serves as the H^+-binding site in this symporter. Since the K_d value for H^+ binding is less than about 30 pM, in the physiological pH range, Glu325 is protonated.

Crystal structures of LacY regardless of inward- or outward-facing conformations consistently show that Glu325 resides in a hydrophobic pocket between helices IX and X (Fig. 5). Glu325 is also in close proximity to an electrostatic and hydrogen-bonding network involving His322 and Lys319 on the same helix, Tyr236 and Asp240 on helix VII, and Arg302 on helix IX (Fig. 5). Glu269 and particularly H322, long thought to be involved in H^+ translocation, interact directly with the galactopyranosyl moiety (Figs. 3, 5). Mutation of Asp269, His322, or Tyr236 causes a marked decrease in sugar affinity (Smirnova et al. 2008). Another salt bridge (Asp237 on Helix VII and Lys358 on helix XI) also lies in the cavity (not shown), but has no direct contact with either site and is not involved in sugar or H^+ binding. This salt bridge is essential for insertion of LacY into the membrane and stability (Kaback et al. 2001).

4 The Alternating-Access Process

An alternating-access type of mechanism has been widely accepted to be involved in membrane transport; thus, membrane transport proteins expose binding site(s) to either side of the membrane through conformational changes. In LacY, both the

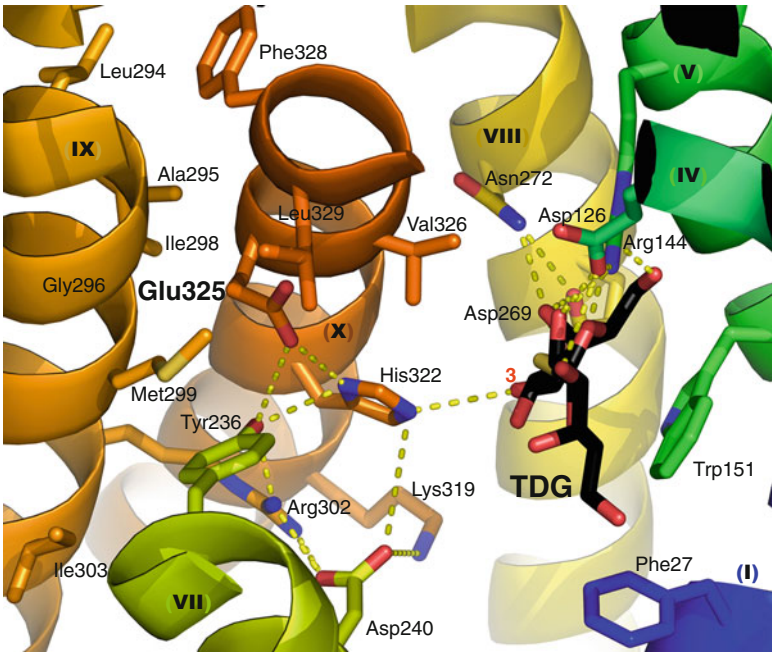


Fig. 5 H^+-binding residue in LacY. Crystal structure of the LacY G46W/G262W mutant in an outward-facing partially occluded conformation with bound TDG [PDB Access ID, 4OAA]. The helices of LacY are colored in *rainbow* and labeled with *Roman numerals*. *Dotted lines* indicate that these polar/charged sidechains and sugar OH groups are in close proximity and may form salt bridge/hydrogen-bonding interactions. The lactose analogue TDG is shown in *black*, and C_3OH is indicated. The H^+-binding residue Glu325 is on helix X

sugar- and H^+-binding sites are located at the apex of either of the periplasmic-open or cytoplasmic-open internal cavities, providing the structural basis for alternating-access process.

The periplasmic- and cytoplasmic-facing conformations with bound galactoside and H^+ reflect only the static states. LacY must dynamically undergo large, global conformational changes to expose both the sugar- and H^+-binding sites on either side of the membrane (Smirnova et al. 2011). Seven independent approaches – site-directed alkylation of single-Cys replacement mutants, thiol cross-linking, single-molecule fluorescence resonance energy transfer, double electron-electron resonance spectroscopy, and time-resolved FRET techniques to detect conformational changes, sugar-binding kinetics, and studies with nanobodies – provide strong independent and converging evidence showing that both periplasmic and cytoplasmic cavities open reciprocally. In the absence of a sugar substrate, LacY favors the inward-facing conformation, the resting state; sugar binding increases the probability of forming intermediate occluded and outward-facing conformations.

5 The H^+/Lactose Symport Mechanism

The lactose transport cycle catalyzed by LacY includes the alternating-access process and several other steps, which can be explained by a simplified eight-step kinetic scheme shown in Fig. 6. Based on an ordered-binding model (Kaczorowski and Kaback 1979), different transport modes will be explained in detail below.

5.1 Lactose Active Transport

The process for the lactose active transport against a lactose concentration gradient driven by $\Delta\tilde{\mu}_{H^+}$ (interior negative and/or alkaline) is indicated in *red arrows* in Fig. 6. LacY is fully protonated at physiological pH values in the resting state due to the high p*K*a value, which primes the sugar-binding site at a relatively higher affinity [1]. The binding of galactoside to protonated LacY from the periplasm [2] induces formation of intermediate [3], in which sugar and H^+ are bound and both cavities are closed in an occluded state. This ternary complex then opens to the cytoplasm [4], sugar dissociates [5] followed by deprotonation [6] to the cytoplasm, the unloaded LacY returns to the outward-facing conformation by steps [7, 8], and protonates immediately [1], ready for the next transport cycle. Note that the ordered mechanism is necessary for the binding of galactoside, and H^+ on first and off second makes it difficult to develop a futile H^+ leak.

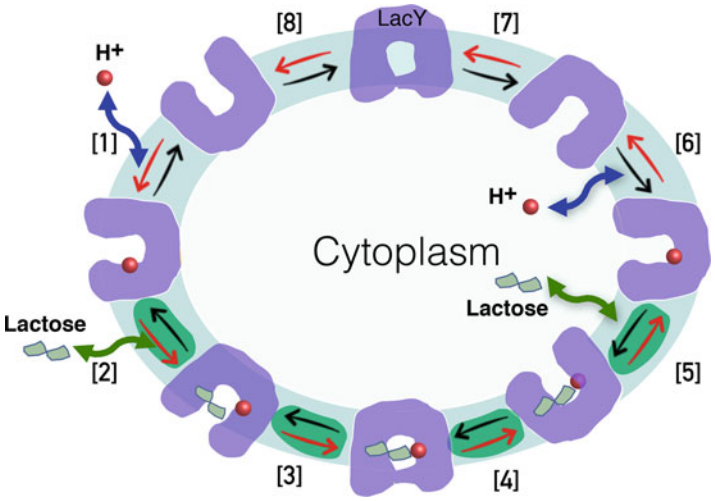


Fig. 6 The H^+/lactose symport mechanism. There are eight kinetic steps as indicated. Sugar and H^+ bind in the middle of the molecule, and LacY moves around the binding sites to release the two substrates by an ordered mechanism on either side of the membrane as indicated by the *arrows*. The alternating-access process only involves the steps [2–5] as highlighted in *green*

LacY drives accumulation of lactose against a 50- to 100-fold concentration gradient (Robertson et al. 1980) with a turnover number of ~20 s^{-1}. Both $\Delta\Psi$ (interior negative) and ΔpH (interior alkaline) have quantitatively same kinetic effect on the transport, with a 50- to 100-fold decrease in K_m value and the same thermodynamic equilibrium. The K_d value for galactosides on either side of the membrane is essentially the same in the absence or presence of $\Delta\tilde{\mu}_{H^+}$ (Guan and Kaback 2004). In the absence of $\Delta\tilde{\mu}_{H^+}$, there is a three to fourfold inhibition of lactose transport in the presence of deuterium oxide (D_2O) indicating that deprotonation is the rate-limiting step (Viitanen et al. 1983; Garcia-Celma et al. 2009; Garcia-Celma et al. 2010; Gaiko et al. 2013). In the presence of $\Delta\tilde{\mu}_{H^+}$, the rate of transport is completely unaffected by D_2O indicating that deprotonation is no longer rate limiting when there is a driving force on the H^+. It is particularly noteworthy that a number of experimental findings indicate strongly that $\Delta\tilde{\mu}_{H^+}$ functions kinetically as a driving force on the H^+, but the alternating-access process is independent of $\Delta\tilde{\mu}_{H^+}$ (Guan and Kaback 2006).

5.2 Lactose Influx or Efflux down a Concentration Gradient

Lactose inwardly directed flux (influx, *red arrows*) or outwardly directed flux (efflux, *black arrows*) down a lactose concentration gradient occurs in a virtually identical fashion starting at step [1] for influx and step [6] for efflux and proceeding via the *red* or *black arrows* around the circle (Fig. 6). These transport modes occur in the absence of $\Delta\tilde{\mu}_{H^+}$, and the limiting step is intracellular deprotonation for step [6] during influx and periplasmic deprotonation for step [1] during efflux. Both reactions exhibit deuterium isotope effects; that is, influx and efflux are inhibited by D_2O.

5.3 Lactose Equilibrium Exchange and Counterflow

Both of these exchange reactions by LacY represent the alternating-access process. Both reactions are driven by lactose concentration gradients that exchange internal lactose for external lactose with the radioactive sugar on the inside (exchange) or outside (counterflow). For equilibrium exchange, lactose concentrations are equal on both sides of the membrane. With counterflow, the internal unlabeled lactose is at a high concentration and the external radioactive lactose is at a much lower concentration. Both reactions do not involve deprotonation. They are essentially unaffected by ambient pH or $\Delta\tilde{\mu}_{H^+}$ and exhibit no deuterium isotope effect. Only steps [2–5] are engaged.

The ordered binding and release of H^+ and galactoside are strongly supported by the behavior of mutants such as E325A that cannot catalyze any transport reactions involving net H^+ translocation (i.e., electrogenic transport) but catalyze equilibrium exchange and counterflow (i.e., electroneutral transport) as the same rate as WT LacY (Carrasco et al. 1986).

6 Future Research

The outcome from a large body of biochemical, biophysical, and structural studies on LacY have provided rich knowledge on H^+/lactose symport mechanism, including high-resolution structural information from different critical conformations of LacY in apo and substrate-bound forms, as well as detailed knowledge on sugar and H^+ binding and symport by this transporter. Several fundamental and mechanistic details still remain largely unknown. What changes resulted from release of sugar during a transport cycle cause LacY deprotonation? What is the structural base for the increase in sugar-binding affinity when Glu325 is protonated? What is the detailed mechanism underlying the coupled transport of the cargo sugar with its coupling cation H^+? It is critical to determine whether or not the conformational changes that occur with the alternating access mechanism are independent of $\Delta\tilde{\mu}_{H^+}$, as appears to be the case for LacY (i.e., does $\Delta\tilde{\mu}_{H^+}$ alter the rate of counterflow or equilibrium exchange with other MFS family members?).

References

Abramson J, Smirnova I, Kasho V, Verner G, Kaback HR, Iwata S (2003) Structure and mechanism of the lactose permease of *Escherichia coli*. Science 301:610–615

Büchel DE, Gronenborn B, Müller-Hill B (1980) Sequence of the lactose permease gene. Nature 283:541–545

Carrasco N, Antes LM, Poonian MS, Kaback HR (1986) Lac permease of *Escherichia coli*: histidine-322 and glutamic acid-325 may be components of a charge-relay system. Biochemistry 25:4486–4488

Chaptal V, Kwon S, Sawaya MR, Guan L, Kaback HR, Abramson J (2011) Crystal structure of lactose permease in complex with an affinity inactivator yields unique insight into sugar recognition. Proc Natl Acad Sci USA 108:9361–9366

Frillingos S, Sahin-Toth M, Wu J, Kaback HR (1998) Cys-scanning mutagenesis: a novel approach to structure function relationships in polytopic membrane proteins. FASEB J 12:1281–1299

Gaiko O, Bazzone A, Fendler K, Kaback HR (2013) Electrophysiological characterization of uncoupled mutants of LacY. Biochemistry 52:8261–8266

Garcia-Celma JJ, Smirnova IN, Kaback HR, Fendler K (2009) Electrophysiological characterization of LacY. Proc Natl Acad Sci USA 106:7373–7378

Garcia-Celma JJ, Ploch J, Smirnova I, Kaback HR, Fendler K (2010) Delineating electrogenic reactions during lactose/H+ symport. Biochemistry 49:6115–6121

Grytsyk N, Sugihara J, Kaback HR, Hellwig P (2017) pKa of Glu325 in LacY. Proc Natl Acad Sci USA 114:1530–1535

Guan L, Kaback HR (2004) Binding affinity of lactose permease is not altered by the H+ electrochemical gradient. Proc Natl Acad Sci USA 101:12148–12152

Guan L, Kaback HR (2006) Lessons from lactose permease. Annu Rev Biophys Biomol Struct 35:67–91

Guan L, Hu Y, Kaback HR (2003a) Aromatic stacking in the sugar binding site of the lactose permease. Biochemistry 42:1377–1382

Guan L, Sahin-Toth M, Kalai T, Hideg K, Kaback HR (2003b) Probing the mechanism of a membrane transport protein with affinity inactivators. J Biol Chem 278:10641–10648

Guan L, Mirza O, Verner G, Iwata S, Kaback HR (2007) Structural determination of wild-type lactose permease. Proc Natl Acad Sci USA 104:15294–15298

Kaback HR (2015) A chemiosmotic mechanism of symport. Proc Natl Acad Sci USA 112:1259–1264

Kaback HR, Sahin-Toth M, Weinglass AB (2001) The kamikaze approach to membrane transport. Nat Rev Mol Cell Biol 2:610–620

Kaczorowski GJ, Kaback HR (1979) Mechanism of lactose translocation in membrane vesicles from *Escherichia coli*. 1. Effect of pH on efflux, exchange, and counterflow. Biochemistry 18:3691–3697

Kumar H, Kasho V, Smirnova I, Finer-Moore JS, Kaback HR, Stroud RM (2014) Structure of sugar-bound LacY. Proc Natl Acad Sci USA 111:1784–1788

Kumar H, Finer-Moore JS, Kaback HR, Stroud RM (2015) Structure of LacY with an alpha-substituted galactoside: connecting the binding site to the protonation site. Proc Natl Acad Sci USA 112:9004–9009

Nie Y, Kaback HR (2010) Sugar binding induces the same global conformational change in purified LacY as in the native bacterial membrane. Proc Natl Acad Sci USA 107:9903–9908

Radestock S, Forrest LR (2011) The alternating-access mechanism of MFS transporters arises from inverted-topology repeats. J Mol Biol 407:698–715

Robertson DE, Kaczorowski GJ, Garcia ML, Kaback HR (1980) Active transport in membrane vesicles from *Escherichia coli*: the electrochemical proton gradient alters the distribution of the lac carrier between two different kinetic states. Biochemistry 19:5692–5702

Sahin-Toth M, Akhoon KM, Runner J, Kaback HR (2000) Ligand recognition by the lactose permease of *Escherichia coli*: specificity and affinity are defined by distinct structural elements of galactopyranosides. Biochemistry 39:5097–5103

Sahin-Toth M, Lawrence MC, Nishio T, Kaback HR (2001) The C-4 hydroxyl group of galactopyranosides is the major determinant for ligand recognition by the lactose permease of *Escherichia coli*. Biochemistry 40:13015–13019

Smirnova IN, Kasho V, Kaback HR (2008) Protonation and sugar binding to LacY. Proc Natl Acad Sci USA 105:8896–8901

Smirnova I, Kasho V, Sugihara J, Choe JY, Kaback HR (2009) Residues in the H(+) translocation site define the pKa for sugar binding to LacY. Biochemistry 48:8852–8860

Smirnova I, Kasho V, Kaback HR (2011) Lactose permease and the alternating access mechanism. Biochemistry 50:9684–9693

Smirnova I, Kasho V, Sugihara J, Kaback HR (2013) Trp replacements for tightly interacting Gly-Gly pairs in LacY stabilize an outward-facing conformation. Proc Natl Acad Sci USA 110:8876–8881

Vazquez-Ibar JL, Guan L, Svrakic M, Kaback HR (2003) Exploiting luminescence spectroscopy to elucidate the interaction between sugar and a tryptophan residue in the lactose permease of *Escherichia coli*. Proc Natl Acad Sci USA 100:12706–12711

Viitanen P, Garcia ML, Foster DL, Kaczorowski GJ, Kaback HR (1983) Mechanism of lactose translocation in proteoliposomes reconstituted with lac carrier protein purified from *Escherichia coli*. 2. Deuterium solvent isotope effects. Biochemistry 22:2531–2536

von Heijne G (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J Mol Biol 225:487–494

Weinglass AB, Whitelegge JP, Hu Y, Verner GE, Faull KF, Kaback HR (2003) Elucidation of substrate binding interactions in a membrane transport protein by mass spectrometry. EMBO J 22:1467–1477

Yousef MS, Guan L (2009) A 3D structure model of the melibiose permease of *Escherichia coli* represents a distinctive fold for Na(+) symporters. Proc Natl Acad Sci USA 106:15291–15296

Role of the BAM Complex in Outer Membrane Assembly

35

Fernando Navarro-Garcia

Contents

1 Introduction 652
2 Preassembled Outer Membrane Proteins 654
3 Chaperones 655
4 The BAM Complex: Structure and Function for Catalyzing OMP Folding 657
4.1 Structure of Bam Proteins 658
4.2 The BAM Complex Catalyzes uOMP Folding and Membrane Insertion 662
5 Research Needs 664
References 665

Abstract

Gram-negative bacteria have a highly evolved cell wall with two membranes constituting a cell envelope consisting of an inner membrane (IM), a periplasmic space, and an outer membrane (OM). Proteins in the bacterial IM contain one or more hydrophobic α-helical transmembrane-spanning domains (TMDs). These co-translationally formed TMDs are accommodated into the IM lipid bilayer through a lateral gate in the Sec complex. While OM proteins (OMPs) reach their destination posttranslationally and contain a unique TMD known as a β-barrel. This amphipathic β-barrel closed cylindrical structure contains 8–26 TMDs that are arranged in a linear antiparallel β-sheet, with the first and last strands interacting at a junction to form this barrel-like shape. OMPs must be transported across the IM, periplasm, and peptidoglycan before they are inserted into the OM to achieve their functional form. The nascent OMPs pass the IM in an unfolded state (uOMPs) with an amino-terminal leader sequence that directs them to the Sec export machinery, which transports the uOMPs into the periplasm, where the lack of ATP is another of

F. Navarro-Garcia (✉)
Department of Cell Biology, Center for Research and Advanced Studies (Cinvestav), Mexico City, Mexico
e-mail: fnavarro@cell.cinvestav.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_35

the various obstacles to overcome. uOMPs are transported across the periplasm, and successive folding and assembly are key for biogenesis, where chaperones and the essential β-barrel assembly machinery (BAM) complex facilitate these processes. In this chapter, I will describe the role of the BAM complex in outer membrane assembly, including the uOMP protection by periplasmic chaperones, and the structural and functional analyses that illustrate how the BAM complex inserts its substrates into the outer membrane.

1 Introduction

The presence of an outer membrane is a remarkable characteristic of Gram-negative bacteria, as well as of eukaryotic organelles such as chloroplast and mitochondria. Gram-negative bacteria have a highly evolved cell wall with two membranes constituting a cell envelope, which consists of an inner membrane (IM), a periplasmic space, and an outer membrane (OM). These two membranes are composed of complex arrays of integral and peripheral proteins, as well as phospholipids and glycolipids (Botos et al. 2017). These membranes are enriched in phospholipids such as phosphatidylethanolamine (PE), phosphatidylglycerol, and small amounts of cardiolipin. The inner membrane is composed by phospholipids in both leaflets, while the OM is highly asymmetric since the inner leaflet contains phospholipids but the outer leaflet consists of lipopolysaccharides (LPS) in association with divalent metal ions (i.e., calcium and magnesium). In between these two membranes, the periplasmic space encompasses layers of peptidoglycan (Fig. 1a), which have an estimated average thickness of 150 Å (Beveridge 1999). The OM, composed of phospholipids and the lipid A part of LPS, forms a hydrophobic core preventing penetration of polar solutes. But unlike other biological membranes, the carbohydrate chains of LPS in the OM also exclude hydrophobic compounds. These sugar chains carry out two main functions, create a polar mesh around bacterial cell preventing access of hydrophobic compounds to the lipid core, and enable intensive lateral interactions between phosphorylated sugar residues with divalent cations by forming bridges to seal the OM (Nikaido 2003). On the other hand, almost all integral membrane proteins can be divided into two structural classes: those with either a α-helical or β-barrel fold. Interestingly, unlike the α-helical membrane proteins, which are found in nearly all membranes, β-barrel membrane proteins are found only within the outer membranes (OMs) of Gram-negative bacteria, mitochondria, and chloroplasts. Both types of fully integrated membrane proteins can serve many functions within the membrane, including nutrient import and export, signaling, motility, and adhesion (Paschen et al. 2003).

Proteins in most of the eukaryotic cell membranes or the bacterial IM contain one or more hydrophobic α-helical transmembrane segments. These membrane-spanning proteins are co-translationally targeted to the endoplasmic reticulum or to the IM, and the hydrophobic membrane-spanning segments are accommodated into the lipid bilayer through a lateral gate in the Sec complex. While in Gram-negative bacteria, most proteins residing in the OM reach their destination posttranslationally

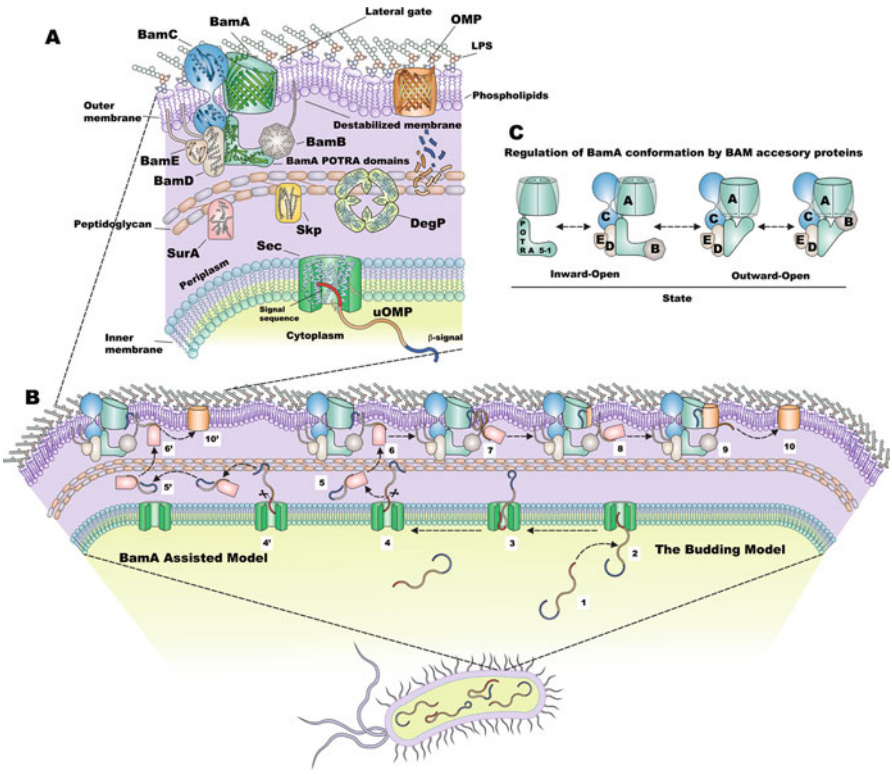


Fig. 1 Two mechanistic models for BAM-mediated OMP biogenesis. (**a**) Gram-negative envelope structure, BAM complex structure, and the main components required for OMP biogenesis. The bacterial envelope contains two membranes, and the outer membrane is an asymmetric bilayer with LPS in its outer leaflet. OMPs contain a signal sequence (red) that targets them to the Sec complex, which translocates them across the inner membrane. Unfolded OMP (uOMP) is bound by periplasmic chaperones (SurA, Skp, and DegP, which also has proteolytic activity) that escort them to the BAM complex. The BAM complex (BamA, BamB, BamC, BamD, and BamE) assembles OMPs into the outer membrane. (**b**) Sequence of events for the two mechanistic models, the BAM-assisted and the budding model. The two models share the first six steps. In the Bam-assisted model (steps indicated by numbers with apostrophes), β-barrel folding begins on the periplasmic side of the outer membrane and is integrated as one unit into the outer membrane by the BAM complex. Thus, OMPs fold themselves and just need a locally disturbed membrane to organically fold into. While in the budding model (steps indicated by normal numbers), β-strands of an incoming uOMP are templated at the open BamA seam (step 7) forming a hybrid BamA/OMP barrel (green/orange, step 8), and once all β-strands are formed (step 9), the new OMP barrel closes and buds off BamA into the outer membrane (step 10). (**c**) Role of the Bam accessory proteins for regulating the conformation of BamA during the OMP biogenesis. Two major conformations have been observed for BamA: the barrel domain of BamA can be in an "inward-open" or "outward-open" conformation. In the inward-open state, the exit pore is occluded, with POTRA5 being located away from the barrel domain to enable access to the lumen from the periplasm. While in the outward-open state, the exit pore is now fully open, as POTRA5 shifts to occlude access to the barrel lumen from the periplasm

and contain a unique membrane-spanning segment known as a β-barrel. This type of membrane-spanning segment is an amphipathic β-sheet that forms a closed cylindrical structure, the β-barrel (Walther et al. 2009). Thus, transmembrane proteins in α-helical structure contain one or more transmembrane-spanning domains (TMDs) that consist of hydrophobic α-helices, whereas monomeric β-barrel proteins contain 8–26 TMDs formed by amphipathic β-strands. The β-barrel name is derived from the conformation of the β-strands, which are arranged in a linear antiparallel β-sheet, with the first strand and last strand interacting at a junction to form this classic barrel-like shape (Noinaj et al. 2017).

Almost all of the integral OM proteins are β-barrel proteins, also known as OMPs (outer membrane proteins), and Gram-negative OMs are heavily packed with OMPs. OMPs play relevant roles in the OM, which are involved in numerous functions such as uptake of nutrients, release of waste materials, secretion of virulence factors, and resistance to host defense systems. OMPs are often responsible for sensing environmental changes and facilitating the downstream modulation of gene expression that this adaptation requires in order to respond to, and exploit, their environmental niches (Vogel and Papenfort 2006). In this way, the OM protects Gram-negative bacteria by serving as the first line of defense that guards against extracellular threats. Thus, the OM is an essential organelle that acts as a selective permeability barrier protecting the cell from harmful chemicals, including detergents and antibiotics, and consequently OM integrity is essential for cell viability (Nikaido 2003). Interestingly, only two OMPs are known to play an essential role in OM biogenesis. Nascent OMPs, in order to achieve their functional form in the OM, must first traverse the periplasm, which is at a 210 Å distance from IM to OM (Matias et al. 2003). This passage through the peptidoglycan layer must be achieved in a largely unfolded state (De Geyter et al. 2016). Then, due to the lack of ATP in the periplasmic space, the subsequent folding and assembly of the OMPs into their functional form relies heavily on free energy changes through chaperone binding and the catalytic properties of the β-barrel assembly machinery (BAM) (Noinaj et al. 2017). In this chapter, I will describe the role of the BAM complex in OM assembly, including the transport of OMP through the IM and its chaperoned periplasmic protection to its folding and insertion into the OM by the BAM complex, as well as the structural details of this complex.

2 Preassembled Outer Membrane Proteins

OM biogenesis is complex process, since the cell must overcome various obstacles, including translocation of the hydrophobic components of the OM across the cytoplasmic or IM and their transport across the aqueous periplasmic space between the OM and the IM. Both LPS and certain OMPs are required for OM integrity and cell viability. Therefore both components must be transported to cross the cell envelope and to be situated correctly in the OM. Additionally, biosynthesis of these components as well as their transport and OM insertion must also be coordinated with cell growth and maintaining the barrier function of this membrane. Unlike

the transport across the IM, which is ATP energy-dependent, transport across the periplasm and insertion into the OM is a challenge because there is no ATP outside the cytoplasm nor any useful ion gradient across the OM (Botos et al. 2017).

OMPs play a central role in the integrity of the OM of Gram-negative bacteria. They must be transported across the IM, periplasm, and peptidoglycan before they are inserted into the OM (Rollauer et al. 2015). These proteins destined for the OM are synthesized by ribosomes in the cytoplasm and fully translated in an unfolded state with an amino-terminal leader sequence that directs them to the general secretory (Sec) export machinery, using the chaperone SecB and the energy of the SecA motor, which transports the nascent OMPs across the IM and into the periplasm (Driessen and Nouwen 2008). Unfolded OMPs (uOMPs) are transported across the periplasm, and successive folding and assembly are key for biogenesis, where chaperones and the essential β-barrel assembly machinery (BAM) complex facilitate these processes (Fig. 1a). In the periplasm, OMPs remain unfolded and must traverse the 165 Å aqueous periplasm in an unfolded but folding-competent state (Murakami et al. 2002), until they reach the asymmetric OM into which they fold. A crucial requirement for folding of OMPs into their native conformation is the lipid bilayer.

The β-barrel folded topology of typical OMPs is composed of a closed cylinder of antiparallel β-strands. Thereby, the native conformation requires that the interior of OMP β-barrels be hydrophilic often water-accessible, whereas the exteriors be hydrophobic and lipid-exposed. Hydrogen bonding allows the interaction of adjacent β-strands, which are covalently connected by short periplasmic turns and longer extracellular loops. The formation of this native and functionally active conformation depends on the OMPs fate in the periplasm; aggregation of uOMP is avoided by the presence of periplasmic chaperones. Thus, these chaperones must prevent uOMP aggregation and its associated cell stress in the absence of an external energy source. Thereby, the thermodynamics and kinetics of interaction between chaperone and uOMP have to be fine-tuned to keep the balance among uOMP biogenesis, folding, trafficking, and degradation in the absence of ATP (Moon et al. 2013; Plummer and Fleming 2016).

3 Chaperones

The biogenesis of OMPs in diderm bacteria involves delivery by periplasmic chaperones, and these protein-chaperone complexes are targeted to the BAM complex, which catalyzes OMP insertion into the OM. Thus, chaperone-stabilized OMPs are escorted across the periplasm to the OM. Once in the periplasm, uOMPs are escorted by chaperones such as the survival factor protein A (SurA), the 17-kDa protein (Skp), the FkpB binding protein A (FkpA), and DegP, a bifunctional chaperone-protease (Costello et al. 2016; Plummer and Fleming 2016). Two major periplasmic pathways have been described to transport OMPs in *E. coli*. One of them is directed by the SurA chaperone, and the other is governed by the trimeric chaperone Skp in cooperation with the bifunctional DegP (Rizzitello et al. 2001). The chaperone SurA appears to be a crucial chaperone during the targeting of OMPs, whereas Skp has a role during the chaperone-mediated biogenesis of some OMPs,

including rescuing mishandled OMPs that are usually chaperoned by SurA (Sklar et al. 2007). However, all the previously mentioned chaperones can function as a network of somewhat redundant chaperones for OMPs. Furthermore, a holistic computational approach (Costello et al. 2016) and recent studies (De Geyter et al. 2016; Moon et al. 2013) indicate a more complicated pathway, suggesting that OMPs may experience complex chaperone networks that are driven by energy scenery in the periplasm (Noinaj et al. 2017). Misfolded OMPs that cannot be rescued are degraded by the periplasmic protease DegP to prevent their accumulation and thus cellular toxicity (Ge et al. 2014; Sklar et al. 2007). Remarkably, Skp has been cross-linked to the IM in spheroplasts (Harms et al. 2001), but cross-linking of Skp to BAM in vivo has not been reported (Sklar et al. 2007), while in vivo SurA has been cross-linked to BamA (Sklar et al. 2007), suggesting that they cooperate sequentially, with Skp interacting first in the OMP folding pathway and then passing its substrates to SurA for transfer to BAM complex (Bos et al. 2007). Recently, a more relevant biological context, showing that the chaperones SurA and Skp have differential effects on the refolding trajectories of the OMP FhuA, was found. While SurA helps the reinsertion of single native β-hairpins into the lipid membrane, Skp significantly decreases misfolded polypeptide conformations (Thoma et al. 2015), suggesting that Skp helps mainly in binding uOMPs to prevent aggregation and that SurA in binding uOMPs and someway affects the uOMP folding pathway (Plummer and Fleming 2016).

Both Skp and DegP employ a similar mechanism to prevent aggregation, which is to protect uOMPs by sequestering them within a demarcated internal uOMP cavity (Plummer and Fleming 2016). Thus, Skp and DegP are both oligomeric chaperones able to accommodate uOMPs into defined internal binding cages (Krojer et al. 2008; Walton and Sousa 2004). When Skp is in uOMP free state (apo-Skp), three α-helical Skp polypeptide chains associate to form a trimeric moiety with a large internal cavity. Through the positively charged monomer tips, the uOMP partner initially contacts the Skp trimer. It is thought that the uOMP makes many weak local interactions within the internal cavity of Skp leading it to a high mobility (Plummer and Fleming 2016). All these numerous interactions result in nanomolar (nM) dissociation constants for binding between Skp and uOMP partners (Moon et al. 2013). The α-helical arms of Skp are sufficiently flexible to accommodate the unstructured uOMP; however the uOMP is not entirely encapsulated but protrudes from openings in the Skp cavity (Zaccai et al. 2016). It is thought that both the bound uOMP and Skp in the complex exist in multiple conformations, all preventing the deleterious uOMP aggregation and protecting it from the aqueous periplasm (Plummer and Fleming 2016). The modeling of Skp-uOMP with a folded soluble domain advocates that uOMPs bind to Skp as an ensemble with the soluble OMP domain in a variety of orientations outside the Skp trimer (Zaccai et al. 2016). This type of uOMP capturing form, called clamp-like mechanism, has also been observed for the periplasmic bifunctional chaperone/protease DegP, which sequesters uOMPs in an internal cage. However, as mentioned above Skp is a functional trimer, while DegP can form a holo-complex composed of up to 24 monomers, which appears large enough to span the entire periplasm (Krojer et al. 2008). This is an interesting fact since the width of the periplasm is on the same length scale as the

size of DegP and other relevant chaperone proteins. Chaperone associations spanning the whole periplasm might provide a solution for the lack of an external energy requirement for uOMP transport across the periplasm, suggesting that this thermal energy-driven diffusion, chaperone-uOMP complexes may easily make the short journey from the IM to the OM (Plummer and Fleming 2016).

Skp can also participate in an alternative pathway to deliver OMPs directly to the OM in a BAM-independent mechanism, since Skp can deliver OMPs to negatively charged synthetic bilayers (McMorran et al. 2013; Patel et al. 2009) and Skp can also fold at least some OMPs into the membranes in vivo (Grabowicz et al. 2016).

A second mechanism for preventing uOMPs from aggregation involves chaperones that lack obvious cage-like structures. The periplasmic prolyl-isomerases SurA and FkpA both are not known to form defined enclosures but are able to bind uOMPs (Bitto and McKay 2003; Saul et al. 2004). The binding mechanism of these chaperones to uOMP partners remains unknown, but the stoichiometry of binding of SurA to FhuA was determined to be 2:1, SurA-uOMP (Thoma et al. 2015). However, both SurA and FkpA may use conformational switching in binding to uOMPs. It has been found that the two parvulin domains of SurA undergo several structural arrangements that mediate SurA activity by modulating populations of SurA in open and closed states (Soltes et al. 2016). In a similar way, in the case of FkpA, the flexibility in one of its N-terminal helices may allow it to exist in many uOMP-binding conformations (Hu et al. 2006).

Recent kinetic simulations of OMP biogenesis by modeling of the flux of unfolded OMPs across the periplasm, incorporating kinetic and thermodynamic data from the literature, suggest that OMPs may make 100 s of interactions on average with SurA, Skp, and other chaperones, which are present in concentrations such that there is always a free chaperone reservoir available for OMP binding (Costello et al. 2016).

In the OM, the only essential OMPs in almost all Gram-negative bacteria are the biogenesis machineries that function to insert LPS and OMPs (Botos et al. 2017). In the case of the OMPs, the dedicate machinery is the BAM complex. In spite of the sequential model for the role of Skp and SurA, it has been found that prefolded BamA, an OMP itself, promotes the release of tOmpA from Skp despite the nM affinity of the Skp-tOmpA complex. This activity is located in the BamA β-barrel domain but is greater when full-length BamA is present, indicating that both the β-barrel and its periplasmic polypeptide transport-associated (POTRA) domains are required for maximal activity. By contrast, SurA is unable to release tOmpA from Skp, providing direct evidence against a sequential chaperone model (Schiffrin et al. 2017).

4 The BAM Complex: Structure and Function for Catalyzing OMP Folding

Once uOMP chaperones finish the voyage across the periplasm, uOMPs must fold into the OM. A multicomponent membrane-embedded complex called the β-barrel assembly machinery (BAM) orchestrates the folding and insertion of new OMPs into

the OM. The BAM complex is formed by five components, including BamA, an OMP itself, which is a multi-domain protein found in all diderm bacteria, and four lipoproteins BamB to BamE, constituting BAM complex responsible for folding and insertion of OMPs into the bacterial OM (Voulhoux et al. 2003; Wu et al. 2005). Thus, BamA is an integral OM protein with an extended periplasmic domain, and BamB, BamC, BamD, and BamE lipoproteins are anchored to the inner leaflet of the OM via an N-terminal lipid anchor (Botos et al. 2017; Wu et al. 2005). However, the different components of BAM complex can vary by species (Noinaj et al. 2017). The periplasmic domain of BamA interacts directly with BamB and BamD, while BamC and BamE interact with BamD, which is supported by the isolation of primarily BamA-BamB and BamC-BamD-BamE modules in vivo (O'Neil et al. 2015; Sklar et al. 2007; Wu et al. 2005). Of these five components, only BamA and BamD are necessary for viability, but all components are required for efficient OMP biogenesis in vivo (Hagan et al. 2010; Sklar et al. 2007; Wu et al. 2005), because BamB, BamC, and BamE single deletions affect OMP content or OM permeability and susceptibility to certain antibiotics (Noinaj et al. 2014).

4.1 Structure of Bam Proteins

BamA contains a transmembrane β-barrel domain, which is thought to play a central role in the assembly and insertion of the incoming β-barrel substrate into the OM. This β-barrel domain consists of a 16-stranded β-sheet enclosing a large water-filled lumen (Albrecht et al. 2014; Ni et al. 2014), while extracellular loops of BamA form a dome on top of BamA. These loops enclose the barrel from the top and prevent solute passage through the BamA pore. One of these extracellular loops (eL6) partially inserts inside the lumen and interacts with β-strands 14–16. An interesting and unique architecture of the β-barrel of BamA is that the seam (the junction site where strands β1 and β16 close the barrel) displays poor hydrogen bonding between these first and last barrel β-strands (Konovalova et al. 2017). This arrangement leads to another intriguing feature, which is that the height of the BamA barrel, dictated by the membrane-spanning hydrophobic belt, is significantly shorter proximal to the seam (9–12 Å) compared to 20 Å on the opposite side of the barrel. Both of these features are key for the mechanisms of BamA function (Konovalova et al. 2017). Additionally, two reported crystal structures show that the strand β16 is partially unzipped from β1, sitting in a bent conformation that was pointing toward the lumen of the barrel domain (Albrecht et al. 2014; Ni et al. 2014). These data indicate that the barrel domain of BamA contains a lateral gate into the membrane, which would create a portal for the insertion of nascent OMPs directly into the OM during biogenesis (Noinaj et al. 2014). Furthermore, an exit pore, that has been proposed to mediate the formation of the extracellular loops of nascent OMPs, was identified (Ni et al. 2014; Noinaj et al. 2014).

On the other hand, the N-terminal periplasmic domain of BamA contains five tandem polypeptide transport-associated (POTRA) domains. Studies have shown that POTRA1–POTRA5 domains mediate the interaction with the other Bam

components (Kim et al. 2007; Wu et al. 2005), acting as a structural scaffold for binding other Bam components and for recruiting uOMPs and chaperones such as SurA (Knowles et al. 2009; Wu et al. 2005). The POTRA domain structures have a βααββ topology consisting of a three-stranded β-sheet positioned against two antiparallel helices. Thus, each of the five POTRAs is structurally similar, with no significant sequence similarity, and the largest difference is with POTRA3 that has an extended loop between α1 and α2 (Gatzeva-Topalova et al. 2008; Kim et al. 2007). Interestingly, the POTRA domains are functionally distinct, despite having very similar structures. Structurally, POTRA1–2 and POTRA3–5 form two subdomains. The relative orientations of POTRA domains within these subdomains are well conserved even when comparing structures from different bacterial species (Gatzeva-Topalova et al. 2010; Noinaj et al. 2013). Additionally, POTRA3–5 has a rigid orientation in solution (Gatzeva-Topalova et al. 2010), and POTRA1–2 behaves as a single rigid unit in solution (Ward et al. 2009). Besides, the orientation between these subdomains varies widely in the crystal structures, apparently due to a flexible hinge between POTRA2 and POTRA3. Whereas, functionally, POTRA3 provides an interaction interface between BamA and BamB (Kim et al. 2007), POTRA5 is important for BamA interaction with the BamCDE subcomplex, and POTRA1 is important for BamA-chaperone SurA interaction (Vuong et al. 2008). Deletion of POTRA1 and POTRA2 is not lethal even though POTRA1 and POTRA2 are important for BamA function. The remaining POTRA domains, however, are essential (Kim et al. 2007). According to the structure of the BAM complex, the BamA POTRA domains do not simply hang in the periplasm, since they form a ringlike structure together with Bam lipoproteins and this ring is predicted to lie parallel to the membrane plane (Bakelar et al. 2016; Gu et al. 2016). The periplasmic domain of BamA interacts directly with BamB and BamD, whereas BamC and BamE interact with BamD (Hagan et al. 2011; Kim et al. 2012; Knowles et al. 2009; O'Neil et al. 2015; Wu et al. 2005). Null mutants of each of the lipoproteins that interact with BamA produce phenotypes ranging from lethality (*bamD*) to significant membrane defects (*bamB*) to barely detectable permeability increases (*bamC* and *bamE*) (Sklar et al. 2007).

BamB is a lipoprotein, which is attached to the periplasmic side of the OM by its N-terminal lipid anchor (Vuong et al. 2008; Wu et al. 2005). BamB plays an important role in membrane protein biogenesis since deletions of BamB result in substantial membrane defects including hypersensitivity to some antibiotics (Charlson et al. 2006). The BamB structure reveals an eight-bladed β-propeller with each propeller composed of four antiparallel β-strands consisting of WD40-like repeats. Three short loops connect the four β-strands within a blade, and longer loops link the blades together. BamB is wider at one end than the other, giving the protein an overall wedge shape. The narrower end also has the longer loops housing some of the most conserved residues of BamB. The BamB loops are numbered consecutively from blade 1 at the N-terminus (Jansen et al. 2015). Several loops in BamB adopt different conformations that depend on its interaction with BamA (bound to BamA or free BamB). The connection of fourth and fifth blades by the inter-blade loop 17 is conformationally flexible (Heuck et al. 2011; Noinaj et al. 2011). The extended BamB loop 13 links the third and fourth blades

of the β-propeller. This loop 13 can be engaged in the interface with BamA, straddling the edge of the β2-strand in BamA POTRA3, which is stabilized by a hydrogen bond between the main chain carbonyl oxygen of BamB Ser-193 and the main chain nitrogen of BamA Leu-247. Thus, loop 13 is well defined in this structure and contributes to the stability of the BamB-BamA interface although it is flexible in the structures of free BamB (Heuck et al. 2011; Noinaj et al. 2011). Extended loops 13 and 17 protruding from one end of the BamB β-propeller contact the face of the POTRA3 β-sheet in BamA. The interface is stabilized by several hydrophobic contacts, a network of hydrogen bonds, and a cation-π interaction between BamA Tyr-255 and BamB Arg-195 (Jansen et al. 2015). The contacts observed in the crystal packing hinted that BamB may bind to unfolded OMPs by β-augmentation and suggested that the eight-bladed β-propeller of BamB will provide a large surface area and multiple binding sites for the β-strands of OMP substrates, serving as a scaffolding protein (Hagan et al. 2010; Noinaj et al. 2011; Roman-Hernandez et al. 2014; Vuong et al. 2008). Mutations that disrupt the BamA-BamB interaction have a phenotype similar to that of BamB deletion, suggesting the function of BamB is dependent on its ability to bind to BamA (Jansen et al. 2015; Vuong et al. 2008; Wu et al. 2005). In vitro studies have further demonstrated the vital role of BamB for the optimal folding of the two OMPs OmpT and EspP and possibly in mediating the delivery of substrate from SurA to the BAM complex, a process that is still not well understood (Hagan et al. 2010; Roman-Hernandez et al. 2014).

BamC is composed of three main domains. At the extreme N-terminus of the protein is a disordered region of about 75 amino acids that is followed by a tandem of 2 globular helix-grip domains, separated from each other by a short flexible linker. Interestingly, neither the N-terminal nor C-terminal domains appear to be required for the interaction of BamC with BamD. However, the BamC-BamD structure revealed that the unstructured N-terminal domain of BamC is essential for the stoichiometric interaction between the two proteins (Kim et al. 2011). This unstructured region binds as an elongated chain across one side of the surface of BamD and sits in the deep groove between tetratricopeptide repeat (TPR) 3 and TPR 4 reminiscent of a "lasso"--type loop across the entire C-terminal face of BamD. Recently a study suggested that part of the structure, including the helix-grip domains of BamC, is exposed on the surface of the bacterial cell but still bound as part of the BAM complex (Webb et al. 2012), defining perhaps the most intriguing aspect of BamC. These data suggest a model in which the N-terminal region of BamC is in the periplasm, bound via its lasso-like structure around the extended face of BamD, while the two globular domains are present on the surface. Structural studies have shown that a conserved short stretch of residues beyond the N-terminal "lasso" region could ideally sit within the membrane because it is composed of largely hydrophobic residues. The authors concluded that this conserved hydrophobic stretch of residues somehow traverses the OM, linking the periplasmic N-terminal to the surface-exposed helix-grip domains at the C-terminal of BamC. Interestingly, this hydrophobic sequence is not predicted to form an α-helix; exactly how this stretch may cross the OM is not known (O'Neil et al. 2015).

BamD consists of five tetratricopeptide repeat (TPR) domains, and it has been suggested that BamD has a role in the activation of BamA and/or interacting with

substrates (Dong et al. 2012; Sandoval et al. 2011). BamD has been shown to directly bind both BamA and BamC and to some partner uOMPs (Hagan et al. 2015; Wu et al. 2005). TPR domains are small helix-turn-helix motifs in which the α-helices are packed in an antiparallel conformation (D'Andrea and Regan 2003). The concave face formed from the TPR domain repeats, as in other proteins, is used to recruit their partner proteins by binding their short C-terminal peptide segments in extended conformations (Wu et al. 2004, 2006). BamD, beside BamA, is the only essential lipoprotein of the BAM complex and interacts directly with BamA. The crystal structure of a fusion between BamD with BamA POTRA4 and POTRA5 showed that TPR3 and TPR4 of BamD mediate the primary interactions with only POTRA5 of BamA, confirming previous studies (Bergal et al. 2016; Wu et al. 2005), and it is the most extensive contact between BamA and BAM lipoproteins. Thus, the long axis of BamD, which is also parallel to the membrane, directly interacts with both POTRA5 (primary interaction) and POTRA1 and 2, forming a ringlike structure below the β-barrel domain of BamA within the periplasm. Although the exact effect of BamD binding to BamA is unclear, it has been suggested that BamD may activate BamA (Ricci et al. 2012). One of the functions of BamD is its important role for substrate recognition and thereby OM protein biogenesis (Hagan et al. 2015).

It has been suggested that BamA POTRA domains and BamD interact with uOMP substrates, proposing that uOMPs are recruited to this periplasmic ring as a holding/folding chamber prior to membrane insertion (Gu et al. 2016). BamD binds uOMP substrates through the binding site within the C-terminal OMP sequences, which is similar to the mitochondrial β-signal. Indeed, substrate recognition is an essential function of BamD, and peptides containing the β-signal to bind BamD inhibit β-barrel assembly and become toxic for the bacteria (Hagan et al. 2015). The important roles of BamD in OM protein biogenesis are highlighted by the trapping of the folding intermediate (Lee et al. 2016) and because deletion of BamA POTRA5 or a disruption of a single salt bridge in a POTRA5-BamD interface is sufficient to fully separate the BamCDE and BamAB subcomplexes (Kim et al. 2007; Plummer and Fleming 2016). All these data suggest that BamA and BamD work together to catalyze folding. Consequently, the function of BamD is beyond to the initial substrate recognition, since BamD remains tighly associated with the substrate all throughout OMP assembly. This fact suggests an active role of BamD during this process (Lee et al. 2016) and must include regulation of BamA conformational cycling through direct interaction with POTRA5 (Rigel et al. 2013). Additional to BamA, BamD interacts with the nonessential components BamC and BamE, which themselves have not been shown to directly bind BamA (Sklar et al. 2007; Wu et al. 2005).

BamE is the smallest component of the BAM complex, and like BamA, it is highly conserved (Kim et al. 2011); BamE contains an ααβββ fold, where a three-stranded antiparallel β-sheet is found packed against a pair of α-helices (Knowles et al. 2011), and an N-terminal posttranslational lipid modification that anchors it to the inner leaflet of the OM (Hagan et al. 2011; Kim et al. 2012). In spite of its highly conserved sequence, BamE is not required for cell viability, and mutants lacking BamE display only minor OM defects. BamE binds BamD by interacting with

primarily TPR4 and TPR5 of BamD but also with POTRA5 of BamA. These consistent data may explain previous studies that reported that BamE enhances the interaction between BamD and BamA (Sklar et al. 2007). BamE possesses a set of electronegatively charged residues important for BamD binding, all lying on one face of the protein, indicating that BamE may interact with the electropositive region of TPR5 of BamD. This would place BamE close enough to cooperatively enhance or stabilize binding of BamD to POTRA5 of BamA (O'Neil et al. 2015).

Interestingly, BamE binds preferentially to phosphatidylglycerol (PG) lipids by using some residues that are also important for binding BamD (Knowles et al. 2011). Binding of BamE to PG does not eliminate binding to BamD and may be cooperative. It has been proposed that this dual protein- and lipid-anchoring mechanism of BamE may promote OMP insertion into the OM. Furthermore, PG enhances the insertion of OMP into the OM, suggesting that BamE contributes by localizing PG into the proximity of the BAM complex for added efficiency. Additionally, BamE seems to work with BamD to control the conformation of BamA (Rigel et al. 2013), since the presence of BamE reduces the extracellular sensitivity of BamA to protease cleavage, while the release or lack of BamE increases protease sensitivity (Rigel et al. 2012). All these data suggest that BamE helps modulate the lateral opening of BamA (Noinaj et al. 2014) due perhaps to that the affinity of BamE for PG might partially destabilize the membrane structure adjacent to BamA, inducing a structural change in BamA (O'Neil et al. 2015). However, the role of BamE in the conformational states of BamA remains unknown. Mutagenesis studies have shown that the loss of BamE compromises the stability of the BAM complex, similarly to BamC, yet the lack of BamE causes only mild OMP assembly defects (Sklar et al. 2007), and BamE is not required for BamA assembly (Hagan et al. 2013).

Altogether, the data indicate that the BamC-BamD-BamE module acts both directly and indirectly to modulate the structural conformation of BamA, making the BAM complex more efficient as a whole in receiving and assembling OMPs into the OM.

4.2 The BAM Complex Catalyzes uOMP Folding and Membrane Insertion

Once uOMPs complete the journey across the periplasm, they must fold into the OM by the BAM complex, and the composition of the bacterial OM plays a crucial role in BAM-catalyzed uOMP folding. In the inner leaflet of the OM occurs uOMP folding in vivo; this leaflet contains lipids with both phosphoethanolamine and phosphoglycerol head groups, which is similar to the bacterial IM composition. These biological head groups delay uOMP folding in vitro, which kinetically retards unassisted uOMP folding (Gessmann et al. 2014). This kinetic barrier prevents uOMP folding into the IM avoiding dissipation of the IM proton gradient essential to sustain cell viability. Thereby, the BAM complex must overcome this same kinetic barrier for uOMP folding into the OM and must also be completed in the absence of an external energy source. In vitro BamA alone can accelerate uOMP folding,

although slower than the entire BAM complex (Plummer and Fleming 2016). BamA acts as an enzyme reducing the activation barrier to uOMP folding in biological membranes, since uOMPs spontaneously insert into synthetic lipid bilayers and BamA accelerates this process. The recognition of uOMPs by BamA involves the conserved C-terminal aromatic signature of uOMP sequences, the β-signal, because its removal inhibits BamA-assisted folding in vitro (Gessmann et al. 2014). Thus, BamA by itself accelerates folding and insertion of model OMPs into liposomes, although at substantially lower rates in vitro (Gessmann et al. 2014; Plummer and Fleming 2016). Further reconstitution of BamA with the BAM lipoproteins increases its efficiency, suggesting that the lipoproteins enhance BamA activity (Hagan et al. 2010; Roman-Hernandez et al. 2014). Likely, the presence of the lipoproteins more efficiently catalyzes conformational changes within the POTRA domains of BamA (Charlson et al. 2006) (Fig. 1c).

Two models were proposed for how the BAM complex mediates the biogenesis of OMPs, but neither has been conclusively demonstrated experimentally to apply to all OMPs (Fig. 1). In fact, it is feasible that different types of OMP interact with the BAM complex in a distinct way, depending on their complexity. In the first one, also called BamA-assisted model (Fig. 1b), uOMPs fold themselves but need a locally disturbed membrane to progressively fold into an OMP. The role of the BAM complex in this process is to function as a catalyst by locally destabilizing the membrane bilayer and traffic the uOMP into close proximity to the primed membrane for insertion into the OM (Noinaj et al. 2017). As mentioned above, β-barrel is a very unusual OMP, displaying a reduced hydrophobic thickness where strands β1 and β16 are joined compared with the opposite side of the β-barrel. This hydrophobic thickness mismatch between BamA and the OM seems to locally thin and disorder the lipid bilayer, creating a region of the bilayer where newly folded OMPs may be inserted more efficiently. Evidence supporting this process include molecular dynamics simulations and electron microscopy (EM) experiments, which have shown a reduced distance between lipid head groups and a corresponding increased disorder of surrounding lipid chains and by EM direct observation using lipid vesicles with BamA inserted (Noinaj et al. 2013; Sinnige et al. 2014). Thus, BamA presumably accomplishes this feat by creating bilayer defects. Folding experiments show that BamA catalyzes the folding/insertion of OMPs in vitro by lowering the energetic barrier imposed by PE lipids found in the inner leaflet of the OM (Gessmann et al. 2014). According to this model and based in biophysical in vitro studies, an uOMP binds to the membrane surface, and at the membrane interface, the OMP secondary structures start to form. Then OMP undergoes a transition step by forming β-hairpins, which penetrate the membrane in a coordinated manner until the barrel is fully formed and integrated into the membrane. This spontaneous folding can happen just in thin membranes made up by the short acyl chain lipids and at the temperature of phase transition or above, establishing the requirement of a membrane defect for an insertion (Danoff and Fleming 2015). Thereby, it was proposed that the BAM complex reduces the kinetic barrier of membrane insertion by creating a membrane defect and/or stabilizing certain transition conformations of the OMP at the membrane (Gessmann et al. 2014).

The second model proposes a more systematic mechanism; in this budding model (Fig. 1b), β-strands of an incoming uOMP are templated at the open BamA seam forming a hybrid BamA/OMP barrel, and once all β-strands are formed, the new OMP barrel closes and buds off BamA into the OM (Noinaj et al. 2017). This model shares the initial steps with the first model, which include importation of the uOMPs into the periplasm and stabilization by chaperones such as SurA and Skp. Then, uOMPs are delivered to the components of the BAM complex, which may include BamB, BamD, and/or the POTRA domains of BamA, leading to folding and insertion that is initiated by the β-signal of the uOMP. When the lateral gate of BamA is open, it is thought that a β-hairpin binds to the exposed N-terminal strand of BamA by β-augmentation. The β-barrel of the OMP is systematically integrated directly into the barrel of BamA for satisfying the requirement that hydrophilic residues face inside the barrel, while hydrophobic residues face outside and mediate interaction with the membrane. The uOMP continues to be incorporated by either strand by strand or by one β-hairpin at a time, but to prevent the formation of a super-pore in the OM, the folded OMP finally "buds" away from the barrel domain of BamA. It is thought that OMP maturation occurs when the first strand of the OMP comes into proximity with the junction site, whereby the last strand of the OMP unlinks from BamA and then couples with its own first strand (Noinaj et al. 2017). The folding process ends by forming an independent OMP barrel, which then diffuses into the OM, whereas the BAM complex is ready for another cycle of OMP folding and insertion.

Finally, a structural model for the fully assembled BAM complex from *E. coli* can provide interesting observations (O'Neil et al. 2015). Accordingly, BamA and the globular domains of BamC are the only surface-exposed structures, whereas BamB, BamD, and BamE are fully within the periplasm. Additionally, BamD TPR4 interacting with POTRA5 of BamA is likely oriented parallel to the membrane. From modeling data, the N-terminal domain of BamD may also interact with other regions of the periplasmic domain of BamA, probably POTRA2. Also, BamE is in the proximity of both POTRA4 and POTRA5 because the interaction of BamE along TPR5 of BamD may link another interaction between BamD and BamA, thereby promoting BamD-BamA apparent affinity. Interestingly, the lateral opening site of BamA is located into the center of the BAM complex just above the POTRA domains, which would be ideal for mediating interaction with uOMPs as they are being folded and inserted into the OM.

5 Research Needs

Several studies of crystal structures complemented by mutagenesis and molecular dynamics simulations have pieced together a structural model for the fully assembled BAM complex. However, further mechanistic experiments are required to know how the BAM complex may function at the OM, and this remains a topic of ongoing research. In particular, it will be stimulating to understand the catalytic mechanism for BAM-mediated OMP folding. Regarding to chaperones it will be interesting to

know the folded state of the substrate OMP when bound to chaperones, the number of chaperones bound to a single OMP, whether there is a relationship between the dependencies on different chaperones and the different folding/insertion mechanisms facilitated by the BAM complex, how the interaction is between a chaperone-OMP complex and the BAM complex, and how uOMPs are moved from chaperones to the membrane via BAM, among other remaining questions related to the role of chaperones in the biogenesis pathway of OMPs.

In order to understand the mechanism of OMP folding/insertion into the OM, an understanding how individual members of the complex functionally interact with incoming substrates and how these interactions contribute to conformational changes in both substrate and BAM complex will be required. Regarding to the function of the BAM complex, it will be interesting to know if this complex actively folds OMPs or just facilitates insertion by local destabilization of the OM or if it is participating in both folding and insertion. Structural studies have also revealed unprecedented conformational changes in BamA into the BAM complex (Fig. 1c), which may play a significant role on BAM function for mediating folding/insertion of uOMPs into the OM. However, more studies are required to determine exactly how these unprecedented conformational changes are regulated. Additionally, the exact role that these conformational changes may play is unknown. They might help to usher uOMPs into the membrane or prime the local membrane for uOMP insertion.

Future work must include experimental tools that integrate information from multi-scale nature studies that combine many experimental techniques to reveal the intricate mechanisms of these highly complex molecular machines for functioning in OMP biogenesis. Thereby, structural biology or biochemistry alone cannot reveal all of the dynamic changes the BAM complex undergoes during the OMP assembly. For instance, structural studies of the BAM complex in the absence of the substrate and away from its native environment require complementary experiments such as isolation and characterization of assembly intermediates. Thus, structural data on kinetically stable intermediates will provide a holistic understanding of the various dynamic events during the assembly reaction. These multi-scale studies will allow to understand the features of these processes and permit the design of molecules that target specific events of the BAM pathway, with potential therapeutic benefits.

Acknowledgment This work was supported by a Consejo Nacional de Ciencia y Tecnología (Conacyt) Grant (221130). I thank Paul Ugalde-Silva for the artistic work in Fig. 1 and Lucia Chavez-Dueñas for compiling the references with EndNote software.

References

Albrecht R, Schutz M, Oberhettinger P, Faulstich M, Bermejo I, Rudel T, Diederichs K, Zeth K (2014) Structure of BamA, an essential factor in outer membrane protein biogenesis. Acta Crystallogr D Biol Crystallogr 70(Pt 6):1779–1789. https://doi.org/10.1107/S1399004714007 482

Bakelar J, Buchanan SK, Noinaj N (2016) The structure of the beta-barrel assembly machinery complex. Science 351(6269):180–186. https://doi.org/10.1126/science.aad3460

Bergal HT, Hopkins AH, Metzner SI, Sousa MC (2016) The structure of a BamA-BamD fusion illuminates the architecture of the beta-barrel assembly machine core. Structure 24(2):243–251. https://doi.org/10.1016/j.str.2015.10.030

Beveridge TJ (1999) Structures of gram-negative cell walls and their derived membrane vesicles. J Bacteriol 181(16):4725–4733

Bitto E, McKay DB (2003) The periplasmic molecular chaperone protein SurA binds a peptide motif that is characteristic of integral outer membrane proteins. J Biol Chem 278(49): 49316–49322. https://doi.org/10.1074/jbc.M308853200

Bos MP, Robert V, Tommassen J (2007) Biogenesis of the gram-negative bacterial outer membrane. Annu Rev Microbiol 61:191–214. https://doi.org/10.1146/annurev.micro.61.080706.093245

Botos I, Noinaj N, Buchanan SK (2017) Insertion of proteins and lipopolysaccharide into the bacterial outer membrane. Philos Trans R Soc Lond Ser B Biol Sci 372(1726). https://doi.org/10.1098/rstb.2016.0224

Charlson ES, Werner JN, Misra R (2006) Differential effects of yfgL mutation on *Escherichia coli* outer membrane proteins and lipopolysaccharide. J Bacteriol 188(20):7186–7194. https://doi.org/10.1128/JB.00571-06

Costello SM, Plummer AM, Fleming PJ, Fleming KG (2016) Dynamic periplasmic chaperone reservoir facilitates biogenesis of outer membrane proteins. Proc Natl Acad Sci USA 113(33): E4794–E4800. https://doi.org/10.1073/pnas.1601002113

D'Andrea LD, Regan L (2003) TPR proteins: the versatile helix. Trends Biochem Sci 28(12): 655–662. https://doi.org/10.1016/j.tibs.2003.10.007

Danoff EJ, Fleming KG (2015) Membrane defects accelerate outer membrane beta-barrel protein folding. Biochemistry 54(2):97–99. https://doi.org/10.1021/bi501443p

De Geyter J, Tsirigotaki A, Orfanoudaki G, Zorzini V, Economou A, Karamanou S (2016) Protein folding in the cell envelope of *Escherichia coli*. Nat Microbiol 1(8):16107. https://doi.org/10.1038/nmicrobiol.2016.107

Dong C, Hou HF, Yang X, Shen YQ, Dong YH (2012) Structure of *Escherichia coli* BamD and its functional implications in outer membrane protein assembly. Acta Crystallogr D Biol Crystallogr 68(Pt 2):95–101. https://doi.org/10.1107/S0907444911051031

Driessen AJ, Nouwen N (2008) Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem 77:643–667. https://doi.org/10.1146/annurev.biochem.77.061606.160747

Gatzeva-Topalova PZ, Walton TA, Sousa MC (2008) Crystal structure of YaeT: conformational flexibility and substrate recognition. Structure 16(12):1873–1881. https://doi.org/10.1016/j.str.2008.09.014

Gatzeva-Topalova PZ, Warner LR, Pardi A, Sousa MC (2010) Structure and flexibility of the complete periplasmic domain of BamA: the protein insertion machine of the outer membrane. Structure 18(11):1492–1501. https://doi.org/10.1016/j.str.2010.08.012

Ge X, Wang R, Ma J, Liu Y, Ezemaduka AN, Chen PR, Fu X, Chang Z (2014) DegP primarily functions as a protease for the biogenesis of beta-barrel outer membrane proteins in the Gram-negative bacterium *Escherichia coli*. FEBS J 281(4):1226–1240. https://doi.org/10.1111/febs.12701

Gessmann D, Chung YH, Danoff EJ, Plummer AM, Sandlin CW, Zaccai NR, Fleming KG (2014) Outer membrane beta-barrel protein folding is physically controlled by periplasmic lipid head groups and BamA. Proc Natl Acad Sci USA 111(16):5878–5883. https://doi.org/10.1073/pnas.1322473111

Grabowicz M, Koren D, Silhavy TJ (2016) The CpxQ sRNA negatively regulates Skp to prevent mistargeting of beta-barrel outer membrane proteins into the cytoplasmic membrane. MBio 7(2):e00312–e00316. https://doi.org/10.1128/mBio.00312-16

Gu Y, Li H, Dong H, Zeng Y, Zhang Z, Paterson NG, Stansfeld PJ, Wang Z, Zhang Y, Wang W, Dong C (2016) Structural basis of outer membrane protein insertion by the BAM complex. Nature 531(7592):64–69. https://doi.org/10.1038/nature17199

Hagan CL, Kim S, Kahne D (2010) Reconstitution of outer membrane protein assembly from purified components. Science 328(5980):890–892. https://doi.org/10.1126/science.1188919

Hagan CL, Silhavy TJ, Kahne D (2011) Beta-barrel membrane protein assembly by the Bam complex. Annu Rev Biochem 80:189–210. https://doi.org/10.1146/annurev-biochem-061408-144611

Hagan CL, Westwood DB, Kahne D (2013) Bam lipoproteins assemble BamA in vitro. Biochemistry 52(35):6108–6113. https://doi.org/10.1021/bi400865z

Hagan CL, Wzorek JS, Kahne D (2015) Inhibition of the beta-barrel assembly machine by a peptide that binds BamD. Proc Natl Acad Sci USA 112(7):2011–2016. https://doi.org/10.1073/pnas.1415955112

Harms N, Koningstein G, Dontje W, Muller M, Oudega B, Luirink J, de Cock H (2001) The early interaction of the outer membrane protein phoe with the periplasmic chaperone Skp occurs at the cytoplasmic membrane. J Biol Chem 276(22):18804–18811. https://doi.org/10.1074/jbc.M011194200.M011194200

Heuck A, Schleiffer A, Clausen T (2011) Augmenting beta-augmentation: structural basis of how BamB binds BamA and may support folding of outer membrane proteins. J Mol Biol 406(5):659–666. https://doi.org/10.1016/j.jmb.2011.01.002

Hu K, Galius V, Pervushin K (2006) Structural plasticity of peptidyl-prolyl isomerase sFkpA is a key to its chaperone function as revealed by solution NMR. Biochemistry 45(39):11983–11991. https://doi.org/10.1021/bi0607913

Jansen KB, Baker SL, Sousa MC (2015) Crystal structure of BamB bound to a periplasmic domain fragment of BamA, the central component of the beta-barrel assembly machine. J Biol Chem 290(4):2126–2136. https://doi.org/10.1074/jbc.M114.584524

Kim S, Malinverni JC, Sliz P, Silhavy TJ, Harrison SC, Kahne D (2007) Structure and function of an essential component of the outer membrane protein assembly machine. Science 317(5840):961–964. https://doi.org/10.1126/science.1143993

Kim KH, Aulakh S, Tan W, Paetzel M (2011) Crystallographic analysis of the C-terminal domain of the *Escherichia coli* lipoprotein BamC. Acta Crystallogr Sect F Struct Biol Cryst Commun 67(Pt 11):1350–1358. https://doi.org/10.1107/S174430911103363X

Kim KH, Aulakh S, Paetzel M (2012) The bacterial outer membrane beta-barrel assembly machinery. Protein Sci Publ Protein Soc 21(6):751–768. https://doi.org/10.1002/pro.2069

Knowles TJ, McClelland DM, Rajesh S, Henderson IR, Overduin M (2009) Secondary structure and (1)H, (13)C and (15)N backbone resonance assignments of BamC, a component of the outer membrane protein assembly machinery in *Escherichia coli*. Biomol NMR Assign 3(2):203–206

Knowles TJ, Browning DF, Jeeves M, Maderbocus R, Rajesh S, Sridhar P, Manoli E, Emery D, Sommer U, Spencer A, Leyton DL, Squire D, Chaudhuri RR, Viant MR, Cunningham AF, Henderson IR, Overduin M (2011) Structure and function of BamE within the outer membrane and the beta-barrel assembly machine. EMBO Rep 12(2):123–128. https://doi.org/10.1038/embor.2010.202

Konovalova A, Kahne DE, Silhavy TJ (2017) Outer membrane biogenesis. Annu Rev Microbiol 71:539–556. https://doi.org/10.1146/annurev-micro-090816-093754

Krojer T, Sawa J, Schafer E, Saibil HR, Ehrmann M, Clausen T (2008) Structural basis for the regulated protease and chaperone function of DegP. Nature 453(7197):885–890. https://doi.org/10.1038/nature07004

Lee J, Xue M, Wzorek JS, Wu T, Grabowicz M, Gronenberg LS, Sutterlin HA, Davis RM, Ruiz N, Silhavy TJ, Kahne DE (2016) Characterization of a stalled complex on the beta-barrel assembly machine. Proc Natl Acad Sci USA 113(31):8717–8722. https://doi.org/10.1073/pnas.1604100113

Matias VR, Al-Amoudi A, Dubochet J, Beveridge TJ (2003) Cryo-transmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and *Pseudomonas aeruginosa*. J Bacteriol 185(20):6112–6118

McMorran LM, Bartlett AI, Huysmans GH, Radford SE, Brockwell DJ (2013) Dissecting the effects of periplasmic chaperones on the in vitro folding of the outer membrane protein PagP. J Mol Biol 425(17):3178–3191. https://doi.org/10.1016/j.jmb.2013.06.017

Moon CP, Zaccai NR, Fleming PJ, Gessmann D, Fleming KG (2013) Membrane protein thermodynamic stability may serve as the energy sink for sorting in the periplasm. Proc Natl Acad Sci USA 110(11):4285–4290. https://doi.org/10.1073/pnas.1212527110

Murakami Y, Imai M, Nakamura H, Yoshimura F (2002) Separation of the outer membrane and identification of major outer membrane proteins from *Porphyromonas gingivalis*. Eur J Oral Sci 110(2):157–162

Ni D, Wang Y, Yang X, Zhou H, Hou X, Cao B, Lu Z, Zhao X, Yang K, Huang Y (2014) Structural and functional analysis of the beta-barrel domain of BamA from *Escherichia coli*. FASEB J 28(6):2677–2685. https://doi.org/10.1096/fj.13-248450

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev MMBR 67(4):593–656

Noinaj N, Fairman JW, Buchanan SK (2011) The crystal structure of BamB suggests interactions with BamA and its role within the BAM complex. J Mol Biol 407(2):248–260. https://doi.org/10.1016/j.jmb.2011.01.042

Noinaj N, Kuszak AJ, Gumbart JC, Lukacik P, Chang H, Easley NC, Lithgow T, Buchanan SK (2013) Structural insight into the biogenesis of beta-barrel membrane proteins. Nature 501(7467):385–390. https://doi.org/10.1038/nature12521

Noinaj N, Kuszak AJ, Balusek C, Gumbart JC, Buchanan SK (2014) Lateral opening and exit pore formation are required for BamA function. Structure 22(7):1055–1062. https://doi.org/10.1016/j.str.2014.05.008

Noinaj N, Gumbart JC, Buchanan SK (2017) The beta-barrel assembly machinery in motion. Nat Rev Microbiol 15(4):197–204. https://doi.org/10.1038/nrmicro.2016.191

O'Neil PK, Rollauer SE, Noinaj N, Buchanan SK (2015) Fitting the pieces of the beta-barrel assembly machinery complex. Biochemistry 54(41):6303–6311. https://doi.org/10.1021/acs.biochem.5b00852

Paschen SA, Waizenegger T, Stan T, Preuss M, Cyrklaff M, Hell K, Rapaport D, Neupert W (2003) Evolutionary conservation of biogenesis of beta-barrel membrane proteins. Nature 426(6968):862–866. https://doi.org/10.1038/nature02208

Patel GJ, Behrens-Kneip S, Holst O, Kleinschmidt JH (2009) The periplasmic chaperone Skp facilitates targeting, insertion, and folding of OmpA into lipid membranes with a negative membrane surface potential. Biochemistry 48(43):10235–10245. https://doi.org/10.1021/bi901403c

Plummer AM, Fleming KG (2016) From chaperones to the membrane with a BAM! Trends Biochem Sci 41(10):872–882. https://doi.org/10.1016/j.tibs.2016.06.005

Ricci DP, Hagan CL, Kahne D, Silhavy TJ (2012) Activation of the *Escherichia coli* beta-barrel assembly machine (Bam) is required for essential components to interact properly with substrate. Proc Natl Acad Sci USA 109(9):3487–3491. https://doi.org/10.1073/pnas.1201362109

Rigel NW, Schwalm J, Ricci DP, Silhavy TJ (2012) BamE modulates the *Escherichia coli* beta-barrel assembly machine component BamA. J Bacteriol 194(5):1002–1008. https://doi.org/10.1128/JB.06426-11

Rigel NW, Ricci DP, Silhavy TJ (2013) Conformation-specific labeling of BamA and suppressor analysis suggest a cyclic mechanism for beta-barrel assembly in *Escherichia coli*. Proc Natl Acad Sci USA 110(13):5151–5156. https://doi.org/10.1073/pnas.1302662110

Rizzitello AE, Harper JR, Silhavy TJ (2001) Genetic evidence for parallel pathways of chaperone activity in the periplasm of *Escherichia coli*. J Bacteriol 183(23):6794–6800

Rollauer SE, Sooreshjani MA, Noinaj N, Buchanan SK (2015) Outer membrane protein biogenesis in Gram-negative bacteria. Philos Trans R Soc Lond Ser B Biol Sci 370(1679). https://doi.org/10.1098/rstb.2015.0023

Roman-Hernandez G, Peterson JH, Bernstein HD (2014) Reconstitution of bacterial autotransporter assembly using purified components. eLife 3:e04234. https://doi.org/10.7554/eLife.04234

Sandoval CM, Baker SL, Jansen K, Metzner SI, Sousa MC (2011) Crystal structure of BamD: an essential component of the beta-barrel assembly machinery of gram-negative bacteria. J Mol Biol 409(3):348–357. https://doi.org/10.1016/j.jmb.2011.03.035

Saul FA, Arie JP, Vulliez-le Normand B, Kahn R, Betton JM, Bentley GA (2004) Structural and functional studies of FkpA from *Escherichia coli,* a cis/trans peptidyl-prolyl isomerase with chaperone activity. J Mol Biol 335(2):595–608

Schiffrin B, Calabrese AN, Higgins AJ, Humes JR, Ashcroft AE, Kalli AC, Brockwell DJ, Radford SE (2017) Effects of periplasmic chaperones and membrane thickness on BamA-catalyzed outer-membrane protein folding. J Mol Biol 429(23):3776–3792. https://doi.org/10.1016/j.jmb.2017.09.008

Sinnige T, Weingarth M, Renault M, Baker L, Tommassen J, Baldus M (2014) Solid-state NMR studies of full-length BamA in lipid bilayers suggest limited overall POTRA mobility. J Mol Biol 426(9):2009–2021. https://doi.org/10.1016/j.jmb.2014.02.007

Sklar JG, Wu T, Kahne D, Silhavy TJ (2007) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. Genes Dev 21(19):2473–2484. https://doi.org/10.1101/gad.1581007

Soltes GR, Schwalm J, Ricci DP, Silhavy TJ (2016) The activity of *Escherichia coli* chaperone SurA is regulated by conformational changes involving a parvulin domain. J Bacteriol 198(6):921–929. https://doi.org/10.1128/JB.00889-15

Thoma J, Burmann BM, Hiller S, Muller DJ (2015) Impact of holdase chaperones Skp and SurA on the folding of beta-barrel outer-membrane proteins. Nat Struct Mol Biol 22(10):795–802. https://doi.org/10.1038/nsmb.3087

Vogel J, Papenfort K (2006) Small non-coding RNAs and the bacterial outer membrane. Curr Opin Microbiol 9(6):605–611. https://doi.org/10.1016/j.mib.2006.10.006

Voulhoux R, Bos MP, Geurtsen J, Mols M, Tommassen J (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. Science 299(5604):262–265. https://doi.org/10.1126/science.1078973

Vuong P, Bennion D, Mantei J, Frost D, Misra R (2008) Analysis of YfgL and YaeT interactions through bioinformatics, mutagenesis, and biochemistry. J Bacteriol 190(5):1507–1517. https://doi.org/10.1128/JB.01477-07

Walther DM, Rapaport D, Tommassen J (2009) Biogenesis of beta-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. Cellular Mol Life Sci CMLS 66(17):2789–2804. https://doi.org/10.1007/s00018-009-0029-z

Walton TA, Sousa MC (2004) Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. Mol Cell 15(3):367–374. https://doi.org/10.1016/j.molcel.2004.07.023

Ward R, Zoltner M, Beer L, El Mkami H, Henderson IR, Palmer T, Norman DG (2009) The orientation of a tandem POTRA domain pair, of the beta-barrel assembly protein BamA, determined by PELDOR spectroscopy. Structure 17(9):1187–1194. https://doi.org/10.1016/j.str.2009.07.011

Webb CT, Selkrig J, Perry AJ, Noinaj N, Buchanan SK, Lithgow T (2012) Dynamic association of BAM complex modules includes surface exposure of the lipoprotein BamC. J Mol Biol 422(4):545–555. https://doi.org/10.1016/j.jmb.2012.05.035

Wu B, Li P, Liu Y, Lou Z, Ding Y, Shu C, Ye S, Bartlam M, Shen B, Rao Z (2004) 3D structure of human FK506-binding protein 52: implications for the assembly of the glucocorticoid receptor/Hsp90/immunophilin heterocomplex. Proc Natl Acad Sci USA 101(22):8348–8353. https://doi.org/10.1073/pnas.0305969101

Wu T, Malinverni J, Ruiz N, Kim S, Silhavy TJ, Kahne D (2005) Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. Cell 121(2):235–245. https://doi.org/10.1016/j.cell.2005.02.015

Wu T, McCandlish AC, Gronenberg LS, Chng SS, Silhavy TJ, Kahne D (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. Proc Natl Acad Sci USA 103(31):11754–11759. https://doi.org/10.1073/pnas.0604744103

Zaccai NR, Sandlin CW, Hoopes JT, Curtis JE, Fleming PJ, Fleming KG, Krueger S (2016) Deuterium labeling together with contrast variation small-angle neutron scattering suggests how Skp captures and releases unfolded outer membrane proteins. Methods Enzymol 566:159–210. https://doi.org/10.1016/bs.mie.2015.06.041

Structure: Function of Transmembrane Appendages in Gram-Negative Bacteria

36

Miguel Ángel Díaz-Guerrero, Meztlli O. Gaytán, and Bertha González-Pedrajo

Contents

1 Introduction 672
2 Transmembrane Appendages 672
2.1 Secretion Systems 673
2.2 Flagella, Pili, and Other Fimbrial Adhesins 675
3 Building Blocks for the Construction of Transmembrane Appendages 676
3.1 T1SS 677
3.2 T2SS 677
3.3 T3SS 678
3.4 T4SS 679
3.5 T5SS 680
3.6 T6SS 681
3.7 Surface Appendages for Motility and Adhesion 681
4 Influence of Lipids on the Structure and Function of Bacterial Appendages 683
5 Research Needs 685
References 686

Abstract

Gram-negative bacteria possess diverse transmembrane appendages that allow them to colonize different ecological niches. An essential prokaryotic strategy used for adaptation to various environments is the secretion of several molecules such as proteins and DNA, which is accomplished by specialized secretion systems that enable substrate transport across the cell envelope.

Miguel Ángel Díaz-Guerrero and Meztlli O. Gaytán have contributed equally to this work.

M. Á. Díaz-Guerrero · M. O. Gaytán · B. González-Pedrajo (✉)
Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico
e-mail: bpedrajo@ifc.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_51

Moreover, bacterial motility and adherence, driven by filamentous surface structures as flagella, pili, and curli, also play a crucial role in bacterial colonization. The construction of these macromolecular complexes faces a physical barrier since they must traverse two distinct lipid membrane bilayers and a peptidoglycan cell wall. Therefore, proteins that display special physicochemical characteristics to assemble within the hydrophobic lipid environment serve as building blocks for the biogenesis of the different transmembrane appendages. Here, we review the architecture and function of the main appendages of diderm bacteria and discuss how the interplay between membrane lipids and proteins influences their assembly and activity.

1 Introduction

Gram-negative bacteria are diderm microorganisms enclosed by two structurally and biochemically distinct lipid bilayers, separated by a periplasmic compartment containing the peptidoglycan cell wall (Fig. 1). The inner membrane (IM) is a phospholipid bilayer whose composition varies among different bacterial species. In *Escherichia coli* it is mainly composed of the amphiphilic lipids, phosphatidylethanolamine and phosphatidylglycerol, and small amounts of cardiolipin. The outer membrane (OM) is highly asymmetric and consists of two distinct layers; the outer leaflet is composed primarily of lipopolysaccharides (LPS) projecting outside and the inner leaflet is formed by glycerophospholipids. The differences between the IM and OM are also reflected in the structure of the integral membrane proteins assembled in each bilayer. While most of the IM proteins are formed by α-helical bundles, OM proteins (OMPs) are β-barrel proteins consisting of multiple β-sheets wrapped into cylinders, which assume a β-barrel conformation. In addition, lipoproteins can be anchored either to the periplasmic leaflet of the IM or to the OM through an N-terminal lipid moiety. These proteinaceous building blocks associate together within the hydrophobic lipid environment of the membranes to create different macromolecular complexes designed to carry out a varied set of cellular tasks, as will be outlined below (Silhavy et al. 2010; Dalbey et al. 2011; Sohlenkamp and Geiger 2016).

2 Transmembrane Appendages

Gram-negative bacteria assemble diverse transenvelope machines such as flagella, pili, curli, and secretion systems. These molecular devices serve critical functions as attachment and invasion, biofilm formation, cell motility, horizontal gene transfer, and transport of small molecules, proteins, or DNA (Dalbey and Kuhn 2012). Surface appendages in Gram-positive bacteria and archaea will not be covered in this review, but readers are referred to extensive reviews on these topics (Jarrell et al. 2013; Anne et al. 2017; Pansegrau and Bagnoli 2017).

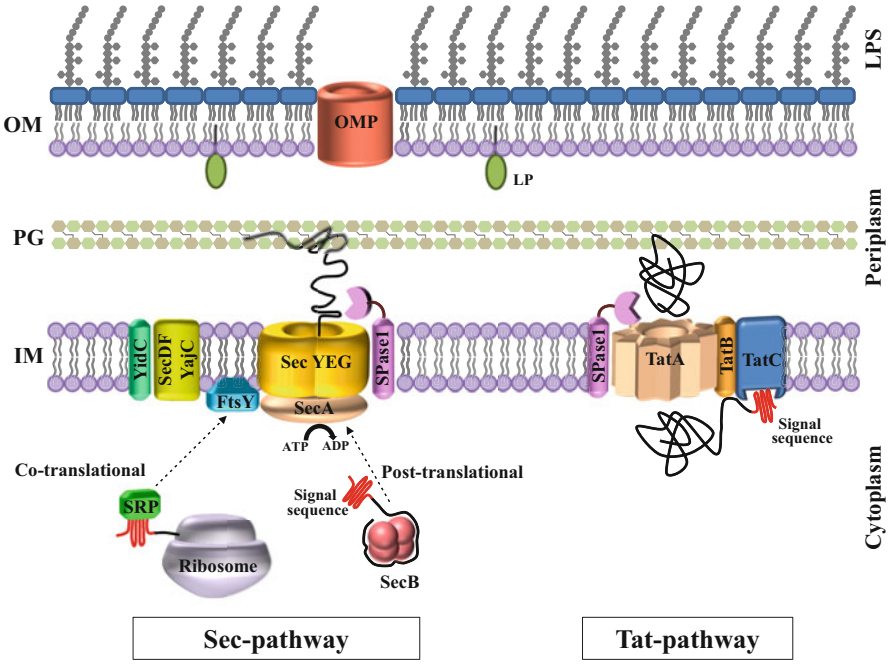


Fig. 1 Schematic diagram of the Gram-negative bacterial cell envelope showing the inner membrane Sec and Tat pathways. The outer and inner membranes (OM and IM) are separated by the periplasmic compartment that contains the peptidoglycan (PG) layer formed by crosslinked sugars and amino acids. The OM is an asymmetric bilayer of glycerophospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet, while the IM is a phospholipid bilayer. Lipoproteins (LP) are mostly found attached to the inner leaflet of the OM, anchored through an N-terminal lipid moiety. With few exceptions, OM proteins (OMP) are β-barrel proteins, while IM proteins are composed of α-helical bundles. Protein integration into or transport across the IM is accomplished by the Sec or Tat pathways. Sec-dependent preproteins are co-translationally targeted to the SecYEG translocon by the signal recognition particle (SRP) and its receptor FtsY or posttranslationally targeted to the SecYEG-SecA complex with the aid of the SecB chaperone (the chaperone-independent pathways are not depicted in the figure). The translocation process is energized by the ATPase SecA. SecDF-YajC and YidC are auxiliary components that increase translocation efficiency and protein insertion into the IM, respectively. Tat-dependent preproteins are targeted to the Tat machinery formed by TatA, TatB, and TatC. Folded proteins are secreted through the TatA pore. Finally, the signal peptidases 1 (SPase1) cleave the signal peptide, releasing the mature proteins

2.1 Secretion Systems

To date, six secretion systems have been extensively studied in Gram-negative bacteria, named type I to type VI secretion systems (abbreviated as TXSS) (Fig. 2). These macromolecular complexes are employed for the transport of different cytoplasmic substrates either to other cell compartments, the extracellular space, or into a target cell. Secreted proteins have multiple functions and play a crucial role in the interplay of bacteria with the environment, e.g., protein translocation into eukaryotic cells is

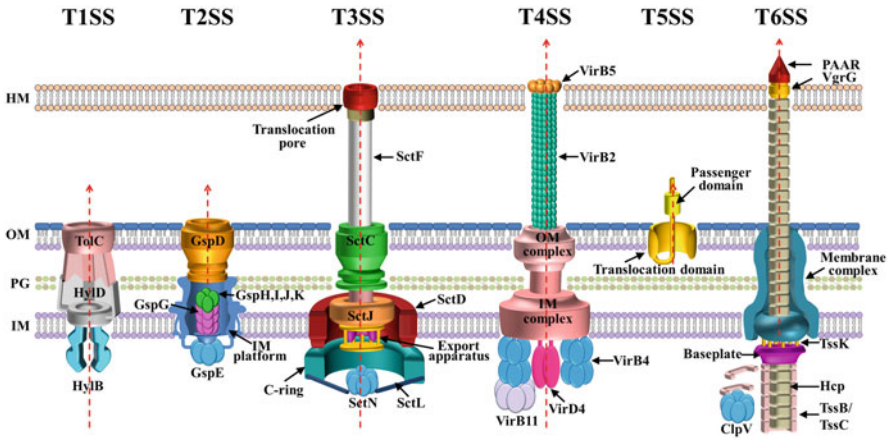


Fig. 2 Schematic models of major secretion systems in Gram-negative bacteria. The general architecture of the type I, II, III, IV, V, and VI secretion systems is depicted. These specialized nanomachines transport substrates across one (T2SS and T5SS), two (T1SS), or three (T3SS, T4SS, and T6SS) phospholipid membranes (substrate delivery pathways are represented by dashed red arrows). These systems comprise protein complexes assembled in the different cell compartments, the cytoplasm, the inner or outer membranes (IM and OM), the peptidoglycan layer (PG), or the host cell membrane (HM). The different components are described in the text

indispensable for the virulence of many bacterial pathogens. Secretion systems can be classified into two different categories: those performing the secretion process in a one-step mechanism (T1SS, T3SS, T4SS, and T6SS) and those whose substrates undergo a two-step secretion process in which they are first transported across the IM through the general secretion (Sec) or Twin-arginine translocation (Tat) pathways and then secreted (T2SS and T5SS) (Costa et al. 2015; Green and Mecsas 2016). The Sec pathway allows the insertion into the IM or translocation into the periplasm of unfolded proteins, in a co- or posttranslational manner (Fig. 1). In the co-translational pathway, the signal recognition particle binds to the N-terminal signal sequence of the nascent protein in complex with the ribosome and targets it to its IM receptor FtsY. In the posttranslational pathway, the preprotein, in complex with the SecB chaperone, is targeted to the SecYEG-SecA complex (Fig. 3, SecYEG-SecA). Preproteins can also be targeted in a chaperone-independent manner either alone or directly through SecA, which binds to the ribosome. Translocation across the IM is energized by the proton-motive force and the ATP hydrolysis of SecA. Protein insertion into the IM is carried out either by the SecYEG translocon alone or together with the YidC insertase (Fig. 3, YidC). The Tat pathway catalyzes the export of fully folded proteins. The signal peptide of Tat substrates contains a twin-arginine motif that is recognized by TatC in the membrane-embedded Tat machinery (TatA, TatB, and TatC). The TatB-TatC hetero-ligomer serves as the primary substrate binding site and is postulated to induce TatA oligomerization and pore formation. In the periplasm, the signal peptidase I (SPase I) cleaves the signal sequence of Sec and Tat substrates to release the mature proteins (Fig. 1) (Patel et al. 2014; Tsirigotaki et al. 2016).

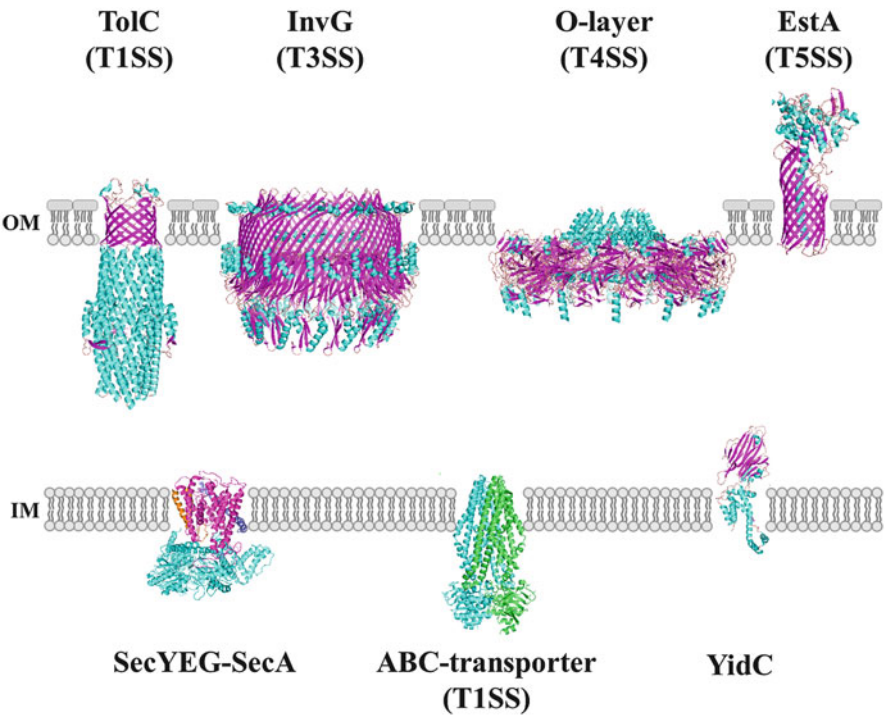


Fig. 3 Structures of membrane-embedded proteins that serve as building blocks for the construction of transmembrane appendages and for protein transport across the inner membrane (IM). Ribbon diagrams of the outer membrane (OM) proteins TolC (PDB: ITQQ), InvG (PDB: 5TCQ), EstA (PDB: 3KVN), as well as the O-layer (PDB: 3JQO) are displayed. α-Helices and β-strands are colored in cyan and magenta, respectively. Structural model of the translocase SecYEG (magenta, blue, and orange) in complex with the ATPase SecA in cyan (PDB: 3DIN). Architecture of an ABC-transporter homodimer (PDB: 5L22); subunits are colored in cyan and green, respectively. Structure of the IM protein YidC (PDB: 3WVF), α-helices and β-strands are colored in cyan and magenta, respectively

2.2 Flagella, Pili, and Other Fimbrial Adhesins

Polymerization of multiple protein subunits promotes the assembly of flagella, pili, and curli fibers (Fig. 4). Flagellar rotation, powered by the influx of protons or sodium ions, drives bacterial motility, allowing bacteria to swim through liquid environments or to swarm on solid surfaces. In addition, flagella are also required for adhesion and biofilm formation in certain bacterial species. Structurally, flagella are thicker and longer than pili, although they are less numerous. Pili are long (1–4 μm) flexible filaments composed of thousands of pilin subunits, essential for various bacterial processes such as adherence to both biotic and abiotic surfaces, auto-aggregation, biofilm formation, cellular invasion, bacteriophage infection, electron transfer, motility, horizontal gene transfer, and DNA uptake during natural

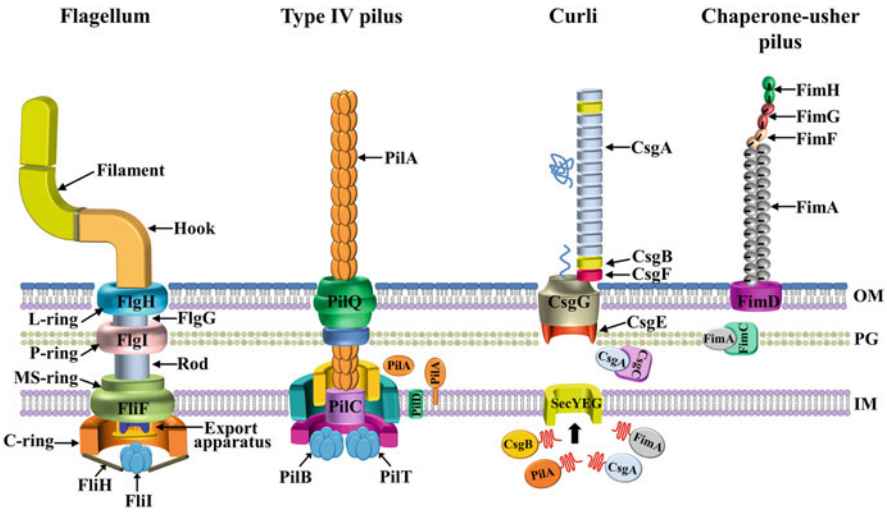


Fig. 4 Schematic representations of the flagellum and different types of pili in Gram-negative bacteria. The general architecture of the extracellular appendages used for motility and adhesion, the flagellum and pili (type IV pili (T4P)), curli, and type I pili (T1P) or chaperone-usher pili, is shown. The early steps in the biogenesis of all these structures involve a Sec-dependent pathway. Next, the flagellum is assembled by a T3SS and the export of subunits through a protein-conducting central channel in the structure. Curli and chaperone-usher pilins are recognized by chaperones in the periplasm and targeted to the outer membrane (OM) secretion machinery. Polymerization of T1P occurs at the OM usher FimD. In the curli pili, it is not known whether curli subunits are incorporated at the proximal or distal end of the fiber. In the T4P, prepilins are inserted into the IM by the Sec machinery, processed by the PilD peptidase, and mature pilins are extracted from the inner membrane (IM) and incorporated into the base of the growing pilus. Components are described in the text

transformation. Furthermore, bacteria belonging to the *Enterobacteriaceae* family assemble highly aggregative and flexible amyloid fibers of 4–7 nm named curli in *E. coli* or thin aggregative fimbriae (Tafi) in *Salmonella* spp. These recently identified adhesins are important for bacteria-bacteria and bacteria-host interactions and have been shown to be involved in biofilm formation and the colonization of inert surfaces such as teflon and stainless steel (Fronzes et al. 2008; Van Gerven et al. 2011; Hospenthal et al. 2017).

3 Building Blocks for the Construction of Transmembrane Appendages

The ordered association of numerous proteins leads to the construction of the bacterial appendages discussed above. Since these nanomachines traverse the cell envelope, specialized integral membrane proteins serve as essential building blocks for their assembly. In the next section we will describe the architecture and function

of the main appendages, emphasizing on the structure of integral membrane proteins and lipoproteins that help build these macromolecular complexes within the hydrophobic lipid bilayers.

3.1 T1SS

The type I secretion system is a tripartite complex composed of two IM components, an ATP-binding cassette (ABC) transporter and a membrane fusion protein (MFP) which functions as a periplasmic adaptor, and an OM protein (OMP) that forms a channel (Fig. 2, T1SS). Together, these pieces assemble a double-membrane spanning and transperiplasmic tunnel-like structure, through which unfolded proteins are secreted in a single ATP hydrolysis-dependent step (Green and Mecsas 2016). The proteins exported by the T1SS greatly vary in size (from the 20 kDa iron scavenger HasA from *Serratia marcescens* to the 900 kDa large adhesion protein LapA from *Pseudomonas fluorescens*) (Thomas et al. 2014) and in function (lipases, proteases, adhesins, toxins, etc.), some of which contribute to the virulence of many plant and animal bacterial pathogens. The prototypical T1SS is the hemolysin (HlyA) export machinery of uropathogenic *E. coli*, formed by HlyB (ABC transporter), HlyD (MFP), and TolC (OMP) (Holland et al. 2005). Most ABC transporters are homodimers, each monomer consisting of two transmembrane domains (TMDs), which form the translocation pathway for substrates across the IM, and two nucleotide-binding domains (Fig. 3, ABC transporter). However, HlyB monomers are formed by only one TMD composed of six predicted transmembrane segments and one ATP-binding domain. Substrates are recognized by this component through a C-terminal uncleavable signal sequence. The MFP HlyD is a trimeric protein predicted to possess an IM single spanning α-helical region and a large periplasmic domain that interacts with TolC. This component also associates with the ABC transporter and participates in substrate recognition. TolC is a homotrimer that forms a short OM β-barrel of 12 strands and a long α-helical domain (four α-helices), which protrudes into the periplasm (10 nm) to form a hydrophilic conduit that narrows toward its periplasmic end to prevent the nonselective passage of folded proteins (Fig. 3, TolC) (Koronakis et al. 2000). Upon substrate binding, HlyB and HlyD associate with TolC, triggering the opening of its α-helical barrel to allow substrate export (Thomas et al. 2014).

3.2 T2SS

The type II secretion system, or general secretory pathway ("Gsp" for a unified nomenclature), exports proteins that are first translocated across the IM by the Sec translocase or the Tat transporter and, in a second step, promotes transport of folded periplasmic proteins through the OM and into the extracellular milieu, by a piston-driven mechanism energized by ATP hydrolysis (Korotkov et al. 2012). A functional T2SS is present in many important human pathogens such as *Acinetobacter*

baumannii, *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Vibrio cholerae* (e.g., cholera toxin is secreted via the T2SS) but is also present in environmental bacteria, some of which promote symbiosis, among other processes. This molecular complex is composed of 12 to 15 distinct proteins that constitute 4 major substructures: an OM channel-forming protein called secretin, a periplasmic pseudopilus, an IM platform, and a homohexameric cytoplasmic ATPase (Fig. 2, T2SS). The OM secretin is a pentadecamer of the GspD protein that forms a gated pore. Each secretin subunit has a conserved membrane-embedded β-barrel C-terminal domain followed by a periplasmic S-domain involved in secretin localization and membrane insertion, and an N-terminal region with four subdomains (N0 to N3), which form a cylindrical structure in the periplasm (Yan et al. 2017; Filloux and Voulhoux 2018). Proteins that belong to the secretin family serve as common building blocks for the biogenesis of different bacterial appendages such as the T3SS and the type IV pilus. In some cases, the assembly of the secretin relies on the assistance of a small lipoprotein known as the pilotin (GspS), which binds to the S-domain. The short pseudopilus in the periplasm is composed of multiple copies of a major pseudopilin, GspG, and four minor pseudopilins (GspH, I, J, and K), and, in contrast to extracellular pilus, this structure remains in the periplasmic space. The IM platform subcomplex, which consists of one polytopic and three bitopic proteins, plays an important role in contacting the OM secretin, the pseudopilus, and is also thought to recruit the oligomeric ATPase (GspE). The latter interaction stimulates the enzymatic activity of GspE, thereby transmitting conformational changes to the IM platform and pseudopilus, which functions as a piston that extends to push secreted substrates through the secretin channel (Thomassin et al. 2017).

3.3 T3SS

The type III secretion system, or injectisome, is a 3.5 MDa complex composed of approximately 25 proteins that assemble a nanosyringe capable of traversing three membranes (Fig. 2, T3SS). This protein export machinery is used by symbiotic and pathogenic Gram-negative bacteria to translocate a myriad of effector proteins directly from the bacterial cytoplasm into a eukaryotic target cell, in an ATP- and proton-motive force-dependent manner (Deng et al. 2017). The injectisome is evolutionarily related to the bacterial flagellum and it has been suggested that it originated from an exaptation process, i.e., the recruitment of flagellar components for a new protein transport function. The T3SS can be divided into three substructures: (i) the extracellular appendages, which consist of a filamentous needle-like structure that serves as a scaffold for the assembly of a tip complex made up of a hydrophilic protein and a translocation pore formed by two hydrophobic proteins that insert into the host cell membrane. In some bacteria, such as enteropathogenic and enterohemorrhagic *E. coli*, instead of a small tip, a large filament is assembled on top of the needle; (ii) the basal body, which comprises three membrane rings connected through a periplasmic rod and which houses the export apparatus, an IM embedded protein complex that controls access of cytosolic proteins to the T3SS

inner channel, (iii) the cytoplasmic components, some of which associate with the IM, as the sorting platform (cytoplasmic C-ring) and the ATPase complex that help in the recruitment and classification of substrates, as well as in energizing the secretion process promoting chaperone-effector dissociation and initial substrate engagement. T3SS substrates are transported in a partially unfolded conformation through a continuous 2.5 nm narrow conduit that extends from the basal body and needle to the host cell. The translocated effectors subvert specific host cell processes to enable bacterial colonization (Gaytan et al. 2016; Deng et al. 2017; Galan and Waksman 2018).

The membrane-embedded protein rings in the basal body are composed of 15 subunits of the OM secretin SctC (homologous to the T2SS-associated secretin) and 24 subunits each of the SctD and SctJ proteins in the IM. The structure of the OM ring, defined at 3.6 Å resolution by single-particle cryoelectron microscopy, showed that it forms a double-layered β-barrel (Fig. 3, InvG) (Worrall et al. 2016). The IM-concentric rings house the export apparatus, formed by the association of five polytopic proteins in the following stoichiometry, 5 SctR, 1 SctS, 1 SctT, 1 SctU, and 9 SctV, which could give a total of 104 TM segments in a membrane patch at the base of the injectisome (Zilkenat et al. 2016). These proteins are highly conserved among different T3SSs (including the flagellar T3SS) and they are essential for protein secretion, although little is known about the structure of their membrane-spanning domains.

3.4 T4SS

The type IV secretion system is an envelope-spanning versatile nanomachine capable of delivering DNA, proteins, or nucleo-protein complexes to the extracellular milieu or into a wide range of target cells (bacterial and eukaryotic cells). The evolutionary origin of this system is the ancestral bacterial conjugation machinery. There are three functional types of T4SSs: one used for transferring DNA through conjugation, which is the main mechanism of antibiotic resistance gene dissemination, one that mediates DNA uptake (transformation) and release, and one engaged in effector protein translocation into target cells. Protein-exporting T4SSs are usually employed by human pathogenic bacteria such as *Helicobacter pylori*, which delivers one effector protein, and *Legionella pneumophila*, which injects over 300 effectors into its eukaryotic target cell. In addition, it has been reported that *Xanthomonas citri* employs its T4SS to kill other Gram-negative bacterial species (Galan and Waksman 2018; Grohmann et al. 2018).

Agrobacterium tumefaciens transfers oncogenic DNA into plant cells via a conjugative T4SS and has been the prototypical bacterium for studying this macromolecular complex, which is composed of 12 proteins named VirB1 to VirB11 and VirD4. The architecture of this secretory system consists of an OM core complex connected by a narrow stalk to an IM complex (Fig. 2, T4SS) (Low et al. 2014). The OM complex is a ring structure formed by 14 subunits each of the proteins VirB7, VirB9, and VirB10. This complex spans both bacterial membranes and is in turn

subdivided into an outer (O-layer) and an inner layer (I-layer), inserting in the OM and IM, respectively (Rivera-Calzada et al. 2013). The O-layer is formed by the protein VirB7 and the C-terminal domains of VirB9 and VirB10, the latter protein forms the inner side of the core complex while VirB7 and VirB9 bind around VirB10. The 14 VirB10 subunits project a double-helical TM region that together form the OM channel. This helical barrel arrangement is infrequent in OM proteins (Fig. 3, O-layer) (Chandran et al. 2009). The I-layer is formed by the N-terminal domains of the VirB9 and VirB10 subunits (Costa et al. 2015). The IM complex consists of a large membrane-embedded platform composed of 12 copies each of VirB3, VirB5, VirB6, and VirB8 and two barrel structures protruding into the cytoplasm, each made up of six subunits of the VirB4 ATPase (Low et al. 2014). The composition of the stalk connecting the OM and IM complexes is still unknown (Galan and Waksman 2018). Furthermore, the pilus of the T4SS is composed of the major pilin subunit VirB2 and the minor pilin VirB5, which is localized at the tip of the pilus and is believed to function as an adhesion protein for cell contact. Protein translocation by the T4SS is powered by the ATP hydrolysis of three energizing ATPases, VirB4, VirB11, and VirD4 (Costa et al. 2015; Grohmann et al. 2018).

3.5 T5SS

The type V secretion system, also known as the autotransporter (AT) system, is one of the simplest pathways for protein secretion because the information required for transport through the OM is present in the secreted substrate polypeptide (or two polypeptides in the two-partner secretion system). Classical ATs consist of an N-terminal signal sequence for export to the periplasm by the Sec apparatus, followed by an N-terminal (secreted or extracellularly exposed) passenger domain and a C-terminal translocation domain that inserts into the OM as a β-barrel, to allow secretion of the passenger domain through the barrel pore (Fig. 2, T5SS). The β-barrel is formed by 12 antiparallel β-strands and its interior is occupied by a linker region that connects it to the passenger domain (Fig. 3, EstA). The energy for transport has been postulated to originate from the passenger domain folding when exiting the pore (Dalbey and Kuhn 2012; Costa et al. 2015; Fan et al. 2016). After transport across the IM, the AT interacts with periplasmic chaperones that prevent its folding during the targeting to the OM β-barrel assembly machinery or Bam complex (formed by the OM protein BamA and four lipoproteins BamB, BamC, BamD, and BamE), which promotes membrane insertion of β-barrel proteins. There is increasing evidence indicating that the Bam complex and, for some ATs, the translocation and assembly module (TAM) complex play a direct role in facilitating passenger domain secretion, and so the autonomous secretion of the AT system has been recently re-evaluated. It has been reported that BamA actively participates in AT secretion through its interaction with the passenger domain (Albenne and Ieva 2017). In many cases, after translocation, the passenger domain is cleaved by an autocatalytic process and released into the extracellular space. In contrast,

non-processed ATs remain associated to the cell envelope via the translocation domain (Fan et al. 2016).

3.6 T6SS

The type VI secretion system is the most recently characterized macromolecular secretion machine, which translocates effector proteins into both prokaryotic and eukaryotic cells in a single step process. This system injects a wide variety of effectors including nucleases, lipases, glycoside hydrolases, amidases, and esterases that are crucial for pathogenicity toward animal and plant hosts as well as to target competitor bacteria (Cianfanelli et al. 2016; Galan and Waksman 2018). The T6SS is formed by 13 essential core components (TssA to TssM), some of which are homologous to the contractile bacteriophage tails. The overall architecture consists of three main structures: a membrane complex, a cytoplasmic baseplate, and a sheathed inner tube (or effector delivery module) (Fig. 2, T6SS). The membrane complex is composed of 10 heterotrimers containing the lipoprotein TssJ, anchored to the OM, and the IM proteins TssM and TssL. This complex is used as a docking station for assembly of the cytoplasmic components (or tail complex), formed by the baseplate, the inner tube, and the tail sheath. The baseplate-like structure, composed of TssA, TssE, TssF, TssG, TssK, and VgrG proteins, associates with the membrane complex and serves as a platform for tube and sheath polymerization. The extended inner tube (1000 nm) is formed by stacked hexamers of the Hcp protein enclosed by a contractile sheath formed by two proteins, TssB and TssC. VgrG is a trimeric protein localized at the membrane end of the Hcp tube forming the tail spike, which is further sharpened into a conical tip by a PAAR repeat-containing protein that binds to the VgrG trimer (Costa et al. 2015; Alteri and Mobley 2016; Gallique et al. 2017; Galan and Waksman 2018). This bacterial weapon delivers effectors using a cell-puncturing mechanism that resembles an inverted phage contractile tail, where, during contraction of the sheath, the inner tube, which is loaded with effectors, VgrG and PAAR, is ejected toward the target cell (Gallique et al. 2017). Finally, the T6SS is disassembled by the cytoplasmic ClpV ATPase, completing the assembly, contraction, and depolymerization cycle necessary for sheath recycling during the injection process (Cianfanelli et al. 2016).

3.7 Surface Appendages for Motility and Adhesion

Many of the building blocks required for the assembly of flagella and pili are homologous to components of the secretion systems. The bacterial flagellum is a rotating organelle used for motility that is assembled through a T3SS (Fig. 4) (Minamino 2014). Although the flagellum and the injectisome differ in overall structure and function, they have a conserved T3S machinery for protein export with several core components that are highly similar between these macromolecular complexes. These proteins are localized in the flagellar basal body, forming the

IM-MS ring (FliF) and the export apparatus (FliP, FliQ, FliR, FlhB, and FlhA) located in a patch of membrane within the MS ring, and also as part of the cytosolic components, the ATPase complex (FliI, FliH, and FliJ) and the cytoplasmic C-ring (FliM and FliN). In contrast, the OM ring structures are different; while in the injectisome a secretin pore is formed, in the flagellum there are two rings, the P ring (FlgI) within the peptidoglycan layer and the L-ring formed by the lipoprotein FlgH in the OM. Once the flagellar core T3SS is assembled, the export of subunits forming the axial structures is initiated, first the periplasmic rod (FliE, FlgB, FlgC, FlgF, and FlgG), then the extracellular hook (FlgE) and hook-associated proteins (FlgK, FlgL, and FliD), and lastly the filament formed by the polymerization of thousands of flagellin monomers (FliC). Substrates are transported through a narrow channel of ~ 2.0 nm and the energy for protein export is provided by both the proton-motive force and ATP hydrolysis. The motor proteins MotA and MotB are assembled in the IM forming the stator complex which is the pathway of proton influx for flagellar rotation (Erhardt et al. 2010; Diepold and Armitage 2015).

Pili or fimbriae are mainly involved in bacterial adhesion to other bacteria, host tissues, and other surfaces, so these appendages are considered important virulence factors. The type IV pili (T4P) is a very versatile surface structure that participates in bacterial adherence, biofilm establishment, microcolonies formation, electron transfer, natural competence, and twitching motility. The T4P is evolutionarily related to the T2SS, where homologous proteins are named pseudopilins. However, in contrast to the periplasmic T2SS pseudopilus, the T4P is a surface-exposed long filament of 4 μm in length and 4–9 nm in diameter. Nonetheless, the assembly of these appendages is highly similar. Prepilins are inserted into the IM by the Sec translocase and then processed by the prepilin peptidase PilD, which cleaves the N-terminal signal sequence. Mature pilins (PilA and minor pilins) are extracted from the IM and assembled into the base of the T4P, which is then extended from an IM assembly platform into a secretin complex (PilQ) embedded in the OM (Fig. 4). The pore complex PilQ from *P. aeruginosa* is made of 14 subunits, whereas InvG (T3SS) and GspD (T2SS) are pentadecameric; however, the overall architecture of the T4P OM secretin is similar to the ones of type II and III secretion systems. The T4P is unique in their ability to extend and retract through the action of the cytoplasmic ATPases PilB and PilT (Dalbey and Kuhn 2012; Gold and Kudryashev 2016; Koo et al. 2016; Hospenthal et al. 2017; Filloux and Voulhoux 2018).

Chaperone-usher pili are present on the surface of many bacterial pathogens. Uropathogenic *E. coli* (UPEC) expresses type 1 and P pili that are crucial for bacterial ascension from the bladder to the upper urinary tract. The type 1 pilus (T1P) of UPEC is assembled through the chaperone-usher pathway and consists of two substructures: a thick helical rod of ~ 2 μm in length composed of the major pilus subunit FimA and a short flexible tip component at the distal end formed by the minor pilus subunits FimF, FimG, and FimH (Fig. 4). Pilin subunits are first transported across the IM through the Sec pathway, and once in the periplasm they are stabilized by the periplasmic chaperone FimC, the chaperone-pilin complex is then recruited to the OM usher FimD, a β-barrel pore that mediates pilus assembly. The pilus subunits possess an incomplete immunoglobulin domain with six β-strands

(instead of seven) which makes the proteins unstable; therefore, the assembly process involves a donation of a β-strand by the chaperone FimC (donor strand complementation) or by an N-terminal extension of the incoming pilus subunit during polymerization (donor strand exchange). Pilin subunits polymerize at the usher pore although it is still unknown what energy source powers the translocation process (Dalbey and Kuhn 2012; Costa et al. 2015; Hospenthal et al. 2017).

Curli is another type of fimbrial adhesin present in enteric bacteria, composed of highly aggregative proteinaceous fibers. These functional amyloid fibers mediate adhesion to human matrix proteins as fibronectin and laminin and also interaction with the host immune system having important roles in pathogenicity and biofilm formation. Similarly to pilins in T1P and T4P, curli subunits are transported through the IM by the Sec pathway. Curli is formed by the major (CsgA) and the minor (CsgB) pilin subunits. In the periplasm these subunits interact with the CsgC chaperone to avoid premature amyloid fiber formation. The OM curli subunit secretion machinery comprises three proteins: the lipoprotein CsgG, which spans the OM and forms a nonameric ring structure of 36 β-barrel strands with an inner diameter of 2 to 4 nm; CsgE, an accessory factor which binds CsgG and functions as the OM pore gate, serving also to target curli subunits to the CsgG channel for secretion; and the extracellular protein CsgF, which interacts with CsgG and stabilizes and localizes the minor curli subunit CsgB (Fig. 4). CsgA is translocated through CsgG in an unfolded manner while CsgB functions as the nucleation point for CsgA homopolymer formation in a mechanism known as nucleation-precipitation (Cao et al. 2014; Evans and Chapman 2014; Costa et al. 2015; Van Gerven et al. 2015).

4 Influence of Lipids on the Structure and Function of Bacterial Appendages

Given that bacterial appendages are assembled within the hydrophobic environment of lipidic membranes, it is not surprising that membrane lipids influence their structure and function. Some examples are described below.

In general, protein secretion is an energy-dependent process in which ATP hydrolysis by specific ATPases is coupled to protein secretion. Therefore, bacteria have developed mechanisms to regulate ATPase activity, to ensure that it occurs at the appropriate time and in the right place. For instance, in the Sec pathway, the ATP hydrolytic activity of SecA, and, in turn, protein translocation, is stimulated by the presence of the acidic phospholipids phosphatidylglycerol (PG) and cardiolipin (CL). These anionic phospholipids are not only essential for SecA ATPase activity but also promote its association with the membrane. It has been shown that lipid clusters enriched with acidic phospholipids, via their negatively charged surface, increase the amount of SecA bound to lipid bilayers. Moreover, CL stabilizes the formation of SecYEG dimers, creating a binding surface for SecA and increasing its ATP turnover (Lill et al. 1990; Breukink et al. 1992; Ulbrandt et al. 1992; Ahn and Kim 1998; Gold et al. 2010). A similar phospholipid activation effect has been

observed on the activity of the so-called secretion ATPases of the type II, III, IV, and VI secretion systems (Krause et al. 2000). The enzymatic activity of EpsE, the T2SS-associated ATPase of *V. cholerae*, is synergistically activated by the phospholipids PG and CL together with the cytoplasmic domain of EpsL, which is an assembly platform component that promotes the association of EpsE to the IM. In contrast, the zwitterionic phospholipid phosphatidylethanolamine (PE) had no effect on the ATPase activity of the EpsE/EpsL complex. The stimulation of ATPase activity occurs through a direct binding of acidic phospholipids to the EpsE/EpsL complex. Thus, it is proposed that the cytoplasmic domain of EpsL could regulate the interaction of the ATPase EpsE with phospholipids, which thereby induces its oligomerization and activation (Camberg et al. 2007). Likewise, the activity of the flagellar ATPase FliI has been shown to be stimulated by acidic phospholipid binding, specifically in the presence of PG and CL, but not with the zwitterionic lipid phosphatidylcholine (Auvray et al. 2002).

In addition to ATPase activity, membrane lipids influence other steps in the translocation process. Proteins that are transported across the IM through the Sec and Tat pathways possess a positively charged N-terminal signal sequence that allows their targeting and secretion. The positively charged signal peptide interacts with the negatively charged phospholipids in the IM, which favors its insertion into the bilayer, thereby facilitating early steps of the translocation process. The insertion of the signal peptide into the IM induces its conformational change to an α-helix (Phoenix et al. 1993; Brehmer et al. 2012). Furthermore, it has been demonstrated that extracted and purified SecYEG complex from *E. coli* lipid membranes is greatly enriched in anionic phospholipids, which are essential for protein translocation since the reconstituted SecYEG complex in liposomes without PG and CL is inactive (Prabudiansyah et al. 2015).

Moreover, lipid posttranslational modification of proteins is a relevant process in the assembly of surface appendages. A null mutant in the phosphoethanolamine (pEtN) transferase EptC of *Campylobacter jejuni* presented a decreased motility, with ~ 95% of the population lacking flagella. This defect is caused by the absence of pEtN modification of the flagellar rod protein FlgG. Thus, the lipid modification of FlgG is proposed to confer structural stability between rod subunits during flagellar assembly (Cullen and Trent 2010; Cullen et al. 2012). EptC belongs to a family of proteins that catalyze the periplasmic decoration of bacterial structures. Another member of this protein family is PptA (pilin phospho-form transferase A), which catalyzes the modification of the major pilin subunit protein, PilE, of the T4P of *Neisseria gonorrhoeae*, with pEtN and phosphocholine. These modifications confer structural stability to its target and provide antigenic diversity (Aas et al. 2006). A phosphoglycerol modification of PilE has also been reported in *N. meningitidis*, which affects T4P-dependent interactions between bacteria, promoting its dissemination during invasive infection (Chamot-Rooke et al. 2011). Interestingly, the recent cryoelectron microscopy structure of the conjugative sex F pilus of the T4SS revealed that it is assembled from stoichiometric protein-phospholipid units. The phospholipid PG interacts with the pilin, whereas PE and CL did not. The lipid head groups are directed toward the pilus lumen, creating a moderately negative

inner conduit which could facilitate transport of the negatively charged ssDNA (Costa et al. 2016).

Finally, the correct function of the YscU protein, an export apparatus component of the *Yersinia pseudotuberculosis* T3SS, depends on its association with lipids. YscU is an IM polytopic protein with a large C-terminal cytoplasmic domain. This protein possesses a positively charged linker domain that connects the membrane and the cytoplasmic domains. The linker region associates with the negatively charged IM undergoing a coil-to-helix transition. However, upon alanine substitution of its positively charged amino acids, the membrane binding affinity was attenuated and the T3SS-dependent protein secretion was significantly reduced (Weise et al. 2014).

5 Research Needs

The successful colonization of different ecological niches relies on the ability of bacteria to adapt to diverse environments and to establish associations with other prokaryotic and eukaryotic cells. The secretion systems and extracellular appendages described in this work play a determinant role in this adaptation process and are crucial for bacterial persistence and survival. In many cases, these transmembrane organelles are employed as virulence weapons against host cells, resulting in the development of multiple diseases that have a dramatic impact on human health. This, together with the increasing prevalence of multidrug-resistant bacterial pathogens, requires the urgent design of novel antimicrobial strategies that specifically target virulence mechanisms instead of viability. Therefore, given the central role of the aforedescribed appendages in pathogenesis, it is of utmost importance to further our knowledge on the structural and mechanistic basis of the assembly and function of these molecular machineries.

The molecular assembly of the different bacterial appendages has been extensively studied. Cryoelectron microscopy has transformed the field of structural biology, creating high-resolution models and providing structural insights of complete protein complexes embedded in the bacterial membranes, e.g., the injectisome and the T4SS core complex. This has unraveled some remarkable structural similarities between the systems. However, atomic-resolution structures of various individual components are still not available, in particular those of the membrane-embedded building blocks, which will be a big challenge for the future. In addition, the influence of membrane lipids on the assembly of these protein complexes remains to be determined.

Membrane lipid composition varies within different bacterial species and under diverse environmental conditions, and it is known to affect the structure and function of membrane-embedded components. Nevertheless, there are only few examples in the literature of the role of lipids in the assembly and function of bacterial appendages. The recent unprecedented finding that the F pilus is composed of a 1:1 stoichiometric phospholipid-protein complex opens the field for future studies on

the mechanisms of pili biogenesis. However, more efforts are required to understand the operation of these nanomachines within the hydrophobic lipid environment. In addition, protein insertion into bacterial membranes also alters the physical properties of the membrane, causing perturbations that might have an impact in cell physiology. This field is still largely unexplored.

Numerous questions and challenges remain for future investigations, such as the detailed molecular mechanisms underlying the energetics of protein secretion and substrate recognition, to elucidate the structure of membrane components, to determine the influence of lipids in bacterial appendages assembly and function, etc. This information will contribute to current efforts in the development of new anti-infective drugs that interfere with the function of these virulence factors.

Acknowledgments Work in our laboratory is supported by grants from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México (DGAPA, UNAM; PAPIIT IN209617) and Consejo Nacional de Ciencia y Tecnología (CONACyT 284081). We acknowledge Dra. Norma Espinosa Sánchez for critical reading of the manuscript and excellent technical assistance.

References

Aas FE, Egge-Jacobsen W, Winther-Larsen HC, Lovold C, Hitchen PG, Dell A, Koomey M (2006) Neisseria gonorrhoeae type IV pili undergo multisite, hierarchical modifications with phosphoethanolamine and phosphocholine requiring an enzyme structurally related to lipopolysaccharide phosphoethanolamine transferases. J Biol Chem 281:27712–27723

Ahn T, Kim H (1998) Effects of nonlamellar-prone lipids on the ATPase activity of SecA bound to model membranes. J Biol Chem 273:21692–21698

Albenne C, Ieva R (2017) Job contenders: roles of the beta-barrel assembly machinery and the translocation and assembly module in autotransporter secretion. Mol Microbiol 106:505–517

Alteri CJ, Mobley HL (2016) The versatile type VI secretion system. Microbiol Spectr 4. https://doi.org/10.1128/microbiolspec.VMBF-0026-2015

Anne J, Economou A, Bernaerts K (2017) Protein secretion in Gram-positive Bacteria: from multiple pathways to biotechnology. Curr Top Microbiol Immunol 404:267–308

Auvray F, Ozin AJ, Claret L, Hughes C (2002) Intrinsic membrane targeting of the flagellar export ATPase FliI: interaction with acidic phospholipids and FliH. J Mol Biol 318:941–950

Brehmer T, Kerth A, Graubner W, Malesevic M, Hou B, Bruser T, Blume A (2012) Negatively charged phospholipids trigger the interaction of a bacterial Tat substrate precursor protein with lipid monolayers. Langmuir 28:3534–3541

Breukink E, Demel RA, de Korte-Kool G, de Kruijff B (1992) SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study. Biochemistry 31:1119–1124

Camberg JL, Johnson TL, Patrick M, Abendroth J, Hol WG, Sandkvist M (2007) Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids. EMBO J 26:19–27

Cao B, Zhao Y, Kou Y, Ni D, Zhang XC, Huang Y (2014) Structure of the nonameric bacterial amyloid secretion channel. Proc Natl Acad Sci U S A 111:E5439–E5444

Chamot-Rooke J, Mikaty G, Malosse C, Soyer M, Dumont A, Gault J, Imhaus AF, Martin P, Trellet M, Clary G, Chafey P, Camoin L, Nilges M, Nassif X, Dumenil G (2011) Posttranslational modification of pili upon cell contact triggers N. meningitidis dissemination. Science 331:778–782

Chandran V, Fronzes R, Duquerroy S, Cronin N, Navaza J, Waksman G (2009) Structure of the outer membrane complex of a type IV secretion system. Nature 462:1011–1015
Cianfanelli FR, Monlezun L, Coulthurst SJ (2016) Aim, load, fire: the type VI secretion system, a bacterial Nanoweapon. Trends Microbiol 24:51–62
Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Microbiol 13:343–359
Costa TRD, Ilangovan A, Ukleja M, Redzej A, Santini JM, Smith TK, Egelman EH, Waksman G (2016) Structure of the bacterial sex F pilus reveals an assembly of a stoichiometric protein-phospholipid complex. Cell 166(1436–1444):e1410
Cullen TW, Trent MS (2010) A link between the assembly of flagella and lipooligosaccharide of the Gram-negative bacterium *Campylobacter jejuni*. Proc Natl Acad Sci U S A 107:5160–5165
Cullen TW, Madsen JA, Ivanov PL, Brodbelt JS, Trent MS (2012) Characterization of unique modification of flagellar rod protein FlgG by *Campylobacter jejuni* lipid A phosphoethanolamine transferase, linking bacterial locomotion and antimicrobial peptide resistance. J Biol Chem 287:3326–3336
Dalbey RE, Kuhn A (2012) Protein traffic in Gram-negative bacteria – how exported and secreted proteins find their way. FEMS Microbiol Rev 36:1023–1045
Dalbey RE, Wang P, Kuhn A (2011) Assembly of bacterial inner membrane proteins. Annu Rev Biochem 80:161–187
Deng W, Marshall NC, Rowland JL, McCoy JM, Worrall LJ, Santos AS, Strynadka NCJ, Finlay BB (2017) Assembly, structure, function and regulation of type III secretion systems. Nat Rev Microbiol 15:323–337
Diepold A, Armitage JP (2015) Type III secretion systems: the bacterial flagellum and the injectisome. Philos Trans R Soc Lond Ser B Biol Sci 370. https://doi.org/10.1098/rstb.2015.0020
Erhardt M, Namba K, Hughes KT (2010) Bacterial nanomachines: the flagellum and type III injectisome. Cold Spring Harb Perspect Biol 2. https://doi.org/10.1101/cshperspect.a000299
Evans ML, Chapman MR (2014) Curli biogenesis: order out of disorder. Biochim Biophys Acta 1843:1551–1558
Fan E, Chauhan N, Udatha DB, Leo JC, Linke D (2016) Type V secretion systems in bacteria. Microbiol Spectr 4. https://doi.org/10.1128/microbiolspec.VMBF-0009-2015
Filloux A, Voulhoux R (2018) Multiple structures disclose the Secretins' secrets. J Bacteriol 200. https://doi.org/10.1128/JB.00702-17
Fronzes R, Remaut H, Waksman G (2008) Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. EMBO J 27:2271–2280
Galan JE, Waksman G (2018) Protein-injection machines in bacteria. Cell 172:1306–1318
Gallique M, Bouteiller M, Merieau A (2017) The type VI secretion system: a dynamic system for bacterial communication? Front Microbiol 8:1454
Gaytan MO, Martinez-Santos VI, Soto E, Gonzalez-Pedrajo B (2016) Type three secretion system in attaching and effacing pathogens. Front Cell Infect Microbiol 6:129
Gold V, Kudryashev M (2016) Recent progress in structure and dynamics of dual-membrane-spanning bacterial nanomachines. Curr Opin Struct Biol 39:1–7
Gold VA, Robson A, Bao H, Romantsov T, Duong F, Collinson I (2010) The action of cardiolipin on the bacterial translocon. Proc Natl Acad Sci U S A 107:10044–10049
Green ER, Mecsas J (2016) Bacterial secretion systems: an overview. Microbiol Spectr 4. https://doi.org/10.1128/microbiolspec.VMBF-0012-2015
Grohmann E, Christie PJ, Waksman G, Backert S (2018) Type IV secretion in Gram-negative and Gram-positive bacteria. Mol Microbiol 107:455–471
Holland IB, Schmitt L, Young J (2005) Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review). Mol Membr Biol 22:29–39
Hospenthal MK, Costa TRD, Waksman G (2017) A comprehensive guide to pilus biogenesis in Gram-negative bacteria. Nat Rev Microbiol 15:365–379
Jarrell KF, Ding Y, Nair DB, Siu S (2013) Surface appendages of archaea: structure, function, genetics and assembly. Life (Basel) 3:86–117

Koo J, Lamers RP, Rubinstein JL, Burrows LL, Howell PL (2016) Structure of the Pseudomonas aeruginosa Type IVa pilus secretin at 7.4 A. Structure 24:1778–1787

Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature 405:914–919

Korotkov KV, Sandkvist M, Hol WG (2012) The type II secretion system: biogenesis, molecular architecture and mechanism. Nat Rev Microbiol 10:336–351

Krause S, Pansegrau W, Lurz R, de la Cruz F, Lanka E (2000) Enzymology of type IV macromolecule secretion systems: the conjugative transfer regions of plasmids RP4 and R388 and the cag pathogenicity island of Helicobacter pylori encode structurally and functionally related nucleoside triphosphate hydrolases. J Bacteriol 182:2761–2770

Lill R, Dowhan W, Wickner W (1990) The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell 60:271–280

Low HH, Gubellini F, Rivera-Calzada A, Braun N, Connery S, Dujeancourt A, Lu F, Redzej A, Fronzes R, Orlova EV, Waksman G (2014) Structure of a type IV secretion system. Nature 508:550–553

Minamino T (2014) Protein export through the bacterial flagellar type III export pathway. Biochim Biophys Acta 1843:1642–1648

Pansegrau W, Bagnoli F (2017) Pilus assembly in gram-positive bacteria. Curr Top Microbiol Immunol 404:203–233

Patel R, Smith SM, Robinson C (2014) Protein transport by the bacterial Tat pathway. Biochim Biophys Acta 1843:1620–1628

Phoenix DA, Kusters R, Hikita C, Mizushima S, de Kruijff B (1993) OmpF-Lpp signal sequence mutants with varying charge hydrophobicity ratios provide evidence for a phosphatidylglycerol-signal sequence interaction during protein translocation across the *Escherichia coli* inner membrane. J Biol Chem 268:17069–17073

Prabudiansyah I, Kusters I, Caforio A, Driessen AJ (2015) Characterization of the annular lipid shell of the Sec translocon. Biochim Biophys Acta 1848:2050–2056

Rivera-Calzada A, Fronzes R, Savva CG, Chandran V, Lian PW, Laeremans T, Pardon E, Steyaert J, Remaut H, Waksman G, Orlova EV (2013) Structure of a bacterial type IV secretion core complex at subnanometre resolution. EMBO J 32:1195–1204

Silhavy TJ, Kahne D, Walker S (2010) The bacterial cell envelope. Cold Spring Harb Perspect Biol 2:a000414

Sohlenkamp C, Geiger O (2016) Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev 40:133–159

Thomas S, Holland IB, Schmitt L (2014) The Type 1 secretion pathway – the hemolysin system and beyond. Biochim Biophys Acta 1843:1629–1641

Thomassin JL, Santos Moreno J, Guilvout I, Tran Van Nhieu G, Francetic O (2017) The trans-envelope architecture and function of the type 2 secretion system: new insights raising new questions. Mol Microbiol 105:211–226

Tsirigotaki A, De Geyter J, Šoštaric N, Economou A, Karamanou S (2016) Protein export through the bacterial Sec pathway. Nat Rev Microbiol 15:21

Ulbrandt ND, London E, Oliver DB (1992) Deep penetration of a portion of *Escherichia coli* SecA protein into model membranes is promoted by anionic phospholipids and by partial unfolding. J Biol Chem 267:15184–15192

Van Gerven N, Waksman G, Remaut H (2011) Pili and flagella biology, structure, and biotechnological applications. Prog Mol Biol Transl Sci 103:21–72

Van Gerven N, Klein RD, Hultgren SJ, Remaut H (2015) Bacterial amyloid formation: structural insights into curli biogensis. Trends Microbiol 23:693–706

Weise CF, Login FH, Ho O, Grobner G, Wolf-Watz H, Wolf-Watz M (2014) Negatively charged lipid membranes promote a disorder-order transition in the Yersinia YscU protein. Biophys J 107:1950–1961

Worrall LJ, Hong C, Vuckovic M, Deng W, Bergeron JRC, Majewski DD, Huang RK, Spreter T, Finlay BB, Yu Z, Strynadka NCJ (2016) Near-atomic-resolution cryo-EM analysis of the Salmonella T3S injectisome basal body. Nature 540:597

Yan Z, Yin M, Xu D, Zhu Y, Li X (2017) Structural insights into the secretin translocation channel in the type II secretion system. Nat Struct Mol Biol 24:177–183

Zilkenat S, Franz-Wachtel M, Stierhof YD, Galan JE, Macek B, Wagner S (2016) Determination of the stoichiometry of the complete bacterial type III secretion needle complex using a combined quantitative proteomic approach. Mol Cell Proteomics 15:1598–1609

Fatty Acid-Binding Proteins, a Family of Lipid Chaperones

37

Masato Furuhashi

Contents

1 Introduction 692
2 Fatty Acid-Binding Proteins (FABPs) 692
3 Adipocyte/Macrophage/Endothelial FABPs 693
3.1 FABP4 (A-FABP/aP2) 693
3.2 FABP5 (E-FABP/mal1) 695
3.3 Combined Deficiency of FABP4 and FABP5 695
4 Secretion and Function of FABP4 and FABP5 696
5 Circulating Concentrations of FABP4 and FABP5 697
6 Ectopic Expression of FABP4 698
7 FABPs as Therapeutic Targets 699
8 Research Needs 699
References 702

Abstract

Lipids are important in various biological functions and the pathogenesis of several diseases. However, the mechanisms between intracellular lipids and their biological targets and signaling pathways are not well understood. Fatty acid-binding proteins (FABPs), a family of lipid chaperones, contribute to systemic metabolic regulation through diverse lipid signaling. Fatty acid-binding protein 4 (FABP4), known as adipocyte FABP (A-FABP) or aP2, and FABP5, known as epidermal FABP (E-FABP) or mal1, are expressed in both adipocytes and macrophages and play important roles in the development of insulin resistance and atherosclerosis in relation to metabolically driven, low-grade, and chronic inflammation, referred to as "metaflammation." Pharmacological

M. Furuhashi (✉)
Department of Cardiovascular, Renal and Metabolic Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan
e-mail: furuhasi@sapmed.ac.jp

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_15

modification of FABP function would be a novel therapeutic strategy for several diseases, including obesity, diabetes mellitus, and atherosclerosis. It has recently been reported that FABP4 is secreted from adipocytes in a nonclassical pathway associated with lipolysis and acts as an adipokine for the development of insulin resistance and atherosclerosis. FABP5 is also secreted from cells, though the mechanism remains unclear. High concentrations of FABP4 and FABP5 are associated with several aspects of metabolic syndrome and cardiovascular events. Furthermore, ectopic expression and function of FABP4 in several types of cells and tissues have recently been shown to be associated with the pathogenesis of diseases. Here, we discuss significant roles of FABP4 and FABP5 among lipid chaperones in physiological and pathophysiological conditions and the possibility of therapeutic targets for metabolic and cardiovascular diseases.

1 Introduction

Trafficking of fatty acids in cells affects many aspects of cellular function. Fatty acids act both as an energy source and as signals for metabolic regulation through enzymatic and transcriptional networks to modulate gene expression, inflammatory and metabolic responses, and growth and survival pathways. Complex shuttling, processing, availability, and removal of lipids regulate lipid signaling by maintaining the balance of lipid species at the target compartments.

2 Fatty Acid-Binding Proteins (FABPs)

Fatty acid-binding proteins (FABPs), a family of intracellular lipid chaperones, regulate lipid trafficking and responses in cells and are linked to metabolic and inflammatory pathways (Furuhashi and Hotamisligil 2008). FABPs are abundantly expressed 14–15-kDa proteins that reversibly bind hydrophobic ligands, such as long-chain fatty acids and other lipids. It has been proposed that FABPs actively facilitate the transport of fatty acids to specific organelles in the cell for lipid oxidation in the mitochondrion or peroxisome; transcriptional regulation in the nucleus; signaling, trafficking, and membrane synthesis in the endoplasmic reticulum (ER); and regulation of enzyme activity and storage as lipid droplets in the cytoplasm.

At least nine different FABP isoforms have been identified. The FABP family includes liver (L-FABP/FABP1), intestinal (I-FABP/FABP2), heart (H-FABP/FABP3), adipocyte (A-FABP/FABP4/aP2), epidermal (E-FABP/FABP5/mal1), ileal (Il-FABP/FABP6), brain (B-FABP/FABP7), myelin (M-FABP/FABP8), and testis (T-FABP/FABP9) isoforms. FABPs have about 15–70% sequence identity between different isoforms and have almost the same three-dimensional structures showing a cap by the helix–loop–helix region and two orthogonal five-stranded

β-sheets by a 10-stranded antiparallel β-barrel structure (Furuhashi and Hotamisligil 2008). The fatty acid-binding pocket is located inside the β-barrel. The opening of the binding pocket is framed on one side by the N-terminal helix–loop–helix cap domain, and usually one long-chain fatty acid can be bound to the interior cavity of FABPs except for FABP1, which can bind two fatty acids. Each FABP has different ligand selectivity and binding affinity for fatty acids because of structural differences.

3 Adipocyte/Macrophage/Endothelial FABPs

Among the FABPs, FABP4, known as adipocyte FABP (A-FABP) or adipocyte P2 (aP2), is highly expressed in adipocytes and accounts for about 1% of all soluble proteins in adipose tissue (Furuhashi and Hotamisligil 2008). FABP5, another FABP known as epidermal FABP (E-FABP), psoriasis-associated FABP (PA-FABP) or mal1, is expressed in several tissues and cells including adipocytes. FABP4 and FABP5 have 52% amino acid similarity and bind to several fatty acids with similar affinity and selectivity. The amount of FABP4 in adipocytes is about 100-fold larger than that of FABP5 in adipocytes. Both FABP4 and FABP5 are also expressed in macrophages and dendritic cells, though the amount of FABP4 in adipocytes is about 10,000-fold larger than that in macrophages. The stoichiometry of FABP4 and that of FABP5 are nearly equal in macrophages under physiological conditions. FABP4 deficiency induces a strong compensatory increase of FABP5 in adipose tissue but not in macrophages or dendritic cells (Hotamisligil et al. 1996; Makowski et al. 2001; Rolph et al. 2006). Other than adipocytes and macrophages, expression of FABP4 and FABP5 is observed in endothelial cells of capillaries and small veins in the heart and kidney (Elmasri et al. 2009; Iso et al. 2013), and FABP4 and FABP5 are involved in transendothelial fatty acid transport into fatty acid-consuming organs (Iso et al. 2013).

3.1 FABP4 (A-FABP/aP2)

Expression of FABP4 is highly induced during adipocyte differentiation and is transcriptionally controlled by peroxisome proliferator-activated receptor (PPAR) γ agonists, fatty acids, insulin, and dexamethasone (Furuhashi and Hotamisligil 2008). Expression of FABP4 is also induced during differentiation from monocytes to macrophages and by treatment with lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate, PPARγ agonists, oxidized low-density lipoprotein, and advanced glycation end products (Furuhashi and Hotamisligil 2008). Treatment with vascular endothelial growth factor-A (VEGF-A) via VEGF-receptor-2 (VEGFR2) or basic fibroblast growth factor (bFGF) induces FABP4 expression in endothelial cells (Elmasri et al. 2009), and FABP4 in endothelial cells has been reported to promote angiogenesis (Elmasri et al. 2012). Interestingly, cellular senescence and oxidative stress induces FABP4 expression in microvascular

endothelial cells (Ha et al. 2006; Lee et al. 2010). Conversely, treatment with omega-3 fatty acids (Furuhashi et al. 2016b) and sitagliptin (Furuhashi et al. 2015a) decreases FABP4 expression in 3T3-L1 adipocytes. Treatment with atorvastatin (Llaverias et al. 2004) and metformin (Song et al. 2010) also reduces FABP4 expression in macrophages.

In an assay for fatty acid-binding affinity, FABP4 generally had higher affinity and selectivity for long-chain fatty acids than did albumin, and linoleic acid, an essential monounsaturated fatty acid, had the highest affinity for FABP4 under a basal condition (Furuhashi et al. 2016a). Under an oxidative condition, the affinity of FABP4 for most of the fatty acids except for palmitic acid was decreased, indicating that palmitic acid, a saturated fatty acid, has relatively high affinity for FABP4 under a specific condition, such as obesity-induced oxidative stress.

The primary sequence of FABP4 does not have a typical nuclear localization signal (NLS) or nuclear export signal (NES) as potential functional domains (Furuhashi and Hotamisligil 2008). However, the NLS and NES could be found in the three-dimensional structure of FABP4 (Ayers et al. 2007). The NLS in FABP4 is activated by closure of the portal loop and perturbation of a swinging doorway region (Gillilan et al. 2007). Non-activating ligands, such as oleic acid and stearic acid, protrude from the portal and prevent its closure, leading to masking of the NLS, while activating ligands, such as linoleic acid, troglitazone, and anilinonaphthalene sulfonate, expose the NLS (Gillilan et al. 2007). Another domain of FABP4 includes a hormone-sensitive lipase (HSL) binding site (Smith et al. 2008). A direct protein-protein interaction between FABP4 and HSL in adipocytes has been reported to regulate lipolysis (Scheja et al. 1999; Shen et al. 1999). Adipocytes in FABP4-deficient mice have been shown to have decreased lipolysis in vitro and in vivo. Interestingly, during experimentally induced lipolysis, FABP4-deficient mice have reduced insulin secretion (Scheja et al. 1999).

FABP4-deficient mice with high-fat diet-induced and genetic obesity show reduced insulin resistance, but there is no effect of FABP4 on insulin sensitivity in lean mice (Hotamisligil et al. 1996). Knockdown of the *Fabp4* gene by RNA interference in dietary obese mice increases body weight and fat mass without significant changes in glucose and lipid homeostasis (Yang et al. 2011), which is similar to the phenotype of FABP4 heterozygous knockout mice on a high-fat diet (Hotamisligil et al. 1996). The remaining expression of FABP4 might maintain some parts of FABP4 function.

FABP4 deficiency protects against atherosclerosis in apolipoprotein E (ApoE)-deficient mice (Makowski et al. 2001). FABP4 in macrophages increases accumulation of cholesterol ester and foam cell formation via inhibition of the PPARγ-liver X receptor α (LXRα)-ATP-binding cassette A1 (ABCA1) pathway and induces inflammatory responses through activation of the inhibitor of nuclear kappa B kinase (IKK)-nuclear factor-kappa B (NF-κB) and c-Jun N-terminal kinase (JNK)-activating protein-1 (AP-1) pathways (Makowski et al. 2005; Hui et al. 2010). FABP4 in dendritic cells also regulates the IKK-NF-κB pathway and T cell priming (Rolph et al. 2006).

3.2 FABP5 (E-FABP/mal1)

FABP5 is expressed most abundantly in epidermal cells of the skin but is also present in other tissues including adipose tissue (adipocytes and macrophages), dendritic cells, and the tongue, mammary gland, brain, kidney, liver, lung, and testis (Furuhashi and Hotamisligil 2008). Since all of these tissues express additional members of the FABP family, it is difficult to investigate the singular function of FABP5. FABP5 deficiency in the epidermis does not alter fatty acid composition of the epidermal membrane, which is an essential component of the water permeability barrier of the skin, and only a minor reduction in transepidermal water loss is observed in FABP5-deficient mice (Owada et al. 2002). Overexpression of FABP5 in a benign nonmetastatic rat mammary epithelial cell line induces metastasis (Jing et al. 2001). Furthermore, FABP5 is expressed in astrocytes, glia of the pre- and perinatal brain, and neurons, and expression of FABP5 is induced following peripheral nerve injury, suggesting a role in the regeneration of neurons (Veerkamp and Zimmerman 2001). It has also been reported that FABP5-deficient mice exhibit impaired working memory and short-term memory in association with reduced brain docosahexaenoic acid (DHA) levels (Pan et al. 2016).

Certain activating fatty acids, including linoleic acid and arachidonic acid, but not non-activators, such as palmitic acid, palmitoleic acid, and sapienic acid, induce translocation of FABP5 from the cytoplasm to the nucleus (Armstrong et al. 2014). Similar to the NLS in FABP4, the NLS has been shown to be present and unmasked on the two helices in the cap domain of FABP5 bound to activating fatty acids (Armstrong et al. 2014).

Expression of FABP5 in macrophages is increased by treatment with toll-like receptor (TLR) agonists: LPS, a TLR4 agonist, and zymosan, a fungal product that activates TLR2 (Furuhashi et al. 2011b, 2014). Expression of FABP5 in endothelial cells is induced by cellular senescence and H_2O_2-induced oxidative stress (Ha et al. 2006; Lee et al. 2010). FABP5 transgenic mice in adipose tissue on a high-fat diet show enhanced basal and hormone-stimulated lipolysis and reduced insulin sensitivity (Hertzel et al. 2002; Maeda et al. 2003). On the other hand, FABP5 deficiency mildly increases systemic insulin sensitivity in dietary and genetic obesity mouse models (Maeda et al. 2003). Ablation of FABP5 suppresses atherosclerosis in LDL receptor-deficient mice on a Western-style hypercholesterolemic diet, and the anti-atherosclerotic effect of FABP5 deletion is associated with reduction of inflammatory response (Babaev et al. 2011).

3.3 Combined Deficiency of FABP4 and FABP5

Mice with combined deficiency of FABP4 and FABP5 (*Fabp4*$^{-/-}$*Fabp5*$^{-/-}$) exhibit protection against type 2 diabetes, fatty liver disease, and atherosclerosis more than do FABP4- or FABP5-deficient mice (Boord et al. 2004; Maeda et al. 2005). The effects of FABP4 and FABP5 on atherosclerosis are mainly due to their actions

in macrophages (Makowski et al. 2001; Babaev et al. 2011). On the other hand, actions of FABP4 and FABP5 in adipocytes and those in macrophages have distinct roles in regulation of insulin sensitivity through metabolic and inflammatory responses (Furuhashi et al. 2008). It has also been demonstrated that *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ mice exhibit defective uptake of fatty acid via capillary endothelial cells of the heart and skeletal muscle with compensatory upregulation of glucose consumption in those tissues during fasting (Syamsunarno et al. 2013). Furthermore, *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ mice show impaired thermogenesis after cold exposure during fasting (Syamsunarno et al. 2014).

Lipidomic analyses showed increased de novo lipogenesis by induction of stearoyl-CoA desaturase-1 (SCD-1) and fatty acid synthase in adipose tissue of *Fabp4*$^{-/-}$*Fabp5*$^{-/-}$ mice, leading to identification of increased palmitoleate (C16:1n7), an unsaturated free fatty acid, as an adipose tissue-derived lipid hormone, referred to as "lipokine," that can decrease fatty liver and increase glucose uptake in skeletal muscle (Cao et al. 2008). Deletion of FABP4 in macrophages also increases de novo lipogenesis pathways through LXRα-mediated SCD-1 activation, resulting in production of palmitoleate and resistance to ER stress (Erbay et al. 2009). Unsaturated fatty acids including palmitoleate modulate histone deacetylation, resulting in decreased basal and LPS-induced expression levels of FABP4 in macrophages (Coleman et al. 2011). Treatment with palmitoleate prevents atherosclerosis in ApoE-deficient mice in relation to reduced ER stress and inflammasome activation (Cimen et al. 2016). In a human study, palmitoleate level was positively correlated with insulin sensitivity assessed by euglycemic-hyperinsulinemic clamp studies after adjustment of age, gender, and adiposity (Stefan et al. 2010). The level of the *trans* isomer of palmitoleate, an exogenous source of C16:1n7, was associated with lower insulin resistance and lower incidence of diabetes mellitus (Mozaffarian et al. 2010).

4 Secretion and Function of FABP4 and FABP5

FABP4 lacks a signal peptide in the N-terminal sequence (Furuhashi and Hotamisligil 2008), which is necessary for the classical secretory pathway, i.e., ER-Golgi-dependent secretion. However, FABP4 is secreted from adipocytes in a nonclassical secretion pathway associated with lipolysis (Cao et al. 2013; Mita et al. 2015). Secretion of FABP4 is also regulated by an intracellular calcium-dependent pathway (Schlottmann et al. 2014). Furthermore, FABP4 is secreted partially by microvesicles derived from adipocytes (Lamounier-Zepter et al. 2009; Kralisch et al. 2014; Mita et al. 2015), an established mechanism for unconventional secretion from adipocytes (Nickel and Rabouille 2009). However, the release of FABP4 via adipocyte-derived microvesicles is a small fraction and conveys a minor activity (Lamounier-Zepter et al. 2009; Mita et al. 2015). It has also been confirmed that

FABP4 is secreted from macrophages (Furuhashi et al. 2016a), though the predominant contributors of circulating FABP4 are adipocytes rather than macrophages (Cao et al. 2013).

Direct effects of exogenous FABP4 have been demonstrated in various types of cells. Treatment with recombinant FABP4 enhances hepatic glucose production in vivo and in vitro (Cao et al. 2013), decreases cardiomyocyte contraction in vitro (Lamounier-Zepter et al. 2009), inhibits expression/activation of endothelial nitric oxide synthase (eNOS) in vascular endothelial cells (Furuhashi et al. 2016a), and increases proliferation/migration of vascular smooth muscle cells (Furuhashi et al. 2016a), glucose-stimulated insulin secretion in pancreatic β cells (Wu et al. 2014), and palmitic acid-dependent inflammatory responses in macrophages, vascular endothelial cells, and vascular smooth muscle cells (Furuhashi et al. 2016a). However, possible receptors of FABP4 have not been identified yet.

Secretome analyses showed that FABP5 is secreted from cells (Hwang et al. 2011; Yamamoto et al. 2016), though the mechanism remains unclear. Transcriptome and metabolome analyses showed that exogenous FABP4 and FABP5 differentially affect transcriptional and metabolic regulation in adipose-derived stem cells near adipocytes (Yamamoto et al. 2016).

5 Circulating Concentrations of FABP4 and FABP5

The concentration of FABP4 is highest among levels of FABP1 ~5 under a physiological condition in a general population without medication (Ishimura et al. 2013). FABP4 level is significantly higher in females than in males, possibly due to the larger amount of body fat in females than in males since there is an independent and strong correlation between FABP4 level and adiposity (Xu et al. 2006; Ishimura et al. 2013). Serum FABP4 level is negatively correlated with estimated glomerular filtration rate (Ishimura et al. 2013), suggesting that FABP4 is eliminated from the circulation mainly by renal clearance. FABP4 level in hemodialysis patients with end-stage kidney disease was about 20 times higher than that in controls with normal renal function and was decreased by 57.2% after hemodialysis (Furuhashi et al. 2011a).

Increased circulating FABP4 levels have been shown to be associated with obesity, insulin resistance, type 2 diabetes mellitus, hypertension, cardiac dysfunction, dyslipidemia, and atherosclerosis (Xu et al. 2006; Ota et al. 2012; Fuseya et al. 2014; Furuhashi et al. 2016d). It has also been reported that serum FABP4 level predicts long-term cardiovascular events (Furuhashi et al. 2011a; von Eynatten et al. 2012). Other than cardiovascular pathologic processes, FABP4 level would be a novel prognostic factor in patients with breast cancer independently of obesity (Hancke et al. 2010).

Several drugs can modify circulating FABP4 levels. Treatment with atorvastatin (Karpisek et al. 2007), a hydroxymethylglutaryl-CoA (HMG-CoA) reductase

inhibitor, several angiotensin II receptor blockers (Furuhashi et al. 2015b), omega-3 fatty acid ethyl esters containing eicosapentaenoic acid (EPA) and DHA (Furuhashi et al. 2016b), and sitagliptin (Furuhashi et al. 2015a), a dipeptidyl peptidase-4 (DPP-4) inhibitor, reduces FABP4 concentrations. On the other hand, treatment with pioglitazone, a PPARγ agonist known as an insulin-sensitizing thiazolidinedione, increases FABP4 levels (Cabre et al. 2007), presumably due to direct activation of PPARγ since the FABP4 gene promoter includes the PPAR response element. Treatment with canagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, paradoxically increases serum FABP4 level in some diabetic patients despite amelioration of glucose metabolism and adiposity reduction, possibly via induction of catecholamine-induced lipolysis in adipocytes (Furuhashi et al. 2016c).

Similar to FABP4, circulating FABP5 has been reported to be detected at levels of about one tenth or less of FABP4 concentrations, and FABP5 levels are associated with components of metabolic syndrome, although the correlation is not as strong as that of FABP4 (Yeung et al. 2008; Ishimura et al. 2013).

6 Ectopic Expression of FABP4

FABP4 is expressed in endothelial cells of capillaries and small veins but not arteries under a physiological condition (Elmasri et al. 2009). FABP4 is markedly induced in regenerated endothelial cells after endothelial balloon denudation in vivo (Lee et al. 2007). Intermittent hypoxia also increases the expression of FABP4 in human aortic endothelial cells (Han et al. 2010). FABP4 is expressed in the aortic endothelium of old, but not young, ApoE-deficient atherosclerotic mice, and chronic treatment with BMS309403, a small molecule FABP4 inhibitor, significantly improves endothelial dysfunction in old ApoE-deficient mice (Hwang et al. 2011). It has also been shown that FABP4 and FABP5 are involved in cellular senescence of vascular endothelial cells (Ha et al. 2006; Lee et al. 2010). Ectopic expression of FABP4 under a pathological condition, but not physiological FABP4 expression, in the endothelium may contribute to the pathogenesis of atherosclerosis and vascular injury.

In normal kidneys, FABP4 is expressed in endothelial cells of the tubulointerstitial peritubular capillary and vein in both the cortex and medulla, but not in glomerular or arterial endothelial cells, under a normal physiological condition (Elmasri et al. 2009). Ectopic expression of FABP4 in the glomerulus has been demonstrated to be associated with progression of proteinuria and renal dysfunction (Tanaka et al. 2014). Among FABPs, FABP1 (L-FABP) is expressed in proximal tubular epithelial cells in the kidney, and urinary FABP1 reflects damage of proximal tubular epithelial cells (Kamijo et al. 2004). Urinary FABP4 has been proposed to be a novel biomarker reflecting glomerular damage (Okazaki et al. 2014), and determination of both FABP1 and FABP4 in urine may enable better characterization of renal injury.

Evidence for the involvement of FABP4 in several kinds of diseases and conditions has been accumulating (Furuhashi and Hotamisligil 2008; Furuhashi et al. 2011b, 2014). FABP4 has been shown to be expressed in lung and bronchoalveolar cells in bronchopulmonary dysplasia, lung lavage cells in sarcoidosis, bronchial epithelial cells in asthma, human placental trophoblasts during placental development, and granulosa cells of the ovary in polycystic ovary syndrome. Furthermore, FABP4 is detected in human ovarian cancer cells at the adipocyte-tumor cell interface, and FABP4 deficiency in mice substantially impairs metastatic tumor growth. FABP4 expression is also detected in lipoblasts of lipoblastoma and liposarcoma, glioblastoma, and urothelial carcinomas.

7 FABPs as Therapeutic Targets

To date, several series of FABP4 inhibitors have been synthesized (Furuhashi and Hotamisligil 2008). The specific FABP4 inhibitor BMS309403 is an orally active small molecule that interacts with the fatty acid-binding pocket within the interior of FABP4 to inhibit binding of endogenous fatty acids (Furuhashi et al. 2007; Sulsky et al. 2007; Furuhashi and Hotamisligil 2008). Treatment with BMS309403 improves insulin resistance, diabetes mellitus, fatty liver disease, and atherosclerosis in experimental models (Furuhashi et al. 2007), indicating that chemical inhibition of FABP4 could be a therapeutic strategy against several aspects of metabolic syndrome. Recent studies have also demonstrated that neutralization of secreted FABP4 with an antibody to FABP4 could be a feasible approach for the treatment of insulin resistance and type 2 diabetes mellitus (Cao et al. 2013; Burak et al. 2015). Other than FABP4, several types of FABP inhibitors have been identified (Berger et al. 2012; Wang et al. 2016), but there have been few detailed examinations using those inhibitors in in vivo and in vitro studies.

8 Research Needs

FABP4 is involved in the regulation of glucose and lipid metabolism in relation to inflammatory and metabolic processes in target cells as shown in Fig. 1. The presence of FABP4 in cells may be beneficial for storing energy in adipocytes, for acting on an immune response in macrophages against pathogens, and for trafficking of fatty acids in capillary endothelial cells. Additionally, secreted FABP4 in association with lipolysis during fasting may regulate hepatic glucose production for survival in a famine. In the contemporary lifestyle with excessive caloric intake and decreased energy expenditure, the presence and induction of FABP4 or enhanced secretion of conformation-changed FABP4, which can bind to

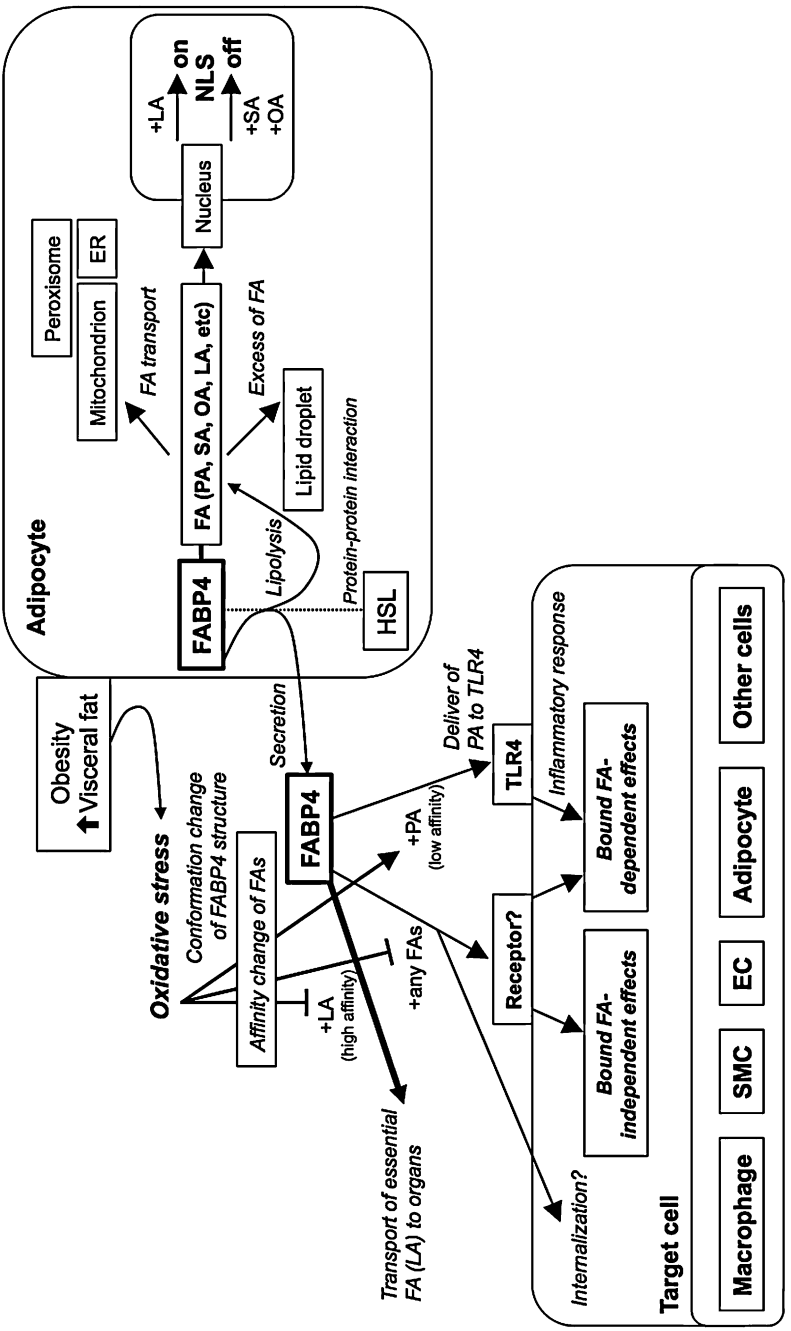


Fig. 1 (continued)

palmitic acid with a relatively high affinity (Furuhashi et al. 2016a), may be rather disadvantageous for regulating inflammatory or metabolic homeostasis. In such conditions, inhibition of FABP4, neutralization/elimination of secreted FABP4, or the use of possible antagonists for unidentified receptors of FABP4 could be an effective therapeutic strategy against metabolic and cardiovascular diseases and possibly other diseases. Further studies are obviously needed to investigate whether chemical or other types of inhibition/neutralization of FABP4 and blocking receptors of FABP4 can be safely used in humans and to show the efficacy of agents for metabolic and cardiovascular diseases. Not only FABP4, a well-studied and characterized isoform among FABPs, but also other FABPs, including FABP5, may offer targeting opportunities as a class for prevention or treatment of other diseases. Much work is still needed to determine the precise applications and indications for other isoforms.

Acknowledgments In relation to this review article, M.F. has been supported by grants from JSPS KAKENHI, MEXT Translational Research Network Program, Uehara Memorial Foundation, SENSHIN Medical Research Foundation, Japan Diabetes Foundation, Takeda Medical Research Foundation, Ono Medical Research Foundation, Takeda Science Foundation, Akiyama Life Science Foundation, Yamaguchi Endocrine Research Foundation, Naito Foundation Natural Science Scholarship, Suhara Memorial Foundation, Kondou Kinen Medical Foundation, and Terumo Foundation for Life Science and Arts. We are grateful to group members of our department for their scientific contribution. The author also regrets the inadvertent omission of many important references due to space limitations.

Fig. 1 Possible intracellular and extracellular functions of FABP4
FABP4 is abundant in the cytosolic fraction of adipocytes and can bind one long-chain fatty acid (*FA*), including palmitic acid (*PA*), stearic acid (*SA*), oleic acid (*OA*), or linoleic acid (*LA*). FABP4 facilitates the transport of FAs to specific organelles in the cell, such as the mitochondrion, peroxisome, endoplasmic reticulum (*ER*), and nucleus, regulates enzyme activity, and stores excess FA as lipid droplets. LA-bound, but not SA- or OA-bound, FABP4 can be moved into the nucleus by unmasking of the nuclear localization signal (*NLS*). The protein-protein interaction between FABP4 and hormone-sensitive lipase (*HSL*) regulates lipolysis, and FABP4 is secreted from adipocytes in a nonclassical pathway associated with lipolysis. FABP4 may act as a carrier of LA, an essential monounsaturated FA, to organs because of a high affinity for FABP4 under normal conditions. Secreted FABP4 may affect several responses in target cells, including macrophages, smooth muscle cells (*SMC*), endothelial cells (*EC*), adipocytes, and other cells, through unidentified receptor-mediated effects in bound FA-dependent and bound FA-independent manners and/or possible internalization of FABP4 into the cell. Obesity and increased visceral fat have been reported to promote oxidative stress. An oxidative stress condition can induce conformation change of the FABP4 structure and decrease affinity of most of the FAs, except for PA, for FABP4. Under a specific condition such as obesity-induced oxidative stress, PA would have relatively high affinity for FABP4, leading to PA-dependent inflammatory responses through unidentified receptors of FABP4 and/or delivery of PA to toll-like receptor 4 (*TLR4*)

References

Armstrong EH, Goswami D, Griffin PR, Noy N, Ortlund EA (2014) Structural basis for ligand regulation of the fatty acid-binding protein 5, peroxisome proliferator-activated receptor beta/delta (FABP5-PPARbeta/delta) signaling pathway. J Biol Chem 289:14941–14954

Ayers SD, Nedrow KL, Gillilan RE, Noy N (2007) Continuous nucleocytoplasmic shuttling underlies transcriptional activation of PPARgamma by FABP4. Biochemistry 46:6744–6752

Babaev VR, Runner RP, Fan D, Ding L, Zhang Y, Tao H, Erbay E, Gorgun CZ, Fazio S, Hotamisligil GS, Linton MF (2011) Macrophage Mal1 deficiency suppresses atherosclerosis in low-density lipoprotein receptor-null mice by activating peroxisome proliferator-activated receptor-gamma-regulated genes. Arterioscler Thromb Vasc Biol 31:1283–1290

Berger WT, Ralph BP, Kaczocha M, Sun J, Balius TE, Rizzo RC, Haj-Dahmane S, Ojima I, Deutsch DG (2012) Targeting fatty acid binding protein (FABP) anandamide transporters – a novel strategy for development of anti-inflammatory and anti-nociceptive drugs. PLoS One 7:e50968

Boord JB, Maeda K, Makowski L, Babaev VR, Fazio S, Linton MF, Hotamisligil GS (2004) Combined adipocyte-macrophage fatty acid-binding protein deficiency improves metabolism, atherosclerosis, and survival in apolipoprotein E-deficient mice. Circulation 110:1492–1498

Burak MF, Inouye KE, White A, Lee A, Tuncman G, Calay ES, Sekiya M, Tirosh A, Eguchi K, Birrane G, Lightwood D, Howells L, Odede G, Hailu H, West S, Garlish R, Neale H, Doyle C, Moore A, Hotamisligil GS (2015) Development of a therapeutic monoclonal antibody that targets secreted fatty acid-binding protein aP2 to treat type 2 diabetes. Sci Transl Med 7:319ra205

Cabre A, Lazaro I, Girona J, Manzanares JM, Marimon F, Plana N, Heras M, Masana L (2007) Fatty acid binding protein 4 is increased in metabolic syndrome and with thiazolidinedione treatment in diabetic patients. Atherosclerosis 195:e150–e158

Cao H, Gerhold K, Mayers JR, Wiest MM, Watkins SM, Hotamisligil GS (2008) Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. Cell 134:933–944

Cao H, Sekiya M, Ertunc ME, Burak MF, Mayers JR, White A, Inouye K, Rickey LM, Ercal BC, Furuhashi M, Tuncman G, Hotamisligil GS (2013) Adipocyte lipid chaperone AP2 is a secreted adipokine regulating hepatic glucose production. Cell Metab 17:768–778

Cimen I, Kocaturk B, Koyuncu S, Tufanli O, Onat UI, Yildirim AD, Apaydin O, Demirsoy S, Aykut ZG, Nguyen UT, Watkins SM, Hotamisligil GS, Erbay E (2016) Prevention of atherosclerosis by bioactive palmitoleate through suppression of organelle stress and inflammasome activation. Sci Transl Med 8:358ra126

Coleman SL, Park YK, Lee JY (2011) Unsaturated fatty acids repress the expression of adipocyte fatty acid binding protein via the modulation of histone deacetylation in RAW 264.7 macrophages. Eur J Nutr 50:323–330

Elmasri H, Karaaslan C, Teper Y, Ghelfi E, Weng M, Ince TA, Kozakewich H, Bischoff J, Cataltepe S (2009) Fatty acid binding protein 4 is a target of VEGF and a regulator of cell proliferation in endothelial cells. FASEB J 23:3865–3873

Elmasri H, Ghelfi E, Yu C-W, Traphagen S, Cernadas M, Cao H, Shi G-P, Plutzky J, Sahin M, Hotamisligil G, Cataltepe S (2012) Endothelial cell-fatty acid binding protein 4 promotes angiogenesis: role of stem cell factor/c-kit pathway. Angiogenesis 15:457–468

Erbay E, Babaev VR, Mayers JR, Makowski L, Charles KN, Snitow ME, Fazio S, Wiest MM, Watkins SM, Linton MF, Hotamisligil GS (2009) Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. Nat Med 15:1383–1391

Furuhashi M, Hotamisligil GS (2008) Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. Nat Rev Drug Discov 7:489–503

Furuhashi M, Tuncman G, Gorgun CZ, Makowski L, Atsumi G, Vaillancourt E, Kono K, Babaev VR, Fazio S, Linton MF, Sulsky R, Robl JA, Parker RA, Hotamisligil GS (2007) Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. Nature 447:959–965

Furuhashi M, Fucho R, Gorgun CZ, Tuncman G, Cao H, Hotamisligil GS (2008) Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice. J Clin Invest 118:2640–2650

Furuhashi M, Ishimura S, Ota H, Hayashi M, Nishitani T, Tanaka M, Yoshida H, Shimamoto K, Hotamisligil GS, Miura T (2011a) Serum fatty acid-binding protein 4 is a predictor of cardiovascular events in end-stage renal disease. PLoS One 6:e27356

Furuhashi M, Ishimura S, Ota H, Miura T (2011b) Lipid chaperones and metabolic inflammation. Int J Inflamm 2011:642612

Furuhashi M, Saitoh S, Shimamoto K, Miura T (2014) Fatty acid-binding protein 4 (FABP4): pathophysiological insights and potent clinical biomarker of metabolic and cardiovascular diseases. Clin Med Insights Cardiol 8:23–33

Furuhashi M, Hiramitsu S, Mita T, Fuseya T, Ishimura S, Omori A, Matsumoto M, Watanabe Y, Hoshina K, Tanaka M, Moniwa N, Yoshida H, Ishii J, Miura T (2015a) Reduction of serum FABP4 level by sitagliptin, a DPP-4 inhibitor, in patients with type 2 diabetes mellitus. J Lipid Res 56:2372–2380

Furuhashi M, Mita T, Moniwa N, Hoshina K, Ishimura S, Fuseya T, Watanabe Y, Yoshida H, Shimamoto K, Miura T (2015b) Angiotensin II receptor blockers decrease serum concentration of fatty acid-binding protein 4 in patients with hypertension. Hypertens Res 38:252–259

Furuhashi M, Fuseya T, Murata M, Hoshina K, Ishimura S, Mita T, Watanabe Y, Omori A, Matsumoto M, Sugaya T, Oikawa T, Nishida J, Kokubu N, Tanaka M, Moniwa N, Yoshida H, Sawada N, Shimamoto K, Miura T (2016a) Local production of fatty acid-binding protein 4 in epicardial/perivascular fat and macrophages is linked to coronary atherosclerosis. Arterioscler Thromb Vasc Biol 36:825–834

Furuhashi M, Hiramitsu S, Mita T, Omori A, Fuseya T, Ishimura S, Watanabe Y, Hoshina K, Matsumoto M, Tanaka M, Moniwa N, Yoshida H, Ishii J, Miura T (2016b) Reduction of circulating FABP4 level by treatment with omega-3 fatty acid ethyl esters. Lipids Health Dis 15:5

Furuhashi M, Matsumoto M, Hiramitsu S, Omori A, Tanaka M, Moniwa N, Yoshida H, Ishii J, Miura T (2016c) Possible increase in serum FABP4 level despite adiposity reduction by canagliflozin, an SGLT2 inhibitor. PLoS One 11:e0154482

Furuhashi M, Omori A, Matsumoto M, Kataoka Y, Tanaka M, Moniwa N, Ohnishi H, Yoshida H, Saitoh S, Shimamoto K, Miura T (2016d) Independent link between levels of proprotein convertase subtilisin/kexin type 9 and FABP4 in a general population without medication. Am J Cardiol 118:198–203

Fuseya T, Furuhashi M, Yuda S, Muranaka A, Kawamukai M, Mita T, Ishimura S, Watanabe Y, Hoshina K, Tanaka M, Ohno K, Akasaka H, Ohnishi H, Yoshida H, Saitoh S, Shimamoto K, Miura T (2014) Elevation of circulating fatty acid-binding protein 4 is independently associated with left ventricular diastolic dysfunction in a general population. Cardiovasc Diabetol 13:126

Gillilan RE, Ayers SD, Noy N (2007) Structural basis for activation of fatty acid-binding protein 4. J Mol Biol 372:1246–1260

Ha MK, Soo Cho J, Baik OR, Lee KH, Koo HS, Chung KY (2006) Caenorhabditis elegans as a screening tool for the endothelial cell-derived putative aging-related proteins detected by proteomic analysis. Proteomics 6:3339–3351

Han Q, Yeung SC, Ip MS, Mak JC (2010) Effects of intermittent hypoxia on A-/E-FABP expression in human aortic endothelial cells. Int J Cardiol 145:396–398

Hancke K, Grubeck D, Hauser N, Kreienberg R, Weiss JM (2010) Adipocyte fatty acid-binding protein as a novel prognostic factor in obese breast cancer patients. Breast Cancer Res Treat 119:367–367

Hertzel AV, Bennaars-Eiden A, Bernlohr DA (2002) Increased lipolysis in transgenic animals overexpressing the epithelial fatty acid binding protein in adipose cells. J Lipid Res 43:2105–2111

Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM (1996) Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. Science 274:1377–1379

Hui X, Li H, Zhou Z, Lam KS, Xiao Y, Wu D, Ding K, Wang Y, Vanhoutte PM, Xu A (2010) Adipocyte fatty acid-binding protein modulates inflammatory responses in macrophages through a positive feedback loop involving c-Jun NH2-terminal kinases and activator protein-1. J Biol Chem 285:10273–10280

Hwang HH, Moon PG, Lee JE, Kim JG, Lee W, Ryu SH, Baek MC (2011) Identification of the target proteins of rosiglitazone in 3T3-L1 adipocytes through proteomic analysis of cytosolic and secreted proteins. Mol Cells 31:239–246

Ishimura S, Furuhashi M, Watanabe Y, Hoshina K, Fuseya T, Mita T, Okazaki Y, Koyama M, Tanaka M, Akasaka H, Ohnishi H, Yoshida H, Saitoh S, Miura T (2013) Circulating levels of fatty acid-binding protein family and metabolic phenotype in the general population. PLoS One 8:e81318

Iso T, Maeda K, Hanaoka H, Suga T, Goto K, Syamsunarno MR, Hishiki T, Nagahata Y, Matsui H, Arai M, Yamaguchi A, Abumrad NA, Sano M, Suematsu M, Endo K, Hotamisligil GS, Kurabayashi M (2013) Capillary endothelial fatty acid binding proteins 4 and 5 play a critical role in fatty acid uptake in heart and skeletal muscle. Arterioscler Thromb Vasc Biol 33:2549–2557

Jing C, Beesley C, Foster CS, Chen H, Rudland PS, West DC, Fujii H, Smith PH, Ke Y (2001) Human cutaneous fatty acid-binding protein induces metastasis by up-regulating the expression of vascular endothelial growth factor gene in rat Rama 37 model cells. Cancer Res 61:4357–4364

Kamijo A, Kimura K, Sugaya T, Yamanouchi M, Hikawa A, Hirano N, Hirata Y, Goto A, Omata M (2004) Urinary fatty acid-binding protein as a new clinical marker of the progression of chronic renal disease. J Lab Clin Med 143:23–30

Karpisek M, Stejskal D, Kotolova H, Kollar P, Janoutova G, Ochmanova R, Cizek L, Horakova D, Yahia RB, Lichnovska R, Janout V (2007) Treatment with atorvastatin reduces serum adipocyte-fatty acid binding protein value in patients with hyperlipidaemia. Eur J Clin Investig 37:637–642

Kralisch S, Ebert T, Lossner U, Jessnitzer B, Stumvoll M, Fasshauer M (2014) Adipocyte fatty acid-binding protein is released from adipocytes by a non-conventional mechanism. Int J Obes (Lond) 38:1251–1254

Lamounier-Zepter V, Look C, Alvarez J, Christ T, Ravens U, Schunck WH, Ehrhart-Bornstein M, Bornstein SR, Morano I (2009) Adipocyte fatty acid-binding protein suppresses cardiomyocyte contraction: a new link between obesity and heart disease. Circ Res 105:326–334

Lee MY, Tse HF, Siu CW, Zhu SG, Man RY, Vanhoutte PM (2007) Genomic changes in regenerated porcine coronary arterial endothelial cells. Arterioscler Thromb Vasc Biol 27:2443–2449

Lee MY, Wang Y, Vanhoutte PM (2010) Senescence of cultured porcine coronary arterial endothelial cells is associated with accelerated oxidative stress and activation of NFkB. J Vasc Res 47:287–298

Llaverias G, Noe V, Penuelas S, Vazquez-Carrera M, Sanchez RM, Laguna JC, Ciudad CJ, Alegret M (2004) Atorvastatin reduces CD68, FABP4, and HBP expression in oxLDL-treated human macrophages. Biochem Biophys Res Commun 318:265–274

Maeda K, Uysal KT, Makowski L, Gorgun CZ, Atsumi G, Parker RA, Bruning J, Hertzel AV, Bernlohr DA, Hotamisligil GS (2003) Role of the fatty acid binding protein mal1 in obesity and insulin resistance. Diabetes 52:300–307

Maeda K, Cao H, Kono K, Gorgun CZ, Furuhashi M, Uysal KT, Cao Q, Atsumi G, Malone H, Krishnan B, Minokoshi Y, Kahn BB, Parker RA, Hotamisligil GS (2005) Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. Cell Metab 1:107–119

Makowski L, Boord JB, Maeda K, Babaev VR, Uysal KT, Morgan MA, Parker RA, Suttles J, Fazio S, Hotamisligil GS, Linton MF (2001) Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. Nat Med 7:699–705

Makowski L, Brittingham KC, Reynolds JM, Suttles J, Hotamisligil GS (2005) The fatty acid-binding protein, aP2, coordinates macrophage cholesterol trafficking and inflammatory activity. Macrophage expression of aP2 impacts peroxisome proliferator-activated receptor gamma and IkappaB kinase activities. J Biol Chem 280:12888–12895

Mita T, Furuhashi M, Hiramitsu S, Ishii J, Hoshina K, Ishimura S, Fuseya T, Watanabe Y, Tanaka M, Ohno K, Akasaka H, Ohnishi H, Yoshida H, Saitoh S, Shimamoto K, Miura T (2015) FABP4 is secreted from adipocytes by adenyl cyclase-PKA- and guanylyl cyclase-PKG-dependent lipolytic mechanisms. Obesity (Silver Spring) 23:359–367

Mozaffarian D, Cao H, King IB, Lemaitre RN, Song X, Siscovick DS, Hotamisligil GS (2010) Trans-palmitoleic acid, metabolic risk factors, and new-onset diabetes in U.S. adults: a cohort study. Ann Intern Med 153:790–799

Nickel W, Rabouille C (2009) Mechanisms of regulated unconventional protein secretion. Nat Rev Mol Cell Biol 10:148–155

Okazaki Y, Furuhashi M, Tanaka M, Mita T, Fuseya T, Ishimura S, Watanabe Y, Hoshina K, Akasaka H, Ohnishi H, Yoshida H, Saitoh S, Shimamoto K, Miura T (2014) Urinary excretion of fatty acid-binding protein 4 is associated with albuminuria and renal dysfunction. PLoS One 9:e115429

Ota H, Furuhashi M, Ishimura S, Koyama M, Okazaki Y, Mita T, Fuseya T, Yamashita T, Tanaka M, Yoshida H, Shimamoto K, Miura T (2012) Elevation of fatty acid-binding protein 4 is predisposed by family history of hypertension and contributes to blood pressure elevation. Am J Hypertens 25:1124–1130

Owada Y, Suzuki I, Noda T, Kondo H (2002) Analysis on the phenotype of E-FABP-gene knockout mice. Mol Cell Biochem 239:83–86

Pan Y, Short JL, Choy KH, Zeng AX, Marriott PJ, Owada Y, Scanlon MJ, Porter CJ, Nicolazzo JA (2016) Fatty acid-binding protein 5 at the blood-brain barrier regulates endogenous brain docosahexaenoic acid levels and cognitive function. J Neurosci 36:11755–11767

Rolph MS, Young TR, Shum BO, Gorgun CZ, Schmitz-Peiffer C, Ramshaw IA, Hotamisligil GS, Mackay CR (2006) Regulation of dendritic cell function and T cell priming by the fatty acid-binding protein AP2. J Immunol 177:7794–7801

Scheja L, Makowski L, Uysal KT, Wiesbrock SM, Shimshek DR, Meyers DS, Morgan M, Parker RA, Hotamisligil GS (1999) Altered insulin secretion associated with reduced lipolytic efficiency in aP2-/- mice. Diabetes 48:1987–1994

Schlottmann I, Ehrhart-Bornstein M, Wabitsch M, Bornstein SR, Lamounier-Zepter V (2014) Calcium-dependent release of adipocyte fatty acid binding protein from human adipocytes. Int J Obes (Lond) 38:1221–1227

Shen WJ, Sridhar K, Bernlohr DA, Kraemer FB (1999) Interaction of rat hormone-sensitive lipase with adipocyte lipid-binding protein. Proc Natl Acad Sci USA 96:5528–5532

Smith AJ, Sanders MA, Juhlmann BE, Hertzel AV, Bernlohr DA (2008) Mapping of the hormone-sensitive lipase binding site on the adipocyte fatty acid-binding protein (AFABP). Identification of the charge quartet on the AFABP/aP2 helix-turn-helix domain. J Biol Chem 283:33536–33543

Song J, Ren P, Zhang L, Wang XL, Chen L, Shen YH (2010) Metformin reduces lipid accumulation in macrophages by inhibiting FOXO1-mediated transcription of fatty acid-binding protein 4. Biochem Biophys Res Commun 393:89–94

Stefan N, Kantartzis K, Celebi N, Staiger H, Machann J, Schick F, Cegan A, Elcnerova M, Schleicher E, Fritsche A, Haring HU (2010) Circulating palmitoleate strongly and independently predicts insulin sensitivity in humans. Diabetes Care 33:405–407

Sulsky R, Magnin DR, Huang Y, Simpkins L, Taunk P, Patel M, Zhu Y, Stouch TR, Bassolino-Klimas D, Parker R, Harrity T, Stoffel R, Taylor DS, Lavoie TB, Kish K, Jacobson BL, Sheriff S, Adam LP, Ewing WR, Robl JA (2007) Potent and selective biphenyl azole inhibitors of adipocyte fatty acid binding protein (aFABP). Bioorg Med Chem Lett 17:3511–3515

Syamsunarno MR, Iso T, Hanaoka H, Yamaguchi A, Obokata M, Koitabashi N, Goto K, Hishiki T, Nagahata Y, Matsui H, Sano M, Kobayashi M, Kikuchi O, Sasaki T, Maeda K, Murakami M, Kitamura T, Suematsu M, Tsushima Y, Endo K, Hotamisligil GS, Kurabayashi M (2013) A critical role of fatty acid binding protein 4 and 5 (FABP4/5) in the systemic response to fasting. PLoS One 8:e79386

Syamsunarno MR, Iso T, Yamaguchi A, Hanaoka H, Putri M, Obokata M, Sunaga H, Koitabashi N, Matsui H, Maeda K, Endo K, Tsushima Y, Yokoyama T, Kurabayashi M (2014) Fatty acid binding protein 4 and 5 play a crucial role in thermogenesis under the conditions of fasting and cold stress. PLoS One 9:e90825

Tanaka M, Furuhashi M, Okazaki Y, Mita T, Fuseya T, Ohno K, Ishimura S, Yoshida H, Miura T (2014) Ectopic expression of fatty acid-binding protein 4 in the glomerulus is associated with proteinuria and renal dysfunction. Nephron Clin Pract 128:345–351

Veerkamp JH, Zimmerman AW (2001) Fatty acid-binding proteins of nervous tissue. J Mol Neurosci 16:133–142; discussion 151–137

von Eynatten M, Breitling LP, Roos M, Baumann M, Rothenbacher D, Brenner H (2012) Circulating adipocyte fatty acid-binding protein levels and cardiovascular morbidity and mortality in patients with coronary heart disease: a 10-year prospective study. Arterioscler Thromb Vasc Biol 32:2327–2335

Wang YT, Liu CH, Zhu HL (2016) Fatty acid binding protein (FABP) inhibitors: a patent review (2012–2015). Expert Opin Ther Pat 26:767–776

Wu LE, Samocha-Bonet D, Whitworth PT, Fazakerley DJ, Turner N, Biden TJ, James DE, Cantley J (2014) Identification of fatty acid binding protein 4 as an adipokine that regulates insulin secretion during obesity. Mol Metab 3:465–473

Xu A, Wang Y, Xu JY, Stejskal D, Tam S, Zhang J, Wat NM, Wong WK, Lam KS (2006) Adipocyte fatty acid-binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome. Clin Chem 52:405–413

Yamamoto T, Furuhashi M, Sugaya T, Oikawa T, Matsumoto M, Funahashi Y, Matsukawa Y, Gotoh M, Miura T (2016) Transcriptome and metabolome analyses in exogenous FABP4- and FABP5-treated adipose-derived stem cells. PLoS One 11:e0167825

Yang R, Castriota G, Chen Y, Cleary MA, Ellsworth K, Shin MK, Tran JL, Vogt TF, Wu M, Xu S, Yang X, Zhang BB, Berger JP, Qureshi SA (2011) RNAi-mediated germline knockdown of FABP4 increases body weight but does not improve the deranged nutrient metabolism of diet-induced obese mice. Int J Obes 35:217–225

Yeung DC, Wang Y, Xu A, Cheung SC, Wat NM, Fong DY, Fong CH, Chau MT, Sham PC, Lam KS (2008) Epidermal fatty-acid-binding protein: a new circulating biomarker associated with cardio-metabolic risk factors and carotid atherosclerosis. Eur Heart J 29:2156–2163

Protein Lipidation, Elucidation by Chemical Proteomics, and Its Functional Roles

38

Gemma Triola

Contents

1 Introduction 708
2 Lipid Modifications of Proteins 710
2.1 Bacterial Lipoproteins 710
2.2 *S*-Prenylation 711
2.3 *N*-Myristoylation 711
2.4 *S*-Acylation 712
2.5 GPI-Anchored Proteins 713
2.6 *C*-Cholesterylation 713
2.7 *C*-Phosphatidylethanolaminylation 713
3 Chemical Proteomics of Lipidated Proteins 714
3.1 Analysis and Identification of *S*-Prenylation 716
3.2 Analysis and Identification of *N*-Myristoylation 717
3.3 Analysis and Identification of *S*-Acylation 718
3.4 Analysis and Identification of Cholesterylation 721
3.5 Analysis and Identification of GPI-Anchor Modification 721
4 Research Needs 722
References 722

Abstract

Protein lipidation regulates the localization and function of membrane-associated proteins. Many efforts have been done in the last decade to develop methods allowing the global analysis of lipidated proteins. The use of bioorthogonal reactions combined with mass spectrometry has provided a powerful method to systematically analyze these modified proteins, thereby revealing the key role of

G. Triola (✉)
Department of Biological Chemistry and Molecular Modelling, Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain
e-mail: gemma.triola@iqac.csic.es

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_50

lipid anchors in controlling protein activity both in health and disease. The knowledge gained in this emerging field will strongly benefit from the recent advances in quantitative chemical proteomics enabling the simultaneous analysis of healthy and diseased patients and leading ultimately to the identification of potential targets for therapeutic interventions.

1 Introduction

Membrane proteins regulate a variety of essential cellular processes, ranging from cell–cell recognition, transport, or signal transduction. As a result, membrane proteins, which account for 40% of the full proteome and are targets for almost 60% of pharmaceutical drugs (Yildirim et al. 2007), are considered attractive targets for drug development.

Proteins located in the membrane can be classified into two broad categories, integral and peripheral proteins. Integral proteins contain membrane-spanning domains embedded in the lipid bilayer. In contrast, peripheral proteins are temporarily bound to the lipid bilayer by specific interactions with membrane lipids. Examples thereof are the recognition of phosphoinositides by the pleckstrin homology (PH) domain (Lemmon 2007), the recruitment of START (steroidogenic acute regulatory (StAR)-related lipid transfer) domains via binding to sphingolipids (Snook et al. 2006), or the covalent attachment of lipids anchors, a process known as protein lipidation (Triola et al. 2012).

The most common lipidation processes include the equipment of proteins with a glycosylphosphatidylinositol anchor (GPI), the myristoylation of *N*-terminal glycines, and the *S*-prenylation or *S*-acylation of cysteine residues (Fig. 1). Except the labile thioesters resulting from *S*-acylation, all these modifications are permanently installed into proteins. Other known lipidation processes are the cholesterylation of members of the hedgehog family or the uncommon conjugation of the yeast autophagy-related protein 8 (Atg8) with phosphatidylethanolamine (Ichimura et al. 2000; Kirisako et al. 2000). Moreover, bacterial proteins can also be posttranslationally modified with three fatty acids attached to a conserved *N*-terminal cysteine (Nakayama et al. 2012).

Protein lipidation regulates protein function, the interaction with effectors, and its location within the cell (Resh 2006b). Moreover, lipidated proteins are crucial for bacterial, viral, and parasite infection processes (Martin et al. 2011), and alterations in the lipidation pattern of membrane-associated proteins have been detected in cancer (Konstantinopoulos et al. 2007; Raju et al. 1996) as well as in neurological disorders (El-Husseini and Bredt 2002). For all these reasons, the characterization and identification of lipidated proteins is an emerging field of research, and the development of strategies enabling their study have become increasingly necessary.

Initially, the study of lipidated proteins was restricted to a limited number of proteins, and it relied on metabolic labeling using radioactive lipids. However, this is a costly and hazardous method, it requires long exposure times, it does not allow quantification, and it is not applicable to the global analysis of proteins. As a result, several alternative strategies have been developed during the last years to overcome

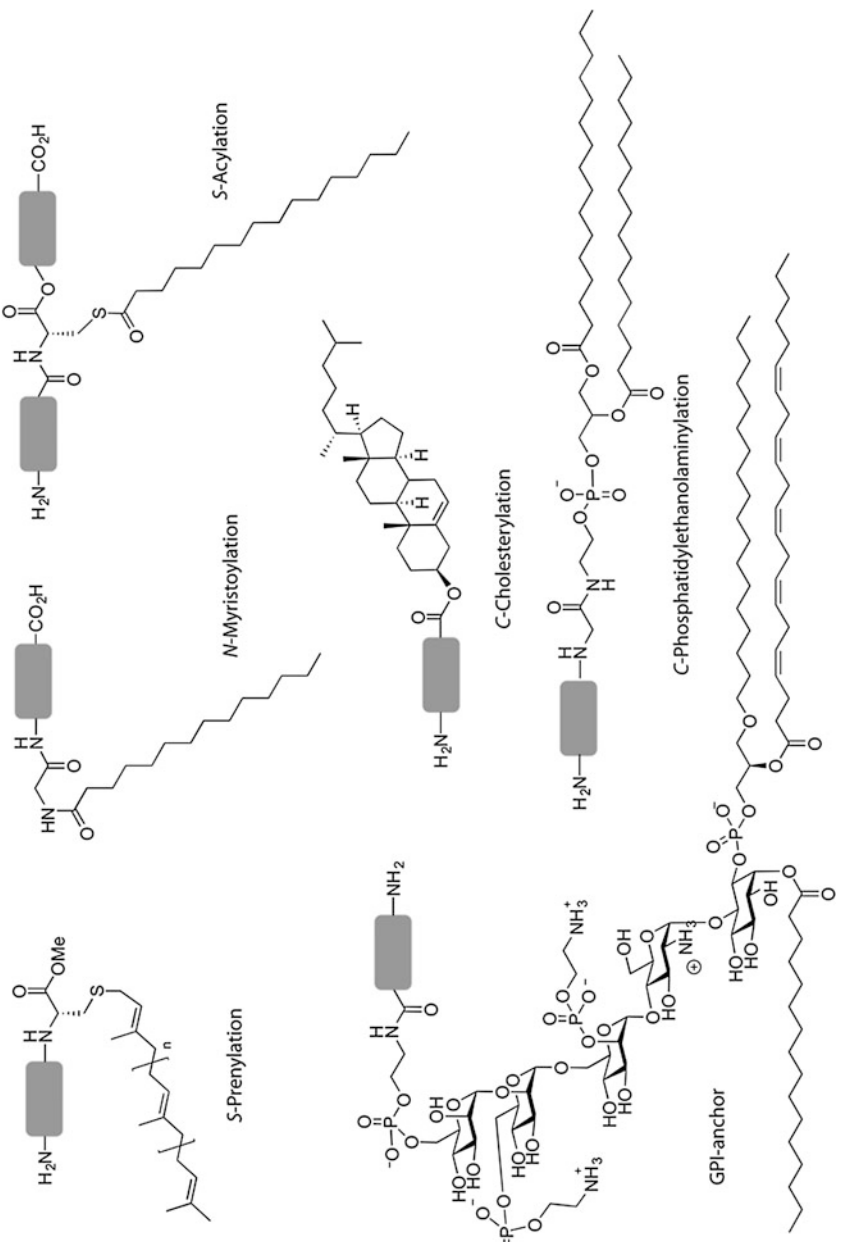


Fig. 1 Most relevant lipid modifications found in proteins. Prenylation of cysteines (either farnesylation ($n = 1$) or geranylgeranylation ($n = 2$) occurs at the *C*-terminus of proteins similarly to *C*-cholesterylation, *C*-phosphatidylethanolamination, or the attachment of GPI anchors. Myristoylation can be mostly found in

these limitations. Hence, the past decade has seen a substantial advancement in the field of lipidated proteins based on the convergence of several key aspects. First, the systematic identification of lipidated proteins has strongly benefited from chemical proteomics, a powerful approach that employs synthetic chemical probes to understand protein function. Second, recent advances in quantitative mass spectrometry-based proteomics have provided crucial information on how lipids modulate protein function and localization. Herein, the most prominent lipid modifications encountered in proteins will be first highlighted. The second part of this chapter will provide an overview of the basic concepts and existing methods for the analysis and identification of lipid-modified proteins.

2 Lipid Modifications of Proteins

2.1 Bacterial Lipoproteins

The biosynthesis of triacylated bacterial proteins starts with the attachment of a diacylglycerol moiety to the sulfhydryl group of a cysteine belonging to a conserved lipobox motif. This step is mediated by a phosphatidylglycerol/prolipoprotein diacylglyceryl transferase (Lgt), and it is followed by the cleavage of the signal peptide located at the *N*-terminus by the prolipoprotein signal peptidase (Lsp) and the acylation of the newly generated amino group by a phospholipid/apolipropotein transacylase (Lnt). These irreversible modifications occur on the periplasmic site of inner membrane, and the modified proteins are then transported to the inner or outer leaflet of the outer membrane or secreted into extracellular milieu (Narita and Tokuda in press). The bacterial lipoprotein genes constitute 1–3% of their total genes and have a crucial role in the maintenance of cell shape and in regulating bacterial virulence, cell wall metabolism, cell division, or antibiotic resistance (Nakayama et al. 2012).

The high complexity of the bacterial lipoproteins has hampered its analysis and characterization. Thus, these lipoproteins present different degrees of fatty acylation (mono-, di-, or triacylated) depending on the growth phase and pH (Kurokawa et al. 2012). Moreover, fatty acid composition depends on the protein and strain background. As example, MALDI–TOF–MS analysis of *Staphylococcus aureus* lipoproteins has revelated that the sn-1 position and the sn-2 position of the diacylglycerol group can be modified with C17:0–C20:0 and with C15:0, respectively, and the cysteine can be acylated with fatty acids from C15:0 to C20:0 (Kurokawa et al. 2012). It is expected that the recent advances in mass spectrometry-based proteomics

Fig. 1 (continued) N-terminal glycine residues although it has been also detected in internal lysine residues, whereas the reversible *S*-acylation occurs at internal cysteine residues. The GPI anchor depicted corresponds to the one present in the human erythrocyte acetylcholinesterase (the structure of these lipids is variable) (Paulick and Bertozzi 2008)

will strongly contribute to the identification and characterization of bacterial lipoproteins (Buddelmeijer 2015).

2.2 *S*-Prenylation

S-prenylation is the attachment of a farnesyl or a geranylgeranyl to a *C*-terminal cysteine (Fig. 1). This step can be catalyzed by three different enzymes, farnesyltransferase (FTase), geranylgeranyltransferase I (GGTase-I), and geranylgeranyltransferase II (GGTase-II). FTase and GGTase-I recognize a *C*-terminal motif known as CAAX box, where C is a cysteine, A are aliphatic amino acids, and X can be any amino acid. After prenylation, Ras-converting enzyme 1 (RCE1) will cleave the last three amino acids, and Icmt (isoprenylcysteine carboxymethyltransferase) will methylate the *C*-terminal isoprenylated cysteine (Ashby 1998).

GGTase II, also known as Rab geranylgeranyl transferase (RabGGTase), acts mainly on Rab proteins, with a crucial role in controlling membrane trafficking within the cell (Itzen and Goody 2011), and mediates the attachment of two geranylgeranyl groups. If the proteins contain the final sequence CXC, they will be methylated at the *C*-terminus, which does not occur in the case of a CC-ending sequence (Smeland et al. 1994).

2.3 *N*-Myristoylation

N-myristoylation is the covalent attachment of the saturated 14-carbon myristic acid to a *N*-terminal glycine and is catalyzed by a *N*-myristoyltransferase (NMT) (Towler et al. 1987) which recognizes the general consensus sequence (Gly-X-X-X-(Ser/Thr/Cys)) (Farazi et al. 2001). This modification occurs mostly co-translationally, and as a result, the lipids are installed on nascent proteins, but it can also take place posttranslationally after a caspase-mediated cleavage of a mature protein that results in the exposure of an internal glycine.

Mammalian species have two NMT enzymes, NMT1 and NMT2. Main differences occur at their *N*-terminal domain and may result in distinct subcellular localization (Giang and Cravatt 1998). It is also possible that these isoforms have different functions, since knockdown of NMT1 decreases the proliferation of the cells around 27% whereas knockdown of NMT2 has no effect on cell division (Ducker et al. 2005). *N*-myristoylated proteins have a broad range of functions including ADP-ribosylation factors (ARFs), α-subunits of G proteins, kinases, and the regulation of signaling cascades. Moreover, virus and bacteria can use the host NMT to increase their infectivity or to achieve maximal virulence (Maurer-Stroh and Eisenhaber 2004; Nimchuk et al. 2000). Examples thereof are the HIV viral proteins Gag and Nef, whose membrane localization is mediated by myristoylation (Furuishi et al. 1997; Gerlach et al. 2010). Fungi and parasitic protozoa possess also an NMT essential for their survival. As a result, NMT has emerged as a potential drug target

for the development of anticancer (Das et al. 2012), antifungal (Zhao and Ma 2014), and antiparasitic agents (Bowyer et al. 2008).

N-myristoylation is not only restricted to *N*-terminal glycines, but it has been also detected in lysine side chains. This modification was first reported over 20 years ago (Stevenson et al. 1992, 1993), but the finding that some members of the sirtuin family (Sirt2, Sirt6) can deacylate long-chain fatty acids from lysine residues and that Sirt6-mediated demyristoylation is required for efficient secretion of TNF-α have suggested more prevalent roles for this modification (Jiang et al. 2013). Additional sirtuin substrates and the enzyme(s) responsible for lysine acylation with long-chain fatty acids have yet to be identified.

2.4 *S*-Acylation

S-acylation is the covalent attachment of fatty acid to cysteine residues, thus forming a thioester linkage. Palmitic acid is the most frequently found lipid, and *S*-acylation is therefore also known as *S*-palmitoylation.

Protein acyl transferases (PATs) catalyze the transfer of palmitate from palmitoyl-CoA to a cysteine residue. PATs were initially identified in *Saccharomyces cerevisiae* (Lobo et al. 2002) and consist of a large family of proteins which contain a common 51 amino acid zinc finger domain with a conserved Asp–His–His–Cys (DHHC) motif. The mechanisms of protein palmitoylation seem to involve a two-step process involving autoacylation of the PAT and subsequent acyl transfer to the substrate protein (Mitchell et al. 2010). Although bioinformatics approaches to predict palmitoylation sites have been developed, no consensus sequence has yet emerged for their protein substrates (Li et al. 2011; Reddy et al. 2016; Ren et al. 2008; Xue et al. 2006).

Due to the labile thioester linkage, *S*-palmitoylation is a fully reversible and dynamic modification with a relatively rapid turnover rate. Hence, deacylation is carried out by acyl protein thioesterases (APT) (Yang et al. 2010) or the lysosomal palmitoyl protein thioesterases (PPT). The high number of DHHC proteins detected (24 in mammals) compared to the number of thioesterases and their different localization in cells suggest that acylation is tighter regulated than deacylation (Rocks et al. 2005, 2010). *S*-palmitoylation is detected not only on cytosolic proteins but also in transmembrane proteins such as G-protein-coupled receptors, immune cell receptors, and ion channels (Resh 2006a). Whereas in soluble proteins this modification is key to ensure stable membrane association, it may regulate the trafficking or the localization of integral membrane proteins in raft-like domains (Levental et al. 2010; Shipston 2011).

S-acylation is usually found in combination with other lipid modifications. Hence, *S*-prenylation and *N*-myristoylation target proteins to discrete membrane compartments and mediate membrane association by increasing protein hydrophobicity. However, these modifications result in only transient membrane association with short half-lives (Silvius and Lheureux 1994). Consequently, palmitoylation often couples with *S*-prenylation or *N*-myristoylation to mediate a stronger and

longer membrane association. Moreover, the reversibility of this lipid modification results in shorter half-life of the *S*-acyl group compared to the half-life of the proteins. As a result, proteins are repeatedly deacylated and reacylated allowing the spatial and temporal regulation of protein activity, stability, and localization as well as the modulation of signaling pathways (Rocks et al. 2010).

2.5 GPI-Anchored Proteins

Another important lipid modification is the posttranslational attachment of a glycosylphosphatidylinositol (GPI) anchor at the *C*-terminus of proteins. The GPI anchor is a complex molecule formed by a phosphoethanolamine, a glycan core, a phosphoinositol, and lipid residues. The complexity of the GPI unit relies on its enormous structural diversity (Levental et al. 2010; Paulick and Bertozzi 2008). Once synthesized, GPI-modified proteins are translocated to the cell surface, where they participate in cell contact, signal transduction, oncogenesis (Sidransky et al. 2008), and immune response (Julius and Marmor 2000). In mammalian cells, approximately 150 proteins are attached to the cell surface by GPI anchors (Fujita and Kinoshita 2010). Furthermore, this lipid modification has been shown to be relevant for prion disease pathogenesis and for parasites, which use the GPI anchor to increase their infectivity (Nagamune et al. 2000). The structural differences detected in GPI units from parasites in contrast to mammals have been also exploited for the generation of vaccines (Seeberger et al. 2008).

2.6 *C*-Cholesterylation

Sonic hedgehog (Shh) is a morphogen with a crucial role in the regulation of cellular proliferation and differentiation and to date the only example of *C*-terminal cholesterylation. To be fully active Shh requires two lipidation steps. The lipidation of Shh starts with the cleavage of an *N*-terminal recognition sequence present in the initial 46 kDa precursor. Then, an autocatalytic process originates a 19 kDa protein that is cholesterylated at the *C*-terminal glycine in a intein-like process (Hall et al. 1997) and *N*-palmitoylated at the *N*-terminal cysteine by the Hedgehog acyl transferase (Buglino and Resh 2008).

2.7 *C*-Phosphatidylethanolaminylation

This uncommon lipidation is in principle restricted to the yeast autophagy-related protein 8 (Atg8) or its mammal orthologues (LC3/GABARAP/GATE-16) that undergo the *C*-terminal conjugation to a phosphatidylethanolamine unit in an ubiquitin-like process (Ichimura et al. 2000; Mizushima et al. 1998). The lipidation of these proteins is essential in the regulation of autophagy, a cellular degradation and recycling mechanism involved in many physiological processes such as the

adaptation to starvation or the host response to infection (Triola 2015). Consequently, this lipidation step has been exploited by the pathogen *Legionella pneumophila*, which uses the bacterial effector protein RavZ to permanently cleave the phosphatidylethanolamine unit from Atg8/LC3 proteins, thereby inhibiting autophagy and facilitating the host cell invasion (Choy et al. 2012).

3 Chemical Proteomics of Lipidated Proteins

Historically, the lack of suitable methods has hampered the study of lipidated proteins. However, large-scale analysis of lipid-modified proteins has been greatly facilitated in the recent years by important advances in mass spectrometry (MS)-based proteomics and by the development of bioorthogonal reactions (Sletten and Bertozzi 2009). Thus, progress in the area of MS has given access to a broad spectrum of methods for the systematic characterization and discovery of lipidated proteins (Triola 2011; Witze et al. 2007). On the other hand, bioorthogonal reactions, i.e., chemical reactions compatible with biomolecules that allow their study in their native settings, have witnessed an enormous development in the last decade and have proven to be versatile tools for exploring lipid function. This approach relies on the use of alkyne or azide-tagged lipid analogues that are accepted as substrates by the lipid transferases in charge of the synthesis of lipidated proteins (Hannoush and Sun 2010). The introduced functional groups provide a chemical hook by which the protein can be then conjugated for visualization or enrichment. Hence, cells or proteins are fed with these lipids, and the resulting tagged proteins are then specifically labeled with fluorescent dyes or affinity handles (e.g., biotin) employing bioorthogonal reactions. The Staudinger ligation and the copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) of azides with terminal alkynes also referred to as Huisgen reaction or click chemistry are among the most commonly employed reactions for bioorthogonal applications. The labeled proteins can be then directly visualized or trapped by affinity chromatography using, for example, avidin-conjugated beads. These enriched proteins will be then digested into peptides that can be fractionated and analyzed by MS for their identification (Fig. 2).

While affinity chromatography combined with MS-based proteomics has been widely used and very successfully applied for the analysis of lipidated proteins, this strategy had to overcome initial major challenges: the study of lipidated proteins by MS is technically demanding because these proteins are often present at low concentrations and require efficient separation methods and the linked fatty acid is unstable during sample preparation and has a reduced ionization efficiency. Thus, several methods have been reported to improve the separation and analysis of lipidated peptides (Kwok et al. 2011). One of the most used methods is the multidimensional protein identification technology (MudPIT) (Link et al. 1999; Washburn et al. 2001), a two-dimensional chromatography separation usually based on a strong ion exchange column followed by a reverse phase chromatography column. The analysis of the peptides can be then performed by MS using a variety of techniques. Tandem MS techniques, including collision-induced dissociation (CID),

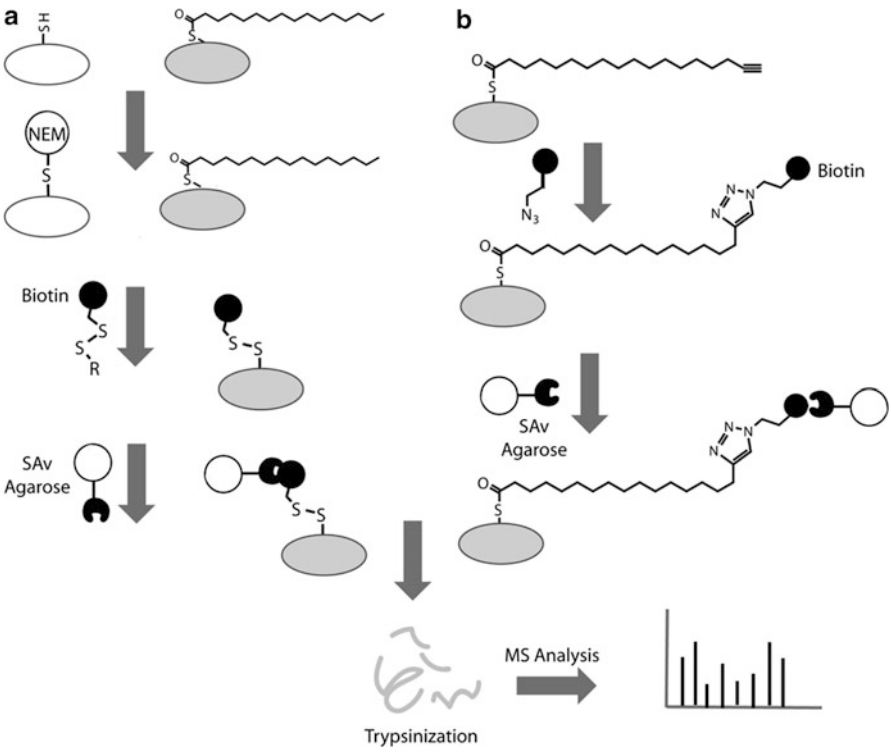


Fig. 2 *S*-acylated proteins can be studied using the acyl-biotin exchange (*ABE*) method (**a**) or through metabolic labeling using azido- or alkyne-tagged lipids (**b**). ABE starts with the blocking of the free sulfhydryls by treatment with *N*-ethylmaleimide (*NEM*) followed by hydrolysis of the existing thioesters and subsequent labeling with a biotin derivative. The labeled proteins can be then enriched using streptavidin agarose (*SAv*) and trypsinized, and the resulting peptides are then analyzed by mass spectroscopy (*MS*). Alternatively, cells or proteins can be fed with alkyne or azide-containing lipids. The tagged proteins are then selectively labeled with biotin derivatives using bioorthogonal reactions, enriched using *SAv* agarose and analyzed. This methodology has been applied to most of the known lipid modifications

electron capture dissociation (ECD), and electron transfer dissociation (ETD), have been largely applied to the study of proteins (Guan 2002; Hoffman and Kast 2006; Ji et al. 2013; Kaczorowska and Cooper 2013). The advent of these advanced MS techniques has enabled the simultaneous analysis and identification of multiple lipidated proteins.

Although all these advances have help to progress our understanding of lipidated proteins by providing qualitative lists of modified proteins, quantitative proteomics is now emerging as a powerful tool enabling a more in-depth analysis of the identified proteins. Consequently, many efforts have been devoted to the development of methods for accurate protein quantification. Label-free quantification was one of the first methods employed, and it correlates protein abundance with spectral counting, that considers both the number of different peptides belonging to a same

protein and the redundant identification of the same peptide in different fractions or with the mass spectrometric signal intensities of the peptides (Liu et al. 2004). Although this method has been widely employed because it does not require isotopic or chemical modification of the peptides and because it can be applied to all type of samples, label-based quantitative methods are currently preferred. Typically, these methods are based on the incorporation of mass difference enabling relative quantifications by measuring relative peak areas of mass spectra. Examples thereof include SILAC (stable isotopic labeling with amino acids in cell culture), SILAM (stable isotope labeling of mammals) (Rauniyar et al. 2013), ICAT (isotope-coded affinity tag), trypsin-catalyzed ^{18}O labeling, and iTRAQ (isobaric tags for relative and absolute quantitation). SILAC relies on the incorporation of isotope-labeled amino acids added directly to cultured cells, what limits its use in primary cells and tissues studies. ICAT uses a trifunctional probe that it is added to the proteins prior to enrichment. This probe contains a cysteine reacting group, an isotope-coded linker, and a biotin for enrichment (Gygi et al. 1999). Quantitative proteomics analyses have been also achieved performing tryptic digestion in the presence of $H_2{}^{18}O$ and $H_2{}^{16}O$ leading to the enzymatic incorporation of isotopes at the *C*-terminus of peptides (Morrison et al. 2015). Another method employed for quantitative proteomics is the iTRAQ, in which peptides are labeled with different isobaric mass tags that during fragmentation produce abundant MS/MS signature ions which relative areas can be employed for protein quantitation (Ross et al. 2004).

3.1 Analysis and Identification of *S*-Prenylation

The profiling of prenylated proteins started with the use of azido-containing farnesyl moieties combined with the bioorthogonal reaction to biotinylated phosphine capture reagents via Staudinger ligation that enabled enrichment and MS-based analysis of the corresponding prenylated proteins (Kho et al. 2004). The azido-tagged probe showed specificity for FTase modified proteins in front of GGTase, and it resulted in the identification of 18 farnesylated proteins. Later, the authors extended this work to use an azido geranylgeranyl analogue for the identification of geranylgeranylated proteins (Chan et al. 2009). Waldmann and Alexandrov also reported the synthesis of isoprenoid pyrophosphates containing azido and diene reacting groups that could be employed for the site-specific modification of proteins by the Staudinger ligation or the Diels–Alder reaction (Nguyen et al. 2007). Later, the authors used a biotin-geranylgeranyl (GerGer) pyrophosphate analogue and an engineered protein prenyltransferases for the proteome-wide analysis of prenylated proteins and to quantify in vivo the effects and specificity of protein prenyltransferase inhibitors (Nguyen et al. 2009). Several additional azide-containing (Berry et al. 2010) and alkyne-containing isoprenoids (Distefano et al. 2010) have been prepared and explored for the labeling and enrichment of prenylated proteins. Since it is generally agreed that lower levels of background labeling are obtained with alkyne-containing reporters, such derivatives have become the reagent of choice for the labeling and profiling of lipidated proteins. Hence, large-scale profiling of prenylated proteins has been

performed in macrophages using an alkyne-containing farnesyl that is accepted by the three prenyltransferases (Charron et al. 2013). More recently, similar studies have been extended to parasites revealing the existence of only 13 prenylated proteins (Suazo et al. 2016). Since the inhibition of protein prenylation is lethal for cultured *Plasmodium falciparum* parasite, these results suggest crucial roles for the identified prenylated proteins. In an alternative approach, metabolic labeling with 8-anilogeranyl diphosphate and subsequent detection of tagged proteins using antibodies specific for the anilogeranyl moiety has also served for the detection of prenylated proteins (Onono et al. 2010).

3.2 Analysis and Identification of *N*-Myristoylation

The proteome-scale characterization of *N*-myristoylated proteins has been mainly performed via metabolic labeling with azido- and alkyne-tagged analogues of myristic acid (Charron et al. 2009; Hannoush and Sun 2010; Heal et al. 2008, 2011b). Since alkyne-containing lipids are usually preferred, tetradec-13-ynoic acid combined with biotin/avidin enrichment and MS proteomics has become the method of choice for the identification and analysis of the myristoylated proteome. Nevertheless, this method has an important limitation due to low specificity of tetradec-13-ynoic acid and its metabolism to long-chain fatty acids that results also in the labeling of *S*-acylated proteins and its incorporation into GPI anchors, thereby complicating the analysis of *N*-myristoylated proteins (Wright et al. 2014). Some strategies have been explored to overcome this initial limitation, including the selection of proteins bearing an *N*-terminal glycine and the use of NMT inhibitors to unambiguously assign real NMT substrates. Global profiling of the *N*-myristoylated proteome performed in human cells during normal cell function and apoptosis applying this technique resulted in the identification of 108 proteins yielding a MG motif. Similar studies were performed combining SILAC-mediated quantification and chemical inhibition of NMT resulting in the identification of 160 *N*-myristoylated proteins, of which 70 could be assigned with high confidence as NMT substrates and 56 were classified as non-substrates (Thinon et al. 2014).

NMT have also emerged as promising target for the treatment of eukaryotic parasites, and this therapeutic interest has strongly contributed to our understanding of *N*-myristoylation. Thus, Tate and coworkers developed a multifunctional probe bearing an azide and a biotin connected by a lysine-containing peptide and applied it to the characterization of *P. falciparum N*-myristoylome and to the validation of NMT as an antiparasitic target. Briefly, cells were fed with tetradec-13-ynoic acid, and the multifunctional linker was attached to the tagged proteins via copper-mediated click chemistry. The tagged proteins were then enriched and released by digestion with trypsin, and the resulting lipid-modified peptides were analyzed by tandem MS. The presence of the lysine in the lipid-modified peptides increases their ionization efficiency and facilitates the unambiguously determination of the site of modification (Wright et al. 2014). More recently, Tate and coworkers extended the development of multifunctional probes to linkers containing arginine, phenylalanine,

or aspartic acid residues cleavable by trypsin, chymotrypsin, and AspN, respectively (Broncel et al. 2015), and employed them to profile protein *N*-myristoylation during *Danio rerio* (zebrafish) embryonic development. Chemical reporters combined with label-free quantification or SILAC-based proteomics have been also employed to identify *N*-myristoylated proteins in different life cycle stages of *Leishmania donovani* (Wright et al. 2015) and *Trypanosoma cruzi*, respectively (Roberts and Fairlamb 2016).

Acylation not only has a crucial role in parasite infection, but it also regulates the host response to microbial and viral infection and can modulate the function of viral proteins. Consequently, global analysis of lipidated proteins has also been performed in infected cells with the aim of getting a better insight into the molecular mechanisms regulating these processes. As an example, characterization of myristoylated and palmitoylated proteins during infection with herpes simplex virus (HSV) revealed a reduction in myristoylation of host proteins and identified an important fraction of palmitoylated viral proteins, suggesting an important role of these proteins in viral pathogenesis (Serwa et al. 2015). Similarly, the effect of HIV-1 infection on protein acylation has been investigated using myristic and palmitic acid derivatives. Global profiling of acylated proteins in both HIV-infected and non-infected cells identified a fraction of palmitoylated and myristoylated proteins that it is significantly altered in infected cells (Colquhoun et al. 2015). Bacterial infection can also target the host myristoylation machinery. This is the case of the *Shigella* virulence factor IpaJ (invasion plasmid antigen J), a cysteine protease that irreversibly cleaves myristoylated glycines of host proteins as a mechanism of pathogenesis. Global myristoylome profiling in cells infected with either wild-type or an Δ*ipaJ Shigella* strain revealed that IpaJ is highly specific, and it acts only on members of the ARF (ADP-ribosylation factors) and ARL (Arf-like) family of GTPases and on the E3 ubiquitin ligase ZNRF2 (Burnaevskiy et al. 2015).

3.3 Analysis and Identification of *S*-Acylation

S-acylation has been mainly studied using two complementary approaches, metabolic labeling with azido or alkynyl fatty acids (Fig. 2b) and the acyl-biotin exchange (ABE) method that exploits the labile nature of the thioester linkage (Fig. 2a) (Drisdel and Green 2004). This latter approach was pioneered by Drisdel and Green, and it relies on the blocking of existent free sulfhydryl groups by *N*-ethylmaleimide (NEM) followed by the hydroxylamine-mediated cleavage of the palmitoyl thioester bonds and the subsequent labeling of the resulting free sulfhydryl with biotin derivatives (Fig. 2a). The labeled proteins can be then enriched using streptavidin agarose and identified by Western blotting or by proteomic analysis. Indeed, one of the first reported *S*-palmitoylome profiling study applied the ABE method to the yeast *S. cerevisiae* leading to the identification of 12 known and 35 new palmitoylated proteins (Roth et al. 2006). Later, this strategy was also employed for the analysis of the rat neural *S*-palmitome detecting 68 known palmitoylated proteins and more than 200 unknown candidates for palmitoylation,

thus uncovering novel functions of *S*-acylated proteins (Kang et al. 2008). This method has been also applied to the profiling of the *S*-palmitome in macrophages (Merrick et al. 2011), human platelets (Dowal et al. 2011), and B lymphocytes (Ivaldi et al. 2012) as well as to gain understanding of the substrate specificity of DHHC members (Huang et al. 2009). Moreover, ABE has also been employed together with quantitative proteomic techniques such as iCAT (Zhang et al. 2008), iTRAQ for identification of the *S*-acylation sites of proteins (Forrester et al. 2011), SILAM for the stable isotope labeling of mouse models of the Huntington's disease (Wan et al. 2013), or the ^{18}O labeling of proteins for the characterization of primary human T cells (Morrison et al. 2015). All in all, ABE is a robust method that has been widely employed for the large-scale analysis of *S*-palmitoylated proteins. In addition, some modifications of the ABE technique have been also reported and employed for the study of *S*-acylated proteins. Examples include the PalmPISC method, the acyl-RAC, and the acyl-PEG exchange. The PalmPISC method was developed combining ABE with trypsin digestion of the biotin-labeled proteins to identify the site of palmitoylation (Yang et al. 2010). This approach was applied to isolated lipid-raft enriched and non-raft membrane fractions and led to the localization of 25 known and 143 novel *S*-acylation candidate sites and the identification of 67 known and 331 novel *S*-acylated protein candidates. The study also revealed that *S*-acylated proteins show greater enrichment in the non-raft fractions than in the lipid raft-enriched fractions. Another modification of the ABE techniques is the resin-assisted capture (acyl-RAC). This technique replaces the biotinylation of free cysteines followed by avidin-based enrichment by the direct conjugation of the proteins to a resin containing thiol-reacting groups. The immobilized proteins can be then on-resin trypsinized and analyzed by mass spectrometry (Forrester et al. 2011). Similarly, acyl-PEG exchange relies on the modification of the free sulfhydryl groups with maleimide-functionalized polyethylene glycol (PEG) reagents. The high molecular weight of the PEG reagents induces a mobility shift that can be monitored by Western blot thus indicating the fraction of unmodified versus *S*-acylated protein and the number of acylation sites (Percher et al. 2016; Yokoi et al. 2016).

Apart from the use of the ABE method, metabolic labeling with azido- or alkynyl-fatty acids combined with bioorthogonal reactions has also strongly contributed the study of *S*-acylated proteins analogously to methods used for the global profiling of *N*-myristoylated and *S*-prenylated proteins (Fig. 2b). Initially, this approach was employed just to visualize fatty-acylated proteins (Charron et al. 2009; Hannoush and Arenas-Ramirez 2009; Kostiuk et al. 2008) and to monitor and correlate *S*-palmitoylation turnover and protein synthesis (Zhang et al. 2010). Martin and Cravatt applied it for the first time for the large-scale profiling of palmitoylated proteins (Martin and Cravatt 2009). This approach involved metabolic labeling of cells with an alkyne-containing 17-octadecynoic acid followed by reaction with a biotin–azide via CuAAC. Proteins were then enriched using streptavidin-coated beads and analyzed by the MudPIT technology leading to identification of 125 high-confidence or 200 medium-confidence palmitoylated proteins. Validation of 18 proteins was then performed by overexpression of fusion proteins tagged with

Flag tags together with metabolic labeling and copper-mediated reaction with rhodamine-azide. Of the 18 proteins analyzed, two proteins could not be validated, but 16 were confirmed to be palmitoylated, thus validating the method developed.

Metabolic labeling has also served to study the role of *S*-acylation in the pathogenesis of viral and fungal infections showing that the DHHC protein PFA4 is essential for the virulence of the yeast *Cryptococcus neoformans* (Santiago-Tirado et al. 2015) and that several *S*-acylated proteins are involved in the mechanism of host cell invasion by the protozoan parasite *Toxoplasma gondii* (Foe et al. 2015).

Quantitative proteomics has been also applied to the study of palmitoylated proteins using tagged lipids. Thus, Cravatt and coworkers employed metabolic labeling with 17-octadecenoic acid combined with SILAC for the characterization of palmitoylation dynamics and the quantification of palmitoylated proteins in mouse T-cell hybridoma cells, revealing that most of the proteins are stably palmitoylated whereas a subset of proteins linked to aberrant cell growth, migration, and cancer is tightly regulated by palmitoylation and depalmitoylation cycles (Martin et al. 2012). More recently, a bifunctional palmitic acid derivative containing a clickable alkyne and a photoactivatable diazirine has been prepared and employed for the identification of interacting partners of *S*-palmitoylated proteins after photo-cross linking experiments coupled with quantitative proteomic analysis. This approach was applied to the identification of binding partners of the interferon-induced transmembrane protein 3 (IFITM3) revealing 12 potential candidates of which two of them, CANX and BCAP31, could be further validated (Peng and Hang 2015).

In summary, two main techniques, ABE and metabolic labeling with tagged fatty acids, have been described and employed for the large-scale profiling of *S*-palmitoylated proteins. Although these techniques have generated a large body of evidence supporting the key role of lipid modification on protein function and localization, both methods present some advantages and limitations that deserve a mention. Hence, ABE can be applied to cell lysates and tissues, and it does not discriminate the nature of the endogenous acyl group. However all the included steps should be quantitative (blocking with NEM, hydrolysis of thioesters linkages) to avoid false-positives, and proteins that contain thioester intermediates, as ubiquitin ligases, will also be enriched using this approach. Metabolic labeling requires the use of fatty acid analogues, and it has a bias toward proteins that undergo a rapid palmitate turnover but enables the study of *S*-palmitoylation dynamics. Recently, both methods have been applied to the characterization of the palmitoylated proteome of *P. falciparum* schizont stage revealing a high degree of complementarity (57.2% of enriched proteins were detected in both approaches), thus showing that these methods may lead to complementary results and suggesting that the combination of both approaches may generate higher confidence datasets (Jones et al. 2012). Finally, all these studies focusing on the *S*-palmitome and its role in the regulation of several cellular processes have generated a large body of relevant information. To facilitate the analysis of all these data generated in the last decade, the *S*-palmitoylated proteins identified in 15 different *S*-acylation profiling studies have been recently

curated into one compendium containing 1838 genes (Sanders et al. 2015). Moreover, enrichment analysis of this compendium has provided additional information indicating a crucial role of *S*-acylated proteins in cancer and neurodegenerative disorders.

3.4 Analysis and Identification of Cholesterylation

Shh has a key role in embryonic development, and it is closely related with cancer progression. As a result, there has been an increasing interest in the development of tools to study Shh cholesterylation. Initially, Tate and coworkers described an azide-containing cholesterol analogue and used it as a substrate for the detection of cholesterylated proteins (Heal et al. 2011a). Other potentially cholesterylated proteins were detected suggesting that this modification may not be restricted to the members of the hedgehog family. Later the same authors described a set of alkyne sterol probes that could get incorporated into cells with a higher affinity and lower toxicity than the azide-containing probe. Moreover, an optimized alkyne sterol probe could be employed for the in vivo visualization of proteins in developing zebra fish embryos and for the SILAC-based quantification of endogenous Shh levels in pancreatic cancer cell lines (Ciepla et al. 2014).

3.5 Analysis and Identification of GPI-Anchor Modification

Proteomic analysis of GPI-anchored proteins has been limited by the low protein abundance and the poor sensitivity of the existing methods. However, despite these limitations and the complexity in structure and function of GPI anchors, some methods have been established for the profiling of GPI-anchored proteins and for the structural analysis of the attached GPI units (Varma and Hendrickson 2010). As example, GPI-anchored proteins have been detected after treatment with a phosphoinositol-specific phospholipase C (PI-PLC) in order to release the GPI units and further analysis of the proteins by MS (Elortza et al. 2003; Masuishi et al. 2013). More recently, the same authors have reported an improved methodology combining titanium dioxide-based affinity purification with hydrogen fluoride treatment for the site-specific analysis of GPI anchoring identifying 49 GPI-anchored proteins and 73 GPI-anchor modification sites (Masuishi et al. 2016). Direct identification of GPI-anchored proteins from the surface of intact cells has been also recently reported. This method relies on the metabolic labeling with an azido sugar analogue and enrichment of the PI-PLC released proteins using an alkyne agarose resin enabling the identification of GPI-anchored proteins without disrupting the cellular structure (Cortes et al. 2014). Alternatively, tetradec-13-ynoic acid has been shown to incorporate into GPI-anchored proteins and can be therefore also employed for the large-scale profiling of GPI-anchored proteins (Wright et al. 2014).

4 Research Needs

Major advances over the last decade in the field of MS-based proteomics have strongly contributed to our current understanding of the role of lipid modifications in regulating a wide range of cellular processes both in health and disease. More precisely, the combination of metabolic labeling with quantitative proteomics has proven particularly powerful, thus providing unique insights into the dynamics and potential druggability of protein lipidation. However, many challenges still lie ahead for the field of protein lipidation such as the discovery of novel lipidated substrates, the identification of new lipid modifications, as well as the detection of the site of modification. Another important field of research is the characterization of protein-lipid interactions, i.e., proteins that specifically interact with the lipid-modified proteins. Moreover, another unmet challenge is to elucidate how the dynamic range of these modifications is modulated during physiological and pathological processes with a particular emphasis on the functional role of protein lipidation in autophagy, infection processes, cancer, and neurodegenerative disorders. Quantitative proteomics has a tremendous potential in this particular field of research enabling the comparison of samples from healthy and diseased patients that may ultimately lead to the identification of potential targets for therapeutic interventions. The development of techniques addressing these issues are poised to make substantial contributions in the field of protein lipidation.

References

Ashby MN (1998) CaaX converting enzymes. Curr Opin Lipidol 9:99–102

Berry AFH, Heal WP, Tarafder AK, Tolmachova T, Baron RA, Seabra MC, Tate EW (2010) Rapid multilabel detection of geranylgeranylated proteins by using bioorthogonal ligation chemistry. ChemBioChem 11:771–773

Bowyer PW, Tate EW, Leatherbarrow RJ, Holder AA, Smith DF, Brown KA (2008) *N*-myristoyltransferase: a prospective drug target for protozoan parasites. ChemMedChem 3:402–408

Broncel M, Serwa RA, Ciepla P, Krause E, Dallman MJ, Magee AI, Tate EW (2015) Multifunctional reagents for quantitative proteome-wide analysis of protein modification in human cells and dynamic profiling of protein lipidation during vertebrate development. Angew Chem Int Ed Engl 54:5948–5951

Buddelmeijer N (2015) The molecular mechanism of bacterial lipoprotein modification – how, when and why? FEMS Microbiol Rev 39:246–261

Buglino JA, Resh MD (2008) Hhat is a palmitoylacyltransferase with specificity for *N*-palmitoylation of Sonic Hedgehog. J Biol Chem 283:22076–22088

Burnaevskiy N, Peng T, Reddick LE, Hang HC, Alto NM (2015) Myristoylome profiling reveals a concerted mechanism of ARF GTPase deacylation by the bacterial protease IpaJ. Mol Cell 58:110–122

Chan LN, Hart C, Guo L, Nyberg T, Davies BS, Fong LG, Young SG, Agnew BJ, Tamanoi F (2009) A novel approach to tag and identify geranylgeranylated proteins. Electrophoresis 30:3598–3606

Charron G, Zhang MZM, Yount JS, Wilson J, Raghavan AS, Shamir E, Hang HC (2009) Robust fluorescent detection of protein fatty-acylation with chemical reporters. J Am Chem Soc 131:4967–4975

Charron G, Li MM, MacDonald MR, Hang HC (2013) Prenylome profiling reveals S-farnesylation is crucial for membrane targeting and antiviral activity of ZAP long-isoform. Proc Natl Acad Sci U S A 110:11085–11090

Choy A, Dancourt J, Mugo B, O'Connor TJ, Isberg RR, Melia TJ, Roy CR (2012) The Legionella effector RavZ inhibits host autophagy through irreversible Atg8 deconjugation. Science 338:1072–1076

Ciepla P, Konitsiotis AD, Serwa RA, Masumoto N, Leong WP, Dallman MJ, Magee AI, Tate EW (2014) New chemical probes targeting cholesterylation of Sonic Hedgehog in human cells and zebrafish. Chem Sci 5:4249–4259

Colquhoun DR, Lyashkov AE, Ubaida Mohien C, Aquino VN, Bullock BT, Dinglasan RR, Agnew BJ, Graham DR (2015) Bioorthogonal mimetics of palmitoyl-CoA and myristoyl-CoA and their subsequent isolation by click chemistry and characterization by mass spectrometry reveal novel acylated host-proteins modified by HIV-1 infection. Proteomics 15:2066–2077

Cortes LK, Vainauskas S, Dai N, McClung CM, Shah M, Benner JS, Correa IR Jr, VerBerkmoes NC, Taron CH (2014) Proteomic identification of mammalian cell surface derived glycosylphosphatidylinositol-anchored proteins through selective glycan enrichment. Proteomics 14:2471–2484

Das U, Kumar S, Dimmock JR, Sharma RK (2012) Inhibition of protein *N*-myristoylation: a therapeutic protocol in developing anticancer agents. Curr Cancer Drug Targets 12:667–692

Distefano MD, DeGraw AJ, Palsuledesai C, Ochocki JD, Dozier JK, Lenevich S, Rashidian M (2010) Evaluation of alkyne-modified isoprenoids as chemical reporters of protein prenylation. Chem Biol Drug Des 76:460–471

Dowal L, Yang W, Freeman MR, Steen H, Flaumenhaft R (2011) Proteomic analysis of palmitoylated platelet proteins. Blood 118:e62–e73

Drisdel RC, Green WN (2004) Labeling and quantifying sites of protein palmitoylation. BioTechniques 36:276–285

Ducker CE, Upson JJ, French KJ, Smith CD (2005) Two *N*-myristoyltransferase isozymes play unique roles in protein myristoylation, proliferation, and apoptosis. Mol Cancer Res 3:463–476

El-Husseini AED, Bredt DS (2002) Protein palmitoylation: a regulator of neuronal development and function. Nat Rev Neurosci 3:791–802

Elortza F, Nuhse TS, Foster LJ, Stensballe A, Peck SC, Jensen ON (2003) Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. Mol Cell Proteomics 2:1261–1270

Farazi TA, Waksman G, Gordon JI (2001) The biology and enzymology of protein *N*-myristoylation. J Biol Chem 276:39501–39504

Foe IT, Child MA, Majmudar JD, Krishnamurthy S, van der Linden WA, Ward GE, Martin BR, Bogyo M (2015) Global analysis of palmitoylated proteins in *Toxoplasma gondii*. Cell Host Microbe 18:501–511

Forrester MT, Hess DT, Thompson JW, Hultman R, Moseley MA, Stamler JS, Casey PJ (2011) Site-specific analysis of protein S-acylation by resin-assisted capture. J Lipid Res 52:393–398

Fujita M, Kinoshita T (2010) Structural remodeling of GPI anchors during biosynthesis and after attachment to proteins. FEBS Lett 584:1670–1677

Furuishi K, Matsuoka H, Takama M, Takahashi I, Misumi S, Shoji S (1997) Blockage of *N*-myristoylation of HIV-1 gag induces the production of impotent progeny virus. Biochem Biophys Res Commun 237:504–511

Gerlach H, Laumann V, Martens S, Becker CFW, Goody RS, Geyer M (2010) HIV-1 Nef membrane association depends on charge, curvature, composition and sequence. Nat Chem Biol 6:46–53

Giang DG, Cravatt BF (1998) A second mammalian *N*-myristoyltransferase. J Biol Chem 273:6595–6598

Guan Z (2002) Identification and localization of the fatty acid modification in ghrelin by electron capture dissociation. J Am Soc Mass Spectrom 13:1443–1447

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999

Hall TMT, Porter JA, Young KE, Koonin EV, Beachy PA, Leahy DJ (1997) Crystal structure of a Hedgehog autoprocessing domain: homology between hedgehog and self-splicing proteins. Cell 91:85–97

Hannoush RN, Arenas-Ramirez N (2009) Imaging the lipidome: omega-alkynyl fatty acids for detection and cellular visualization of lipid-modified proteins. ACS Chem Biol 4:581–587

Hannoush AN, Sun J (2010) The chemical toolbox for monitoring protein fatty acylation and prenylation. Nat Chem Biol 6:498–506

Heal WP, Wickramasinghe SR, Bowyer PW, Holder AA, Smith DF, Leatherbarrow RJ, Tate EW (2008) Site-specific N-terminal labelling of proteins *in vitro* and *in vivo* using *N*-myristoyl transferase and bioorthogonal ligation chemistry. Chem Commun (4):480–482

Heal WP, Jovanovic B, Bessin S, Wright MH, Magee AI, Tate EW (2011a) Bioorthogonal chemical tagging of protein cholesterylation in living cells. Chem Commun 47:4081–4083

Heal WP, Wright MH, Thinon E, Tate EW (2011b) Multifunctional protein labeling via enzymatic N-terminal tagging and elaboration by click chemistry. Nat Protoc 7:105–117

Hoffman MD, Kast J (2006) Mass spectrometric characterization of lipid-modified peptides for the analysis of acylated proteins. J Mass Spectrom 41:229–241

Huang K, Sanders S, Singaraja R, Orban P, Cijsouw T, Arstikaitis P, Yanai A, Hayden MR, El-Husseini A (2009) Neuronal palmitoyl acyl transferases exhibit distinct substrate specificity. FASEB J 23:2605–2615

Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M et al (2000) A ubiquitin-like system mediates protein lipidation. Nature 408:488–492

Itzen A, Goody RS (2011) GTPases involved in vesicular trafficking: structures and mechanisms. Semin Cell Dev Biol 22:48–56

Ivaldi C, Martin BR, Kieffer-Jaquinod S, Chapel A, Levade T, Garin J, Journet A (2012) Proteomic analysis of S-acylated proteins in human B cells reveals palmitoylation of the immune regulators CD20 and CD23. PLoS One 7:e37187

Ji Y, Leymarie N, Haeussler DJ, Bachschmid MM, Costello CE, Lin C (2013) Direct detection of S-palmitoylation by mass spectrometry. Anal Chem 85:11952–11959

Jiang H, Khan S, Wang Y, Charron G, He B, Sebastian C, Du J, Kim R, Ge E, Mostoslavsky R et al (2013) SIRT6 regulates TNF-alpha secretion through hydrolysis of long-chain fatty acyl lysine. Nature 496:110–113

Jones ML, Collins MO, Goulding D, Choudhary JS, Rayner JC (2012) Analysis of protein palmitoylation reveals a pervasive role in *Plasmodium* development and pathogenesis. Cell Host Microbe 12:246–258

Julius M, Marmor MD (2000) The function of GPI-anchored proteins in T cell development, activation and regulation of homeostasis. J Biol Regul Homeost Agents 14:99–115

Kaczorowska MA, Cooper HJ (2013) Electron capture dissociation and collision induced dissociation of S-dipalmitoylated peptides. J Am Soc Mass Spectrom 24:1224–1227

Kang RJ, Wan JM, Arstikaitis P, Takahashi H, Huang K, Bailey AO, Thompson JX, Roth AF, Drisdel RC, Mastro R et al (2008) Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. Nature 456:904–909

Kho Y, Kim SC, Jiang C, Barma D, Kwon SW, Cheng JK, Jaunbergs J, Weinbaum C, Tamanoi F, Falck J et al (2004) A tagging-via-substrate technology for detection and proteomics of farnesylated proteins. Proc Natl Acad Sci U S A 101:12479–12484

Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, Ohsumi M, Takao T, Noda T, Ohsumi Y (2000) The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol 151:263–275

Konstantinopoulos PA, Karamouzis MV, Papavassiliou AG (2007) Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. Nat Rev Drug Discov 6:540–555

Kostiuk MA, Corvi MM, Keller BO, Plummer G, Prescher JA, Hangauer MJ, Bertozzi CR, Rajaiah G, Falck JR, Berthiaume LG (2008) Identification of palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue. FASEB J 22:721–732

Kurokawa K, Kim MS, Ichikawa R, Ryu KH, Dohmae N, Nakayama H, Lee BL (2012) Environment-mediated accumulation of diacyl lipoproteins over their triacyl counterparts in *Staphylococcus aureus*. J Bacteriol 194:3299–3306

Kwok Y, Sung WC, Lin AL, Liu HH, Chou FA, Hsieh SS, Leng CH, Chong P (2011) Rapid isolation and characterization of bacterial lipopeptides using liquid chromatography and mass spectrometry analysis. Proteomics 11:2620–2627

Lemmon MA (2007) Pleckstrin homology (PH) domains and phosphoinositides. Biochem Soc Symp 7:81–93

Levental I, Grzybek M, Simons K (2010) Greasing Tteir way: lipid modifications determine protein association with membrane rafts. Biochemistry 49:6305–6316

Li YX, Shao YH, Deng NY (2011) Improved prediction of palmitoylation sites using PWMs and SVM. Protein Pept Lett 18:186–193

Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR 3rd (1999) Direct analysis of protein complexes using mass spectrometry. Nat Biotechnol 17:676–682

Liu HB, Sadygov RG, Yates JR (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76:4193–4201

Lobo S, Greentree WK, Linder ME, Deschenes RJ (2002) Identification of a Ras palmitoyltransferase in *Saccharomyces cerevisiae*. J Biol Chem 277:41268–41273

Martin BR, Cravatt BF (2009) Large-scale profiling of protein palmitoylation in mammalian cells. Nat Methods 6:135–138

Martin DDO, Beauchamp E, Berthiaume LG (2011) Post-translational myristoylation: fat matters in cellular life and death. Biochimie 93:18–31

Martin BR, Wang C, Adibekian A, Tully SE, Cravatt BF (2012) Global profiling of dynamic protein palmitoylation. Nat Methods 9:84–U205

Masuishi Y, Nomura A, Okayama A, Kimura Y, Arakawa N, Hirano H (2013) Mass spectrometric identification of glycosylphosphatidylinositol-anchored peptides. J Proteome Res 12:4617–4626

Masuishi Y, Kimura Y, Arakawa N, Hirano H (2016) Identification of glycosylphosphatidylinositol-anchored proteins and omega-sites using TiO_2-based affinity purification followed by hydrogen fluoride treatment. J Proteome 139:77–83

Maurer-Stroh S, Eisenhaber F (2004) Myristoylation of viral and bacterial proteins. Trends Microbiol 12:178–185

Merrick BA, Dhungana S, Williams JG, Aloor JJ, Peddada S, Tomer KB, Fessler MB (2011) Proteomic profiling of S-acylated macrophage proteins identifies a role for palmitoylation in mitochondrial targeting of phospholipid scramblase 3. Mol Cell Proteomics 10:M110.006007

Mitchell DA, Mitchell G, Ling Y, Budde C, Deschenes RJ (2010) Mutational analysis of *Saccharomyces cerevisiae* Erf2 reveals a two-step reaction mechanism for protein palmitoylation by DHHC enzymes. J Biol Chem 285:38104–38114

Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M, Ohsumi Y (1998) A protein conjugation system essential for autophagy. Nature 395:395–398

Morrison E, Kuropka B, Kliche S, Brugger B, Krause E, Freund C (2015) Quantitative analysis of the human T cell palmitome. Sci Rep 5:11598

Nagamune K, Nozaki T, Maeda Y, Ohishi K, Fukuma T, Hara T, Schwarz RT, Sutterlin C, Brun R, Riezman H et al (2000) Critical roles of glycosylphosphatidylinositol for *Trypanosoma brucei*. Proc Natl Acad Sci U S A 97:10336–10341

Nakayama H, Kurokawa K, Lee BL (2012) Lipoproteins in bacteria: structures and biosynthetic pathways. FEBS J 279:4247–4268

Narita SI, Tokuda H (in press) Bacterial lipoproteins; biogenesis, sorting and quality control. Biochim Biophys Acta

Nguyen UTT, Cramer J, Gomis J, Reents R, Gutierrez-Rodriguez M, Goody RS, Alexandrov K, Waldmann H (2007) Exploiting the substrate tolerance of farnesyltransferase for site-selective protein derivatization. ChemBioChem 8:408–423

Nguyen UTT, Guo Z, Delon C, Wu YW, Deraeve C, Fraenzel B, Bon RS, Blankenfeldt W, Goody RS, Waldmann H et al (2009) Analysis of the eukaryotic prenylome by isoprenoid affinity tagging. Nat Chem Biol 5:227–235

Nimchuk Z, Marois E, Kjemtrup S, Leister RT, Katagiri F, Dangl JL (2000) Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. Cell 101:353–363

Onono FO, Morgan MA, Spielmann HP, Andres DA, Subramanian T, Ganser A, Reuter CW (2010) A tagging-via-substrate approach to detect the farnesylated proteome using two-dimensional electrophoresis coupled with western blotting. Mol Cell Proteomics 9:742–751

Paulick MG, Bertozzi CR (2008) The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. Biochemistry 47:6991–7000

Peng T, Hang HC (2015) Bifunctional fatty acid chemical reporter for analyzing S-palmitoylated membrane protein-protein interactions in mammalian cells. J Am Chem Soc 137:556–559

Percher A, Ramakrishnan S, Thinon E, Yuan X, Yount JS, Hang HC (2016) Mass-tag labeling reveals site-specific and endogenous levels of protein S-fatty acylation. Proc Natl Acad Sci U S A 113:4302–4307

Raju RVS, Moyana TN, Sharma RK (1996) Increased expression of *N*-myristoltransferase in human colonic tumors. FASEB J 10:1309–1309

Rauniyar N, McClatchy DB, Yates JR 3rd (2013) Stable isotope labeling of mammals (SILAM) for *in vivo* quantitative proteomic analysis. Methods 61:260–268

Reddy KD, Malipeddi J, DeForte S, Pejaver V, Radivojac P, Uversky VN, Deschenes RJ (2016) Physicochemical sequence characteristics that influence S-palmitoylation propensity. J Biomol Struct Dyn1–14

Ren J, Wen L, Gao X, Jin C, Xue Y, Yao X (2008) CSS-Palm 2.0: an updated software for palmitoylation sites prediction. Protein Eng Des Sel 21:639–644

Resh MD (2006a) Palmitoylation of ligands, receptors, and intracellular signaling molecules. Sci STKE 2006:re14

Resh MD (2006b) Trafficking and signaling by fatty-acylated and prenylated proteins. Nat Chem Biol 2:584–590

Roberts AJ, Fairlamb AH (2016) The *N*-myristoylome of *Trypanosoma cruzi*. Sci Rep 6:31078

Rocks O, Peyker A, Kahms M, Verveer PJ, Koerner C, Lumbierres M, Kuhlmann J, Waldmann H, Wittinghofer A, Bastiaens PIH (2005) An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. Science 307:1746–1752

Rocks O, Gerauer M, Vartak N, Koch S, Huang ZP, Pechlivanis M, Kuhlmann J, Brunsveld L, Chandra A, Ellinger B et al (2010) The palmitoylation machinery is a spatially organizing system for peripheral membrane proteins. Cell 141:458–471

Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S et al (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3:1154–1169

Roth AF, Wan JM, Bailey AO, Sun BM, Kuchar JA, Green WN, Phinney BS, Yates JR, Davis NG (2006) Global analysis of protein palmitoylation in yeast. Cell 125:1003–1013

Sanders SS, Martin DD, Butland SL, Lavallee-Adam M, Calzolari D, Kay C, Yates JR 3rd, Hayden MR (2015) Curation of the mammalian palmitoylome indicates a pivotal role for palmitoylation in diseases and disorders of the nervous system and cancers. PLoS Comput Biol 11:e1004405

Santiago-Tirado FH, Peng T, Yang M, Hang HC, Doering TL (2015) A single protein *S*-acyl transferase acts through diverse substrates to determine cryptococcal morphology, stress tolerance, and pathogenic outcome. PLoS Pathog 11:e1004908

Seeberger PH, Kamena F, Tamborrini M, Liu XY, Kwon YU, Thompson F, Pluschke G (2008) Synthetic GPI array to study antitoxic malaria response. Nat Chem Biol 4:238–240

Serwa RA, Abaitua F, Krause E, Tate EW, O'Hare P (2015) Systems analysis of protein fatty acylation in herpes simplex virus-infected cells using chemical proteomics. Chem Biol 22:1008–1017

Shipston MJ (2011) Ion channel regulation by protein palmitoylation. J Biol Chem 286:8709–8716

Sidransky D, Nagpal JK, Dasgupta S, Jadallah S, Chae YK, Ratovitski EA, Toubaji A, Netto GJ, Eagle T, Nissan A et al (2008) Profiling the expression pattern of GPI transamidase complex subunits in human cancer. Mod Pathol 21:979–991

Silvius JR, Lheureux F (1994) Fluorometric evaluation of the affinities of isoprenylated peptides for lipid bilayers. Biochemistry 33:3014–3022

Sletten EM, Bertozzi CR (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. Angew Chem Int Ed Engl 48:6974–6998

Smeland TE, Seabra MC, Goldstein JL, Brown MS (1994) Geranylgeranylated Rab proteins terminating in Cys–Ala–Cys, but not Cys–Cys, are carboxyl-methylated by bovine brain membranes *in-vitro*. Proc Natl Acad Sci U S A 91:10712–10716

Snook CF, Jones JA, Hannun YA (2006) Sphingolipid-binding proteins. Biochim Biophys Acta 1761:927–946

Stevenson FT, Bursten SL, Locksley RM, Lovett DH (1992) Myristyl acylation of the tumor necrosis factor alpha precursor on specific lysine residues. J Exp Med 176:1053–1062

Stevenson FT, Bursten SL, Fanton C, Locksley RM, Lovett DH (1993) The 31-kDa precursor of interleukin 1 alpha is myristoylated on specific lysines within the 16-kDa N-terminal propiece. Proc Natl Acad Sci U S A 90:7245–7249

Suazo KF, Schaber C, Palsuledesai CC, Odom John AR, Distefano MD (2016) Global proteomic analysis of prenylated proteins in *Plasmodium falciparum* using an alkyne-modified isoprenoid analogue. Sci Rep 6:38615

Thinon E, Serwa RA, Broncel M, Brannigan JA, Brassat U, Wright MH, Heal WP, Wilkinson AJ, Mann DJ, Tate EW (2014) Global profiling of co- and post-translationally *N*-myristoylated proteomes in human cells. Nat Commun 5:4919

Towler DA, Eubanks SR, Towery DS, Adams SP, Glaser L (1987) Amino-terminal processing of proteins by *N*-myristoylation – substrate-specificity of *N*-myristoyl transferase. J Biol Chem 262:1030–1036

Triola G (2011) The protein lipidation and its analysis. J Glycom Lipidom. https://doi.org/10.4172/2153-0637.S4172-4001

Triola G (2015) Chemical tools for modulating autophagy. Tetrahedron 71:387–406

Triola G, Waldmann H, Hedberg C (2012) Chemical biology of lipidated proteins. ACS Chem Biol 7:87–99

Varma Y, Hendrickson T (2010) Methods to study GPI anchoring of proteins. ChemBioChem 11:623–636

Wan J, Savas JN, Roth AF, Sanders SS, Singaraja RR, Hayden MR, Yates JR 3rd, Davis NG (2013) Tracking brain palmitoylation change: predominance of glial change in a mouse model of Huntington's disease. Chem Biol 20:1421–1434

Washburn MP, Wolters D, Yates JR (2001) Large-scale analysis of the yeast proteome by multi-dimensional protein identification technology. Nat Biotechnol 19:242–247

Witze ES, Old WM, Resing KA, Ahn NG (2007) Mapping protein post-translational modifications with mass spectrometry. Nat Methods 4:798–806

Wright MH, Clough B, Rackham MD, Rangachari K, Brannigan JA, Grainger M, Moss DK, Bottrill AR, Heal WP, Broncel M et al (2014) Validation of *N*-myristoyltransferase as an antimalarial drug target using an integrated chemical biology approach. Nat Chem 6:112–121

Wright MH, Paape D, Storck EM, Serwa RA, Smith DF, Tate EW (2015) Global analysis of protein *N*-myristoylation and exploration of *N*-myristoyltransferase as a drug target in the neglected human pathogen *Leishmania donovani*. Chem Biol 22:342–354

Xue Y, Chen H, Jin CJ, Sun ZR, Yao XB (2006) NBA-Palm: prediction of palmitoylation site implemented in Naive Bayes algorithm. BMC Bioinformatics 7:458

Yang W, Di Vizio D, Kirchner M, Steen H, Freeman MR (2010) Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. Mol Cell Proteomics 9:54–70

Yildirim MA, Goh KI, Cusick ME, Barabasi AL, Vidal M (2007) Drug–target network. Nat Biotechnol 25:1119–1126

Yokoi N, Fukata Y, Sekiya A, Murakami T, Kobayashi K, Fukata M (2016) Identification of PSD-95 depalmitoylating enzymes. J Neurosci 36:6431–6444

Zhang J, Planey SL, Ceballos C, Stevens SM Jr, Keay SK, Zacharias DA (2008) Identification of CKAP4/p63 as a major substrate of the palmitoyl acyltransferase DHHC2, a putative tumor suppressor, using a novel proteomics method. Mol Cell Proteomics 7:1378–1388

Zhang MM, Tsou LK, Charron G, Raghavan AS, Hang HC (2010) Tandem fluorescence imaging of dynamic S-acylation and protein turnover. Proc Natl Acad Sci U S A 107:8627–8632

Zhao C, Ma S (2014) Recent advances in the discovery of *N*-myristoyltransferase inhibitors. ChemMedChem 9:2425–2437

Membrane-Disrupting Proteins

39

Jeremy H. Lakey and Gregor Anderluh

Contents

1 Introduction 730
2 The Importance of Secondary Structure 731
3 The Alpha Helix 731
4 Beta Structure 734
5 Tryptophan 736
6 Specific Lipid Interactions 737
7 Conclusion 738
8 Research Needs 738
References 738

Abstract

The cell membrane is vulnerable to attack from various external toxins and environmental threats. The ability to damage membranes of target cells has evolved across biology as a way to procure food and defend against disease or attack from other species. The membrane disruption is mostly carried out by proteins, and, although they originate from across the full spectrum of biological diversity, they share common mechanisms at the molecular level. This chapter uses specific examples to show how the secondary structure of proteins

J. H. Lakey (✉)
Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK
e-mail: jeremy.lakey@ncl.ac.uk; j.h.lakey@ncl.ac.uk

G. Anderluh
Laboratory for Molecular Biology and Nanobiotechnology, National Institute of Chemistry, Ljubljana, Slovenia
e-mail: gregor.anderluh@ki.si

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_53

determines the various modes of action and how, starting from simple physico-chemical interactions, specific molecular recognition has arisen to increase the effectiveness of the toxins.

1 Introduction

Proteins that damage membranes are generally involved in either the promotion of, or inhibition of, pathogenesis in its broadest sense. From bacteria to bees and sea anemones to mammals, membrane damage is used for attack and defense (Anderluh and Lakey 2008). Rearrangement of eukaryotic membranes during normal cell turnover or their destruction during programmed cell death can also use some similar mechanisms, but these are less well understood and also not of direct relevance to this review.

Whatever the reason for their evolution, the proteins' main target is disruption of the impermeability of the cell membrane (Anderluh and Lakey 2008). The membrane surrounding the cell functions by creating a tight barrier between the cytoplasm and the exterior. This is achieved by a 3–5 nm hydrophobic barrier that has to be overcome by water-soluble molecules. Disruption of this barrier has grave consequences for most cells, although their sensitivity varies. Bacteria, with their small internal volumes, often unfriendly external environments and single membrane, which carries out many functions from nutrient uptake to energy conversion, are much more sensitive to small increases in permeability than eukaryotic cells. Bacteria are thus sensitive to a range of membrane-damaging molecules from amphipathic helices in so-called lytic peptides to large pore-forming toxins such as colicins which penetrate the two barriers of gram-negative bacteria.

Eukaryotic cells from multicellular organisms are relatively large, in protected environments and possessors of a whole range of specialized membrane compartments (Lakey et al. 1994b; Iacovache et al. 2008). It is possible to damage an animal cell which lacks the cell walls found in plants or fungi by colloid osmotic lysis. This occurs when the cell's water permeability is increased by toxin-induced pores. The cell is full of large macromolecules such as proteins, DNA, etc. which act as impermeable colloids which will not equilibrate across the bilayer. On the other hand, water and small solutes enter the cell in response to a lowered water potential and concentration gradients, respectively. The cell is unable to mitigate this flux and increase in volume until it lyses. Nevertheless, it has been found that membrane-penetrating proteins whose targets are eukaryotic often have subtle effects in intracellular compartments which do not rely upon the bursting of the cell (Iacovache et al. 2008). The clearest examples of these are the toxin delivering proteins which penetrate the target cell membrane in order to deliver cargo enzymes that target intracellular sites with extreme selectivity. Examples of these are diphtheria, anthrax, PMT (Pasteurella multocida toxin), cholera, and verotoxins. Even bacterial toxins which appear to rely upon pore formation alone have effects upon cells at concentrations which are considered sub-lytic and may depend upon their effects upon

intracellular compartments; these include aerolysin, alpha hemolysin, and the increasingly medically important LukF toxin from *Staphylococcus aureus*. In both groups, toxins have evolved to use intracellular compartments by receptor-mediated endocytosis. The delivery group appears to cross the intracellular membranes efficiently and does not appear to cause observable damage to the lipid bilayer itself (Iacovache et al. 2008).

This requirement for endocytosis reveals a feature of many membrane-damaging proteins which is their reliance upon surface receptors. The toxins must have the ability to be water and membrane soluble and have been termed Janus proteins after the Roman god who could look both into the past and the future and was often depicted upon doorways looking both outward and inside the house. To make the transition from water to the membrane, the proteins must have an affinity for some aspect of the membrane surface. The most obvious of these are proteinaceous cell surface receptors first recognized by Paul Ehrlich (1913). However, many membrane-damaging molecules have the ability to interact directly with the membrane bilayer itself. This chapter will cover the transition of membrane-damaging proteins from aqueous to membrane state and address some basic features of this process.

2 The Importance of Secondary Structure

Insertion of proteins into membranes is dependent upon the efficient masking of the peptide bond. In every n-residue peptide/protein, there are (n-1) peptide bonds (not taking into account the imide bonds of X-proline linkages), so they are both universal and are as numerically important as side chains. If alone they will expose an amide proton and carbonyl oxygen, which, because of the nature of the peptide bond, are highly polarized hydrogen bond donors and acceptors, respectively. This makes them unfavorable membrane components unless they can replace water as a hydrogen-bonding partner with something else. In fact burial of a hydrogen bond in the membrane incurs an energy cost approximating to that of charged side chains. The most efficient way to ensure the neutralization of the peptide bond polarity is to form hydrogen bonds with other peptide bonds by the formation of an alpha helix or beta sheet structure (Fig. 1). It is because of this that an understanding of the role of secondary structure in membrane-damaging peptides and proteins is so crucial. In the majority of cases, the proteins fall into one or other secondary structure family, and this is understandable when their relative properties are understood.

3 The Alpha Helix

Alpha helices, as will be exemplified by the bee venom peptide melittin, create a hydrogen-bonding network that is contained within one contiguous section of polypeptide (Fig. 1a). Thus, if there are flexible links between the helices in large proteins, these can act as separate structural units (Fig. 1b) only interacting if they

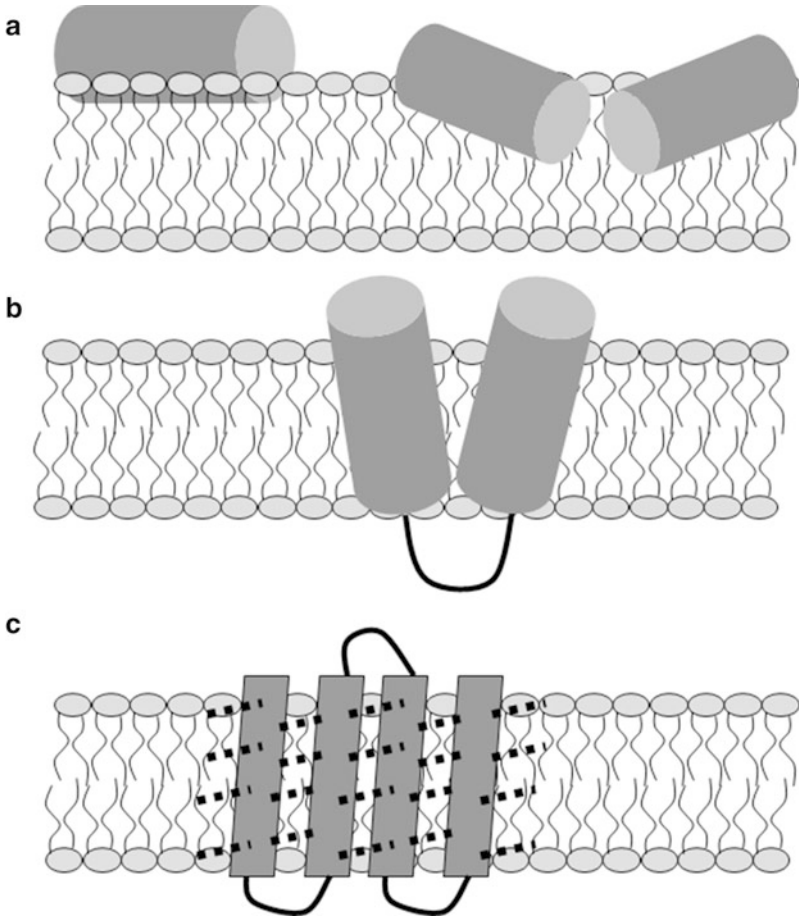


Fig. 1 Protein interactions with membranes. (**a**) Helices can adopt a surface-bound arrangement or form more deeply inserting structures. This is regulated by helix hydrophobicity, density of peptides at the surface, and lipid interactions. (**b**) The same general rules affect proteins made of groups of helices except that the helix-helix links restrict the rearrangement, and because their peptide bonds may not be hydrogen bonded, they may be unstable within the membrane core. (**c**) Beta structure forms hydrogen bonds between strands (*dotted lines*), but at the end of a beta sheet, the structure cannot expose edges to the bilayer core. Thus, beta barrels are more usual (see Fig. 3c)

have complementary surfaces defined by their side chains, which always point outward from the helix. The individual helices can be water soluble, hydrophobic, or as with melittin amphipathic. In the case of poly-helical membrane-damaging proteins, the elements arrange themselves according to similar rules but in this case guided by the inter-helix links (Kolter et al. 2005).

The alpha helix thus represents the simplest form of protein secondary structure for insertion into membranes. Its hydrophobicity is determined by the side chains which point outward from the helix, and here the nature of the membrane interaction

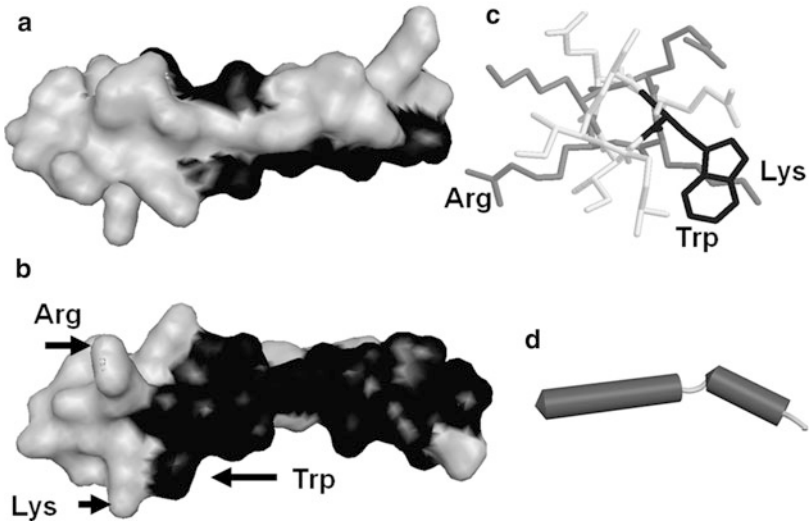


Fig. 2 Details of melittin, an example of a cationic membrane-damaging peptide. (**a**, **b**) Two different views of the helical form of melittin with the hydrophobic surfaces colored *dark*. (**c**) End view with the membrane interaction surface pointing downward and key Trp and cationic side chains highlighted. (**d**) Cartoon of helix structure showing the break in the peptide caused by the proline 14 residue

is defined. The example chosen here is melittin, although there are many examples of so-called lytic peptides (Bechinger 2004).

Melittin is a 26-residue cationic peptide from the venom of the European honeybee (*Apis mellifera*) (Fig. 2) (Raghuraman and Chattopadhyay 2007). The helix forms an amphipathic structure and can make a molecule with the required Janus behavior (Fig. 2a, b). Every third to fourth residue will point in one direction, and if hydrophobic, the amphipathic helix motif can be observed readily even in the primary structure. In some cases, such as melittin, the peptide may be unfolded in solution and then adopt the helix only when bound to the membrane (Bechinger 2004). In this way, the water solubility of the peptide is high in water due to the free peptide bonds, and then it can adopt the necessary hydrophobic peptide in the membrane. The attraction to the membrane surface is thus an important step, and the peptide must first be attracted to the hydrophobic membrane by other means. In most cases, the lytic peptides are cationic, and since most membranes bear a net negative charge, it is their lysine and arginine side chains that promote the first interactions. The long side chains of lysine and arginine also provide hydrophobic groups and thus play a critical amphiphilic role in the membrane interaction (Fig. 2c). Cationic antimicrobial peptides are in fact an essential part of innate immunity in both plants and animals (Bechinger and Lohner 2006). The other specific interaction is driven by tryptophan. The cartoon representation of melittin (Fig. 2d) reveals a break in the helix, and this is due to a proline at position 14. This creates a flexible backbone and is a feature of several lytic peptides. The lytic

peptides have varied roles in membrane damage. In bee venom, for example, they induce phospholipase activity, but more generally they can induce pores which can kill microbial and eukaryotic cells. There are several models of how the initial surface binding develops into pore formation. The fundamental two types are a transmembrane barrel formed by amphipathic helices, barrel stave, or wormhole models with their water-soluble surfaces facing the pore lumen and a carpet/detergent model where the density of lytic peptides at the membrane surface causes a break in the membrane structure. The tight binding of peptides to lipids and the dynamic states have suggested a toroidal pore in which the structure is a mixture of lipids and proteins (Fig. 1a). The membrane-disrupting proteins which are composed of helices (Lakey et al. 1994b; Kolter et al. 2005; Iacovache et al. 2008) comprise various combinations of hydrophobic, hydrophilic, and amphipathic helices. Examples such as the pore-forming colicins are folded in water solution with the hydrophobic sections buried (Fig. 3b) and then upon membrane binding unfold to allow the hydrophobic components to insert into the membrane and form membrane-disrupting structures. Usually the helix content is largely unchanged, and a rearrangement is the main feature of the insertion step (Lakey et al. 1994b; Anderluh and Lakey 2008).

4 Beta Structure

Beta-form membrane-damaging toxins can be separated into those in which the beta structure remains outside of the membrane and those in which it forms the membrane-penetrating structure. In the former, such as the example of equinatoxin from the sea anemone *Actinia equina*, the beta structure provides a rigid scaffold to which are attached lipid-binding and membrane-insertion structures (Fig. 3a). In the latter, the beta domain penetrates the membrane and had to conform to the limitations of polypeptide in the membrane (Fig. 3c). The nature of beta secondary structure is determined by hydrogen bonding between the peptide bonds of separate beta strands. The strands may be consecutive in the primary structure, but in all cases, at least a pair (minimally a beta hairpin) is needed to define beta structure. Whereas the free edge of a beta sheet in soluble proteins can interact with water, upon membrane insertion the peptides need to make complete sets of hydrogen-bonding interactions. This is illustrated in Fig. 1c, and it is clear that a flat beta sheet cannot be energetically stable in the hydrophobic core of the bilayer. The solution to this, found in all beta sheet membrane-disrupting proteins, is the rolling up of the beta sheet into a beta barrel. Thus, the free edges of the sheet become just another strand-strand hydrogen-bonding network. The structure is the same as outer membrane proteins from bacteria and mitochondria, but in general these barrels are composed of one polypeptide. In membrane-damaging toxins, the water to membrane-bound step involves an essential oligomerization, and thus, the membrane-penetrating beta barrel is composed of beta hairpins from several monomers. The clearest examples are the heptameric pore-forming toxins such as alpha hemolysin (Fig. 3c). The 14-stranded barrel is formed by beta hairpins

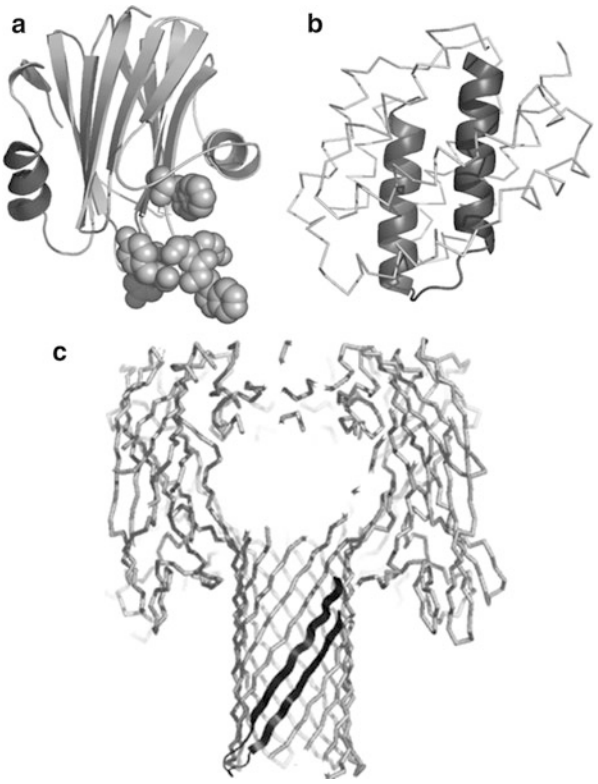


Fig. 3 Structures of membrane-damaging proteins (Anderluh and Lakey 2008). (**a**) Equinatoxin has a rigid beta sandwich core which does not penetrate the membrane. A tryptophan-rich loop (space-filling representation) binds the membrane interface and contains a sphingomyelin-binding pocket, while the amphipathic (melittin-like) N-terminal helix (*left*) moves downward into the membrane core to create a pore in an oligomer. (**b**) Colicin pore-forming domain, an antibacterial toxin from *Escherichia coli.* This is an example of a helical water-soluble toxin that rearranges its structure to create a membrane-spanning pore. The *dark* helices are fully hydrophobic and kept soluble by the amphipathic melittin-like peptides around them. (**c**) The alpha hemolysin from *Staphylococcus aureus* pore is composed of seven monomers each of which contributes a single beta hairpin (*highlighted dark*) to the barrel that crosses the membrane. The outside of the barrel exposes hydrophobic side chains, while the alternate side chains are hydrophilic and point inward making a nonspecific water-filled pore

from seven monomers. These hairpins are often unrecognizable in the water-soluble monomer which first binds to the surface via a receptor-binding step followed by a tryptophan-dependent interfacial interaction. Once in an oligomeric state, the prepore develops into the membrane-inserted form by extending the hydrophobic barrel into the bilayer. The side chains of a beta structure alternately point inward and outward down a strand, and thus, if every other side chain is hydrophobic, it ensures that the final structure is membrane compatible. This also means that the unassembled hairpin is only semihydrophobic and does not detract

from the water solubility of the soluble monomer. The cholesterol-dependent cytolysins produced by bacteria display a different arrangement in which the number of monomers is much greater (>30), and thus, large ring-shaped pores are formed in the target cell membrane.

5 Tryptophan

The role of tryptophan in the movement of peptides from water to membrane has proven to be surprisingly important. As it is the largest side chain and clearly hydrophobic, the likelihood that it would play a major role in membrane insertion could have easily been taken for granted. On closer inspection, however, it displays properties that have proven essential in the activity of several membrane-damaging proteins. The replacement of the single tryptophan in melittin significantly reduces activity. In the actinoporin equinatoxin II (EqtII) (Fig. 3a), tryptophan residues are present on the loop which first interacts with the membrane, and their replacement inhibits both binding to artificial membranes and in vivo toxicity (Hong et al. 2002). As in most biological interactions, the on-rate for binding is diffusion limited and possibly affected in the short range by electrostatics (e.g., cationic peptides) which can accelerate the approach over the last few nm and even play a role in orienting the protein correctly at the membrane surface (Lakey et al. 1994a). Nevertheless, it is the off-rate that really determines the observed affinity, and the insertion process requires an initial surface binding to provoke the structural changes that accompany the main insertion event. Specific side chains are thus fundamental for keeping the toxin at the membrane interface long enough for the second largely irreversible stage of insertion to occur. Often the tryptophans provide a fingerhold on the membrane by providing a way to catch hold of this generic interface. The next stages of EqtII membrane insertion are the specific binding to a sphingomyelin headgroup followed by the insertion of an amphipathic (melittin-like) helix, but this will not occur without the initial tryptophan interaction. The explanation for the behavior of tryptophan has largely been provided by the work of White and colleagues (Wimley and White 1996; Yau et al. 1998) who developed more definitive versions of the original amino acid hydrophobicity scales. White and co introduced the interfacial scale. This was supported by measurements on standard peptides in which the central residue was changed for each of the 20 possible variants. The tryptophan behavior is thought to be due to pi electrons which favorably interact with the interface (Yau et al. 1998). On integral membrane proteins, tyrosines are also found with their hydroxyl groups pointing outward across the interface, but because this orientation makes them unlikely anchors for invading proteins, it is tryptophan which is clearly the most important in interfacial binding.

The loop of EqtII is also rich in Tyr and Phe (Hong et al. 2002), and this pattern is also found in quite unrelated proteins such as the cholesterol-dependent cytolysins and anthrax toxin (Anderluh and Lakey 2008) or the sphingomyelinase from *Listeria* (Openshaw et al. 2005). In cholesterol-dependent cytolysins, the loop is well

characterized by X-ray crystallography and has been studied in depth (Anderluh and Lakey 2008). It is thought to be involved in the cholesterol binding and is another example of adopting an interfacial interaction in order to bind a poorly exposed membrane-buried receptor.

6 Specific Lipid Interactions

In some cases the interactions with lipids are highly specific and essential. In the case of EqtII, sphingomyelin is essential for full activity of the toxin, and it is interesting to note that the host organism *A. equina* (and other anemones) has a sphingomyelin analog which may protect them from their own toxin. Recognition of sphingomyelin is not straightforward as its headgroup phosphorylcholine is identical to the abundant phospholipid phosphatidylcholine which is ineffective as a receptor for EqtII. Measuring protein-lipid interactions is more difficult than studying protein interactions with soluble ligands, and a range of approaches which tackle this particular challenge, including dot blots and surface plasmon resonance techniques, have been performed. Using mutagenesis to remove residues from the large loop of EqtII (Bakrac et al. 2008) showed that recognition of the sphingomyelin-specific backbone structure was achieved by the interaction of the aromatic rings of Trp112 and Tyr 113 with the distinctive hydroxyl and amide groups of the sphingomyelin ceramide moiety. This was confirmed by NMR and molecular dynamics studies (Weber et al. 2015) and supports the suggestion that following the Trp insertion which is specific for interfacial interactions, and described in the previous section, these residues then provide a specific molecular recognition event which ensures that the toxin is associated with the lipids that will ensure the most efficient formation of pores in the membrane. While sphingomyelin and cholesterol are associated with ordered lipid raft areas, it appears that EqtII and related toxins preferentially bind to the borders or even areas of disordered lipids (Gilbert et al. 2014).

In general, pore-forming colicins interact with their initial target membrane, the outer surface of gram-negative bacteria, via high-affinity interactions with protein receptors such as BtuB and Cir, before progressing to disrupt the inner membrane and kill the cells. Recently it was shown that colicin N was unique in that it interacted with a membrane lipid. Gram-negative bacteria have an outer and inner membrane, and the outer leaflet of the former is composed entirely of hexa- or hepta-acylated anionic glycolipids called lipopolysaccharides. Colicin N was shown to be dependent upon a minimum size of LPS called Rc-LPS; removal of glucose and heptose moieties from this structure leads to cells which are resistant to the toxin (Johnson et al. 2014). As the colicin cannot bind to truncated LPS molecules in surface plasmon resonance assays, it appears that the initial membrane penetration requires recognition of the distal parts of the lipid. Thus, recent data have indicated that essential, specific lipid interactions by membrane-disrupting proteins can rely upon recognition of either water or membrane exposed regions.

7 Conclusion

Membrane-damaging proteins travel to their target as water-soluble precursors and thus must drive their own membrane binding and insertion steps. The critical phase of interaction is the interfacial binding, and this is driven by cationic and tryptophan interactions but may be followed by highly specific lipid-binding steps. The subsequent step is reliant upon hiding the peptide bonds from the membrane core, and this is achieved by the specific formation of secondary structure with hydrophobic side chains exposed in specific patterns.

8 Research Needs

To fully understand the mechanisms of these proteins, we need more highly resolved structural data. One crucial aspect is the need to resolve static structures with highly resolved lipids, and this is difficult to obtain from X-ray crystallography. Although cryoelectron microscopy is helping with the protein complexes, it cannot give us a clear picture of the protein-lipid interactions. Sometimes we will be lucky to trap lipids in such structures, but other structural and spectroscopic methods need to be developed to give us a better idea of both the high-resolution structural interactions and the dynamics of lipids in the vicinity of these proteins.

References

Anderluh G, Lakey JH (2008) Disparate proteins use similar architectures to damage membranes. Trends Biochem Sci 33(10):482–490

Bakrac B, Gutierrez-Aguirre I, Podlesek Z, Sonnen AFP, Gilbert RJC, Macek P, Lakey JH, Anderluh G (2008) Molecular determinants of sphingomyelin specificity of a eukaryotic pore-forming toxin. J Biol Chem 283(27):18665–18677

Bechinger B (2004) Structure and function of membrane-lytic peptides. Crit Rev Plant Sci 23 (3):271–292

Bechinger B, Lohner K (2006) Detergent-like actions of linear amphipathic cationic antimicrobial peptides. Biochim Biophys Acta 1758(9):1529–1539

Ehrlich P (1913) Address in pathology chemotherapeutics: scientific principle, methods and results. Lancet 182(4694):445–451

Gilbert RJC, Serra MD, Froelich CJ, Wallace MI, Anderluh G (2014) Membrane pore formation at protein-lipid interfaces. Trends Biochem Sci 39(11):510–516

Hong Q, Gutierrez-Aguirre I, Barlic A, Malovrh P, Kristan K, Podlesek V, Macek P, Gonzalez-Mañas J-M, Lakey JH, Anderluh G (2002) Two-step membrane binding by equinatoxin II, a pore-forming toxin from the sea anemone, involves an exposed aromatic cluster and a flexible helix. J Biol Chem 277(44):41916–41924

Iacovache I, van der Goot FG, Pernot L (2008) Pore formation: an ancient yet complex form of attack. Biochim Biophys Acta 1778(7–8):1611–1623

Johnson CL, Ridley H, Marchetti R, Silipo A, Griffin DC, Crawford L, Bonev B, Molinaro A, Lakey JH (2014) The antibacterial toxin colicin N binds to the inner core of lipopolysaccharide and close to its translocator protein. Mol Microbiol 92(3):440–452

Kolter T, Winau F, Schaible UE, Leippe M, Sandhoff K (2005) Lipid-binding proteins in membrane digestion, antigen presentation, and antimicrobial defense. J Biol Chem 280(50):41125–41128

Lakey JH, Parker MW, Gonzalez Manas JM, Duche D, Vriend G, Baty D, Pattus F (1994a) The role of electrostatic charge in the membrane insertion of colicin A: calculation and mutation. Eur J Biochem 220:155–163

Lakey JH, van der Goot FG, Pattus F (1994b) All in the family: the toxic activity of colicins. Toxicology 87:85–108

Openshaw AEA, Race PR, Monzo HJ, Vazquez-Boland JA, Banfield MJ (2005) Crystal structure of SmcL, a bacterial neutral sphingomyelinase C from Listeria. J Biol Chem 280(41):35011–35017

Raghuraman H, Chattopadhyay A (2007) Melittin: a membrane-active peptide with diverse functions. Biosci Rep 27(4–5):189–223

Weber DK, Yao S, Rojko N, Anderluh G, Lybrand TP, Downton MT, Wagner J, Separovic F (2015) Characterization of the lipid-binding site of equinatoxin II by NMR and molecular dynamics simulation. Biophys J 108(8):1987–1996

Wimley WC, White SH (1996) Experimentally determined hydrophobicity scale for proteins at membrane interfaces. Nat Struct Biol 3(10):842–848

Yau WM, Wimley WC, Gawrisch K, White SH (1998) The preference of tryptophan for membrane interfaces. Biochemistry 37(42):14713–14718

Modeling Lipid Membranes

40

Pouyan Khakbaz, Viviana Monje-Galvan, Xiaohong Zhuang, and Jeffery B. Klauda

Contents

1 Introduction 742
2 Brief History and Review of Lipid Force Fields for Membranes 744
3 Computational Approaches 746
4 What Can Be Simulated? 748
5 Research Needs 754
References 755

Abstract

Molecular modeling of lipid membranes has been an evolving field over the last 40 years. This chapter provides a brief historical background of simulations and provides an introductory overview of computational membrane modeling at the molecular level. The development of lipid force fields (FFs) at various levels (atomistic to coarse grained) has allowed for accurate descriptions of membrane properties. The current diversity in lipids available in FFs currently allows researchers to model representative membrane models across the biota spectrum. Modeling is not limited to lipids, and many functional studies of cellular membranes focus on membrane-associated proteins that reside in or interact with the

P. Khakbaz · V. Monje-Galvan · X. Zhuang
Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD, USA
e-mail: pkhakbaz@umd.edu; vmonje@umd.edu; xiaohongzhuang1924@gmail.com

J. B. Klauda (✉)
Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD, USA

Biophysics Program, University of Maryland, College Park, MD, USA
e-mail: jbklauda@umd.edu

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_52

surface of the membrane. The field of molecular membrane modeling is in an exciting stage to grow and investigate a wide array of biological phenomena.

1 Introduction

Cell membranes are essential to all living organisms as they separate internal from external material and act as a barrier to unwanted chemicals (van Meer et al. 2008). These membranes contain lipid molecules that are amphiphilic and can self-assemble into bilayer structures that present the hydrophilic head groups to the water phase and protect the hydrocarbon interior from water exposure. The membrane profile consists of five regions going from extra-membrane to the interior as roughly defined as bulk water, water/head group interface, head group, head group/hydrocarbon interface, and hydrocarbon. Although all membranes consist of these basic regions, variations exist due to the diversity of lipids present in the membrane, in the lipid head group, and in the acyl chains (van Meer et al. 2008). These variations can influence the thickness of the membrane, formation of lateral phase changes (lipid rafts (van Meer et al. 2008)), and interaction with proteins. Membranes are not limited to lipids and are well known to contain a multitude of proteins. The *fluid mosaic model* originally proposed by Singer and Nicholson in 1972 suggests a membrane dilute in proteins with proteins matching their hydrophobic exterior to that of an unperturbed lipid matrix (Engelman 2005; Singer and Nicolson 1972). This simple model has been challenged with more up-to-date research that demonstrates the membrane is much more concentrated with proteins (Engelman 2005; Takamori et al. 2006) with likely variation in thickness due to demixing of lipids and/or proteins.

Focusing on the lipid membrane, there are many methods to quantify structural and dynamic characteristics of membranes. Phase changes that exist in lipid membranes from liquid crystalline (L_α) to gel are commonly reported using differential scanning calorimetry (DSC) (Mannock et al. 2010). In membranes with cholesterol, bilayers can phase separate to liquid-ordered (L_o) and liquid-disordered (L_d) phases that can be characterized thermodynamically with DSC but also spatially with Förster resonance energy transfer (FRET) probes (Feigenson 2009) or X-ray/neutron scattering (Pan et al. 2013). FRET generally requires domains of the μm size to be optically resolved, and scattering techniques require extensive sampling for shorter timescales that limit its general use. X-ray and neutron scattering can also be used to probe the profile density of bilayers to provide insight into membrane thickness and functional groups location for single- (Nagle and Tristram-Nagle 2000) and multi-component lipid membranes (McIntosh and Simon 1994; Mojumdar et al. 2015). Scattering requires structural model fit to raw experimental form factors (Klauda et al. 2006), which can make it difficult to probe the membrane profile structure of multicomponent membranes. Molecular-level structure and dynamics can be probed with NMR using lipids that are isotopically labeled. Since lipids reside in a membrane plane, this order can be used to investigate head group and chain order with

respect to the bilayer normal. Deuterium order parameters (S_{CD}) are commonly used to provide such structure of lipids in membranes and allow for quantification of single- to multicomponent membranes. Typically these are done using lipids with fully deuterated acyl chains (Nagle and Tristram-Nagle 2000), but singly deuterated carbons have also been used for exact signal assignment (Yasuda et al. 2014). The S_{CD} can provide general information on lipid chain order, fluidity, and phase changes (Nagle and Tristram-Nagle 2000) that may exist in lipid membranes. In addition to membrane order, NMR can be used to investigate dynamical motions in lipids with spin-lattice relaxation rates (R_1) or its inverse (T_1). ^{13}C (Carbon-13) is commonly used to probe internal and collective motions of lipids (Klauda et al. 2008a). ^{31}P (Phosphate-31) can also be used to look at rotational motions that exist in lipids with the aid of interpretation from molecular simulations (Klauda et al. 2008b). NMR and other techniques can be applied to probe lateral motions of lipids in bilayers to quantify lipid diffusion constants (Gawrisch et al. 2002). There are various other experimental techniques that can probe bilayers, e.g., vibrational sum frequency generation (VSFG) (Ishiyama et al. 2016) and Raman microscopy (Ando et al. 2015), but many of these require interpretation of results with molecular-based models.

Although experimental techniques offer a detailed description of lipid bilayers, many techniques require models to interpret results (from X-ray, neutron, NMR, and VSFG measurements) (Klauda et al. 2006). This chapter focuses on the computational models of membranes to aid in our understanding of lipid bilayers and associated proteins. The advantage of molecular simulations is that details at the molecular and atomic level are directly obtained using mathematical models to represent these molecules and how they interact. This allows for direct study of bilayer structure and dynamics without the introduction of artificial labels that some experiments require. Careful interpretation of results is required based on validation of models against existing experimental measures. Moreover, computational simulations can be limited to a certain length- and timescales depending on the selected approach. The underlying force field (FF, mathematical representation of molecular interactions) is obtained to best match a set of experimental metrics, but one cannot fully expect exact comparisons to experiment due to FF inaccuracies in parameterization or functional assumptions. However, as we describe below, many cases demonstrate high accuracy of lipid FFs, and this is an exciting time in which membrane modeling can begin to probe models that better represent how these membranes behave *in vivo*.

This chapter is subdivided into four sections to provide some background in modeling membranes with the aid of different computational techniques. In Sect. 2, a brief history is presented on molecular simulation of lipid membranes with a focus on different FFs that have been developed to describe lipid structure and interactions. Section 3 contains an overview of computational approaches that utilize FFs to probe membrane function. Section 4 reviews some examples of what can be simulated using current methods for membranes and membrane-associated proteins. We conclude this chapter on potential future directions for this field in Sect. 5.

2 Brief History and Review of Lipid Force Fields for Membranes

Although experimental papers on lipid membranes have been published for over 70 years, the use of computational models is more recent. Molecular dynamics (MD) was originally published for simple hard spheres by Alder and Wainwright in 1956 and extended to a realistic Lennard-Jones (LJ) fluid in 1964 (Rahman 1964). The ability to semi-accurately model lipids required two decades of development, and by the late 1980s, initial efforts were made in molecular simulations of bilayers to compare with suggested previous experimentally based models (Pastor 1994). All-atom MD (AA-MD) at the 100-ps timescale was used to investigate short timescale motions and demonstrate that membrane viscosity is related to interactions at the interface (Venable et al. 1993). As computational power increased, studies of lipid membranes using MD also increased, and further comparison with experiments continued (Feller 2000). At the turn of the century, MD simulations were routinely run and able to probe the 10-ns timescale. This timescale allowed for comparison of MD simulations with many experimental measures and thus suggested the need for further development of lipid FFs.

We will now briefly review the lipid FFs available for molecular simulations starting from those that represent every atom. The focus here will be on common FFs and is not exhaustive in order to provide the reader with a brief overview of the history of lipid FF development. One of the more detailed methods to represent lipids is to explicitly include all atoms (AAs) in the FF (including hydrogen). The CHARMM family of FFs has been one that has developed from the initial stages of AA-MD (Feller 2000; Pastor 1994) to recent modifications. Details of the functional form and parametrization are left to the reader to further investigate (Klauda et al. 2008c); the aim here is to provide a brief overview of improvements in this FF. The first modification (CHARMM27, C27) (Feller and MacKerell 2000) focused on modifying interaction parameters, partial atomic charges, and torsional profiles of the hydrocarbon and phosphate to improve the lipid FF. This was subsequently improved with a focus on using higher-level quantum mechanical methods to obtain more robust torsional profiles of alkanes and improved comparison with acyl chain S_{CD}s and T_1s (Klauda et al. 2005, 2008a). The main deficiency of this and other FFs was the inability to accurately represent the lateral density (surface area/lipid). The CHARMM36 (C36) lipid FF solved this issue and is accurate for structural properties for a range of lipids in comparison with NMR and X-ray experiments (Klauda et al. 2010). This FF has been extended to a wide range of lipids observed *in vivo*, i.e., sterols (Lim et al. 2012), polyunsaturated fatty acids (Klauda et al. 2012), phosphatidylinositols (Li et al. 2009), sphingolipids/ceramides (Venable et al. 2014), unique acyl chains in bacterial lipids (Lim and Klauda 2011; Pandit and Klauda 2012), and lipopolysaccharides (Wu et al. 2013). The C36FF has allowed for more complex membrane models to be simulated (see Sect. 4) due to its ability to accurately represent structure in such membranes.

There are other AA lipid FFs that have been modified recently to best match experiment. Stockholm lipids (Slipids) (Jämbeck and Lyubartsev 2012, 2013) used

the C36 FF as a starting point for optimization but further adjusted non-bonded parameters using a scheme that is more compatible with the AMBER family of FFs. The AMBER community also has developed a lipid FF (LIPID14) compatible with its corresponding protein and nucleic acid FF (Dickson et al. 2014). The Slipids and LIPID14 FFs are comparable in accuracy to C36 (with slightly less lipid diversity) but should only be used with the AMBER family of FFs, as there are variations on how non-bonded parameters are modeled between biomolecular FFs.

Lipid FFs with AA representations are computationally demanding due to the inclusion of all hydrogens. An alternative is to use a united-atom (UA) approach where nonpolar hydrogens are combined with a non-hydrogen atom; for example, the CH, CH_2, and CH_3 groups would be considered as a single interaction site. This simplification greatly reduces the computational demand while maintaining near atomic details. The most common family of UA FFs used for lipid membranes is GROMOS (Chandrasekhar et al. 2003; Schuler et al. 2001). There are many versions of this FF with varying level of accuracy (please see the following review for more details (Lyubartsev and Rabinovich 2016)). The CHARMM community has also developed a UA-acyl chain FF for lipids and surfactants (C36UA) that is of comparable accuracy to the AA C36 lipid FF (Lee et al. 2014). Further development and testing are underway to demonstrate the general applicability of C36UA for most lipids available in the C36 AA lipid FF.

Even with UA FF, timescales are typically limited to μs on traditional computational resources for systems with up to a few hundred lipids. To probe larger membrane models or longer timescales, coarse-grained (CG) FFs have been developed to combine multiple non-hydrogen atoms into a single interaction site. This technique loses atomic detail but allows for molecular simulations of phase changes with a relatively minimal computational cost (Risselada and Marrink 2008). The most widely used CG FF for biomolecular simulations has been the MARTINI FF (Marrink et al. 2007; Monticelli et al. 2008). This FF generally follows a four-to-one mapping of non-hydrogen atoms for lipids and proteins (four non-hydrogen atoms represented by a single interaction site including the attached hydrogens). This scheme is also used for water, where a single water site represents four waters. CG-MD not only reduces the interaction sites compared to UA and AA but also allows longer simulation time steps (20–40 vs. 2fs) enhancing the length of these simulations. These CG-MD simulations can provide details on equilibrium conditions in a bilayer, but the simulation time does not correspond to "real time," and thus accurate dynamical information cannot be obtained with this approach. The MARTINI FF has been parametrized based on matching experimental partitioning measurements. The Shinoda-DeVane-Klein (SDK) CG FF is an alternative to MARTINI that has been parameterized using results from AA-MD simulations (Shinoda et al. 2010).

The other end of the CG lipid FFs spectrum is the recent development of a polarizable lipid FF that improves upon assumptions in traditional AA-MD (with constant atomic partial charges) (Chowdhary et al. 2013). These simulations offer potential improvements in representing chemical changes of molecules in differing environments where fixed-charge models fail. The development of the polarizable

FF for broad biomembrane research is limited and requires more optimization to a wider variety of lipid types.

3 Computational Approaches

The most common technique to running molecular simulations is the use of Newton's equation of motion $\boldsymbol{F} = m\boldsymbol{a}$, also known as Newton's second law, where $\boldsymbol{F}$ is the force and $\boldsymbol{a}$ is acceleration. This chapter only introduces the key parts of this technique; the reader should consult additional resources to gain a full knowledge of this technique (Allen and Tildesley 1987; Frenkel and Smit 2002). The FFs described above are used to predict the movement of atoms via the calculation of the force exerted on them. This requires initial conditions that are typically based on randomly assigning velocities to atoms that conform to a Boltzmann-like distribution to represent the desired temperature. With this initial condition, the system naturally evolves to motions based on interaction between atoms in molecules. AA-MD requires time steps that are shorter than the fastest motions and, typically, with constrained hydrogens to their attached atoms, i.e., 2 femtoseconds (fs). Therefore, if you desire to perform a simulation of 2 μs, 10^9 steps are needed for this simulation. The main advantage of UA- and CG-MD is that these simulations each can be run with longer time steps, thus extending the time range one can cover by using these methods (Fig. 1). AA-MD can reach the μs-timescale on moderately sized systems (under 50,000 atoms on up-to-date computational resources in order to obtain data in a few months), whereas CG-MD easily reaches this timescale and beyond (equivalent to one million atoms). The time needed for these simulations depends on the system size, as for each time step the computer code must calculate all given interactions – with more atoms more computations are required. In addition, considering the electronic structure of molecules with quantum mechanical (QM) methods limits the ability to reach beyond the picosecond timescale. It is crucial when developing a project that one considers the desired timescale/length scale to ensure that the computational technique is appropriate.

There have been other methods proposed to enhance conformational sampling at the cost of losing information on timescales. This has been important, for example, to studies in protein folding in a water solvent (Snow et al. 2005). Temperature replica exchange (TREX) (Snow et al. 2005; Sugita and Okamoto 1999) is a common approach for non-membrane system and requires many simulations run at different temperatures to allow for sampling to quickly hop over transitional barriers. In general, this is not possible with lipid membranes as they are only stable over a limited temperature range in which that enhancement will not be achieved. A corresponding technique for membranes is to use surface tension (not temperature) instead as the random walk (Mori et al. 2013). This approach was shown to enhance lateral diffusion of lipids and enhanced sampling of tilt angles of a transmembrane peptide. Other methods have been proposed to reduce transitional barriers such as transition-tempered metadynamics (Sun et al. 2016), adaptive biasing force (Hénin et al. 2010), accelerated MD (Wang et al. 2011), and replica exchange with solute

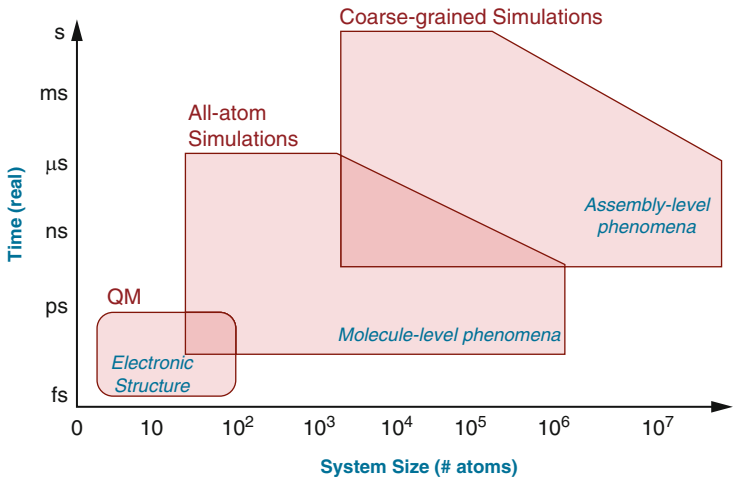


Fig. 1 Size and time plot for various methods used in molecular simulation

tempering (Huang and García 2014). Each of these methods has their own approach to enhancement but allows the use of existing FFs and has generally been used to investigate demixing in lipid membranes, free energy of chemical partitioning in membranes, and peptide conformations in model membranes.

An alternative technique to enhanced sampling is the use of hybrid multi-scale simulations that treat important regions at a higher level than surrounding regions. These techniques require a careful and verified method to combine simulations at multiple scales. One of the main assumptions in the FFs described above is that chemical bonds are not broken. To probe chemical reactions, such as ionization of an amino acid in a protein, one approach is to use a hybrid QM molecular mechanics (MM) technique that focus on a small region at the QM level (Riccardi et al. 2010), allowing for full-length proteins to be probed partially at this level. Switching from the QM scale is the use of UA-MD in conjunction with CG FFs. The PACE approach (Han and Schulten 2012) combines a UA protein model with MARTINI solvent and lipids to allow for a more detailed description of the protein and allow for its conformational flexibility. Similar procedures have been taken for AA/CG multi-scale modeling (Shi et al. 2006); the use of multi-scale modeling from the CG to mesoscopic level has been a focus of the Voth group (Shi and Voth 2005). These approaches have been used to look at lipid demixing and phase separation in model membranes (Shi and Voth 2005).

Another technique to enhance motions in membranes to probe interactions with proteins has been the recent development of the highly mobile membrane-mimetic (HMMM) model (Ohkubo et al. 2012). HMMM utilizes AA-MD but replaces full-length lipids with short-chained lipids and represents the hydrophobic core by an organic solvent. This simulation is especially useful for studying peptides and proteins that bind to the periphery of the membrane where long timescales are required with traditional AA-MD.

4 What Can Be Simulated?

With current computational power on high performance computing resources, there is a wide range of topics that can be probed using molecular simulations of model membranes. Initially, as described in Sect. 2, short simulations of single-component lipid membranes were the main focus of model membrane research. One challenge was setting up these membranes to start close to the natural state. The CHARMM-GUI *Membrane Builder* (Jo et al. 2007; Wu et al. 2014a) has allowed for easy setup of these systems. Currently, single-lipid membranes containing 100 lipids can easily be simulated for several 100 ns with multiple replicas using high performance computational clusters, e.g., running on three nodes with 20 cores each. Although not fully representing *in vivo* membranes, these single-component membranes allow for development and refinement of lipid force field parameters (Klauda et al. 2010, 2012; Pandit and Klauda 2012; Venable et al. 2014) and have also been used to interpret experimental findings (Klauda et al. 2006, 2008a, b). These studies also allow for validation of lipid force field parameters for simulating more realistic membrane model and also serve as a control to compare effects of adding different lipids to the bilayer.

Recently, there has been some interest in going beyond a description of the liquid crystalline (L_α) lipid bilayer to simulating more ordered phases in bilayers. It is known that for most fully saturated lipids, there are phase changes from the L_α to an intermediate phase known as the ripple (P_β') and then at even lower temperatures to a gel structure (L_β') (Akabori and Nagle 2015). Most MD simulations have focused on the L_α phase as this is similar to the most common structure seen in nature, i.e., the liquid-disordered phase. However, studies have looked into phase changes in lipids either biasing the initial structure to that of the L_β' (Essmann et al. 1995; Venable et al. 2000) or probing structural changes in response to heating or cooling (Khakbaz and Klauda 2016; Leekumjorn and Sum 2007). The structural changes that potentially exist in these saturated phosphatidylcholine (PC) bilayers are shown in Fig. 2. The ripple phase is stable over a limited range of temperature (Akabori and Nagle 2015), roughly over 10 °C, and MD simulations have suggested a interdigitated structure of the minor arm and a major arm that looks gel-like (Khakbaz and Klauda 2016; Risselada and Marrink 2008).

More complex model membranes have also been studied considering a mixture of two to three lipids. These are still simplifications of naturally occurring membranes but can offer insights into phase changes and structural properties of membranes. It is well known that the addition of cholesterol to lipid membranes with saturated acyl chains will result in a condensing effect, i.e., the surface area per lipid will decrease and the bilayer will become more laterally dense (Edholm and Nagle 2005). Phospholipid AA-MD simulations with cholesterol have probed the structure of lipid bilayers at conditions that represent the liquid-disordered (L_d) and liquid-ordered (L_o) phases (Lim and Klauda 2011; O'Connor and Klauda 2011). For membranes with a majority of cholesterol (to model ocular lens membranes), it was found that these membranes can form weblike structures of cholesterol-only domains

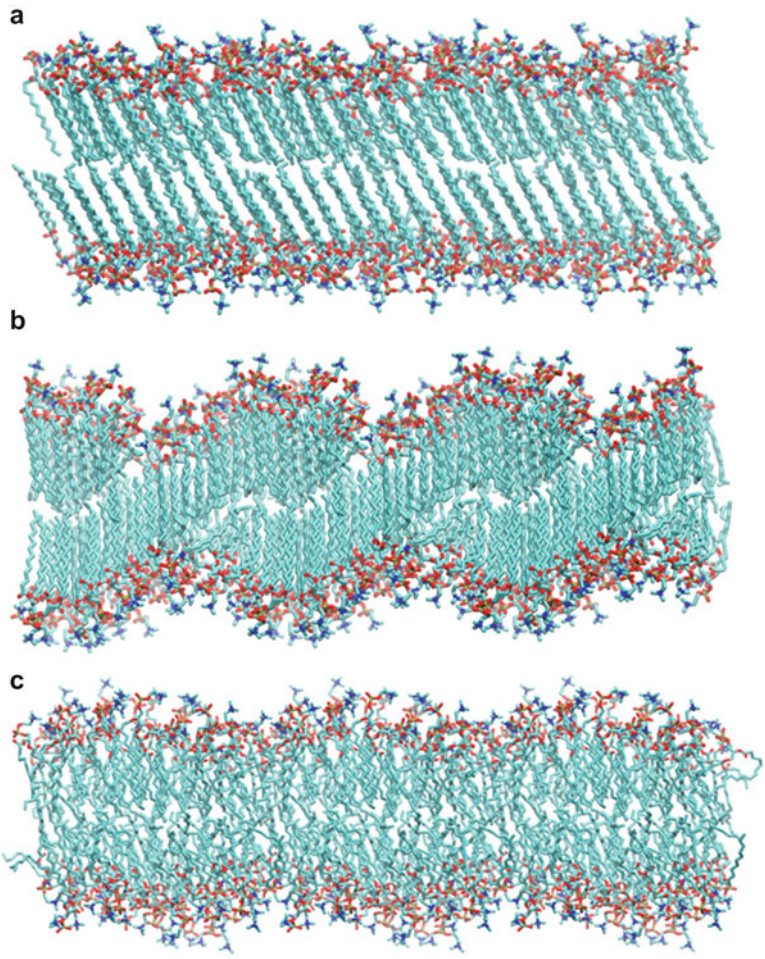


Fig. 2 End snapshots of the 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) bilayer (water is not shown but included in the AA-MD C36 simulation). (**a**) L_{β}' (gel) structure at 273 K after 500 ns, (**b**) P_{β}' (*ripple*) structure at 275 K after 500 ns, and (**c**) L_{α} (liquid crystalline) at 303 K after 300 ns

(O'Connor and Klauda 2011). Ternary lipid simulations have been used initially with CG-MD (Risselada and Marrink 2008) and more recently with AA-MD (Sodt et al. 2014, 2015) to probe L_d/L_o phase coexistence.

Recently, there has been a focus on developing model membranes of cells across the biota spectrum. CG FFs, particularly MARTINI, have reached a stage in which many of the biologically relevant lipids have been parameterized. This is also the case with the AA FF developed by the CHARMM community. Ingólfsson et al. performed CG-MD with the MARTINI FF on model membranes with 63 lipid types that were distributed with asymmetric leaflet composition (Ingólfsson et al. 2014). This work found that nanodomains existed with gangliolipids in the outer leaflet and

phosphatidylinositols for the inner leaflet. CG-MD has also been used to model membranes of plants and cyanobacteria (van Eerden et al. 2015). Using AA-MD the structure of model membranes of chlamydia (Lim and Klauda 2011), of the inner and outer membranes of *E. coli* (Khakbaz and Klauda 2015; Pandit and Klauda 2012; Wu et al. 2014b), and of yeast organelle membranes (Monje-Galvan and Klauda 2015) was also simulated. The structure of *E. coli* membranes was investigated by mimicking the composition changes during the colony growth cycle (Khakbaz and Klauda 2016). An example structure for the stationary phase of *E. coli* is shown in Fig. 3 and its associated electron density profile (EDP). The EDP shows the densest region of the membrane (the lipid head group region) and the least dense one (the center of the bilayer). This bacterial membrane can be compared to the model membrane of the endoplasmic reticulum (ER) of yeast (Fig. 3). The thicknesses of the model membranes for yeast and *E. coli* are similar, but the profile shows subtle differences. Yeast has larger head groups for its lipids, and thus the density's maximum is higher. The yeast model also contains more chain unsaturation, and thus the density at the center is lower.

In addition to modeling realistic membranes in the cell, there has been increased interest in probing the effects of lipid peroxidation and how this influences membrane structure. When free radicals chemically attack lipids (peroxidation), they react with double bonds and cleave these to form hydroperoxides (Yin et al. 2011). These ultimately form water-liking groups that can exist near the hydrophobic interior. MD simulations on oxidized lipids have shown that membranes with oxidized lipids decrease their lateral density and readily form hydrogen bonds with water (Wong-ekkabut et al. 2007). However, at high concentrations of aldehyde-modified lipids (>75%), these can form a water pore. We have also seen complex water pore formation in chemically modified lipids with ester groups in the acyl chain (Villanueva et al. 2013) (Fig. 4a, b). These lipids allow for water pore formation even at lower concentrations (30% of the ester-modified lipids), which lead us to suggest that having a mixed location of oxidation in the acyl chain will allow for pore formation at physiological concentrations of lipid oxidation (Villanueva et al. 2013).

Cell membrane damage is not limited to chemically modified lipids and can occur when chemicals partition into the lipid membrane. There has been a strong interest in using microbes to produce biofuels and certain chemicals (Dunlop 2011). In the United States, petroleum for cars routinely has ethanol as an additive (up to 10%), which is produced by microbes. However, microbe cell membranes can be damaged from what they produce or the associated by-products. Manipulation of the cell membrane lipids can allow for increased tolerance to various chemicals (Henderson and Block 2014; Liu et al. 2013). Until recently, the primary focus for ethanol has been how this alcohol influences single-lipid PC membranes to represent mammalian membranes (Dickey et al. 2009; Terama et al. 2008). More current studies have focused on the effect of the lipid head group on the tolerance of membranes to ethanol stress (Konas et al. 2015). Ethanol easily protrudes deeply into model membranes (Fig. 4c, d) at 12 mol%. An anionic lipid (phosphatidylserine, PS) was found to act as a protectant compared to zwitterionic lipids. However, when mixed

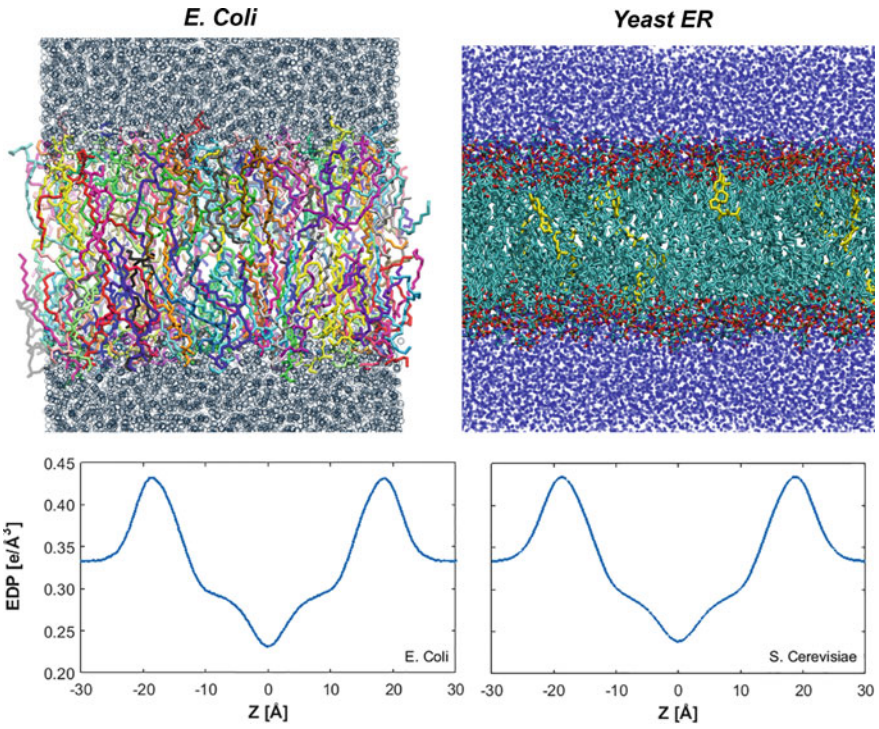


Fig. 3 (*left/top*) Snapshot of the *E. coli* membrane model for the stationary phase (Khakbaz and Klauda 2015). Water is in *dark blue* and lipids are in various colors. (*right/top*) Snapshot of the ER of yeast with water in *blue*, ergosterol in *yellow*, and remaining is lipids (Monje-Galvan and Klauda 2015). (*bottom*) The corresponding EDPs as a function of bilayer depth with $z = 0$ to represent the center of the bilayer

with phosphatidylethanolamine (PE), more ethanol penetrated into the bilayer compared to the single-component membrane. This result suggested that accurate models with representative lipids are needed to fully probe the cellular response to ethanol stress and are currently a focus in our lab.

Modeling cellular membrane ultimately requires the inclusion of transmembrane (TM) or peripheral membrane proteins (PMPs). The development of accurate lipid FFs has been crucial to allow for studies of these membrane-associated proteins. If interested, the reader can look at several review articles that cover some recent findings using molecular simulations of proteins in membranes (Li et al. 2015; Monje-Galvan and Klauda 2016; Stansfeld and Sansom 2011). TM channel and primary/secondary transporters have been the main focus of molecular simulations as these have a wide array of applications in biology. Water and ion channel proteins have been extensively investigated and are still an active area of computational research. Secondary active transporters (SATs) have complex conformational changes in transporting larger substrates. These proteins have outer regions that

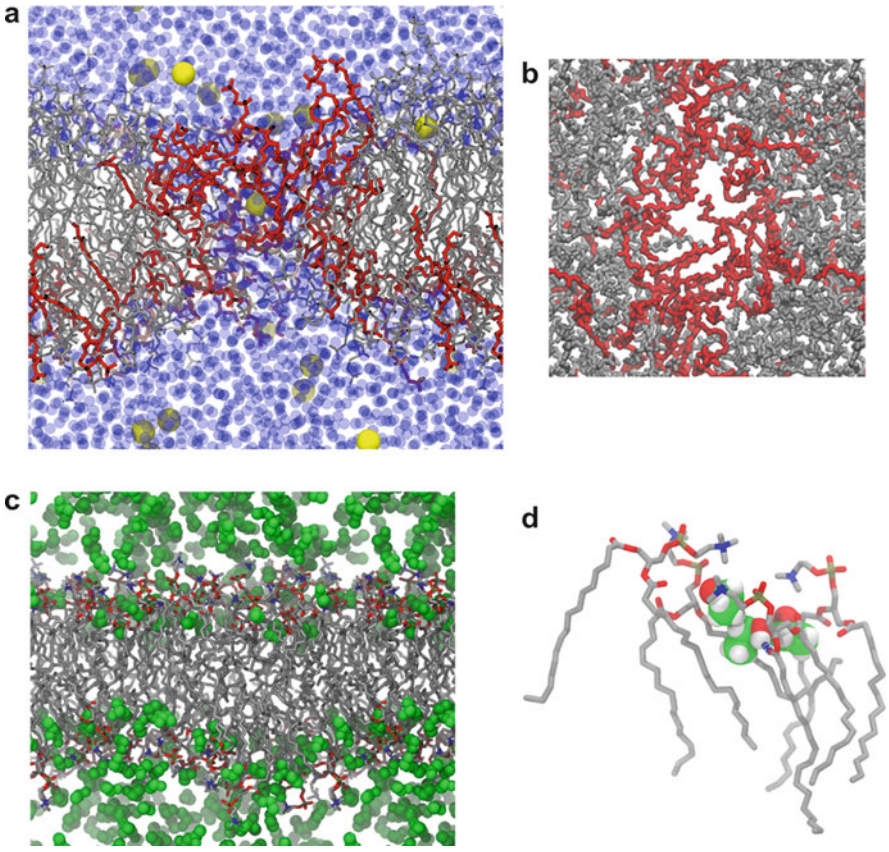


Fig. 4 (**a**) Cross-sectional view end snapshot of ester-modified lipid (in *red*) at 30% in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine(POPC, in *gray*) with water in *blue* and Na^+ in *yellow* (Villanueva et al. 2013). (**b**) Same system as (**a**) but now looking down on the membrane with the pore formed in *white*. (**c**) Ethanol (12%, *green*) in a POPC bilayer (*cross-sectional view*) (Konas et al. 2015). (**d**) Same system as C but zoomed-in view of ethanol (*green*, *C*; *white*, *H*; *red, oxygen*) interacting with POPC.

are attracted to the hydrophobic and hydrophilic sections of the membrane (Fig. 5). For lactose permease (LacY) of *E. coli*, an enhanced sampling method was used to probe conformational changes from the inward- to outward-facing states (Pendse et al. 2010). AA-MD was used to relax this structure in a native-like membrane as lipid-protein interactions can be important to protein structure.

Membrane-associated proteins that do not form TM domains, such as PMPs, have also been the focus of molecular simulations (Monje-Galvan and Klauda 2016). Lipids actively recruit proteins to the membrane surface either by specific or non-specific (electrostatic) interactions. We have focused on a lipid transport protein known as the oxysterol-binding protein homologue (Osh) in yeast. Probing

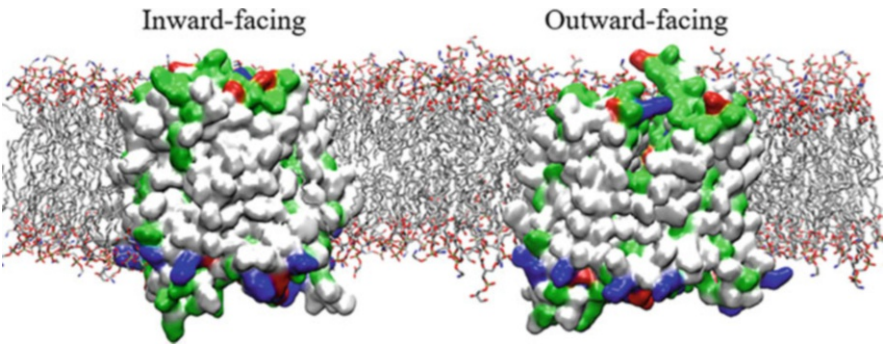


Fig. 5 These snapshots show the surface rendering of LacY in an equilibrated bilayer (*red/gray*). These two open-state structures are color coded with *white* (nonpolar), *green* (polar), *red* (acidic), and *blue* (basic)

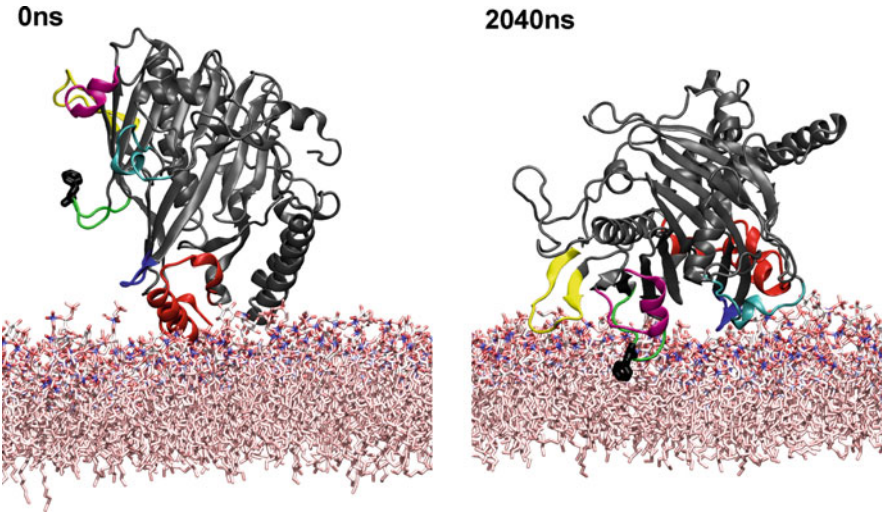


Fig. 6 MD simulations of Osh4 binding to the TGN membrane (beginning and end) (Monje-Galvan and Klauda 2016). Certain regions are colored: ALPS-like motif (*red*), Phe-loop (*green* with Phe3 in *black*), and cationic loops (*yellow* and *blue*)

lipid-protein interactions with experiments is difficult, but our μs AA-MD simulations have provided insight into the full-length Osh4 binding to model trans-Golgi network (TGN) and ER membranes. Snapshots of Osh4 binding to a TGN membrane are shown in Fig. 6. The protein uses non-specific interactions (cationic residues attracted to anionic lipids) to promote and stabilize binding to the membrane. Additional stabilization occurs with the Phe-loop penetration into the hydrophobic core of the membrane.

5 Research Needs

The computational biology field with applications to modeling cellular membranes is an exciting research area destined to significantly grow and probe more complex and larger biological systems and assemblies. The advancing computational power has allowed this field to explore many biologically relevant length and timescales. There is a continual need to develop accurate membrane models. It is well known that different strains of bacteria or growth conditions modify the cell membrane composition. This may be important to consider in future membrane modeling if changes dramatically influence cell membrane structure and dynamics.

Of particular focus in the field will be developing membrane models to represent cellular membranes in mammalian organs. For example, the complex membranes of the neurological system with sphingo- and glycolipids have yet to be fully studied. Lipid FF parameters and simple model systems have been tested, but these studies did not include diverse membrane models to represent cellular membranes that exist in neurons in the central and peripheral nervous system. Such studies will be necessary to fully probe ion channel protein function, neurological disorders, neurological autoimmune disorders, pathogenic attack of neurons, and mechanisms for myelin sheath formation. The ocular lens membrane also makes up for an interesting research need in understanding the complex structure of these unique membranes with a majority of cholesterol and how this relates to cataract formation (early onset or later in life). Another example is developing membrane models of the stratum corneum that form lipid multilayers with unique structures suggested from experiments (Mojumdar et al. 2015) and are not fully understood. These are only a few examples in which there is a need for further research using computational models for cellular membranes.

The need in membrane research goes beyond just lipid-only membranes but also includes further progression of research in membrane-associated proteins. The majority of current efforts at the AA-MD side have been on rather small membrane-associated proteins (less than 500 residues). However, there are many large complex proteins that form oligomers and super structures that are ripe for computational study. Some bacteria secrete α-hemolysin to act as a toxin and cause cell death. This large protein has also been studied as a way to measure molecules that pass through its pore. Initial simulations on this protein offer some insight into its function (Balijepalli et al. 2013), but more extensive work is needed to develop a tool to aid in biochemical, peptide, and nucleic acid sensing. Much larger neurotransmitter receptors such as the serotonin 5-HT3 receptor consist of a roughly 450-amino-acid protein that complexes into a pentamer. These gated-channel proteins have a wide range of importance in brain function and mental disorders. We have reached a critical stage in computational resources that these proteins can be simulated (Guros and Klauda 2016). Signaling proteins that have large intracellular and extracellular domains are also ripe for future studies. We have been focusing on a class of proteins known as plexins that are actively involved in various signaling controlling processes such as bone or cancer growth. Most membrane-associated research has focused on limited regions of the protein (Barton et al. 2016; Zhang

et al. 2015). However, with the recently discovered structure of the extracellular domain (Kong et al. 2016), there is strong potential for the study of dimer formation of this 1,200+ amino-acid protein using molecular simulations.

Acknowledgments Some of the research presented here and time spent on writing this work was supported by the NSF grant MCB-1149187.

References

Akabori K, Nagle JF (2015) Structure of the DMPC lipid bilayer ripple phase. Soft Matter 11:918–926

Allen MP, Tildesley DJ (1987) Computer simulations of liquids. Clarendon Press, Oxford

Ando J, Kinoshita M, Cui J, Yamakoshi H, Dodo K, Fujita K, Murata M, Sodeoka M (2015) Sphingomyelin distribution in lipid rafts of artificial monolayer membranes visualized by Raman microscopy. Proc Natl Acad Sci 112:4558–4563

Balijepalli A, Robertson JW, Reiner JE, Kasianowicz JJ, Pastor RW (2013) Theory of polymer-nanopore interactions refined using molecular dynamics simulations. J Am Chem Soc 135:7064–7072

Barton R, Khakbaz P, Bera I, Klauda JB, Iovine MK, Berger BW (2016) Interplay of specific trans- and juxtamembrane interfaces in plexin A3 dimerization and signal transduction. Biochemistry 55:4928–4938

Chandrasekhar I, Kastenholz M, Lins RD, Oostenbrink C, Schuler LD, Tieleman DP, van Gunsteren WF (2003) A consistent potential energy parameter set for lipids: dipalmitoylphosphatidylcholine as a benchmark of the GROMOS96 45A3 force field. Eur Biophys J Biophys Lett 32:67–77

Chowdhary J, Harder E, Lopes PEM, Huang L, MacKerell AD, Roux B (2013) A polarizable force field of dipalmitoylphosphatidylcholine based on the classical drude model for molecular dynamics simulations of lipids. J Phys Chem B 117:9142–9160

Dickey AN, Yim W-S, Faller R (2009) Using ergosterol to mitigate the deleterious effects of ethanol on bilayer structure. J Phys Chem B 113:2388–2397

Dickson CJ, Madej BD, Skjevik ÅA, Betz RM, Teigen K, Gould IR, Walker RC (2014) Lipid14: the amber lipid force field. J Chem Theory Comput 10:865–879

Dunlop MJ (2011) Engineering microbes for tolerance to next-generation biofuels. Biotechnol Biofuels 4:32

Edholm O, Nagle JF (2005) Areas of molecules in membranes consisting of mixtures. Biophys J 89:1827–1832

Engelman DM (2005) Membranes are more mosaic than fluid. Nature 438:578–580

Essmann U, Perera L, Berkowitz ML (1995) The origin of the hydration interaction of lipid bilayers from MD simulation of dipalmitoylphosphatidylcholine membranes in gel and liquid crystalline phases. Langmuir 11:4519–4531

Feigenson GW (2009) Phase diagrams and lipid domains in multicomponent lipid bilayer mixtures. Biochim Biophys Acta Biomembr 1788:47–52

Feller SE (2000) Molecular dynamics simulations of lipid bilayers. Curr Opin Colloid Interface Sci 5:217–223

Feller SE, MacKerell AD Jr (2000) An improved empirical potential energy function for molecular simulations of phospholipids. J Phys Chem B 104:7510–7515

Frenkel D, Smit B (2002) Understanding molecular simulations: from algorithms to applications. Academic, San Diego

Gawrisch K, Eldho NV, Polozov IV (2002) Novel NMR tools to study structure and dynamics of biomembranes. Chem Phys Lipids 116:135–151

Guros NB, Klauda JB (2016) Characterizing nanopore-polymer interactions and cys-loop protein receptor gating. Biophys J 110:630a–631a

Han W, Schulten K (2012) Further optimization of a hybrid united-atom and coarse-grained force field for folding simulations: improved backbone hydration and interactions between charged side chains. J Chem Theory Comput 8:4413–4424

Henderson CM, Block DE (2014) Examining the role of membrane lipid composition in determining the ethanol tolerance of *Saccharomyces cerevisiae*. Appl Environ Microbiol 80:2966–2972

Hénin J, Fiorin G, Chipot C, Klein ML (2010) Exploring multidimensional free energy landscapes using time-dependent biases on collective variables. J Chem Theory Comput 6:35–47

Huang K, García AE (2014) Acceleration of lateral equilibration in mixed lipid bilayers using replica exchange with solute tempering. J Chem Theory Comput 10:4264–4272

Ingólfsson HI, Melo MN, van Eerden FJ, Arnarez C, Lopez CA, Wassenaar TA, Periole X, de Vries AH, Tieleman DP, Marrink SJ (2014) Lipid organization of the plasma membrane. J Am Chem Soc 136:14554–14559

Ishiyama T, Terada D, Morita A (2016) Hydrogen-bonding structure at zwitterionic lipid/water interface. J Phys Chem Lett 7:216–220

Jämbeck JPM, Lyubartsev AP (2012) An extension and further validation of an all-atomistic force field for biological membranes. J Chem Theory Comput 8:2938–2948

Jämbeck JPM, Lyubartsev AP (2013) Another piece of the membrane puzzle: extending slipids further. J Chem Theory Comput 9:774–784

Jo S, Kim T, Im W (2007) Automated builder and database of protein/membrane complexes for molecular dynamics simulations. PLoS One 2:e880

Khakbaz P, Klauda JB (2015) Probing the importance of lipid diversity in cell membranes via molecular simulation. Chem Phys Lipids 192:12–22

Khakbaz P, Klauda J (2016) Probing the ripple phase of lipid bilayers using molecular simulations. Biophys J 110:86a

Klauda JB, Brooks BR, MacKerell AD Jr, Venable RM, Pastor RW (2005) An Ab Initio study on the torsional surface of alkanes and its effect on molecular simulations of alkanes and a DPPC bilayer. J Phys Chem B 109:5300–5311

Klauda JB, Kučerka N, Brooks BR, Pastor RW, Nagle JF (2006) Simulation-based methods for interpreting X-ray data from lipid bilayers. Biophys J 90:2796–2807

Klauda JB, Eldho NV, Gawrisch K, Brooks BR, Pastor RW (2008a) Collective and noncollective models of NMR relaxation in lipid vesicles and multilayers. J Phys Chem B 112:5924–5929

Klauda JB, Pastor RW, Brooks BR, Roberts MF, Redfield AG (2008b) Rotation of lipids in membranes: MD simulation, ^{31}P spin-lattice relaxation, and rigid-body dynamics. Biophys J 94:3074–3083

Klauda JB, Venable RM, MacKerell AD, Pastor RW (2008c) Considerations for lipid force field development. In: Scott EF (ed) Computational modeling of membrane bilayers. Elsevier, London, pp 1–48

Klauda JB, Venable RM, Freites JA, O'Connor JW, Mondragon-Ramirez C, Vorobyov I, Tobias DJ, MacKerell AD, Pastor RW (2010) Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. J Phys Chem B 114:7830–7843

Klauda JB, Monje V, Kim T, Im W (2012) Improving the CHARMM force field for polyunsaturated fatty acid chains. J Phys Chem B 116:9424–9431

Konas RM, Daristotle JL, Harbor NB, Klauda JB (2015) Biophysical changes of lipid membranes in the presence of ethanol at varying concentrations. J Phys Chem B 119:13134–13141

Kong Y, Janssen BJ, Malinauskas T, Vangoor VR, Coles CH, Kaufmann R, Ni T, Gilbert RJ, Padilla-Parra S, Pasterkamp RJ, Jones EY (2016) Structural basis for plexin activation and regulation. Neuron 91:548–560

Lee S, Tran A, Allsopp M, Lim JB, Hénin J, Klauda JB (2014) CHARMM36 united-atom chain model for lipids and surfactants. J Phys Chem B 118:547–556

Leekumjorn S, Sum AK (2007) Molecular studies of the gel to liquid-crystalline phase transition for fully hydrated DPPC and DPPE bilayers. BBA-Biomembr 1768:354–365

Li Z, Venable RM, Rogers LA, Murray D, Pastor RW (2009) Molecular dynamics simulations of PIP2 and PIP3 in lipid bilayers: determination of ring orientation, and the effects of surface roughness on a Poisson-Boltzmann description. Biophys J 97:155–163
Li J, Wen PC, Moradi M, Tajkhorshid E (2015) Computational characterization of structural dynamics underlying function in active membrane transporters. Curr Opin Struct Biol 31:96–105
Lim JB, Klauda JB (2011) Branching at the iso- and anteiso-positions in complex chlamydia membranes: a molecular dynamics study. Biochim Biophys Acta Biomembr 1808:323–331
Lim JB, Rogaski B, Klauda JB (2012) Update of the cholesterol force field parameters in CHARMM. J Phys Chem B 116:203–210
Liu P, Chernyshov A, Najdi T, Fu Y, Dickerson J, Sandmeyer S, Jarboe L (2013) Membrane stress caused by octanoic acid in Saccharomyces cerevisiae. Appl Microbiol Biotechnol 97:3239–3251
Lyubartsev AP, Rabinovich AL (2016) Force field development for lipid membrane simulations. Biochim Biophys Acta Biomembr 1858:2483–2497
Mannock DA, Lewis RNAH, McMullen TPW, McElhaney RN (2010) The effect of variations in phospholipid and sterol structure on the nature of lipid–sterol interactions in lipid bilayer model membranes. Chem Phys Lipids 163:403–448
Marrink SJ, Risselada HJ, Yefimov S, Tieleman DP, de Vries AH (2007) The MARTINI force field: coarse grained model for biomolecular simulations. J Phys Chem B 111:7812–7824
McIntosh TJ, Simon SA (1994) Long- and short-range interactions between phospholipid/ganglioside GM1 bilayers. Biochemistry 33:10477–10486
Mojumdar EH, Gooris GS, Barlow DJ, Lawrence MJ, Deme B, Bouwstra JA (2015) Skin lipids: localization of ceramide and fatty acid in the unit cell of the long periodicity phase. Biophys J 108:2670–2679
Monje-Galvan V, Klauda JB (2015) Modelling yeast organelle membranes and how lipid diversity influences bilayer properties. Biochemistry 54:6852–6861
Monje-Galvan V, Klauda JB (2016) Peripheral membrane proteins: tying the knot between experiment and computation. Biochim Biophys Acta Biomembr 1858:1584–1593
Monticelli L, Kandasamy SK, Periole X, Larson RG, Tieleman DP, Marrink S-J (2008) The MARTINI coarse-grained force field: extension to proteins. J Chem Theory Comput 4:819–834
Mori T, Jung J, Sugita Y (2013) Surface-tension replica-exchange molecular dynamics method for enhanced sampling of biological membrane systems. J Chem Theory Comput 9:5629–5640
Nagle JF, Tristram-Nagle S (2000) Structure of lipid bilayers. Biochim Biophys Acta-Rev Biomembr 1469:159–195
O'Connor JW, Klauda JB (2011) Lipid membranes with a majority of cholesterol: applications to the ocular lens and aquaporin 0. J Phys Chem B 115:6455–6464
Ohkubo YZ, Pogorelov Taras V, Arcario Mark J, Christensen Geoff A, Tajkhorshid E (2012) Accelerating membrane insertion of peripheral proteins with a novel membrane mimetic model. Biophys J 102:2130–2139
Pan J, Heberle FA, Petruzielo RS, Katsaras J (2013) Using small-angle neutron scattering to detect nanoscopic lipid domains. Chem Phys Lipids 170–171:19–32
Pandit KR, Klauda JB (2012) Membrane models of *E. coli* containing cyclic moieties in the aliphatic lipid chain. Biochim Biophys Acta Biomembr 1818:1205–1210
Pastor RW (1994) Molecular dynamics and Monte Carlo simulations of lipid bilayers. Curr Opin Struct Biol 4:486–492
Pendse PY, Brooks BR, Klauda JB (2010) Probing the periplasmic-open state of lactose permease in response to sugar binding and proton translocation. J Mol Biol 404:506–521
Rahman A (1964) Correlations in the motion of atoms in liquid argon. Phys Rev 136:A405–A411
Riccardi D, Yang S, Cui Q (2010) Proton transfer function of carbonic anhydrase: insights from QM/MM simulations. Biochim Biophys Acta (BBA) – Proteins Proteomics 1804:342–351
Risselada HJ, Marrink SJ (2008) The molecular face of lipid rafts in model membranes. Proc Natl Acad Sci 105:17367–17372

Schuler LD, Daura X, Van Gunsteren WF (2001) An improved GROMOS96 force field for aliphatic hydrocarbons in the condensed phase. J Comput Chem 22:1205–1218
Shi Q, Voth GA (2005) Multi-scale modeling of phase separation in mixed lipid bilayers. Biophys J 89:2385–2394
Shi Q, Izvekov S, Voth GA (2006) Mixed atomistic and coarse-grained molecular dynamics: simulation of a membrane-bound ion channel. J Phys Chem B 110:15045–15048
Shinoda W, DeVane R, Klein ML (2010) Zwitterionic lipid assemblies: molecular dynamics studies of monolayers, bilayers, and vesicles using a new coarse grain force field. J Phys Chem B 114:6836–6849
Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175:720–731
Snow CD, Sorin EJ, Rhee YM, Pande VS (2005) How well can simulation predict protein folding kinetics and thermodynamics? Annu Rev Biophys Biomol Struct 34:43–69
Sodt AJ, Sandar ML, Gawrisch K, Pastor RW, Lyman E (2014) The molecular structure of the liquid-ordered phase of lipid bilayers. J Am Chem Soc 136:725–732
Sodt AJ, Pastor RW, Lyman E (2015) Hexagonal substructure and hydrogen bonding in liquid-ordered phases containing palmitoyl sphingomyelin. Biophys J 109:948–955
Stansfeld Phillip J, Sansom Mark SP (2011) Molecular simulation approaches to membrane proteins. Structure 19:1562–1572
Sugita Y, Okamoto Y (1999) Replica-exchange molecular dynamics method for protein folding. Chem Phys Lett 314:141–151
Sun R, Dama JF, Tan JS, Rose JP, Voth GA (2016) Transition-tempered metadynamics is a promising tool for studying the permeation of drug-like molecules through membranes. J Chem Theory Comput 12:5157–5169
Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846
Terama E, Ollila OHS, Salonen E, Rowat AC, Trandum C, Westh P, Patra M, Karttunen M, Vattulainen I (2008) Influence of ethanol on lipid membranes: from lateral pressure profiles to dynamics and partitioning. J Phys Chem B 112:4131–4139
van Eerden FJ, de Jong DH, de Vries AH, Wassenaar TA, Marrink SJ (2015) Characterization of thylakoid lipid membranes from cyanobacteria and higher plants by molecular dynamics simulations. Biochim Biophys Acta Biomembr 1848:1319–1330
van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9:112–124
Venable RM, Zhang YH, Hardy BJ, Pastor RW (1993) Molecular-dynamics simulations of a lipid bilayer and of hexadecane – an investigation of membrane fluidity. Science 262:223–226
Venable RM, Brooks BR, Pastor RW (2000) Molecular dynamics simulations of gel (LβI) phase lipid bilayers in constant pressure and constant surface area ensembles. J Chem Phys 112:4822–4832
Venable RM, Sodt AJ, Rogaski B, Rui H, Hatcher E, MacKerell AD, Pastor RW, Klauda JB (2014) CHARMM all-atom additive force field for sphingomyelin: elucidation of hydrogen bonding and of positive curvature. Biophys J 107:134–145
Villanueva DY, Lim JB, Klauda JB (2013) Influence of ester-modified lipids on bilayer structure. Langmuir 29:14196–14203
Wang Y, Markwick PRL, de Oliveira CAF, McCammon JA (2011) Enhanced lipid diffusion and mixing in accelerated molecular dynamics. J Chem Theory Comput 7:3199–3207
Wong-ekkabut J, Xu Z, Triampo W, Tang IM, Peter Tieleman D, Monticelli L (2007) Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamics study. Biophys J 93:4225–4236
Wu Emilia L, Engström O, Jo S, Stuhlsatz D, Yeom Min S, Klauda Jeffery B, Widmalm G, Im W (2013) Molecular dynamics and NMR spectroscopy studies of E. coli lipopolysaccharide structure and dynamics. Biophys J 105:1444–1455

Wu Emilia L, Fleming Patrick J, Yeom Min S, Widmalm G, Klauda Jeffery B, Fleming Karen G, Im W (2014) E. coli outer membrane and interactions with OmpLA. Biophys J 106:2493–2502

Wu EL, Cheng X, Jo S, Rui H, Song KC, Lee J, Davila-Contreras EM, Beaven AH, Monje-Galvan V, Venable RM, Klauda JB, Im W (2014) CHARMM-GUI membrane builder toward realistic biological membrane simulations. J Comput Chem 35:1997–2004

Yasuda T, Kinoshita M, Murata M, Matsumori N (2014) Detailed comparison of deuterium quadrupole profiles between sphingomyelin and phosphatidylcholine bilayers. Biophys J 106:631–638

Yin H, Xu L, Porter NA (2011) Free radical lipid peroxidation: mechanisms and analysis. Chem Rev 111:5944–5972

Zhang L, Polyansky A, Buck M (2015) Modeling transmembrane domain dimers/trimers of plexin receptors: implications for mechanisms of signal transmission across the membrane. PLoS One 10:e0121513

Part IV

Membrane Homeostasis

Membrane Formation and Regulation

41

Megan E. Ericson and Charles O. Rock

Contents

1 Introduction: An Overview of Phospholipid Production and Regulation 764
2 Fatty Acid Synthesis: Initiation 764
3 Fatty Acid Synthesis: Elongation 766
4 Formation and Modification of Unsaturated Fatty Acids 767
5 Transcriptional Regulation 769
6 Biochemical Regulation 771
7 Research Needs 771
References 772

Abstract

Membrane homeostasis in bacteria ensures survival in rapidly changing environments. Most prokaryotes utilize the essential type II fatty acid synthesis to generate lipid species required for membrane formation and adaptation. This highly conserved system consists of multiple enzymes that each catalyzes a specific reaction. FAS II is an energy-intensive process and regulatory control at both transcriptional and biochemical levels ensuring the pathway output matches the demand for new membrane. This review summarizes the steps of FASII, the key regulatory steps, and checks and balances that maintain membrane homeostasis.

M. E. Ericson · C. O. Rock (✉)
Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA
e-mail: megan.ericson@stjude.org; charles.rock@stjude.org

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_54

1 Introduction: An Overview of Phospholipid Production and Regulation

Most bacterial species encode a cohort of conserved proteins collectively known as type II fatty acid synthesis (FAS II) system. Products of FAS II are incorporated into phospholipids required for construction of the membrane. *Escherichia coli* FAS II enzymes and phospholipid synthesis were the paradigms for all bacteria until the genomics revolution revealed the diversity of bacterial enzymes connected to lipid production. Covering the full diversity of FAS in bacteria is outside the scope of this text, but more comprehensive reviews are available (Marrakchi et al. 2002; Cronan 2003; Zhang et al. 2003; Lu et al. 2004; Zhang and Rock 2008, 2015; Parsons and Rock 2013). Phospholipid synthesis is divided into four modules by clustering enzymes that drive the formation of key products (Fig. 1). Lipid synthesis begins with the initiation module, and each product of the initiation module becomes a new fatty acid. The elongation module lengthens the carbon chain of the fatty acid (FA) and serves as the branch point for unsaturated fatty acid (UFA) production in bacteria (Lu et al. 2004). The intermediates of these modules are all bound to acyl carrier protein (ACP) as thioesters attached to its phosphopantetheine prosthetic group. Initiation and elongation modules constitute FAS II and are the most energy-intensive steps of lipid formation (Zhang and Rock 2008). Acyl-ACP produced by the elongation cycle will become the substrate for enzymes from the acyltransfer module to place into the one- or two-position of glycerol-3-phosphate to form phosphatidic acid (Yao and Rock 2013). Lastly, the membrane phospholipid module creates diverse phospholipid species using phosphatidic acid as a precursor (Kanfer and Kennedy 1964; Parsons and Rock 2013). FAS II is tightly regulated at the transcriptional level by proteins that most often act as repressors (Zhang and Rock 2009). Pathway-derived ligands bind to these factors and alter their affinity for DNA. Long-chain-acyl-ACP inhibition of multiple FAS II proteins presents an example of biochemical regulation (Heath and Rock 1995). Although acyl-ACP plays a key regulatory role, many questions remain about the control of FAS II, and the answers are important to areas like biofuel production and antibiotic development (Parsons and Rock 2011; Janssen and Steinbüchel 2014).

2 Fatty Acid Synthesis: Initiation

The initiation module determines the amount of FA made by bacteria. The key players in the initiation module are acetyl-CoA carboxylase (AccABCD), FabD, FabH, and ACP (Fig. 1). ACP is an acidic, 8.86 kDa protein with the unique ability to interact specifically with all of the enzymes in FASII. This protein-protein interaction occurs via affinity between a cluster of negative residues on the surface of ACP and corresponding positive residues adjacent to the active sites of FAS II enzymes (White et al. 2005). The initiation module begins with catalysis of acetyl-CoA conversion into malonyl-CoA by AccABCD. Two distinct reactions complete this process. First, the ATP-dependent carboxylation of biotin attached to biotin

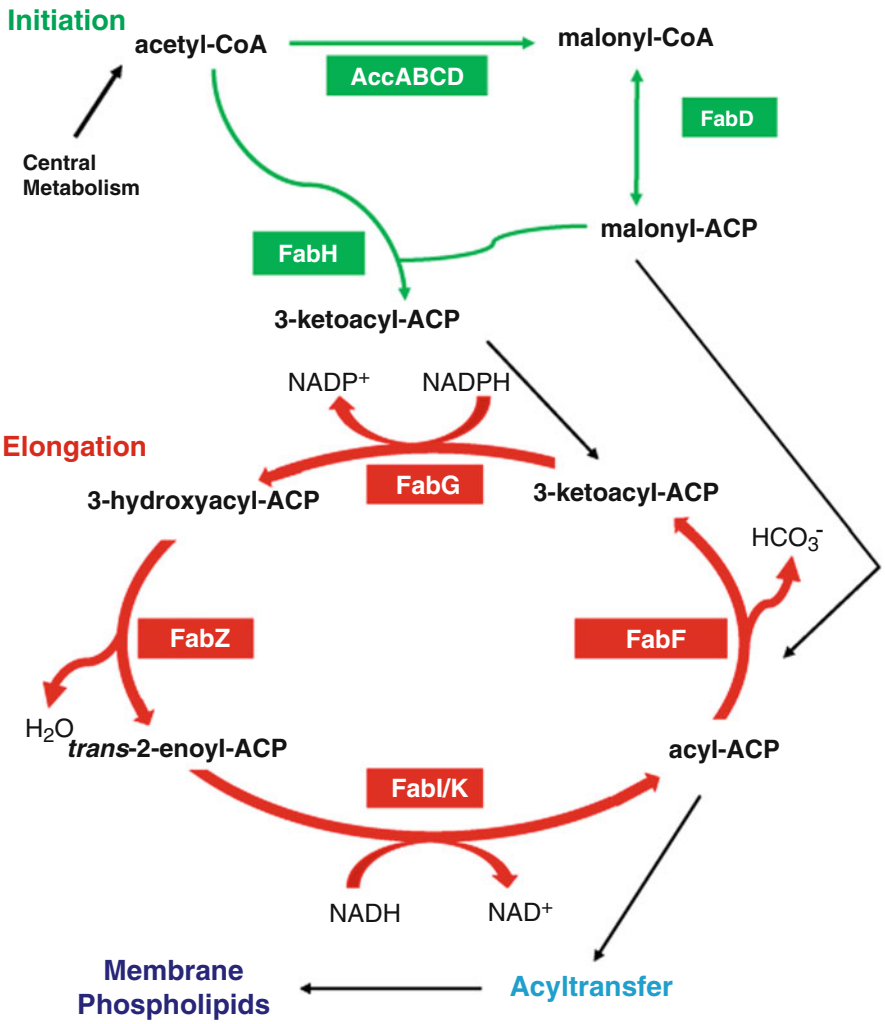


Fig. 1 *Lipid metabolism in bacteria.* The initiation module consists of acetyl-CoA carboxylase (*AccABCD*) , FabD, FabH, and acyl carrier protein (*ACP*). ACP is a small, acidic protein that carries FAS II intermediates between enzymes in the pathway. The 3-ketoacyl-ACP product of the initiation module enters the elongation cycle that extends the fatty acid chain by two carbons with each turn. When the acyl chain reaches a certain length, it can no longer be used by FabB/F and becomes substrate for the enzymes in the acyltransfer module. The acyltransfer module produces phosphatidic acid (PA) which is the precursor to phospholipids

carboxy carrier protein. Next, the carboxyl group from carboxybiotin is transferred to acetyl-CoA to form malonyl-CoA. It is thought that all four proteins form a complex in vivo but dissociate upon cell disruption (Cronan and Waldrop 2002). Overexpression of AccABCD resulted in a fivefold increase in FA synthesis in an *E. coli* system where lipid and protein synthesis had been uncoupled (Davis et al.

2000) demonstrating that AccABCD is biochemically regulated. The malonate group must be transferred to ACP before it can be used in FAS II. FabD, the malonyl-CoA/ACP transacylase, catalyzes a rapid equilibrium reaction to form malonyl-ACP. This malonyl-ACP serves as a substrate for 3-ketoacyl-ACP synthase III (FabH) and is also used as a building block in the elongation module. FabH is a ping-pong enzyme with a Cys-His-Asn catalytic triad. However, the enzyme that initiates FAS II in *Pseudomonas aeruginosa*, FabY, has an atypical Cys-His-His active site triad (Yuan et al. 2012). The FabH/FabY reaction commits FAS II to the production of a new fatty acid. *E. coli* FabH is specific for acetyl-CoA-based substrates, but homologues in other bacterial species have differences in the acyl-CoA binding pocket structure leading to lipid diversity between species. The FabH enzymes of gram-positive bacteria use five-carbon-branched chain acyl-CoAs resulting in *anteiso*- and *iso*-branched chain FA, while *Mycobacterium tuberculosis* FabH prefers long-chain acyl-CoAs which produce long-chain mycolic acids (Li et al. 2005).

3 Fatty Acid Synthesis: Elongation

Four proteins, FabG, FabZ, FabI/K, and FabF/B, make up the elongation module (Fig. 1). One cycle through the module increases the chain length of a growing FA by two carbons. Many intermediates and by-products of the elongation module serve essential functions in bacteria such as the enzyme cofactors lipoic acid and biotin (Jordan and Cronan 1997; Lin et al. 2010), lipid A that anchors lipopolysaccharide in gram-negative bacteria (Raetz et al. 2007), and extracellular molecules like quorum-sensing *N*-acyl homoserine lactones and quinolone signals (More et al. 1996; Bredenbruch et al. 2005). Elongation begins with the NADPH-dependent reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP by the 3-hydroxyacyl-ACP reductase, FabG (Fig. 1). FabG enzymes are strikingly similar between organisms indicating that this enzyme does not define FA structure. Next, the hydroxyacyl-ACP product of FabG undergoes a dehydration reaction catalyzed by FabZ to form *trans*-2-enoyl-ACP. FabZ acts as a homodimer that adopts a hot dog fold. Enoyl-ACP reductase, or FabI/K, reduces the C2-C3 double bond in *trans*-2-enoyl-ACP to form acyl-ACP in an NAD(P)H-dependent manner. FabI/K acts as the rate-determining enzyme for the elongation module. FabZ reactions favor the 3-hydroxyacyl-ACP over the enoyl-ACP intermediate implying that FabI pulls the elongation cycle in FAS II to completion. Acyl-ACP formed by FabI is utilized as a substrate for either the 3-ketoacyl-ACP synthase I or II (FabB or FabF, respectively) enzymes for another round of elongation or enzymes in the acyltransferase module for phospholipid synthesis. FabB/FabF takes malonyl-ACP formed in the initiation module as the carbon source for a condensation reaction with the growing acyl-ACP. FabF is the only elongation enzyme present in most bacteria. FabB is always found in bacteria that also express FabA and has an essential role in the formation of unsaturated fatty acids. Both 3-ketoacyl-ACP synthase enzymes contain a thiolase fold and a catalytic triad of Cys-His-His residues. The condensing enzymes' inability to bind to

acyl-ACP greater than 18 carbons is the major determinant of FA chain length. FA chain length and unsaturation define the fluidity and thickness of the membrane phospholipid bilayer.

4 Formation and Modification of Unsaturated Fatty Acids

Membrane fluidity must be maintained to optimize essential functions, and many bacteria use UFA as a means to achieve homeostasis. Bacteria that synthesize UFA employ a variety of methods to produce these lipids. *E. coli* requires a phospholipid pool containing at least 15 percent UFA and encodes FabA and FabB to form UFA in the elongation module (Fig. 2a). FabA is a bifunctional enzyme capable of both the dehydration of 3-hydroxyacyl-ACP to *trans*-2-enoyl-ACP and the subsequent isomerization of *trans*-2-enoyl-ACP to *cis*-3-enoyl-ACP. FabA and FabZ share some sequence homology, overall topology, and nearly identical active sites. The FabA isomerization reaction prefers a specific substrate, *trans*-2-decenoyl-ACP, due to an active site tunnel that perfectly fits a ten-carbon acyl-ACP. FabB, the ketoacyl-ACP synthase I always present in conjunction with FabA, is the rate-limiting enzyme in this de novo form of UFA formation. FabB continues the formation of UFA by elongating *cis*-3-decenoyl-ACP to bypass the reductase step. Mutations in either FabA or FabB in *E. coli* result in UFA auxotrophs. The FabA/B system is well represented in γ-proteobacteria, but not in other bacteria. Gram-positive bacteria like *Streptococcus pneumoniae* utilize the FabM enzyme to produce UFA. FabM catalyzes the same isomerization reaction as FabA but has a different structure related to the isomerases of FA β-oxidation. FabM competes specifically with the *trans*-2-enoyl-ACP reductase FabK for substrate. Other bacteria contain FabZ-like enzymes that isomerize *trans*-2-decenoyl-ACP in the elongation module. *Enterococcus faecalis* encodes a bifunctional FabZ-like enzyme, FabN, and *Aerococcus viridans* expresses FabQ, yet another bifunctional dehydratase/isomerase that has the ability to make polyunsaturated fats by partnering with a FabB (Bi et al. 2013). The defining feature that enables a dehydratase to also perform an isomerase reaction is unknown, but it seems that the shape of the active site tunnel may impact this activity.

Several bacterial species, including *E. coli* and *M. tuberculosis*, convert the double bond in UFA in phospholipids to a cyclopropane ring (CFA) (Grogan and Cronan 1997) (Fig. 2b). UFA-containing phospholipids are substrates as the decrease of UFA in stationary phase membranes corresponds with rising levels of CFA. Cyclopropane fatty acid synthase (Cfa) catalyzes the reaction using *S*-adenosylmethionine (AdoMet) as the carbon donor. The use of AdoMet illustrates the energy cost to form cyclopropane rings as formation of each AdoMet requires 3 ATP molecules. Cfa protein increases dramatically during stationary phase when *cfa* transcription is upregulated. RpoS, a sigma factor active after log phase growth, promotes *cfa* expression in stationary phase. Cfa is extremely unstable and has a half-life of around 5 min in vivo. Biological significance of CFA formation is not completely understood, but it is clear that the CFAs are less reactive than UFAs and mutants lacking CFA do not recover well from freeze-thaw or acid shock treatments.

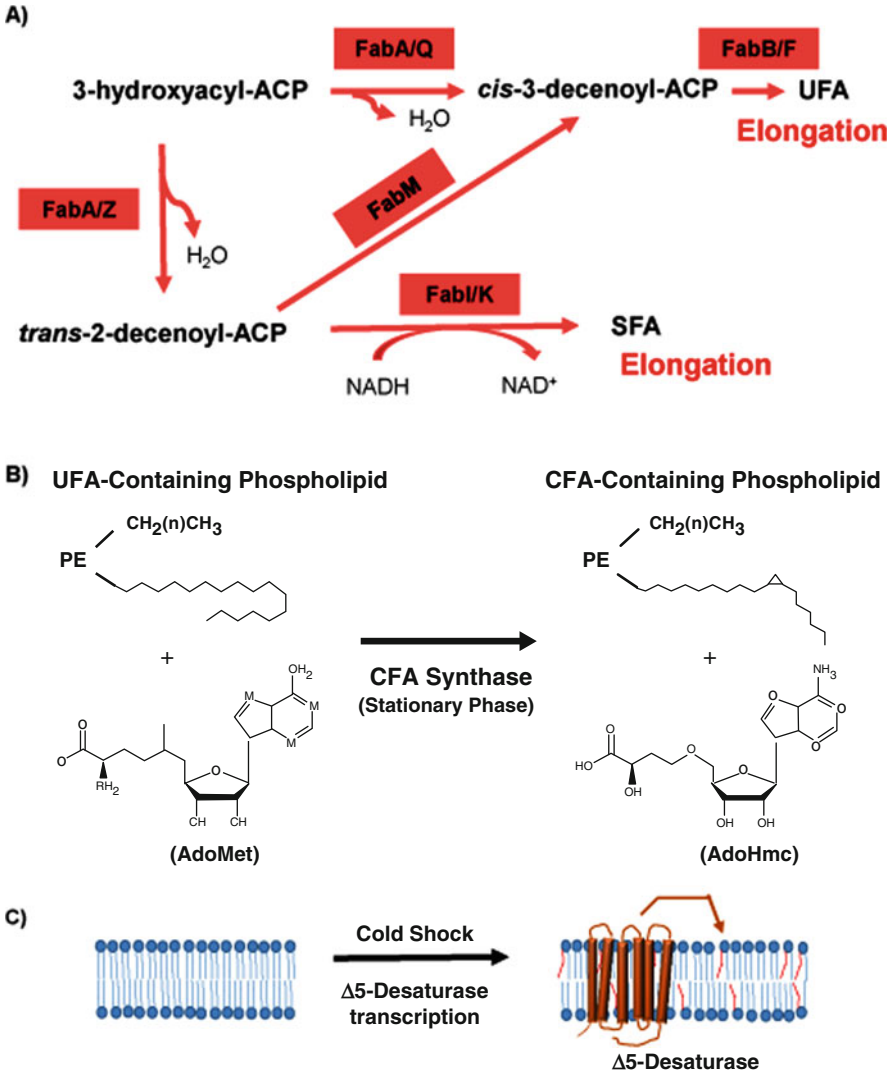


Fig. 2 *Unsaturated fatty acid synthesis in bacteria* (**a**) Formation of double bonds in the elongation module. There are two different modes of unsaturated fatty acid (*UFA*) synthesis in the elongation cycle. *E. coli* and other gram-negative bacteria contain the bifunctional dehydratase/isomerase enzyme FabA. FabB is coupled to FabA and performs a rate-limiting step in UFA synthesis. Other bacteria have a FabZ-like protein called FabQ that is coupled to FabF. *S. pneumoniae* contains the isomerase FabM that competes with FabK for substrate. FabF elongates the product of the FabM reaction. (**b**) Cyclopropane ring formation. Cyclopropane fatty acid (*CFA*) synthase converts the double bond in preexisting UFAs in phospholipids into a cyclopropane ring using S-adenosylmethionine (*AdoMet*) as a carbon donor. Transcription of *cfa* occurs in all stages of growth, but an RpoS site in the promoter results in strong expression in stationary phase. Formation of a cyclopropane ring during stationary phase protects the reactive double bond in the UFA. This does not occur in all bacteria. (**c**) *Bacillus subtilis* desaturase (Δ5-Des). The single desaturase in

Decreasing temperatures prompt immediate changes in membrane fluidity. The membrane becomes more ordered, and increasing the UFA content is a typical way to maintain homeostasis. *E. coli* responds by upregulating FabB to accelerate de novo UFA synthesis. Many gram-positive bacteria do not form UFA but contain branched-chain SFAs that have a similar impact on membrane fluidity as UFA (Mantsch et al. 1985). *Bacillus subtilis,* however, expresses a single iron, oxygen-dependent desaturase that introduces a *cis*-double bond at the Δ5 position of a SFA already in the membrane (Mansilla and de Mendoza 2005) (Fig. 2c). This Δ5-Des is under the control of the two-component system DesKR. DesK, the sensor kinase, is shifted to a position capable of activating DesR when membrane fluidity is decreased from a temperature or phospholipid composition change. DesR promotes transcription of the *des* gene to produce Δ5-Des, which introduces double bonds into the membrane.

5 Transcriptional Regulation

Transcriptional control relies on FA ligands binding to repressors/activators and altering this affinity to DNA (Zhang and Rock 2009). FA cofactors provide a form of feedback regulation as transcriptional activity occurs in response to the supply of FA chains for phospholipid synthesis. One example is FabR in *E. coli* (Fig. 3a). FabR represses the expression of *fabA/B*, the genes encoding the core enzymes required for UFA production. FabR binds to both unsaturated and saturated acyl-ACP and acyl-CoA enabling it to finely regulate the production of UFA based on the UFA to SFA ratio. UFA-ligands prompt binding of FabR to the *fabA/B* promoter and subsequent repression, while SFA ligands result in the reverse. FapR of *B. subtilis* was the first identified transcription factor of FAS II genes in gram-positive pathogens (Schujman et al. 2003). FapR binds to the promoters in the *fab* regulon and represses gene expression (Fig. 3b). Malonyl-CoA binding lowers FapR affinity for DNA and enables expression of the *fab* genes. FapR knockout cells do not display dramatic alterations in lipid content. FabT of *S. pneumoniae* represses expression of the *fab* regulon when bound to long-chain acyl-ACP (Lu and Rock 2006) (Fig. 3c). FabT is able to bind to acyl-ACP of all chain lengths, but only long-chain acyl-ACP is capable of instigating structural changes that ensure DNA binding. FabT deletion strains display fatty acid chain length increases and a decrease in the UFA to SFA ratio. Control of gene expression by transcription factors impacts FAS II activity, but it is clear that it is not the only form of regulation. Most importantly, overexpression of FAS II enzymes does not result in a corresponding increase in membrane demonstrating the importance of biochemical regulation.

Fig. 2 (continued) B. subtilis forms a *cis*-double bond at the fifth position carbon in the FA chain of an existing phospholipid in an aerobic reaction. Δ5-Des activity is induced by reduced membrane fluidity that activates the two-component DesKR system to stimulate transcription of *des*

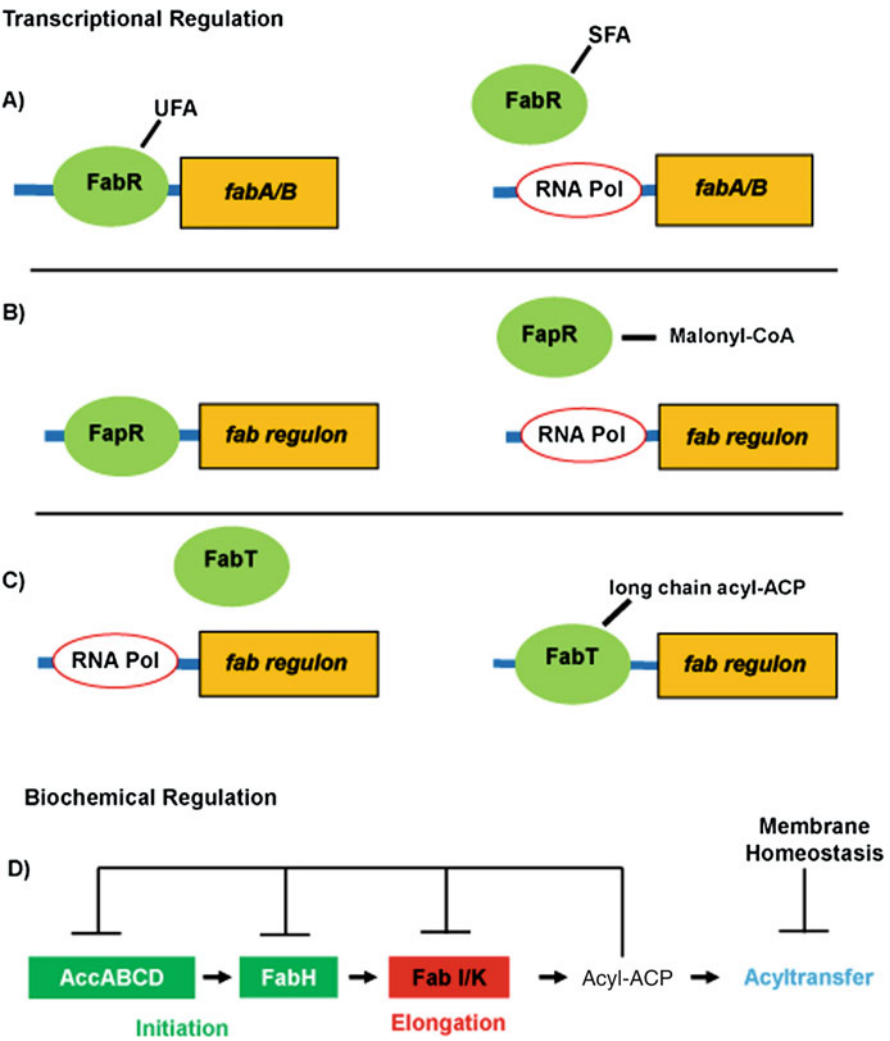


Fig. 3 *Regulation of fatty acid synthesis in bacteria*. (**a**) FabR, a transcriptional repressor in *Escherichia coli*, controls the expression of the FabA and FabB proteins. UFA in acyl-CoA or acyl-ACP thioesters bound to FabR promotes FabR-DNA interactions and inhibits expression of FabAB. (**b**) FapR is a repressor found in gram-positive bacteria and controls the 12 gene *fab* regulon. Malonyl-CoA binding lowers the affinity of FapR for DNA allowing transcription of fatty acid biosynthesis genes. (**c**) FabT represses fatty acid biosynthesis gene transcription in *Streptococcus pneumoniae*. Long-chain acyl-ACP binding to FabT increases its affinity for DNA and represses expression of the fab genes. (**d**) Long-chain acyl-ACP inhibits AccABCD, FabH, and FabI. Long-chain acyl-ACP accumulation may stem from a decrease in activity of the acyltransfer module which is thought to be coordinated with cell growth

6 Biochemical Regulation

Biochemical regulation based on product feedback inhibition plays an important role in determining the FA output of FAS II. Long-chain acyl-ACP inhibition of AccABCD, FabH, and FabI in *E. coli* is an example of this regulation (Heath and Rock 1996; Davis and Cronan 2001) (Fig. 3d). Short-circuiting this feedback loop is a key to the efficient production of biofuels in bacteria (Janssen and Steinbüchel 2014). These regulatory points have been well documented in *E. coli* but have yet to be demonstrated in other bacteria. Bacteria that incorporate extracellular fatty acids via a fatty acid kinase system, such as the pathogen *Streptococcus pneumoniae*, convert exogenous FA to acyl-ACP via an enzyme from the acyltransferase module (Parsons et al. 2011). Buildup of this exogenous acyl-ACP appears to have the same inhibitory impact on Acc as pools of malonyl-CoA drop precipitously. In *Staphylococcus aureus*, when exogenous oleic acid is present, malonyl-CoA abundance is unaltered, but the production of fatty acid decreases by half pointing to FabH regulation. The feedback inhibition of FabI by acyl-ACP modulates the rate of FAS II. Regulation of all of these enzymes by acyl-ACP points toward the importance of enzymes from the acyltransfer module as the key regulatory step in lipid synthesis. For example, the importance of PlsX is clear in *B. subtilis* as PlsX knockouts cease all phospholipid and fatty acid synthesis (Paoletti et al. 2007). Lipid production must also be regulated in relation to the synthesis of other macromolecules to maintain a constant protein-lipid ratio in the membrane. Inhibition of PlsB activity in *E. coli* by ppGpp, a signaling molecule that regulates protein translation, is the only known example. An interaction between SpoT, which triggers formation of ppGpp, and ACP is thought to prompt an increase in the production of ppGpp which can then inhibit activity of PlsB (Battesti and Bouveret 2006). There remain many open questions regarding the connection between FA synthesis and production of other macromolecules, inhibition of FabH in a variety of bacteria, and the importance of acyltransferase enzymes that require further investigation.

7 Research Needs

Decades of research have provided structural and mechanistic understanding of most FAS II enzymes. Identified FAS II regulatory controls demonstrate that feedback occurs at both the transcriptional and biochemical levels. Future research on the biochemistry of FAS II enzymes should focus on the regulatory mechanisms that coordinate FAS II activity with the demand for membrane phospholipid. Of particular interest will be to build on the structural and mechanistic understanding of FAS II enzymes to determine the structures of the regulatory complexes. PlsX, poised between FA production and its conversion into phospholipid, presents itself as an obvious enzyme to provide feedback on cell FA requirements (Lu et al. 2006). It is

curious that PlsX is expressed in almost all bacteria regardless of the presence of its partner protein PlsY. What purpose could these bacteria have for PlsX and its presumed production of acyl-phosphate? Identification of new enzymes that interact with FAS II, like fatty acid kinase, may reveal additional control points for FAS II regulation (Parsons et al. 2014). Likewise, a deeper understanding of the regulatory systems in FAS II will provide new insight for antibiotic development (Parsons and Rock 2011). As regulatory processes of FAS II are deciphered, they are sure to be as diverse and nuanced as the bacterial phospholipid species produced.

Acknowledgments This work was supported by the National Institutes of Health Grants GM034496 (C.O.R.), Cancer Center Support Grant CA21765, and the American Lebanese Syrian Associated Charities.

References

Battesti A, Bouveret E (2006) Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. Mol Microbiol 62(4):1048–1063

Bi H, Wang H, Cronan JE (2013) FabQ, a dual-function dehydratase/isomerase, circumvents the last step of the classical fatty acid synthesis cycle. Chem Biol 20(9):1157–1167

Bredenbruch F, Nimtz M, Wray V, Morr M, Muller R, Haussler S (2005) Biosynthetic pathway of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines. J Bacteriol 187(11):3630–3635

Cronan JE Jr (2003) Bacterial membrane lipids: where do we stand? Annu Rev Microbiol 57:203–224

Cronan JE Jr, Waldrop GL (2002) Multi-subunit acetyl-CoA carboxylases. Prog Lipid Res 41 (5):407–435

Davis MS, Cronan JE Jr (2001) Inhibition of *Escherichia coli* acetyl coenzyme A carboxylase by acyl-acyl carrier protein. J Bacteriol 183:1499–1503

Davis MS, Solbiati J, Cronan JE Jr (2000) Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J Biol Chem 275 (37):28593–28598

Grogan DW, Cronan JE Jr (1997) Cyclopropane ring formation in membrane lipids of bacteria. Microbiol Mol Biol Rev 61(4):429–441

Heath RJ, Rock CO (1995) Regulation of malonyl-CoA metabolism by acyl-acyl carrier protein and b-ketoacyl-acyl carrier protein synthases in *Escherichia coli*. J Biol Chem 270:15531–15538

Heath RJ, Rock CO (1996) Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in *Escherichia coli*. J Biol Chem 271:1833–1836

Janssen HJ, Steinbüchel A (2014) Fatty acid synthesis in *Escherichia coli* and its applications towards the production of fatty acid based biofuels. Biotechnol Biofuels 7(1):7

Jordan SW, Cronan JE Jr (1997) A new metabolic link; the acyl carrier protein of lipid synthesis donates lipoic acid to the pyruvate dehydrogenase complex in *Escherichia coli* and mitochondria. J Biol Chem 272:17903–17906

Kanfer J, Kennedy EP (1964) Metabolism and function of bacterial lipids II. Biosynthesis of phospholipids in *Escherichia coli*. J Biol Chem 239:1720–1726

Li Y, Florova G, Reynolds KA (2005) Alteration of the fatty acid profile of *Streptomyces coelicolor* by replacement of the initiation enzyme 3-ketoacyl acyl carrier protein synthase III (FabH). J Bacteriol 187(11):3795–3799

Lin S, Hanson RE, Cronan JE (2010) Biotin synthesis begins by hijacking the fatty acid synthetic pathway. Nat Chem Biol 6(9):682–688

Lu Y-J, Rock CO (2006) Transcriptional regulation of fatty acid biosynthesis in *Streptococcus pneumoniae*. Mol Microbiol 59:551–566

Lu Y-J, Zhang Y-M, Rock CO (2004) Product diversity and regulation of type II fatty acid synthases. Biochem Cell Biol 82:145–155

Lu Y-J, Zhang Y-M, Grimes KD, Qi J, Lee RE, Rock CO (2006) Acyl-phosphates initiate membrane phospholipid synthesis in gram-positive pathogens. Mol Cell 23(5):765–772

Mansilla MC, de Mendoza D (2005) The *Bacillus subtilis* desaturase: a model to understand phospholipid modification and temperature sensing. Arch Microbiol 183(4):229–235

Mantsch HH, Madec C, Lewis RNAH, McElhaney RN (1985) Thermotropic phase behavior of model membranes composed of phosphatidylcholine containing iso-branched fatty acids. 2. Infrared and 31P NMR spectroscopic studies. Biochemistry 24:2440–2446

Marrakchi H, Zhang Y-M, Rock CO (2002) Mechanistic diversity and regulation of type II fatty acid synthesis. Biochem Soc Trans 30:1050–1055

More MI, Finger LD, Stryker JL, Fuqua C, Eberhard A, Winans SC (1996) Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science 272(5268):1655–1658

Paoletti L, Lu Y-J, Schujman GE, de Mendoza D, Rock CO (2007) Coupling of fatty acid and phospholipid synthesis in *Bacillus subtilis*. J Bacteriol 189:5816–5824

Parsons JB, Rock CO (2011) Is bacterial fatty acid synthesis a valid target for antibacterial drug discovery? Curr Opin Microbiol 14:544–549

Parsons JB, Rock CO (2013) Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res 52:249–276

Parsons JB, Frank MW, Subramanian C, Saenkham P, Rock CO (2011) Metabolic basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis inhibitors. Proc Natl Acad Sci U S A 108(37):15378–15383

Parsons JB, Broussard TC, Bose JL, Rosch JW, Jackson P, Subramanian C, Rock CO (2014) Identification of a two-component fatty acid kinase responsible for host fatty acid incorporation by *Staphylococcus aureus*. Proc Natl Acad Sci U S A 111:10532–10537

Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in Gram-negative bacteria. Annu Rev Biochem 76:295–329

Schujman GE, Paoletti L, Grossman AD, de Mendoza D (2003) FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis. Dev Cell 4(5):663–672

White SW, Zheng J, Zhang Y-M, Rock CO (2005) The structural biology of type II fatty acid biosynthesis. Annu Rev Biochem 74:791–831

Yao J, Rock CO (2013) Phosphatidic acid synthesis in bacteria. Biochim Biophys Acta 1831 (3):495–502

Yuan Y, Schdeva M, Leeds JA, Meredith TC (2012) Fatty acid biosynthesis in *Pseudomonas aeruginosa* is initiated by FabY: a new class of b-ketoacyl-acyl carrier protein synthases. J Bacteriol 194(19):5171–5184

Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6:222–233

Zhang Y-M, Rock CO (2009) Transcriptional regulation in bacterial membrane lipid synthesis. J Lipid Res 50:S115–S119

Zhang Y-M, Rock CO (2015) Fatty acid and phospholipid biosynthesis in prokaryotes. In: Ridgway ND, RS ML (eds) Biochemistry of Lipids, Lipoproteins and Membranes, 6th edn. Elsevier, Amsterdam, pp 73–112

Zhang Y-M, Marrakchi H, White SW, Rock CO (2003) The application of computational methods to explore the diversity and structure of bacterial fatty acid synthase. J Lipid Res 44:1–10

Regulation of Membrane Lipid Homeostasis in Bacteria upon Temperature Change

42

M. C. Mansilla and D. de Mendoza

Contents

1 Introduction 776
2 Modulation of Membrane Fluidity 776
3 Changes in the Proportion of Branched-Chain Fatty Acids 777
4 Introduction of Double Bonds in Fatty Acids 778
4.1 The Anaerobic Pathway 780
4.2 Bacterial Desaturases 781
5 Lipid: A Remodeling Induced by Cold Shock 783
6 Research Needs 784
References 785

Abstract

Bacteria precisely remodel the fluidity of their membrane bilayer via the incorporation of proportionally more unsaturated fatty acids (or fatty acids with analogous properties, such as branched-chain fatty acids) as growth temperature decreases. This process, termed homeoviscous adaptation, is suited to disrupt the order of the lipid bilayer and optimizes the performance of a large array of cellular physiological processes at the new temperature. As such, microbes have developed molecular strategies to sense changes in membrane fluidity, provoked by a decrease in environmental temperature, and initiate cellular responses that upregulate the

M. C. Mansilla (✉)
Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Universidad Nacional de Rosario, Rosario, Argentina
e-mail: mansilla@ibr-conicet.gov.ar

D. de Mendoza
Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Universidad Nacional de Rosario, Rosario, Argentina
e-mail: demendoza@ibr-conicet.gov.ar

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_56

biosynthesis of either unsaturated, terminally branched, or shorter-chain fatty acids. In this review we describe some of the basic molecular strategies that bacteria use to sense temperature. While the activities of all biomolecules are altered as a function of temperature, the thermosensors we focus on here are molecules whose temperature sensitivity provides information about the thermal environment that is used to trigger an appropriate adjustment of membrane architecture. We also discuss selected examples of membrane and lipopolysaccharide remodeling induced by cold that involves changes in the activity of fatty acid biosynthetic enzymes or the expression of acyltransferases that modify the lipid A.

1 Introduction

Bacteria are poikilothermic organisms and when environmental temperatures suddenly drop below physiological conditions, they must adapt to the new physical situation in order to survive. The cytoplasmic membrane is critically affected by a temperature decrease because upon cooling membrane lipids become ordered, and membrane fluidity depends largely on the packing of the lipids acyl chains. When fatty acyl chains are packed in a disordered state, membranes are fluid and hydrated, and this condition is termed the liquid-crystalline state (Cevc and Marsh 1987). However, if the fatty acyl array becomes ordered, the membrane becomes more rigid and thicker, water permeability decreases, and the membranes are less hydrated. This is called the gel phase. The transition between both phases is temperature dependent and occurs in a cooperative fashion. The temperature at the midpoint of this transition is called the transition temperature (Tm). The Tm of a lipid bilayer is a function of membrane lipid composition and, in organisms lacking cholesterol as bacteria, mainly depends on the fatty acid composition of the membrane lipids. Acyl chain packing is disrupted by branches and by *cis*-double bonds, which introduce kinks in the chains, thereby decreasing the Tm. In addition, longer acyl chains have stronger interactions between them, which increases Tm.

For normal function of membrane lipid bilayers, they need to be largely fluid, and bacteria must regulate their phase transition in response to a temperature decrease. Without regulation, bacteria shifted from a high to a low environmental temperature would have more rigid membranes, and vital functions such as transport, energy generation, and signal transduction will be disturbed (Phadtare 2004; Mansilla and de Mendoza 2005).

2 Modulation of Membrane Fluidity

Bacteria can acclimate to changing environmental temperatures by remodeling their membrane lipid composition in order to modify the transition temperature of the membrane, maintaining a fluid state array of the fatty acyl chains. This mechanism of

regulation is called homeoviscous adaptation (de Mendoza and Cronan 1983). In all cases examined, as the temperature drops, a higher proportion of fatty acyl chains which exerts lower interactions between them are incorporated into the membrane. Specific changes include introduction of double bonds, reduction of the average chain length, increase of the methyl branching, or the change from *iso*- to *anteiso*-branching pattern. These modifications can be performed directly on the acyl chains of preexisting phospholipids, as seen in unsaturation by desaturases, giving a fast cold response. The other modifications require de novo synthesis of different fatty acids and subsequent incorporation into the membrane.

3 Changes in the Proportion of Branched-Chain Fatty Acids

Branched-chain fatty acids (BCFA), as straight-chain fatty acids, are synthesized by type II fatty acid synthetase (FASII) (Parsons and Rock 2013). The difference between the two pathways relies in the respective primers used in the first elongation step, catalyzed by 3-ketoacyl-acyl carrier protein (ACP) synthase III (KASIII or FabH). This enzyme catalyzes a decarboxylative condensation between an acyl coenzyme (CoA) primer and malonyl-ACP (Parsons and Rock 2013). In bacteria that generate only straight-chain fatty acids, acetyl-CoA is the preferred substrate of FabH. BCFA synthesis requires short branched-chain α-keto acids as primer sources. These keto acids are derivatives of the branched-chain amino acids valine, leucine, and isoleucine. These amino acids suffer a deamination, catalyzed by an amino-transferase, followed by an oxidative decarboxylation of the resulting ketoacid, catalyzed by branched-chain ketoacid dehydrogenase (BDKH)(Kaneda 1991). The BCFA precursors are isobutyryl-CoA, isovaleryl-CoA, and 2-methylbutyryl-CoA, products of the catabolism of valine, leucine, and isoleucine, respectively. The primer derived from isoleucine produces *anteiso*-branched fatty acids, while the other primers produce the *iso*-branched species. The methyl group of the BCFA affects acyl chain packing, so the proportion of BCFA affects membrane fluidity. It has been established that *anteiso*-branched fatty acids promote a more fluid bilayer structure than the *iso*-branched fatty acids, because the methyl branch is located deeper inside the membrane. For example, the phase transitions of chemically synthesized diacylphosphatidylcholine samples containing diacyl n-C_{15}, *iso*-C_{15}, and *anteiso*-C_{15} are 34.2, 6.5, and −16.5 °C, respectively (Kaneda 1991). This physicochemical difference among normal-, *iso*-, and *anteiso*-branched fatty acids correlates with the positional preference of their incorporation into two positions of membrane phospholipids. Among C_{15} fatty acids, *anteiso*-C_{15} has a higher ability to lower the Tm than other fatty acids synthesized by *Bacillus subtilis* and *Staphylococcus aureus*; thus in these bacteria, it is incorporated preferentially into the 2 position of membrane phospholipids (Kaneda 1991; Parsons et al. 2013). In contrast, n-C_{15} fatty acid is incorporated primarily into the 1 position of phospholipids, while iso-C_{15} fatty acid is incorporated into both, the 1 and 2 positions (Kaneda 1991).

In response to cold stress, bacteria that produce BCFA modify their *iso*/*anteiso* ratio to maintain membrane fluidity. It has been reported that exogenous isoleucine supply is essential for the survival of *B. subtilis* JH642 cells after a cold shock. As the rate of isoleucine synthesis in this strain is below the requirements for BCFA *anteiso*-precursors, in the absence of exogenous supplementation with this amino acid, it is unable to perform the switch to a higher proportion of *anteiso*-C_{15} and *anteiso*-C_{17}, required for normal growth at low temperatures (Klein et al. 1999). In addition, a *B. subtilis* mutant in lipoate synthase, *lipA,* which is unable to synthesize lipoyl-BKDH and therefore to generate BCFA precursors, is impaired to grow in minimal media at 37 °C, even if it is supplemented with isoleucine (Martin et al. 2009). Growth at this temperature can be restored by the addition of any one of the BCFA precursors, but this mutant is cold sensitive if *anteiso*-branched fatty acid precursors are not provided (Martin et al. 2009).

Listeria monocytogenes, which typically contains more than 90% of BCFA in their membranes, combines two modes of adaptation of fatty acid composition when grown at temperatures of 10 °C or below. These bacteria, when shifted to low temperatures, not only change the branching pattern from *iso* to *anteiso* but also produce a shortening of the fatty acid chain length: *anteiso*-C_{15} increases to constitute more than 60% of the total fatty acids in phospholipids (Annous et al. 1997). Mutants in the BKDH cluster (*bkd*) of *L. monocytogenes* showed cold sensitivity (Zhu et al. 2005). At 26 °C, 2-methylbutyrate and isovalerate stimulated the growth of the mutants, but at 10 °C, only 2-methylbutyrate could restore their growth (Zhu et al. 2005).

How do bacterial cells regulate *iso*/*anteiso* ratio when temperature goes down? A tentative interpretation is that the condensing enzyme FabH is involved in such regulation. In vitro measurements of the initial rates of FabH-catalyzed condensation of malonyl-ACP and different branched-chain acyl-CoAs showed that the condensing enzyme of *L. monocytogenes* prefers 2-methylbutyryl-CoA over the other primers at 30 °C. This selectivity becomes even more pronounced at 10 °C, leading to an increased production of *anteiso*-branched-chain fatty acids during low-temperature adaptation, as outlined in Fig. 1a (Singh et al. 2009). The thermal regulation of *anteiso*-branched-chain fatty acids seems to be an intrinsic property of the activity of FabH, rather than a higher availability of 2-methylbutyryl-CoA at lower temperatures, since acyl-CoA pool levels were reduced similarly for all fatty acid precursors (Singh et al. 2009).

4 Introduction of Double Bonds in Fatty Acids

Unsaturated fatty acids (UFAs) have a much lower transition temperature than saturated fatty acids (SFAs) because the steric hindrance imparted by the rigid kink of the *cis*-double bond results in much poorer packing of the acyl chains, resulting in disordered membranes that remain fluid at lower temperatures (de Mendoza and Cronan 1983). There are two major mechanisms by which bacteria

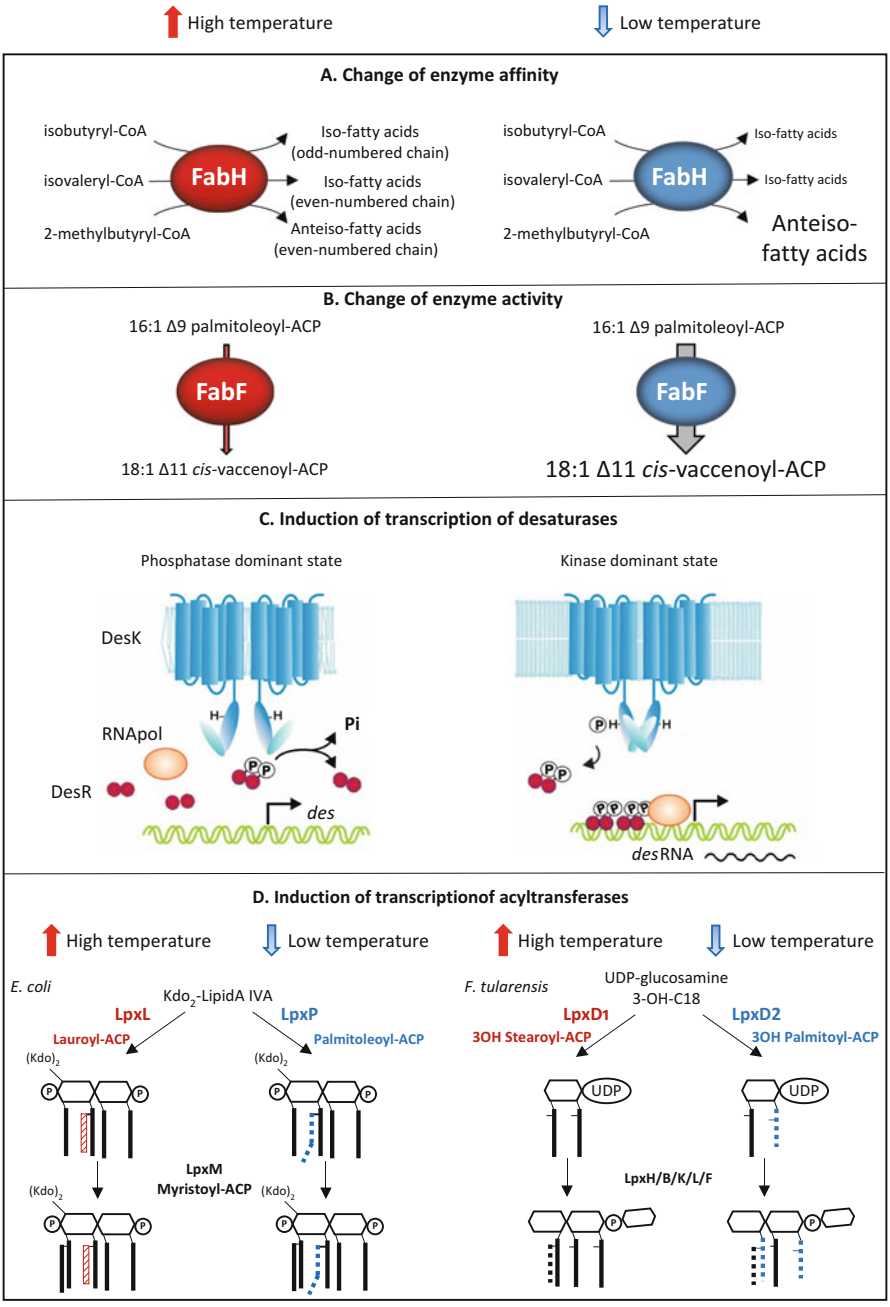


Fig. 1 Different bacterial strategies for membrane lipid remodeling after cold shock. (**a**) At low temperatures *Listeria monocytogenes* condensing enzyme FabH shows higher affinity for 2-methylbutyryl-CoA, the primer of anteiso-fatty acids, leading to a higher proportion of these packing-disrupter fatty acids in the membrane. (**b**) The production of UFAs by the anaerobic

synthesize UFAs: most of them, including *Escherichia coli*, synthesize UFAs anaerobically (Mansilla et al. 2004), whereas some prokaryotes such as cyanobacteria, bacilli, mycobacteria, and pseudomonads use an oxygen-dependent fatty acid desaturation pathway (Phetsuksiri et al. 2003; Mansilla and de Mendoza 2005; Zhu et al. 2006).

4.1 The Anaerobic Pathway

The anaerobic pathway of UFA biosynthesis was extensively studied in the *E. coli* model system and has been covered in several comprehensive reviews (Magnuson et al. 1993; Mansilla et al. 2004; Zhang and Rock 2008). Briefly, at constant temperature, in *E. coli*, the proportion of *cis*-UFA is determined by competition for intermediates at a specific branch point in the pathway, which occurs at the FabA enzyme step. FabA defines a distinct protein family of dehydratases that converts the β-hydroxy to the *trans*-2-decenoyl-ACP intermediate and functions as an isomerase that establishes a rapid equilibrium between *trans*-2- and *cis*-3-decenoyl-ACP at the 10-carbon stage. The SFA/UFA ratio depends on the ratio between the use of *cis*-3-decenoyl-ACP by FabB and the use of *trans*-2-decenoyl-ACP by FabI. The introduction of the double bond at the 10-carbon intermediate and the subsequent elongation steps give rise to the two major UFAs that are found in bacteria: Δ9–16:1 and Δ11–18:1 (also known as *cis*-vaccenate). The FabB-condensing enzyme has the unique catalytic property of elongating *cis*-3-decenoyl-ACP, whereas FabF (a FabB isozyme) does not. Thus, both FabA and FabB are required for UFA synthesis, while FabF is involved in the elongation of Δ9–16:1 to Δ11–18:1. There is a biochemical mechanism that allows the elongation cycle to respond to the environmental temperature. This mechanism was carefully studied in *E. coli*, and FabF was identified as a key player in the alteration of membrane composition in response to temperature change (Fig. 1b). At low temperatures, *E. coli* FabF promotes the production of Δ11–18:1, whereas at higher temperatures, FabF functions poorly and the formation of Δ11–18:1 is reduced (for a review, see de Mendoza and Cronan 1983).

Fig. 1 (continued) pathway in *Escherichia coli* is increased at low temperature due to the increased activity of FabF, the enzyme responsible for the elongation of 16:1Δ9 palmitoleoyl-ACP to 18:1Δ11 *cis*-vaccenoyl-ACP. (**c**) Transcription of the gene encoding the sole desaturase of *Bacillus subtilis* is regulated by a two-component system, composed of the histidine kinase DesK and the response regulator DesR. DesK is able to sense the increase of membrane thickness produced by the temperature drop, acquiring a kinase-dominant state. DesK-mediated phosphorylation of DesR results in transcriptional activation of *des*. (**d**) Induction of specific acyltransferases of lipid A synthesis. In *E. coli* the gene encoding LpxP is highly transcribed after cold shock, and the UFA palmitoleate (C16:1; *dashed lines*) is then incorporated instead of laurate (C12:0; *striped rectangles*). *Solid rectangles*: myristic acid (C14:0). In *Francisella tularensis* the acyltransferase LpxD2 is highly expressed at 21 °C, resulting in incorporation of shorter-chain fatty acids to lipid A (palmitic acid, C16:0; *dashed lines*), instead of longer fatty acids (stearic acid, C18:0; *solid rectangles*)

4.2 Bacterial Desaturases

The other known pathway of UFA synthesis is performed by fatty acid desaturases, a special type of oxygenases that can remove two hydrogens from a full-length fatty acyl aliphatic chain, catalyzing the formation of a double bond in the substrate. They function only in aerobic organisms, since they require molecular oxygen, iron cofactors, and two reducing equivalents for catalysis (Altabe et al. 2013).

4.2.1 A Sensor of Membrane Thickness in Bacteria

In addition to altering protein conformation directly, temperature changes can affect protein activity as a secondary consequence of structural alterations in intimately associated molecules. In the case of the bacterial cold sensor DesK, for example, temperature-dependent changes in the lipid membrane appear to be the primary mediators of the protein's thermal responsiveness (for a recent review, see de Mendoza 2014; Saita et al. 2016). DesK is an integral membrane-associated histidine kinase, which is at the top of the signaling cascade of a regulatory pathway that controls the synthesis of UFAs in *B. subtilis*. In vivo and in vitro experiments have demonstrated that DesK acts as a kinase at cold temperatures, autophosphorylating a conserved histidine residue within its kinase domain. The phosphoryl group is then transferred to the receiver aspartic acid in the DNA-binding response regulator DesR. Phosphorylation of DesR triggers the reorganization of its quaternary structure, a key event necessary to activate the transcription of the *des* gene that encodes the acyl-lipid desaturase Δ5-Des. UFAs, the end products of Δ5-Des activity, promote a more fluid membrane that appears to switch DesK from the kinase to the phosphatase state. Consequently, the concentration of phospho-DesR declines, and transcription of *des* is terminated.

The activation of DesK upon cooling appears to be intimately related to changes in membrane structure. Among other effects, cooling causes an increment in membrane thickness by decreasing disorder of lipids. Indeed, experimental evidence supports the idea that DesK senses the thickening of the membrane as temperature drops: (i) the protein exhibits increased kinase activity when reconstituted into vesicles of longer acyl chains (Martin and de Mendoza 2013; Cybulski et al. 2010); (ii) DesK kinase activity is stimulated in bacteria with a higher proportion of long-chain fatty acids in their membranes, whereas an increment in the amount of short-chain fatty acids stimulates its phosphatase activity (Porrini et al. 2014); and (iii) computational modeling and simulations strongly supported the concept that membrane thickness is the main driving force for signal sensing and that it acts by inducing helix stretching and rotation promoting a kinase-competent state (Saita et al. 2015). In addition, structure-guided mutagenesis established that stabilization/destabilization of a 2-helix coiled coil (2-HCC), which connects the transmembrane sensory domain of DesK to its cytosolic catalytic region, is crucial to control it signaling state (Saita et al. 2015). On the basis of the above-described evidence, the following model was proposed for DesK cold sensing: Upon drop in temperature, the membrane becomes thicker and more structured, imposing on the 2-HCC a stress that results in its conversion from the relaxed phosphatase state into the autokinase

competent state, inducing Δ5-Des (Fig. 1c). Such a regulatory mechanism is well suited to maintain levels of membrane fluidity within an optimal range.

4.2.2 CasKR Regulates Optimal Unsaturation of *B. cereus* Fatty Acids

Bacillus cereus sensu *lato* is composed of a set of ubiquitous strains, including human pathogens that can survive in a wide range of temperatures, can grow in refrigerated food, and sometimes cause food-associated illness. *B. cereus* membrane lipids contain a high proportion of UFAs at 37 °C (about 27%, mostly of them containing a double bond at position Δ10), while at low temperatures, there is an increase in the UFA content to 45%, due to the synthesis of Δ5 as well as Δ5,10 di-UFAs. These reactions are catalyzed by two acyl-lipid desaturases DesA (Δ5 desaturase) and DesB (Δ10 desaturase) (Chazarreta et al. 2013).

The cold shock induction of UFA synthesis in *B. cereus* could be due to an increase in the expression and/or in the desaturation activities of both desaturases at lower temperatures. Interestingly, the DesK-DesR pathway described in *B. subtilis* (de Mendoza 2014) is absent in *B. cereus.* This suggests that the thermal regulatory mechanisms controlling the expression of *B. cereus* desaturase genes are different from those described for the *B. subtilis* Δ5 Des. Recently, a two-component system named CasKR, which is essential for *B. cereus* cold adaptation, was identified (Diomandé et al. 2014). Transcriptomic analysis of the Δ*casKR* mutant revealed that *desA* and *desB* genes, encoding DesA and DesB acyl-lipid desaturases, were downregulated in this strain. This mutant showed lower Δ5 UFA content compared with the parental strain, which could be responsible for its cold-sensitive phenotype (Diomandé et al. 2015). Although cold induction of *desA* transcription is dependent on the CasKR system, CasR does not interact in vitro with the *desA* promoter, suggesting that the regulation is indirect (Diomandé et al. 2016). Clearly, much more work is necessary to understand the role of the CasKR system in the regulation of fatty acid desaturation in *B. cereus*.

4.2.3 A Cold Sensor that also Responds to Multiple Stress Conditions in Cyanobacteria

Cyanobacteria also respond to cold shock increasing the proportion of UFAs in membrane lipids. This response is the result of the enhanced transcription of the desaturase genes. In *Synechocystis* PCC6803, three out of four genes coding for desaturases (*desA*, *desB*, and *desD*) are cold inducible. The mechanism of cold-induced transcription of *desA*, encoding Δ12 desaturase, is still unknown, but *desB* and *desD* genes (encoding ω-3 and Δ6 desaturase, respectively) are part of the regulon controlled by the membrane-bound histidine kinase Hik33 and its cognate regulators (Sinetova and Los 2016).

Hik33 has two membrane-spanning domains, a HAMP linker, a leucine zipper, and a PAS domain located at its amino terminus, while the carboxy-terminus contains a highly conserved histidine kinase catalytic domain. This histidine kinase, together with Rre26 (RpaB), is able to regulate the expression of ~20 cold-inducible genes (Sinetova and Los 2016). Besides, Hik33 is also involved in signal perception of salt, osmotic, and oxidative stress, in combination with Rre31 and other unidentified response regulators. It was proposed that induction of cold-inducible

genes is favored by the interaction of Hik33 with Ssl3451, a homologue of SipA. This small protein enhances the kinase phosphorylation at low temperature, which would lead to a greatest preference of Hik33 to the Rre26 response regulator (Sinetova and Los 2016). It is worth to note that the cold induction of *desB* mediated by Hik33 is red light dependent: there is no induction of *desB* transcription if cells are in the dark. However, it does not require the activity of the photosynthetic apparatus. Regulation of *desB* transcription after cold shock is affected by another regulatory system, the Ser/Thr kinase SpkE. The mutant lacking SpkE shows a similar transcription pattern to the Hik33-deficient mutant, including the absence of *desB* induction by cold (Sinetova and Los 2016).

The cold-regulated induction of desaturase gene expression in *Synechocystis* seems to be similar to the pathway controlled by DesK/DesR in *B. subtilis* (de Mendoza 2014). However, some differences must be highlighted: Hik33 is a global cold sensor involved in the expression of several genes that are induced at low temperature (Sinetova and Los 2016), while DesK/DesR system regulates exclusively the expression of the *des* gene (Beckering et al. 2002). In addition, the analysis of the transcriptome of *Synechocystis* indicated that Hik33 is also involved in the sensing of other kinds of stresses (hyperosmotic stress, salt stress, and peroxydative stress), while DesK does not respond to those stimuli. Using a chimeric histidine kinase, Hik33n–SphSc, Shimura et al. (2012) demonstrated that localization of the kinase on the membrane might be necessary for kinase activity in vivo, regardless of the sequence of the transmembrane helices, proposing that the mechanism for cold sensing in Hik33 would be different from that in DesK.

5 Lipid: A Remodeling Induced by Cold Shock

The outer membrane (OM) of Gram-negative bacteria is also affected by cold shock. Although the overall structure of the OM is considered a lipid bilayer, its composition is distinct from that of the cytoplasmic membrane. The major component of the outer leaflet of OM is a unique glycolipid called lipopolysaccharide (LPS), which has three structural regions: O-antigen, core, and lipid A. Lipid A, the hydrophobic anchor of LPS, is not a typical glycerol lipid, but instead the fatty acids are connected through hydroxyl and amine groups of a disaccharide composed of glucosamine phosphate (Raetz et al. 2007). Gram-negative bacteria with both environmental and mammalian reservoirs can synthesize modified forms of lipid A in response to environmental stimuli and temperature change.

E. coli lipid A usually contains six acyl chains. Above 30 °C four (*R*)-3-hydroxymyristoyl groups (C14:0) are attached to the glucosamine disaccharide, and then two short saturated acyl chains (laurate, C12:0, and myristate, C14:0) are transferred by the sequential action of LpxL and LpxM acyl transferases, respectively, to the (*R*)-3-hydroxymyristoylgroup of the distal unit. When cells are cooled to 12 °C, the UFA palmitoleate (C16:1) is incorporated in lipid A instead of laurate, comprising around 11% of their fatty acid chains. In contrast, only traces of palmitoleate can be detected in 30 °C grown cells (Raetz et al. 2007). A similar effect on lipid A composition was observed in *Salmonella typhimurium* cells after cold shock (Wollenweber et al. 1983).

In *E. coli*, palmitoleoyl-ACP-dependent acylation of the precursor $(Kdo)_2$-lipid IV_A is catalyzed by a third lipid A acyltransferase, LpxP, which is homologous to LpxL. The level of *lpxP* mRNA increases by several orders of magnitude after a cold shock, which correlates with the induction of more than 30-fold of LpxP activity when cells are shifted from 30 °C to 12 °C (Raetz et al. 2007) (Fig. 1d, left side). Since the melting point of *cis*-9-pamitoleic acid is 0.5 °C, whereas that of lauric acid is 44.2 °C (Small 1986), the acylation of lipid A with palmitoleate instead of laurate might therefore function to adjust outer membrane fluidity in *E. coli* cells shifted to low temperatures. As expected, a mutant lacking *lpxP* showed increased sensitivity to antibiotics at low temperature, evidencing that the integrity of its OM is altered during cold shock (Raetz et al. 2007). Similarly, the bacterial pathogen *Francisella tularensis*, the cause of tularemia, a highly contagious pulmonary disease of humans and animals, remodels its membrane lipid A according to growth temperature. *Francisella* cells express preferentially a particular acyltransferase, LpxD1 or LpDx2, under different environmental temperature conditions. This bacterium expresses a unique tetra-acylated LPS at all growth temperatures, but at 37 °C LpxD1 incorporates 18-C acyl chains at the 2 and 2′ positions of lipid A, while at 21 °C LpxD2 adds shorter acyl chains (16-C) to these positions, increasing OM fluidity in colder environments (Fig. 1d, right side). Both the transcriptional and enzymatic levels of the individual acyltransferases are regulated by the temperature shift (Li et al. 2012).

Yersinia pestis, the causative agent of the deadly disease called Bubonic plague, also synthesizes an alternative lipid A at different growth temperatures. At the mammalian host temperature (37 °C), this bacterium produces and exports primarily tetra-acylated lipid A to the outer membrane (Kawahara et al. 2002). However, during growth at flea vector temperature (21 °C), *Y. pestis* generates primarily hexa-acylated lipid A modified with C12 and C16:1, a form that resembles lipid A produced by *E. coli* at 12 °C (Kawahara et al. 2002). This bacterium lacks *lpxL* and expresses *lpxM* and *lpxP* homologues only below 21 °C (Rebeil et al. 2006). Consequently, upon entry into the rodent host, *Y. pestis* synthesizes mainly tetra-acylated lipid A, a variant with poor Toll-like receptor 4-stimulating activity, which enables bacteria to evade detection by the mammalian innate immune system (Montminy et al. 2006).

6 Research Needs

Although considerable efforts have made in dissecting the mechanism by which the *B. subtilis* DesK-DesR system control desaturase expression, it will be particularly interesting to determine exactly how the membrane component of this transcriptional regulatory system senses membrane viscosity and transmits the signal to the transcriptional apparatus.

The mechanism by which the biosynthesis of BCFAs is upregulated after a downshift in growth temperature remains to be elucidated. BCFAs are not confined to bacteria but also exist in fungi, plants, and animals. Thus, understanding the

regulation of the synthesis of these fatty acids in bacteria could have impact in other organisms where their role (roles) remains to be fully appreciated. Finally, understanding the temperature regulation of lipid A fatty acid composition will facilitate the future biomedical investigation of outer membrane homeostasis in major human pathogens.

Acknowledgments This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (FONCYT). M.C. Mansilla and D. de Mendoza are Career Investigators of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

References

Altabe SG, Mansilla MC, de Mendoza D (2013) Remodeling of membrane lipids by bacterial desaturases. In: Ntambi JM (ed) Stearoyl-CoA desaturase genes in lipid metabolism. Springer, New York, pp 209–231

Annous BA, Becker LA, Bayles DO, Labeda DP, Wilkinson BJ (1997) Critical role of anteiso-C15:0 fatty acid in the growth of at low temperatures. Appl Environ Microbiol 63:3887–3894

Beckering CL, Steil L, Weber MH, Volker U, Marahiel MA (2002) Genome wide transcriptional analysis of the cold shock response in *Bacillus subtilis*. J Bacteriol 184:6395–6402

Chazarreta Cifré L, Alemany M, de Mendoza D, Altabe S (2013) Exploring the biosynthesis of unsaturated fatty acids in *Bacillus cereus* ATCC 14579 and functional characterization of novel acyl-lipid desaturases. Appl Environ Microbiol 79:6271–6279

Cvec G, Marsh D (1987) Phospholipid bilayers: physical principles and models. Wiley-Interscience, New York, pp 2–28

Cybulski LE, Martin M, Mansilla MC, Fernandez A, de Mendoza D (2010) Membrane thickness cue for cold sensing in a bacterium. Curr Biol 20:1539–1544

de Mendoza D (2014) Temperature sensing by membranes. Annu Rev Microbiol 68:101–116

de Mendoza D, Cronan JE (1983) Thermal regulation of membrane lipid fluidity in bacteria. Trends Biochem Sci 8:49–52

Diomandé SE, Chamot S, Antolinos V, Vasai F, Guinebretiere MH, Bornard I, Nguyen-the C, Broussolle V (2014) The CasKR two-component system is required for the growth of mesophilic and psychrotolerant *Bacillus cereus* strains at low temperatures. Appl Environ Microbiol 80:2493–2503

Diomandé SE, Nguyen-the C, Abee T, Tempelaars MH, Broussolle V, Brillard J (2015) Involvement of the CasK/R two-component system in optimal unsaturation of the *Bacillus cereus* fatty acids during low-temperature growth. Int J Food Microbiol 213:110–117

Diomandé SE, Doublet B, Vasaï F, Guinebretiére M-H, Broussolle V, Brillard J (2016) Expression of the genes encoding the CasK/R two-component system and the DesA desaturase during Bacillus cereus cold adaptation. FEMS Microbiol Lett 363:pii: fnw174

Kaneda T (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288–302

Kawahara K, Tsukano H, Watanabe H, Lindner B, Matsuura M (2002) Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. Infect Immun 70:4092–4098

Klein W, Weber MH, Marahiel MA (1999) Cold shock response of *Bacillus subtilis*: isoleucine-dependent switch in the fatty acid branching pattern for membrane adaptation to low temperatures. J Bacteriol 181:5341–5349

Li Y, Powell DA, Shaffer SA, Rasko DA, Pelletier MR, Leszyk JD, Scott AJ, Masoudi A, Goodlett DR, Wang X, Raetz CR, Ernst RK (2012) LPS remodeling is an evolved survival strategy for bacteria. Proc Natl Acad Sci U S A 109:8716–8721

Magnuson K, Jackovski S, Rock CO, Cronan JE Jr (1993) Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiol Rev 57:522–542

Mansilla MC, de Mendoza D (2005) The *Bacillus subtilis* desaturase: a model to understand phospholipid modification and temperature sensing. Arch Microbiol 183:229–235

Mansilla MC, Cybulski LE, Albanesi D, de Mendoza D (2004) Control of membrane lipid fluidity by molecular thermosensors. J Bacteriol 186:6681–6688

Martin M, de Mendoza D (2013) Regulation of *Bacillus subtilis* DesK thermosensor by lipids. Biochem J 451:269–275

Martin N, Lombardia E, Altabe SG, de Mendoza D, Mansilla MC (2009) A lipA (yutB) mutant, encoding lipoic acid synthase, provides insight into the interplay between branched-chain and unsaturated fatty acid biosynthesis in *Bacillus subtilis*. J Bacteriol 191:7447–7455

Montminy SW, Khan N, McGrath S, Walkowicz MJ, Sharp F, Conlon JE, Fukase K, Kusumoto S, Sweet C, Miyake K, Akira S, Cotter RJ, Goguen JD, Lien E (2006) Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. Nat Immunol 7:1066–1073

Parsons JB, Rock CO (2013) Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res 52:249–276

Phadtare S (2004) Recent developments in bacterial cold-shock response. Curr Issues Mol Biol 6:125–136

Phetsuksiri B, Jackson M, Scherman H, McNeil M, Besra GS, Baulard AR, Slayden RA, DeBarber AE, Barry CE III, Baird MS, Crick DC, Brennan PJ (2003) Unique mechanism of action of the thiourea drug isoxyl on *Mycobacterium tuberculosis*. J Biol Chem 278:53123–53130

Porrini L, Cybulski L, Altabe SG, Mansilla MC, de Mendoza D (2014) Cerulenin inhibits unsaturated fatty acids synthesis in *Bacillus subtilis* by modifying the input signal of DesK thermosensor. Microbiol Open 3:213–224

Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76:295–329

Rebeil R, Ernst RK, Jarrett CO, Adams KN, Miller SI, Hinnebusch BJ (2006) Characterization of late acyltransferase genes of *Yersinia pestis* and their role in temperature-dependent lipid A variation. J Bacteriol 188:1381–1388

Saita E, Abriata LA, Tsai YT, Trajtenberg F, Lemmin T, Buschiazzo A, Dal Peraro M, de Mendoza D, Albanesi D (2015) A coiled coil switch mediates cold sensing by the thermosensory protein DesK. Mol Microbiol 98:258–271

Saita E, Albanesi D, de Mendoza D (2016) Sensing membrane thickness: lessons learned from cold stress. Biochim Biophys Acta 1861:837–846

Shimura Y, Shiraiwa Y, Suzuki I (2012) Characterization of the subdomains in the N-terminal region of histidine kinase Hik33 in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Cell Physiol 53:1255–1266

Sinetova MA, Los DA (2016) New insights in cyanobacterial cold stress responses: Genes, sensors, and molecular triggers. Biochim Biophys Acta 1860:2391–2403

Singh AK, Zhang YM, Zhu K, Subramanian C, Li Z, Jayaswal RK, Gatto C, Rock CO, Wilkinson BJ (2009) FabH selectivity for anteiso branched-chain fatty acid precursors in low-temperature adaptation in *Listeria monocytogenes*. FEMS Microbiol Lett 301:188–192

Small DM (1986) General properties of lipids conferred by the aliphatic chain. In: Hanahan DJ (ed) The physical chemistry of lipids, Handbook of lipid research, vol 4. Plenum, New York, pp 21–42

Wollenweber HW, Schlecht S, Lüderitz O, Rietschel ET (1983) Fatty acid in lipopolysaccharides of *Salmonella* species grown at low temperature. Identification and position. Eur J Biochem 130:167–171

Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6:222–233

Zhu K, Bayles DO, Xiong A, Jayaswal RK, Wilkinson BJ (2005) Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain-keto acid dehydrogenase. Microbiology 151:615–623

Zhu K, Choi KH, Schweizer HP, Rock CO, Zhang YM (2006) Two aerobic pathways for the formation of unsaturated fatty acids in *Pseudomonas aeruginosa*. Mol Microbiol 60:260–273

Membrane Homeostasis in Bacteria upon pH Challenge

43

Christian Sohlenkamp

Contents

1 Introduction 788
2 Modification of Fatty Acids Composition 789
3 Changes in Membrane Lipid Types 791
3.1 Formation of Aminoacylated Derivatives of Phosphatidylglycerol 792
3.2 Presence of Hopanoids 793
3.3 Increase of (Hydroxylated) Ornithine Lipids 794
3.4 Presence and Increase of Sphingolipids 795
3.5 Modifications of Lipopolysaccharide Structure 795
3.6 Modifications of Cardiolipin Concentration 796
4 Conclusions and Research Needs 796
References 796

Abstract

Bacteria are frequently exposed to acid stress. In order to survive these unfavorable conditions, they have evolved a set of resistance mechanisms, which include the pumping of protons out of the cytoplasm, the production of ammonia, proton-consuming decarboxylation reactions, and modifications of the membrane lipid composition. In this chapter, I will discuss the changes in membrane composition that have been described in bacteria to be part of the adaptation to acid stress conditions. The cytoplasmic membrane is a major barrier to proton influx in acid-treated cells. However, there is no single one membrane adaptation used by all

C. Sohlenkamp (✉)
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico
e-mail: chsohlen@ccg.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_57

bacteria in response to acid stress. Rather, different bacteria seem to use different strategies to adjust their membrane lipid composition in response to an increase in proton concentration.

1 Introduction

Bacteria are frequently exposed to varying environmental conditions such as, for example, changes in temperature, pH, or osmotic conditions and the presence or absence of nutrients and toxins. In order to cope with these stresses and to survive, they have adopted different response mechanisms. Examples for acidic environments in which bacteria are found are acidic soils, the gastric compartments of animals, the plant rhizosphere, and lytic vacuoles inside macrophages. In unprotected cells, a decrease in intracellular pH causes lowered enzyme activities, protein unfolding and denaturation, DNA damage, and membrane damage (Lund et al. 2014). The presence of an acid stress response provides the bacteria with an advantage in these otherwise life-threatening conditions. The most common mechanisms to resist acidic conditions are the pumping of protons out of the cytoplasm, the production of ammonia, proton-consuming decarboxylation reactions, and modifications of the membrane lipid composition (Kanjee and Houry 2013; Lund et al. 2014). In this chapter I will discuss the changes in membrane composition that have been described in bacteria to be part of the adaptation to acid stress conditions.

There is a lot of diversity of membrane lipid structures in the bacterial world, and there is no such thing as a typical bacterial membrane lipid composition (Sohlenkamp and Geiger 2016). Membranes are formed by amphiphilic lipids which in most cases and conditions are studied glycerophospholipids, but other lipids such as sphingolipids, hopanoids, and aminoacyl lipids have also been described. Membrane properties are defined in large part by the fatty acid structures that are incorporated into membrane lipids, although they also depend on other factors, such as headgroup composition. The cytoplasmic membrane is a major barrier to proton influx in acid-treated cells, and the modification of phospholipids in the inner membrane is a way to decrease proton permeability. Therefore, the ability to adjust membrane properties by modifying the types of fatty acids that are present in membrane lipids by altering the structure of preexisting membrane lipids or synthesizing new lipids is an important factor for bacterial survival under changing environmental conditions (Parsons and Rock 2013). As there is no typical bacterial membrane lipid composition, there is no single one membrane adaptation used by all bacteria in response to acid stress. Rather, different bacteria seem to use different strategies to adjust their membrane lipid composition in response to acid stress. Existing strategies can be classified in two groups: the modification of existing membrane lipids allows a quick response to acid stress, whereas the de novo synthesis of membrane lipids is a slower response.

2 Modification of Fatty Acids Composition

Membrane properties are defined in large part by the fatty acid structures that are incorporated into membrane lipids. The fatty acid composition of the membrane lipids depends on the growth conditions and the growth phase of the culture. The probably best-known example of how growth conditions affect the fatty acid composition is the maintenance of a similar membrane fluidity at different growth temperature (homeoviscous adaptation): membranes of *Escherichia coli* cells grown at 16 °C have a higher proportion of unsaturated fatty acids to saturated fatty acids than the membranes of cells grown at 37 °C. These adjustments allow that the fluidity of the membrane stays approximately constant at both growth temperatures although the fatty acid composition is changed. The acyl chain composition of the membrane lipids determines the viscosity of the membrane and adjustments in acyl chain composition are made to maintain the required biophysical properties. The proton permeability of the membrane of mesophilic bacteria is also controlled by the lipid composition.

Under conditions of acid stress and in the stationary growth phase, a conversion of unsaturated fatty acids to cyclopropane fatty acids (CFAs) (Fig. 1) has been observed in *E. coli* and several other bacteria. CFAs are formed from unsaturated fatty acids by adding a methyl group from *S*-adenosylmethionine (SAM) to the double bond, a reaction catalyzed by the enzyme cyclopropane fatty acid synthase (Cfa) (Chang and Cronan 1999; Kim et al. 2005). Transcription of the *cfa* gene is induced in *E. coli* under acidic conditions (Chang and Cronan 1999). *E. coli* membranes lacking CFAs have been shown to be more permeable to protons and showed a decreased ability to extrude protons (Shabala and Ross 2008), and mutants deficient in Cfa are very sensitive to a shift to low pH, but this sensitivity can be overcome by feeding CFAs exogenously. CFAs have biophysical properties similar to unsaturated fatty acids, but apparently they are more stable to environmental insults, such as acid stress. The acid tolerance of individual *E. coli* strains appears to be correlated with membrane CFA content (Brown et al. 1997).

Mutants deficient in synthesis of CFA also have been described and characterized in other bacteria, such as *Salmonella typhimurium*, *Sinorhizobium meliloti*, and *Brucella abortus* (Kim et al. 2005; Basconcillo et al. 2009; Palacios-Chaves et al. 2012). Gene expression under acid stress conditions is induced in *S. meliloti*, or mutants deficient in synthesis of CFA are affected in survival under acidic growth conditions in *Brucella* and *Salmonella*. CFAs in *S. meliloti* increase by 10–15% under acid stress, and the transcription of the *cfa2* gene is induced under these conditions (Basconcillo et al. 2009). A *Salmonella* mutant deficient in CFA production was sensitive to low pH. Kim et al. (2005) concluded that unsaturated fatty acids as well as CFAs are necessary for full acid resistance in *Salmonella*, although CFAs contribute more to acid resistance. In *Chlorobium tepidum*, the inhibitor sinefungin, an analog of *S*-adenosyl-L-methionine, was used to show the importance of CFAs in glycolipids for the acid resistance of chlorosomes (Mizoguchi et al. 2013). Among *Helicobacter* isolates those identified as gastric colonizers apparently tend to

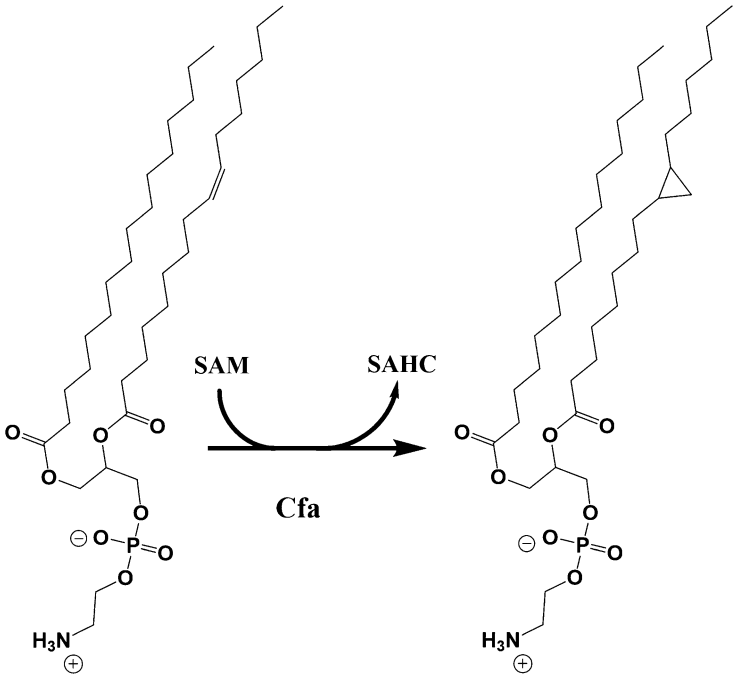


Fig. 1 Cyclopropanation of membrane lipid-bound unsaturated fatty acids. Depending on the growth phase or in response to acid stress, many bacteria can convert monounsaturated fatty acid into cyclopropane fatty acids (*CFAs*). These CFAs have biophysical properties similar to unsaturated fatty acids but are more stable to environmental stress conditions, such as low pH. Cyclopropanation occurs on membrane lipid-bound fatty acids, not on free fatty acids. In this example modification of the common phospholipid phosphatidylethanolamine is shown. The enzyme cyclopropane fatty acid synthase uses the methyl donor *S*-adenosylmethionine (*SAM*). There is no known mechanism to reverse the cyclopropanation of fatty acids; their content is diluted when the cells reenter logarithmic growth (Zhang and Rock 2008)

produce large amounts of CFAs. The isolates identified as intestinal colonizers do not produce large amounts of CFAs (Haque et al. 1996). But the presence/absence of CFA does not always influence acid resistance: *Pseudomonas putida* DOT-T1E mutants deficient in CFA formation were not affected under acidic growth conditions (Pini et al. 2009).

It is known that the cyclopropane bond is more stable than a double bond and that its presence affects proton permeability of the membrane, but the mechanism of how CFAs increase the resistance to acid stress is not completely clear yet. A molecular dynamics simulation has suggested that CFAs may fulfill a dual function: they stabilize membranes against adverse conditions while at the same time promoting their fluidity (Poger and Mark 2015).

Other adjustments in the fatty acyl composition of bacteria occurring under acid stress conditions also have been described. A few bacteria, especially gram-positives can also form branched chain fatty acids in addition to linear fatty acids. These

usually occur as iso- or anteiso-fatty acids. In general, the anteiso-fatty acids promote a more fluid membrane structure than the iso-fatty acids, because the methyl branch is further from the end of the fatty acid (Zhang and Rock 2008). *Listeria monocytogenes* has been shown to modify their iso- to anteiso-fatty acid ratio in response to temperature and pH stress. Under acid stress conditions, the formation of anteiso-fatty acids is increased in *L. monocytogenes* (Giotis et al. 2007).

In response to acidification, *Streptococcus mutans* increases the proportion of long chained monounsaturated fatty acids in its membrane with a concomitant decrease in short chained saturated fatty acids (Fozo et al. 2004; Fozo and Quivey 2004a). It has been shown that monounsaturated fatty acids are essential for survival at low pH (Fozo and Quivey 2004b). A *fabM* mutant deficient in the formation of unsaturated fatty acids is extremely sensitive to low pH in comparison to the wild type. This phenotype can be relieved by adding long-chain monounsaturated fatty acids to the medium (Fozo and Quivey 2004b). Similarly, in *Lactobacillus casei* ATCC 4646, an increase in long-chain monounsaturated fatty acids was observed when grown at acid pH, but in this strain also, the cyclopropane fatty acid lactobacillic acid was accumulated. Apparently, the acidic conditions caused a shift toward longer fatty acids, leading the authors to suggest that increased fatty acid length is an important membrane alteration to increase survival in acidic environments (Fozo and Quivey 2004b). A different response was reported for *L. casei* ATCC 334 (now called *Lactobacillus paracasei*): an increase in saturated and cyclopropane fatty acids was observed, whereas monounsaturated fatty acids showed a decrease (Broadbent et al. 2010).

Cyclopropane fatty acids apparently also play an important role in acidophilic bacteria such as *Acidithiobacillus ferrooxidans* and *Thiobacillus* sp. Levin (1971) and Kerger et al. (1986) have reported that the fatty acid profile of *Thiobacillus* species is characterized by high levels (>60%) of CFAs. Similar levels of CFAs are also reported in *A. ferrooxidans*, although lowering the pH from the optimal pH 2.0 further does not increase the amount of CFAs formed (Mykytczuk et al. 2010).

3 Changes in Membrane Lipid Types

In addition to modifications of fatty acids, other membrane modifications have been described in acid-stressed bacteria. There is a lot of diversity of membrane lipid structures in the bacterial world. Not all bacteria can form the same lipids and have an identical lipid composition. This might be the reason why different bacteria seem to use different strategies to adjust membrane lipid composition to changing environmental conditions. Most membrane lipids are glycerophospholipids, but other lipids such as sphingolipids and aminoacyl lipids have also been described. A function for a specific membrane lipid or membrane lipid modification under acid stress conditions has been usually concluded from the observation of increased formation of the specific lipid under acidic conditions or from the phenotype of mutants deficient in the formation of a specific membrane lipid. The presence of the following membrane lipids or an increase in their concentration under acidic stress

conditions has been related to a possible function in acid stress response: (A) formation of aminoacylated derivatives of phosphatidylglycerol, (B) presence of hopanoids, (C) increase of (hydroxylated) ornithine lipids, (D) presence and increase of sphingolipids, (E) modifications of lipopolysaccharide structure, and (F) modifications of cardiolipin concentration.

3.1 Formation of Aminoacylated Derivatives of Phosphatidylglycerol

The anionic phospholipid phosphatidylglycerol (PG) can be aminoacylated in some bacteria. Amino acids such as arginine, lysine, or alanine are transferred from a charged tRNA to the glycerol headgroup of phosphatidylglycerol thereby modifying an anionic lipid depending on the amino acid transferred into a zwitterionic lipid or a cationic lipid (Slavetinsky et al. 2016) (Fig. 2). This amino acid transfer is catalyzed

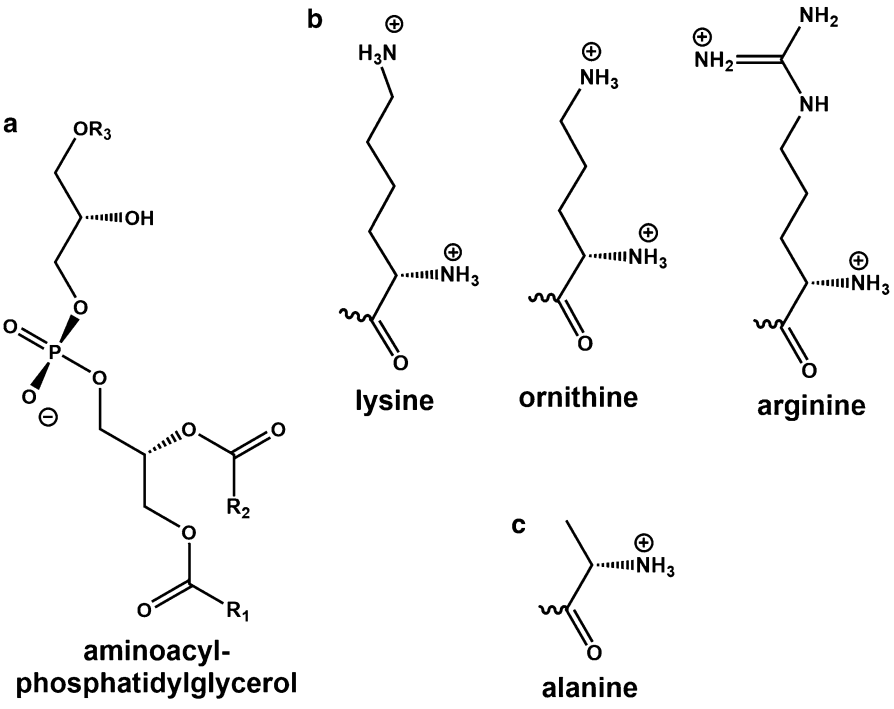


Fig. 2 Structure and headgroup variation of aminoacylated phosphatidylglycerol. Depending on which amino acid is ester bound to phosphatidylglycerol, the resulting lipid can be cationic (lysine, ornithine, arginine) or zwitterionic (alanine). (**a**) phosphatidylglycerol backbone and (**b** and **c**) amino acids that have been found in aminoacyl phosphatidylglycerol in different species. R_1 and R_2 alkyl chains of *sn1*- and *sn2*-bound fatty acids and R_3 amino acid ester bound to the glycerol headgroup of PG (shown in **b** and **c**)

by enzymes called multiple peptide resistance factor (MprF) or low pH inducible (LpiA). MprF has also been shown to have a flippase activity, flipping the cationic lipid lysyl-PG (LPG) from the cytoplasmic layer of the membrane to the periplasmic layer of the membrane (Ernst et al. 2009; Slavetinsky et al. 2012). In *Staphylococcus aureus*, *Enterococcus faecalis*, and *Rhizobium tropici*, the formation of the cationic phospholipid LPG is induced under acidic growth conditions (Houtsmuller and van Deenen 1965; Vinuesa et al. 2003; Sohlenkamp et al. 2007), and *P. aeruginosa* synthesizes significant amounts of alanyl-PG (APG) when exposed to acidic growth conditions but not under neutral growth conditions (Klein et al. 2009). Consistently, in a transcriptional study of the *Sinorhizobium meliloti* acid stress response, the *lpiA* gene was among the strongest induced genes (Hellweg et al. 2009). The transcriptional induction of the *mprF/lpiA* gene and the increase in the formation of the different aminoacylated PGs in different species indicate a possible function under acid stress, but growth of *P. aeruginosa* mutants deficient in APG was not affected under acidic growth conditions (Klein et al. 2009). In gram-negative bacteria, the gene encoding for LpiA is in most cases forming an operon with a gene coding for a putative serine lipase that has been shown to be a periplasmic protein in *Agrobacterium tumefaciens* and *P. aeruginosa* (Kang et al. 1994; Arendt et al. 2013). Often genes encoded in operons encode enzymes acting in the same metabolic pathway. Consistently, in *P. aeruginosa*, the enzyme PA0919 encoded by the second gene of this operon appears to have some minor APG hydrolase activity that should lead to the liberation of alanine in the periplasm (Arendt et al. 2013). It is not clear how this activity might be conferring acid resistance to the bacteria.

3.2 Presence of Hopanoids

Bacteriohopanepolyols, generally referred to as hopanoids, are triterpenoic, pentacyclic compounds. They are widespread in bacteria and can be found in the cytoplasmic and in the outer membrane. They are structurally similar to sterols such as cholesterol and are often considered their functional analogs in bacteria. The enzyme squalene-hopene cyclase is responsible for the formation of the simplest hopanoids, diplotene or diplopterol, from squalene. This basic structure can be modified leading to structural variations present in hopanoids: some are methylated, some are unsaturated, and the functionalization of the side chain can vary greatly (Sohlenkamp and Geiger 2016). It has been proposed that the presence of hopanoids enhances the membrane stability, decreases the membrane permeability, and increases stress tolerance in a few bacteria. A few studies have suggested that hopanoids are necessary for maintaining membrane integrity as well as coping with external stresses: ethanol tolerance in *Zymomonas mobilis*, oxygen diffusion in *Frankia*, and prevention of water diffusion in *Streptomyces* spores (Berry et al. 1993; Horbach et al. 1991; Poralla et al. 1984, 2000). In recent years many of the genes involved in hopanoid synthesis and modification have been identified, and mutants deficient in these genes have been constructed. Whereas *Burkholderia cenocepacia* and *Rhodopseudomonas palustris* mutants deficient in hopanoid formation showed

increased sensitivity to low pH and other environmental stresses; *Streptomyces scabies* mutants were not affected (Welander et al. 2009; Schmerk et al. 2011; Seipke and Loria 2009). In *Bradyrhizobium diazoefficiens* 2-methylated hopanoids contribute to growth under plant cell-like microaerobic conditions and acidic conditions in the free-living state. Hopanoids with a side chain are also required under various stress conditions including high temperature, low pH, and others (Kulkarni et al. 2015).

In vitro experiments have shown that hopanoids are efficient in condensing artificial membranes and enhancing the viscosity of liposomes (Poralla et al. 1984). It was demonstrated that lipid A undergoes a pH-dependent change in order to become less fluid at lower pH and to approach a gel state possibly causing the membrane to become leaky. Hopanoids are capable of interacting with lipid A and inhibiting this phase transition while at the same time helping to form a highly ordered bilayer (Sáenz et al. 2012, 2015). Hopanoid-producing gram-negative bacteria with disrupted hopanoid synthesis exhibit retarded growth at low pH (Welander et al. 2009; Schmerk et al. 2011).

Recently hopanoids have been found covalently bound to lipid A in a photosynthetic *Bradyrhizobium* strain (Silipo et al. 2014). These hopanoid lipid A hybrids reinforced the stability and rigidity of the outer membrane. A hopanoid-deficient strain displayed increased sensitivity to stressful conditions and reduced the ability to survive intracellularly in the host plant (Silipo et al. 2014).

Interestingly, the gram-negative bacteria lacking hopanoids were more sensitive to abiotic stresses than the respective wild type, whereas this was not the case for the gram-positive *S. scabies*. As there is no lipid A in *S. scabies*, hopanoid probably have a different function in the latter by interacting with other cellular structures, and this might be also the reason for the differences in phenotypes observed.

3.3 Increase of (Hydroxylated) Ornithine Lipids

Ornithine lipids (OLs) are amino acyl lipids, composed of an ornithine headgroup to the α-amino group of which a 3-hydroxy fatty acyl group is amide-bound. A secondary fatty acid is ester linked to the 3-hydroxy group of the first fatty acid. It has been predicted that OLs can be formed by about half of the sequenced bacteria, but they are absent from eukaryotes and archaea (Vences-Guzmán et al. 2015). OLs can be hydroxylated in various positions, but especially the 2-hydroxylation of the secondary fatty acid has been related to an increased acid and temperature resistance (Rojas-Jimenez et al. 2005; Gonzalez-Silva et al. 2011; Vences-Guzman et al. 2011). When *R. tropici* is grown at pH 4.5, more hydroxylated OLs are accumulated compared to growth at neutral pH. Mutants deficient in OlsC, the OL hydroxylase responsible for this modification, present a drastic growth phenotype at low pH (Vences-Guzmán et al. 2011). Another OL hydroxylase called OlsD was described in *B. cenocepacia* that is responsible for the 2-hydroxylation of the amide-linked fatty acid (Gonzalez-Silva et al. 2011). This species of OLs cannot be detected when cells are grown at neutral pH, but small amounts accumulate when the cells are grown at

pH 4.0, indicating that expression of the *olsD* gene is induced under low pH conditions. Both OlsC and OlsD are homologs of the lipid A 2-hydroxylase LpxO responsible for the alpha-hydroxylation of the 3′-myristic acid residue occurring in *S. typhimurium* lipid A under conditions mimicking the inside of vacuoles of macrophages (Gibbons et al. 2000, 2008). Nikaido has speculated that the presence of additional hydroxyl group in membrane lipids (like the ones introduced by OlsC, OlsD, and LpxO) might allow for the formation of additional hydrogen bonds which would cause a decrease in membrane fluidity and membrane permeability (Nikaido 2003).

3.4 Presence and Increase of Sphingolipids

Sphingolipids were thought for a long time to be absent from bacteria. Apparently, only a few bacteria mainly belonging to the *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) group of bacteria and to the alphaproteobacteria are able to form sphingolipids. Evidence that the presence of sphingolipids in bacteria is related to an increased stress resistance comes from *Acetobacter malorum*. Ogawa et al. (2010) have shown that the sphingolipid content of its membrane increases under acidic growth conditions and at increased temperature (Ogawa et al. 2010). Only minor amounts of sphingolipid can be detected when the cells are grown at neutral pH, but ceramide formation is strongly induced at pH 4 or lower. No mutants deficient in sphingolipid synthesis have been constructed to confirm these suggestions.

A *Saccharomyces cerevisiae* mutant that formed sphingolipids only in the presence of the long-chain base phytosphingosine was sensitive to various abiotic stresses in the absence of sphingolipids: it failed to grow at 37 °C, in the presence of high salt concentrations and at low pH (Patton et al. 1992). A possible explanation for these phenotypes is that sphingolipids have a function in the response to abiotic stress.

3.5 Modifications of Lipopolysaccharide Structure

In general the outer membrane is not considered to play an important role in protecting bacteria against acidic conditions. Therefore there are only few reports relating to the importance of this structure under low pH stress. In *Shigella flexneri*, a pathogen causing diarrheal disease and dysentery among young children, it was shown that a mutant lacking O antigen was significantly more sensitive to extreme acid conditions than the wild type. In this bacterium, moderate acidic conditions induced the addition of phosphoethanolamine to the 1-phosphate group of lipid A. This modification contributed to an increased resistance to extreme acid conditions (Martinić et al. 2011).

The ability of *Salmonella typhimurium* to adapt to the acidic pH and the low divalent cation concentrations found inside macrophage vacuoles is critical for the

infection process. Among other changes occurring in the cells, lipid A is modified with phosphoethanolamine and 4-amino-arabinose, the acyl chains of lipid A are remodeled, and LpxO introduces a 2-hyroxylation on the 3′-secondary myristoyl chain (Gibbons et al. 2008; Henderson et al. 2016).

3.6 Modifications of Cardiolipin Concentration

In *Streptococcus mutans* the transcription of the gene encoding cardiolipin (CL) synthase is induced under acidic conditions, and *S. mutans* mutants deficient in CL formation are acid sensitive. In *S. mutans* wild-type monounsaturated fatty acids are important for acid resistance (Fozo and Quivey 2004a, b), and CL is the membrane lipid with major concentrations of these monounsaturated fatty acids. In *S. mutans* mutants deficient in cardiolipin synthase (Cls) that only reduced amounts of these fatty acids can be detected (MacGilvray et al. 2012). It is possible that the acid sensitive phenotype of the Cls mutants is caused by the decrease in monounsaturated fatty acids in the membrane. Furthermore it was observed that the absence of CL reduced acid resistance but does not prevent an acid-adaptive response (MacGilvray et al. 2012).

4 Conclusions and Research Needs

When bacteria are exposed to acid stress, they have to adapt to these adverse conditions, one important response is to make their membrane less permeable to protons. This apparently can be reached by making the membrane less fluid, denser, or thicker. Clearly, the most common membrane modification occurring in bacteria in response to acid stress is the cyclopropanation of monounsaturated fatty acids. Other common responses involve hopanoid formation and the introduction of hydroxyl groups into existing membrane lipids such as ornithine lipids, lipid A, or sphingolipids. Work is needed to completely understand the molecular mechanisms especially how the interactions between membrane molecules affect membrane properties such as proton permeability.

Acknowledgments Work in the laboratory was supported by grants to C.S. from SEP-CONACyT (237713) and PAPIIT-UNAM (IN202413, IN208116).

References

Arendt W, Groenewold MK, Hebecker S, Dickschat JS, Moser J (2013) Identification and characterization of a periplasmic aminoacyl-phosphatidylglycerol hydrolase responsible for *Pseudomonas aeruginosa* lipid homeostasis. J Biol Chem 288:24717–24730

Basconcillo LS, Zaheer R, Finan TM, McCarry BE (2009) Cyclopropane fatty acyl synthase in *Sinorhizobium meliloti*. Microbiology-SGM 155:373–385

Berry AM, Harriott OT, Moreau RA, Osman SF, Benson DR, Jones AD (1993) Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. Proc Natl Acad Sci U S A 90:6091–6094

Broadbent JR, Larsen RL, Deibel V, Steele JL (2010) Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. J Bacteriol 192:2445–2458

Brown JL, Ross T, McMeekin TA, Nichols PD (1997) Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. Int J Food Microbiol 37:163–173

Chang Y-Y, Cronan JE Jr (1999) Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol Microbiol 33:249–259

Ernst CM, Staubitz P, Mishra NN, Yang S-J, Hornig G, Kalbacher H, Bayer AS, Kraus D, Peschel A (2009) The bacterial defensing resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog 5(11):e1000660. https://doi.org/10.1371/journal.ppat.1000660

Fozo EM, Quivey RG Jr (2004a) Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. Appl Environ Microbiol 70:929–936

Fozo EM, Quivey RG Jr (2004b) The *fabM* gene product of *Streptococcus mutans* is responsible for the synthesis of monounsaturated fatty acids and is necessary for survival at low pH. J Bacteriol 186:4152–4158

Fozo EM, Kajfasz JK, Quivey RG Jr (2004) Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol Lett 238:291–295

Gibbons HS, Lin S, Cotter RJ, Raetz CRH (2000) Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, a new Fe (II)/alpha-ketoglutarate-dependent dioxygenase homologue. J Biol Chem 275:32940–32949

Gibbons HS, Reynolds CM, Guan Z, Raetz CRH (2008) An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. Biochemistry 47:2814–2825

Giotis ES, McDowell DA, Blair IS, Wilkinson BJ (2007) Role of branched-chain fatty acids in pH stress tolerance in *Listeria monocytogenes*. Appl Environ Microbiol 73:997–1001

Gónzalez-Silva N, López-Lara IM, Reyes-Lamothe R, Taylor AM, Sumpton D, Thomas-Oates J, Geiger O (2011) The dioxygenase-encoding *olsD* gene from *Burkholderia cenocepacia* causes the hydroxylation of the amide-linked fatty acyl moiety of ornithine- membrane lipids. Biochemistry 50:6396–6408

Haque M, Hirai Y, Yokota K, Mori N, Jahan I, Ito H, Hotta H, Yano I, Kanemasa Y, Ogawa K (1996) Lipid profile of *Helicobacter* spp.: presence of cholesteryl glucoside as a characteristic feature. J Bacteriol 178:2065–2070

Hellweg C, Pühler A, Weidner S (2009) The time course of the transcriptomic response of *Sinorhizobium meliloti* 1021 following a shift to acid pH. BMC Microbiol 9:37

Henderson JC, Zimmerman SM, Crofts AA, Boll JM, Kuhns LG, Herrera CM, Trent SM (2016) The power of asymmetry: architecture and assembly of the gram-negative outer membrane lipid bilayer. Annu Rev Microbiol 70:755–778

Horbach S, Neuss B, Sahm H (1991) Effect of azasqualene on hopanoid biosynthesis and ethanol tolerance of *Zymomonas mobilis*. FEMS Microbiol Lett 79:347–250

Houtsmuller UM, van Deenen LL (1965) On the amino acid esters of phosphatidyl glycerol from bacteria. Biochim Biophys Acta 106:564–576

Kang HW, Wirawan IG, Kojima M (1994) Cellular localization and functional analysis of the protein encoded by the chromosomal virulence gene (*acvB*) of *Agrobacterium tumefaciens*. Biosci Biotechnol Biochem 58:2024–2032

Kanjee U, Houry WA (2013) Mechanisms of acid resistance in *Escherichia coli*. Annu Rev Microbiol 67:65–81

Kerger BD, Nichols PD, Antworth CP, Sand W, Bock E, Cox JC, Langworthy TA, White DC (1986) Signature fatty acids in the polar lipids of acid-producing *Thiobacillus* spp.: methoxy, cyclopropyl, alpha-hydroxy-cyclopropyl and branched and normal monoenoic fatty acid. FEMS Microbiol Ecol 38:67–77

Kim BH, Kim S, Kim HG, Lee J, Lee IS, Park YK (2005) The formation of cyclopropane fatty acids in *Salmonella enterica* serovar typhimurium. Microbiol-SGM 151:209–218

Klein S, Lorenzo C, Hoffmann S, Walther JM, Storbeck S, Piekarski T, Tindall BJ, Wray V, Nimtz M, Moser J (2009) Adaptation of *Pseudomonas aeruginosa* to various conditions includes tRNA-dependent formation of alanyl-phosphatidylglycerol. Mol Microbiol 71:551–565

Kulkarni G, Busset N, Molinaro A, Gargani D, Chaintreuil C, Silipo A, Giraud E, Newmann DK (2015) Specific hopanoid classes differentially affect free-living and symbiotic states of *Bradyrhizobium diazoefficiens*. MBio 6:e01251-15

Levin RA (1971) Fatty acids of *Thiobacillus thiooxidans*. J Bacteriol 108:992–995

Lund P, Tramonti A, De Biase D (2014) Coping with low pH: molecular strategies in neutralophilic bacteria. FEMS Microbiol Rev 38:1091–1125

MacGilvray ME, Lapek JD, Friedman AE, Quivey RG Jr (2012) Cardiolipin biosynthesis in *Streptococcus mutans* is regulated by response to external pH. Microbiol-SGM 158:2133–2143

Martinić M, Hoare A, Contreras I, Álvarez SA (2011) Contribution of the lipopolysaccharide to resistance of *Shigella flexneri* 2a to extreme acidity. PLoS One 6:e25557

Mizoguchi T, Tsukatani Y, Harada J, Takasaki S, Yoshitomi T, Tamiaki H (2013) Cyclopropane-ring formation in the acyl groups of chlorosome glycolipids is crucial for acid resistance of green bacterial antenna systems. Bioorg Med Chem 21:3689–3694

Mykytczuk NCS, Trevors JT, Ferroni GD, Leduc LG (2010) Cytoplasmic membrane fluidity and fatty acod composition of *Acidothiobacillus ferrooxidans* in response to pH stress. Extremophiles 14:427–441

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

Ogawa S, Tachimoto H, Kaga T (2010) Elevation of ceramide in *Acetobacter malorum* S24 by low pH stress and high temperature stress. J Biosci Bioeng 109:32–36

Palacios-Chaves L, Zuñiga-Ripa A, Gutiérrez A, Gil-Ramírez Y, Conde-Álvarez R, Moriyón I, Iriarte M (2012) Identification and functional analysis of the cyclopropane fatty acid synthase of *Brucella abortus*. Microbiology 158:1037–1044

Parsons JB, Rock CO (2013) Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res 52:249–276

Patton JL, Srinivasan B, Dickson RC, Lester RL (1992) Phenotypes of sphingolipid-dependent strains of *Saccharomyces cerevisiae*. J Bacteriol 174:7180–7184

Pini C-V, Bernal P, Godoy P, Ramos J-L, Segura A (2009) Cyclopropane fatty acids are involved in organic solvent tolerance but not in acid stress resistance in *Pseudomonas putida* DOT-T1E. Microb Biotechnol 2:253–261

Poger D, Mark AE (2015) A ring to rule them all: the effect of cyclopropane fatty acids on the fluidity of lipid bilayers. J Phys Chem B 119:5487–5495

Poralla K, Hartner T, Kannenberg E (1984) Effect of termperature and pH on the hopanoid content of *Bacillus acidocaldarius*. FEMS Microbiol Lett 23:253–256

Poralla K, Muth G, Hartner T (2000) Hopanoids are formed during transition from substrate to aerial hyphae in *Streptomyces coelicolor* A3(2). FEMS Microbiol Lett 189:93–95

Rojas-Jiménez K, Sohlenkamp C, Geiger O, Martínez-Romero E, Werner D, Vinuesa P (2005) A ClC chloride channel homolog and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance. Mol Plant-Microbe Interact 18:1175–1185

Sáenz JP, Sezgin E, Schwille P, Simons K (2012) Functional convergence of hopanoids and sterols in membrane ordering. Proc Natl Acad of Sci USA 109:14236–14240

Sáenz JP, Grosser D, Bradley AS, Lagny TJ, Lavrynenko O, Broda M, Simons K (2015) Hopanoids as functional analogues of cholesterol in bacterial membranes. Proc Natl Acad Sci U S A 112:11971–11976

Schmerk CL, Bernards MA, Valvano MA (2011) Hopanoid production is required for low-pH tolerance, antimicrobial resistance, and motility in *Burkholderia cenocepacia*. J Bacteriol 193:6712–6723

Seipke RF, Loria R (2009) Hopanoids are not essential for growth of *Streptomyces scabies* 87-22. J Bacteriol 191:5216–5223

Shabala L, Ross T (2008) Cyclopropane fatty acids improve *Escherichia coli* survival in acidified minimal media by reducing membrane permeability to H^+ and enhanced ability to extrude H^+. Res Microbiol 159:458–461

Silipo A, Vitiello G, Gully D, Sturiale L, Chaintreuil C, Fardoux J, Gargani D, Lee H-I, Kulkarni G, Busset N, Marchetti R, Palmigiano A, Moll H, Engel R, Lanzetta R, Paduano L, Parrilli M, Chang W-S, Holst O, Newman DK, Garozzo D, D'Errico G, Giraud E, Molinaro A (2014) Covalently linked hopanoid-lipid a improves outer-membrane resistance of a *Bradyrhizobium* symbiont of legumes. Nature Comm 5:5106. https://doi.org/10.1038/ncomms6106

Slavetinsky CJ, Peschel A, Ernst CM (2012) Alabyl-phosphatidylglycerol and lysyl-phosphatidylglycerol are translocated by the same MprF flippases and have similar capacities to protect against the antibiotic daptomycin in *Staphylococcus aureus*. Antimicrob Ag Chemother 56:3492–3497

Slavetinsky C, Kuhn S, Peschel A (2016) Bacterial aminoacyl phospholipids- biosynthesis and role in basic cellular processes and pathogenicity. Biochim Biophys Acta. https://doi.org/10.1016/j.bbalip.2016.11.013

Sohlenkamp C, Geiger O (2016) Bacterial membrane lipids: diversity in structures and pathways. FEMS Microbiol Rev 40:133–159

Sohlenkamp C, Galindo-Lagunas KA, Guan Z, Vinuesa P, Robinson S, Thomas-Oates J, Raetz CRH, Geiger O (2007) The lipid lysyl-phosphatidylglycerol in is present in membranes of *Rhizobium tropici* CIAT899 and confers increased resistance to polymyxin B under acidic growth conditions. Mol Plant-Microbe Interact 20:1421–1430

Vences-Guzmán MA, Guan Z, Ormeño-Orrillo E, González-Silva N, López-Lara IM, Martínez-Romero E, Geiger O, Sohlenkamp C (2011) Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899. Mol Microbiol 79:1496–1514

Vences-Guzmán MA, Guan Z, Escobedo-Hinojosa WI, Bermúdez-Barrientes JR, Geiger O, Sohlenkamp C (2015) Discovery of a bifunctional acyltransferase responsible for ornithine lipid synthesis in *Serratia proteamaculans*. Environ Microbiol 17:1487–1496

Vinuesa P, Neumann-Silkow F, Pacios-Bras C, Spaink HP, Martínez-Romero E, Werner D (2003) Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. Mol Plant-Microbe Interact 16:159–168

Welander PV, Hunter RC, Zhang L, Sessions AL, Summons RE, Newman DK (2009) Hopanoids play a role in membrane integrity and pH homeostasis in *Rhodopseudomonas palustris* TIE-1. J Bacteriol 191:6145–6156

Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6:222–233

Contributions of Membrane Lipids to Bacterial Cell Homeostasis upon Osmotic Challenge

44

T. Romantsov and J. M. Wood

Contents

1 Introduction 802
2 Osmoregulation of Bacterial Membrane Phospholipid Composition 802
3 Lipids in Bacterial Cell Biology 803
3.1 Physical Properties of Lipids and Membranes 806
3.2 Distributions of Phospholipids in Bacterial Cytoplasmic Membranes 809
4 Protein-Lipid Interactions: Their Impact on the Distribution and Function of Osmoregulatory Proteins 811
4.1 Tools for the Detection of Protein-Lipid Complexes and Their Subcellular Distributions 812
4.2 Osmosensing Transporters 813
5 Research Needs 817
References 817

Abstract

Changing environmental osmotic pressure causes transmembrane water fluxes that may impair cellular functions. Bacteria mitigate water fluxes by controlling the solute content of their cytoplasm. Increasing osmotic pressure triggers solute synthesis or uptake via osmosensing transporters, whereas osmotic downshock triggers solute release via mechanosensitive channels. Membrane lipids are implicated in the subcellular localization and function of membrane-based osmoregulatory systems. Zwitterionic phosphatidylethanolamine (PE) and anionic phosphatidylglycerol (PG) and cardiolipin (CL) are the predominant phospholipids in most bacteria, but their proportions vary widely. For many species, anionic lipids increase in proportion during cultivation in high salinity media. Evidence suggests that interactions among anionic lipid headgroups and

T. Romantsov (✉) · J. M. Wood (✉)
Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada
e-mail: romantst@uoguelph.ca; jwood@uoguelph.ca

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_58

cytoplasm-exposed areas of osmosensory transporters ProP, BetP, and OpuA are fundamental to their osmosensory response. CL-dependent targeting of transporter ProP to the CL-rich environment at the poles of *Escherichia coli* cells further modulates the osmolality response. Protein-lipid interactions are also fundamental to the gating of mechanosensitive channels MscL and MscS by membrane tension. Future work should encompass further characterization of the impacts of lipid composition on key physical properties of the membrane, as well as the regulation of lipid composition and membrane properties in response to environmental cues. The roles of lipids in the structural mechanisms of osmosensing and mechanosensitive channel gating are not fully understood. Osmosensory systems provide useful paradigms for the study of both protein-lipid interactions and the role of subcellular localization in bacterial lipid and protein function.

1 Introduction

Changing environmental osmotic pressure causes transmembrane water efflux or influx that may impair cellular functions. Such water flux occurs within seconds because most phospholipid membranes are highly water permeable and cell membranes include aquaporins. Periods of minutes are required for ensuing changes to cell structure. Osmotic upshifts cause immediate cellular dehydration, concentrating and crowding cellular constituents, whereas osmotic downshifts cause water influx that dilutes cytoplasmic constituents, increases cytoplasmic volume, and may strain the cytoplasmic membrane and cell wall to the point of lysis.

Bacteria use the following osmoregulatory systems to tolerate changes in the osmotic pressure (Π) (or, at constant temperature, the osmolality (Π/RT)). Increasing osmolality triggers active solute uptake via osmosensing transporters and forestalls dehydration. Increased membrane tension due to osmotic downshock opens mechanosensitive channels to release cytoplasmic solutes and prevent cell lysis. Membrane lipids are clearly implicated in the function and subcellular localization of osmosensing transporters and mechanosensitive channels. Here we review evidence that bacterial membrane lipid composition is adjusted in response to osmotic stress. In addition, examples drawn from bacterial osmoregulation show how phospholipids participate in cellular organization and osmoregulatory protein function.

2 Osmoregulation of Bacterial Membrane Phospholipid Composition

To determine phospholipid composition, lipids are extracted from whole bacteria with chloroform/methanol (Bligh and Dyer 1959). Lipid analysis has then been accomplished by thin layer chromatography (TLC, e.g., Romantsov et al. 2007),

liquid chromatography coupled to mass spectrometry (LC-MS, e.g., Oliver et al. 2014; Romantsov et al. 2009b), or directly by flow injection MS (e.g., Sevin and Sauer 2014). TLC of ^{32}P or ^{33}P-labeled phospholipids provides quantitative headgroup analysis but does not reveal the acyl chain compositions of phospholipids. Quantitative MS can be used to determine both headgroup and acyl chain composition (e.g., Oliver et al. 2014; Sevin and Sauer 2014). Complete descriptions of cellular phospholipid compositions can also be obtained via MS analysis of material from TLC plates (Lin et al. 2007). Different results have been reported by researchers using these different methods. For example, no cardiolipin (CL) was detected with flow injection MS-based analysis of *Escherichia coli* phospholipids (Sevin and Sauer 2014), whereas CL is routinely detected by TLC-based analysis (Cronan 2003; Romantsov et al. 2007) and LC-MS (Oliver et al. 2014; Romantsov et al. 2009b). There is clearly scope to combine these methods and determine the origins of the differing results.

Bacterial polar lipid extracts include phospholipids derived from the cytoplasmic membrane for Gram-positive organisms and the cytoplasmic membrane plus the inner leaflet of the outer membrane for Gram-negative organisms. Zwitterionic and anionic phospholipids (phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) plus CL, respectively) are present, and the proportion of anionic phospholipid increases with the salinity of the growth medium for diverse Gram-positive and Gram-negative organisms (Table 1). The proportion of CL increases in *E. coli* and *Bacillus subtilis* (Table 1) and in *Rhodobacter sphaeroides* (Catucci 2004; Tsuzuki 2011). In other bacteria, the proportion of PG rises dramatically (*B. subtilis, Oceanomonas baumannii*, and *Lactococcus lactis*) (Table 1).

Bacterial phospholipid composition also depends on the growth phase and the growth medium. For example, the proportion of PG increased dramatically in stationary phase when *B. subtilis* and *Listeria monocytogenes* were cultivated on rich media (LB and BHI, respectively), whereas the proportion of PG in *E. coli* cultivated on MOPS minimal medium decreased in stationary phase (Table 1). Furthermore, Oliver et al. reported unusually high levels of PE in *E. coli* cells that were cultivated on rich medium (LB) to mid-exponential phase (Oliver et al. 2014). More detailed comparisons should be made with careful attention to the bacterial growth conditions and analytical methods (including their accuracy and precision). Headgroup and acyl chain composition are equally important because they determine both lipid phase behavior and protein-lipid interactions, as discussed below.

3 Lipids in Bacterial Cell Biology

Light and electron microscopy revealed the widely understood differences in cell wall structure among bacterial groups (e.g., Gram-positive and Gram-negative bacteria). Fluorescence microscopy now achieves live cell imaging with improved spatial and temporal resolution, revealing that neither the cytoplasm nor the cytoplasmic membrane is isotropic and membrane lipids play key roles in cellular organization (Shapiro et al. 2009). Here we outline relevant physical properties of

Table 1 Impact of growth medium salinity on bacterial phospholipid content

Organism	Growth medium[a]		Growth phase	Headgroup (mole %)[b]			Acyl chains[e]	Refs[f]
	Base	NaCl (M)		Species	LOM	HOM		
Bacillus subtilis	LB	1.5	NT[c]	PE PG LPG CL	20 42 14 24	17 32 5 46	↑ straight (vs branched) saturated chains ↓ saturation	1, 2
	LB	NT	Exp	PE PG LPG CL	25 35 11 29	NT NT NT NT	NT	3
			Stat	PE PG LPG CL	2 65 5 28	NT NT NT NT		
Escherichia coli	MOPS	0.3[d]	Exp	PE PG CL	79 18 4	74 18 8	↑ odd numbers of carbon atoms	4, 5
			Stat	PE PG CL	91 3 6	86 5 9	NT	
	M9	0.45	Exp	PE PG Q8	89 11 0	77 22 1	NT	6
	LB	NT	Exp	PE PG CL PA	93.4 4.4 2.0 0.1	NT	NT	7
Lactococcus lactis	CDM	0.3	Exp	NT	NT	NT	↑ cyclopropane ↑ saturation	8

Oceanomonas baumannii	TSB	2.1	Mid-Exp	PE PG CL	71 23 6	44 48 8	↑ saturation ↓ length	9
	MSM	1.2	Mid-Exp	PE PG CL	63 20 17	51 40 5	↓ saturation ↑ C16, ↓C12, C18	10
Vibrio sp. DSM14379	PYE	1.7	Late Exp	PE+LPE PG+CL	78 22	68 32	↑ saturation	11
Listeria monocytogenes	BHI	NT	Exp	PG LysPG CL LysCL	13 73 9 0	NT	NT	3
			Stat	PG LysPG CL LysCL	43 27 17 9			
Halobacillus halophilus	G10	3	Late Exp	NT	NT	NT	↑C15	12

[a]The growth media were *BHI* brain heart infusion medium; *CDM* chemically defined medium, *G10* G10 minimal medium, *LB* Luria-Bertani medium, *MOPS* MOPS-based minimal medium, *MSM* minimal salts medium, *PYE* peptone yeast extract, *TSB* tryptone soya broth, *M9* M9 minimal medium

[b]Bacteria were cultivated in the indicated base medium (low osmolality medium, LOM) and in the same medium supplemented with NaCl at the indicated concentration (high osmolality medium, HOM) to the indicated growth phase. The phospholipids were *CL* cardiolipin or diphosphatidylglycerol, *LPE* lysophosphatidylethanolamine, *LPG* lysophosphatidylglycerol, *LysCL* lysylcardiolipin, *LysPG* lysylphosphatidylglycerol, *PA* phosphatidic acid, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *Q8* ubiquinone 8

[c]NT is not tested

[d]The phospholipid compositions of bacteria cultivated in media adjusted to equivalent osmolalities with NaCl and sucrose were the same (Tsatskis et al. 2005)

[e]↑ is increased; ↓ is decreased

[f]The references are 1 (López et al. 2000), 2 (López et al. 2006), 3 (Dare et al. 2014), 4 (Romantsov et al. 2007), 5 (Romantsov et al. 2009b), 6 (Sevin and Sauer 2014), 7 (Oliver et al. 2014), 8 (Guillot et al. 2000), 9 (Brown et al. 2000), 10 (Brown et al. 2003), 11 (Danevcic et al. 2005), and 12 (Lopalco et al. 2013)

lipids and membranes. Then we discuss relevant imaging techniques and evidence that lipids are anisotropically distributed within bacterial cytoplasmic membranes.

3.1 Physical Properties of Lipids and Membranes

The prevalent phospholipids PE, PG, and CL have been implicated in bacterial cell biology, while isoprenoid lipids such as hopanes, carotenoids, and ubiquinones may also be involved (Agmo Hernández et al. 2015; Barak and Muchova 2013; Bramkamp and Lopez 2015; Lin and Weibel 2016).

Biophysical tools that include light scattering, fluorescence, nuclear magnetic resonance, electron paramagnetic resonance, infrared and Raman spectroscopies, differential scanning calorimetry, fluorescence, electron and atomic force microscopies, micropipette aspiration, and tether pulling with optical tweezers are all used to characterize lipid and membrane properties (Gennis 1989; Yeagle 2011). The origin and physiological significance of each property can be deduced by comparing data obtained with pure lipids (single species and mixtures), lipid extracts from biological material, and intact biomembranes. However, analysis of lipid contributions to membrane properties is complicated because they depend on lipid-protein ratios, and the lipid proportions must vary reciprocally: it is impossible to increase the proportion of one lipid without decreasing the proportion of another.

The physical properties of membranes, listed and defined in Table 2, depend on the proportions and the headgroup and acyl chain compositions of the constituent phospholipids, on the proportions and identities of isoprenoid lipids, on the protein content of the membrane, on interactions among proteins and lipids, and on the ambient temperature, pressure, and solvent composition (including ion composition, ionic strength, and pH) (Gennis 1989; Yeagle 2011). Most properties reflect the packing of the molecules in each leaflet of the membrane bilayer and therefore correlate with the shapes of individual lipid molecules (Table 2). Essentially, PE and CL are conical (their headgroups smaller in cross-sectional area in the membrane plane than their acyl chains) whereas PG and phosphatidylcholine (PC) are cylindrical. The net negative charges of the CL and PG headgroups render their properties more sensitive to solvent pH and ion composition than those of PE and PC (Lewis and McElhaney 2000). In addition, unsaturation and cyclopropyl groups increase the cross-sectional area of the acyl chains relative to that of the headgroups. Properties affected by lipid shape include phase behavior, membrane fluidity, lateral pressure, and lateral and transverse lipid asymmetry. Molecular dynamic simulations are increasingly used to study the behavior of bacterial lipids (e.g., Ingólfsson et al. 2016; Khakbaz and Klauda 2015; Parkin et al. 2015; Wu et al. 2014).

Much of the extensive literature on lipid properties pertains to membranes rich in PC, which is abundant in mammals and rare in bacteria, whereas PE is the most common and abundant zwitterionic phospholipid in bacteria. PC and PE differ in shape (cylindrical versus conical) and in the hydrogen bonding capacities and charge densities of their headgroups. Thus further biophysical analysis of bacterial membrane models is required. The physical properties of membranes that include CL or

Table 2 Physical properties of lipids and membranes

Property	Definition
Lipid shape	The cross-sectional area of a headgroup relative to that of the acyl chains in an amphipathic lipid; in these terms lipids are inverted cones (e.g., lysophospholipids), cylinders (e.g., PC, PG, CL), or cones (e.g., PE, CL)
Lipid polymorphism	The abilities of lipids to aggregate in different ways, giving rise to different shapes including micelles, bilayers, and hexagonal and cubic phases
Lipid phase	A physically distinct form of a lipid or membrane, including the lamellar gel (L_β), lamellar liquid/crystalline (L_α), inverted hexagonal II (H_{II}), and inverted cubic (Q_{II}) phases
Fluidity	A measure of the resistance to movement (translational or rotational diffusion) of molecules in the membrane. For an isotropic liquid, fluidity is the inverse of viscosity
Transverse asymmetry	Differences in lipid composition between the leaflets of a membrane bilayer
Lateral asymmetry (phase separation)	Variations in lipid composition (and hence phase) along the plane of the membrane bilayer
Intrinsic curvature	The tendency of a membrane monolayer to curve (rather than being planar)
Lateral pressure (intrinsic strain)	Pressure in the plane of the membrane due to the frustration of intrinsic curvature on formation of a planar membrane bilayer
Tension	Membrane strain (normalized area change) due to a mechanical stress imposed on the membrane
Surface charge	The net charge on the membrane surface (usually negative), which in turn reflects the pH and ion composition of the bulk solvent and determines the pH and ion composition of the membrane-proximal solvent

the isoprenoid lipid ubiquinone are discussed below. These lipids are selected because CL is clearly implicated in the subcellular localization and function of bacterial osmoregulatory proteins (Barak and Muchova 2013; Lin and Weibel 2016), while ubiquinone was recently implicated in salinity tolerance for *E. coli* (Sevin and Sauer 2014).

CL is a relatively small component (less than 10 mol %) of the cytoplasmic membranes of most bacteria. CL differs from other phospholipids in being comprised of a single, nonionic headgroup (glycerol) shared between two phosphatidate moieties. That dual tethering constrains the glycerol headgroup and decreases the capacity of its free hydroxyl for intra- or intermolecular interactions. Recent work (Lewis and McElhaney 2009; Olofsson and Sparr 2013) contradicts the earlier conclusion that intramolecular hydrogen bonding occupies the headgroup hydroxyl and elevates the pK_a of one of the CL phosphates (Kates et al. 1993). Thus CL is a conical lipid (Table 2) that bears two negative charges at physiological pH (Lewis and McElhaney 2009), and the headgroup hydroxyl is available for interaction with solvent constituents, including proteins. The physical properties of CL in vivo are exquisitely dependent on its solvent environment (particularly the presence of

counter-cations), its acyl chain composition, and its lipid environment (e.g., the high proportion of PG and other cylindrical, anionic lipid characteristics of many Gram-positive bacteria versus the high proportion of zwitterionic, conical PE characteristic of many Gram-negative bacteria). The lamellar gel to lamellar liquid crystalline (L_β/L_α) phase transition temperatures of CL bilayers exceed those of PG bilayers with the same saturated acyl chains, under the same solvent conditions (Lewis and McElhaney 2009). Repulsions among anionic headgroups mitigate the tendency for the conical CL molecule to increase intrinsic strain and promote non-lamellar lipid phases. Conversely, the tendency for CL to form the non-bilayer, hexagonal H_{II} phase is favored by headgroup charge neutralization (Lewis and McElhaney 2009). CL was relatively immiscible with PG (Benesch et al. 2015) or PE (Frias et al. 2011) in model systems with matched, unsaturated myristoyl side chains. However these side chains are not representative of bacterial membrane lipids, and the conditions favoring phase separation were not physiological.

The polycyclic isoprenoid cholesterol is well known as a membrane stabilizer that broadens phospholipid phase transitions and decreases membrane fluidity. Cholesterol is absent from most bacterial membranes, but similar roles may be assumed by bacterial homologues denoted hopanoids (Sáenz et al. 2012). Bacteria also contain ubiquinones and noncyclic isoprenoids, including carotenoids. Ubiquinones (Q) are widely recognized as electron and proton carriers in respiratory chains and as antioxidants. Recent evidence suggests that they may also stabilize membrane structure (Agmo Hernández et al. 2015).

As noted above (Table 1), ubiquinone 8 (Q8) accumulates in *E. coli* cells grown at high salinity (0.4–0.5 M NaCl) (Sevin and Sauer 2014). The growth of Q8-deficient bacteria was impaired at low salinity, and such bacteria were also less salinity tolerant than those retaining Q8. The growth impairment at low salinity resulted from impaired respiration, whereas the decreased salinity tolerance did not arise from impaired respiration or oxidative stress tolerance. The authors postulated that Q8 conferred osmotolerance by enhancing membrane stability.

Most biophysical studies of ubiquinone employed Q10-supplemented PC as a model for mammalian mitochondrial membranes and were motivated by efforts to understand the role of Q10 in mitochondrial electron transport (Agmo Hernández et al. 2015; Kaurola et al. 2016) (reviewed by Quinn 2012). Extensive data support location of Q in the membrane core, between the leaflets of the phospholipid bilayers, with the quinone ring and isoprenoid tail in the membrane plane. However, data also indicate that Q may intercalate among the phospholipids with the ring at the polar interface between the phospholipid headgroups and acyl chains, the tail extending parallel to the acyl chains. Tails that include six or more isoprenoid units and oxidation of the headgroup to the less hydrophilic, quinone form favor the former disposition, whereas shorter isoprenoid tails and a reduced, more polar quinol headgroup favor the latter. Data suggest that the presence of Q in the membrane core decreases membrane stability, increasing membrane thickness, fluidity, and solute permeability, while its intercalation among the phospholipids contributes to membrane stability, decreasing fluidity, permeability, and detergent susceptibility. In addition, isoprenoid compounds may contribute to lipid lateral

Table 3 Fluorescent probes employed to image live bacteria

Name	Chemical structure	Target(s)	Representative reference(s)[a]
DAPI	4′,6-diamidino-2-phenylindole	DNA	
FM4-64	(N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide	Membrane	1
Laurdan	6-Dodecanoyl-2-dimethylaminonaphthalene	Membrane	2
NAO	10-N-nonyl acridine orange	Anionic lipid/ cardiolipin	3
Nile Red	9-(diethylamino)-5H-benzo[α]phenoxazin-5-one	Membrane	2

[a]The references are 1 (Fishov and Woldringh 1999; Zaritsky et al. 1999), 2 (Strahl et al. 2014), and 3 (Mileykovskaya and Dowhan 2000; Mileykovskaya et al. 2001; Oliver et al. 2014)

phase separation, thereby complicating the interpretation of biophysical data. A better understanding of the role of Q in salinity tolerance will be gained by correlating the disposition of bacterial Q (e.g., Q8) with its impact on membrane physical properties, including phase separation, using in vitro systems that simulate bacterial membranes.

3.2 Distributions of Phospholipids in Bacterial Cytoplasmic Membranes

Cytoplasmic membrane phospholipids are not randomly distributed over the surfaces of bacterial cells. Nonrandom lipid distribution may occur on various area scales. Some lipids bind tightly and specifically to membrane proteins, thereby ensuring lipid enrichment within the membrane area so defined (see Sect. 4). There is strong evidence that particular proteins and processes in eukaryotic cells are associated with lipid rafts which are small (up to 0.2 μm) sterol- and sphingolipid-enriched, detergent-resistant domains. Some evidence suggests that structures analogous to lipid rafts, though different in lipid composition, exist in bacterial membranes (Boekema et al. 2013; Bramkamp and Lopez 2015). Here we discuss evidence that anisotropic lipid distribution in bacterial cytoplasmic membranes yields membrane domains with different lipid compositions on larger area scales, up to approximately 0.5 μm.

The locations of phospholipids within live bacteria can be detected via microscopy based on fluorescent probes (Table 3). FM4-64 is widely used as a non-specific membrane stain but it is positively charged, hence unlikely to cross membranes and likely to associate preferentially with anionic lipids. NAO has played a particularly important role in bacterial cell biology because it can be used to detect CL-rich membrane domains (Mileykovskaya and Dowhan 2000; Romantsov et al. 2007). Cells show green fluorescence when the cationic acridine headgroup of NAO associates non-specifically with anionic phospholipid headgroups and the nonpolar

nonyl side chain is anchored in the membrane. At low NAO/phospholipid ratios, the dye associated with CL-rich domains is detected via the red, excimer fluorescence that arises from π-π interactions between stacked acridine rings associated with adjacent CL molecules (Mileykovskaya et al. 2001; Oliver et al. 2014). In addition, a variety of phospholipid analogues are available in which a fluorophore replaces one or more acyl chains, or a fluorophore is attached to an acyl chain or the lipid headgroup. Available lipid-linked fluorophores include diphenylhexatriene (DPH), nitrobenzoxadiazole (NBD), pyrene, and 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) (most also used to measure bacterial membrane properties other than spatial distribution). These probes are assumed to distribute in the membrane like their phospholipid homologues. Recently de Wit et al. reported that label-free, interferometric light scattering microscopy can also be used to detect variations in membrane refractive index due to lateral lipid phase separation (de Wit et al. 2015).

Focusing primarily on PE and PG, Fishov and his colleagues provided direct and indirect evidence for lipid phase separation in the membranes of *E. coli* and *B. subtilis* based on the fluorescence of membrane probes FM4-64 (fluorescence microscopy) (Fishov and Woldringh 1999), diphenylhexatriene (fluorescence anisotropy), and laurdan (6-dodecanoyl-*N,N*-dimethyl-2-naphthylamine, sensitivity to solvent polarity) (Vanounou et al. 2002) and pyrene-labeled phospholipids PY-PE (2-pyrenedecanoyl-phosphatidylethanolamine) and PY-PG (2-pyrenedecanoyl-phosphatidylglycerol) (pyrene excimer formation) (Vanounou et al. 2003). Various laboratories provided direct evidence that the membranes at the cell poles and septa of *E. coli* and *B. subtilis* are enriched in CL (reviewed in Barak and Muchova 2013; Lin and Weibel 2016; Matsumoto et al. 2006; Romantsov et al. 2009b). This evidence was derived from both NAO-based fluorescence microscopy (Mileykovskaya and Dowhan 2000; Renner and Weibel 2011; Romantsov et al. 2007) and phospholipid analysis of minicells (small, spherical cells, representative of the cell poles, that arise from aberrant division of *E. coli* cells) (Koppelman et al. 2001; Romantsov et al. 2008). Using bacteria devoid of CL and high NAO/lipid ratios, Oliver et al. associated red NAO excimer fluorescence with concentration of PG at *E. coli* cell poles (Oliver et al. 2014). Their data highlight the need for careful use of this dye and reinforce the earlier conclusion that PE and PG are differently distributed in *E. coli* (Vanounou et al. 2003). Finally, the detection of helical structures with FM4-64 and related probes has been attributed to the presence of helical PG-rich membrane domains in *B. subtilis* (Barak and Muchova 2013; Barák et al. 2008).

The origins of the CL- and PG-rich domains discussed above remain uncertain. Postulated mechanisms include spontaneous lipid microphase separation, location-specific lipid synthesis, and protein-directed lipid localization. Mathematical modeling suggests that the CL-rich domains at cell poles may arise from spontaneous, transverse and lateral, lipid phase separation. Due to its conical shape, CL is proposed to partition into the more negatively curved inner leaflet of the cytoplasmic membrane at the cell poles and away from the less negatively curved inner leaflet of the sidewall membrane or the positively curved outer leaflet (Huang et al. 2006; Mukhopadhyay et al. 2008). This model was supported when CL localization was

correlated with curvature in *E. coli* spheroplasts with engineered shapes (Renner and Weibel 2011). However important questions remain. First, does transverse lipid asymmetry occur in bacterial cytoplasmic membranes (Huijbregts et al. 2000) and does CL preferentially occupy the inner leaflet of the cytoplasmic membrane at the poles of *E. coli* cells? Second, does the model accurately represent the physiological context of CL? Huang et al. modeled a two-component membrane comprised of a non-CL component with an intrinsic curvature of zero and CL with an intrinsic curvature in the range -0.2 to -0.5 nm^{-1} (Huang et al. 2006). The intrinsic curvature of PG is low. However PE, with an intrinsic curvature estimated at -0.5 nm^{-1} (Lewis and Cafiso 1999), constitutes approximately 70 mole percent of the phospholipid in the cytoplasmic membrane of *E. coli* (Cronan 2003). Thus, depending on the acyl chain compositions and the divalent cation composition of the solvent, PE could have a greater tendency than CL to concentrate spontaneously at the cell poles. Thirdly, is the model consistent with the polar localization of CL in organisms that have diverse PE/PG ratios (e.g., *E. coli* and *B. subtilis*)? Most physical properties of lipids correlate with their molecular shapes. Thus it would also be informative to determine which of those properties correlate with subcellular lipid localization.

Fluorescence microscopy revealed that a GFP-ClsA (green fluorescent protein-cardiolipin synthase A) fusion protein concentrated at the septa of *B. subtilis* cells, and a putative targeting sequence was identified (Kusaka et al. 2016; Nishibori et al. 2005). If CL diffusion through the membrane were restricted, CL synthesis at the septum may be sufficient to yield bacteria with CL concentrated at the cell septa and (after division) at the cell poles. The subcellular distribution of *E. coli* ClsA has not been reported, and the putative targeting sequence of *B. subtilis* ClsA is not well conserved in the *E. coli* protein. Although CL was enriched at the hyphal tips, branch points, and anucleate regions of *Streptomyces coelicolor* cells, a ClsA-EGFP was randomly distributed (Jyothikumar et al. 2012).

With respect to protein-directed lipid localization, the transertion hypothesis posits that membrane domains arise from the coupled transcription, translation, and membrane insertion of proteins, particularly along the cells' sidewalls (Fishov and Norris 2012; Roggiani and Goulian 2015). In addition, there is evidence that the MreB cytoskeleton alters physical properties of the adjacent cytoplasmic membrane in *B. subtilis* (Strahl et al. 2014). This membrane potential-dependent effect influences the localization of other proteins and may foster protein-dependent lipid domain formation.

4 Protein-Lipid Interactions: Their Impact on the Distribution and Function of Osmoregulatory Proteins

Like phospholipids, cytoplasmic membrane proteins are not isotropically distributed in bacteria. Nonrandom protein distribution occurs on a variety of area scales and is protein specific (Table 4). Particular lipids may be associated with scattered homo- or

Table 4 Some processes involving anisotropic membrane lipid and protein distribution

Process	Key player		Subcellular localization	Reference (s)[a]
	Name	Function		
Chemoreception	Tsr	Chemoreceptor	Cell poles	1
Osmotic stress tolerance	MscL	Mechanosensitive channel	None	2, 3
	MscS	Mechanosensitive channel	Cell poles	3
	ProP	Osmosensing transporter	Cell poles	3, 4
Respiration	Many Proteins	Respiratory chain components	Patches	5
Cell division	FtsZ	Divisome placement	Septum	6
Sporulation	SpoVM	Curvature sensing	Spore	7, 8
Sugar metabolism	EI, HPr	General components of the phosphoenolpyruvate-sugar phosphotransferase system	Cell poles	9

[a]The references are 1 (Maddock and Shapiro 1993), 2 (Norman et al. 2005), 3 (Romantsov et al. 2009a), 4 (Romantsov et al. 2007), 5 (Magalon and Alberge 2016), 6 (Busiek and Margolin 2015), 7 (Ramamurthi et al. 2009; Shapiro et al. 2009), 8 (Gill et al. 2015), and 9 (Govindarajan et al. 2013)

hetero-oligomeric protein complexes of various sizes (e.g., respiratory chain complexes), or they may be associated with proteins that reside at specific subcellular sites for obvious reasons (e.g., proteins involved in cell division and sporulation). For some proteins, concentration at the cell poles is well established, but the rationale for that targeting is not obvious (e.g., chemoreceptors, components EI and HPr of the phosphoenolpyruvate-sugar phosphotransferase systems). Here we summarize techniques for the detection of lipid-protein interactions and nonrandom protein distribution. Then we provide specific examples of those phenomena associated with the osmotic stress response.

4.1 Tools for the Detection of Protein-Lipid Complexes and Their Subcellular Distributions

Membrane protein-lipid interactions, membrane protein localization, and the functional significance of those phenomena are best deduced by comparing the properties of purified proteins, protein-lipid complexes like proteoliposomes, fabricated from detergent-solubilized proteins and lipid extracts or pure lipids, intact biomembranes, and intact cells. It is challenging to detect physiologically relevant interactions among membrane components because analyses usually begin with organic solvent- or detergent-mediated solubilization that disrupts at least some lipid-lipid and lipid-protein interactions. Stable protein-lipid interactions may be detected via X-ray crystallography (Koshy and Ziegler 2015) or mass spectrometry of detergent-solubilized proteins or protein complexes (Laganowsky et al. 2014). Weaker protein-lipid interactions can be inferred if either element perturbs the nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), or Fourier

transform infrared (FTIR) spectrum of the other (e.g., Guler et al. 2015). Such work employs appropriately labeled, purified proteins and liposomes or membrane mimetics that include detergents, micelles, bicelles, and nanodiscs.

Electron microscopy plays an important role in elucidating cellular organization. However, improvements in fluorophore labeling and in spatial and temporal resolution have made fluorescence microscopy the preferred tool for detecting protein localization in bacteria. Fluorescent protein (FP) tagging remains most popular because it permits in vivo imaging of cells in which target proteins are uniformly labeled with appropriate fluorophores. A wide variety of FPs, tailored for particular applications, are available (Shaner et al. 2005). However other data must corroborate information obtained in this way because FP-tagged proteins may be subject to incorrect folding or proteolytic degradation and protein tags may alter target protein localization or function (Boekema et al. 2013; Landgraf et al. 2013; Margolin 2012). In vivo labeling can also be achieved by treating CCPGCC-tagged proteins with biarsenical fluorophore FlAsH-EDT_2 (fluorescein arsenical helix binder-ethylenediamine$_2$) or ReAsH-EDT_2 (Griffin et al. 2000). Reagents for in vivo labeling of His_n-tagged proteins are also under development (Lai et al. 2015). These small peptide tags are less likely to interfere with protein function or localization than large fluorescent proteins, but the reagents may be unstable, and it may be difficult to attain high labeling efficiencies because they do not easily cross bacterial cell membranes. Although it is not compatible with in vivo imaging, immunofluorescence microscopy remains an important method for the detection of untagged, native proteins. Finally, protein localization at the cell poles could be investigated by comparing minicells, produced by asymmetric division of rod-shaped cells with division defects, with their normal counterparts.

Application of the listed techniques has revealed that proteins assume particular subcellular distributions in bacteria (Table 4). As for lipids, there is intense interest in the mechanisms underlying anisotropic protein distribution (reviewed by (Laloux and Jacobs-Wagner 2014; Treuner-Lange and Sogaard-Andersen 2014). This section will end with discussions of the roles of lipids in function and subcellular localization for osmosensing transporters and mechanosensitive channels.

4.2 Osmosensing Transporters

For osmosensing transporters, the rate of osmolyte (substrate) uptake is a sigmoid function of the osmolality in cells and, after purification and reconstitution, in proteoliposomes (Wood 2007). The paradigmatic osmosensing transporters ProP of *E. coli*, BetP of *Corynebacterium glutamicum*, and OpuA of *Lactococcus lactis* differ in evolutionary origin, molecular structure, and energy coupling mechanism (Table 5) (Wood 2011). Available data suggest that each is similar in structure and transport mechanism to its paralogues that are not osmosensors, and each includes one or more extended cytoplasmic domains that are implicated in osmosensing.

Table 5 Properties of the paradigmatic osmosensing transporters

Transporter	Organism	Phylogenetic group	Energy coupling mechanism	Available structures (PDB IDs)	Refs[a]
ProP	*E. coli*	Major facilitator superfamily	H^+-osmolyte symport	1R48 (C-terminal domain)	1
BetP	*C. glutamicum*	Betaine-choline-carnitine transporter family	Na^+-osmolyte symport	2WIT, 3P03, 4AIN, 4C7R, 4DOJ, 4LLH (entire protein plus lipid)	2, 3
OpuA	*L. lactis*	ATP-binding cassette family	Transport-coupled ATP hydrolysis	3L6G, 3L6H (betaine-binding domain)	4

[a]The references are 1 (Zoetewey et al. 2003), 2 (Koshy and Ziegler 2015), 3 (Koshy et al. 2013), and 4 (Wolters et al. 2010)

4.2.1 The Osmosensory Mechanism

Cell dehydration simultaneously changes many cellular properties: it may decrease turgor pressure, altering strain in the cytoplasmic membrane and cell wall, while it increases the concentration of each individual cytoplasmic solute, the collective concentration of cytoplasmic ions, and the crowding of cytoplasmic molecules. Thus elucidation of an osmosensory mechanism entails identifying the cellular property detected by the transporter as well as understanding how variations to that property modulate transporter structure and function (Wood 1999). Early experiments showed that osmosensory transporter activity is not determined by turgor pressure or membrane tension (Poolman et al. 2004).

Proteoliposomes reconstituted with the purified proteins were exploited to measure the effects of the external and internal solvents on transporter activity (reviewed in Kramer 2010; Kramer et al. 2010; Wood 2011). Diverse membrane-impermeant solutes, including electrolytes and nonelectrolytes, affected transporter activity similarly when applied externally to attain the same osmolality. This response was phospholipid sensitive: the osmolality at which each transporter activates is a direct function of the anionic lipid content of the host membrane (PG, or PG plus CL). All three transporters activate as the concentration of inorganic ions, but not of neutral organic solutes, increases at their cytoplasmic surface (in the proteoliposome lumen). In each case, a high luminal ion concentration (in the range 0.2–0.4 M) is required to attain half maximal transporter activity. High molecular weight polymers facilitate the salt activation of ProP and OpuA, suggesting that those transporters are also affected by osmotically induced macromolecular crowding.

For ProP and OpuA, osmotic activation is a non-specific cation effect (physiologically, the predominant cation would be K^+) (Culham et al. 2012, 2016; Gul and Poolman 2013; Karasawa et al. 2013). Coulombic, cation effects could favor an increase in local membrane surface charge density, folding of anionic, cytoplasm-

exposed domains, and/or the juxtaposition of anionic protein and membrane surfaces (Culham et al. 2012; Karasawa et al. 2013). Candidate protein sequences have been identified (Karasawa et al. 2011; Wood 2015). Crystal structures are not available for the membrane-integral domains of ProP and OpuA, so it is not clear whether they bind lipids tightly and whether transporter-bound and/or bulk lipids are involved in their activation.

High-resolution structures have provided a wealth of information about the transport mechanism of BetP and important context for studies of osmosensing (Koshy et al. 2013 and references cited therein). PG molecules associated with the detergent-solubilized protein are found in the core of the BetP trimer and on its periphery. In contrast to H^+ symporter ProP and ABC transporter OpuA, BetP is a Na^+ symporter that responds to osmotically induced increases in luminal K^+ concentration (not to Na^+ concentration) in proteoliposomes. Researchers propose that BetP specifically senses the osmotically induced increase in cytoplasmic K^+ concentration via its interaction with cytoplasm-exposed portions of the protein and the membrane lipid. Evidence suggests that osmotically induced changes to physical properties of the lipid are also required (Maximov et al. 2014). Recently Güler et al. used FTIR spectroscopy to examine protein-lipid interactions in two-dimensional crystals of BetP with *C. glutamicum* lipids (Guler et al. 2015).

The proposed osmosensory mechanisms for ProP, OpuA, and BetP are converging despite the key observation that the osmotic activation of BetP is K^+-specific whereas that of ProP and OpuA is not. The avoidance of secondary effects is a major challenge when solutes are provided at high concentrations for analyses of osmosensory mechanisms (e.g., Culham et al. 2016). Despite rigorous efforts to avoid such issues, perhaps the apparent cation specificity of BetP arises because ions exert effects on the coupling mechanism other than adjustment of the medium osmolality.

4.2.2 Subcellular Localization and Osmosensory Transporter Function

In addition to their role in osmosensing, phospholipids are implicated in the subcellular localization of *E. coli* ProP. The CL content of bacteria in exponential phase cultures varies with the growth medium osmolality because *clsA*, encoding one of three CL synthases in *E. coli*, is osmotically induced (Romantsov et al. 2007; Tsatskis et al. 2005). Bacteria that are $clsA^-$ retain residual CL (less than 1 mol %); CL localization at the cell poles was found in approximately 75% of $clsA^+$ bacteria, but not in $clsA^-$ bacteria (Romantsov et al. 2009a). Localization of tagged ProP at *E. coli* cell poles and septa was detected by fluorescence microscopy of FlAsH-labeled cells (Romantsov et al. 2007). The fraction of cells with ProP concentrated at the cell poles varied from 4% to 51% as the CL content of the bacteria was modulated by growing $clsA^-$ and $clsA^+$ strains in media with different osmolalities (Romantsov et al. 2008). The proportion of PG in $clsA^-$ bacteria varied from 25 mol% to 30 mol% with the growth medium osmolality. ProP was concentrated at the cell poles in only a small fraction of these cells (5–10%), and no reproducible trend with PG content could be detected (Romantsov et al. 2008, 2009a). Thus the polar localization of ProP was CL dependent, not anionic lipid dependent. FlAsH labeling was also employed to

investigate the polar localization of other membrane proteins (Romantsov et al. 2009a). CL-dependent localization at the cell poles was particular to ProP (see further discussion in Sect. 4.2.3). Amino acid replacements had analogous effects on MVCCPGCC-ProP localization in vivo, and association of a peptide replicating the ProP C-terminal domain with liposomes comprised of a polar lipid extract from *E. coli* in vitro (Romantsov et al. 2008 and unpublished data). Thus association of the extended cytoplasmic, C-terminal domain of ProP with CL headgroups may confer CL-dependent polar localization on ProP.

The physiological role of CL-dependent ProP localization may be to adjust the phospholipid environment, and hence the function, of the transporter. As noted above, the osmolality at which ProP activates is directly proportional to the anionic lipid content of the membrane; the proportion of CL in the membrane, and presumably its proportion at the cell poles, is a direct function of the growth medium osmolality (Romantsov et al. 2008; Tsatskis et al. 2005). Thus polar localization places ProP in a CL-rich environment, adjusting its osmolality response to encompass ambient conditions.

4.2.3 Mechanosensitive Channels

The mechanosensitive (MS) channels of large (MscL) and small (MscS) conductance of *E. coli* serve as paradigms for the two evolutionary families of MS channels present in bacteria (Booth and Blount 2012). Studies of MscL first established the *force from lipid principle* that MS channels open as phospholipid membrane tension (the normalized membrane area change) increases in response to mechanical stress; this channel gating does not require interactions with other proteins, including the cytoskeleton (Anishkin et al. 2014). MS channels are therefore central to the study of protein-lipid interactions. Available crystal structures for the homopentameric MscL and the homohexameric MscS channel have complemented extensive biochemical analyses to yield insights regarding the structural transitions during channel gating. Nevertheless, the mechanosensory mechanism remains controversial (Battle et al. 2015; Rasmussen 2016; Wang et al. 2008; Zhang et al. 2016). Current efforts focus on the roles of the following phenomena in channel opening: (1) the interplay among membrane tension, the lateral pressure profile of the phospholipid bilayer, and channel structure (Gullingsrud and Schulten 2004); (2) changes in interdigitation of lipid acyl chains within the channel proteins, revealed by a recent, high-resolution crystal structure of MscS (Pliotas et al. 2015); and (3) hydrophobic interactions with the lipid acyl chains (including hydrophobic mismatch) and electrostatic or hydrogen bonding interactions with the lipid headgroups (Zhong and Blount 2014 and references cited therein).

Most species possess only one, highly conserved MscL and multiple, less conserved MscS (or MscS-like) channels. MscL, MscS, and the five MscS-like proteins in *E. coli* have MS channel activity and can individually protect *E. coli* from osmotic downshock (Edwards et al. 2012 and references cited therein). Osmotic shock tolerance is usually conferred by MscL and a single MscS, but the roles of additional channels may be revealed via further analysis of channel protein expression

(Bialecka-Fornal et al. 2012) and phenotypes associated with the magnitude and rate of osmotic stress (Bialecka-Fornal et al. 2015).

Noting the abundance of MscL channels in *E. coli* (Häse et al. 1997), Boucher et al. proposed that MS channel proteins may contribute to osmotic stress tolerance by increasing the elasticity of bacterial membranes (Boucher et al. 2009). Large clusters of *E. coli* MscL channels were observed in vitro (Grage et al. 2011), but visualization of MscL-GFP (Norman et al. 2005) and MscL-CCPGCC (with FlAsH labeling) (Romantsov et al. 2009a) yielded little evidence for subcellular localization of MscL in vivo. In contrast, FlAsH-based fluorescence microscopy revealed some CL-dependent localization of MscS-CCPGCC (Romantsov et al. 2009a), and the cytoplasmic MscS domain was reported to interact with cell division protein FtsZ (Koprowski et al. 2015). Further work will be required to further substantiate these observations and elucidate the physiological role of the observed behavior.

5 Research Needs

Abundant evidence indicates that lipid-protein interactions are fundamental to the functions of osmoregulatory systems. Neither lipids nor proteins are randomly distributed within bacterial cytoplasmic membranes. Thus the lipid environment of a bacterial membrane protein may depend on its subcellular location. The physical properties of membranes are key determinants of membrane protein localization and function. Analysis of models for mammalian membranes revealed that their physical properties are exquisitely sensitive to membrane lipid composition. Complete qualitative and quantitative descriptions of bacterial membrane compositions (phospholipid headgroups, phospholipid acyl chains, and isoprenoid lipids) and the physical properties of bacterial membrane lipids are required. That information will support further testing of hypotheses regarding lipid-protein interactions in osmoregulatory protein function and subcellular localization. Structural analyses that reveal the roles of lipid-protein interactions in osmosensory transport and mechanosensitive channel gating are in progress. Thus these systems provide useful paradigms for the study of both protein-lipid interactions and the role of subcellular localization in bacterial lipid and protein function.

Acknowledgments The authors are grateful for helpful discussions with Ronald N. McElhaney (University of Alberta), Robert S. Hodges (University of Colorado), Kerwyn C. Huang (Stanford University), Tariq Akhtar and Leonid Brown (University of Guelph), and members of the Wood laboratory. We also thank the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes for Health Research for financial support.

References

Agmo Hernández V, Eriksson EK, Edwards K (2015) Ubiquinone-10 alters mechanical properties and increases stability of phospholipid membranes. Biochim Biophys Acta 1848:2233–2243

Anishkin A, Loukin SH, Teng J, Kung C (2014) Feeling the hidden mechanical forces in lipid bilayer is an original sense. Proc Natl Acad Sci U S A 111:7898–7905

Barak I, Muchova K (2013) The role of lipid domains in bacterial cell processes. Int J Mol Sci 14:4050–4065

Barák I, Muchová K, Wilkinson AJ, O'Toole PJ, Pavlendová N (2008) Lipid spirals in *Bacillus subtilis* and their role in cell division. Mol Microbiol 68:1315–1327

Battle AR, Ridone P, Bavi N, Nakayama Y, Nikolaev YA, Martinac B (2015) Lipid–protein interactions: lessons learned from stress. Biochim Biophys Acta 1848:1744–1756

Benesch MGK, Lewis RNAH, McElhaney RN (2015) On the miscibility of cardiolipin with 1,2-diacyl phosphoglycerides: binary mixtures of dimyristoylphosphatidylglycerol and tetramyristoylcardiolipin. Biochim Biophys Acta 1848:2878–2888

Bialecka-Fornal M, Lee HJ, DeBerg HA, Gandhi CS, Phillips R (2012) Single-cell census of mechanosensitive channels in living bacteria. PLoS One 7:e33077

Bialecka-Fornal M, Lee HJ, Phillips R (2015) The rate of osmotic downshock determines the survival probability of bacterial mechanosensitive channel mutants. J Bacteriol 197:231–237

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Boekema EJ, Scheffers DJ, van Bezouwen LS, Bolhuis H, Folea IM (2013) Focus on membrane differentiation and membrane domains in the prokaryotic cell. J Mol Microbiol Biotechnol 23:345–356

Booth IR, Blount P (2012) The MscS and MscL families of mechanosensitive channels act as microbial emergency release valves. J Bacteriol 194:4802–4809

Boucher PA, Morris CE, Joos B (2009) Mechanosensitive closed-closed transitions in large membrane proteins: osmoprotection and tension damping. Biophys J 97:2761–2770

Bramkamp M, Lopez D (2015) Exploring the existence of lipid rafts in bacteria. Microbiol Mol Biol Rev 79:81–100

Brown GR, Sutcliffe IC, Bendell D, Cummings SP (2000) The modification of the membrane of *Oceanomonas baumannii* when subjected to both osmotic and organic solvent stress. FEMS Microbiol Lett 189:149–154

Brown GR, Sutcliffe IC, Cummings SP (2003) Combined solvent and water activity stresses on turgor regulation and membrane adaptation in *Oceanimonas baumannii* ATCC 700832. Antonie Van Leeuwenhoek 83:275–283

Busiek KK, Margolin W (2015) Bacterial actin and tubulin homologs in cell growth and division. Curr Biol 25:R243–R254

Catucci L, Depalo N, Lattanzio VMT, Agostiano A, Corcelli, A (2004) Neosynthesis of cardiolipin in *Rhodobacter sphaeroides* under osmotic stress. Biochem 43:15066–15072.

Cronan JE (2003) Bacterial membrane lipids: where do we stand? Annu Rev Microbiol 57:203–224

Culham DE, Meinecke M, Wood JM (2012) Impacts of the osmolality and the lumenal ionic strength on osmosensory transporter ProP in proteoliposomes. J Biol Chem 287:27813–27822

Culham DE, Shkel IA, Record MT, Wood JM (2016) Contributions of Coulombic and Hofmeister effects to the osmotic activation of *Escherichia coli* transporter ProP. Biochemistry 55:1301–1313

Danevcic T, Rilfors L, Strancar J, Lindblom G, Stopar D (2005) Effects of lipid composition on the membrane activity and lipid phase behaviour of *Vibrio* sp. DSM14379 cells grown at various NaCl concentrations. Biochim Biophys Acta 1712:1–8

Dare K, Shepherd J, Roy H, Seveau S, Ibba M (2014) LysPGS formation in *Listeria monocytogenes* has broad roles in maintaining membrane integrity beyond antimicrobial peptide resistance. Virulence 5:534–546

de Wit G, Danial JSH, Kukura P, Wallace MI (2015) Dynamic label-free imaging of lipid nanodomains. Proc Natl Acad Sci U S A 112:12299–12303

Edwards MD, Black S, Rasmussen T, Rasmussen A, Stokes NR, Stephen T-L, Miller S, Booth IR (2012) Characterization of three novel mechanosensitive channel activities in *Escherichia coli*. Channels 6:272–281

Fishov I, Norris V (2012) Membrane heterogeneity created by transertion is a global regulator in bacteria. Curr Opin Microbiol 15:724–730

Fishov I, Woldringh C (1999) Visualization of membrane domains in *Escherichia coli*. Mol Microbiol 32:1166–1172

Frias M, Benesch MGK, Lewis RNAH, McElhaney RN (2011) On the miscibility of cardiolipin with 1,2-diacyl phosphoglycerides: binary mixtures of dimyristoylphosphatidylethanolamine and tetramyristoylcardiolipin. Biochim Biophys Acta 1808:774–783

Gennis RB (1989) Biomembranes: molecular structure and function. Springer, New York

Gill RL, Castaing J-P, Hsin J, Tan IS, Wang X, Huang KC, Tian F, Ramamurthi KS (2015) Structural basis for the geometry-driven localization of a small protein. Proc Natl Acad Sci 112:E1908–E1915

Govindarajan S, Elisha Y, Nevo-Dinur K, Amster-Choder O (2013) The general phosphotransferase system proteins localize to sites of strong negative curvature in bacterial cells. mBio 4:00443-13

Grage SL, Keleshian AM, Turdzeladze T, Battle AR, Tay WC, May RP, Holt SA, Contera SA, Haertlein M, Moulin M et al (2011) Bilayer-mediated clustering and functional interaction of MscL channels. Biophys J 100:1252–1260

Griffin BA, Adams SR, Jones J, Tsien RY (2000) Fluorescent labeling of recombinant proteins in living cells with FlAsH. Methods Enzymol 327:565–578

Guillot A, Obis D, Mistou M-Y (2000) Fatty acid membrane composition and activation of glycine-betaine transport in *Lactococcus lactis* subjected to osmotic stress. Int J Food Microbiol 55:47–51

Gul N, Poolman B (2013) Functional reconstitution and osmoregulatory properties of the ProU ABC transporter from *Escherichia coli*. Mol Membr Biol 30:138–148

Guler G, Gartner RM, Ziegler C, Mantele W (2015) Lipid-protein interactions in the regulated betaine symporter BetP probed by infrared spectroscopy. J Biol Chem 291:4295–4307

Gullingsrud J, Schulten K (2004) Lipid bilayer pressure profiles and mechanosensitive channel gating. Biophys J 86:3496–3509

Häse CC, Minchin RF, Kloda A, Martinac B (1997) Cross-linking studies and membrane localization and assembly of radiolabelled large mechanosensitive ion channel (MscL) of *Escherichia coli*. Biochem Biophys Res Commun 232:777–782

Huang KC, Mukhopadhyay R, Wingreen NS (2006) A curvature-mediated mechanism for localization of lipids to bacterial poles. PLoS Comput Biol 2:e151

Huijbregts RP, de Kroon AI, de Kruijff B (2000) Topology and transport of membrane lipids in bacteria. Biochim Biophys Acta 1469:43–61

Ingólfsson HI, Arnarez C, Periole X, Marrink SJ (2016) Computational 'microscopy' of cellular membranes. J Cell Sci 129:257–268

Jyothikumar V, Klanbut K, Tiong J, Roxburgh JS, Hunter IS, Smith TK, Herron PR (2012) Cardiolipin synthase is required for *Streptomyces coelicolor* morphogenesis. Mol Microbiol 84:181–197

Karasawa A, Erkens GB, Berntsson RPA, Otten R, Schuurman-Wolters GK, Mulder FAA, Poolman B (2011) Cystathionine β-Synthase (CBS) domains 1 and 2 fulfill different roles in ionic strength sensing of the ATP-binding cassette (ABC) transporter OpuA. J Biol Chem 286:37280–37291

Karasawa A, Swier LJYM, Stuart MCA, Brouwers J, Helms B, Poolman B (2013) Physicochemical factors controlling the activity and energy coupling of an ionic strength-gated ATP-binding cassette (ABC) transporter. J Biol Chem 288:29862–29871

Kates M, Syz JY, Gosser D, Haines TH (1993) pH-dissociation characteristics of cardiolipin and its 2′-deoxy analogue. Lipids 28:877–882

Kaurola P, Sharma V, Vonk A, Vattulainen I, Róg T (2016) Distribution and dynamics of quinones in the lipid bilayer mimicking the inner membrane of mitochondria. Biochim Biophys Acta 1858:2116–2122

Khakbaz P, Klauda JB (2015) Probing the importance of lipid diversity in cell membranes via molecular simulation. Chem Phys Lipids 192:12–22

Koppelman C-M, den Blaauwen T, Duursma MC, Heeren RMA, Nanninga N (2001) *Escherichia coli* minicell membranes are enriched in cardiolipin. J Bacteriol 183:6144–6147

Koprowski P, Grajkowski W, Balcerzak M, Filipiuk I, Fabczak H, Kubalski A (2015) Cytoplasmic domain of MscS interacts with cell division protein FtsZ: a possible non-channel function of the mechanosensitive channel in *Escherichia coli*. PLoS One 10:e0127029

Koshy C, Ziegler C (2015) Structural insights into functional lipid–protein interactions in secondary transporters. Biochim Biophys Acta 1850:476–487

Koshy C, Schweikhard ES, Gartner RM, Perez C, Yildiz O, Ziegler C (2013) Structural evidence for functional lipid interactions in the betaine transporter BetP. EMBO J 32:3096–3105

Kramer R (2010) Bacterial stimulus perception and signal transduction: response to osmotic stress. Chem Rec 10:217–229

Kramer R, Nicklisch S, Ott V (2010) Use of liposomes to study cellular osmosensors. Methods Mol Biol 606:21–30

Kusaka J, Shuto S, Imai Y, Ishikawa K, Saito T, Natori K, Matsuoka S, Hara H, Matsumoto K (2016) Septal localization by membrane targeting sequences and a conserved sequence essential for activity at the COOH-terminus of *Bacillus subtilis* cardiolipin synthase. Res Microbiol 167:202–214

Laganowsky A, Reading E, Allison TM, Ulmschneider MB, Degiacomi MT, Baldwin AJ, Robinson CV (2014) Membrane proteins bind lipids selectively to modulate their structure and function. Nature 510:172–175

Lai Y-T, Chang Y-Y, Hu L, Yang YH, Chao A, Du Z-Y, Tanner JA, Chye M-L, Qian C, Ng K-M et al (2015) Rapid labeling of intracellular His-tagged proteins in living cells. Proc Natl Acad Sci U S A 112:2948–2953

Laloux G, Jacobs-Wagner C (2014) How do bacteria localize proteins to the cell pole? J Cell Sci 127:11–19

Landgraf D, Okumus B, Chien P, Baker TA, Paulsson J (2013) Segregation of molecules at cell division reveals native protein localization. Nat Methods 9:480–486

Lewis JR, Cafiso DS (1999) Correlation between the free energy of a channel-forming voltage-gated peptide and the spontaneous curvature of bilayer lipids. Biochemistry 38:5932–5938

Lewis RNAH, McElhaney RN (2000) Surface charge markedly attenuates the nonlamellar phase-forming propensities of lipid bilayer membranes: calorimetric and 31P-nuclear magnetic resonance studies of mixtures of cationic, anionic, and zwitterionic lipids. Biophys J 79:1455–1464

Lewis RNAH, McElhaney RN (2009) The physicochemical properties of cardiolipin bilayers and cardiolipin-containing lipid membranes. Biochim Biophys Acta 1788:2069–2079

Lin TY, Weibel DB (2016) Organization and function of anionic phospholipids in bacteria. Appl Microbiol Biotechnol 100:4255–4267

Lin S-Y, Huang M-Z, Chang H-C, Shiea J (2007) Using electrospray-assisted laser desorption/ionization mass spectrometry to characterize organic compounds separated on thin-layer chromatography plates. Anal Chem 79:8789–8795

Lopalco P, Angelini R, Lobasso S, Köcher S, Thompson M, Müller V, Corcelli A (2013) Adjusting membrane lipids under salt stress: the case of the moderate halophilic organism *Halobacillus halophilus*. Environ Microbiol 15:1078–1087

López CS, Heras H, Garda H, Ruzal S, Sánchez-Rivas C, Rivas E (2000) Biochemical and biophysical studies of *Bacillus subtilis* envelopes under hyperosmotic stress. Int J Food Microbiol 55:137–142

López CS, Alice AF, Heras H, Rivas EA, Sánchez-Rivas C (2006) Role of anionic phospholipids in the adaptation of *Bacillus subtilis* to high salinity. Microbiology 152:605–616

Maddock JR, Shapiro L (1993) Polar location of the chemoreceptor complex in the *Escherichia coli* cell. Science 259:1717–1723

Magalon A, Alberge F (2016) Distribution and dynamics of OXPHOS complexes in the bacterial cytoplasmic membrane. Biochim Biophys Acta 1857:198–213

Margolin W (2012) The price of tags in protein localization studies. J Bacteriol 194:6369–6371

Matsumoto K, Kusaka J, Nishibori A, Hara H (2006) Lipid domains in bacterial membranes. Mol Microbiol 61:1110–1117

Maximov S, Ott V, Belkoura L, Kämer R (2014) Stimulus analysis of BetP activation under in vivo conditions. Biochim Biophys Acta 1838:1288–1295

Mileykovskaya E, Dowhan W (2000) Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange. J Bacteriol 182:1172–1175

Mileykovskaya E, Dowhan W, Birke RL, Zheng D, Lutterodt L, Haines TH (2001) Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces. FEBS Lett 507:187–190

Mukhopadhyay R, Huang KC, Wingreen NS (2008) Lipid localization in bacterial cells through curvature-mediated microphase separation. Biophys J 95:1034–1049

Nishibori A, Kusaka J, Hara H, Umeda M, Matsumoto K (2005) Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes. J Bacteriol 187:2163–2174

Norman C, Liu ZW, Rigby P, Raso A, Petrov Y, Martinac B (2005) Visualisation of the mechanosensitive channel of large conductance in bacteria using confocal microscopy. Eur Biophys J 34:396–402

Oliver PM, Crooks JA, Leidl M, Yoon EJ, Saghatelian A, Weibel DB (2014) Localization of anionic phospholipids in *Escherichia coli* cells. J Bacteriol 196:3386–3398

Olofsson G, Sparr E (2013) Ionization constants pK_a of cardiolipin. PLoS One 8:e73040

Parkin J, Chavent M, Khalid S (2015) Molecular simulations of Gram-negative bacterial membranes: a vignette of some recent successes. Biophys J 109:461–468

Pliotas C, Dahl ACE, Rasmussen T, Mahendran KR, Smith TK, Marius P, Gault J, Banda T, Rasmussen A, Miller S et al (2015) The role of lipids in mechanosensation. Nat Struct Mol Biol 22:991–998

Poolman B, Spitzer JJ, Wood JM (2004) Bacterial Osmosensing: roles of membrane structure and electrostatics in lipid-protein and protein-protein interactions. Biochim Biophys Acta 1666:88–104

Quinn PJ (2012) Lipid–lipid interactions in bilayer membranes: married couples and casual liaisons. Prog Lipid Res 51:179–198

Ramamurthi KS, Lecuyer S, Stone HA, Losick R (2009) Geometric cue for protein localization in a bacterium. Science 323:1354–1357

Rasmussen T (2016) How do mechanosensitive channels sense membrane tension? Biochem Soc Trans 44:1019–1025

Renner LD, Weibel DB (2011) Cardiolipin microdomains localize to negatively curved regions of *Escherichia coli* membranes. Proc Natl Acad Sci U S A 108:6264–6269

Roggiani M, Goulian M (2015) Chromosome-membrane interactions in bacteria. Annu Rev Genet 49:115–129

Romantsov T, Helbig S, Culham DE, Gill C, Stalker L, Wood JM (2007) Cardiolipin promotes polar localization of osmosensory transporter ProP in *Escherichia coli*. Mol Microbiol 64:1455–1465

Romantsov T, Stalker L, Culham DE, Wood JM (2008) Cardiolipin controls the osmotic stress response and the subcellular location of transporter ProP in *Escherichia coli*. J Biol Chem 283:12314–12323

Romantsov T, Battle AR, Hendel JM, Martinac B, Wood JM (2009a) Protein localization in *Escherichia coli* cells: comparison of cytoplasmic membrane proteins ProP, LacY, ProW, AqpZ, MscS, and MscL. J Bacteriol 192:912–924

Romantsov T, Guan Z, Wood JM (2009b) Cardiolipin and the osmotic stress responses of bacteria. Biochim Biophys Acta 1788:2092–2100

Sáenz JP, Sezgin E, Schwille P, Simons K (2012) Functional convergence of hopanoids and sterols in membrane ordering. Proc Natl Acad Sci U S A 109:14236–14240

Sevin DC, Sauer U (2014) Ubiquinone accumulation improves osmotic-stress tolerance in *Escherichia coli*. Nat Chem Biol 10:266–272

Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. Nat Methods 2:905–909

Shapiro L, McAdams HH, Losick R (2009) Why and how bacteria localize proteins. Science 326:1225–1228

Strahl H, Burmann F, Hamoen LW (2014) The actin homologue MreB organizes the bacterial cell membrane. Nat Commun 5:3442

Treuner-Lange A, Sogaard-Andersen L (2014) Regulation of cell polarity in bacteria. J Cell Biol 206:7–17

Tsatskis Y, Khambati J, Dobson M, Bogdanov M, Dowhan W, Wood JM (2005) The osmotic activation of transporter ProP is tuned by both its C-terminal coiled-coil and osmotically induced changes in phospholipid composition. J Biol Chem 280:41387–41394

Tsuzuki M, Moskvin OV, Kuribayashi M, Sato K, Retamal S, Abo M (2011) Salt stress-induced changes in the transcriptome, compatible solutes, and membrane lipids in the facultatively phototrophic bacterium *Rhodobacter sphaeroides*. Appl Environ Microbiol 77:7551–7559.

Vanounou S, Pines D, Pines E, Parola AH, Fishov I (2002) Coexistence of domains with distinct order and polarity in fluid bacterial membranes. Photochem Photobiol 76:1–11

Vanounou S, Parola AH, Fishov I (2003) Phosphatidylethanolamine and phosphatidylglycerol are segregated into different domains in bacterial membrane. A study with pyrene-labelled phospholipids. Mol Microbiol 49:1067–1079

Wang W, Black SS, Edwards MD, Miller S, Bartlett W, Dong C, Naismith J, Booth IR (2008) The structure of an open form of an *E. coli* mechanosensitive channel reveals the molecular basis of gating. Science 321:1179–1183

Wolters JC, Berntsson RP, Gul N, Karasawa A, Thunnissen AM, Slotboom DJ, Poolman B (2010) Ligand binding and crystal structures of the substrate-binding domain of the ABC transporter OpuA. PLoS One 5:e10361

Wood JM (1999) Osmosensing by bacteria: signals and membrane-based sensors. Microbiol Mol Biol Rev 63:230–262

Wood JM (2007) Bacterial osmosensing transporters. Methods Enzymol 428:77–107

Wood JM (2011) Bacterial osmoregulation: a paradigm for the study of cellular homeostasis. Annu Rev Microbiol 65:215–238

Wood JM (2015) Bacterial responses to osmotic challenges. J Gen Physiol 145:381–388

Wu EL, Cheng X, Jo S, Rui H, Song KC, Davila-Contreras EM, Qi YF, Lee JM, Monje-Galvan V, Venable RM et al (2014) CHARMM-GUI membrane builder toward realistic biological membrane simulations. J Comput Chem 35:1997–2004

Yeagle PL (2011) The structure of biological membranes, 3rd edn. CRC Press, Boca Raton

Zaritsky A, Woldringh C, Fishov I, Vischer NO, Einav M (1999) Varying division planes of secondary constrictions in spheroidal *Escherichia coli* cells. Microbiology 145:1015–1022

Zhang XC, Liu Z, Li J (2016) From membrane tension to channel gating: a principal energy transfer mechanism for mechanosensitive channels. Protein Sci 25:1954–1964

Zhong D, Blount P (2014) Electrostatics at the membrane define MscL channel mechanosensitivity and kinetics. FASEB J 28:5234–5241

Zoetewey DL, Tripet BP, Kutateladze TG, Overduin MJ, Wood JM, Hodges RS (2003) Solution structure of the C-terminal antiparallel coiled-coil domain from *Escherichia coli* osmosensor ProP. J Mol Biol 334:1063–1076

Membrane Homeostasis upon Nutrient (C, N, P) Limitation

45

F. Schubotz

Contents

1 Introduction 824
2 Membrane Homeostasis upon Phosphorus Limitation 825
2.1 Photosynthetic Organisms 825
2.2 Chemosynthetic Organisms 829
3 Membrane Homeostasis upon Nitrogen Limitation 832
3.1 Photosynthetic Organisms 832
3.2 Chemosynthetic Organisms 834
4 Membrane Homeostasis upon Carbon Limitation 834
4.1 Photosynthetic Organisms 834
4.2 Chemosynthetic Organisms 835
5 Regulation of Lipid Remodeling 836
5.1 Plants 836
5.2 Algae 838
5.3 Bacteria 839
6 Environmental Relevance and Research Needs 841
References 842

Abstract

Natural environments are dynamic systems where organisms have to constantly acclimate to fluctuations in external conditions, including the bioavailability of essential nutrients carbon (C), nitrogen (N), and phosphorus (P). Membrane lipid plasticity plays an important role to adjust to these environmental challenges by either reducing the cellular need of these macronutrients or to help protect the cell and keep it viable during a prolonged state of reduced energy supply. When P limited, many organisms are able to replace their phospholipids with non-phosphorus containing glycolipids or aminolipids, liberating P for other

F. Schubotz (✉)
MARUM and Department of Geosciences, University of Bremen, Bremen, Germany
e-mail: schubotz@uni-bremen.de

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_59

cellular processes. Under N depletion, a common stress response is the increased production of triacylglycerol (TAG) lipids that serve as energy and carbon storage until nutrients become available again. During severe C starvation, the cell switches to survival mode and membrane lipids are remodeled to conserve energy and stabilize the cell against external stressors, but are also degraded and serve as an endogenous carbon and energy supply. These homeostatic adjustments are found among all domains of life with different specificities and play decisive roles during natural selection of populations in marine and terrestrial ecosystems.

1 Introduction

Carbon (C), nitrogen (N), and phosphorus (P) are required elements for life. While carbon forms the backbone of biomolecules, phosphorus and nitrogen are needed for the build-up of macromolecules such as proteins, DNA, membrane lipids, and low-molecular weight metabolites involved in energy transfer, such as ATP. In nature, the bioavailability of these essential macronutrients is often scarce and thus growth limiting. As a consequence, organisms have developed adaptive strategies that either improve acquisition and uptake of these nutrients or conserve their use. Membrane lipid homeostasis plays a central role in maintaining optimal cell function despite large external fluctuations in substrate concentrations or other stressors (Parsons and Rock 2013; Harayama and Riezman 2018). Homeostatic regulations can occur via modulation of membrane lipid head group composition, for instance by replacing phospholipids with non-P containing glycolipids or aminolipids under P limitation, making phosphorus available for other cellular processes. This regulation emerged as prominent stress response during P scarcity in phototrophic organisms such as microalgae and cyanobacteria living in the oligotrophic open ocean (Van Mooy et al. 2009) and since has been extended to various marine heterotrophic bacteria (Sebastian et al. 2016) and is effective in plants and soil bacteria (Siebers et al. 2015; López-Lara and Geiger 2016). Nutrient limitation also stimulates the accumulation of storage lipids, in particular triacylglycerols (TAG) in lipid droplets located within the cell. TAG serve as energy and carbon storage molecules and provide a glycerolipid reservoir for the rapid synthesis and lipid remodeling of membrane lipids under both standard and stress conditions (Zienkiewicz et al. 2016). The increased production of TAG under N limitation in many plants, unicellular phototrophs and oleaginous heterotrophic organisms has garnered much interest in using these organisms for biofuel production (Du and Benning 2016). A third type of homeostatic control is the modification of the core lipid structures, which may alter membrane fluidity and thus permeability. Upon C starvation, cellular responses includes rigorous remodeling of fatty acyl side chains, for instance, decreasing the unsaturation level, which makes the membrane less permeable and thus conserves energy (Kjelleberg et al. 1987). Furthermore, membrane lipid catabolism is upregulated and biomolecules that are not essential for function and cellular maintenance are internally scavenged (DiRusso and Nyström 1998; Yu 1999). The aim of this chapter is to give an introduction into the versatile membrane lipid

adaptations and regulatory cellular networks that eukarya, bacteria, and archaea have in place to cope with C, N, and P limitation and how these flexible adjustments may have aided these organisms to evolve in their respective environmental niches.

2 Membrane Homeostasis upon Phosphorus Limitation

Uptake of phosphorus into cells occurs predominantly through its inorganic form phosphate. In most soils, phosphate concentrations are low (on average ca. 1 μM) as most of the phosphate is readily converted to organic compounds by microbes or becomes insoluble due to sorption to mineral cations (Ruttenberg 2014). In aquatic systems, phosphate concentrations are even lower and range in the nanomolar to micromolar range, with lowest concentrations observed in the oligotrophic open ocean and maximal values of around 4 μM detected in the deeper ocean or upwelling regions (Karl 2014). The marine environment is thus considered to be in a state of chronic phosphate starvation. To increase nutrient uptake, some phytoplankton can also utilize dissolved organic P and enzymatically access dissolved organic P. Similarly, fungi and bacteria exploit organic phosphorus sources and can mobilize insoluble P by enzymatic activity (Ruttenberg 2014). Plants also have many regulatory mechanisms in place to obtain P despite low soil solution concentrations. These include forming symbiotic associations with mycorrhizal fungi and rhizobial bacteria, modifying their root architecture to optimize nutrient uptake, or intracellular regulation of P-containing biomolecules (Chiou and Lin 2011). Hereby, membrane phospholipid substitution was identified as a significant reservoir of internal P as phospholipids can comprise as much as one-third of total organic P in plants (Poirier et al. 1991). In microalgae, phospholipid substitutions were shown to support around 16% of total cellular P demand under P limitation (Van Mooy et al. 2009; Martin et al. 2010).

2.1 Photosynthetic Organisms

While cellular membranes of mammals, yeast, and most bacteria are dominated by phospholipids, glycolipids are the predominant membrane lipids of phototrophs (López-Lara and Geiger 2016; Harayama and Riezman 2018; Kalisch et al. 2016). In plants and algae, glycolipids are found in the photosynthetic membranes of chloroplasts, where they are part of the four canonical thylakoid membrane lipids, which include the two galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the sulfolipid sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG) as the sole phospholipid (reviewed in Kalisch et al. 2016). In contrast to the highly conserved lipid composition of thylakoid membranes, nonphotosynthetic, extraplastidial membranes are comprised of much more diverse lipid groups, including many different nitrogen-containing phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Fig. 1) and in lower amounts phosphatidyl-(*N*)-methylethanolamine (PME),

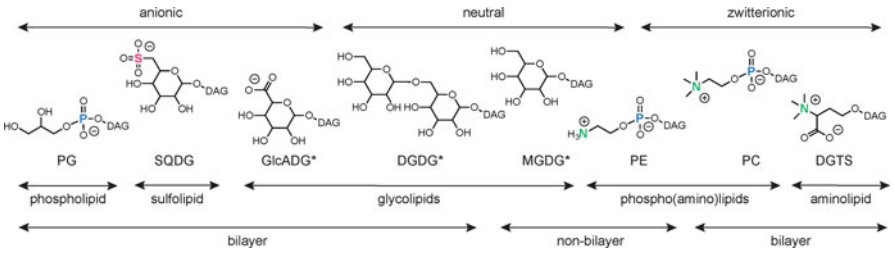


Fig. 1 Common head groups of membrane forming lipids in photosynthetic and chemosynthetic organisms and their respective bilayer-forming properties and charges at physiological pH. Presence of phosphorus (P), nitrogen (N), or sulfur (S) within the head groups are marked in bold letters. *DAG* diacylglycerol, *PG* phosphatidylglycerol, *SQDG* sulfoquinovosyldiacylglycerol, *GlcADG* glucuronosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *MGDG* monogalactosyldiacylglycerol, *PE* phosphatidylethanolamine, *PC* phosphatidylcholine, *DGTS* diacylglyceryl-(*N,N,N*)-trimethylhomoserine. *These glycolipids can also contain different types of hexose sugars. (Figure adapted from Boudière et al. 2014)

phosphatidyl-(*N,N*)- dimethylethanolamine (PDME), or phosphatidylserine (PS). In addition to these phospholipids, many algae, lower plants, and some photosynthetic bacteria synthesize aminolipids that contain an ether-linked betaine moiety in their head group (Dembitsky 1996). Three types of these betaine lipids have been described: diacylglycerol-(*N,N,N*)-trimethylhomoserine (DGTS), diacylglycerol-hydroxymethyl-(*N,N,N*)-trimethyl-ß-alanine (DGTA), and diacylglycerol-carboxyhydroxymethylcholine (DGCC).

During normal growth, the occurrence of galactolipids is restricted to plastic membranes. Here, they play important structural and functional roles, constructing the matrix of the thylakoids in which the photosystems are embedded and as integral components of these protein complexes (Boudière et al. 2014). While MGDG, DGDG, and PG are essential for photosynthetic function, the loss of SQDG does not always impair photosynthesis but instead helps maintain growth under P-limiting conditions. This was first demonstrated for the anoxygenic photosynthetic bacterium *Rhodobacter sphaeroides* (Benning et al. 1993) but has since been extended to cyanobacteria, green algae, diatoms, and plants (Güler et al. 1996; Essigmann et al. 1998; Van Mooy et al. 2009; Cañavate et al. 2016). Due to an observed reciprocal relationship of the two anionic lipids SQDG and PG during P limitation, it was suggested that SQDG may function as a substitute lipid for PG while allowing to maintain an optimal membrane lipid charge in the thylakoids (Boudière et al. 2014) (Figs. 1 and 2). As a beneficial consequence, the P that was originally bound in phospholipids is now available for other cellular biochemical functions. A similar reciprocal relationship was observed for the two zwitterionic lipids PC and betaine lipids (DGTS, DGTA or DGCC), where betaine lipids replaced PC under P limitation in unicellular algae and anoxygenic photosynthetic bacteria (Benning et al. 1995; Van Mooy et al. 2009). This further indicates that maintaining a balanced membrane charge is important during phospholipid replacement in photosynthetic organisms. However, it should be noted that even though biophysical properties of

substitute lipids may be similar, a complete replacement may not always be possible. For the cyanobacterium *Synechocystis* sp. PCC6803, minimal amounts of PG remained necessary to sustain photosynthesis even under P-limited conditions (Sato et al. 2000).

In most microalgae, namely, green algae, diatoms, and eustigmatophytes, phospholipid substitutions under P limitation follow the expected trends of betaine lipid: PC and SQDG:PG increases (typically between 2- and 30-fold) (Fig. 2). However, cryptophytes and some haptophytes maintain near constant levels of SQDG and betaine lipids even though they significantly reduce phospholipid contents, resulting in unchanged or less pronounced SQDG:PG and BL:PC replacements (Cañavate et al. 2016). An exception to this is the common oceanic haptophyte *Emiliania huxleyi,* which exhibited a respective 10- to 40-fold increase of DGCC:PC and SQDG:PG when grown under P limitation demonstrating the large intraspecific variability in membrane lipid plasticity (Shemi et al. 2016). This demonstrates that phospholipid substitutions and their biochemical controls are highly diverse and taxa-dependent and may be a consequence of differential niche adaptions of microalgae to their respective environments.

Besides expected increases in substitute lipid ratios, *E. huxleyi* also exhibited increases in the galactolipids MGDG and DGDG during P limitation (Shemi et al. 2016). Most of the examined microalgae displayed no notable changes in the contribution of MGDG and DGDG (Martin et al. 2010; Cañavate et al. 2016), apart from the diatom *Phaeodactylum tricornutum,* where DGDG increased while MGDG decreased (Abida et al. 2014). Increase in DGDG amounts is also a common response of seed plants under P limitation, where accumulation of DGDG was not only observed in the thylakoids but also in extraplastidial membranes, where it apparently substitutes for phospholipids PC and PE (Härtel et al. 2000; Nakamura 2013). Under normal growth conditions, the ratio of MGDG to DGDG is tightly regulated in thylakoid membranes which have been attributed to their respective non-bilayer to bilayer-forming properties (Boudière et al. 2014). Due to its small head group, MGDG has a conical shape and forms hexagonal phases resulting in a preference to form non-bilayers, while the bulkier head group of DGDG results in a cylindrical shape, forming a lamellar phase in mixtures with water resulting in its bilayer forming properties (Dowhan et al. 2016). These lipid architectural properties are crucial for protein folding and insertion in membranes, thus the balance of non-bilayer and bilayer lipids is important during adjustment to changing external conditions. However, the impact of changes in MGDG:DGDG ratios in algae and plants on the photosynthetic machinery has so far not been explored.

Under P-limited growth, some phototrophs also newly synthesize non-galactose glycolipids. Hereby, the appearance of a dihexose glycolipid glucosylgalactosyldiacylglycerol (GlcGalDG) was reported for the anoxygenic photosynthetic bacterium *Rhodobacter sphaeroides* (Benning et al. 1995) and the accumulation of the anionic glycolipid glucuronosyldiacylglycerol (GlcADG) was reported for different seed plants (Okazaki et al. 2015). While the importance of galactolipids over glucolipids in thylakoid membranes was established under normal growth (Hölzl et al. 2005), it remains unclear which functions these newly synthesized glucolipids have under

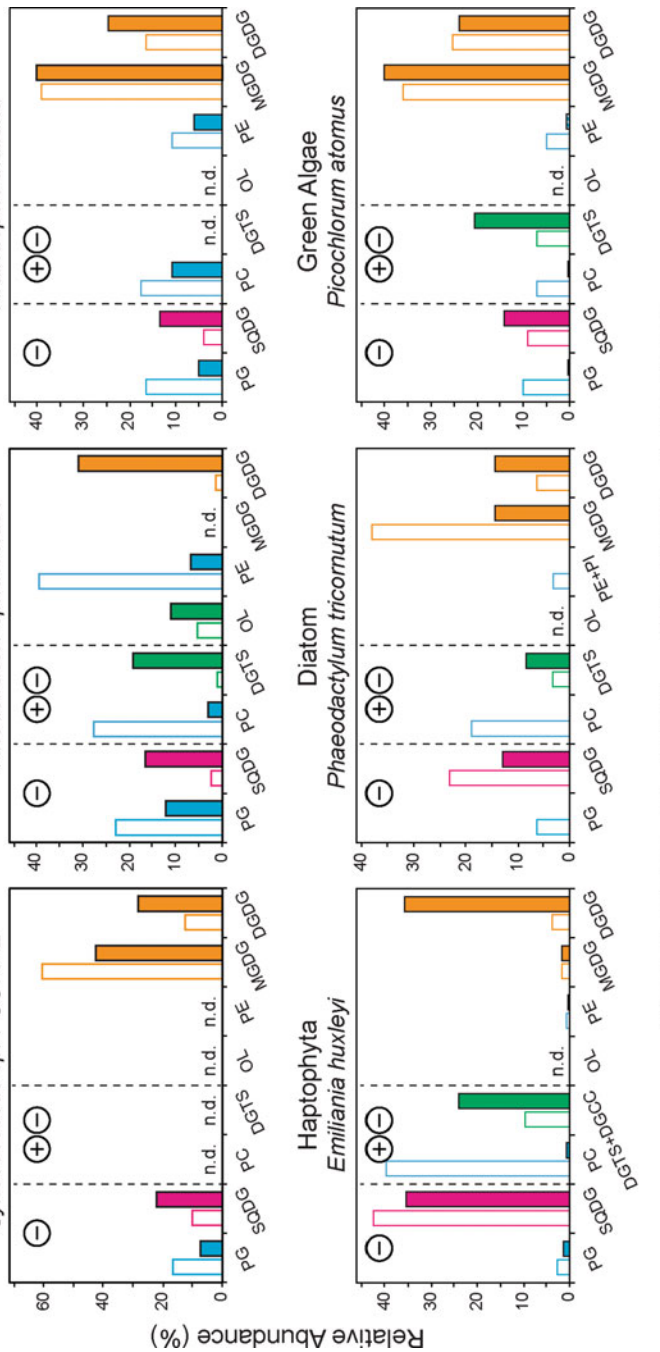


Fig. 2 Membrane lipid composition of representative photosynthetic organisms (bacteria, plants, and algae) grown under P-replete (open symbols) and P-limited (filled symbols) conditions (data from Benning et al. 1993, 1995; Güler et al. 1996; Essigmann et al. 1998; Abida et al. 2014; Shemi et al. 2016, and Cañavate et al. 2016). The common anionic phospholipid substitutions (PG:SQDG) and zwitterionic substitute lipids (PC:betaine lipids) are depicted next to each other. For abbreviations see Fig. 1 and text, n.d. - not detected.

phosphate starvation. Apart from modifications in the head groups, another important stress response when facing P deprivation is the accumulation of storage lipids, such as TAG lipids which can amount up to 50% of cell dry weight in freshwater and marine algae (Zienkiewicz et al. 2016; Mühlroth et al. 2017). TAG are stored in lipid droplets located in the cytoplasm or chloroplast. They do not fulfill structural roles in cells, but instead provide a cellular carbon and energy storage and a reservoir for fatty acids that may be quickly made available for resynthesis of complex lipids once nutrient availability is again increased (see also Sects. 3.1 and 5.1).

2.2 Chemosynthetic Organisms

Bacteria and yeast predominantly contain phospholipids under normal growth conditions (López-Lara and Geiger 2016; Harayama and Riezman 2018), but when facing reduced external P supply these organisms also have the ability to replace phospholipids with non-phosphorus lipids. In contrast to phototrophic organisms, bacteria synthesize a higher variety of glycolipids (Hölzl and Dörmann 2007), many of which only appear under phosphorus limited growth. While glycolipids are mostly absent in Gram-negative bacteria under normal growth, Gram-positive bacteria contain abundant glycolipids that are comprised of a variety of different mono and di-hexose head groups, typically mixtures of glucose, galactose, or mannose (Hölzl and Dörmann 2007). In Gram-positive bacillus species, it was first observed that phospholipids and glycolipids of the same charge may be interchangeable during P-limited growth (Minnikin et al. 1971). Hereby, anionic phospholipids (PG and diphosphatidylglycerol, DPG) were replaced with anionic GlcADG, while the neutral/zwitterionic phospholipid PE was replaced with the neutral glycolipid diglucosyldiacylglycerol (DGlcDG). Similar charge-related phospholipid-glycolipid replacements under P limitation have since been extended to many other bacteria (Fig. 3), including Gram-negative bacteria that typically do not synthesize glycolipids under normal conditions (Geske et al. 2012; Carini et al. 2015; Bosak et al. 2016). Some bacteria and fungi are also able to synthesize DGTS as a sole betaine lipid, so far neither DGTA nor DGCC have been reported in these organisms (Geiger et al. 2010; Riekhof et al. 2014; López-Lara and Geiger 2016). DGTS synthesis in non-phototrophic bacteria was first observed in *Sinorhizobium meliloti,* an alphaproteobacterium of the Rhizobium clade that is involved in plant-symbiosis (Geiger et al. 1999). Under P-limiting conditions, DGTS newly appeared together with SQDG apparently substituting for the zwitterionic and anionic phospholipids PC and PG, respectively. It has now been established that a wide range of marine and terrestrial bacteria, including members of the Gram-negative alpha- and gammaproteobacteria, bacteriodetes and the Gram-positive actinobacteria can synthesize DGTS, which may functionally replace zwitterionic phospholipids PC, PDME, PME, or PE under P-limiting conditions (Geske et al. 2012; Yao et al. 2015; Sebastian et al. 2016). Notably, some of these organisms synthesize DGTS also under P replete conditions. Many fungi also contain DGTS under normal growth and are able to replace the bulk of their phospholipids, in particularly PC with DGTS in

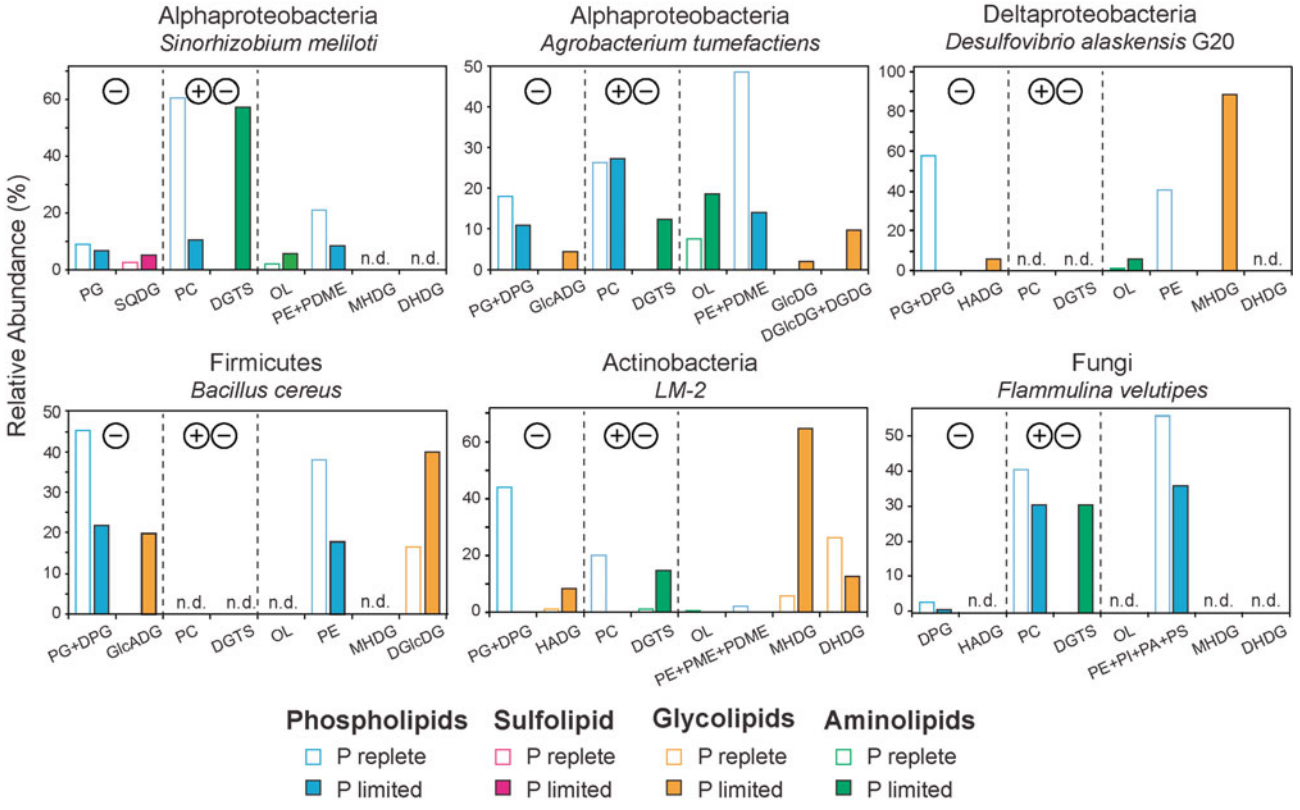


Fig. 3 Membrane lipid composition of representative chemotrophic organisms grown under P-replete (open symbols) and P-limited (filled symbols) conditions (data from Minnikin et al. 1971; Geiger et al. 1999; Semeniuk et al. 2014; Senik et al. 2015; Yao et al. 2015 and Bosak et al. 2016). The common anionic phospholipid substitutions (PG:SQDG) and zwitterionic substitute lipids (PC:DGTS) are depicted next to each other. Hexose sugar type was not determined in these studies. For abbreviations see Fig. 1 and text, n.d. - not detected

response to P starvation (Riekhof et al. 2014; Senik et al. 2015). Hereby, a complete replacement of PC with DGTS is possible as growth was not hindered in PC-lacking mutants of the yeast *S. cerevisiae* (Riekhof et al. 2014).

Another class of aminolipids that are suggested to play a role under P-limited growth in bacteria are ornithine lipids (OL). OL are non-phosphorus containing lipids that do not occur in eukarya or archaea but are common constituents of bacterial membranes under normal growth (López-Lara and Geiger 2016). They do not contain a glycerol backbone, but instead are comprised of a 3-hydroxy fatty acyl group attached in amide linkage to an ornithine head group with an additional fatty acid ester-linked at the 3-hydroxy group of the first fatty acid. Increases of OL were observed for some bacteria when grown under P-limited conditions (Geiger et al. 1999) and zwitterionic OL seemingly plays an important role in sustaining membrane lipid charge under P limitation in bacteria that lack the ability to synthesize DGTS (López-Lara et al. 2005). Notably, pathways of phospholipid remodeling remain to be explored due to the structural difference of OL from regular membrane-forming glycerolipids.

SQDG does not seem to be a common anionic substitute lipid in bacteria, instead hexuronic acid diacylglycerol glycolipids (HADG, sugar types were not determined in these studies) predominantly appears in P-limited bacteria when anionic phospholipid (PG and DPG) contents are decreased (Carini et al. 2015; Yao et al. 2015; Bosak et al. 2016). For bacteria that do not contain the genetic capacity to synthesize DGTS, it was observed that mixtures of HADG and monohexose glycolipids (MHDG, sugar types were not determined in these studies) comprise a substantial part of total lipids under P depletion (Carini et al. 2015; Bosak et al. 2016). Although cell replication was sustained in these organisms under P limitation, growth and fitness was significantly impaired as shown for the anaerobic sulfate-reducing bacterium *Desulfovibrio alaskensis* G20 (Bosak et al. 2016). So far, little is known on the specific functional roles of phospholipids and their substitute lipids in bacterial membranes. It can be assumed that the replacement of the zwitterionic lipids PC and PE with uncharged lipids, such as MHDG, largely maintains membrane lipid charge and the non-bilayer forming MHDG may replace the non-bilayer-forming PE functionally. Nevertheless, the ratio of anionic to zwitterionic/neutral and non-bilayer to bilayer forming substitute lipids does not always reflect the original phospholipid configuration under normal growth (Fig. 3). Since very often cell shape and membrane ultrastructure is significantly altered during P limitation (Van Mooy et al. 2009; Bosak et al. 2016), it remains to be explored if membrane lipid charge or lipid shape is more crucial for maintaining membrane integrity or if other biophysical properties of lipids are of relevance. Besides charge maintenance and bilayer forming properties, other important biophysical functions of lipids include differences in melting point that are important for maintaining membrane fluidity, their involvement in membrane curvature or bending and lipid-lipid and lipid-protein interactions due to, e.g., variations in van der Waals forcing or hydrogen bonding (Harayama and Riezman 2018).

There are only very limited studies on membrane lipid responses to changes in external nutrient supply in archaea. A comprehensive survey of the lipidome of

thaumarchaeota, which are environmentally relevant ammonium-oxidizing archaea, showed that members of this archaeal clade contain abundant glycolipids with intrinsic low phospholipid contents, often lower than 10% (Elling et al. 2017). It is thus assumed that these archaea are inherently adapted to low nutrient and phosphorus supply, which would explain their abundant presence in oligotrophic environments. Phospholipid content can be higher and more diverse in euryarchaeota (Koga and Nakano 2008). Meador et al. (2014) investigated the response of the thermophilic methanogen *T. kodakarensis* to P limitation and surprisingly did not observe glycolipids during growth in P-reduced media, instead phospholipids dominated. However, it should be noted that growth was not sustainable below 40 μM phosphate, indicating that this organism is generally not adapted to low phosphate levels.

3 Membrane Homeostasis upon Nitrogen Limitation

Similar to phosphorus, nitrogen is mainly acquired by plants, algae, and microorganisms through its inorganic form as nitrate or ammonium. In addition, microbes and some algae can also utilize organic nitrogen sources such as amino acids, purines and amides like urea (Geisseler et al. 2010), and diazotrophic bacteria are able to fix nitrogen from dinitrogen gas (N_2). Primary productivity in the open ocean and in terrestrial environments is often considered to be nitrogen limited (Xu et al. 2012; Moore et al. 2013). The loss of N occurs mainly through microbial denitrification processes that convert bioavailable nitrogen to N_2, in soils also leaching and run-off contributes to significant losses. Similar to adaptations to low P contents, N deficiency induces a variety of cellular responses, such as increasing uptake via enzyme activity, reducing the cellular N demand by proteome changes (Grzymski and Dussaq 2011; Xu et al. 2012), or accumulation of triacylglycerol (TAG) storage lipids. Despite these responses, continuous N deprivation typically leads to cell growth cessation (Sharma et al. 2012; Du and Benning 2016). Since TAG-producing organisms are of increasing interest for biofuel production, significant research has been dedicated to further understand the underlying mechanisms of TAG production under N limitation (Fig. 4).

3.1 Photosynthetic Organisms

A common response of algae, plants, and cyanobacteria to nitrogen limitation is the sequestration of carbon into TAG in cellular lipid droplets. TAG accumulation has been observed in several species of green microalgae, diatoms, haptophytes, red algae, and eustigmatophytes and can amount up to 50% of dry weight (Breuer et al. 2012; Sharma et al. 2012) (Fig. 4). It thus represents a significant storage of carbon and energy for the cell. In general, N deprivation triggers a faster and higher TAG accumulation than P deprivation and is ca. fivefold higher than during normal growth (Abida et al. 2014; Degraeve-Guilbault et al. 2017). Accumulation of TAG

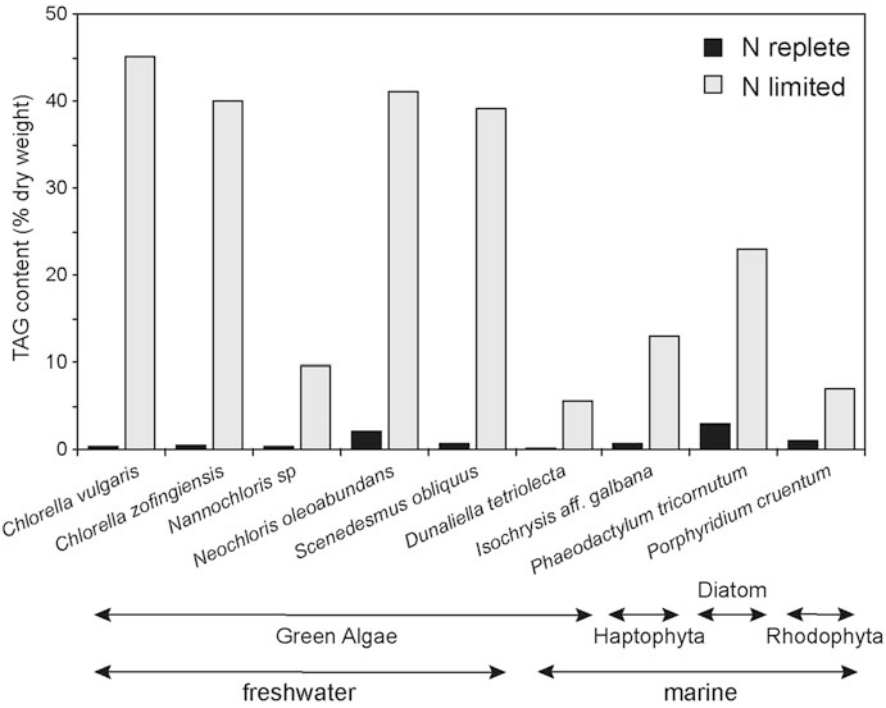


Fig. 4 Triacylglycerol (TAG) accumulation (% dry weight) of representative freshwater and marine microalgae grown under N-replete (black bars) N-limited (grey bars) conditions. (Figure modified after Breuer et al. 2012)

and fatty acid phytyl esters was also reported to occur in seed plants (Gaude et al. 2007; Du and Benning 2016). Besides functioning as a carbon and energy storage, TAG production may also serve as a fatty acid reservoir for resynthesis of complex lipids when conditions improve (Zienkiewicz et al. 2016). TAG production predominantly occurs through de novo fatty acid biosynthesis and with only a small proportion originating from recycling of membrane glycerolipids. This is why TAG synthesis is also thought to serve as a major sink for photoassimilates that are no longer consumed by the decreased growth metabolism. Reduced cell growth and biomass production consumes less NADPH than is produced during photosynthesis; this may result in the production of harmful reactive oxygen species that may damage cell components. During TAG production and accompanying fatty acid synthesis, excess NADPH is consumed via the generation of $NADP^+$ (cf. Sharma et al. 2012).

Interestingly, the amounts of N-containing lipids, such as PC or PE, are typically not reduced under N deprivation. Instead, a common response in diatoms and plants is an increase in DGDG contents, with a sometimes concomitant decrease in MGDG (Gaude et al. 2007; Abida et al. 2014; Degraeve-Guilbault et al. 2017). The increased amounts of DGDG have been hypothesized to stabilize the thylakoid bilayer

structure after nitrogen-recycling induced protein-loss and concurrent membrane destabilization (Degraeve-Guilbault et al. 2017). However, these galactolipid response mechanisms and their functional roles require further interrogation.

3.2 Chemosynthetic Organisms

Not many studies have investigated the sole effects of N limitation on the lipid composition of chemosynthetic organisms. In the yeast *Cryptococcus curvatus,* similar trends as in microalgae towards the accumulation of free fatty acids were observed when grown under N-limited conditions (Hassan et al. 1996), indicating that also nonphotosynthetic organisms respond to N limitation by storage lipid accumulation. Investigations of nitrogen-starved *Bacillus subtilis* observed a catabolic overproduction of NADPH, similar to the overproduction of photoassimilates in phototrophs (Rühl et al. 2012). These authors, however, did not look at the presence or absence of TAG and de novo production of fatty acids, which may scavenge the overproduced NADPH.

4 Membrane Homeostasis upon Carbon Limitation

Carbon limitation in autotrophs is not as critical as for heterotrophic organisms since CO_2 for carbon fixation is ubiquitously present in the atmosphere. Autotrophic plants, however, can experience C limitation as they also contain many non-photosynthetic organs, such as roots, stems, or flowers that can undergo carbohydrate limitation if photosynthesis is reduced due to changes in light intensity or through attack by pathogens (Yu 1999). Carbon limitation is of notable concern for many heterotrophic microorganisms that live in deeper soil or sediment depths and in the deep ocean where organic matter becomes increasingly recalcitrant and harder to access. These organisms may often be found in a state of dormancy, where they reduce cellular activity and maintenance to a minimum until substrates become available again (Kjelleberg et al. 1987; Jørgensen and Marshall 2016).

4.1 Photosynthetic Organisms

A common response to carbohydrate (glucose or sucrose) limitation in non-photosynthetic plant cells includes the scavenging of alternative C sources from cellular compounds such as starch, lipids, proteins, or other cellular material, also referred to as autophagy. Hereby, phospholipid and glycolipid levels can decrease to more than half of their normal level (Journet et al. 1986). Catabolic breakdown of membrane phospholipids and glycolipids to first fatty acids and subsequently acetyl-CoA through *β*-oxidation provides NADH that can be used for ATP production during C-limited growth. Lee et al. (1998) could show that phospholipase D (PLD) and lipolytic acyl hydrolase were activated at the early part of starvation, followed by

ß-oxidation and enzymes involved in the glyoxylate cycle, which synthesized carbohydrates from acetyl-CoA. During starvation-induced autophagy, membrane lipids are also required for the formation of autophagosomes, which are double membrane vesicles that encapsulate cytoplasmic material and deliver it for degradation and recycling to the lysosomes (Xie and Klionsky 2007). The core functional units of autophagy are conserved in plants, yeasts, and mammals with numerous *AuTophaGy* (ATG) genes involved in the formation of the autophagosome (Hayward and Dinesh-Kumar 2011; Aguilar-López and Funes 2018). Hereby, the phospholipid PI is first phosphorylated to phosphatidylinositol 3-phosphate (PI3P) and as PI3P plays an essential role during the initiation of the autophagosome, while the phospholipid PE is needed for autophagosome expansion. Membrane lipid catabolism and autophagy-induced nutrient remobilization is an effective strategy for cellular life-extension during C starvation periods and under normal conditions is a housekeeping process to remove damaged cell material during cell maintenance (Slávikova et al. 2005).

4.2 Chemosynthetic Organisms

Starvation responses, mainly upon glucose deprivation, have been studied in bacteria, predominantly in *Escherichia coli,* some proteobacteria and spore-forming bacillus species, and in yeast. To prolong survival upon carbon-source depletion, yeast cells activate the same conserved core Atg protein complexes involved in autophagy as plants and other eukarya (Aguilar-López and Funes 2018). As described above (Sect. 4.1), membrane lipids play hereby both an important role in autophatogosome formation and as endogenous energy and carbon source during cellular self-digestion.

Similar to C-starved eukaryotic cells, bacteria in stationary phase activate genes involved in the utilization of alternative carbon sources, such as lipids and proteins, but also exhibit significant changes in membrane composition (Kjelleberg et al. 1987). Fatty acid degradation *(fad)* genes involved in (*i*) the activation of endogenous fatty acids released from membrane phospholipids (Pech-Canul et al. 2011) and (*ii*) in *ß*-oxidation (DiRusso and Nyström 1998) become activated during carbon starvation. This ultimately generates acetyl-CoA, a source of carbon and energy for the cell (Fig. 7b, see Sect. 5.3 for details). Self-digestion during bacterial growth-arrest in stationary phase is accompanied by a significant cell reduction, also referred to as dwarfing, as parts of the cell envelope are being degraded (Nyström 2004). Membrane lipid degradation and a concomitant decline in fatty acid and phospholipid biosynthesis may lead to an overall decrease in phospholipid levels by over 90% (Hood et al. 1986). A common microbial response to starvation is the cyclopropanation of previously monounsaturated membrane lipid fatty acids by the cyclopropane fatty acid synthase (CFA) (Grogan and Cronan 1997) or a general change in the level of unsaturations to a more saturated form (Tavormina et al. 2016). These changes in the fatty acyl side chains decrease the fluidity and permeability properties of membranes and thus physically stabilize cells and protect them from the external

environment. Modifications in head group composition during starvation include accumulation of DPG attended by an increase in cardiolipin synthase activity and a decrease in PG (Hiraoka et al. 1993; Tavormina et al. 2016). Cardiolipin synthase null mutants survive the stationary phase poorly, underlining a decisive role of DPG in cell survival (Hiraoka et al. 1993). Other changes in the cell envelope include the acylation of lipid A with an additional C16 fatty acyl chain by palmitoyl transferase (PagP) (Houser et al. 2015). This response may be attributed to increasing the hydrophobic molecules on the cell surface, which may ultimately favor the adhesion and aggregation of potential substrate molecules (Kjelleberg et al. 1987). These membrane lipid modifications therefore not only fortify and shield the cell from external stressors but also increase substrate acquisition during prolonged starvation.

Many bacteria that live in the deep ocean and deeper parts of marine sediments have a tendency to synthesize ether lipid-containing phospholipids seemingly replacing phospholipids with fatty acyl core structures (Schubotz et al. 2009; Evans et al. 2017). The production of ether lipids makes the cell less permeable and more robust, and may reduce energy-costly membrane lipid remodeling (Valentine 2007). This is also why it is thought that archaea, which exclusively synthesize ether lipids, may be inherently better adapted to survive in low energy yielding environments.

5 Regulation of Lipid Remodeling

5.1 Plants

Extensive research has been performed in elucidating pathways involved in lipid remodeling under P starvation in the flowering plant Arabidopsis. There are two dominant pathways described in seed plants that liberate DAG backbones from phospholipids for galactolipid remodeling: (1) the NPC (nonspecific phospholipase C) pathway which cleaves the entire phosphocholine head group of PC in one step via the enzymes phospholipase C (PLC), and (2) the PLD/PAP pathway, which first cleaves off choline via phospholipase D (PLD) and in a second step induces the loss of phosphate via phosphatidic acid phosphatase (PAP) (Fig. 5). Details of these pathways have been reviewed (Moellering and Benning 2011; Nakamura 2013). A third and less studied pathway observed in Arabidopsis, the LAH/GDPD pathway, involves the activity of lipid acyl hydrolase (LAH) through patatin-related type A phospholipases (PLAs) and glycerophosphoryldiester phosphodiesterases (GDPDs) to release free acetyl-CoAs and glycerol-3-phosphate (G3P) (Nakamura 2013), which in turn can be utilized for de novo synthesis of phosphorus-free lipids. Angkawijaya and Nakamura (2017) demonstrated that the enzymes PECP1 and PS2 were involved in dephosphorylating the PC head group to phosphate and choline, making phosphorus available for other cellular processes. However, breakdown of PC via these enzymes is not the only cellular phosphate source during P starvation, since a PECP1/PS2-deficient mutant that did not show accumulation of choline was not growth-affected. Further research is warranted to elucidate which

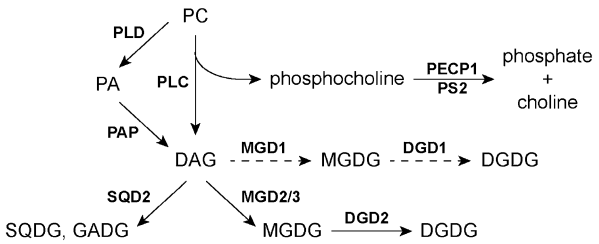


Fig. 5 A metabolic map of phospholipid to glycolipid and sulfolipid conversion during phosphorus limitation in seed plants. Dashed lines indicate predominant biosynthetic pathway under P-replete conditions. For abbreviations see Fig. 1 and text

mechanisms are involved in providing phosphorus to the cell via the breakdown of phospholipids.

Under P limitation in plants, DGDG contents increase in the chloroplast membranes and in extraplastidial membranes, including the tonoplasts and mitochondrial membranes. Like all thylakoid glycolipids, DGDG is exclusively synthesized within the chloroplast, therefore the observed lipid remodeling must involve extensive lipid trafficking of DGDG across membranes (Moellering and Benning 2011; Nakamura 2013). Synthesis of galactolipids under normal conditions is primarily performed via the MGDG synthase MGD1 localized to the inner chloroplast envelope and the DGDG synthase DGD1 localized to the outer envelope (Moellering and Benning 2011; Kalisch et al. 2016). Under phosphate deprivation, DAG produced during the NPC pathway in extraplastidial membranes is exclusively provided to the outer chloroplast envelope, where MGD2 and DGD2 are activated to synthesize DGDG (Nakamura 2013; Kalisch et al. 2016) (Fig. 5). DAG produced by the PLD/PAP pathway is still transferred to the inner envelope of chloroplasts and regularly galactosylated by MGD1 and DGD1, although some portion of DAG used by the outer envelope-localized galactolipid biosynthetic genes may also be derived from PLD/PAP-mediated DAG production (Nakamura 2013). The lipid transport systems that are involved in trafficking DGDG to extraplastidial membranes are still not very well characterized. Kelly et al. (2016) could show that the N-terminal sequence of DGD1 (NDGD1) is required for galactolipid transfer between the inner and outer chloroplast envelope membrane. Furthermore, it has not yet been elucidated how PG is converted to SQDG; however, differences in their respective acyl moieties point to their reassembly during lipid remodeling (Nakamura 2013). The enzyme SQD2 which is involved in SQDG biosynthesis is also responsible for GlcADG formation under phosphate deprivation (Okazaki et al. 2015).

TAG production in plants during nutrient depletion is mainly assumed to occur through the acyl-CoA dependent Kennedy pathway (see Sect. 5.2), alternatively an acyl editing cycle performed by PC:DAG cholinephosphotransferase may be employed that provides PC-modified fatty acids for de novo DAG/TAG synthesis (Bates and Browse 2012).

5.2 Algae

The current mechanistic understanding of membrane lipid turnover and TAG production in algae during nutrient depletion is still very limited and largely relies on genome predictions that have yet to be experimentally verified (Zienkiewicz et al. 2016; Mühlroth et al. 2017). To date, three major pathways of TAG synthesis via DAG have been described in microalgae (reviewed in Zienkiewicz et al. 2016). Two of them, (1) the Kennedy pathway and the (2) monoacylglycerol pathway are acyl-CoA dependent and an acyl-CoA-independent pathway is mediated via (3) phospholipid:diacylglycerol acyltransferase (PDAT) (Fig. 6). In the Kennedy pathway DAG is formed from glycerol-3-phosphate (G3P) by subsequent acylation and dephosphorylation reactions. The first acylation occurs via the enzyme glycerol-3-phosphate-acyltransferase (GPAT), which forms lysophosphatidic acid; the second acylation is performed by the enzyme lysophosphatidic acid acyltransferase (LPAAT) to form phosphatidic acid (PA). PA is then dephosphorylated by a phosphatase to form diacylglycerol (DAG). As opposed to the Kennedy pathway the monoacylglycerol pathway requires only two steps in the conversion from G3P to DAG. First, monoacylglycerol (MAG) is synthesized from G3P via GPAT4 and GPAT5 acyltransferases and then MAG is converted to DAG by a bifunctional monoacyl/diacylglycerol acyltransferase (M/DGAT) (Petrie et al. 2012). In both acyl-CoA-dependent pathways, TAG are then formed by acylation of DAG using acyl groups from the acyl-CoA pool via the activity of M/DGAT. In the third pathway of TAG synthesis, phospholipids can function as an acyl donor and the cleaved fatty acids can be directly incorporated into TAG via the acylation of DAG by the activity of PDAT, yielding lyso-phospholipids in the process. The PDAT-mediated pathway of TAG formation was first described in yeast and plants

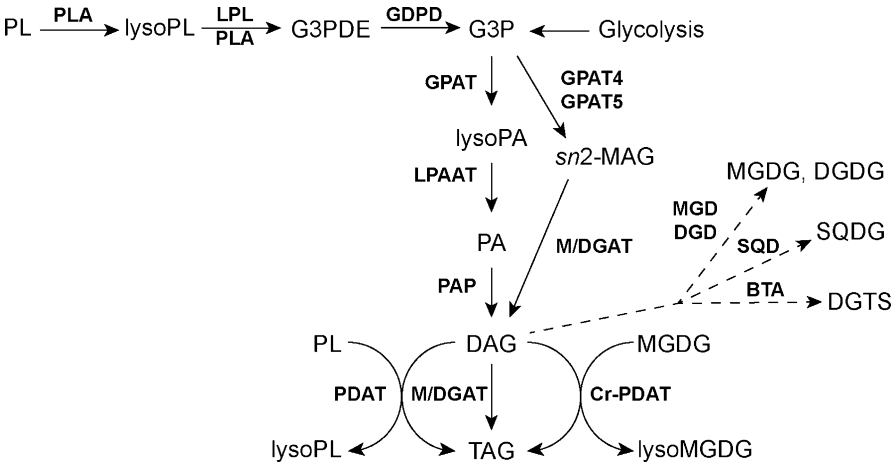


Fig. 6 Proposed pathways of phospholipid remodeling and triacylglycerol (TAG) accumulation during nutrient limitation in microalgae. Dashed line represents remodeling of phospholipid (PL)-derived diacylglycerol (DAG) to glycolipids or betaine lipids. For abbreviations see Fig. 1 and text

(Dahlqvist et al. 2000) and PDAT encoding genes have been identified in all microalgae studied so far, including green algae, diatoms, and heterokonts (Zienkiewicz et al. 2016). In the green algae *Chlamydomonas reinhardtii,* Cr-PDAT was first described as a homologous enzyme to PDAT of plants that acts as a galactolipid:DAG acyltransferase, transferring a fatty acyl group from MGDG to DAG to form TAG and lyso-MGDG (Yoon et al. 2012). Recently, it was suggested that besides phospholipids and galactolipids, also DGTS may be a candidate source lipid for TAG production (Popko et al. 2016). However, the genes involved in the conversion from DGTS to TAG have not yet been described and so far only a transfer of fatty acids from DGTS via the action of a phospholipase A_2 has been suggested (Popko et al. 2016).

Gene expression analysis in the heterokont *N. oceananica* under P limitation indicated that P mobilization through membrane lipid degradation was mediated through two different pathways under exponential and stationary growth (Mühlroth et al. 2017). During exponential growth, phospholipids were stepwise degraded to G3P via the LHA/GDPD pathway and patatin-like PLA activity (see also Sect. 5.1), and the substitute lipids DGTS and SQDG were synthesized in extraplastidial membranes and plastidial membranes, respectively. During stationary growth under P limitation, genes involved in phospholipid biosynthesis via the Kennedy pathway as well as TAG synthesis were upregulated and phospholipids were immediately recycled to TAG via PDAT activity.

5.3 Bacteria

In bacteria, phospholipid remodeling studies in the root-nodule forming Gram-negative soil bacteria *S. meliloti* revealed the activity of phospholipase C (PlcP) to be primarily involved in the conversion of zwitterionic phospholipids PE and PC to DGTS under P limitation (Zavaleta-Pastor et al. 2010). Hereby, PlcP initiates the degradation of PE and PC to DAG, which in turn can be utilized also for the synthesis of glycosidic substitute lipids, such as SQDG, GlcADG, or MGlcDG (Carini et al. 2015; Sebastian et al. 2016) (Fig. 7a). The liberated head groups phosphocholine and phosphoethanolamine can then be utilized as an intracellular phosphorus source (Zavaleta-Pastor et al. 2010). Expression of genes involved in the remodeling of phospholipids are induced by the two-component system PhoR/PhoB and include the genes for PlcP and for the synthesis of non-phosphorus lipids, e.g., *btaA* and *btaB* for the synthesis of DGTS from DAG (Zavaleta-Pastor et al. 2010). PlcP does not show homology to any of the phospholipases C or D involved in the degradation of phospholipids under P limitation in plants and also differs from extracellular bacterial phospholipase C (Zavaleta-Pastor et al. 2010). A study of diverse marine heterotrophic bacteria, including alphaproteobacteria and flavobacteria, that contain homologues to PlcP from *S. meliloti* showed expression of *plcP* under P starvation. The direct involvement in lipid remodeling was shown by construction of deletion mutants that were not able to synthesize DGTS (Sebastian et al. 2016). These authors have thus demonstrated that PlcP is

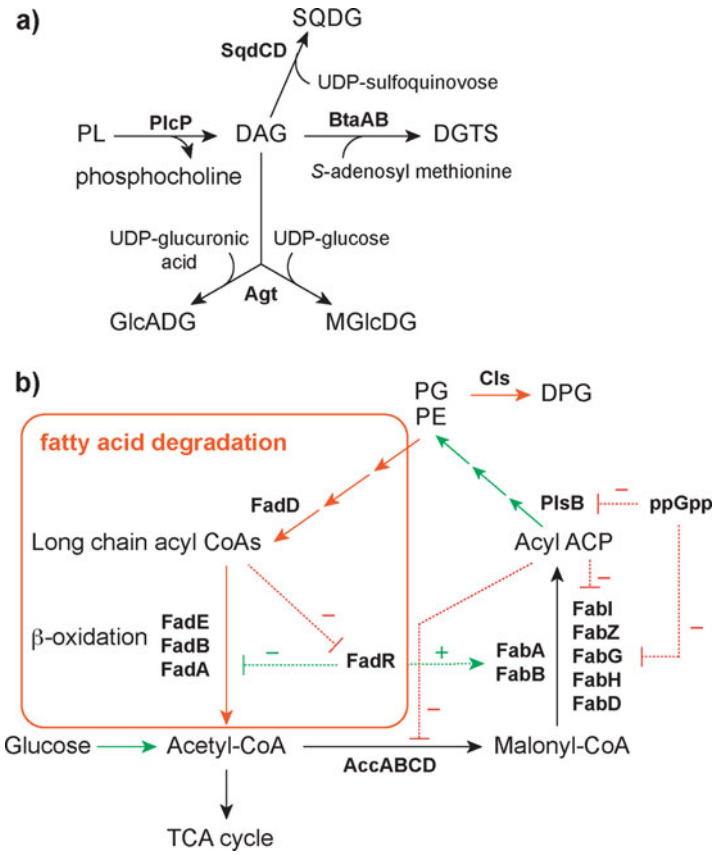


Fig. 7 (**a**) Proposed pathways of non-phosphorus containing lipid biosynthesis via phospholipids and phospholipase C (PlcP) in bacteria. (**b**) Simplified schematic of regulation of lipid metabolism in *E. coli* (after DiRusso and Nyström 1998; Fujita et al. 2007; Janßen and Steinbüchel 2014). Green lines represent pathways only active under carbon replete conditions; orange lines are pathways activated under carbon starvation. Dashed lines indicate positive and negative control (activation or repression) of gene expression or enzyme activity. For abbreviations see Fig. 1 and text

central to the lipid remodeling response to P deficiency in many different environmentally relevant bacteria. In addition, PlcP homologues were found to be ubiquitous in *Tara* Oceans metagenomes with phylogenetic associations to diverse bacterial phyla including alphaproteobacteria, gammaproteobacteria, and flavoproteobacteria (Sebastian et al. 2016). Homologous enzymes to glycosyltransferase Agt that were shown to be involved in MGlcDG and GlcADG synthesis in bacteria (Semeniuk et al. 2014; Sebastian et al. 2016) and *btaA* and *btaB* genes involved in the biosynthesis of betaine lipid were found in the vicinity of the *plcP*. It remains to be shown whether these environmental PlcP homologues show a similar substrate specificity towards zwitterionic phospholipids PE and PC as in *S. meliloti* or whether they might also be involved in the degradation of other phospholipids.

An important regular of fatty acid metabolism during bacterial starvation is the bifunctional transcription factor FadR that is both involved in regulation of genes involved in fatty acid biosynthesis *(fab*) and degradation (My et al. 2015). Figure 7b depicts important enzymes and regulatory components involved in lipid metabolism in *E. coli* under glucose replete and glucose starved conditions (reviewed in DiRusso and Nyström 1998 and Janßen and Steinbüchel 2014). Under normal growth conditions, *fad* genes that degrade fatty acids to acetyl-CoA are repressed by FadR. Upon carbon-source depletion, elevated levels of long chain acyl CoAs cause inhibition of FadR-dependent repression, which activates the *fad* genes involved in *ß*-oxidation generating acetyl-CoA. Carbon starvation also increases levels of the global transcriptional regulator cyclic-AMP receptor protein-cAMP complex, which additionally induce the activation of *fad* genes (DiRusso and Nyström 1998; Janßen and Steinbüchel 2014). An increase in levels of the signal molecule ppGpp (guanosine tetraphosphate) during C depletion decreases phospholipid biosynthesis by enzyme inhibition of PlsB (glycerol-3-phosphate acyltransferase), which catalyzes the first acyl transferase reaction during phospholipid biosynthesis (Fig. 7b); ppGpp also inhibits transcription of many *fab* genes (DiRusso and Nyström 1998; Janßen and Steinbüchel 2014). As a consequence of PlsB inhibition by ppGpp acyl-ACP levels increase, which results in feedback inhibition of enzymes involved in both the initiation (AccABCD, acetyl-CoA carboxylase and FabH, 3- ketoacyl-ACP synthase III) and elongation (FabI, enoyl-ACP reductase) of fatty acid biosynthesis (Heath and Rock 1996). It was first demonstrated in *S. meliloti* and *E. coli* that FadD (acyl-CoA synthase) is not only involved in transport and activation of exogenous fatty acids to acyl-CoA esters but also in the activation of endogenous free fatty acids that are released from membrane phospholipids during stationary phase for subsequent degradation by *ß*-oxidation (Pech-Canul et al. 2011). FadR-dependent derepression was also suggested to be involved in CL accumulation as *fadR* null mutants synthesize more CL and less PG in C starved bacteria; however, the role in *cls* transcription or cardiolipin synthase activity remains to be elucidated (Vanderwinkel et al. 1976).

While the FadR transcription factor has been mostly studied in *E. coli,* homologues are present in other Gram-negative and Gram-positive bacteria (Zhang and Rock 2016). Additional transcription factors FabR and FabT regulate fatty acid and phospholipid synthesis in Gram-positive *B. subtilis* and *Streptococcus pneumoniae,* respectively (Fujita et al. 2007); however, fatty acid degradation pathways have not been well studied. This highlights the complex regulation of fatty acid metabolism in bacteria and that many biochemical pathways remain to be explored.

6 Environmental Relevance and Research Needs

Phototrophs are evolutionally adapted to nutrient poor environments, which is for instance manifested in the evolutionary preserved biosynthesis of glycolipids in cyanobacteria to primary plasmids in plants and algae (Kalisch et al. 2016). This review demonstrated that also many chemotrophic organisms have similar

capabilities in synthesizing non-P containing "phototroph-type" glyco- and aminolipids that may enhance their competition when facing P limitation in the environment. However, it is still unclear why chemotrophs switch back to synthesizing phospholipids under normal growth conditions, while phototrophs do not. Using phospholipids as an internal P storage may be one reason; in addition, non-P lipids may not fully substitute for all functional roles of phospholipids in the membrane as is indicated by the multiple ultrastructural changes and growth impairment during P limitation. Our understanding of structural and functional roles of membrane lipids in bacteria are still very limited and their detailed examination is only at the beginning (López-Lara and Geiger 2016). Since even closely related bacterial species show vast differences in their genetic potential for, e.g., betaine lipid biosynthesis, a challenge remains to understand the complex evolution history when, how, and why bacteria acquired the ability to synthesize non-P containing lipids: was it mainly through lateral gene transfer or are these more ancient biosynthetic pathways that have been lost over time? In addition, insights how bacteria and other microgranisms manage growth and survival under carbon and energy starved conditions in the environment can be gained by further elucidating the regulatory networks involved during cellular self-digestion. Finally, a better understanding of lipid remodeling in oleaginous organisms and identification of regulatory cascades controlling TAG accumulation under stress conditions would also greatly benefit the continuous efforts to bioengineer microalgae for biofuel production (Du and Benning 2016).

In order to appraise the evolutionary importance of membrane homeostasis during natural selection of populations in ecosystems, we need to gain a better understanding of environmental distributions of complex lipids. While oxygenated and photic systems are typically dominated by glycolipids as would be expected by their abundant presence in photosynthetic organisms (Van Mooy et al. 2006, 2009), patterns of phospholipid, glycolipid, or aminolipid abundance in anoxic environments are often hard to reconcile with what is known from cultures (Schubotz et al. 2009; Evans et al. 2017). If nothing less, this calls for revised culturing approaches of environmentally relevant microorganisms by growing them under conditions that are representative for the environments where they have been isolated from.

Acknowledgments This work was supported in part by the Central Research Development Fund of the University of Bremen, Germany.

References

Abida H, Dolch L-J, Meï C, Villanova V, Conte M, Block MA, Finazzi G, Bastien O, Tirichine L, Bowler C, Rébeillé F, Petroutsos D, Jouhet J, Maréchal E (2014) Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in *Phaeodactylum tricornutum*. Plant Physiol 167:118–136

Aguilar-López JL, Funes S (2018) Autophagy in stationary phase of growth. In: Geiger O (ed) Biogenesis of fatty acids, lipids and membranes, Handbook of hydrocarbon and lipid microbiology. Springer Nature Switzerland AG, pp 1–18

Angkawijaya AE, Nakamura Y (2017) Arabidopsis PECP1 and PS2 are phosphate starvation-inducible phosphocholine phosphatases. Biochem Biophys Res Comm 494:397–401

Bates PD, Browse J (2012) The significance of different diacylgycerol synthesis pathways on plant oil composition and bioengineering. Front Plant Sci 3:147

Benning C, Beatty JT, Prince RC, Somerville CR (1993) The sulfolipid sulfoquinovosyldiacylglycerol is not required for photosynthetic electron transport in *Rhodobacter sphaeroides* but enhances growth under phosphate limitation. Proc Natl Acad Sci USA 90:1561–1565

Benning C, Huang ZH, Gage DA (1995) Accumulation of a novel glycolipid and a betaine lipid in cells of *Rhodobacter sphaeroides* grown under phosphate limitation. Arch Biochem Biophys 317:103–111

Bosak T, Schubotz F, de Santiago-Torio A, Kuehl JV, Carlson HK, Watson N, Daye M, Summons RE, Arkin AP, Deutschbauer AM (2016) System-wide adaptations of *Desulfovibrio alaskensis* G20 to phosphate-limited conditions. PLoS One 11:e0168719

Boudière L, Michaud M, Petroutsos D, Rébeillé F, Falconet D, Bastien O, Roy S, Finazzi G, Rolland N, Jouhet J, Block MA, Maréchal E (2014) Glycerolipids in photosynthesis: composition, synthesis and trafficking. Biochim Biophys Acta 1837:470–480

Breuer G, Lamers PP, Martens DE, Draaisma RB, Wijffels RH (2012) The impact of nitrogen starvation on the dynmics of triacylglycerol accumulation in nine microalgal strains. Bioresour Technol 124:217–226

Cañavate JP, Armada I, Hachero-Cruzado I (2016) Interspecific variability in phosphorus-induced lipid remodelling among marine eukaryotic phytoplankton. New Phytol 213:700–713

Carini P, Van Mooy BAS, Thrash JC, White A, Zhao Y, Campbell EO, Fredricks HF, Giovannoni SJ (2015) SAR11 lipid renovation in response to phosphate starvation. Proc Natl Acad Sci USA 112:7767–7772

Chiou T-J, Lin S-I (2011) Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol 62:185–206

Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne S (2000) Phospholipid: diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci USA 97:6487–6492

Degraeve-Guilbault C, Bréhélin C, Haslam R, Sayanova O, Marie-Luce G, Jouhet J, Corellou F (2017) Glycerolipid characterization and nutrient deprivation-associated changes in the green picoalga *Ostreococcus tauri*. Plant Physiol 173:2060–2080

Dembitsky V (1996) Betaine ether-linked glycerolipids: chemistry and biology. Prog Lipid Res 35:1–51

DiRusso CC, Nyström T (1998) The fats of Escherichia coli during infancy and old age: regulation by global regulators, alarmones and lipid intermediates. Mol Microbiol 27:1–8

Dowhan W, Bogdanov M, Mileykovskaya E (2016) Functional roles of lipids in membranes. In: Ridgway ND, McLeod RS (eds) Biochemistry of lipids, lipoproteins and membranes. Elsevier, Boston, pp 1–40

Du Z-Y, Benning C (2016) Triacylglycerol accumulation in photosynthetic cells in plants and algae. In: Nakamura Y, Li-Biesson Y (eds) Lipids in plant and algae development. Springer, Cham, pp 179–205

Elling FJ, Könneke M, Nicol GW, Stieglmeier M, Bayer B, Spieck E, La Torre De JR, Becker KW, Thomm M, Prosser JI, Herndl GJ, Schleper C, Hinrichs K-U (2017) Chemotaxonomic characterisation of the thaumarchaeal lipidome. Environ Microbiol 19:2681–2700

Essigmann B, Güler S, Narang RA, Linke D, Benning C (1998) Phosphate availability affects the thylakoid lipid composition and the expression of SQD1, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 95:1950–1955

Evans TW, Wörmer L, Lever MA, Lipp JS, Lagostina L, Lin Y-S, Jørgensen BB, Hinrichs K-U (2017) Size and composition of subseafloor microbial community in the Benguela upwelling area examined from intact membrane lipid and DNA analysis. Org Geochem 111:86–100

Fujita Y, Matsuoka H, Hirooka K (2007) Regulation of fatty acid metabolism in bacteria. Mol Microbiol 66:829–839

Gaude N, Bréhélin C, Tischendorf G, Kessler F, Dörmann P (2007) Nitrogen deficiency in Arabidopsis affects galactolipid composition and gene expression and results in accumulation of fatty acid phytyl esters. Plant J 49:729–739

Geiger O, Röhrs V, Weissenmayer B, Finan TM, Thomas-Oates JE (1999) The regulator gene phoB mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-N,N,N-trimethylhomoserine in *Rhizobium (Sinorhizobium) meliloti*. Mol Microbiol 32:63–73

Geiger O, González-Silva N, López-Lara IM, Sohlenkamp C (2010) Amino acid-containing membrane lipids in bacteria. Prog Lipid Res 49:46–60

Geisseler D, Horwath WR, Joergensen RG, Ludwig B (2010) Pathways of nitrogen utilization – a review. Soil Biol Biochem 42:2058–2067

Geske T, vom Dorp K, Dormann P, Holzl G (2012) Accumulation of glycolipids and other nonphosphorous lipids in *Agrobacterium tumefaciens* grown under phosphate deprivation. Glycobiology 23:69–80

Grogan DW, Cronan JE (1997) Cyclopropane ring formation in membrane lipids of bacteria. Microbiol Mol Biol Rev 61:429–441

Grzymski JJ, Dussaq AM (2011) The significance of nitrogen cost minimization in proteomes of marine microorganisms. ISME J 6:71–80

Güler S, Seeliger A, Härtel H, Renger G, Benning C (1996) A null mutant of *Synechococcus* sp. PCC7942 deficient in the sulfolipid sulfoquinovosyl diacylglycerol. J Biol Chem 271:7501–7507

Harayama T, Riezman H (2018) Understanding the diversity of membrane lipid composition. Nat Rev Molec Cell Microbiol 19:281–296

Härtel H, Dormann P, Benning C (2000) DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. Proc Natl Acad Sci USA 97: 10649–10654

Hassan M, Blanc PJ, Granger L-M, Pareilleux A (1996) Influence of nitrogen and iron limitations on lipid production by *Cryptococcus curvatus* grown in batch and fed-batch culture. Process Biochem 31:355–361

Hayward AP, Dinesh-Kumar SP (2011) What can plant autophagy do for an innate immune response? Annu Rev Phytopathol 49:557–576

Heath RJ, Rock CO (1996) Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in Escherichia coli. J Biol Chem 271:1833–1836

Hiraoka S, Matsuzaki H, Shibuya I (1993) Active increase in cardiolipin synthesis in the stationary phase growth phase and its physiological significance in *Escherichia coli*. FEBS Lett 336:221–224

Hölzl G, Zähringer U, Warnecke D, Heinz E (2005) Glycoengineering of cyanobacterial thylakoid membranes for future studies on the role of glycolipids in photosynthesis. Plant Cell Physiol 46:1766–1778

Hölzl G, Dörmann P (2007) Structure and function of glycoglycerolipids in plants and bacteria. Prog Lipid Res 46:225–243

Hood MA, Guckert JB, White DC, Deck F (1986) Effects of nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein levels in *Vibrio cholerae*. Appl Environ Microbiol 52:788–793

Houser JR, Barnhart C, Boutz DR, Carroll SM, Dasgupta A, Michener JK, Needham BD, Papoulas O, Sridhara V, Sydykova DK, Marx CJ, Trent MS, Barrick JE, Marcotte EM, Wilke CO (2015) Controlled measurement and comparative analysis of cellular components in *E. coli* reveals broad regulatory changes in response to glucose starvation. PLoS Comput Biol 11: e1004400

Janßen HJ, Steinbüchel A (2014) Fatty acid synthesis in *Escherichia coli* and its application towards the production of fatty acid based biofuels. Biotechnol Biofuels 7:7

Jørgensen BB, Marshall IPG (2016) Slow microbial life in the seabed. Ann Rev Mar Sci 8:311–332

Journet EP, Bligny R, Douce R (1986) Biochemical changes during sucrose deprivation in higher plant cells. J Biol Chem 261:3193–3199

Kalisch B, Dörmann P, Hölzl G (2016) DGDG and glycolipids in plants and algae. In: Nakamura Y, Li-Biesson Y (eds) Lipids in plant and algae development. Springer, Cham, pp 51–83

Karl DM (2014) Microbially mediated transformations of phosphorus in the sea: new views of an old cycle. Ann Rev Mar Sci 6:279–337

Kelly AA, Kalisch B, Hölzl G, Schulze S, Thiele J, Melzer M, Roston RL, Benning C, Dörmann P (2016) Synthesis and transfer of galactolipids in the chloroplast envelope membranes of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 113:10714–10719

Kjelleberg S, Hermansson M, Mårdén P, Jones GW (1987) The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. Annu Rev Microbiol 41:25–49

Koga Y, Nakano M (2008) A dendrogram of archaea based on lipid component parts composition and its relationship to rRNA phylogeny. Syst Appl Microbiol 31:169–182

Lee SH, Chae HS, Lee TK, Kim SH, Shin SH, Cho BH, Cho SH, Kang BG, Lee WS (1998) Ethylene-mediated phospholipid catabolic pathway in glucose-starved carrot suspension cells. Plant Physiol 116:223–229

López-Lara IM, Geiger O (2016) Bacterial lipid diversity. Biochim Biophys Acta 1862:1287–1299

López-Lara IM, Gao J-L, Soto MJ, Solares-Pérez A, Weissenmayer B, Sohlenkamp C, Verroios GP, Thomas-Oates J, Geiger O (2005) Phosphorus-free membrane lipids of *Sinorhizobium meliloti* are not required for the symbiosis with Alfalfa but contribute to increased cell yields under phosphorus-limiting conditions of growth. Mol Plant Microbe Interact 18:973–982

Martin P, Van Mooy BA, Heithoff A, Dyhrman ST (2010) Phosphorus supply drives rapid turnover of membrane phospholipids in the diatom *Thalassiosira pseudonana*. ISME J 5:1057–1060

Meador TB, Gagen EJ, Loscar ME, Goldhammer T, Yoshinaga MY, Wendt J, Thomm M, Hinrichs K-U (2014) Thermococcus kodakarensis modulates its polar membrane lipids and elemental composition according to growth stage and phosphate availability. Front Microbiol 5:10

Minnikin DE, Abdolrahimzadeh H, Baddiley J (1971) The interrelation of polar lipids in bacterial membranes. Biochim Biophys Acta 249:651–655

Moellering ER, Benning C (2011) Galactoglycerolipid metabolism under stress: a time for remodeling. Trends Plant Sci 16:98–107

Moore CM, Mills MM, Arrigo KR, Berman-Frank I, Bopp L, Boyd PW, Galbraith ED, Geider RJ, Guieu C, Jaccard SL, Jickells TD, La Roche J, Lenton TM, Mahowald NM, Maranon E, Marinov I, Moore JK, Nakatsuka T, Oschlies A, Saito MA, Thingstad TF, Tsuda A, Ulloa O (2013) Processes and patterns of oceanic nutrient limitation. Nat Geosci 6:701–710

Mühlroth A, Winge P, Assimi El A, Jouhet J, Maréchal E, Hohmann-Marriott MF, Vadstein O, Bones AM (2017) Mechanisms of phosphorus acquisition and lipid class remodeling under P limitation in a marine microalga. Plant Physiol 175:1543–1559

My L, Ghandour Achkar N, Viala JP, Bouveret E (2015) Reassessment of the genetic regulation of fatty acid synthesis in *Escherichia coli:* global positive control by the functional dual regulator FadR. J Bacteriol 197:1862–1872

Nakamura Y (2013) Phosphate starvation and membrane lipid remodeling in seed plants. Prog Lipid Res 52:43–50

Nyström T (2004) Stationary-phase physiology. Annu Rev Microbiol 58:161–181

Okazaki Y, Nishizawa T, Takano K, Ohnishi M, Mimura T, Saito K (2015) Induced accumulation of glucuronosyldiacylglycerol in tomato and soybean under phosphorus deprivation. Physiol Plant 155:33–42

Parsons JB, Rock CO (2013) Bacterial lipids: metabolism and membrane homeostasis. Prog Lipid Res 52:249–276

Pech-Canul À, Nogales J, Miranda-Molina A, Àlvarez L, Geiger O, Soto MJ, López-Lara IM (2011) FadD is required for utilization of endogenous fatty acids released from membrane lipids. J Bacteriol 193:6295–6304

Petrie JR, Vanhercke T, Shrestha P, Tahchy El A, White A, Zhou X-R, Liu Q, Mansour MP, Nichols PD, Singh SP (2012) Recruiting a new substrate for triacylglycerol synthesis in plants: the monoacylglycerol acyltransferase pathway. PLoS One 7:e35214

Poirier Y, Thoma S, Somerville C, Schiefelbein J (1991) Mutant of *Arabidopsis* deficient in xylem loading of phosphate. Plant Physiol 97:1087–1093

Popko J, Herrfurth C, Feussner K, Ischebeck T, Iven T, Haslam R, Hamilton M, Sayanova O, Napier J, Khozin-Goldberg I, Feussner I (2016) Metabolome analysis reveals betaine lipids as major source for triglyceride formation, and the accumulation of sedoheptulose during nitrogen-starvation of *Phaeodactylum tricornutum*. PLoS One 11:e0164673

Riekhof WR, Naik S, Bertrand H, Benning C, Voelker DR (2014) Phosphate starvation in fungi induces the replacement of phosphatidylcholine with the phosphorus-free betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine. Eukar Cell 13:749–757

Rühl M, Le Coq D, Aymerich S, Sauer U (2012) ^{13}C-flux analysis reveals NADPH-balancing transhydrogenation cycles in stationary phase of nitrogen-starving *Bacillus subtilis*. J Biol Chem 287:27959–27970

Ruttenberg KC (2014) The global phosphorus cycle. In: Karl DM, Schlesinger WH (eds) Treatise on Geochem, 2nd edn, Vol. 10, Biogeochem. Elsevier, Oxford, UK, pp 499–558

Sato N, Hagio M, Wada H, Tsuzuki M (2000) Requirement of phosphatidylglycerol for photosynthetic function in thylakoid membranes. Proc Natl Acad Sci USA 97:10655–10660

Schubotz F, Wakeham SG, Lipp JS, Fredricks HF, Hinrichs K-U (2009) Detection of microbial biomass by intact polar membrane lipid analysis in the water column and surface sediments of the Black Sea. Environ Microbiol 11:2720–2734

Sebastian M, Smith AF, González JM, Fredricks HF, Van Mooy B, Koblížek M, Brandsma J, Koster G, Mestre M, Mostajir B, Pitta P, Postle AD, Sánchez P, Gasol JM, Scanlan DJ, Chen Y (2016) Lipid remodelling is a widespread strategy in marine heterotrophic bacteria upon phosphorus deficiency. ISME J 10:968–978

Semeniuk A, Sohlenkamp C, Duda K, Holzl G (2014) A bifunctional glycosyltransferase from Agrobacterium tumefaciens synthesizes monoglucosyl and glucuronosyl diacylglycerol under phosphate deprivation. J Biol Chem 14:10104–10114

Senik SV, Maloshenok LG, Kotlova ER, Shavarda AL, Moiseenko KV, Bruskin SA, Koroleva OV, Psurtseva NV (2015) Diacylglyceryltrimethylhomoserine content and gene expression changes triggered by phosphate deprivation in the mycelium of the basidomycete *Flammulina velutipes*. Phytochemistry 117:34–42

Sharma KK, Schuhmann H, Schenk PM (2012) High lipid induction in microalgae for biodiesel production. Energies 5:1532–1553

Shemi A, Schatz D, Fredricks HF, Van Mooy BAS, Porat Z, Vardi A (2016) Phosphorus starvation induces membrane remodeling and recycling in *Emiliania huxleyi*. New Phytol 211:886–898

Siebers M, Dörmann P, Hölzl G (2015) Membrane remodelling in phosphorus-deficient plants. In: Plaxton WC, Lambers H (eds) Annu plant rev vol 48: phosphorus metabolism in plants, 1st edn. Wiley, Chichester, pp 237–263

Slávikova S, Shy G, Yao Y, Glozman R, Levanony H, Pietrokosvski S, Elazar Z, Galili G (2005) The autophagy-associated *Atg8* gene family operates both under favourable growth coniditions and under starvation stresses in Arabidopsis plants. J Exp Bot 56:2839–2849

Tavormina PL, Kellermann MY, Antony CP, Tocheva EI, Dalleska NF, Jensen AJ, Valentine DL, Hinrichs K-U, Jensen GJ, Dubilier N, Orphan VJ (2016) Starvation and recovery in the deep-sea methanotroph *Methyloprofundus sedimenti*. Mol Microbiol 103:242–252

Valentine DL (2007) Adaptations to energy stress dictate the ecology and evolution of the archaea. Nat Rev Microbiol 5:316–323

Van Mooy BAS, Rocap G, Fredricks HF, Evans CT, Devol AH (2006) Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. Proc Natl Acad Sci USA 103:8607–8612

Van Mooy BAS, Fredricks HF, Pedler BE, Dyhrman ST, Karl DM, Koblížek M, Lomas MW, Mincer TJ, Moore LR, Moutin T, Rappé MS, Webb EA (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 458:69–72

Vanderwinkel E, Dee Vliegher M, Fontaine M, Charles D, Denamur F, Vandervoorde D, De Kegel D (1976) Septation deficiency and phospholipid pertubation in *Escherichia coli* genetically constitutive for the beta oxidation pathway. J Bacteriol 127:1389–1399

Xie Z, Klionsky DJ (2007) Autophagosome formation: core machinery and adaptations. Nat Cell Biol 9:1102–1109

Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63:153–182

Yao M, Elling FJ, Jones C, Nomosatryo S, Long CP, Crowe SA, Antoniewicz MR, Hinrichs K-U, Maresca JA (2015) Heterotrophic bacteria from an extremely phosphate-poor lake have conditionally reduced phosphorus demand and utilize diverse sources of phosphorus. Environ Microbiol 18:656–667

Yoon K, Han D, Li Y, Sommerfeld M, Hu Q (2012) Phospholipid: diacylglycerol acyltransferase is a multifunctional enzyme involved in membrane lipid turnover and degradation while synthesizing triacylglycerol in the unicellular green microalga *Chlamydomonas reinhardtii*. Plant Cell 24:3708–3724

Yu SM (1999) Cellular and genetic responses of plants to sugar starvation. Plant Physiol 121:687–693

Zavaleta-Pastor M, Sohlenkamp C, Gao JL, Guan Z, Zaheer R, Finan TM, Raetz CRH, López-Lara IM, Geiger O (2010) *Sinorhizobium meliloti* phospholipase C required for lipid remodeling during phosphorus limitation. Proc Natl Acad Sci USA 107:302–307

Zhang Y-M, Rock CO (2016) Fatty acid and phospholipid biosynthesis in prokaryotes. In: Ridgway ND, McLeod RS (eds) Biochemistry of lipids, lipoproteins and membranes. Elsevier, Amsterdam, pp 73–112

Zienkiewicz K, Du Z-Y, Ma W, Vollheyde K, Benning C (2016) Stress-induced neutreal lipid biosynthesis in microalga – molecular cellular and phyisological insights. Biochim Biophys Acta 1861:1269–1281

Autophagy in Stationary Phase of Growth

46

José L. Aguilar-López and Soledad Funes

Contents

1 Introduction 850
2 General View on Autophagy 853
3 Autophagy During Regulation of Lifespan 856
4 Lipids in the Control of Autophagy 857
5 Formation of Lipid Droplets for Lipid Homeostasis 859
6 Autophagy During the Control or Carbohydrate Reserves 860
7 Autophagy-Like Processes in Bacteria 861
8 Research Needs 862
References 863

Abstract

The growth of microorganisms can be modeled depending on the nutrient availability of the surrounding medium in different stages. Stationary phase is characterized by a general depletion of nutrients and an excess of potentially harmful molecules accumulated during the exponential phase of growth. This situation causes a characteristic stress response where lipid metabolism is essential. Autophagy is a eukaryotic self-degradative process started by various cues when cells enter the stationary phase of growth, and is characterized by degradation of cytosolic contents either damaged or simply used for recycling of biological building blocks. Lipid metabolism is essential for this process since the molecules which are going to be degraded are engulfed by double membrane vesicles that later fuse with the vacuole or the lysosome. In bacteria, there are also several

J. L. Aguilar-López · S. Funes (✉)
Departamento de Genética Molecular, División de Investigación Básica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico, Ciudad de Mexico, Mexico
e-mail: joseluis0988@gmail.com; sfunes@ifc.unam.mx

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8_60

catabolic processes which specifically degrade lipids or proteins and change the metabolic state of the cells in order to resist the stress produced during stationary phase. In this chapter, we discuss the general lipid metabolism associated with entrance of microorganisms to stationary phase that modulate cell fate.

1 Introduction

The growth of microorganisms such as bacteria, microalgae, or yeasts can be modeled with four different phases (Mitchison 2003; Nyström 2004; Navarro Llorens et al. 2010): (1) A lag phase, which indicates an adjustment period where microorganisms adapt to a new environment; (2) an exponential phase or log phase, where cells tend to divide actively at a constant rate and thereby the population doubles with consecutive time periods; (3) a **stationary phase**, where the growth rate and the death rate are equal, resulting in a horizontal plateau in the growth curve; and (4) a decline or death phase (Fig. 1). Each of these phases, as well as the maximum density reached by the culture, depends largely on the components of the growth media and other factors such as temperature or pH. In addition, cells of each of these phases can be characterized by particular metabolic states.

When microorganisms are maintained in media with unrestricted availability of nutrients, like the so-called standard laboratory conditions, cells keep the exponential phase of growth until they exhaust growth-supporting substrates. At this point, the death of cells that have lost viability provides nutrients that support division of the remaining viable cells (Navarro Llorens et al. 2010). This stationary phase can be considered an operational definition where the culture displays no net increase in cell number (Huisman et al. 1996; Nyström 2004). The entrance to stationary phase is associated to metabolic responses that essentially allow cells to contend with nutrient starvation. In bacteria as well as in yeast, it has been observed that following the entrance to stationary phase, cells stop dividing but still preserve their viability. Therefore, if they are again placed on a nutrient-rich medium the division cycle can resume.

Entrance of *Escherichia coli* cells to stationary phase is accompanied by several responses. Starved bacteria become smaller and more spherical or ovoid compared to cells from log phase, exhibit a condensed chromosome, and dimerized, hence translationally inactive, ribosomes (Książek 2010). One of the first responses activated upon entrance to stationary phase is also the expression of RpoS (σ^S), a sigma subunit of RNA polymerase that replaces the vegetative sigma factor (RpoD) under many stress conditions and which works as a master regulator for the expression of several stress-response genes that are also involved in the survival throughout stationary phase (Hengge-Aronis 2002). In addition, changes on the lipid composition of the membranes have been reported which affect their fluidity: In Gram negative bacteria like *E. coli* or *Salmonella typhimurium*, one of the phenotypic changes during stationary phase occur within the inner membrane. Under rich growing conditions, the membranes in these organisms contain monounsaturated fatty acids, predominantly palmitoleic acid (16:1) and *cis*-vaccenic acid (18:1), with

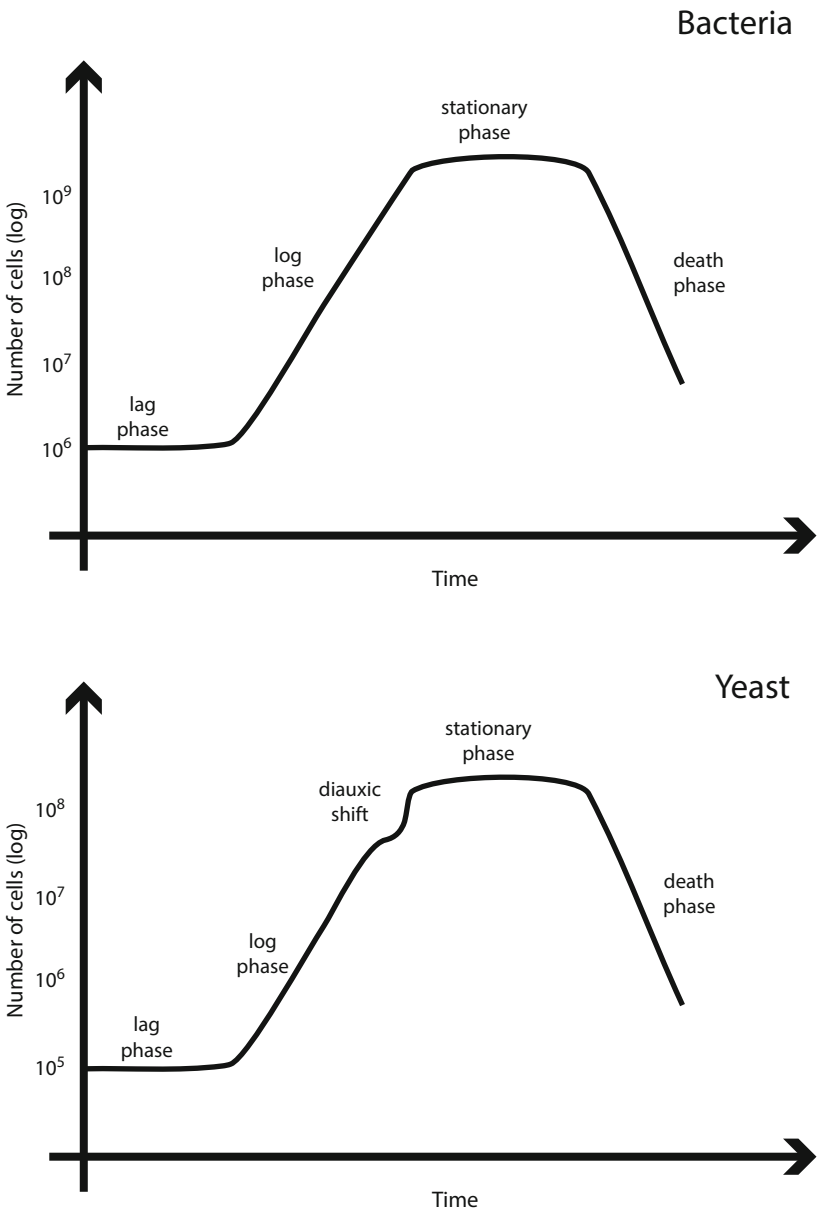


Fig. 1 Growth of microorganisms can be described by four different phases when grown in rich-nutrient environments. After an adaptation period (lag phase), the number of cells increases exponentially and their growth can be modeled exponentially (log phase). When nutrients are exhausted, the growth rate and the death rate are equal, resulting in a plateau in the growth curve (stationary phase). Finally, the cell population declines rapidly (death phase). The upper panel indicates an example of a growth curve of bacteria and the lower of yeast. In the latter it is possible to observe the diauxic shift produced at the end of the log phase when the metabolism changes from glycolytic to respiratory

a relative abundance of almost 50%; upon entrance to stationary phase their relative amount decreases to less than 5%. This reduction of monounsaturated fatty acids is accompanied by a corresponding increase in the levels of cyclopropane fatty acid (CFA) derivatives, from 10% during exponential phase to nearly 50%. The synthesis of cyclopropane fatty acids is a modification of the unsaturated fatty acids present into the already membrane-localized phospholipid molecules catalyzed by the CFA-synthase. The increase on cyclopropane fatty acids during stationary phase relates to an increased activity of CFA-synthase whose transcription is regulated by RpoS (σ^S). In addition, the ratio of phosphatidylglycerol to phosphatidylethanolamine increases, cardiolipin levels also increase, and phosphatidylserine amounts decrease (Huisman et al. 1996; Grogan and Cronan 1997; Wang and Cronan 1994).

In the yeast *Saccharomyces cerevisiae,* the entrance into stationary phase is usually a response to a depletion of a carbon source in the medium. In this phase, cells become quiescent and are arrested into the G_0 phase of the cell cycle. Similar responses can be observed also if the cells are starved for nitrogen or phosphate; however, the molecular mechanisms that regulate the responses depending on the type of nutrient lacking in the medium are not identical (Galdieri et al. 2010). Yeast cells of the stationary phase exhibit thick cell walls and contain more abundant mitochondria, their genetic material is organized in distinct condensed chromosomes, their overall transcription rate is three to five times lower than cells growing in exponential phase, and translation is lowered to about 0.3% (Galdieri et al. 2010). Due to the metabolic responses associated with the entrance to stationary phase, it has also been considered to study **chronological lifespan (CLS)** by assessing the length of time a cell remains viable following cell cycle arrest; where viable means that a cell can re-enter the cell cycle in response to addition of essential nutrients (Arlia-Ciommo et al. 2014). The signal transduction pathways that are responsible for orchestrating the transition from exponential growth to stationary phase also affect **aging** of yeast cells.

In the particular case of yeast cultures growing on fermentable carbon sources, cells go through an additional phase between the logarithmic and the stationary phase. When glucose becomes limiting, a **diauxic shift** occurs and the culture switches to respiratory growth. When the ethanol becomes also scarce, the cells stop dividing and the yeasts become resistant to many stresses, including heat and oxidative stress, and are able to survive in this state for several months. The cells show also lowered metabolic rates in this stage (Longo et al. 1999). In chronological **lifespan** studies, lifespan is measured by the ability of these nondividing cells to maintain viability over time (Tissenbaum and Guarente 2002).

Exit from the exponential phase and the transition to the diauxic shift and later to stationary phase is regulated by proteinase kinase A (PKA), **TOR1**, Snf1, and Rim15 signaling pathways. Each of these pathways sense changes in availability of nutrients: from an environment where glucose is abundant to a situation where cells utilize ethanol and finally to a phase where no carbon source is available. PKA and TOR1 pathways are negative regulators of transition into the diauxic shift, and Snf1 and Rim15 are positive regulators. PKA occupies a crucial position in response of cells to glucose and couples cell cycle progression and growth. TOR1 responds to

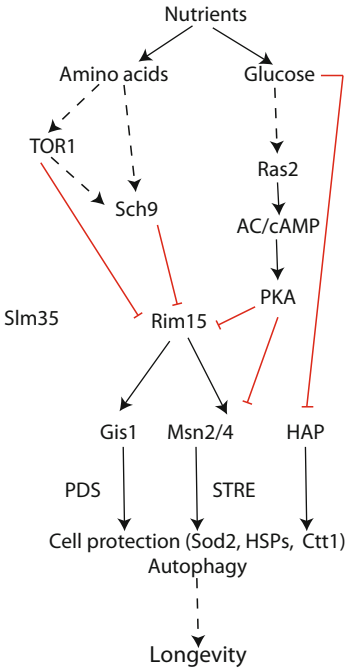


Fig. 2 Signaling pathways regulate lifespan in response to the levels of nutrients. Different signaling pathways that respond to changing levels of nutrients such as TOR1, Ras2 and Sch9, converge in the protein kinase Rim15. Transcription factors responding to stress signals such as Msn2, Msn4 and Gis1, activate stress response genes enhancing cell protection and eventually leading to cell longevity. Continuous lines indicate direct interactions between components; dashed lines indicate that the denoted interaction can be modulated by unidentified intermediaries; black lines indicate an activation in the presence of nutrients whereas the red lines indicate inhibitory interactions

growth conditions and availability of nutrients, primarily a nitrogen source (Fig. 2) (Galdieri et al. 2010).

2 General View on Autophagy

Autophagy is a self-degradative process conserved amongst eukaryotic organisms that involves targeting of cytosolic material to the endo-lysosomal system for degradation (Fig. 3) (Lippai and Szatmári 2017). This process plays an important role during cell maintenance, removing misfolded or aggregated proteins, as well as eliminating damaged organelles such as mitochondria, endoplasmic reticulum, and peroxisomes. In addition, autophagy is needed to maintain the balance between energy sources during critical growth stages and stress caused by nutritional depletion, such as the situation reached at the stationary phase where the nutrients originally present have been already consumed (Glick et al. 2010).

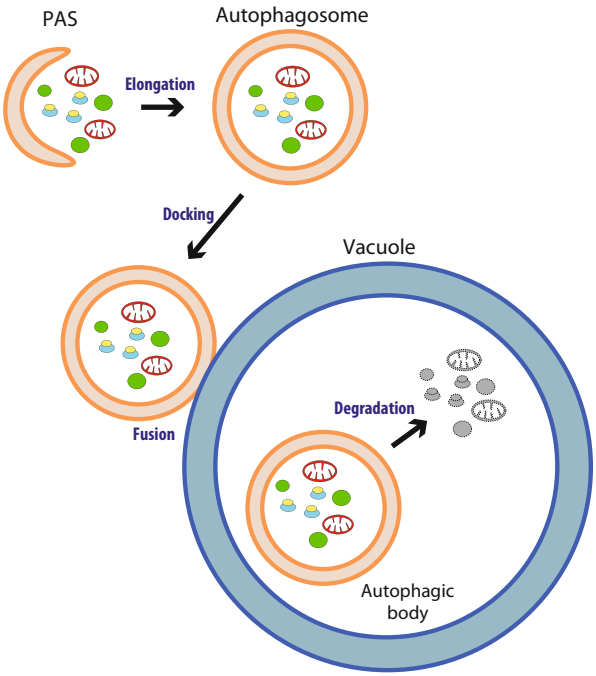


Fig. 3 Macroautophagy in yeast is a process that can degrade cytosolic contents for recycling biological blocks. During macroautophagy, portions of the cytoplasm and organelles are sequestered and engulfed by portions of the membrane that elongate around a phagophore assembly site or preautophagosomal structure (*PAS*). When the vesicles are sealed they are known as autophagosomes and directed towards the vacuole where they dock and their membranes fuse. The internalized autophagosome inside the vacuole is known as autophagic body. The membrane and its contents are degraded by the action of hydrolases within the vacuole

Three major autophagy pathways have been identified: (1) macroautophagy; (2) microautophagy; and (3) autophagy mediated by chaperones, which has been identified only in mammals. Macroautophagy is characterized by the formation of a double membrane vesicle forming the phagophore and the autophagosome. In contrast, during microautophagy the targets are directly sequestered by invagination of the vacuole membrane. Both macroautophagy and microautophagy include selective and nonselective processes. The study of the molecular machineries regulating autophagy has allowed the identification of more than 30 autophagy-related genes or Atg's in both yeast and higher eukaryotes (Klionsky and Codogno 2013).

One of the key events during the process of autophagy is the formation of the autophagosome, the membranous structure that engulfs intracellular material and eventually fuses to the vacuole to degrade its contents. Most of the Atg-proteins identified so far are involved in the autophagosome formation (Mizushima et al. 2011). Each step is under the control of specific autophagy complexes whose activity is directly or indirectly regulated by stress signaling pathways (Fig. 4) (Antonioli et al. 2017).

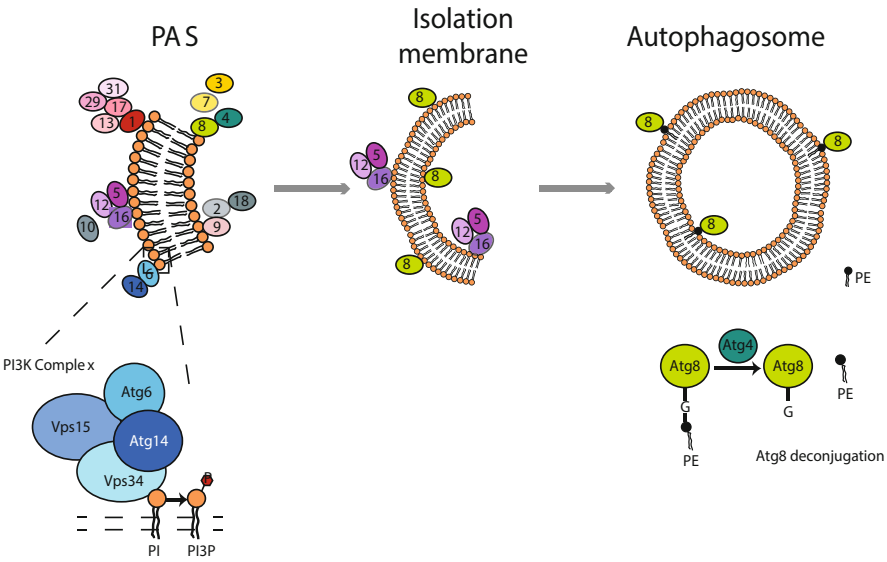


Fig. 4 Formation of the autophagosome is directed by a sequential mechanism that involves several core Atg-proteins. One of the mechanisms activated during the stationary phase where the nutrients become scarce, is the formation of autophagosomes. The first step is the activation of the Atg1-kinase complex (Atg1-Atg13-Atg17-Atg29-Atg31), followed by the recruitment of the PI3K complex, the conjugation system Atg12-Atg5, and vesicles containing Atg9-Atg2-Atg18. All these constitute the phagophore assembly site (*PAS*). The elongation of the isolation membrane and the closure of the autophagosome is guided by the action of the conjugation systems Atg12-Atg5-Atg16 and Atg8-PE. Atg8-PE must be deconjugated and recycled through Atg4 action. See text for details

Following autophagy induction in yeast, Atg protein complexes are recruited in a hierarchic order around the **phagophore assembly site (PAS)** where the elongation and eventual closure of the double-membrane autophagosomes occurs. The first assembly step involves a complex formed by Atg1-Atg13-Atg17-Atg29 and Atg31. Atg1 is a serine-threonine kinase whose kinase activity is essential for the formation of the autophagosome. The Atg1-Atg13-Atg17-Atg29 complex recruits Atg9 and the autophagy-specific PI3K complex, which in turn recruit the Atg2-Atg18 complex and, finally, the Atg ubiquitin-like conjugation systems Atg8 and Atg12. Upon activation, Atg12 forms a multimeric complex with Atg5 and Atg16 thought to induce curvature into the growing phagophore. On the other hand, Atg8 requires the covalent conjugation to phosphatidylethanolamine (PE) by Atg4-Atg7-Atg3 and the Atg12-Atg5-Atg16 complex in order to anchor the autophagosomal membranes. Atg8 anchoring to the membranes regulates expansion of the phagophore and participates later in cargo recruitment to the autophagosome by interacting with cargo binding (Glick et al. 2010; Noda and Inagaki 2015; Schaaf et al. 2016).

Once the autophagosome has reached a diameter between 300 and 900 nm, it is directed to the vicinity of the vacuole, docks with the vacuolar membrane, and fuses

with this organelle. The internal vesicle of the autophagosome is then released into the vacuolar lumen, and this intravacuolar vesicle is now called the autophagic body. Is in this location where vacuolar hydrolases degrade the limiting membrane of the autophagic body, allowing access to its cytoplasmic contents and their recycling into molecular building blocks (Abeliovich and Klionsky 2001).

Nutrient deficiency can positively regulate autophagy through different signaling pathways, including those where PKA, TOR1, and Snf1 are involved. These routes can control initiation of the autophagosome formation independently from each other (Stephan et al. 2009; Mizushima et al. 2011). The TOR1 complex is considered as the central negative regulator of macroautophagy (Noda and Ohsumi 1998; Cutler et al. 1999). Inactivation of TOR1, either by deleting the *TOR1* gene or by the addition of rapamycin, stimulates autophagy even in rich environments (Loewith et al. 2002).

3 Autophagy During Regulation of Lifespan

Regulation of lifespan is a complex and multifactorial biological response guided by genetic, environmental, and stochastic factors which determine cellular degeneration and progressive decline of physiological functions until cell death. As yeast cells age, they show an exponential increase in mortality rate over time, similar to human aging. Yeast aging has been modeled in two distinct ways: replicative lifespan and chronological lifespan. *S. cerevisiae* is a budding yeast that divides asymmetrically, giving rise to a large mother cell and a small daughter cell. **Replicative lifespan** in yeast is determined by the number of divisions that each mother cell can undergo, hence lifespan is then said to have ended when the mother cell no longer divides. In contrast, chronological yeast aging is measured using stationary phase cultures as the ability of non-dividing cells to maintain viability over time (Tissenbaum and Guarente 2002).

Lifespan in diverse organisms can be extended by a reduction on the activity of nutrient-sensing pathways. In yeast, a reduction in the activity of either PKA and/or TOR1 signaling pathways causes an increase in both chronological and replicative lifespan. In addition to the direct signals produced by nutrients, there are additional stimuli that can modulate the activity of the components in those pathways. For example, the Sch9 kinase, which is a component of the TOR1 pathway (Fig. 2), can be directly phosphorylated by the Pkh1/2 kinases, whose activity is in turn regulated by phytosphingosine (PHS), an intermediate in sphingolipid metabolism. Down-regulation of sphingolipid synthesis results therefore in a decreased activity of Sch9 and a concomitant chronological lifespan extension. All these indicates that Sch9 may function as an integration point for both, nutrient and sphingolipid-derived signals during regulation of lifespan regulation (Sampaio-Marques et al. 2014).

Although the TOR1 signaling pathway has been most studied when modulating responses to nutrient availability, it has also been linked with longevity. Due to the multicomponent structure of this pathway, it is suggested that the environmental effects on longevity can be mediated, directly or indirectly, by affecting TOR1

signaling pathway (Lushchak et al. 2017). Many studies that analyzed the extension of lifespan linked to nutrient-sensing pathways have reported the up-regulation of antioxidant enzymes, such as the superoxide dismutase Sod2 that scavenges superoxide anions. In addition, the pro-longevity effects attributed to the activation of a general stress response by decreasing the nutrient sensing pathways activity seem to be associated with an increase of mitochondrial function. In fact, it has been established that the lack of mitochondrial respiration severely affects the survival of stationary phase cells and thus the chronological lifespan (Sampaio-Marques et al. 2014).

4 Lipids in the Control of Autophagy

Lipids are fundamental components of the cell that are constantly recycled and redistributed to maintain cellular energy homeostasis (Singh et al. 2009). These processes of recycling are mainly mediated by the **lysosomes**, which mediate the degradation of lipids to redistribute their catabolites and support different cellular functions. Therefore, degradation of lipids occurs through autophagy. Besides maintaining lipid homeostasis, the process of autophagy involves a complex sequence of membrane modifications from its early stages during autophagosome formation and until late stages during the fusion of autophagosomes with the vacuole. As a result, lipids also play an important role in the control of autophagy (Dall'Armi et al. 2013).

As mentioned above, the TOR1 complex negatively regulates the initiation of autophagy, and the phosphatidylinositol (PI) 3-kinases play an essential role in this signaling cascade, controlling the nucleation and elongation of autophagosome formation. The autophagosomal membranes are characterized by containing phosphatidylinositol 3-phosphate (PI3P) as specific lipid, which is formed by phosphorylation of the PI on the 3′ position of the inositol ring by the PI3K complex, accumulated in particular regions of the preautophagosomal structure (PAS) (Shibutani and Yoshimori 2014). PI3K inhibition by wortmannin, LY294002, or 3-methyl adenine (3-MA) blocks autophagy and compromise the sorting of soluble vacuolar hydrolases (Robinson et al. 1988; Blommaart et al. 1997).

The vacuolar protein sorting 34 (Vps34) is the only PI3-kinase identified in yeast *S. cerevisiae*. Vps34 uses exclusively phosphatidylinositol (PI) as a substrate, exerting its regulatory function by producing PI3P to recruit effector proteins with PI3P-binding domains such as Atg18, Atg21, and Hsv2 (Burman and Ktistakis 2010; Jaber and Zong 2013). Vps34 is part of two PI3 kinase complexes involved in different processes. On one hand, Vps34 can interact with Vps15, Vps30/Atg6, and Atg14 to form the class III PI3-kinase complex I, which participates in autophagosome biogenesis. Vps15 is a serine threonine kinase that phosphorylates Vps34 and thereby guides its recruit to the membrane. The activity of Vps15 is essential for complex formation, while the lipid kinase activity of Vps34 is not. Atg14 is also important for complex localization to the PAS. The association of Vps34 with Vps15, Atg6 and Vps38 is also linked to the protein transport to the

vacuole through the PI3-kinase complex II, a process that is not involved in autophagy (Stack et al. 1995; Kihara et al. 2001).

PI3P binding proteins (called PI3P effectors) are characterized by having a FYVE, WD40, or PX domain, through which they bind to PI3P. This domain is also used to determine the localization of PI3P during different growth phases or metabolic conditions in yeast (Gillooly et al. 2000). PI3P and its effector proteins play an important function during autophagosome biogenesis controlling the curvature and size of the autophagosome (Table 1) (Obara et al. 2008). Compared with the external membrane, the autophagosomal inner membrane is specifically enriched with PI3P. Whether this asymmetry with respect to the PI3P content is the result of a rapid metabolism of PI3P by phosphatases in the outer cellular membrane or if it is simply absent from the external membrane, it is currently unknown. In yeast, during the log phase of growth, PI3P is found in abundance in endosomes and in the vacuolar membrane. However, during the transition to stationary phase, a massive transport of PI3P towards the vacuole occurs. This transport is dependent of the membrane dynamics related to autophagy (Obara and Ohsumi 2011).

At the beginning of the autophagosome formation during stationary phase, PI3P indirectly regulates the formation of the Atg8-phosphatidylethanolamine (Atg8-PE) complex within the autophagosome membrane. PE is the main phospholipid needed for autophagosome expansion, and its conjugation with Atg8 is determined by the physiological pH and the presence of acidic phospholipids. The regulation of Atg8-PE assembly coordinates the membrane formation and the PAS expansion. Before Atg8-PE conjugation, the cytosolic form of Atg8 is cleaved by Atg4, which in turn, is also responsible for complex Atg8-PE removal. This complex is one of the main markers used in autophagy studies. The deconjugation of Atg8 from PE, mediated by Atg4, is necessary for a correct disassembly of PAS and recycling of Atg proteins. If Atg8 and PE cannot be separated, the formation of the

Table 1 Lipid binding proteins involved in autophagy in yeast

Lipid-binding protein	Interacting lipid	Function
Atg3	PE	Role in formation of Atg8-phosphatidylethanolamine conjugates, which are involved in membrane dynamics during autophagy
Atg5	PE	Undergoes conjugation with Atg12 to form a complex involved in Atg8 lipidation
Atg12	PE	Conjugated to Atg5 to form a complex involved in Atg8 lipidation
Atg18	PI3P; PI (3,5)P2	Required for vesicle formation in autophagy
Atg21	PI3P; PI (3,5)P2	Required for vesicle formation in the cytoplasm-to-vacuole targeting (Cvt) pathway
Atg27	PI3P	Type I membrane protein involved in autophagy
Hsv2	PI(3,5)P2	Plays a role in micronucleophagy

Based on Knævelsrud and Simonsen (2012). Protein function annotated according to the *Saccharomyces* Genome Database (www.yeastgenome.org)

autophagosome is compromised (Oh-oka et al. 2008; Longatti and Tooze 2009; Simonsen and Tooze 2009; Nair et al. 2012).

Also phosphatase-mediated PI3P-PI turnover is important during autophagosome formation. In yeast, Ymr1 phosphatase activity is required for the disassembly of Atg proteins in the membrane of the autophagosome and during fusion with the vacuole (Cebollero et al. 2012).

5 Formation of Lipid Droplets for Lipid Homeostasis

Lipid droplets (LDs) are specialized monolayer organelles for lipid homeostasis that increase their number and size during the stationary phase and also act as scaffolds for autophagosome biogenesis. As a store of triacylglycerol (TAG) and steryl esters (STE), the LDs must be hydrolyzed in the vacuole to produce fatty acids as an energy source. In vacuoles isolated from cells grown in nitrogen starvation, a 10-fold increase in the amount of lipids is observed compared to cells in the log phase, this phenomenon causes a massive internalization of LDs during stationary phase (Singh et al. 2009; van Zutphen et al. 2014; Gao and Goodman 2015).

In addition to eukaryotic organisms, LDs are present in many prokaryotic microorganisms such as *Mycobacterium*, *Streptomyces*, *Rhodococcus*, and *Nocardia*. In bacteria, during the exponential growth phase the synthesized fatty acids are mainly used for biogenesis of phospholipids. While in stationary phase the fatty acids are synthesized and accumulated as TAG inclusions in the cytoplasm (Alvarez and Steinbüchel 2002; Fujimoto et al. 2008).

When the cell requires energy sources during the stationary phase, the LDs are directed to the vacuole in a process better known as lipophagy. Long periods of starvation increase the number of LDs in autophagosomes. Lipophagy acts then as a regulator of the size and number of LDs in basal conditions and as a mechanism of survival under stress conditions (Weidberg et al. 2009).

It has been described that the degradation of LDs occurs by microautophagy, although some authors indicate that it may also be by macroautophagy. In mouse models, it has been observed that the LDs are engulfed in the autophagosome through the activity of the LC3-PE complex (Atg8-PE in yeast). However, in microorganisms such as yeast the degradation of LDs does not require this complex (Walther and Farese 2012; van Zutphen et al. 2014).

The LDs are grouped in clusters near the vacuole and when targeted by microautophagy to the vacuole they are degraded by lipases inside this organelle like Atg15 in *S. cerevisiae* and *Fusarium graminearum.* This degradation depends on the Atg core proteins Atg1, Atg3, Atg4, Atg5, Atg7, Atg10, Atg12, and Atg16, and elimination of any of these proteins compromises lipophagy, e.g., in absence of Atg1 the LDs remain in the cytosol and are degraded by cytosolic lipolysis (Epple et al. 2001; Nguyen et al. 2011). In yeast, during starvation or stationary phase, cytosolic lipases such as Tgl3 and Tgl4 decrease considerably, whereas the activity of vacuolar lipase Atg15 increases supporting the degradation of LDs by autophagy to generate energy and promote cellular survival. In addition to Atg proteins, the enzymes Dga1

and Lro1, as well as Are1 and Are2, which are responsible for the synthesis of TAG and STE, respectively, are also essential for autophagy under nitrogen starvation (Shpilka et al. 2015; Ward et al. 2016).

The fusion of the LDs to the vacuole depends on the formation of vacuolar microdomains, which are regions where the membrane of the vacuole is found in a liquid-ordered phase rich in sterols. It has been reported that these microdomains are only formed during the stationary phase (Wang et al. 2014).

As LDs are dynamic organelles, one of the main questions is how they are recognized in order to be degraded via autophagy. It is suggested that protein Aup1 (ancient ubiquitous 1) integrates to the surface of the LDs and is recognized by the autophagosome by ubiquitination marks made by E2 ubiquitin conjugates in its C-terminal region (Spandl et al. 2011). One of the main regulators of docking and degradation of LDs is the vacuolar membrane GTPase of the Rab family Ypt7 in *S. cerevisiae*, homologous to Rab7 in mammals. To regulate LDs dynamics under low nutrient conditions, Ypt7 is associated with LDs. The absence of Ypt7 causes an increase in the amount of fatty acids and in the number of LDs, as well as morphological changes and intra LDs contact and fusion. In addition to Ypt7, Vm13, the H subunit of the V1 part of the vacuolar H^+-ATPase (V-ATPase), and the complex HOPS (Homotype fusion and vacuole protein sorting), control cell fatty acid content and LDs number (Bouchez et al. 2015; Schroeder et al. 2015). As mentioned, the degradation of LDs also occurs in the cytosol by the means of lipolysis through neutral lipases. Recent studies indicate that there is a cellular cross talk among autophagic pathways and neutral lipases. In organisms where chaperone-mediated microautophagy is present, autophagy promotes cytosolic lipolysis through the exposure of the LDs core by the degradation of LD-associated proteins (Cingolani and Czaja 2016).

6 Autophagy During the Control or Carbohydrate Reserves

During late stages of the logarithmic phase, yeast synthesizes glycogen to be used in the stationary phase. It is at this stage or under low nutrient conditions where glycogen accumulates as a reserve of glucose and energy. Genetic studies have shown that genes *ATG1* and *ATG13*, involved in autophagy, are regulated by the protein kinase Snf1. Snf1, homologue of the mammalian AMP-kinase, controls glucose repression process and glycogen accumulation during stationary phase (Lillie and Pringle 1980; Zhang and Cao 2017).

Snf1 has also been associated with the control of autophagy at the transcriptional level, since its activity controls the nuclear localization of the Gln3 and Msn2 transcription factors. Snf1 regulates the subcellular localization of Msn2 in response to glucose. When Snf1 is active, Msn2 remains in the cytosol and in response the STRE (Stress response element) regulated genes are not activated. Phosphorylation of Gln3 is mediated by Snf1, a reaction that may be critical for both nutrient sensing and starvation responses (Bertram et al. 2002; Mayordomo et al. 2002; Cebollero and Reggiori 2009).

7 Autophagy-Like Processes in Bacteria

In bacteria, the process of autophagy has an equivalent process that is known as Dwarfing. This refers to a continuous size reduction of cells observed after the completion of reductive division. It is the result of degradation of endogenous material and this degradation includes the cell envelope, especially the cytoplasmic membrane and cell wall. Importantly, this process is triggered by starvation and is considered the bacterial equivalent to autophagy in eukaryotes (Nyström 2004). In the same way as autophagy is essential for lifespan extension in eukaryotic organisms, self-digestion seems to be an important survival mechanism of starved bacteria.

Some pathways have been identified to modulate responses to the starvation conditions characteristic of the entrance to stationary phase:

1. Lipid balance and lipid degradation. Lipid balance is a crucial aspect for survival during stationary phase. The lipid content and distribution between membrane leaflets is crucial when adapting to stressful conditions. For example, the increased accumulation of phospholipids in the outer leaflet of the outer membrane in *E. coli* triggers cell death during stationary phase. It was proposed that this process is a result of lipid loss from the outer membrane through vesiculation at cell-division sites, prompting a migration of phospholipids from the inner membrane to the outer membrane eventually leading to ruptures from the inner membrane and release of the cytosolic contents and cell lysis (Sutterlin et al. 2016). On the other hand, lipid degradation is a crucial process for cells experiencing the stress caused by stationary phase. Lipid degradation upon changing availability of fatty acids in the environment is modulated by responses activated by the bifunctional transcription factor FadR. When cells enter stationary phase, the genes required to degrade fatty acids to acetyl-CoA regulated by FadR become activated. This degradation could provide bacteria with carbon and energy during stress situations via digestion of membrane constituents (Nyström 2004; Zhang and Rock 2009).
2. Catabolic control. Dwarfing of carbon-starved *E. coli* cells is accompanied by an increased synthesis of glycolytic enzymes and a reduced production of enzymes involved in the TCA cycle. This response in carbon-starved stationary phase is regulated by the ArcA/ArcB system. Repression of aerobic metabolism upon stationary phase correlates with upregulation of *arcA,* and cells lacking ArcA fail to perform reductive division early during starvation and lose viability at an accelerated rate after a few days in stationary phase (Nyström 2004).
3. Protein degradation. One of the responses of *E. coli* to starvation and to growth arrest is the protein synthesis in the absence of exogenous carbon. The source of amino acids for this synthesis seems to come from degradation of already existing proteins. This process has been called protein-dependent autophagy, and it has been suggested that oxidation during stationary phase might be the mechanism in which *E. coli* targets specific proteins for degradation (Nyström 2004).

All these autodegenerative processes are activated during entrance of *E. coli* and other bacteria to stationary phase, and their activity is directly linked to the survival of the cells, measured by the ability to reinitiate cell division if the surrounding conditions change to a nutrient-rich environment.

8 Research Needs

The relevance of lipids as essential components of membranes has long been recognized. For example, in recent years it has been observed that the phospholipids that constitute a membrane not only determine the dynamics of that membrane but also modulate the function of the embedded proteins by specific interactions or modifications to each polypeptide. However, their importance as signaling molecules or as active players during cell development is far from being understood.

The study of microorganisms in stationary phase has become an important tool to understand the basis of cell responses when entering stress conditions where nutrients are scarce. In the particular case of autophagy, it is clear that lipids are not merely the accompanying biomolecules to the selective process of intracellular degradation, but they work together with Atg-core proteins during the key stages of autophagosome formation. Autophagy and lipid metabolism are conserved processes. A profound evaluation of the physiological and molecular mechanisms that control these processes during stationary phase is important to understand cell function and response. One of the main questions around this topic is how lipids control the formation of the autophagosome. Nowadays, it is still debated whether it occurs through vesicles or by direct contact with other membranes. In addition, it remains to be elucidated how cells control the autophagy rate during late stages of growth and how the vacuoles export accumulated fatty acids. In addition, it is still unclear how the lipids from other membranes, like those from organelles such as mitochondria or peroxisomes, modulate the responses that lead to their specific autophagy (i.e., mitophagy and pexophagy, respectively).

There are several examples pointing the relevance of asymmetric lipid distribution among different leaflets of a membrane and how this particular distribution is important during signaling cascades or for maintaining cell integrity. Despite that, the precise machineries involved during the exchange of phospholipids in stationary phase as well as the molecular mechanism that regulates this exchange remains largely unknown.

Lipids play also an important role during stationary phase as emergency carbon source via lipophagy of lipid droplets within the vacuole. What molecular cues trigger the formation of lipid droplets is a mechanism that is still not understood.

In addition, the study of how lipids regulate stress responses in bacteria is limited. In particular, it will be interesting to understand how lipid interactions regulate the activity of two-component systems that sense external signals and communicate them to the signaling cascades inside the cell. Furthermore, how lipid dynamics affects tolerance to stress, regulate remodeling processes, and regulate lifespan will

provide invaluable information which could be applied in several aspects both medical and biotechnological.

There is no doubt that lipids play an essential role during stationary phase, both as storage molecules as well as part of the signaling cascades and modulators of stress responses. It will be exciting to unravel the molecular details from these processes and clarify how they are important for cells to survive stress conditions.

Acknowledgments Our work is supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT, México-CB 237344) and the Dirección General de Asuntos del Personal Académico (DGAPA-UNAM, México IN202715).

References

Abeliovich H, Klionsky DJ (2001) Autophagy in yeast: mechanistic insights and physiological function. Microbiol Mol Biol Rev 65:463–479. Table of contents. https://doi.org/10.1128/MMBR.65.3.463-479.2001

Alvarez HM, Steinbüchel A (2002) Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol 60:367–376. https://doi.org/10.1007/s00253-002-1135-0

Antonioli M, Di Rienzo M, Piacentini M, Fimia GM (2017) Emerging mechanisms in initiating and terminating autophagy. Trends Biochem Sci 42:28–41. https://doi.org/10.1016/j.tibs.2016.09.008

Arlia-Ciommo A, Piano A, Leonov A et al (2014) Quasi-programmed aging of budding yeast: a trade-off between programmed processes of cell proliferation, differentiation, stress response, survival and death defines yeast lifespan. Cell Cycle 13:3336–3349. https://doi.org/10.4161/15384101.2014.965063

Bertram PG, Choi JH, Carvalho J et al (2002) Convergence of TOR-nitrogen and Snf1-glucose signaling pathways onto Gln3. Mol Cell Biol 22:1246–1252. https://doi.org/10.1128/MCB.22.4.1246-1252.2002

Blommaart EF, Krause U, Schellens JP et al (1997) The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem 243:240–246

Bouchez I, Pouteaux M, Canonge M et al (2015) Regulation of lipid droplet dynamics in *Saccharomyces cerevisiae* depends on the Rab7-like Ypt7p, HOPS complex and V1-ATPase. Biol Open 4:764–775. https://doi.org/10.1242/bio.20148615

Burman C, Ktistakis NT (2010) Regulation of autophagy by phosphatidylinositol 3-phosphate. FEBS Lett 584:1302–1312. https://doi.org/10.1016/j.febslet.2010.01.011

Cebollero E, Reggiori F (2009) Regulation of autophagy in yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1793:1413–1421. https://doi.org/10.1016/j.bbamcr.2009.01.008

Cebollero E, van der Vaart A, Zhao M et al (2012) Phosphatidylinositol-3-phosphate clearance plays a key role in autophagosome completion. Curr Biol 22:1545–1553. https://doi.org/10.1016/j.cub.2012.06.029

Cingolani F, Czaja MJ (2016) Regulation and functions of autophagic lipolysis. Trends Endocrinol Metab 27:696–705. https://doi.org/10.1016/j.tem.2016.06.003

Cutler NS, Heitman J, Cardenas ME (1999) TOR kinase homologs function in a signal transduction pathway that is conserved from yeast to mammals. Mol Cell Endocrinol 155:135–142

Dall'Armi C, Devereaux KA, Di Paolo G (2013) The role of lipids in the control of autophagy. Curr Biol 23:R33–R45. https://doi.org/10.1016/j.cub.2012.10.041

Epple UD, Suriapranata I, Eskelinen EL, Thumm M (2001) Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. J Bacteriol 183:5942–5955. https://doi.org/10.1128/JB.183.20.5942-5955.2001

Fujimoto T, Ohsaki Y, Cheng J et al (2008) Lipid droplets: a classic organelle with new outfits. Histochem Cell Biol 130:263–279. https://doi.org/10.1007/s00418-008-0449-0

Galdieri L, Mehrotra S, Yu S, Vancura A (2010) Transcriptional regulation in yeast during diauxic shift and stationary phase. OMICS 14:629–638. https://doi.org/10.1089/omi.2010.0069

Gao Q, Goodman JM (2015) The lipid droplet-a well-connected organelle. Front Cell Dev Biol 3:49. https://doi.org/10.3389/fcell.2015.00049

Gillooly DJ, Morrow IC, Lindsay M et al (2000) Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. EMBO J 19:4577–4588. https://doi.org/10.1093/emboj/19.17.4577

Glick D, Barth S, Macleod KF (2010) Autophagy: cellular and molecular mechanisms. J Pathol 221:3–12. https://doi.org/10.1002/path.2697

Grogan DW, Cronan JE (1997) Cyclopropane ring formation in membrane lipids of bacteria. Microbiol Mol Biol Rev 61(4):429–441

Hengge-Aronis R (2002) Recent insights into the general stress response regulatory network in *Escherichia coli*. J Mol Microbiol Biotechnol 4:341–346

Huisman GW, Siegele DA, Zambrano MM, Kolter R (1996) Morphological and physiological changes during stationary phase. In: Neidhardt FC, Curtis R III, Ingraham JL, ECC L, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol 2, 2nd edn. ASM Press, Washington, DC, pp 1672–1682

Jaber N, Zong W-X (2013) Class III PI3K Vps34: essential roles in autophagy, endocytosis, and heart and liver function. Ann N Y Acad Sci 1280:48–51. https://doi.org/10.1111/nyas.12026

Kihara A, Noda T, Ishihara N, Ohsumi Y (2001) Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. J Cell Biol 152:519–530

Klionsky DJ, Codogno P (2013) The mechanism and physiological function of macroautophagy. J Innate Immun 5:427–433. https://doi.org/10.1159/000351979

Knævelsrud H, Simonsen A (2012) Lipids in autophagy: constituents, signaling molecules and cargo with relevance to disease. Biochim Biophys Acta 1821:1133–1145. https://doi.org/10.1016/j.bbalip.2012.01.001

Książek K (2010) Let's stop overlooking bacterial aging. Biogerontology 11:717–723. https://doi.org/10.1007/s10522-010-9278-3

Lillie SH, Pringle JR (1980) Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J Bacteriol 143:1384–1394

Lippai M, Szatmári Z (2017) Autophagy-from molecular mechanisms to clinical relevance. Cell Biol Toxicol 33:145–168. https://doi.org/10.1007/s10565-016-9374-5

Loewith R, Jacinto E, Wullschleger S et al (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 10:457–468

Longatti A, Tooze SA (2009) Vesicular trafficking and autophagosome formation. Cell Death Differ 16:956–965. https://doi.org/10.1038/cdd.2009.39

Longo VD, Liou LL, Valentine JS, Gralla EB (1999) Mitochondrial superoxide decreases yeast survival in stationary phase. Arch Biochem Biophys 365:131–142. https://doi.org/10.1006/abbi.1999.1158

Lushchak O, Strilbytska O, Piskovatska V et al (2017) The role of the TOR pathway in mediating the link between nutrition and longevity. Mech Ageing Dev. https://doi.org/10.1016/j.mad.2017.03.005

Mayordomo I, Estruch F, Sanz P (2002) Convergence of the target of rapamycin and the Snf1 protein kinase pathways in the regulation of the subcellular localization of Msn2, a transcriptional activator of STRE (stress response element)-regulated genes. J Biol Chem 277:35650–35656. https://doi.org/10.1074/jbc.M204198200

Mitchison JM (2003) Growth during the cell cycle. Int Rev Cytol 226:165–258

Mizushima N, Yoshimori T, Ohsumi Y (2011) The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol 27:107–132. https://doi.org/10.1146/annurev-cellbio-092910-154005

Nair U, Yen W-L, Mari M et al (2012) A role for Atg8-PE deconjugation in autophagosome biogenesis. Autophagy 8:780–793. https://doi.org/10.4161/auto.19385

Navarro Llorens JM, Tormo A, Martínez-García E (2010) Stationary phase in gram-negative bacteria. FEMS Microbiol Rev 34:476–495. https://doi.org/10.1111/j.1574-6976.2010.00213.x

Nguyen LN, Bormann J, Le GTT et al (2011) Autophagy-related lipase FgATG15 of *Fusarium graminearum* is important for lipid turnover and plant infection. Fungal Genet Biol 48:217–224. https://doi.org/10.1016/j.fgb.2010.11.004

Noda NN, Inagaki F (2015) Mechanisms of autophagy. Annu Rev Biophys 44:101–122. https://doi.org/10.1146/annurev-biophys-060414-034248

Noda T, Ohsumi Y (1998) Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 273:3963–3966

Nyström T (2004) Stationary-phase physiology. Annu Rev Microbiol 58:161–181. https://doi.org/10.1146/annurev.micro.58.030603.123818

Obara K, Ohsumi Y (2011) PtdIns 3-kinase orchestrates autophagosome formation in yeast. J Lipids 2011:498768–498769. https://doi.org/10.1155/2011/498768

Obara K, Noda T, Niimi K, Ohsumi Y (2008) Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in *Saccharomyces cerevisiae*. Genes Cells 13:537–547. https://doi.org/10.1111/j.1365-2443.2008.01188.x

Oh-oka K, Nakatogawa H, Ohsumi Y (2008) Physiological pH and acidic phospholipids contribute to substrate specificity in lipidation of Atg8. J Biol Chem 283:21847–21852. https://doi.org/10.1074/jbc.M801836200

Robinson JS, Klionsky DJ, Banta LM, Emr SD (1988) Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol Cell Biol 8:4936–4948

Sampaio-Marques B, Burhans WC, Ludovico P (2014) Longevity pathways and maintenance of the proteome: the role of autophagy and mitophagy during yeast ageing. Microb Cell 1:118–127. https://doi.org/10.15698/mic2014.04.136

Schaaf MBE, Keulers TG, Vooijs MA, Rouschop KMA (2016) LC3/GABARAP family proteins: autophagy-(un)related functions. FASEB J 30:3961–3978. https://doi.org/10.1096/fj.201600698R

Schroeder B, Schulze RJ, Weller SG et al (2015) The small GTPase Rab7 as a central regulator of hepatocellular lipophagy. Hepatology 61:1896–1907. https://doi.org/10.1002/hep.27667

Shibutani ST, Yoshimori T (2014) A current perspective of autophagosome biogenesis. Cell Res 24:58–68. https://doi.org/10.1038/cr.2013.159

Shpilka T, Welter E, Borovsky N et al (2015) Lipid droplets and their component triglycerides and steryl esters regulate autophagosome biogenesis. EMBO J 34:2117–2131. https://doi.org/10.15252/embj.201490315

Simonsen A, Tooze SA (2009) Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. J Cell Biol 186:773–782. https://doi.org/10.1083/jcb.200907014

Singh R, Kaushik S, Wang Y et al (2009) Autophagy regulates lipid metabolism. Nature 458:1131–1135. https://doi.org/10.1038/nature07976

Spandl J, Lohmann D, Kuerschner L et al (2011) Ancient ubiquitous protein 1 (AUP1) localizes to lipid droplets and binds the E2 ubiquitin conjugase G2 (Ube2g2) via its G2 binding region. J Biol Chem 286:5599–5606. https://doi.org/10.1074/jbc.M110.190785

Stack JH, DeWald DB, Takegawa K, Emr SD (1995) Vesicle-mediated protein transport: regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast. J Cell Biol 129:321–334

Stephan JS, Yeh Y-Y, Ramachandran V et al (2009) The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. Proc Natl Acad Sci U S A 106:17049–17054. https://doi.org/10.1073/pnas.0903316106

Sutterlin HA, Shi H, May KL et al (2016) Disruption of lipid homeostasis in the Gram-negative cell envelope activates a novel cell death pathway. Proc Natl Acad Sci U S A 113:E1565–E1574. https://doi.org/10.1073/pnas.1601375113

Tissenbaum HA, Guarente L (2002) Model organisms as a guide to mammalian aging. Dev Cell 2:9–19

Walther TC, Farese RV (2012) Lipid droplets and cellular lipid metabolism. Annu Rev Biochem 81:687–714. https://doi.org/10.1146/annurev-biochem-061009-102430

Wang AY, Cronan JE (1994) The growth phase dependent synthesis of cyclopropane fatty acids in *Escherichia coli* is due to an RpoS (KatF) dependent promoter plus enzyme instability. Mol Microbiol 11:1009–1017

Wang C-W, Miao Y-H, Chang Y-S (2014) A sterol-enriched vacuolar microdomain mediates stationary phase lipophagy in budding yeast. J Cell Biol 206:357–366. https://doi.org/10.1083/jcb.201404115

Ward C, Martinez-Lopez N, Otten EG et al (2016) Autophagy, lipophagy and lysosomal lipid storage disorders. Biochim Biophys Acta 1861:269–284. https://doi.org/10.1016/j.bbalip.2016.01.006

Weidberg H, Shvets E, Elazar Z (2009) Lipophagy: selective catabolism designed for lipids. Dev Cell 16:628–630. https://doi.org/10.1016/j.devcel.2009.05.001

Zhang N, Cao L (2017) Starvation signals in yeast are integrated to coordinate metabolic reprogramming and stress response to ensure longevity. Curr Genet 26:4818–4815. https://doi.org/10.1007/s00294-017-0697-4

Zhang Y-M, Rock CO (2009) Transcriptional regulation in bacterial membrane lipid synthesis. J Lipid Res 50(Suppl):S115–S119. https://doi.org/10.1194/jlr.R800046-JLR200

van Zutphen T, Todde V, de Boer R et al (2014) Lipid droplet autophagy in the yeast *Saccharomyces cerevisiae*. Mol Biol Cell 25:290–301. https://doi.org/10.1091/mbc.E13-08-0448

Index

A
ABC complex, 130
ABC-export systems, 303
ABC transporters, 677
Abiotic stress, 115
ACC, *see* Acetyl-CoA carboxylase (ACC)
4-Acetamido-4`-maleimidylstilbene-2,2`-disulfonic acid, 612
Acetate, 26
Acetoacetyl-ACP, 394
Acetoacetyl-CoA, 488
Acetobacter, 124
Acetobacterium woodii, 28
Acetogen(s), 26
Acetogenic bacteria, 26
Acetylation, 300–301
Acetyl-CoA, 4, 26, 61
Acetyl-CoA carboxylase (ACC), 47, 262, 394, 404, 764, 765
  gene expression, 415
Acetyl-CoA synthase (ACS), 30
N-Acetylgalactosamine, 166
N-Acetylglucosamine, 167, 301
Acholeplasma laidlawii, 528, 530
Acidic phospholipids, 684
Acidification, 493, 791
Acidophilic bacteria, 791
Acid tolerance, 92
Acinetobacter baumannii, 603
Acinetobacter baylyi, 451
AcpM, 422
AcpXL, 49
Actinia equina, 734
Actin-like protein, 583
Actinobacteria, 17, 447, 448
Actinoporin, 736
Activated fatty acid, 246
Activated sludge community, 492
Activator of fatty acid biosynthetic genes, 398
Active transport, 639
Acyl-ACP, 392, 404, 532, 769, 771
Acyl-ACP hydrolase, 246
Acylation, 325
  N-Acylation, 299
  S-Acylation, 712–713
N-Acylhomoserine lactone, 274, 279–280
Acyl biotin exchange, 718
Acyl carrier protein (ACP), 46, 91, 128, 187, 241, 261, 297, 392, 764
Acyl chain, 803
Acyl-CoA, 392, 399, 451, 532
Acyl-CoA carboxylases
  in actinomycetes, 414–415
  genetic and biochemical regulation, 415
  subunits composition and biochemical reaction of, 412
1-Acylglycerol-3-phosphate, 396
1-Acyl-glycerol-3-phosphate acyltransferase, 91
Acyl homoserine lactones, 51
Acyl moieties, 497
Acyloxyacyl lipids, 115
Acyloxyacyl structure, 110, 117
Acyl-PEG, 719
Acyl-phosphate, 91, 392, 396, 404
N-Acylphosphatidylethanolamine (*N*-acyl-PE), 580
Acyl protein thioesterases, 712
Acyl-RAC, 719
N-Acylsphinganine, 126
Acyltransferase(s), 334, 396
  O-Acyl transferase, 112
  N-Acyl transferase, 112, 299
Acyltransferase enzyme, 416
Acyltransfer module, 764

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50430-8

Adamantyl urea, 200
Adenosine triphosphate (ATP), 247
Adenosylmethionine, 191
S-Adenosylmethionine, 95, 279
  diacylglycerol 3-amino-3-carboxypropyl transferase, 100
  diacylglycerylhomoserine-*N*-methyltransferase, 100
Adhesins, 676
Adipocyte FABP (A-FABP), 693
Adipocyte P2 (aP2), 693
ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase CD38, 628
Aerobic desaturation, 49, 189
Aerobic dynamic feeding (ADF), 493
Aerobic organisms, 781
Aerolysin, 731
A-factor, *see* Autoregulatory factor
AFN-1252, 52
Aggregatibacter actinomycetemcomitans, 604
Aging, 354, 852
Agro-forestry residues, 493
Agro-industrial wastes, 447
Alanyl-PG, 793
Alarmones, 397
Albaflavenone, 286
Alcaligenes eutrophus, 489
Aldehyde, ferredoxin oxidoreductase (AOR), 35
Alkaline ceramidases, 348
Alkane, 286
Alkane-derived signal, 284–285
3-Alkanoic acids (3HAs), 487
Alk-1-enyl ether, 526
Alkyl-alcohols, 501
2-Alkyl-4(1H)-quinolone, 282
Alkylpyrones, 141, 437
Alkyl-quinolone, 282
Alkylquinone, 143, 439–441
Alkylresorcinols, 141, 437, 440
Allosteric mechanism, 396
Alpha-helix, 731
Alpha-hemolysin, 731
Alternative 2-methyl 3-erythritol-4-phosphate pathway, 65
Alternative mevalonate pathway, 62
Alternative route, 62
Amidation, 222
Aminoacylated cardiolipin, 92
5-Aminolevulinate synthase, 128
Amino lipids, 110, 118
2-Amino-3-oxobutyrate coenzyme A ligase, 128
8-Amino-7-oxononanoate synthase, 128
AmJ, 225
Amphipathic α-helix, 534
Amphipathic molecule, 576
Amphiphilic membrane lipid, 549
Amphiphilic phospholipids, 546
Amyloid fibers, 683
Anaerobic desaturation, 190
Anaerobic pathway, 780
Anhydrotetracycline (aTC), 618
8-Anilogeranyl diphosphate, 717
Anionic charges, 550
Anionic phospholipid, 580, 684, 803
Anisotropic lipid distribution, 809
Anoxic photosynthesis, 491
Anteiso branched-chain fatty acids, 397, 404
Anteiso-branched fatty acids, 777
Anteiso chain, 528
Anteiso fatty acids, 791
Anthrax toxin, 736
Antibacterials, 226, 228
Antibiotic(s), 66, 78, 157, 159, 172, 174
Antibiotic resistance, 679
Antimicrobial agents, 603
Anti-microbial peptides, 733
Antiparallel β-strands, 655
Anti-proliferative signal, 349
Aquaporins, 802
Arabidopsis thaliana, 146
Arabinosylation, 300
Arbuscular mycorrhization, 305
Archaeal polar lipids, 541
Archaeaol, 541
Archaetidyl-*myo*-inositolphosphate, 547
Are1p, 512
Are2p, 512
Arthrobacter nicotianae, 12
Aspergillus nidulans, 11
Astroglial glutamate transporter type 1 (GLT), 632
Asymmetry, 632
Atmospheric chemistry, 79
ATPase activity, 683
ATP-binding cassette (ABC) transporter, 303, 677
Aur1p, 347
Autoimmune and inflammatory diseases, 174
Autoinducer, 274
Autokinase competent state, 782
Autolysins, 172
Autophagy, 713, 853–856, 860
  lifespan regulation, 856–857
  lipids, 857–859

Autoregulatory factor, 280
Autotransporter (AT), 680
Autotrophic growth, 36
Azido-or alkynyl-fatty acids, 719
Azotobacter vinelandii, 140–142, 436, 437

B
BacA, 227
Bacillus, 598
  B. anthracis, 587
  B. cereus sensu lato, 782
  B. subtilis, 535, 577, 582, 781, 803
Bacitracin, 229
Bacteria, 527, 730
  adhesion, 173
  cell division, 581
  flagellum, 678
  hosts, 454
  inclusion, 484
  osmoregulation, 802
  virulence, 278
  volatile compound, 285–287
  weapon, 681
Bacterial dialkylether glycerophospho inositol synthase (BEPIS), 94
Bacterial sphingolipids
  biosynthesis of, 125–130
  function of, 131–132
  localization and transport of, 130–131
  structure of, 125–126
Bacteriochlorophylls, 76
Bacteriophage tails, 681
Bacteriorhodopsins, 76
Bacteriovorax stolpii, 128
Bacteroides, 124
Bacteroides fragilis, 587
Bacteroidetes, 17
Bactoprenol, 72
Baeyer-Villiger oxidation, 192
BamA, 658
BamA-assisted model, 663
BamB, 659
BamC, 660
BamD, 660
BamE, 661
β-Barrel, 654, 658
β-Barrel assemblymachinery (BAM) complex, 658, 680
  Bam protein structure, 658–662
  uOMP folding and membrane insertion, 662–664
Beauveria bassiana, 12
Bee, 730, 731
Benzofuran, 199
Betaine lipid, 100, 826
Betaine lipid diacylglyceryl trimethylhomoserine, 98
Beta structure, 734
BetP, 813
Bicelles, 813
Bicell model, 369
Bifunctional palmitic acid, 720
Binding affinity, 642
Bioactive sphingolipids, 353
BioC-BioH, 264
Biochemical regulation, 769–771
  enzyme activity by temperature, 397
  negative feedback, 396
Biocompatibility, 494
Biodegradability, 494
BioF, 267
Biofilm, 195, 276
Biofilm formation, 173, 586
Biofuels, 447
BioI, 264
Biological membranes, 526
Bioorthogonal reactions, 714
BioQ, 269
BioR, 269
Bioremediation, 500
Biosurfactants, 238
Biosynthesis, 175, 293
Biotechnological, 386
Biotechnological applications, 447
Biotin, 260
Biotin carboxylase, 412
Biotin carboxyl carrier protein (BCCP), 264, 412
Biotin-dependent proteins, 412
Biotin-protein ligase, 268
Biotin synthase (BioB), 268
Biotinyl-5′-AMP, 268
BirA, 267
Borrelia burgdorferi, 587
Bradyrhizobium japonicum, 95
Branched-chain 2-oxoacid dehydrogenase complex, 49
Branched-chain amino acids, 49
Branched chain fatty acids (BCFA), 44, 49, 420, 777–778, 790
Branched-chain ketoacid dehydrogenase (BDKH), 777
Branching pattern, 778
Broad substrate specificity, 547
Budding model, 369, 664

Bulking, 492
Burkholderia cenocepacia, 113
2,3-Butanediol, 286
γ-Butyrolactone, 280–281

C
Ca^{2+} homeostasis, 352
Caldarchaeol, 541
Cancer, 342
Candida, 14
Capnine, 132
Carbamoylation, 301
Carbohydrate, 484
Carbon and energy sources, 487
Carbon-based internal reserve, 485
Carbon monoxide dehydrogenase (CODH), 28
Carbonosome, 463–465, 468, 471, 487
Carbon to nitrogen ratios, 498
α-Carboxy-acyl-CoAs, 411
Carboxylesterase, 267
Carboxyltransferase (CT), 412
Carboxysome, 485
Cardiolipin/phosphatidylethanolamine synthase, 95
Cardiolipin (CL), 89, 92–94, 329, 527, 555, 577, 683, 792, 796, 809
Cardiolipin (CL) synthase A, 583
Carnitine, 11
Carnitine palmitoyltransferase 2 (CPT-2), 11
β-Carotene, 78
Carotenoid(s), 78, 587, 808
CasKR, 782
Catalysis, 334
Catalysts, 175
Cationic antimicrobial peptide, 92
Caulobacter, 128
Cdc28-Cyclin B, 327
CDP-alcohol phosphatidyltransferase, 542
CDP-alcohol phosphotransferase, 91
CDP-choline, 97
CDP-diacylglycerol (CDP-DAG), 90–91, 532
  ER mitochondria encounter structure (ERMES), 330–331
  mitochondrial transport, phosphatidylserine and phosphatidylethanolamine, 332
  Ups1-Mdm35 complex, 331–332
CDP-DAG synthase, 546
CDP-diacylglycerol synthase (CdsA), 532
CDP-diglyceride synthase, 91
CDP-ethanolamine pathway, 95
CDP-inositol, 94
Cell cycle arrest, 350
Cell division, 193
  and separation, 173
Cell envelope of bacteria, 218
Cell membrane(s), 742
  damage, 750
Cell poles, 810
Cell-to-cell communication, 279
Cellular lipases, 453
Cellular metabolism, 376
Cellular organization, 803
Cell wall, 71
  architecture, 186
  fluidity, 186
  permeability barrier, 186
Cenancestor, 68
Ceramide, 126
Ceramide phosphoethanolamine, 125
Ceramide phosphoglycerol, 125
Ceramide phosphoinositol, 125
Ceramide synthases, 346
Cerilipin, 114
Chain length, 777
Channel gating, 816
Chaperones, 655–657
Chaperone-usher pili, 682
Charge balance rule, 560, 616, 621, 624, 630, 633
Chemical feedstocks, 4
Chemical proteomics, 710
Chemiosmotic machine, 639
Chemoreceptors, 812
Chemosynthetic organisms
  carbon limitation, 835–836
  nitrogen limitation, 834
  phosphorus limitation, 829–832
Chitin backbone, 294
Chitin hydrolases, 307–308
Chitin oligomers (COs), 304
Chlorophylls, 76
Chlorosome, 485
Cho2, 332
Choanoflagellate, 133
Cholera AutoInducer-1, 284
Cholestenone, 534
Cholesterol, 534, 576, 737
Cholesterol dependent cytolysins, 736
Cholesterylation, 713
Choline kinase, 97
Choline phosphate, 97
Chromatograms, 242
Chronological lifespan (CLS), 354, 852
Claisen condensation, 127

Claisen-type condensation, 193
Clostridium, 101
C. aceticum, 27
C. acetobutylicum, 530
C. autoethanogenum, 28
C. beijerinckii, 529, 530
C. butyricum, 529
C. difficile LTA, 166
CO/H2/CO2 mixture (synthesis gas), 35
CO_2, 26
Cold-sensitivity, 778
Cold sensor, 782
Cold sensor DesK, 781
Cold stress, 778
Colicin Ia channel, 620, 621
Colicin M, 225
Collision, 549
Colloid osmotic lysis, 730
Colwellia, 15
Compatible solutes, 491
Complex fatty acids, 5
Complex lipochitin oligosaccharides, 293
Complex sphingolipids, 349
Concentration gradient, 582
Condensing enzyme FabH, 778
Confocal microscopy, 582
Conformational change, 640
Conical lipid, 807
Copolymer of 3-HB and 3-HV (P(3HB-co-3HV)), 489
Copper-catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC), 714
Cord factor, 203
Cortical actin cytoskeleton, 584
Corynebacteriales, 184
Corynebacterium, 8
Corynebacterium glutamicum, 813
Coulombic effects, 814
4-coumaroyl-HSL, 280
Counterflow, 640
Cryoelectron microscopy, 684
Cryo-protective function, 491
Crystal structure, 638
Csg1p, 347
CTP, 325
CTP/phosphocholine cytidylyltransferase, 97
Cubic phase *Qα*,, 528
Cupriavidus necator, 458, 459, 462–465, 467, 469, 488
C5 universal building blocks, 61
Curli, 683
Cyanobacteria, 782
Cyanophycin, 484
Cyclic-di-adenosine-monophosphate (c-di-AMP), 172
Cyclic dimeric guanosine monophosphate, 278
Cyclohexane, 529
Cyclopropanation, 796
Cyclopropane bond, 790
Cyclopropane fatty acid, 49, 789
Cyclopropane fatty acid synthase (Cfa), 49, 101, 767, 835
Cyclopropane fatty acyl residue, 101
Cys-scanning mutagenesis, 638
Cysteate, 132
Cysteine residues, 612, 615
Cytochrome bc_1, 566
Cytochrome *c*, 567
Cytochrome *c* oxidase, 566
Cytochromes, 75
Cytokines, 174
Cytokinesis, 352, 581
Cytolipin, 115
Cytophaga-flavobacterium-bacteroidetes (CFB), 113, 795
Cytoplasmic components, 599
Cytoplasmic membrane, 89, 124
Cytosolic fatty acid pool, 400

D
DAG kinases, 325
D-alanine, 168, 169
DAPA synthase (BioA), 268
N-Deacetylated COs, 304
Deacetylation, 296
Deacylation of steryl esters, 516
Decanoyl-ACP, 48
cis-3-decenoyl-ACP, 47, 531
DegP, 656
Dehydratase(s), 189
Dehydratase/isomerase enzyme, 190
Dehydrogenase/isomerase activities, 396
Δ11-18:1, 780
De novo fatty acid biosynthesis, 450
De novo synthesis, 319
Δ5−Des, 769
Desaturase, 204, 769
Δ5-desaturase, 401
Δ9 desaturase (DesA), 400
Des gene, 781
Desiccation, 446
DesK, 401
DesKR, 400
DesKR two-component regulator, 398

DesKR two-component system, 401
DesR, 401, 769
DesT, 398, 400
Detergent, 812–813
Detergent-resistant membranes (DRM), 586
Dethiobiotin, 268
Deuterium isotope effects, 647
Deuterium oxide, 647
Dga1p, 511, 517–520
DHHC, 712
Diacylglycerol (DAG), 98, 168, 365, 498
  recycling, 169–170
Diacylglycerol acyltransferase, 448
Diacylglyceryl-homoserine, 100
Diacylglyceryl-*N,N,N*-trimethylhomoserine, 100
Dialkylether glycerol, 94
Dialkylether glycerophosphoinositide, 94
Dialkylresorcinol(s), 146, 282
7,8-Diaminopelargonic acid, 268
Diastereomers, 550
Diatoms, 9
Diauxic shift, 852
Differential distribution of pathways, 66
Diffusible signal factor, 276
Digeranylgeranyl-G1P, 544
Diglucosyldiacylglycerol (DGlcDAG), 528
Diglycosyl diradylglycerol (DGDG), 527
Dihydroceramide, 126, 128
Dihydroceramide desaturase, 347
DiI-C12, 585
Dimethylallyl diphosphate, 61
Disaccharides, 638
Disordered membranes, 778
Diterpenes, 70
Division, 549
Divisome, 557
Dlt proteins, 169
dltXABCD gene operon, 171
DNA-binding domain, 398
DNA-binding response regulator DesR, 781
cis-2-dodecenoic acid, 276
Double bond(s), 777
cis-Double bond, 397
Double electron–electron resonance spectroscopy, 645
Downstream processing, 494
Dpl1p, 349
Drug-tolerant bacteria, 203
Dual topologies, 625
DXP pathway, 63
DXR-I, 64
DXR-II, 64

E
Ech complex (energy-conserving hydrogenase), 32
Ectomycorhiza, 305
Effector proteins, 678
Efflux, 640
*egs*A, 543
Elastomer, 487
Electrochemical H^+ gradient, 639
Electrogenic transport, 647
Electron bifurcation, 30
Electron carriers, 440
Electroneutral transport, 647
Electrostatic and hydrogen-bonding network, 644
Electrostatic interaction, 577
Electrostatic surface potentials, 641
Elongation, 780
Elongation condensing enzyme, 47, 394
Elongation module, 764
Elongation of fatty acids, 394
Embden-Mayerhoff-Parnas pathway (EMP-pathway, glycolysis), 37
Emiliania huxleyi, 827
Endocytosis, 352
Endogenous fatty acids, 392
Endoplasmic reticulum (ER), 611
Endosomal sorting complex required for transport, 598
Endotoxin, 152–154
Energetic content, 497
Energy density, 486
Energy metabolism, 588
Energy reserve, 486
Enhance conformational sampling, 746
Enhanced biological phosphorus removal, 485
Enoyl-ACP reductase, 47, 395
Enterotoxigenic *E. coli* B7A, 587
Enzymatic redundancy, 245
Epidermal FABP (E-FABP), 693
Equilibrium exchange, 640
Equinatoxin, 734
Ergosterol, 72, 380, 384, 585
ER integral membrane protein, 328
ER mitochondria encounter structure (ERMES), 330
Escherichia coli, 9, 152, 155, 157, 453, 529, 555, 577, 612, 617, 620, 623, 625, 638, 780, 803, 808, 810–811
  FadR transcriptional regulatory mechanism, 399
Esters of fatty acid, 484
Ethanolamine, 95

Ethanolamine-P, 530
Ether-type lipids, 72
Ethionamide, 198
Eukaryotic target cell, 679
Eustigmatophytes, 9
Evolutionary origin, 67
Excimer fluorescence, 810
Exogenous fatty acids, 392, 393, 404
Export apparatus, 679
Export machineries, 205
Extracellular matrix, 203
Extracellular membrane vesicles (EMVs), 598
Extramembrane domains (EMD), 559, 610, 612, 622, 623, 625, 627, 632

F
FabA/FabB pathway, 395, 766, 767
FabD, 766
FabF, 397, 766, 780
FabG, 766
FabH, 531, 766
FabI/K, 766
FabM, 767
FabN, 767
FABPs, *see* Fatty acid-binding proteins (FABPs)
FABP4
  circulating concentrations, 697
  combined deficiency of, 695–696
  deficiency, 693, 694
  ectopic expression, 698–699
  expression of, 693–694
  intracellular and extracellular functions of, 701
  secretion and function, 696
  stoichiometry of, 693
FABP5
  circulating concentrations, 697–698
  combined deficiency of, 695
  expression, 695
  secretion and function, 696–697
FabR, 398, 400, 769, 770
FabT, 398, 769, 770
FabT, of *Streptococcus pneumoniae*, 403
FabX mechanism, 404
FabZ, 766, 767
FadR, 398
FadR transcriptional factor, 398
F-antigen, 166
FapR, 398, 769, 770
  of *Bacillus subtilis*, 401–403
fap regulon, 402
Farnesol, 586
Farnesyl diphosphate, 586
Farnesyl transferase, 711
FAS II regulatory controls, 771
FasR, 394
$FasR_{CG}$, 425
$FasR_{SC}$, 425
FA synthase type II system (FAS II), 411
Fat-storage inducing transmembrane (FIT) proteins, 514
Fatty acid(s), 44, 141, 260, 392, 446
  for antiobiotic development, 51–52
  biodiesel production, 52
  biosynthesis of, 4–9
  branched-chain, 44
  chemical feedstocks, 4
  degradation, 10–12
  elongation of, 394
  glycerolipids, 9–10
  membranes, 4, 9, 12
  modifications, 47–51
  monoenoic, 12
  polyenoic, 15
  Pyrococcus furiosus, 4
  research, 53
  saturated, 12
  signaling molecules, 4
  in *Thermotoga maritima*, 4
  type II fatty acid biosynthesis pathway, 46
  unsaturated, 44
Fatty acid-binding proteins (FABPs), 692–693
  adipocyte/macrophage/endothelial, 693–696
  isoforms, 692
  as therapeutic targets, 699 (*see also* Fatty acid-binding proteins (FABPs))
Fatty acid biosynthesis, 394–396
  FadR degradation in *E. coli*, 398–400
  transcriptional regulation of, 398
Fatty acid biosynthesis regulator (FabR), 400
Fatty acid desaturases, 781
Fatty acid ester, 279
Fatty acid α-hydroxylases (Fah), 130
Fatty acid kinase, 392
Fatty acid synthase (FAS), 8, 187
  type II fatty acid synthase (FAS II), 46, 394
Fatty acid synthesis
  in *Corynebacterium*, 421
  elongation, 766–767
  initiation module, 764–766
  in *Mycobacterium*, 421–423

Fatty acid synthesis (*cont.*)
regulation, 424–427
in *Streptomyces*, 419–421
type II, 764
Fatty acyl, 296–299
Fatty acyl-ACP, 240, 246, 248
Fatty acyl alcohol, 451
Fatty acyl-CoA, 240, 246
Fatty acyl-CoA reductase(s), 239, 451
Fatty alcohol, 239, 243
Fatty aldehyde, 239, 451
Fatty aldehyde dehydrogenase, 241, 245
Fatty aldehyde reductase, 239
Fatty aldehydes, 248
Feast and famine, 493
Feed-back regulator, 393
Feed-forward control mechanism, 402
Fem transferases, 224
Ferredoxin, NADPH oxidoreductase (Nfn), 30
F1Fo-ATP synthases, 32
Filamentation phenotype, 171
Fimbriae, 682
FkpB binding protein A (FkpA), 657
Flagellar basal body, 681
Flagellum, 681
Flavin, 31
Flavolipin, 115, 117
Fld1p, 514
Flip-flopping membrane proteins
biological relevance of TMD reorientations, 628–631
Charge Balance Rule, 621–624
membrane protein topological duality and lipid-dependent reversibility, of topological interconversion, 624–627
monitoring membrane protein and lipid flip-flops, 628
monitoring TMD topology dynamics and interconversions *in vitro*, 627–628
pathogenic mutations, 632
research needs, 632–633
SCAMTM, 611–621
TMD protein structure, 611
Flippase, 92
Floc-forming bacteria, 492
Fluid mosaic model, 576, 742
Fluorescein arsenical helix binder-ethylenediamine$_2$, 813
Fluorescence microscopy, 803
Fluorescent emission, 579
FM 4–64, 579
Foamy macrophages, 202
Folding pathway, 656
Force field (FF), 743
Force from lipid principle, 816
Förster resonance energy transfer (FRET), 628
Fosmidomycin, 67
Francisella tularensis, 784
FtsZ, 171
Fucosylation, 299–300
Functional classes of isoprenoids, 71
Functional membrane microdomains, 89, 586
Fungi, 305–308
Fusion, 549

G
α-Gal, 168
Galactolipids, 826
β-D-galactopyranosyl 1-thio-β-D-galactopyranoside, 641
Galactoside/H^+ symport, 639
G1 arrest, 350
Gas vesicle, 485
Gel phase ($L\beta$), 528
Gene expression analysis, 839
General secretory pathway, 677
General stress response, 397
Genetic engineering, 452
Genome mining, 441
Genome-scale metabolic models (GEMs), 386
constraint-based modeling, 377–378
flux balance analysis, 378–379
flux variability analysis, 379
generation of, 376–377
in silico modeling, 379
metabolic engineering, 379
model reconstruction, 377
Genomic databases, 309
Genotoxic response, 350
Geosmin, 71, 78, 286
Geranylgeranyl-PP, 544
Geranylgeranyltransferase, 711
Gibbs standard free energy change, 548
GlcN, 168
Global warming, 38
Gluconobacter, 128
Gluconobacter cerinus, 113
D-Glucopyranosides, 638
Glucosamine, 166
D-Glucose, 638
Glucose, 641
Glucosylgalactosyldiacylglycerol, 827
Glutamine lipids, 115
Glycerol, 452
Glycerol acetal, 529

Glycerol-1-phosphate, 89
Glycerol-3-phosphate, 89, 396, 532
Glycerol-3-phosphate acyltransferases (GPATs), 91, 319
Glycerolipids, 9, 316
Glycerol monomycolate, 201
Glycerol-phosphate acyltransferases, 51
Glycerophosphocholine, 97
Glycerophospholipid, 88, 90, 316, 365, 576
Glyco-cholesterol derivatives, 587
Glycogen, 484
Glycolipid(s), 825
Glycolipid anchor, 165
Glycosphingolipid, 576
Glycosylalditol-phosphate polymers, 166
Glycosyl diacylglycerol, 99
Glycosylphosphatidylinositol (GPI), 713
Glycosylphosphatidylinositol (GPI)-anchored proteins, 351, 713
Glycosyltransferase, 99, 169
Glyoxylate shunt enzymes, 398
G1P, 542–544
G1P dehydrogenase, 543
G3P dehydrogenase, 543
GPT2/GAT1, 319
Gram-negative, 577
Gram-negative bacteria, 124, 672, 730
Gram-positive, 597, 598, 604
Gram-positive bacteria, 397, 531, 577
GtlA (Lmo0933), 169
Guanosine penta-and tetraphosphate (ppGpp), 397
GW module protein, 172

H

HadA–HadB–HadC, 423
β-Hairpins, 656
Halophiles, 241, 491
Hcp tube, 681
Head-to-tail condensation, 190
Heatstress response, 353
Helicobacter pylori, 587
Heliothis zea, 12
2-Helix coiled coil (2-HCC), 781
Helix packing, 640
Helix-turn-helix motif, 402
HemeA, 75
HemeO, 75
Hemiterpene synthases, 71
Hemolysin, 677
Heptadecanol, 251
2-Heptyl-3-hydroxy-4(1H)-quinolone, 282
Hereditary sensory and autonomic neuropathy type I (HSAN1), 342
Heterologous genes, 454
Hexadecanol and hexadecane, 502
High affinity, 632
High cell density processes, 497
High resolution mass spectrometry, 354
Histidine kinase, 781
Histidine kinase Hik33, 782
Histidine kinase sensor, 279
Histidine zipper, 251
HMG-CoA reductase (HMGR), 61
  class 1 (HMGR-I), 64
  class 2 (HMGR-II), 64
Holo-ACP synthase, 46
Homeostatic regulations, 824
Homeoviscous adaptation, 777
Homoacetogenesis, 37
Homoviscous adaptation, 789
Hopane, 534
Hopanoids, 74, 587, 788, 792–794, 796, 808
Host cationic antimicrobial peptides, 173
Host cells invasion, 173
Host-specificity, 293
Hot-dog fold, 402
Human pathogens, 785
Hybrid multi-scale simulations, 747
Hydrocarbons, 549
Hydrogen-bonding interactions, 642
Hydrogen-dependent CO2 reductase (HDCR), 28
Hydrophilic compound, 549
Hydrophobic interaction, 577
Hydrophobic mismatch, 816
3-Hydroxyacyl-ACP, 394
3-Hydroxyacyl-ACP dehydratases, 47, 395
3-Hydroxybuyric acid (3HB), 486
3-Hydroxy-decanoyl-ACP, 48
β-Hydroxydecanoyl-ACP, 531
α-Hydroxyketone, 284
Hydroxylamine, 718
3-Hydroxylated fatty acids, 49
S-2-Hydroxylation, 49
27-Hydroxyoctacosanoic acid, 49
3-Hydroxypentadecane-4-one, 284
3-Hydroxytridecane-4-one, 284
Hysteresis, 396

I

IclR, 398
IDI-I, 65
IDI-II, 65

I-layer, 680
Immune response, 230
Immunofluorescence microscopy, 813
Immunostimulatory activity, 201
Increased sensitivity to antibiotics, 784
Indolecarboxamide, 200
Induced-fit mechanism, 642
Industrial and urban wastes, 493
Industrial by-products, 493
Industry, 447
Infection, 292
Inflammation, 173
Influx, 640
Inhibition of FabI, 771
In-host prenylation, 77
Initiation module, fatty acid synthesis, 764, 765
Injectisome, 678
Innate immune system, 92, 125, 784
Inner membrane, 594, 599, 672
Ino2-Ino4-Opi1 yeast circuit, 328
Inositol/choline-responsive elements (UASINO), 328
Inositolphosphorylceramide (IPC), 347, 352
Integral membrane proteins, 672
Inter-kingdom communication, 275
Inter-species communication, 275
Intracellular inclusion, 484
Intracellular storage, 484
Intracytoplasmic lipid-bodies, 500
Intraluminal vesicles, 598
Intra-species signaling, 278
Intrinsic affinity, 581
Intrinsic curvature, phosphatidylglycerol, 811
Intrinsic strain, 808
Invariant natural killer T cells (iNKT), 131
Invasion plasmid antigen J (IpaJ), 718
Inverted hexagonal H_{II} phase, 528
Inverted topology, 616
Inward-open conformation, 641
Ion homeostasis, 172
Ipt1p, 347
Iron-sulfur clusters, 31
Isc1, 348
iso/*anteiso* ratio, 778
Isoamyl alcohol, 249
Isobaric tags for relative and absolute quantitation (iTRAQ), 716
2-Isocapryloyl-3R-hydroxymethyl-γ-butyrolactone, 280
Iso chain, 528
Isoenzymes, 449
Iso fatty acids, 791
Isoleucine, 777
Isomerase (IDI), 61
cis-trans isomerase, 101, 531
cis-trans isomerization, 531
Isoniazid, 52, 197
Isopentenylation, 77
Isopentenyl diphosphate, 61
Isopentenyl phosphate kinase, 62
Isoprene, 79
Isoprenoid(s), 58
Isoprenoid lipids, 806
Isotope-coded affinity tag (ICAT), 716
Isoxyl, 198

J
Jasmonate pathway, 17
Jojoba oil, 453

K
KAPA synthase, 268
KasA and KasB, 422
Kennedy pathway, 447, 498
β-Ketoacyl-acyl carrier protein synthase III, 247
β-Ketoacyl synthase, 416
7-Keto-8-aminopelargonic acid, 267
3-Ketodihydrosphingosine reductase, 344

L
L430, 118
L654, 118
Label-based quantitative methods, 716
Label-free quantification, 715
lac operon, 638
Lactobacillic acid, 49, 791
Lactococcus lactis, 813
Lactose permease (LacY), 559, 618, 620, 623, 625, 627, 628
Lactose symport mechanism, 646–647
LAG1 and LAC1, 346
Lands' cycle, 324
Lantibiotics, 229
LASSs/CerSs, 346
Last universal common ancestor (LUCA), 26, 541
Lateral gene transfer, 67
Lateral phase separation, 809, 810
Lateral pressure profile, 806, 816
Laurdan, 585
Legionella, 284

Legionella autoinducer-1, 284
Legionella pneumophila, 97
Leprosy, 182
LicA, 171
LicB permease, 171
Lifestyle change, 278
Lignocellulosic raw materials, 452
N-Linked fatty acyl chain, 296, 297
Linum usitatissimum, 15
Lipases, 324
Lipid, 484, 485, 526
  bilayer, 88, 554
  carrier, 225–228
  component part, 547
  degradation, 486
  divide, 547
  domains, 88, 563–564
  homeostasis, 426
  importer, 195
  inclusion bodies, 450, 454
  microdomains, 534, 626
  peroxidation, 750
  pools, 334
  raft, 88, 534, 576, 809
  staining techniques, 576
  translocase, 579
Lipid A, 51, 124, 783–784, 794, 795
  acylation, 152
  EptA, 156
  lipopolysaccharide, 157–158
  modifications, 154–155
  PagP, 156–157
  research, 159
Lipid A-hydroxylase, 113
Lipid-driven differentiation, 550
Lipid droplet(s) (LDs), 318, 510, 859–860
  biogenesis models, 368–371
  Dga1p, 517–520
  formation, 513–514
  structure of, 367–368
  TAG lipases, 515–516
Lipid I, 218
Lipid II, 218
Lipidic signal, 274
Lipid metabolism in yeast
  oleaginous yeasts, 383
  omics data analysis, 385–386
  S. cerevisiae, 380–383
  target prediction, 384–385
"Lipid" mutants, 618
Lipidomic approaches, 334
Lipid phosphate phosphatases (LPP), 325
Lipid remodeling
  algae, 838–839
  bacteria, 839–841
  plants, 836–837
Lipoate-protein ligase, 263
Lipogenesis, 454
Lipoic acid, 260
Lipolysis, 326
Lipopeptides, 229
Lipopolysaccharide (LPS), 124, 154, 157, 577, 737, 792, 795–796
  transport, 130
Lipoproteins, 672
Liposomes, 813
Lipoteichoic acid (LTA), 98
  biosynthesis of, 168–171
  functions, 171–174
  molecular structure, 164–168
  structural complexity, 175
  target for anti-infective, probiotics and vaccines, 174–175
  type I, 166
  type IV, 166
Lipoyl group, 260
Lipoyl synthase, 263
Liquid crystalline phase, 528
Liquid-ordered phase formation, 587
Listeria, 94
Listeria monocytogenes, 397, 531, 778
Liver kinase B1 (LKB1), 630
Ljungdahl, L. G., 28
Localization of specific factors, 173
Long-chain acyl-ACP, 396, 403, 404
Long-chain acyl-CoA, 399
  carboxylases, 414–415
  synthetase, 392
Long-chain alcohols, 500
Long-chain aliphatic alcohols, 286
Long chain base (LCB), 342
  kinases Lcb4/5, 348
Long chain fatty acid, 12
Long-chain fatty acid methyl ester, 279
Long-chain length PHA (LCL-PHAs), 488
Long-chain organic acid (LCOAs), 488
Long-chain saturated acyl-ACP, 400
Long-chain unsaturated fatty acyl thioesters, 400
Low-to-zero value substrate, 502
LpDx2 acyltransferase, 784
lpiA, 793
LpxD1 acyltransferase, 784
LpxL acyl transferase, 783
LpxM acyl transferase, 783

lpxM and *lpxP*, 784
LpxO, 795, 796
LpxP acyl transferase, 784
Lro1p, 511
L-serine, 547
LtaA, 168
LTA, *see* Lipoteichoic acid (LTA)
LtaS, 169
LuxI synthase, 279
LuxR receptor, 279
Lysine lipids, 114
LysoPA acyltransferases (LPAATs), 323
Lysophosphatidic acid (LPA), 532
Lysosomes, 857
Lysyl-PG, 793
Lysyl-phosphatidylglycerol, 92
Lysyl-tRNA, 92
LytR-CpsA-Psr (LCP) family of proteins, 170

M
MabR, 394, 425
Macromolecular crowding, 814
Magnetosome, 485
Major non-polymorphic histocompatibility complex, 132
3-(*N*-maleimidylpropionyl) biocytin, 612
Malonyl-ACP, 394, 396
Malonyl-ACP methyl ester, 265
Malonyl-CoA, 4, 47, 394, 396, 402, 403, 414
  ACP transacylase, 47, 262, 394
Malonyl-condensation, 297
Malonyl-phosphopantetheine moiety, 403
Maltose binding protein, 241, 251
Mannose-(inositol-P)2-ceramide (M(IP)2C), 347, 352
Mannose-inositol-phosphoceramide (MIPC), 347, 352
Marine environment, 825
Marine snow, 238
Marinobacter, 238
Marinobacter hydrocarbonoclasticus, 451
MarR regulators, 403
Mass spectrometry analysis, 323
Mass spectrometry-based proteomics, 710
Mechanosensitive channels, 813
Mechanosensitive channels of large (MscL), 816–817
Mechanosensitive channels of small (MscS), 816
Medium and long chain-fatty acyl and fatty alcohols, 501
Medium-chain length PHA (MCL-PHAs), 488
Melittin, 731
Membrane, 4, 9, 12, 154, 156, 157
  compartmentalization, 585
  depolarization, 585
  domains, 809
  EMVs, 598–599
  fluidity, 392, 397, 806
  lipid, 392, 554, 610, 618, 620, 623, 625, 626, 633
  lipid catabolism, 824
  OMVs (*see* Outer membrane vesicles (OMVs))
  remodeling, 115
  targeting sequence, 564, 577
  tension, 802
  thickness, 401, 781, 808
  trafficking, 585
  vesicles, 597, 599, 602
  viscosity, 784
Membrane-forming enzymes, 546
Membrane fusion protein (MFP), 677
Membrane homeostasis, 769
  carbon limitation, 834–836
  nitrogen limitation, 832–834
  phosphorus limitation, 825–832
Membrane protein, 554
  folding, 632
  flip-flopping (*see* Flip-flopping membrane proteins)
  topogenesis, 627
  topologies, 626
Membrane regions with increased fluidity, 583
Menaquinones, 75
Meromycolic acids, 422
Meromycolic chain, 184
Mesorhizobium, 114
Mesorhizobium loti, 99
Metabolic balance, 501
Metabolic engineering, 453
Metabolic network, 376
Metabolic water, 486
Metabolism, 317
Methionine salvage pathway, 65
N-Methylation, 296
Methyl-branched fatty acids, 397
Methyl-branched isoprenoid chain, 549
Methyl branching, 777
2-Methylbutyryl-CoA, 397, 531
Methyl-β-cyclodextrin (MβCD), 627, 628
11-Methyl-*cis*-2-dodecenoic acid, 276
2-Methyl 3-erythritol-4-phosphate (MEP) pathway, 63
Methyl 3-hydroxymyristate, 279

Methyl 3-hydroxypalmitate, 279
Methyl ketone, 286
Methylmalonyl-CoA, 414
Methyltransferase, 191
  N-Methyltransferase, 114
  O-Methyltransferase, 265
Mevalonate (MVA) pathway, 61–63, 544
Micelles, 813
Microbes, 14
Microbial fuel cells, 238
Microbial mats, 491
Microbiome, 16, 17
Minicell, 580, 810
Minor phospholipids, 89
Min system, 581
Mitis group, 168
Mitochondria, 11, 329, 566–567
  function, 351
  transport, 332–333
Mitochondrial contact sites (MICOS), 333
Mixed microbial communities, 492
Mixed microbial culture (MMC), 492
Model membranes of cells, 749
Molecular dynamics (MD), 744
Molecular simulation, 743
Monoacylglyerol lipase, 516, 519
Monoenoic fatty acids, 12–15
  cis and *trans*, 12
  etymologies of, 14
  microbes, 14
Mono-functional protein, 416
Monogalactosyldiacylglycerol (MGDG), 827
Monoglucosyldiacylglycerol (MGlcDAG), 528
Monomeric composition, 494
Monooxygenase EthA, 198
Monoterpenes, 70
Monounsaturated fatty acid (MUFA), 317
Moorella thermoacetica, 28
Mortierella alpina, 384
MraY, 220
MreB cytoskeleton, 583, 811
mRNA, 243
MsbA flippase, 130
mtFabH, 422
Mucor circinelloides, 384
Multicellular rosette, 133
Multidimensional protein identification technology (MudPIT), 714
Multi-drug resistant transporter EmrE, 620
Multifunctional polypeptides, 416
MurG, 222
MurJ, 224
Mustela vison, 15
Mutagenesis, 248
Myc factors, 305
Mycobacterial cell envelope, 417
Mycobacterium, 8, 394, 421
  M. smegmatis, 436, 440
  M. tuberculosis, 184, 450, 580
Mycolic acid, 4, 394, 411
  biogenesis, 197–200
  biological functions, 200–204
  biosynthetic machinery and regulation, 187–197
  conformational packing, 186–187
  definition, 182
  research, 204–205
  structure, 184–186
  synthesis inhibition, 182
Mycoloyltransferases, 194
Mycomembrane, 182
Mycoplasma pneumoniae, 99
Myo-inositol, 547
N-Myristoylation, 711–712
Myristoyltransferase, 12
N-myristoyltransferase (NMT) enzymes, 711
Myxococcus xanthus, 125, 128, 602, 604

N
Na^+-dependent glutamate/aspartate transporter (GLAST), 632
Nannochloropsis gaditana, 9
Nanobodies, 645
Nanodiscs, 813
Native-oil producers, 454
Natural killer T cells, 125
Natural products, 58
Navicula, 14
Negative control, 302
Negative feedback, 396
Negatively curved inner leaflet, cardiolipin, 810
Negative regulators, 195
Neisseria meningitidis, 605
Nem1/Spo7 complex, 326
Neutral lipids, 446
Nile Red, 584
Nisin, 229
Nitrogen fixation, 292, 491
Nitrogen-fixing bacteria, 491
4-Nitrophenyl-α-D-galactopyranoside (α-NPG), 641
10-*N*-Nonyl acridine orange (NAO), 579
Nocardia, 8
nodA-like genes, 308
nod boxes, 302

Nod factors, 292, 296, 297, 299, 301, 303, 304
Nod genes regulation, 301–302
Nodulation competitiveness, 92
Non-bilayer prone lipids, 562–563
Non-catalytic site, 248
Non-fermentable carbon sources, 326
Non-halogenated solvents or ionic liquids, 502
Nonionic detergents, 586
Nonpolar lipids, 497
  degradation, 514–519
  steryl ester synthesis, 512
  triacylglycerol synthesis, 511
Non-reducing end, 301
Non-tuberculous, 184
nSMase2, 348
N-terminal lipid anchor, 658, 659
N-terminal signal sequence, 684
Nuclear factor-erythroid 2 (NF-E2)-related factor (Nrf1), 631
Nucleoid occlusion (NOC) system, 581

O
Octadecenoic fatty acids, 16
Octanoic acid, 260
Octanoyl-ACP, protein *N*-octanoyltransferase, 263
O-layer, 680
Oleagenicity, 454
Oleaginous yeasts, 383–384
Oleochemicals, 447
Oligoisoprenylated nucleic acids, 77
Oligomerisation, 734
OlsBA, 113
OlsC, 113, 794
OlsD, 114, 794
OlsF, 113
Omics data analysis, 379–380
OMVs, *see* Outer membrane vesicles (OMVs)
One-component systems, 398
Open biological systems, 492
Operator sequences, 398
Opi1, 328
Opi3, 332
OpuA, 813
Ordered-binding model, 646
Ornithine-containing lipid, 98
Ornithine lipids, 792, 794, 831
  synthesis, 110–114
Oscillatory movement, 582
Osmolality, 814
Osmolytes, 581
Osmoprotectant, 172
Osmosensing transporters, 813–817
Osmosensory mechanism, 814
Osmotic pressure, 802
Osmotic stress response, 581
Osteoclastogenesis, 132
Outer membrane, 89, 116, 672
Outer membrane vesicles (OMVs), 594
  biofilm formation, 602–603
  mechanisms of OMV biogenesis, 600–601
  pathogenesis, 603
  predatory behavior, 604
  regulation of OMV biogenesis, 601–602
  structures, composition and function, 599–600
Outward-open conformation, 641
Overall fold, 640–645
Overexpression, 452
β-Oxidation, 453, 501
  of fatty acids, 398
  pathway, 52
Oxidative stress tolerance, 808
3-Oxoacyl-ACP reductase, 47, 394
3-Oxoacyl-ACP-synthase I, 47, 394
3-Oxoacyl-ACP-synthase II, 47, 394
3-Oxoacyl-ACP synthase III, 47, 262, 394
2'-Oxoalkylresorcinolic acid synthase (ORAS), 441
Oxoamine synthases, 127
Oxoester, 500
N-3-Oxohexanoyl-HSL, 279
3-Oxo-sphinganine, 126, 128
Oxygen limited processes, 497
Oxygen-requiring desaturases, 400

P
Pah1 regulation, 326
Palmitic acid, 392
Palmitoleoyl-ACP-dependent acylation, 784
Palmitoleyl-ACP, 47, 395
Palmitoyl-ACP, 246, 396, 404
S-Palmitoylation, 712
Palmitoyl-CoA, 323, 396
Palmitoyl transferase, 836
PalmPISC method, 719
Pantothenic acid, 396
Pathogenesis, 603–604
Pathogenic mutations, 632
PE-methyltransferases, 332
Peptidoglycan, 170, 218, 228–230, 597, 599, 600, 602
Peptidoglycan biosynthesis precursor lipid II, 585

Peptidoglycan cell wall, 577
biosynthetic machinery, 584
Peptidoglycan layer, 682
Peripheral membrane proteins (PMPs), 751
Peripheral protein, 577
Periplasmic chaperones, 655
Periplasmic glucan, 98
Phaeodactylum tricornutum, 827
Phagophore assembly site (PAS), 855
Phase behaviour, 806
Phase changes, 748
Phase separation, 549, 550
Phase transition temperatures, 808
Phasin, 487
PHA synthase, 488
Phenolic lipids, 140
biosynthetic machinery in bacteria, 440–441
distribution, 436
fungal and plant type III PKSs for biosynthesis, 441
Phenylalanine permease (PheP), 620
Pho80-Pho85, 327
PhoB, 116
Polyhydroxyalkanoates (PHAs) granule, 487
PHA-producing organisms, 492
Pho80-Pho85 kinases, 327
Phosphatases, 227
Phosphatidate phosphatase, 325
Phosphatidic acid (PA), 10, 91, 318, 364, 396, 498, 532, 577
phosphatase, 323, 452
transporter, 331
Phosphatidyethanolamine, 684
Phosphatidylcholine (PC), 95, 555, 684, 806, 808
Phosphatidylcholine synthase, 97
Phosphatidylethanolamine (PE), 89, 95, 332, 527, 555, 557, 618, 623, 625, 627
Phosphatidylethanolaminylation, 713
Phosphatidylglycerol (PG), 89, 91, 169, 329, 527, 555, 557, 662, 683, 825–827
Phosphatidylglycerol phosphate, 91
Phosphatidylglycerol phosphate synthase, 91
Phosphatidylinositol, 94–95, 555
Phosphatidylinositol phosphate, 94
Phosphatidylinositol phosphate synthase, 94
Phosphatidylinositols, 383
Phosphatidyl *N*-methylethanolamine, 529
Phosphatidylserine (PS), 95, 332, 527, 556
Phosphatidylserine decarboxylase Psd1, 331
Phosphatidylserine synthase, 95
Phosphoanhydride bond, 94
Phosphocholine, 684
Phosphodiester-linked units, 164
Phosphoethanolamine, 684
3-Phosphoinositide-dependent kinase-1 (PDK1), 353
Phosphoinositide-dependent protein kinase 1 (PDK1), 630
Phosphoinositol ether lipid, 94
Phospholipase C, 98
Phospholipase D (PLD), 91, 834
Phospholipid(s), 124, 316, 380, 452, 526, 554, 764
Phospholipid acyl-chain desaturase, 101
Phospholipid *N*-methyltransferase, 95
Phospholipid scramblase 1 (PLSC1), 631
Phosphomevalonate kinase, 544
4-Phosphopantetheine, 8
Phosphorelay, 278
Phosphorus-free lipid, 98
Phosphorus-limiting condition, 97
Phosphorylation, 323, 630
Phosphotransferase, 228
Photorhabdus clumping factor, 283
Photosynthetic membrane, 99
Photosynthetic organisms
carbon limitation, 834–835
nitrogen limitation, 832–834
phosphorus limitation, 825–829
Pichia pastoris, 368
Pili, 675, 682
Pilin VirB2 subunit, 617
Pilotin, 678
Pimeloyl-CoA synthetase (BioW), 267
Piperidinol derivative, 200
Pkh1/2, 353
Pkh-Ypk pathway, 354
PKS, *see* Polyketide synthase (PKS)
Planctomycete, 114, 117
Plasmalogen, 101, 529
Plasma membrane, 218
Plasticity, 64
Platelet-activating factor receptor, 97
Platencin, 52
Platensimycin, 52
PlsB, 396, 404, 532
PlsC, 532
PlsX/PlsY system, 396, 532, 771
PmrC, 156
Polar head group-attaching enzymes, 547–548
Polar localization, 815–816
Polar region, 579
Poles, 193
Poly(3-hydroxybutyrate) (P(3HB)), 486

Polyenoic fatty acids, 15–17
classification, 15
jasmonate pathway, 17
potential isomers, 15
signaling cascades, 17
Polyester, 486
1,3-Polyglycerolphosphate [poly(GroP)], 164
Polyhydroxyalkanoate (PHA), 458, 466
carbonosome, 464
cycle, 460–461, 465–467
granules, 458, 460, 465
metabolic pathways for synthesis, 459–460
synthase, 459–463, 465, 468, 470
Polyisoprenoid lipids, 587
Polyketides, 436
Polyketide synthase (PKS), 140, 187, 411
Polymerization degree (DP), 294
Polyphosphate, 484
Polyphosphate-accumulating organism, 485
Polyunsaturated fatty acids (PUFAs), 9, 531
Polyunsaturated α-MAs, 184
Positive Inside Rule, 622, 623, 626, 633, 641
Positively curved outer leaflet, cardiolipin, 810
Post-insertional changes, 622
Post-translational modification, 684
Potassium transport, 172
POTRA domain, 659
ppGpp, 404, 771
4′-Phosphopantetheine, 46
PRELI, 331
Prenylation, 77
S-Prenylation, 711
Prenylphosphates, 70
Prenyl transferase, 68, 225, 546
Primary endosymbiosis, 68
Primary metabolites, 59
Probiotics, 174
Pro-growth signal, 349
Promoter, 302
ProP, 813
Propilin (pVirB2), 617
Propionyl-CoA carboxylases, 414
Protein
acyl transferases, 712
lipidation, 708
and lipid flip-flops, 628
phosphorylation, 197
secretion, 679
topogenesis, 610, 622, 626, 627, 630, 633
translocation, 680
translocation machinery, 580
Protein data bank, 241
Protein kinase A (PKA), 327
Protein kinase C (PKC), 327
Protein palmitoyl thioesterases, 712
Protein-protein interaction, 415
Proteobaceteria, 17
Proteolipid domain, 588
Proteoliposome, 627, 813, 814
Proteomic studies, 334
Pseudomonas, 458, 460–465, 467
P. aeruginosa, 400, 602
P. putida, 460–462, 464–467, 469–473, 529, 531, 580, 602
P. syringae, 531
Pseudomonas Quinolone Signal, 282
Pseudopilus, 678
Psoriasis-associated FABP (PA-FABP), 693
Pss gene, 533
Pyridoxal 5′-phosphate, 127
Pyrite (FeS_2), 548
Pyrococcus furiosus, 4
Pyruvate, 30
Pyruvate dehydrogenase, 260
Pyruvate ferredoxin oxidoreductase, 30

Q
Quality control mechanism, 546
Quantitative headgroup analysis, 803
Quinol, 808
Quinolone, 282
Quinone, 74, 808
Quorum sensing, 274
Quorum signals, 594

R
Ralstonia, 279
Rate-limiting step, 647
ω3:ω6 ratio, 15
RavZ, 714
Readily biodegradable carbon sources, 493
Recruitment of non-homologous enzyme, 67
Red light-dependent, 783
Reducing end, 299–301
Reductive acetyl-CoA pathway, 27
Regulator of pathogenicity factor (*rpf*), 277
Regulatory circuits, 454
Regulatory network, 386, 450
Relaxed phosphatase state, 781
Remediation and lipid's waste valorisation, 502
Replication, 581
Replicative lifespan, 856
Repressors, 302
Resistance to stress, 491

Respirasome, 567
Response regulator, 279
Retinal, 76
Rhizobia, 292
Rhizosphere, 292, 492, 788
Rhodobacter nitrogen fixation, 32
Rhodobacter sphaeroides, 826
Rhodococcus, 410, 450, 499
  R. jostii RHA1, 245
  R. opacus, 497
R-3-hydroxybutyryl-CoA (3HB), 488
Rigid kink, 778
RNA polymerase, 397
RNA polymerase cofactor DksA, 397
RNA-Seq, 243
Rnf complex, 32
Rod-shaped bacteria, 584
Rosette colonies, 133
Rosette-inducing factors, 133
Rre26, 782
Rre31, 782
RT-qPCR, 243

S
Saccharomyces, 380, 383
Saccharomyces cerevisiae, 316, 367, 380, 384, 385, 510, 555, 795
  catalyzation, 510
  Dga1p, 511
  polypeptides in nonpolar lipid metabolism, 517–519
  TAG lipases, 514
Salinibacter sulfonolipid, 133
Salinity, 803
Salinity tolerance, 807
Salt bridge, 644
Saturated fatty acid (SFA), 12, 317
  etymologies of, 13
  eukaryotic cells, 12
  phospholipids and storage lipids, 12
Scs2, 328
Scs7p, 347
SCT1/GAT2, 319
Sea anemones, 730
SecA-dependent protein translocation, 615
Sec export machinery, 655
SecG, 615, 616
Secondary active transporters, 638
Secondary endosymbiosis, 68
Secondary metabolism, 59
Secondary metabolites, 78–79
Secondary structure, 731
Secondary transporters, 557
Second messenger, 98
Sec protein translocation apparatus, 586
Secretin, 678
Secretion, 302–303, 673–674
Secretion system, 677–681
Secretome analyses, 697
SecYEG translocon, 615, 674
Segregation of chromosome, 581
Self-defensive protection, 498
Sensory domain, 398
Septa, 193
Septal regions, 580
Septum, 173
Ser/Thr and Tyr protein kinases and phosphatases, 426
Ser/Thr kinase SpkE, 783
Serine/threonine protein kinases, 197
Serineglycine lipid, 115, 117
Serine palmitoyl transferase (SPT), 126, 344, 587
Serratia, 114
Seventeen kilodalton protein (Skp), 655–657
Shedding microvesicles, 598
Shewanella, 10
Short chain fatty acids, 12
Short-chain length PHA (SCL-PHAs), 488
Short-chain organic acid (SCOA), 488
Shortening, 778
Siderophores, 238
Signal, 784
β Signal, 661
Signaling cascades, 15
Signaling molecules, 4
Signaling pathways, 354
Signal peptidase I, 674
Signal recognition particle, 674
Signal transduction, 585
Single-component membranes, 748
Single molecule fluorescence resonance energy transfer, 645
Sinorhizobium meliloti, 110
Sirtuins, 712
Sit4, 350
Site-directed alkylation, 645
Small metal binding protein, 251
Small molecule inhibitor, 174
Sonic hedgehog, 713
Sorangium cellulosum, 125
Sorghum bicolor, 145
Sorgoleone, 145
Spatio-temporal localization of proteins, 585
Specific lipid interactions, 737

Sphinganine, 126
Sphingobacterium, 124
Sphingoid base, 124
Sphingolipid(s), 380, 788, 792, 795
  interacting domains, 355
  metabolism, 342, 344–349
Sphingolipidoses, 342
Sphingolipid targets, 355
Sphingomonas, 124
Sphingomonas paucomobilis, 125
Sphingomyelin, 534, 737
Sphingomyelinase, 736
Sphingosine-1-P, 349
Sphingosine-1-P lyase, 349
Spills of vegetable oils, 12
Spiral-like structures, 582
Spodoptera fragiterda, 12
Spontaneous lipid microphase separation, 810
Sporopollenin, 146
Sporulation, 491
SpoT, 397
Ssl3451, 783
Stable isotopic labeling of mammals (SILAM), 716
Stable isotopic labeling with amino acids in cell culture (SILAC), 716
Standard free energy change, 94
Staphylococcus aureus, 52, 393, 403, 587
Starvation conditions, 197
State of higher virulence, 97
Stationary phase, 850
Staudinger ligation, 714
Sterol esters, 383
Steryl ester (SE), 364, 367
  degradation, 516–517
  hydrolase, 516–517
  hydrolysis, 370
  synthesis, 512–513
Stigmatella aurantiaca, 128
*S*tomatin, *P*rohibitin, *F*lotillin and *H*flK/C (SPFH), 584, 586
Storage compound, 485
Storage lipids, 425
Straight chain, 549
Streptococcus pneumoniae, 168, 392
  FabT of, 403
Streptomyces, 280, 419, 449
  S. coelicolor, 811
  S. griseus, 142–143, 436, 438–439
Stress response pathways, 587
Substituents, 166
Substituted Cysteine Accessibility Method (SCAM™), 612, 614, 616, 617, 621
ε Subunit, 414
Sucrose permease (CscB), 625
Sugar-binding kinetics, 645
Suicide inactivator, 642
Sulfation, 300
Sulfolipid, 99
Sulfolipid sulfoquinovosyldiacylglycerol (SQDG), 825–826
Sulfonolipids, 132–134
Sulfoquinovosyl diacylglycerol, 98
Sulfur cycle, 491
Sulfur globule, 484
Supercomplex imaging, 568
Sur2p, 346
Surface enhanced infrared absorption spectroscopy, 644
Surface metabolism, 541, 548, 550
Survival factor protein A (SurA), 655
Symbiotic, 292
Symbiotic plasmid, 293
Synechococcus, 9, 99
Synechocystis, 783
Synthesis gas, 35
Synthetase (BioD), 268
Synthetic biology, 452
System biology, 355

T
TacF transporter, 171
TAG-accumulating machinery, 450
TagO, 171
TAG, *see* Triacylglycerols (TAG)
Tailor-made production, 502
Tandem MS techniques, 714
Taurine, 114
Taxonomic group, 579
Taxonomic markers, 58
Teichoic acids, 164
Teixobactin, 229
Terpene synthases, 70–73
Terpenoid, 58, 285
Tetradec-13-ynoic acid, 717
Tetratricopeptide repeat, 660
TetR family, 400
Thermodynamic equilibrium, 647
Thermoplasma-type MVA pathway, 62
Thermoplastic, 487
Thermotoga maritima, 4
Thermotolerance, 353
Thiacetazone, 198
Thin aggregative fimbriae, 676
Thioesterase, 52, 241, 246, 247, 396, 452

Thioester bond, 46
Thiolactomycin, 199
Thiol cross-linking, 645
Thiophene, 199
Thraustochytrid, 10
Three-carbon backbone, 549
Three-step process, 493
Time course, 303–304
Time-resolved FRET, 645
TolC, 677
Toll-like receptors (TLR), 131
Toll-like receptor 2, 118
Toll-like receptor 4, 117
Toluene, 531
Topogenic signal, 630
Topology, 303, 334, 559, 611
  constraints, 610
  duality, 624
  dynamics, 614–621, 627
  heterogeneity, 625
  inversion, 561, 616
  membrane protein, 626
  SecG, 616
TOR1, 852, 856, 857
Transacylase activity, 247
Transcription, 245
Transcriptional regulation, 385, 769
  DesT, 401
  of fatty acid biosynthesis, 398–400
  unsaturated fatty acid formation, 400–401
Transcriptional regulators, 204
Transcriptional repressor, 415
Transcription factors, 398
Transcriptomic analysis, 782
Trans-2-decenoyl-ACP, 47, 531
Trans-2-enoyl-ACP, 394
Transenvelope machines, 672
Transertion hypothesis, 811
Trans-fatty acids, 529
Translocation and assembly module (TAM) complex, 680
Translocon channel, 624
Transmembrane domains (TMD), 559, 610, 632
  biological relevance of, 628
  protein structure, 611
  topology dynamics and interconversions *in vitro*, 627
Transmembrane region, 577
Transmembrane-spanning domains (TMD), 654
Transmembrane (TM), 751
L,D-Transpeptidase, 224
Transporter, trehalose monomycolate, 205
Trans-unsaturated fatty acids, 51
Trans-unsaturated fatty acyl residue, 101
Transversal asymmetry, 579
Transverse lipid asymmetry, 806, 811
Trehalose dimycolate, 194
Treponema, 97
Triacylglycerides, 316, 380
Triacylglycerols (TAG), 10, 44, 364, 383, 497, 824
  bacterial, 447–450
  cell factories, 498
  degradation of, 370
  lipases, 514–519
  lipid formation, 513–514
  metabolic engineering, 451–454
  synthesis, 365, 511–512
  synthesis by bacteria, 447
TRIAP1, 331
Triclosan, 52
Trimethylornithine lipids (TMOL), 117
Trypanosomes, 12
Tryptophan, 736
TSC10, 344
Tuberculosis, 182
Turgor pressure, 814
Turnover number, 647
Twin-arginine translocation (Tat) pathways, 674
Two-component system, 278, 279, 782
Twofold pseudo-symmetry, 641
Type 1 pilus (T1P), uropathogenic *E. coli*, 682
Type III polyketide synthase, 140, 436, 437
Type I secretion system, 677
Type II secretion system, 677–678
Type III secretion system, 678–679
Type IV secretion system, 97, 679–680
Type V secretion system, 680–681
Type VI secretion system, 681

U

Ubiquinone(s), 75, 567, 805, 806
UDP-glucosyltransferase, 99
UDP-sulfoquinovose, 99
Undecaprenol-phosphate, 170
Undecaprenyl-phosphate, 218
Undecaprenyl phosphate, 72
Unfolded OMPs, 655
Unsaturated archaetidic acid, 544
Unsaturated fatty acids (UFAs), 44, 395, 400, 404, 767–769, 778, 789
  α,β-unsaturated fatty acids, 51, 297
  cis-unsaturated fatty acids, 529

Unsaturated fatty acids (UFAs) (*cont.*)
cis-2-unsaturated fatty acids, 276
UppS, 225
Ups1-Mdm35 complex, 332
Ups2, 332
Ursa maritimus, 15

V
cis-Vaccenic acid, 397, 531
Vaccine protective antigen, 174
Vacuolar sorting protein (Vps4), 598
Vancomycin, 228
Vegetative cell, 580
Vernicia fordii, 15
Very long chain fatty acids (VLCFAs), 10
Vesicular-budding model, 370
Vibrio, 15
Vibrioferrin, 238
Vibrio fischeri, 279
Virulence in mice, 202
Vitamin A, 76
VLCFA synthase complex, 346
Volatile short-chain fatty acid, 286

W
Wächtershäuser, G., 541, 548
Wall teichoic acid (WTA), 164
Water soluble precursors, 738
Wax ester, 10, 238, 242, 500
biosynthesis in bacteria, 450–451
profile, 250
synthase, 241, 247, 448
synthesis by bacteria, 447
Wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT), 498
Wood, H.G., 28
Wood-Ljungdahl pathway, 27
Wzy transmembrane protein family, 170

X
Xanthomonas, 276
Xanthomonas campestris, 94
X-ray crystallography, 640

Y
Yarrowia lipolytica, 368, 383, 385
Yeast, 364, 367, 511, 514, 518
genetics, 355
S. cerevisiae, 343
Yersinia pestis, 784
Ypc1, 348
YpfP, 168

Z
Z-ring, 581
Zwitterionic PE, 116