Handbook of
Hydrocarbon and Lipid Microbiology
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Howard Goldfine *Editor*

Health Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids

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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.

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Howard Goldfine
Editor

Health Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids

With 81 Figures and 12 Tables

Springer

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

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Preface

The consequences of microbial interactions with lipids is a subject of great consequence with many ramifications. As the title of this volume notes, there are many types of lipids, including hydrocarbons, oils, neutral lipids (di- and tri-acylglycerols), phospholipids, sphingolipids, and sterols. Other volumes in this series focus on the biogenesis of lipids and membranes, the aerobic utilization of hydrocarbons, oils, and lipids, the production of fuels and chemicals, and the biodegradation and bioremediation of oils and other lipids.

In this volume the focus is on the many interactions of microbes with lipids and their consequences for human health. Some of these occur on or in the skin, the primary organ for host defense against infection. Others occur in the intestine where ingested lipids come into contact with the human microbiome, which itself is influenced by lipids and hydrocarbons in the diet. Bacteria produce short-chain fatty acids from sugars and complex carbohydrates, and these affect intestinal activity and have other health consequences. Sterols are metabolized by microbes in the human intestine; among these the metabolism of bile acids by the gut microbiota results in physiologically active compounds with effects throughout the body.

Mycobacteria with their waxy, lipid-rich outer membrane represent a special case. Two chapters are devoted to the complexity of these lipids and their effects on the interactions of these organisms with the host. Many bacterial pathogens including both Gram-negative and Gram-positive species express lipases, phospholipases, and sphingomyelinases which either provide needed nutrients or manipulate the host in ways that promote infectious processes. These interactions are discussed in detail in two chapters.

A number of bacterial species that are capable of degrading hydrocarbons are important human pathogens; among them are species in the *Pseudomonas*, *Burkholderia*, and *Acinetobacter* genera. A separate chapter is devoted to this topic.

I am greatly indebted to all the authors who have selflessly summarized the current states of affairs with respect to these topics. The scientific literature grows exponentially, and it is extremely important to have authoritative reviews that encapsulate specific areas related to the microbiology of lipids and the roles of specific lipids, their digestion, and the importance of lipid-degrading enzymes in host-pathogen interactions. It is not a simple matter to ask active scientists to take the

large amount of time needed to write a cohesive and current review of a complex field. The authors have provided these critical updates for which we are all grateful.

I would also like to express my thanks to series editor Kenneth Timmis and to Sylvia Blago and Simone Giesler at Springer for all that they have done to bring this volume together.

Philadelphia, PA, USA
March 2020

Howard Goldfine, Ph.D.

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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 Volume Editor

Dr. Howard Goldfine received his B.S. degree from the City College of the City University of New York and his Ph.D. from the University of Chicago. After postdoctoral fellowships in the laboratories of Earl Stadtman at the NIH and Konrad Bloch at Harvard University, he joined the faculty of the Department of Microbiology and Immunology at Harvard Medical School. He moved to the University of Pennsylvania as an Associate Professor of Microbiology in the School of Medicine and subsequently was promoted to Full Professor. He has held a Macy Foundation Faculty Scholar Award, a Fogarty Senior International Fellowship, and a fellowship from the Japan Society for the Promotion of Science and has been a Visiting Professor at the University of Basel and University College London. He is President of the newly organized International Plasmalogen Society. In recognition of his research accomplishments he was elected a Fellow of the American Academy of Microbiology in 1988 and of the American Association for the Advancement of Science in 1998.

Dr. Goldfine has had a notable career in biochemistry and microbiology over the past half century. During this time he has made important contributions to our understanding of bacterial fatty acid and phospholipid biosynthesis, the assembly of the outer membranes of Gram-negative bacteria, the regulation of biophysical properties of bacterial membranes, and the structures, biosynthesis, and functions of bacterial ether lipids. In a second major phase of his research career his laboratory played a large role in elucidating the roles of phospholipases in bacterial pathogenesis with a focus on *Listeria monocytogenes*.

Contributors

Bauke Albada Laboratory for Organic Chemistry, Wageningen University and Research, Wageningen, The Netherlands

Michael R. Barer Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK

Department of Clinical Microbiology, University Hospitals Leicester NHS Trust, Leicester, UK

Katrin Anne Becker Department of Molecular Biology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Rich Boden School of Biological and Marine Sciences, Sustainable Earth Institute, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK

Peter Bossier Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Ghent, Belgium

Ryan Boudreau Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

Patrick J. Brennan Mycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA

Malcolm Brodlie Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK

Holger Brüggemann Department of Biomedicine, Aarhus University, Aarhus, Denmark

Charles C. Caldwell Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

Hélène Cazzola UMR7025 CNRS Enzyme and Cell Engineering Laboratory, Sorbonne Universités, Université de Technologie de Compiègne, Compiègne, France

Destini Cordner Department of Chemical and Environmental Engineering, University of Cincinnati, Cincinnati, OH, USA

Fernando de la Cruz Intergenomic Group, Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) (UC-CSIC), Santander, Spain

Thomas Ebensen Department of Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Braunschweig, Germany

Michael J. Edwards Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

Barbara Fuchs Global Research Immunology, In vivo Biology, ALK, Hørsholm, Denmark

Philippe Gérard Micalis Institute, INRA, AgroParisTech, University Paris-Saclay, Jouy-en-Josas, France

Beatriz Galán Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas (CIB) (CSIC), Madrid, Spain

José L. García Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas (CIB) (CSIC), Madrid, Spain

Department of Applied Systems Biology and Synthetic Biology, Instituto de Biología Integrativa de Sistemas (I2Sysbio) (CSIC-UV), Valencia, Spain

Aaron Gardner Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK

Natalie J. Garton Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK

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

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

Charlotte Grootaert Laboratory of Food Chemistry and Human Nutrition, Ghent University, Ghent, Belgium

Erich Gulbins Department of Molecular Biology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

Carlos Alberto Guzmán Department of Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Braunschweig, Germany

Lauren A. Hinkel Department of Microbiology and Molecular Genetics, Larner College of Medicine, College of Agriculture and Life Sciences, University of Vermont, Burlington, VT, USA

Jacob Hogg Department of Chemical and Environmental Engineering, University of Cincinnati, Cincinnati, OH, USA

Nicola J. Holden Cellular and Molecular Sciences, James Hutton Institute, Dundee, Scotland, UK

Lesley Hoyles Department of Bioscience, Nottingham Trent University, Nottingham, UK

John Katsaras Large Scale Structures Group, Joint Institute for Neutron Sciences, Oak Ridge National Laboratory, Oak Ridge, TN, USA

Joseph Leopoldo Q. Laranja Aquaculture Department, Southeast Asian Fisheries Development Center, Iloilo, Philippines

Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Ghent, Belgium

Xiang Li Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX, USA

José Luis Martínez Centro Nacional de Biotecnología (CNB), CSIC, Madrid, Spain

David E. Minnikin School of Biosciences, Institute of Microbiology and Infection, University of Birmingham, Edgbaston, Birmingham, UK

Jonathan D. Nickels Department of Chemical and Environmental Engineering, University of Cincinnati, Cincinnati, OH, USA

Juan Nogales Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas (CIB) (CSIC), Madrid, Spain

Department of Systems Biology, Centro Nacional de Biotecnología (CNB) (CSIC), Madrid, Spain

Marta Ribeiro LEPABE – Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, Faculdade de Engenharia da Universidade do Porto, Porto, Portugal

Fernando Rojo Centro Nacional de Biotecnología (CNB), CSIC, Madrid, Spain

Yannick Rossez UMR7025 CNRS Enzyme and Cell Engineering Laboratory, Sorbonne Universités, Université de Technologie de Compiègne, Compiègne, France

Claire Rossi UMR7025 CNRS Enzyme and Cell Engineering Laboratory, Sorbonne Universités, Université de Technologie de Compiègne, Compiègne, France

María Santos-Merino Intergenomic Group, Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) (UC-CSIC), Santander, Spain

Kai Schulze Department of Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Braunschweig, Germany

Aaron P. Seitz Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

Manuel Simões LEPABE – Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, Faculdade de Engenharia da Universidade do Porto, Porto, Portugal

Tom Van de Wiele Center for Microbial Ecology and Technology, Ghent University, Ghent, Belgium

R. John Wallace Rowett Institute, University of Aberdeen, Aberdeen, UK

Matthew J. Wargo Department of Microbiology and Molecular Genetics, Larner College of Medicine, College of Agriculture and Life Sciences, University of Vermont, Burlington, VT, USA

Yang Zhang Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX, USA

Part I

Lipids, Membranes, Infection, and Immunity

Lipid Rafts in Bacteria: Structure and Function

1

Jonathan D. Nickels, Jacob Hogg, Destini Cordner, and John Katsaras

Contents

Abstract

"Lipid raft" is the term applied to transient lipid domains organized laterally within the plane of biological membranes. These structures are thought to range in size from tens to hundreds of nanometers and to be compositionally enriched in high-melting temperature lipids. These characteristics create different physical environments within the "rafts" and in the continuous lipid phase that surrounds them. Based on the principle of hydrophobic mismatch and other properties, membrane proteins preferentially partition into, or out of, the raft, facilitating protein interactions, which in turn regulate downstream cellular processes.

J. D. Nickels · J. Hogg · D. Cordner
Department of Chemical and Environmental Engineering, University of Cincinnati, Cincinnati, OH, USA
e-mail: jonathan.nickels@uc.edu; hoggjb@mail.uc.edu; cordnedj@mail.uc.edu

J. Katsaras (✉)
Large Scale Structures Group, Joint Institute for Neutron Sciences, Oak Ridge National Laboratory, Oak Ridge, TN, USA
e-mail: katsarasj@ornl.gov

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Though better understood in the context of the eukaryotic cell membrane, microbial membranes also appear to utilize similar principles to organize their membranes – though their lipid compositions differ considerably from those of eukaryotic membranes. Raft-like structures have been directly observed in microorganisms such as *Bacillus subtilis*, and a number of functional roles for rafts are being investigated. Among these roles is the organization of proteins associated with antibiotic resistance in *Staphylococcus aureus*, further highlighting the importance of lipids and lipid rafts, in biology and medicine.

1 Introduction

Microorganisms have an emerging and fascinating role to play in the story of lipid rafts. That story begins with the notion that the cell membrane is not the archetypical homogeneous fluid mosaic that was envisioned by Singer and Nicholson (1972). Through the 1970s (Yu et al. 1973; Shimshick and McConnell 1973) and 1980s (Simons and Van Meer 1988), evidence of lipid sorting and detergent-resistant regions within the cell membrane gained wider acceptance which resulted in the proposal of the lipid raft hypothesis (Simons and Ikonen 1997) in the late 1990s. Evidence for lateral organization of microbial membranes emerged in the literature shortly thereafter (Matsumoto et al. 2006), though studies had indicated of the possibility of lateral phase separation earlier (Linden et al. 1973). For example, a heterogeneous distribution of lipids was observed in the septa of mycobacteria (Christensen et al. 1999). In *E. coli*, membrane domains were visualized in dividing cells (Fishov and Woldringh 1999) with the poles and septa enriched in cardiolipin (Mileykovskaya and Dowhan 2000). Cardiolipin domains were also observed in *B. subtilis* (Kawai et al. 2004), and more recently isotopic labeling and neutron scattering methods directly probed the lateral heterogeneity of the acyl chains of lipids in *B. subtilis (*Nickels et al. 2017b*)* (Fig. 1).

The lipid raft hypothesis asserts that regions within the cell membrane differ in lipid composition and physical properties. Membrane proteins are thought to partition preferentially between lipid domains and the continuous phase that surrounds them, enhancing certain associations of proteins while suppressing others (Sezgin et al. 2017b; Lingwood and Simons 2010). These domains are thought to facilitate a range of biochemical processes (Brown and London 1998), including cell adhesion and migration (del Pozo et al. 2004), cell recognition (Pierce 2002), protein sorting (Lingwood and Simons 2010; Jacobson et al. 2007), synaptic transmission (Hering et al. 2003), cytoskeletal organization (Villalba et al. 2001), signal transduction (Simons and Toomre 2000), and apoptosis (Gajate and Mollinedo 2001). Recently, it has been suggested that lipid rafts function as reservoirs of high-melting temperature membrane components, causing rafts to act as buffers of membrane physical properties (Nickels et al. 2019), a concept which may be applicable to other environmental perturbation

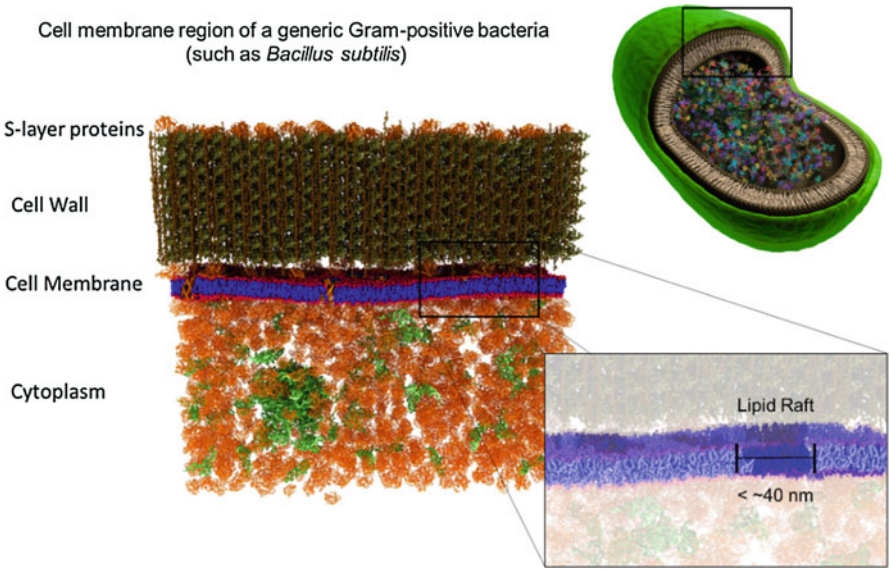

Fig. 1 The bacterial cell membrane for a gram-positive organism such as *B. subtilis*. Gram-positive organisms possess a single-cell membrane, surrounded by a cell wall, and do not typically contain membrane-bound organelles. Bacterial membranes also contain lateral heterogeneities consistent with lipid rafts, which recent experiments indicated are around 40 nm in diameter. (Adapted from Nickels et al. 2017b)

such as solvents or small amphiphilic molecules. Because of these biological roles, rafts have been studied extensively in both animal cells (Verkleij et al. 1973; Allen et al. 2007) and microbes (López and Kolter 2010; García-Fernández et al. 2017; Nickels et al. 2017b). But the canonical view of lipid rafts is a mammalian/eukaryotic perspective. Microbial membranes differ from those of eukaryotes in terms of composition, implying that the lipids domains between eukaryotes and prokaryotes differ in composition, even though they may perform similar roles.

As a result, a new frontier of understanding in lateral membrane organization involving bacterial membranes is rapidly approaching. Recent studies suggest lipid rafts are of interest to medical researchers for a variety of phenomena, specifically bacterial and viral pathogenesis (van der Goot and Harder 2001; Dick et al. 2012; García-Fernández et al. 2017; Epand and Epand 2009). For example, work by Lopez (García-Fernández et al. 2017) shows that targeting raft organization using statin class drugs diminishes antibiotic resistance in methicillin-resistant *Staphylococcus aureus* – a pathogen that is often hospital-associated and notoriously difficult to treat, due to its resistance to common antibiotics.

In this chapter, we will discuss the evolving story of lipid rafts in microorganisms and describe some of the current concepts of lipid raft structure and function in microbial systems.

2 Structure of Lipid Rafts

We can consider lipid raft structure from a number of perspectives. The physical size of domains is an obvious metric to describe rafts in any system, but one can also consider the lipid composition and protein content. Each perspective is a valid way to view the structure embedded within the cellular membrane and to frame how rafts contribute to biological function.

From a eukaryotic perspective, lipid rafts are commonly understood as being heterogeneous (Pike 2004) and dynamic (Samsonov et al. 2001) structures ranging in size between 10 and 100 nanometers (nm) (Pralle et al. 2000) and composed primarily of "high-melting temperature" lipids, but also rich in sterols, carbohydrates, and proteins (Brown and London 2000). However, microbial systems deviate from this picture in several important respects. Compositionally, many microbial systems lack the sphingolipids and sterols found in eukaryotic cells. Bacterial cells are also substantially smaller and possess cell walls and other complex membrane-associated structures, such as flagella.

The size of lipid rafts has been a contentious question since the articulation of the lipid raft hypothesis. Much of the debate stems from an inability to detect nanoscale structures due to limitations in the methods available to study them. More specifically, with the optical resolution limits and the lack of clear rafts in systems which were thought to contain rafts, it was speculated that lipid rafts were nanoscopic in size (Varma and Mayor 1998) quite early on. Besides their small size, rafts present little contrast with the surrounding lipid membrane, presenting an additional analytical challenge in determining a definitive size. However, it is highly unlikely that there is any definitive size for lipid rafts at all. Evidence from model lipid mixtures shows that domain size varies strongly with lipid composition (de Almeida et al. 2005). It is important to keep in mind, therefore, that rafts are expected to be heterogeneous both within a single-cell based due to local composition and curvature fluctuation and across many types of biological membrane which vary widely in composition (Van Meer et al. 2008). This is important to provide context to the reported ~40 nm size of rafts observed for *Bacillus subtilis* cell membranes (Nickels et al. 2017b) which certainly does not reflect a universal lipid raft size, but rather an average of raft sizes within this specific organism at this specific temperature. The sensitivity of raft size to local composition is illustrated in a simple comparison of model binary and ternary lipid mixtures which produce ordered domain ~20–100 nm and $>$ ~75–100 nm in size, respectively (de Almeida et al. 2005). And while additional factors such as mechanical properties and the inclusion of other molecules also influence the size of these domains, it is widely thought that lipid-lipid interactions are the dominant force.

2.1 Methods

Determining the structural details of lateral lipid organization and lipid rafts is an analytical challenge. There is little inherent contrast between lipid rafts and the

surrounding lipids which can be used for observation. The experimental and computational approaches to understanding rafts in microbial systems are broadly similar to those that have been used to study eukaryotic rafts and model membranes. There are a range of direct physical methods performed ex vivo or on model systems such as mass spectrometry (Kraft et al. 2006), nuclear magnetic resonance (NMR) (Veatch et al. 2004), various fluorescence spectroscopy (Bacia et al. 2004; Schwille et al. 1999) and microscopy (Zipfel et al. 2003; Baumgart et al. 2007b; Stöckl and Herrmann 2010; Zacharias et al. 2002; Korlach et al. 1999; Baumgart et al. 2003) methods, atomic force microscopy (AFM) (Yuan et al. 2002; Johnston 2007; Tokumasu et al. 2003; Goksu et al. 2009), and x-ray (Mills et al. 2008) and neutron scattering (Pencer et al. 2005; Nickels et al. 2015a, 2017b; Heberle et al. 2013).

Among these methods for observing lateral organization of lipid bilayer components, fluorescence-based approaches are possibly the most widely used. Although limited by the diffraction limit, fluorescence microscopy (Stöckl and Herrmann 2010) is able to interrogate domain size, shape, and physical properties (Baumgart et al. 2003) based on the partitioning of dye molecules into the different phases (Klymchenko and Kreder 2014) and the diffusion and ligand binding of raft lipid analogs in model and cellular plasma membranes (Sezgin et al. 2012). Fluorescence recovery after photobleaching (FRAP) (Wu et al. 1977) and fluorescence correlation spectroscopy (Bacia et al. 2004) are particularly useful approaches for studying lateral diffusion in bilayers (Derzko and Jacobson 1980) and distinguishing the liquid-ordered phase in raft-forming models (Mouritsen and Jørgensen 1994; Almeida et al. 1992). FRAP works by photobleaching a defined area and measuring the rate at which fluorophores diffuse back into the bleached spot. Because of the small size of lipid rafts, many tools have been employed to probe length scales smaller than the diffraction limit. Confocal and two-photon fluorescence (Gaus et al. 2003) microscopy have been used to directly image large domains, and Förster resonance energy transfer (FRET) (Jares-Erijman and Jovin 2003) has proven extremely useful for studying domain formation and phase behavior (Zhao et al. 2007; de Almeida et al. 2005; Feigenson 2009), as well as identifying raft-associating molecules (Kenworthy et al. 2000). Similarly, other multiphoton methods (Bagatolli and Gratton 2000), a range of super-resolution microscopy techniques (Simons and Gerl 2010), and single molecule (Schütz et al. 2000) approaches are also being developed to overcome the diffraction limit of optical techniques.

Vibrational spectroscopy is another workhorse method that has been applied to questions of lipid organization (Mendelsohn and Moore 1998). The vibrational frequencies of specific functional groups are sensitive to the local environment of lipids and proteins. Because of this, shifts in vibrational frequency and relative amplitudes can be descriptive of lipid order, given sufficient signal to noise ratios. The most widespread vibrational spectroscopy method, infrared spectroscopy (IR), has been used in several studies to detect raft-like domains in model membrane systems (Schultz and Levin 2008; Lewis and McElhaney 2013). Raman scattering has been used on lipid systems for many years (Czamara et al. 2015) with recent developments being applied to asymmetric and laterally organized lipid bilayers.

Tip-enhanced Raman spectroscopy is an attractive way to enhance the observable signal, especially when combined with isotopic substitutions (Opilik et al. 2011). Multiphoton Raman methods, such as coherent anti-Stokes Raman spectroscopy (Potma and Xie 2005; Li et al. 2005), have also been used to observe lateral demixing in the plane of model lipid membranes. Multiphoton methods may provide some advantages over standard Raman in terms of lowering excitation power, resulting in less potential damage to the sample. Nonlinear methods, such as sum frequency vibrational spectroscopy, have been used to study bilayer asymmetry (Brown and Conboy 2013) and lipid phase behavior (Liu and Conboy 2004). Lipid asymmetry is another key organizational feature of the cell membrane (Nickels et al. 2015b). This method clearly observes a spectral indication of bilayer asymmetry, but the resulting flip-flop rates appear to be quite a bit faster than other methods (Heberle et al. 2016). This may be due to the presence of defects in the supported lipid bilayers needed for this method. It has been known for several decades that pore formations dramatically increase the rate of lipid flip-flop (Fattal et al. 1994). Lipid diffusion through such defects would be a competing mechanism with true transmembrane flip-flop in such systems.

Here we focus on experimental approaches used to study microbial membranes, but molecular dynamics (MD) simulations are another important approach that is beginning to be used. We mention this because the next two methods we will discuss, nuclear magnetic resonance (NMR) and scattering (x-ray and neutron), are the most conducive to comparison with MD simulation. The time and length scales of the resulting observations are nearly the same as what current simulations can probe. NMR, specifically solid-state NMR for lipid systems, provides detailed information about molecular identity, local structure and structural uncertainty, and molecular motions (Davis 1983; Seelig and Seelig 1974) based on the response of atoms to an applied magnetic field. Several different elements (Henderson et al. 1974; Petrache et al. 2000; Davis 1983) are used for NMR in the direct study of lipids, commonly including ^{1}H, ^{2}H, ^{13}C, and ^{31}P, and there are many methods and protocols within the overall umbrella of NMR. The addition of membrane insoluble paramagnetic compounds is useful for determination of bilayer asymmetry (Solomon 1955; Bloembergen 1957). If a paramagnetic shift reagent is added to the bulk solution outside of the vesicle, such as a lanthanide ion, there will be a detectible shift in the NMR spectra for one leaflet (Su et al. 2008; Solomon 1955; Bloembergen 1957). Shift reagents, also used to track asymmetric membrane protein insertion (Su et al. 2008), were recently been used to track lipid flip-flop in asymmetric vesicles (Heberle et al. 2016), addressing some potential issues described in the preceding paragraph.

For the case of lateral organization, ^{2}H NMR has played an important role in quantifying the compositional changes within phase separating model lipid mixtures (Veatch et al. 2004). It should be noted that these authors were not comfortable ascribing this result to strict phase coexistence, citing the possibility of critical fluctuations within a single phase. Subsequent observations of nanoscopic compositional heterogeneities in similar model lipid compositions using scattering methods indicate that there is a population with ensemble properties of a nanoscopic size and

local compositions resembling the distinct lipid phases. Carbon-deuterium order parameters of lipid acyl chains are a tremendously useful reporter on the structural order of the fatty acid tails in the hydrophobic region of a lipid bilayer. This parameter is then an obvious target to connect NMR experimental results and MD simulations describing lipid acyl chain order because the carbon-deuterium order parameter reflects the average angle between the C-D bond and the bilayer normal. The results of NMR and MD simulations seem to be in good agreement at present, and while comparisons of order parameters for lipid head groups are not yet well modeled, current efforts can be followed at http://nmrlipids.blogspot.com. Parameters describing lipid acyl chain order can also be obtained by fluorescence depolarization (Heyn 1979), yet the requirement of a fluorescent probe does introduce an outside element, perturbing the system and potentially affecting the result. NMR has the benefit of using isotopic labels which are far less perturbing. Using such labeling, one may access the T1 relaxation time revealing similar information about lipid ordering (Brown et al. 1983). Heteronuclear, or cross polarization, methods where polarization is transferred between two atoms are another strategy for obtaining information about the ordering of the lipid tails (Hong et al. 1995). The use of magic angle spinning (MAS) conditions should also be mentioned as a way of dramatically enhancing the signal of the system and avoiding the necessity of oriented samples (Gross et al. 1997). MD simulation and NMR are also well matched for the description of local motions of lipids in bilayers. MD simulations can compute diffusion confidents directly from the atomic displacements in their trajectories. The pulsed field gradient method has been used to measure the lateral diffusion rate of lipids and cholesterol in the plane of the bilayer (Orädd and Lindblom 2004; Scheidt et al. 2005). This has been applied to the dynamical properties of the lipids within model raft-forming mixtures as well (Orädd et al. 2005; Lindblom et al. 2006).

Neutron and x-ray scattering are powerful structural biology techniques that have been used extensively in the determination of the lipid membrane structure (Marquardt et al. 2015) (Fig. 2). Scattering methods probe important molecular length scales (Å to 100s of nm), and inelastic scattering techniques are able to probe molecular motions occurring from the order of 100s of nanoseconds to fractions of a picosecond. The coherent elastic scattering of both x-rays and neutrons reflects the pair distribution functions of the atoms making up the sample. Such pair distribution functions reflect the molecular structure and can be directly computed from MD simulations. Indeed, the lamellar structure of certain lipid phases (Luzzati and Husson 1962) and liquid-like conformations of the lipid tails in the hydrophobic region (Luzzati and Husson 1962; Tardieu et al. 1973; Engelman 1971) were observed by x-ray diffraction. X-ray diffraction played a critical role in quantifying the linear relationship between acyl chain length and bilayer thickness in lipid bilayers (Lewis and Engelman 1983) and remains a critical structural technique for biomembranes (Nagle and Tristram-Nagle 2000). X-ray diffraction is sensitive to the existence of lateral organization for some lipid systems (Koenig et al. 1997), including complex lipid mixtures (Majewski et al. 2001). The way that lateral organization can be implied from x-ray scattering observations (Heftberger et al.

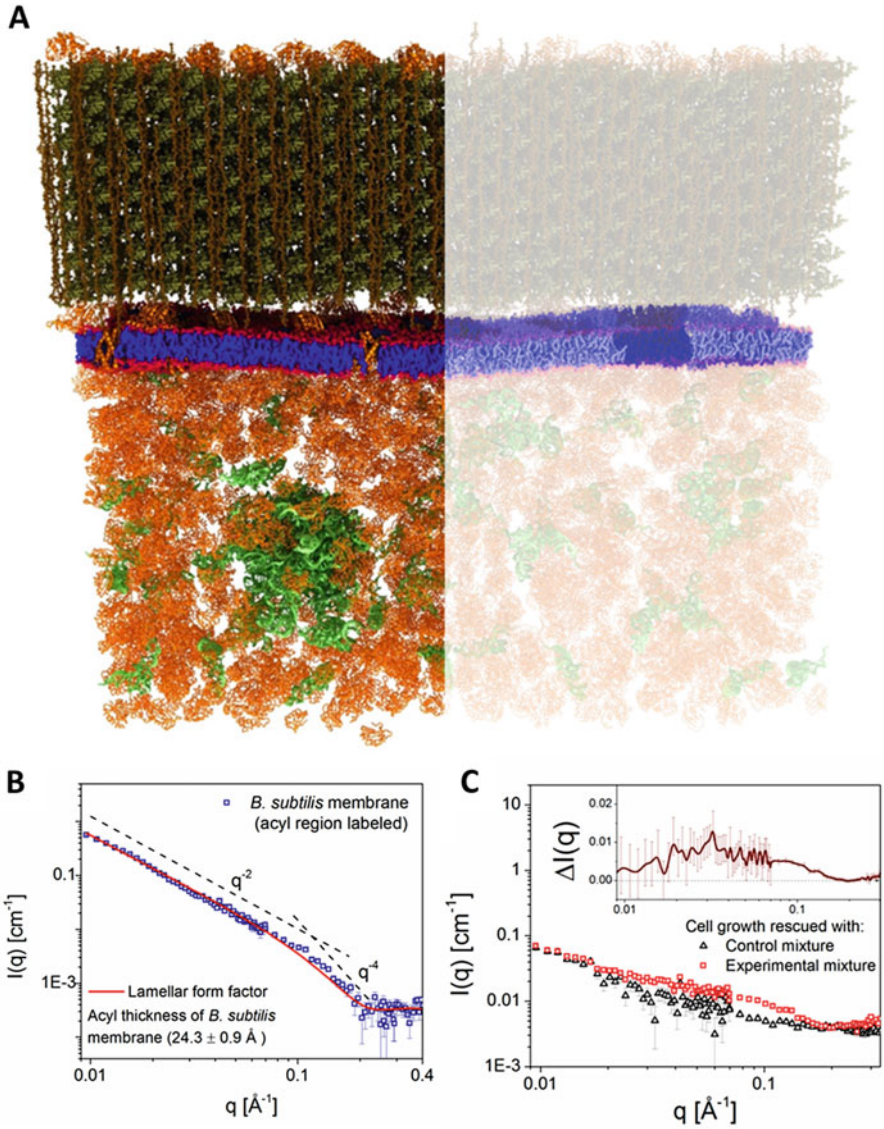

Fig. 2 Neutron scattering is an emerging method for the study of lipid rafts in bacteria. (**a**) Neutrons scatter differently from the isotopes of hydrogen, ^{1}H and ^{2}H (deuterium). By careful isotopic substitutions, it is possible to manipulate the scattering contrast of an entire living bacterial cell, such that contrast between rafts and the surrounding membrane is observed. (**b**) The lamellar form factor of the cell membrane can be observed by labeling a partially deuterated cell's membrane with ^{1}H fatty acids, revealing the bilayer thickness in the living cell. (**c**) Raft structures can be detected based on differences in partitioning of neutron scattering length density matched fatty acid mixtures in the cell membrane. Here we see the excess scattering induced by unequal partitioning of the isotopes of hydrogen. (Adapted from Nickels et al. 2017b)

2015) is usually the presence of multiple d-spacings at lower scattering wave vectors.

Similar to x-ray scattering, neutron scattering is a diffraction technique that can probe length scales from 100s of nm to Å; however, there are some important distinctions. Firstly, neutrons scatter from the atomic nucleus with a strength denoted in the nuclear scattering length, *b*, whereas x-rays scatter from the electron density. Secondly, thermal and cold neutrons are more often used to study dynamics via inelastic scattering. The observed changes in momentum (differences in the velocity of the incident and scattered neutron) can be directly related to the collective and self-motions of the atoms in the sample (Bee 1988). Note that inelastic scattering of x-rays is also a useful method to probe atomic motions, but typically at very high frequencies and longer wave vectors than neutrons, making the method less useful for lipids. The neutron scattering length, *b*, varies widely between elements and even between the isotopes of a single element (Sears 1992), in contrast to x-ray scattering where electron density varies systematically with atomic number. This has two important implications in regard to the study of lipids and complex lipid structures. Firstly, this means that there is a substantial contribution to the observed scattering from low atomic number atoms such as hydrogen. This matters because lipids are so hydrogen-rich. Indeed, neutrons have been a vital tool in studying the detail structure of biomembranes (Büldt et al. 1978, 1979; Zaccai et al. 1979), including the location of another hydrogen-rich molecule in biological systems, water (Zaccai et al. 1975; Nickels and Katsaras 2015), and the motions of water around biointerfaces (Toppozini et al. 2015; Nickels et al. 2012; Perticaroli et al. 2017; König et al. 1994; Settles and Doster 1996; Swenson et al. 2008). The inelastic scattering of neutrons provides additional information in the time domain about the motions of lipid molecules over a wide dynamic range from the collective undulations (Watson and Brown 2010; Zilman and Granek 1996) and thickness fluctuations (Woodka et al. 2012) of the bilayer, to lateral diffusion of lipids (Pfeiffer et al. 1989) and local motions of the individual lipids (Pfeiffer et al. 1989; König et al. 1992; Swenson et al. 2008), to the vibrational regime (Rheinstädter et al. 2004). These motions are correlated to important physical properties of lipid membranes, such as the bending modulus (Zilman and Granek 1996).

The second important point is isotopic substitution, specifically the isotopes of hydrogen (^{1}H) and deuterium (^{2}H). These molecules can be deployed to achieve "contrast-matching" conditions (Jacrot 1976). In the case of complex lipid compositions, contrast matching typically means introducing deuterium substituted lipids in carefully calculated amounts into specific lipid phases. Calculations of atomic/isotopic content are used to match the scattering length density of a water/heavy water solvent mixture to a single given phase (Nickels et al. 2015a) or the theoretical match point of bilayer when perfectly mixed (Heberle et al. 2013; Nickels et al. 2017b; Pencer et al. 2005). In the latter case, a null scattering condition should be observed, and deviations from this imply lateral demixing of the bilayer. This approach has been used in other guises for many applications however, for example, in the study of proteins (Jacrot 1976), lipids (Knoll et al. 1981), nucleic acids

(Pardon et al. 1975), polymers (Nickels et al. 2016), and other materials. There is recent work using this kind of neutron scattering data to carefully study the sizes of lipid domains using these contrast-matching approaches (Heberle et al. 2013; Nickels et al. 2015a). Indeed, contrast-matched vesicles are useful to study how the modulus of nanoscopic lipid domains is distinct from the surround phase of phase-separated bilayer (Nickels et al. 2015a). This work suggested that it is possible that phase separation can be influenced by the local distribution of elastic moduli. Again, the observed dynamical features are directly connected to the time dependence of the self and pair correlation functions, properties accessible in MD simulations. By defining those correlation functions for specific modes, one can extract dynamical terms analogous to these experimental observations.

Not surprisingly, technical limitations frustrate many of these powerful physical techniques in the biologically relevant living cellular environment. Ex vivo approaches to get around these limitations, such as the study of giant plasma membrane vesicles derived from living cells via chemical treatment, show clear indications of large-scale liquid-liquid phase separation (Baumgart et al. 2007a). Also, natural lipid extracts (Nickels et al. 2017a) and synthetic model lipid mixtures which exhibit microscopic liquid-liquid phase coexistence (Veatch and Keller 2002, 2003; Dietrich et al. 2001; Edidin 2003) have made careful study of the physical processes behind lipid rafts a tractable problem (Jacobson et al. 2007).

It is clearly desirable to study lipid rafts in living cell membranes, resulting in a number of experimental approaches being employed. Early in the study of lipid rafts, detergent resistance was found to be a useful way to isolate raft lipids and raft cargo in both model lipid mixtures and cells (Ahmed et al. 1997). Detergent resistance (Lingwood and Simons 2007; London and Brown 2000) uses various detergents, such as Triton-X100, to solubilize regions of the cell membrane. Interestingly, not all portions of the membrane are disrupted resulting in detergent-soluble and detergent-resistant membrane fractions. These fractions can subsequently be interrogated via mass spectrometry and other biochemical assays (Lingwood and Simons 2007). A recent example of this approach might be the isolation of staphyloxanthin in the detergent-resistant *Staphylococcus aureus* membrane (García-Fernández et al. 2017). Another indirect method which was important in the early study of lipid rafts is cholesterol depletion. Cholesterol depletion studies demonstrated changes in cellular behavior or activity (Kabouridis et al. 2000) based on the removal of cholesterol, presumably from lipid rafts. However, the vast majority of microorganisms do not contain sterols in their cell membranes, instead using functional analogs such as hopanoids (Sáenz et al. 2012) and isoprenoid lipids (Boucher et al. 2004; García-Fernández et al. 2017). High-resolution, two-photon fluorescence spectroscopy is one such approach at tracking lipid raft cargo in living cells (Gaus et al. 2003). The method is based on the differential partitioning of the small hydrophobic dye laurodan (Owen et al. 2012). Other fluorescence-based methods using protein-linked molecules showed the cycling of raft-associated membrane components in living cells (Nichols et al. 2001). These methods provide powerful, if indirect, evidence of the existence of lipid rafts and optical length scale structural information. Recently, neutron scattering was used to directly observe lateral demixing in the cell

membrane of a living bacterium, confirming the presence of nanoscopic heterogeneities consistent with the concept of lipid rafts (Nickels et al. 2017b).

2.2 Composition

The composition of lipid rafts is often understood in the context of high cholesterol and sphingomyelin/high-melting lipids. This is a eukaryote centric view, however, and does not apply to the majority of microbial membranes. From a bacterial membrane perspective, one frequently thinks lipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (CL) are common in the membrane of *E. coli* (Gidden et al. 2009; Parsons and Rock 2013), the gram-negative bacterial model, and in that of *B subtilis* (Gidden et al. 2009; Nickels et al. 2017a; Den Kamp et al. 1969), a typical gram-positive model organism. Yet, there are many other molecules to consider (Fig. 3). Microbial membranes are overwhelmingly diverse in terms of their lipid compositions (Sohlenkamp and Geiger 2016), even in the model organisms such as *E. coli* and *B. subtilis*. In the context of lipid rafts, these other lipids, such as hopanoids and prenyl lipids, are important as analogs to sterols (Sáenz et al. 2012). Other varieties of lipid include

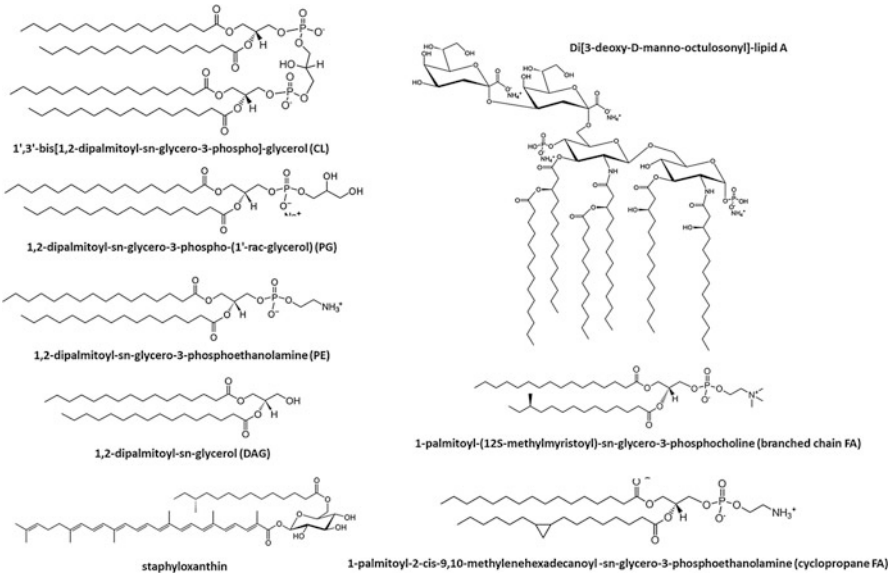

Fig. 3 Bacteria contain a wide range of lipids. This includes typical glycerophospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (CL), as well as the metabolic intermediate diacylglycerol (DAG). Terpenoids and prenyl lipids (like staphyloxanthin) are found in many bacterial membranes, some of which are thought to be important analogs to sterols. Lipopolysaccharides are typically found in the outer membrane of gram-negative species. A range of fatty acids are also employed across bacterial species, including branched chain and cyclopropane fatty acids

ornithine lipids, sulfonolipids, or the many varieties of glycolipids (GL). Free fatty acids and other small molecules can also be found in some bacterial membranes. Ether lipids are also found in a range of bacterial species. Common in archaea, bacterial ether lipids contain fatty acyl and acyl modifications in the *sn*-1 and *sn*-2 positions. The distribution of these and other less frequent bacterial membrane components across the range of organisms is summarized well by Sohlenkamp and Geiger (Sohlenkamp and Geiger 2016) in Table 1 of their excellent review.

Glycerophospholipids are the most common lipid species in most cell membranes. This group includes those mentioned above, PE, PG, and CL, as well as other molecules such as phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), and the lysyl versions of these lipids, such as lysyl-PG (LPG). Diacylglycerol (DAG) is another component of bacterial membranes and an important metabolic intermediate in lipid synthesis (Carrasco and Mérida 2007). DAG can be enzymatically modified to obtain phosphatidic acid (PA) and subsequently cytidine diphosphate-diacylglycerol (CDP-DAG) which is the central metabolite in the production of glycerophospholipids. Because of this, DAG is the backbone of GLs and the anchor for lipoteichoic acid, an important structural component of the cell wall (Percy and Gründling 2014). Lipopolysaccharides (LPS), such as Lipid A, are typically found in the outer membrane of gram-negative bacteria. LPS is often a toxin that triggers innate immunity (Alexander and Rietschel 2001) in humans and is reported to cluster in mammalian lipid rafts (Pfeiffer et al. 2001).

Local differences between the global lipid composition and the local composition are of course the central feature of lateral organization (Fig. 4). The physical properties of lipids drive such differences, with acyl thickness and spontaneous curvature mismatches as potential driving forces for raft formation. DAG, for instance, may have a role in raft formation due to its negative curvature, while

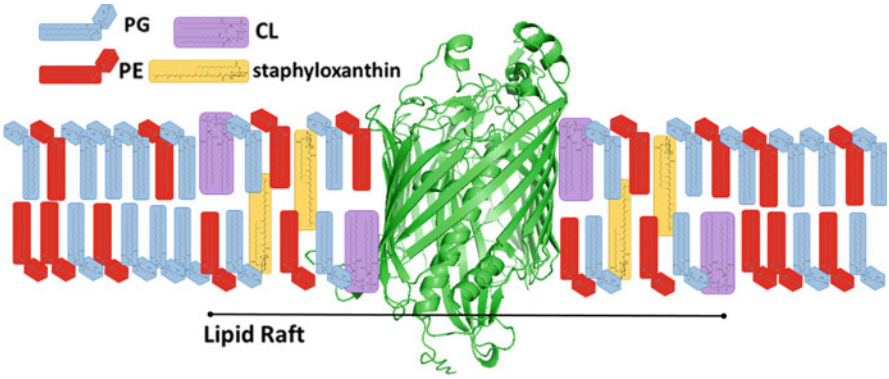

Fig. 4 Schematic of a lipid raft with associated transmembrane protein. This simplified composition belies the reality that much remains to be discovered about the varied compositions of lipid rafts in bacteria. Some bacterial membranes are thought to contain rafts enriched in phosphatidylethanolamine (PE) (Nishibori et al. 2005), cardiolipin (CL) (Mileykovskaya and Dowhan 2000), or some terpenoids, like staphyloxanthin (Sáenz et al. 2012) (protein shown is a LPS transporter from *Pseudomonas aeruginosa* (Botos et al. 2016))

lysyl lipids have a large positive spontaneous curvature. Other lipids, such as CL (Mileykovskaya and Dowhan 2000) and PE (Nishibori et al. 2005), have been demonstrated to localize in the cell membrane of bacterial cells. But the correlation from raft-forming lipid mixtures is still being investigated in living bacteria, and most anticipate that there will be a far greater diversity of raft-forming compositions than is seen in mammalian membranes.

The dependence of rafts upon sterols in eukaryotes, typically cholesterol in humans, is a distinguishing feature of lateral organization. Ergosterol is thought to regulate membrane properties to much the same degree in fungi, protozoans, and yeasts (Xu et al. 2001), with observations that a membrane with little to no cholesterol will exhibit little to no raft formation (Rothberg et al. 1990). Sterols are major component of the membrane in eukaryotes, and their presence and absence throughout the lipid bilayer plays an important role in the functions and behaviors of not only lipid rafts but with the membrane as a whole. Their amphipathic structure allows them to interact with both the nonpolar acyl chain regions and polar head group regions of the cellular membrane. Sterols also have a substantial influence over the biophysical properties of the membrane, such as bilayer fluidity, thickness (Kusumi et al. 1983; Vance and Vance 1985) permeability, bending modulus, and protein complex conglomeration (Haines 2001; Nickels et al. 2015a; Simons and Toomre 2000).

Unsurprisingly, prokaryotes are thought to utilize a range of compounds to perform similar functions. Sterols are produced in a limited number of microorganisms; while some organisms have been shown to scavenge sterols from the extracellular environment. We will discuss one such case for *B. burgdorferi* later in this chapter. Isoprenoid lipids, also called terpenoids, are a broad class of compounds that are thought to play some role in raft formation in bacterial membranes. Their functional convergence has been long speculated, and experimental evidence is now emerging (Sáenz et al. 2012). In fact, sterols are synthesized from isoprenoid precursors such as squalene.

In prokaryotic membranes isoprenoid lipids are increasingly being associated with rafts and raft-associated cellular functions, such as membrane trafficking, protein localization, and signal transduction (Lopez and Koch 2017). Hopanoids are a specific class of isoprenoid lipid – triterpenoids – which has been strongly implied to function analogously to sterols. Hopanoids are a diverse set of molecules found widely among bacteria (Rohmer et al. 1984). Diplopterol is one example used by Saenz and coworkers (Sáenz et al. 2012) to illustrate experimentally the biophysical similarity. They compared the membrane ordering effect in model sphingomyelin/cholesterol and sphingomyelin/diplopterol bilayers. This is not a universal effect across all hopanoids, however, as was pointed out subsequently in simulations of PC lipid bilayers containing diplotene or bacteriohopanetetrol. There, the authors showed how the latter molecule accumulated in the bilayer midplane, having no ordering effect at all (Poger and Mark 2013). However, such effects are also seen for the location of vitamin E (Marquardt et al. 2013) as a function of chain length, so it is possible that bacteriohopanetetrol would partition differently in other model bilayers.

Carotenoids are another subclass of isoprenoid lipid – also called tetraterpenoids – based on eight isoprene units. Carotenoids are important molecules in photosynthetic organisms due to their light-absorbing properties, especially in the 400–550 nm wavelength range. Carotenoids typically span the lipid bilayer and do not have as many ring structures as hopanoids. Nonetheless, carotenoids have also been implicated in the maintenance of lipid rafts. Garcia-Fernandez et al. showed that carotenoids, or absence thereof, are a key factor in formation of rafts using a carotenoid-deficient *Staphylococcus aureus* mutant. They demonstrate the pivotal role rafts play in the mechanism of antibiotic resistance (García-Fernández et al. 2017).

Proteins are another major component of the cell membrane, with integral membrane proteins accounting for 20–30% of genes (Krogh et al. 2001). This is true for bacteria as much as for eukaryotes (Daley et al. 2005). In the context of this chapter, we are interested in the organization of membrane proteins and their recruitment to rafts. Membrane proteins broadly come in two categories: integral membrane proteins and peripheral membrane proteins. Integral membrane proteins can only be removed by membrane disruption. This includes proteins which cross the full bilayer like ion channels or receptors involved in cell adhesion or active transport of metabolites. Transmembrane domains typically contain hydrophobic residues and have conserved sequences, making their identification possible from sequence analysis tools. Peripheral membrane proteins are those which can be extracted from the membrane without fully disrupting the bilayer and do not typically cross the full bilayer but are instead associated with one leaflet through acylation, ionic interactions, or specific affinity to membrane components.

Membrane proteins are sensitive to bilayer thickness (Andersen and Koeppe 2007), and this is thought to be the energetic driver of protein partitioning between raft and "sea" phases of the cell membrane. These phases present different hydrophobic thickness which will provide better solvation matching to the hydrophobic portions of membrane proteins. Because of this, there are structural motifs which have become associated and are potentially predictive for proteins which are found in lipid rafts. The set of proteins described by the acronym, SPFH, is one such domain, describing the group of proteins stomatin, prohibitin, flotillin, and HflK/C (Browman et al. 2007). Evidence has shown that proteins containing these SPFH segments may interact with other proteins to localize binding partners to the raft and impact their organization once inside it (Langhorst et al. 2005, 2007; Browman et al. 2007). Another important category of raft-associated proteins is the glycosylphosphatidylinositol (GPI)-anchored proteins. These are integral membrane proteins, typically containing long acyl chain and doubly acyl chain modifications; and though it varies greatly based on activity, GPI-anchored proteins have been reported to exist within rafts at concentrations up to 15 times greater than the surrounding membrane (Varma and Mayor 1998).

3 Functions of Lipid Rafts

Lipid rafts are the subject of ongoing research, and their role in biological systems is varied and evolving. Some aspects of lipid rafts remain under debate, such as the mechanism of raft formation, phase behavior (Feigenson 2009), or active biological

process (Yethiraj and Weisshaar 2007; Turner et al. 2005). Yet the core concept connects lipid organization to protein co-localization, and protein co-localization to cellular function is now widely accepted. The physical properties of coexisting lipid phases and their incorporated proteins create distinct characteristics of both raft and non-raft regions. It is hypothesized (Nickels et al. 2019) that rafts may function to stabilize physical properties against rapid changes in temperature or solvent. Membrane thickness, curvature, tension, and other properties are products of these interactions. Further, the partitioning of proteins in the bilayer based on their structural features along with active sorting methods allows for the regulation of a range of cellular processes. This mediation influences signaling pathways, transport mechanisms, cellular division, and other membrane functions.

3.1 Defining Membrane Physical Properties

The understanding of lipid rafts as coexisting lipid phases in the cell membrane plays a key role in defining the physical properties of rafts and maintaining the properties of the cell membrane as a whole. As noted above, bacterial membranes are compositionally diverse (Sohlenkamp and Geiger 2016), with a given organism using potentially hundreds of lipids. In order to simplify the system for biophysical study, model lipid bilayers have emerged as an excellent resource for the study of lipid bilayer phase separation, with a rich literature emerging since the year 2000. Commonly using ternary lipid mixtures (Feigenson 2009; Veatch and Keller 2003; Marsh 2009) containing one high-melting lipid, one low-melting lipid, and cholesterol, these studies identified the liquid-ordered phase, Lo, and the liquid-disordered phase, Ld, as analogs to the biologically relevant raft phase. L_O and L_D phases have numerous physical differences beyond their composition, such as the local lipid order (acyl chain, tilt, splay), lateral compressibility, hydrophobic thickness, and bending modulus (Nickels et al. 2015a). Moreover, the precise combination of these properties contribute to the interfacial energy between the phases, quantified in the line tension and partition coefficients of the various molecules within the bilayer (Honerkamp-Smith et al. 2008; Kuzmin et al. 2005; García-Sáez et al. 2007).

As might be guessed from the nomenclature, lipids in the Lo phase are more ordered as defined by a number of order parameters. In general, this means that lipid acyl tails in the Lo phase have on average a more *trans* configuration and are more likely to be oriented parallel to the bilayer normal. In the case of typical diacyl lipid molecule, the acyl tails have parallel orientations with respect to each other when compared to lipids in the Ld phase. This ties quite directly into differences in bilayer thickness, compressibility, and bending modulus. The bending modulus describes the resistance of the bilayer to deformation out of the plane of the bilayer and is directly related to the lateral compressibility and bilayer thickness. The bending modulus and the related Gaussian modulus have an influence on the global bilayer free energy (Helfrich 1973) and have been indicated as one potential driver of lateral organization (Seul and Andelman 1995; Nickels et al. 2015a).

Membrane protein hydrophobic mismatch is a common concept for discussion of lipid rafts discussed in the prior and subsequent sections of this chapter; but lateral

pressure profiles exerted upon membrane proteins are another potentially important influence of lipid rafts. For example, it has been suggested that the insertion of ketamine in lipid bilayers changes the opening probability for model ion channels (Jerabek et al. 2010), supporting the lateral pressure mechanism of anesthesia (Cantor 1997). Changes in protein activity based upon conformational changes induced by the lateral pressure profile of the lipid environment are another way that lipid rafts can influence cellular behavior. Finally, bending modulus and the diffusivity of proteins within the membrane can be influenced by the existence of distinct lipid phases. Indeed, partitioning of lipids between phases continually changes as a function of temperature leading to a thermal buffering of bilayer rigidity and bilayer fluidity (Nickels et al. 2019).

3.2 Protein Partitioning

The functionality of lipid rafts is premised upon their role as a mechanism of preferential protein sorting. The origins of this mechanism are rooted in fundamental thermodynamic interactions. When two membrane domains with differing hydrophobic thickness form in the presence of a protein, the protein will preferentially accumulate within the bilayer region which more closely matches their hydrophobic thickness (Fig. 5). This will tend to co-localize proteins with similar characteristics, leading to a clear mechanism by which multiple proteins can be segregated together in a certain membrane region.

The physical characteristics that help define the hydrophobic thickness of a lipid raft are governed by a combination of factors which depend upon composition of lipids found in the specified domain, namely, the lengths of the acyl tails of its component lipids, the chemistry of the lipid head groups and their interaction with water, and the lateral packing of the constituent lipids. The less that membrane proteins and lipids must adapt to reduce any exposure of hydrophobic regions to the aqueous environment outside of the bilayer, the more energetically advantageous the protein partitioning will be. Various mechanisms are employed in order to minimize these mismatches and resultant strain energies. For example, small conformational changes in trans- and intramembrane proteins can alter their overall length and reduce the impact of these mismatches. Additionally, local deformation of the lipid bilayer allows for reduction of line tension at the phase boundary.

Protein partitioning into a lipid raft is just one step in sequence of events leading to various cellular functions. A raft associated protein frequently collocates with other proteins to form protein complexes. Detergent resistance has identified a number of protein complexes prokaryotic cells. In *B. subtilis* the resistant protein complexes include signaling proteins, biosynthesis machinery, and transporter complexes (López and Kolter 2010; Niño and Marc 2013). Further study revealed that there was direct interaction between these proteins and flotillins, like FloT and FloA, indicating co-localization of specific proteins. Functional dependences were observed as protein function was inhibited when flotillins (FloT and FloA) were not present (López and Kolter 2010; Niño and Marc 2013; Schneider et al. 2015).

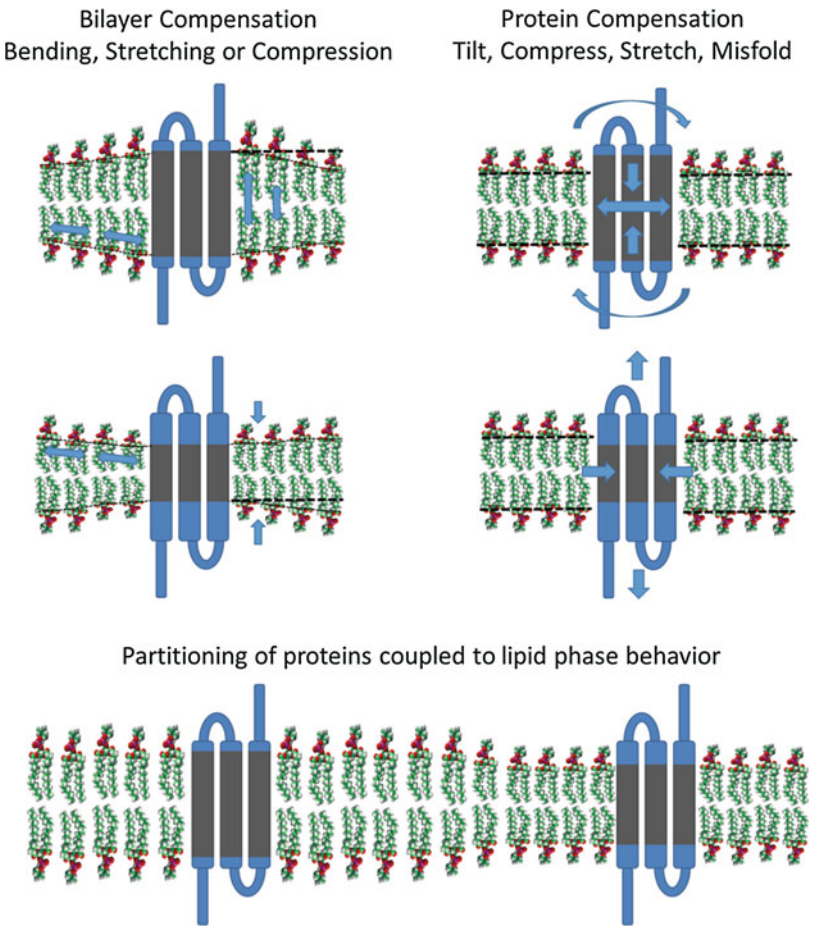

Fig. 5 Protein partitioning in lipid rafts is the result of many interactions between cell membrane lipids and membrane integral and associated proteins. Hydrophobic mismatch is thought to be among the strongest drivers determining the raft affinity proteins

Flotillins belong to the SPFH (stomatin/prohibitin/flotillin/HflK/C) superfamily of proteins, which share a region of undetermined function but similar sequence (Tavernarakis et al. 1999). Flotillins are distinct in their coiled structures at the C-terminal (Schroeder et al. 1994).

In many single-cell organisms, flotillins are thought to mark lipid raft locations (Fig. 6). Through the use of electron microscopy and staining-based methods, these proteins can be easily observed to localized domains across the cellular membrane (Lang and Philp 1998; Stuermer et al. 2001). These domains tend to be uniform in diameter and very similar in size to lipid rafts, approx. 100 nm across. While they relatively evenly spaced across cellular membranes, they tend to conglomerate more at cellular contact sites (Stuermer et al. 2001). Flotillins have been observed to

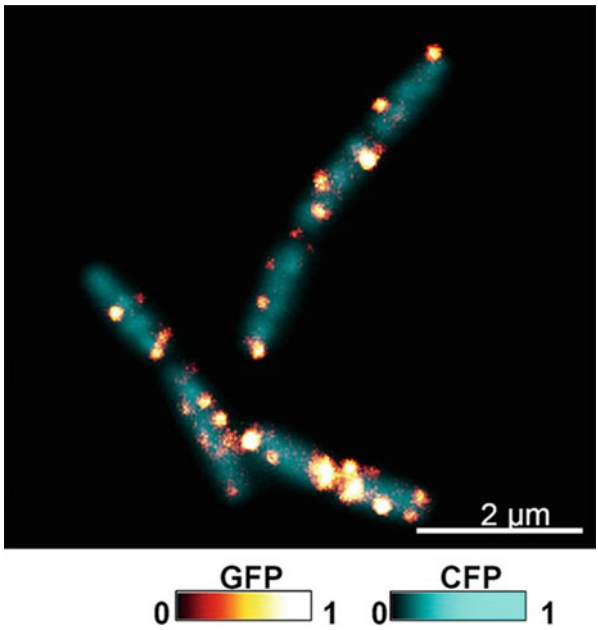

Fig. 6 Localization of flotillin-GFP fusion protein to membrane rafts in *B. subtilis* cells expressing P_{spac}-*cfp* reporter. Fluorescent microscopy image from Bramkamp and Lopez (2015)

co-localize with other GPI-anchored proteins like F3/contactin and others, suggesting that they are involved in the ferrying of GPI-anchored proteins across the cell membrane. Additionally, they appear to closely interact with kinases, transport, and several types of receptor signaling mechanisms (Stuermer et al. 2001; Wakasugi et al. 2004).

3.3 Selected Examples

We now discuss several specific examples of rafts, or raft-like domains, either in the membranes of, or interacting with, microorganisms. Lipid rafts in both eukaryotes and bacteria alike play important roles in the mechanism of pathology. Their ability to co-localize specific complexes of proteins is especially useful as a vector for cellular interaction and invasion of a host cell, or responding to an environmental challenge, such as an antibiotic. Moreover, microbial pathogens have been able to evolve many complex strategies to avoid immunological detection and responses within their hosts.

3.3.1 *Vibrio cholera* and Cholera

Vibrio cholera is a gram-negative bacterium that causes the disease chorea. This bacterium produces a protein toxin called cholera toxin and is associated with a membrane binding B-subunit. CTxB (cholera toxin B-subunit) has become an important and frequently studied example of raft-associated proteins. CTxB can bind up to five GM1 gangliosides, by targeting the toxin to host cells. The GM1

gangliosides tend to be associated with lipid raft regions of the plasma membrane, and subsequent endocytosis can occur through GM1-rich caveola structures (Parton 1994) or clathrin-coated pits (Nichols 2003). Once internalized, a reverse translocation from the plasma membrane to the Golgi complex occurs (Nichols et al. 2001). The A1 and A2 subunits of CTxB are cleaved, triggering the activation of the A1 enzymatic fragment. This fragment activates and locks the G protein of $G_s\alpha$ in a GTP-bound form, resulting in a continuous activation and stimulation of the enzyme adenylate cyclase to produce cAMP. This triggers a large excretion of water and ions out of infected cells through the opening of cAMP sensitive channels.

The targeting mechanism of CTxB to GM1-enriched areas of the plasma membrane allows them to be used as lipid raft markers (Nichols et al. 2001) in a productive use of the molecule. Due to the multivalent binding, the toxin has the capability of recognizing the underlying membrane structure by cross-linking small and ephemeral lipid rafts (Day and Kenworthy 2015). The enrichment of their receptors in rafts suggests that bacterial products such as toxins bind preferentially to detergent-resistant highly ordered plasma membrane regions to access the cell (Sezgin et al. 2017a). This suggests that CTxB actively changes the properties of the membrane upon binding. Fluorescence resonance energy transfer (FRET) microscopy can then be used to detect the presence of lipid rafts. FRET was detected between molecules of the glycosphingolipids GM1 labeled with cholera toxin B-subunit and between antibody-labeled GPI-anchored proteins, exhibiting these raft markers are in submicrometer proximity in the plasma membrane. In the plasma membrane, lipid rafts either exist only as transiently stabilized structures or, if stable, comprise at most a minor fraction of the cell surface (Kenworthy et al. 2000). CTxB thus serves as a useful model for understanding the properties and functions of protein-stabilized domains.

3.3.2 *Borrelia burgdorferi*

While each bacterial and eukaryotic membrane has its own unique composition of lipids in order to facilitate lipid raft and protein complex formation, some bacteria have been found to go to extreme lengths to create proper functioning conditions for lipid rafts. One example is that of *Borrelia burgdorferi*. Known as the bacteria that cause Lyme disease, the *Borrelia* genus was found to contain types of glycolipids rarely found in bacterial cells and thought only to be used in eukaryotic cellular bilayers. Of three specific glycolipids identified in *B. burgdorferi*, two were found to contain cholesterol. These glycolipids were identified as cholesteryl 6-O-acyl-β-D-galactopyranoside (ACGal), cholesteryl-β-D-galactopyranoside (CGal), and mono-α-galactosyl-diacylglycerol (MGalD) (Ben-Menachem et al. 2003). Upon investigation these glycolipids were also found to exist in other *Borrelia* species as well, showing a similarity in functional regulation of the lipid bilayer across the entire genus. This presence of cholesterols in the *Borrelia* genus suggests that the characteristic microdomains of sterol-, or sterol analog, rich areas found in eukaryotic bilayers may be found inside bacterial bilayers as well. Further, the complexes of proteins found in the liquid-ordered phase of *B. burgdorferi* suggest a sensory or signaling function due to the environmental factors (LaRocca et al. 2010).

Fascinatingly, *B. burgdorferi* lack the cellular machinery to produce these molecules. This means that the acquisition of sterols for the outer membranes of these cells is a process which must be undergone via lipid transfer from a host eukaryotic cell. This is supported by its inability to synthesize long-chain-saturated, unsaturated fatty acids, or cholesterol analogs, paired with the cell's lipid membrane concentration mirroring those of its host fluid or tissue (Johnson 1977). The similarity in lipid composition of *B. burgdorferi* and its eukaryotic host allow for lipid-lipid interactions between the two membranes, especially in lipid raft domains, where the similar interactions would be highest. These spirochetes use this interaction as an important mechanism for nutrition acquisition, which includes the sterol and other fatty acid stripping from the host cell (Crowley et al. 2013). This interaction was observed by Crowley through the use of fluorescently labeled fatty acids before and after *Borrelia* interaction with host cells. The presence of labeled sterols on both host and *B. burgdorferi* after incubation confirmed this hypothesis (Crowley et al. 2013). This mechanism is thought to be responsible for the cellular damage that results from Lyme disease.

3.3.3 *Staphylococcus aureus*

The human pathogen *S. aureus*, commonly known as MRSA, is a notable example of complex microdomain interactions. This bacterium uniquely expresses a single flotillin, FloA (Marshall and Wilmoth 1981; Pelz et al. 2005; Wieland et al. 1994), and has been able to develop an ability to successfully resist antibiotic treatments, causing its iconic hard to treat infections in hospitals. Antibiotics such as methicillin (a β-lactam antibiotic) have been shown to be ineffective versus strains of *S. aureus* via the production of low-affinity penicillin-binding protein (PBP2a). This protein, by binding with the antibiotic, is able to prevent it from inhibiting PBP active sites, which are essential to cellular division. In this way PBP2a allows MRSA strains to replicate and grow in the presence of antibiotics (Kreiswirth et al. 1993).

A key component of this mechanism is FloA, as it identified to be the singular flotillin driving protein recruitment in these microdomains (Marshall and Wilmoth 1981; Pelz et al. 2005). Preferential binding of FloA to staphyloxanthin (a lipid produced in *S. aureus*) leads to its oligomerization in staphyloxanthin-rich microdomains. As these areas grow and assemble FMMs, PBP2a is taken as protein cargo of FloA, concentrated in these regions, and is ultimately used to inhibit antibiotics (García-Fernández et al. 2017). As FloA conglomeration increases scaffold-forming activities in the bilayer, an increase in PBP2a organization is also seen. This relationship is important because the flotillin dependence of PBP2a localization means that the lack of FloA causes inhibition of protein recruitment. By disrupting the FMM formation in *S. aureus*, the antibiotic resistance of MRSA bacteria was decreased significantly, and conventional antibiotic therapy again became effective (García-Fernández et al. 2017).

4 Research Needs and Conclusions

The term "microbial membrane" refers to an almost impossible diverse array of biomembranes. There are a huge variety of lipid compositions (Sohlenkamp and Geiger 2016) that need to be investigated, and efforts to describe the properties of the

molecules involved in microbial lipid rafts should be supported. Isoprenylated species, cyclic and branched chain fatty acids, methylated lipid head group chemistries, and glycosylated lipids anchoring a cell wall all differ substantially from the eukaryotic picture. Understanding properties like the hydrophobic thickness and spontaneous curvature of these molecules will enable a more direct comparison to the better studied, mammalian centric, semi-canonical view of lipid rafts.

Beyond compositional considerations, another vital and broad-reaching research need is for robust and reliable methods for detecting membrane domains and identifying protein content. Fluorescent strategies relying on co-localization of proteins have convincingly proven the existence and functional roles of lipid domains (Kenworthy et al. 2000). Unfortunately, the resolution limits of optical microscopy impact the utility of these methods in determining the sizes of these structures. Advances in super-resolution microscopy are overcoming these challenges (Huang et al. 2008; Hess et al. 2006), but the need for exogenous fluorescent probes remains potentially problematic.

Neutron scattering has been a valuable, and fluorophore free, tool for studying lipid raft structure and physical properties both in vitro (Nickels et al. 2015a) and in vivo (Nickels et al. 2017b). These studies rely upon exploiting the differences in scattering length density of deuterated and hydrogenated molecules. Because microbial membranes have differing compositions from eukaryotes, the commercially available deuterated lipids and fatty acids are insufficient, making the availability of deuterated microbial lipids and lipid precursors as another pressing need. Lipid extracts contain the rich compositional diversity of the biological membrane, and many microbes can tolerate the highly deuterated culture conditions needed to produce deuterated biomass. Because of this, lipid extracts are a great source of some deuterated materials, as well as being excellent ways to study the physical properties of native microbial cell membranes (Nickels et al. 2017a).

Lipid rafts are currently understood as an organizational motif in the cell membrane of living cells. Rafts are thought to preferentially sort protein cargo based on the physical properties of the membrane phases. The biochemistry that this organization facilitates is deeply involved in the regulation of cellular activity, making these structures critical for proper cellular function and potential avenues for pathogenic interactions.

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Lipids of Clinically Significant Mycobacteria

2

David E. Minnikin and Patrick J. Brennan

Dedication: To the memory of Philip Draper (1936–2019), a scientist of the highest integrity whose outstanding research in lipid chemistry and biochemistry was fundamental in the characterization of the leprosy bacillus and related mycobacteria.

Contents

D. E. Minnikin (✉)
School of Biosciences, Institute of Microbiology and Infection, University of Birmingham, Edgbaston, Birmingham, UK
e-mail: d.e.minnikin@bham.ac.uk

P. J. Brennan
Mycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA
e-mail: Patrick.Brennan@ColoState.EDU

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Abstract

Mycobacterial pathogens, such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and the "non-tuberculous mycobacteria," are characterized by the presence of a robust cell envelope that influences pathogenicity but also provides targets for antimycobacterial drugs. A key cell envelope component is a lipid rich mycobacterial outer membrane (MOM) involving "free" lipids interacting with a "bound" layer of high molecular weight (70–90 carbons) long-chain fatty acids, the mycolic acids. The principal free lipids range from the highly hydrophobic phthiocerol dimycocerosate waxes through related glycosyl phenolphthiocerol dimycocerosates (phenolic glycolipids) to strongly antigenic hydrophilic glycopeptidolipids and lipooligosaccharides. The mycolic acids are covalently linked to a special arabinogalactan polysaccharide, which is attached to the basal cell wall structural peptidoglycan. The specialized mycobacterial inner membrane (MIM) is rich in characteristic glycophospholipids, the phosphatidylinositol mannoside family, which are extended into important lipopolysaccharides, the lipomannans, and lipoarabinomanns. The origins and structures of the full range of lipids, from clinically significant mycobacteria, are detailed.

1 Introduction

1.1 Mycobacterial Pathogens

Mycobacterium tuberculosis and *Mycobacterium leprae* are dominant pathogens, infecting the human race during the past millennia. Recently, a distinct leprosy agent has been characterized and provisionally labelled "*Mycobacterium lepromatosis*" (Han et al. 2008; Singh et al. 2015), and even more recently, another uncultivable related animal pathogen, "*Mycobacterium uberis*" has been reported (Benjak et al. 2018). An opportunist human pathogen, *Mycobacterium haemophilum*, has strong

phylogenetic links with leprosy bacilli (Besra et al. 1991; Donoghue et al. 2018). Currently, there are seven recognizable clades of *M. tuberculosis sensu stricto*, but there are also a number of closely-related mycobacteria that have been grouped together in the "*M. tuberculosis* complex" (Niemann and Supply 2014). This complex also includes animal pathogens, *Mycobacterium bovis* being the best documented (Smith et al. 2006). A distinct smooth morphology tubercle bacillus, labelled "*Mycobacterium canettii*," is regarded as being on the periphery of the *M. tuberculosis* complex, but with likely ancestral links (van Soolingen et al. 1997; Supply and Brosch 2017). Other significant mycobacterial pathogens are included in the so-called nontuberculous mycobacteria (NTMs) (Falkinham 2015; Claeys and Robinson 2018). NTMs have been comprehensively reviewed by Turenne (2019) who emphasized the absolute necessity for validating novel taxa, such as "*M. lepromatosis*," "*M. uberis*," and "*M. canettii*," before referring to them as established species.

Mycobacterium kansasii and *Mycobacterium gastri* are opportunists that produce tuberculosis-like human disease (Philley et al. 2016; Johnston et al. 2017). A very broad taxon, infecting humans and animals, was often referred to as the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* (MAIS) complex, but more recently, it has been simply termed the *Mycobacterium avium* complex (MAC) (Falkinham 2015; Philley et al. 2016; Turenne 2019; Verma 2019). Humans and fish can suffer from *Mycobacterium marinum* infections (Johnson and Stout 2015) and a closely related pathogen, *Mycobacterium ulcerans*, causes human "buruli ulcers" (Sizaire et al. 2006; Chany et al. 2013). *Mycobacterium fortuitum* and related members of the original "*M. fortuitum* complex," such as *Mycobacterium peregrinum*, are opportunist human agents (Falkinham 2015; Prevots and Marras 2015). Cattle diseases result from infection with related *Mycobacterium farcinogenes* and *Mycobacterium senegalense* (Chamoiseau 1979; Turenne 2019). The *Mycobacterium abscessus* complex consists of a group of rapidly growing, inherently drug-resistant, nontuberculous mycobacteria, differentiated into three subspecies, *M. abscessus*, *M. massiliense*, and *M. bolleti*, responsible for a wide spectrum of skin and soft-tissue diseases, central nervous system infections, bacteremia, and ocular and other infections (Medjahed et al. 2010; Tortoli et al. 2018). *Mycobacterium abscessus* is a serious pathogen for patients with cystic fibrosis (Parkins and Floto 2015; Martiniano and Nick 2015; Skolnik et al. 2016; Ryan and Byrd 2018) and closely related *Mycobacterium chelonae* can infect heart valve transplants (Wallace 1994). Opportunist pathogens include *Mycobacterium gordonae*, *Mycobacterium malmoense*, *Mycobacterium mucogenicum*, *Mycobacterium szulgai*, and *Mycobacterium xenopi* (Rombouts et al. 2009; Prevots and Marras 2015).

1.2 Mycobacterial Lipids

As a consequence of having two distinct membrane organelles in the cell envelope (Minnikin 1982; Brennan and Nikaido 1995; Hoffmann et al. 2008; Zuber et al. 2008; Daffé and Reyrat 2008; Daffé and Zuber 2014; Minnikin et al. 2015), mycobacterial lipids are remarkably diverse. The cytoplasmic membrane is a bilayer

based on polar lipids, but including high proportions of characteristic glycophospholipids known as phosphatidylinositol mannosides (PIMs). This distinct composition warrants recognition of this organelle as a special mycobacterial inner membrane (MIM) (Bansal-Mutalik and Nikaido 2014; Minnikin et al. 2015). The inner leaflet of the mycobacterial outer membrane (MOM) is based on a monolayer of long-chain mycolic acids (MAs), covalently bound to terminal arabinose units presented by an arabinogalactan-peptidoglycan macromolecule. A wide variety of free lipid types are considered to interact with the MOM platform, mainly contributing to the MOM outer leaflet. The origins and structures of the individual lipid classes are detailed in the following sections.

The special character of mycobacterial lipids was revealed by Rudolf J. Anderson and colleagues in the 1920s and 1930s (see Anderson 1939, 1940, 1941, 1943). Methods were not available to enable the determination of precise chemical structures, though significant progress was made by Edgar Lederer and coworkers (see Asselineau 1966; Lederer 1967, 1971). Technological advances in infra-red spectrometry and chromatography allowed discriminatory examination of mycobacterial lipid fractions (Smith et al. 1954, 1957, 1960a, b; Smith and Randall 1965). The mainly glycolipid fractions were designated as characteristic "Mycosides" (Smith et al. 1960b). Mycosides A, B, and G are phenolic glycolipids from *M. kansasii*, *M. bovis*, and *M. marinum*, respectively (Smith and Randall 1965; Navalkar et al. 1965). The labels mycosides C and D were applied to families of glycopeptidolipids, expressed by the MAIS complex (Smith et al. 1957, 1960a, b; Smith and Randall 1965). Mycosides F, from *M. fortuitum* (Fregnan et al. 1961), were later shown to be acyl trehaloses (Gautier et al. 1992; Hamid et al. 1993; Sempere et al. 1993).

A number of substantial early reviews included general surveys of mycobacterial lipids (Anderson 1939, 1940, 1941, 1943; Asselineau 1966; Lederer 1967, 1971; Goren 1972; Ratledge 1976; Barksdale and Kim 1977; Asselineau and Asselineau 1978; Goren and Brennan 1979; Minnikin and Goodfellow 1980; Minnikin 1982). Later, highly informative reviews appeared (McNeil et al. 1989; Minnikin 1991; McNeil and Brennan 1991; Brennan and Nikaido 1995; Daffé and Draper 1998; Daffé and Lemassu 2000; Minnikin et al. 2002; Draper and Daffé 2005; Kremer and Besra 2005). Recent general surveys are available (Daffé and Reyrat 2008; Daffé and Zuber 2014; Jackson 2014; Minnikin et al. 2015; Lowary 2016; Chiaradia et al. 2017; Singh et al. 2018).

2 Mycolic Acids (MAs) and Conjugates

Mycolic acids (MAs) are 3-hydroxy-2-alkyl-branched long-chain fatty acids produced by mycobacteria and related taxa, the so-called "mycolata" (Chun et al. 1996). Anderson and co-workers identified mycolic acids, in pioneering studies (Stodola et al. 1938; Anderson 1941). The essential 3-hydroxy-2-alkyl-branched nature of TB mycolic acids was defined by Asselineau and Lederer (1950), explaining how, on pyrolysis, cleavage produces straight-chain hexacosanoate and long-chain aldehydes. In accord with accepted chemical usage, the long-chain aldehydes were

designated as "meromycolic aldehydes" from the Ancient Greek *méros*, denoting a part or portion (Morgan and Polgar 1957). Distinct structural types are found in mycobacteria with variations in the meromycolate chain (Etémadi 1967a, b; Minnikin 1982; Daffé et al. 1983; Marrakchi et al. 2008, 2014; Verschoor et al. 2012; Minnikin et al. 2015; Daffé et al. 2017).

Cyclopropane rings were identified in mycobacterial MAs (Gastambide-Odier et al. 1964), with later distinction between *cis*- and *trans*-cyclopropane rings (Minnikin 1966; Minnikin and Polgar 1967a, b, c). MAs with no meromycolate oxygen functions are termed α-mycolic acids (~C_{70}–C_{90}); smaller (C_{66} and C_{68}) α′-mycolates are also encountered. Ketomycolates are widespread, often accompanied by methoxy- or wax-ester mycolates, all in the C_{80}–C_{90} size range. These oxygenated MAs have either *cis*- or *trans*- double bonds or cyclopropane rings, the *trans*- unsaturations being accompanied by an adjacent methyl branch. Minor amounts of extended oxygenated MAs are encountered (Qureshi et al. 1978; Takayama et al. 1979; Watanabe et al. 2002), currently being labelled XL-MAs (Slama et al. 2016). Epoxymycolates (Daffé et al. 1981b; Minnikin et al. 1980, 1982b) and ω – 1 methoxymycolates (Luquín et al. 1987, 1990, 1991) have a more limited distribution in certain rapid-growing mycobacteria. MAs are mainly covalently bound structural MOM components, but are also found in free lipid conjugates (Asselineau 1966; Minnikin 1982; Brennan and Nikaido 1995; Daffé and Zuber 2014; Marrakchi et al. 2008, 2014; Minnikin et al. 2015; Daffé et al. 2017).

2.1 Mycolic Acids (MAs)

The major MAs, typical of the *M. tuberculosis* complex, are shown in Fig. 1A. The di-*cis*-cyclopropyl α-MAs of *M. tuberculosis* have a characteristic short 11- and 13-carbon chain between the 3-hydroxy group and the proximal *cis*-cyclopropane (Fig. 1A), contrasting with other mycobacteria that usually have 17-carbon chains (Fig. 1B–D). Additionally, TB α-MAs have a 20 carbon terminal chain, with the others having 18 carbons, and the chain in 2-postion has 24 rather than 22 carbons (Fig. 1B–D). Such subtle differences in functional group location influence conformational behavior, with TB α-MAs packing in a more extended manner than α-MAs from *M. kansasii* (Villeneuve et al. 2010; Minnikin et al. 2015). It is appropriate, therefore, to assign a distinguishing label "TB-α" to the α-MAs from members of the *M. tuberculosis* complex (Fig. 1A: a, b). In many publications, such importance of MA alkyl chain length is ignored; these errors are often perpeptuated, as exemplified by Marrakchi et al. (2014) and Daffé et al. (2017).

It is notable that in the major *trans*-cyclopropyl methoxy and keto MAs, from the *M. tuberculosis* complex, the adjacent methyl branch is distal (Fig. 1A: e, i). In the minor *trans*-alkene methoxy and keto MAs (Fig. 1A: f, j), however, the adjacent methyl branch is proximal (Fig. 1A: e, i). Similar proximal methyl *trans*-alkene keto MAs are found in *M. avium* (Fig. 1D) and *M. marinum*, where they are accompanied by corresponding methoxy MAs (Fig. 1E). The MAs of *M. kansasii* (Fig. 1B), *M. leprae* (Fig. 1C), and *M. avium* (Fig. 1D) have mainly a 22-carbon chain in

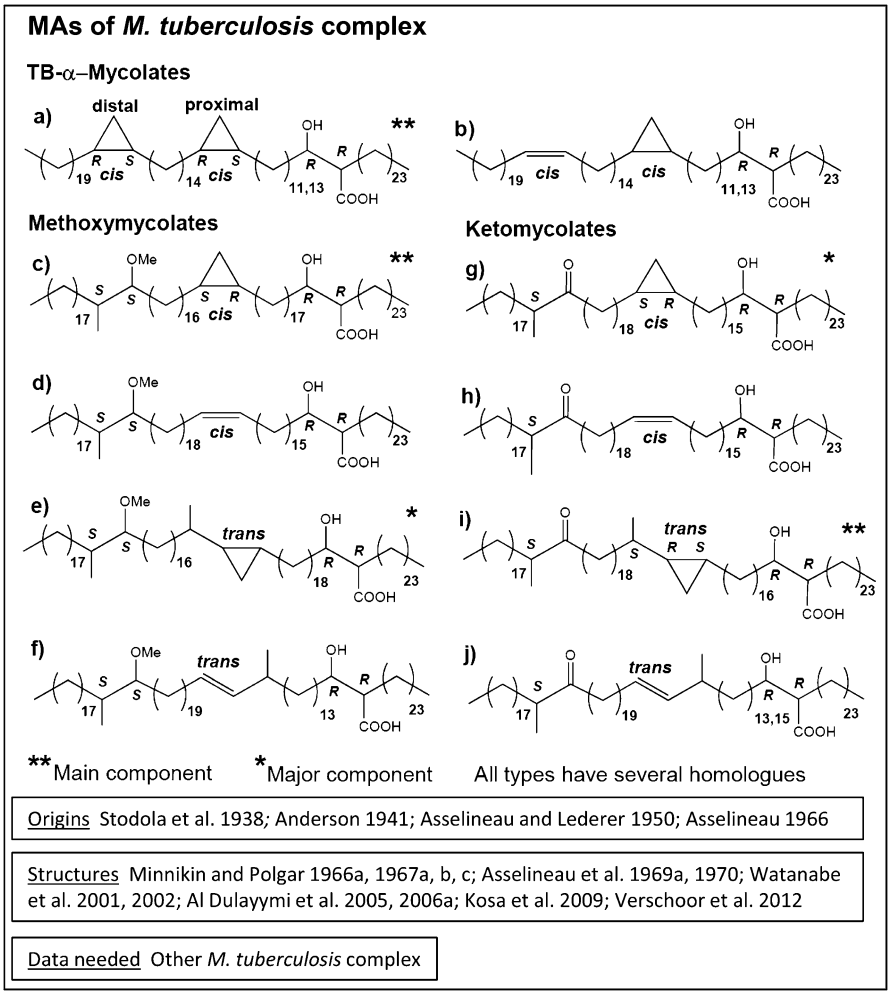

Fig. 1A Mycolic acids (MAs) of the *Mycobacterium tuberculosis* complex

2-position. In *M. kansasii*, α-, methoxy-, and keto MAs provide an overall pattern similar to *M. tuberculosis*, with the above mentioned subtle differences in α-MA structure (Fig. 1B).

The *M. leprae* MAs are similar to those of *M. kansasii*, but lack a methoxy component (Fig. 1C) (Minnikin et al. 1985a); an earlier report indicated a minor methoxymycolate (Daffé et al. 1981a). The *M. avium* complex has α- and keto-MAs similar to those of *M. kansasii*, but methoxy-MAs are replaced by wax-ester MAs (Fig. 1D). The MAs from *M. marinum* and *M. ulcerans* (Fig. 1E) lack cyclopropanation in the oxygenated MAs, the methyl branch, adjacent to *trans*-alkenes in methoxy- and keto-MAs, being on the proximal side (Daffé et al. 1991a). It is also notable that, as in TB α-MAs (Fig. 1A), the dicyclopropyl α-MAs of *M. marinum*

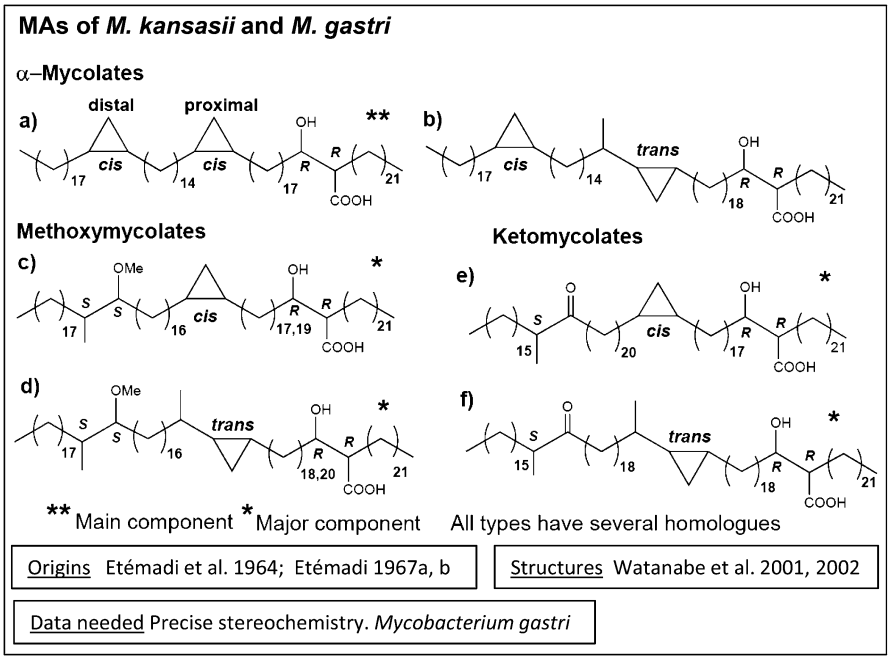

Fig. 1B Mycolic acids (MAs) of *Mycobacterium kansasii* and *Mycobacterium gastri*

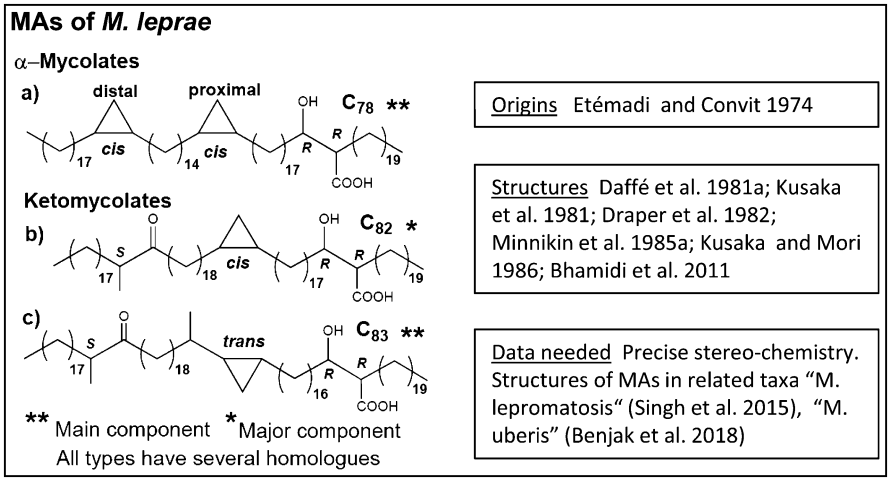

Fig. 1C Mycolic acids (MAs) of *Mycobacterium leprae*

have a short 13-carbon chain between the 3-OH group and the proximal cyclopropane (Fig. 1E). Conformational investigations should be made on all the *M. marinum* MA classes as in previous studies (Villeneuve et al. 2005, 2007, 2010, 2013). It would be interesting to see if the α-MAs of *M. marinum* perform in an analogous

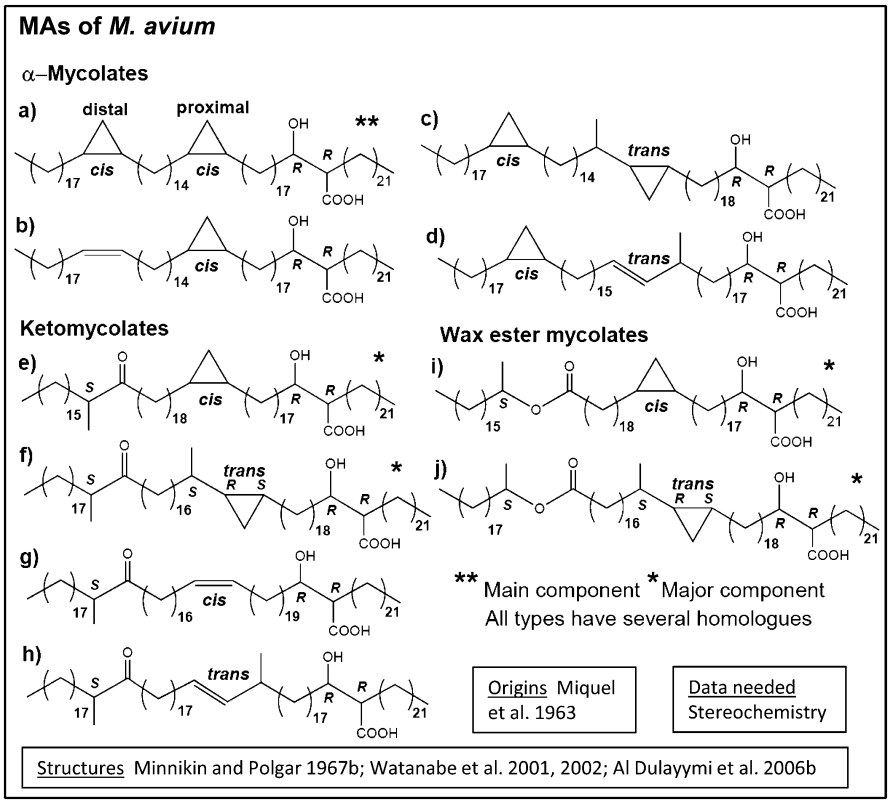

Fig. 1D Mycolic acids (MAs) of the *Mycobacterium avium* complex (MAC)

manner to the TB α-MAs and if an adjacent proximal methyl group facilitates folding of *M. marinum trans*-alkene methoxy and keto MAs in the same way that a distal methyl branch assists folding of a *trans*-cyclopropane oxygenated MAs from the *M. tuberculosis* complex (Villeneuve et al. 2013). Several isolates of *M. ulcerans* appear to lack methoxymycolates (Daffé et al. 1984).

Rapidly growing mycobacteria, such as *M. chelonae*, *M. abscessus*, and members of the so-called "*M. fortuitum* complex," have characteristic MA patterns (Fig. 1F, G). The MAs of *M. chelonae* and *M. abscessus* are limited to comparable amounts of α- and α′-MAs (Fig. 1F) (Minnikin et al. 1982a). The α′-MAs are relatively simple with a single *cis*-alkene; however, there are two varieties of α-MAs, one having two *cis*-double bonds (α_1) and the other (α_2) containing a *trans*-alkene with an adjacent proximal methyl branch (Fig. 1F). The main MAs of *M. fortuitum*, and related mycobacteria, are also α_1- and α_2-MAs (Fig. 1G), accompanied occasionally by minor proportions of α′-MAs in *M. farcinogenes* and *M. senegalense* (Ridell et al. 1982), but rarely in *M. fortuitum* (Minnikin et al. 1980, 1984a; Daffé et al. 1983). Epoxy-MAs (Fig. 1G) in the *M. fortuitum* complex were first recognized as acid

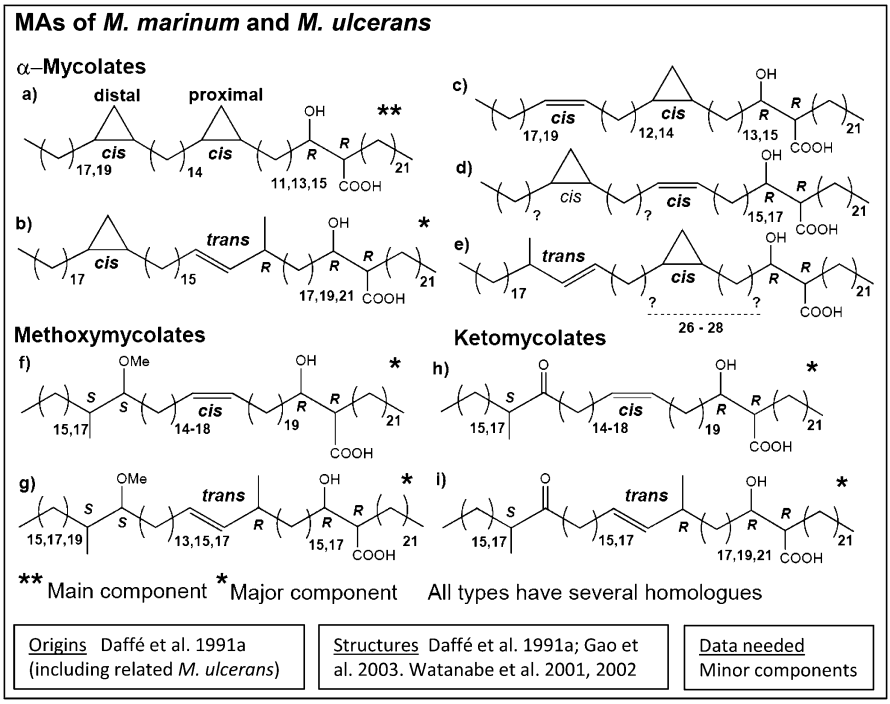

Fig. 1E Mycolic acids (MAs) of *Mycobacterium marinum* and *Mycobacterium ulcerans*

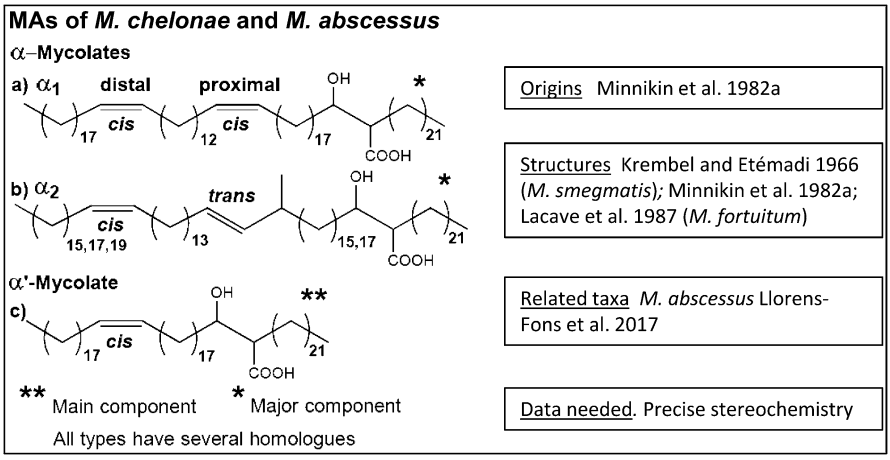

Fig. 1F Mycolic acids (MAs) of *Mycobacterium chelonae* and *Mycobacterium abscessus*

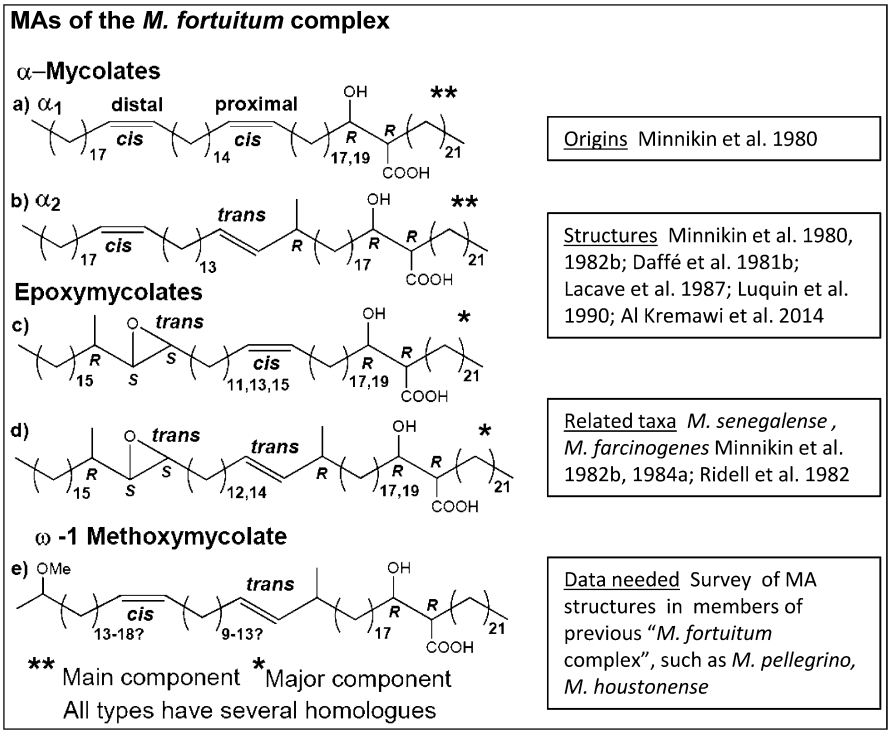

Fig. 1G Mycolic acids (MAs) of the *Mycobacterium fortuitum* complex

methanolysis products (Minnikin et al. 1980; Ridell et al. 1982) and later characterized as having epoxide rings (Daffé et al. 1981b; Minnikin et al. 1982b, 1984a; Lacave et al. 1987). An unknown minor mycolate, labelled "K," was recorded in *M. fortuitum* (Minnikin et al. 1980, 1984a) and in *M. senegalense* (Ridell et al. 1982), *M. peregrinum* (Minnikin et al. 1984a), and *Mycobacterium porcinum* (Luquín et al. 1987). Using an environmental organism, it was shown that mycolate "K" is a novel ω – 1 methoxymycolate (Fig. 1G) (Luquín et al. 1990), and its presence was confirmed in *M. fortuitum*, *M. porcinum*, *M. peregrinum*, and *M. senegalense* (Luquín et al. 1991).

2.2 Trehalose Mono- and Dimycolates (TMMs and TDMs)

Trehalose dimycolates (TDMs) were isolated from tubercle bacilli and an association with cording morphology resulted in the loose term "cord factors" being assigned to these lipids (Bloch 1950; Asselineau 1966; Goren 1975; Asselineau and Asselineau 1978). The essential structures of TDMs were established (Noll, 1956; Noll et al. 1956) and early detailed analyses undertaken by Adam et al. (1967) and Strain et al.

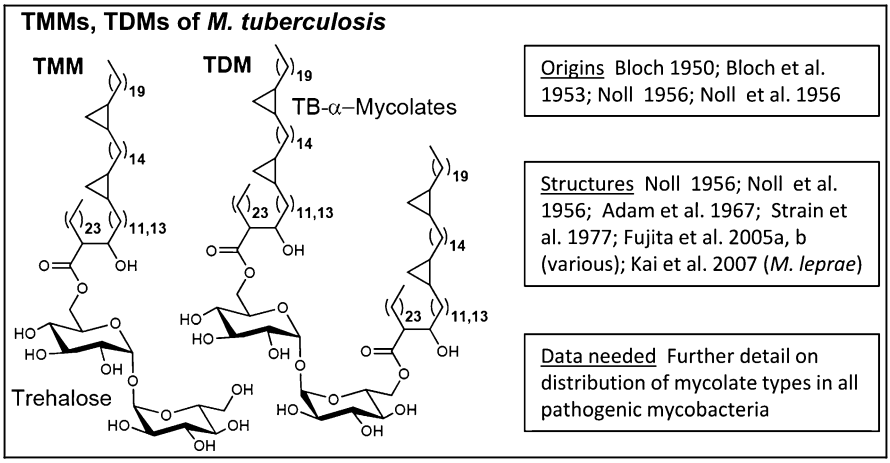

Fig. 2 Trehalose mono- and dimycolates (TMMs, TDMs) of *Mycobacterium tuberculosis*

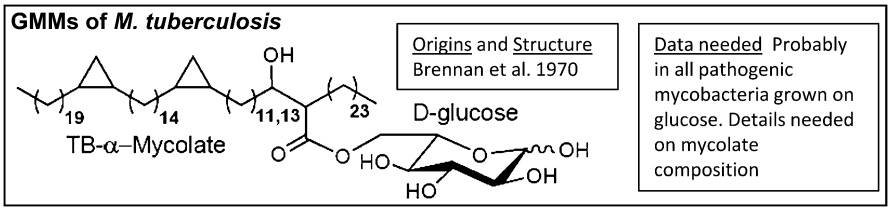

Fig. 3 Glucose monomycolates (GMMs) of *Mycobacterium tuberculosis*

(1977). These two latter studies demonstrated, for the first time, that certain *M. bovis* BCG strains lacked methoxymycolates, a phenomenon only clarified later (Minnikin et al. 1984b). In mass spectrometric studies, the complexity of trehalose monomycolates (TMMs) (Fujita et al. 2005a) and TDMs (Fujita et al. 2005b) from a range of mycobacteria was displayed, including *M. leprae* (Kai et al. 2007). Representative structures of TDMs and TMMs from *M. tuberculosis* are shown in Fig. 2. Trehalose mycolates have a clear metabolic role in the transfer of mycolic acids into the mycobacterial cell wall (Belisle et al. 1997; Kremer and Besra 2005; Takayama et al. 2005).

2.3 Other Mycolic Acid Esters

2.3.1 Glucose Monomycolates (GMMs)

Glucose monomycolates (GMMs) (Fig. 3) were reported by Brennan et al. (1970) in mycobacteria when glucose is included in growth media. Similarly, *M. avium*

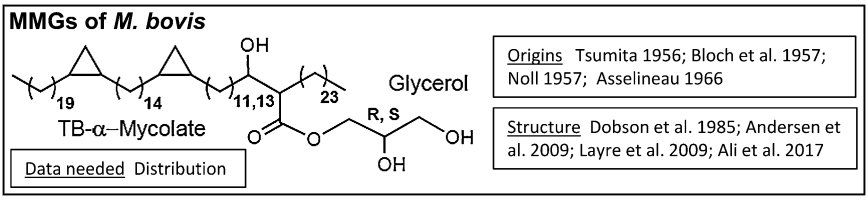

Fig. 4 Monomycoloyl glycerols (MMGs) of *Mycobacterium bovis*

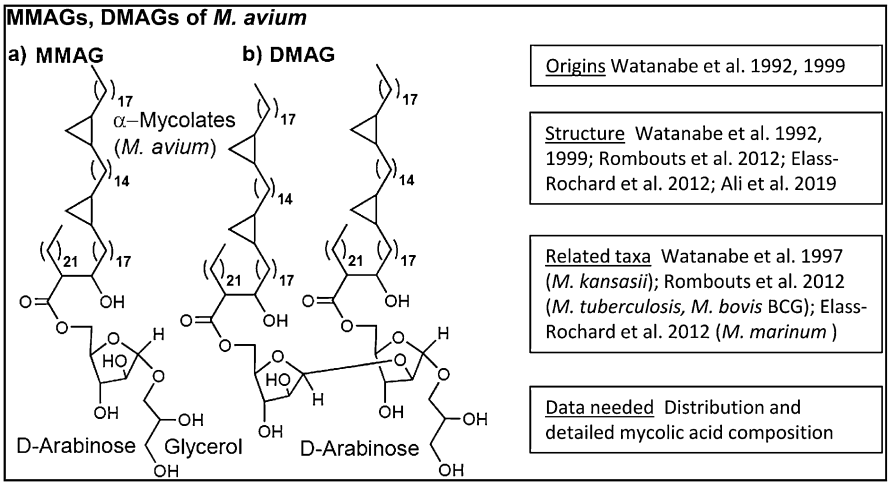

Fig. 5 Mycoloyl mono- and diarabinoglycerols (MMAGs, DMAGs) of *Mycobacterium avium*

and *M. tuberculosis* grown on fructose gave glycolipids based on fructose (Itoh and Suzuki 1974).

2.3.2 Monomycoloyl Glycerols (MMGs, GroMMs)

Monomycoloyl glycerols (MMGs, GroMMs) (Fig. 4) are expressed in members of the *M. tuberculosis* complex, particularly *M. bovis* (Tsumita 1956; Bloch et al. 1957; Noll 1957; Asselineau 1966; Dobson et al. 1985; Andersen et al. 2009), with the mycolic acid composition reflecting that of whole cells. The natural MMGs from *M. bovis* BCG included both glycerol isomers, with the R-isomer showing enhanced antigenic properties (Layre et al. 2009).

2.3.3 Mono- and Dimycoloyl Diarabinoglycerols (MMAGs, DMAGs)

Mono- and dimycoloyl diarabinoglycerol glycolipids (MMAG, DMAG) (Fig. 5) were initially characterized from the MAIS complex (Watanabe et al. 1992, 1999), but they are more widespread including *M. tuberculosis* (Rombouts et al. 2012) and

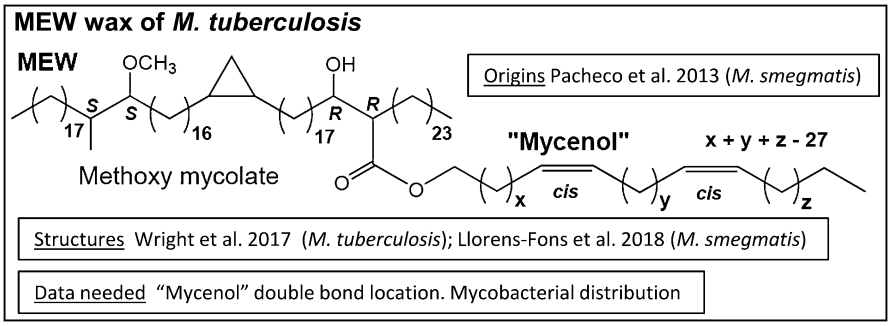

Fig. 6 Mycenyl mycolate ester wax (MEW) of *Mycobacterium tuberculosis*

M. marinum (Elass-Rochard et al. 2012). Synthetic studies indicated that these lipids are based on substituted glycerols with L-configuration (Ali et al. 2019).

2.3.4 Mycenyl Mycolate Ester Waxes (MEWs)

Investigations of *mmpL11* lipid transporter proteins in *M. smegmatis* revealed the presence of partially characterized mycolate ester waxes (MEWs), based on long-chain alcohols (Pacheco et al. 2013; Melly and Purdy 2019). MEWs, based principally on methoxy mycolates, were characterized from *M. tuberculosis* (Fig. 6) (Wright et al. 2017). The unsaturated long-chain alcohols are a new class of mycobacterial lipids, deserving of a recognizable label, so the term "mycenols" is proposed. Related MEWs from *M. smegmatis* have been analyzed in more detail (Llorens-Fons et al. 2018).

3 Multimethyl-Branched and Polyunsaturated Fatty Acid Esters of Trehalose

Trehalose is an effective scaffold for a range of glycolipids, esterified with fatty acids other than mycolic acids (Asselineau 1966; Asselineau and Asselineau 1978; Khan et al. 2012). In *M. tuberculosis*, related di-, tri-, and pentaacyl trehaloses (DATs, TATs, and PATs) include dextrorotatory fatty acids whose methyl branches have *S*-configuration (Minnikin et al. 1985b, 2002; Daffé et al. 1988a; Muñoz et al. 1997; Jackson et al. 2007). Acyl trehaloses (DATs and TATs) are also produced by *M. fortuitum* (Gautier et al. 1992; Hamid et al. 1993; Sempere et al. 1993). Sulfoglycolipds (SGLs), based on trehalose, have very long multimethyl branched phthioceranic and hydroxyphthioceranic acyl chains. The trehalose polyphleates (TPPs) have similarly long polyunsaturated fatty acids. In contrast, glycosylated acylated trehaloses (lipooligosaccharides, LOSs) have relatively short methyl-branched fatty acids, but usually longer oligosaccharide units.

3.1 Di-, Tri-, and Pentaacyl Trehaloses (DATs, TATs, and PATs)

Early extraction of mycobacterial lipids used ethanol-diethyl ether to both kill the bacteria and provide a lipid extract that included phosphatides and other low-melting "fats" that were easily saponified (see Anderson 1939, 1940; Aebi et al. 1953; Asselineau 1966). Chloroform extraction of the bacterial residue yielded higher-melting "waxes," which required extended saponification to release long-chain components (see Sect. 4.2). Facile saponification of *M. tuberculosis* "fats" gave a crude dextrorotatory fatty acid, labelled "phthioic acid" (Anderson 1929). These multimethyl-branched fatty acids were characterized as mycolipenic (phthienoic) (Chanley and Polgar 1950), mycolipodienoic (Coles and Polgar 1969), mycolipanolic (Coles and Polgar 1968), and mycosanoic (Cason et al. 1964) acids (Minnikin 1982). A "3-hydroxy-C_{27}-acid," corresponding to mycolipanolic acid, was recorded by Asselineau (1966), citing unpublished work by C Asselineau, J-C Promé, and J Asselineau. The nature of the parent lipids was revealed as diacyl and pentaacyl trehaloses (DATs and PATs), in a focused systematic search (Minnikin et al. 1985b, 2002). Structures of the DATs were elaborated (Fig. 7A) (Besra et al. 1992a; Baer 1993), along with those of the triacyl trehaloses (TATs) (Muñoz et al. 1997) and PATs (Daffé et al. 1988a) (Fig. 7B). More than 30 molecular species of DATs from *M. tuberculosis* H37Rv were recently revealed (Frankfater et al. 2019).

2-Methyloctadec-2-enoic acid was found in *M. fortuitum* (Valero-Guillén et al. 1987) and its location in specific DAT and TATs (Fig. 8) established (Gautier et al. 1992; Hamid et al. 1993; Sempere et al. 1993). These acyl trehaloses are the

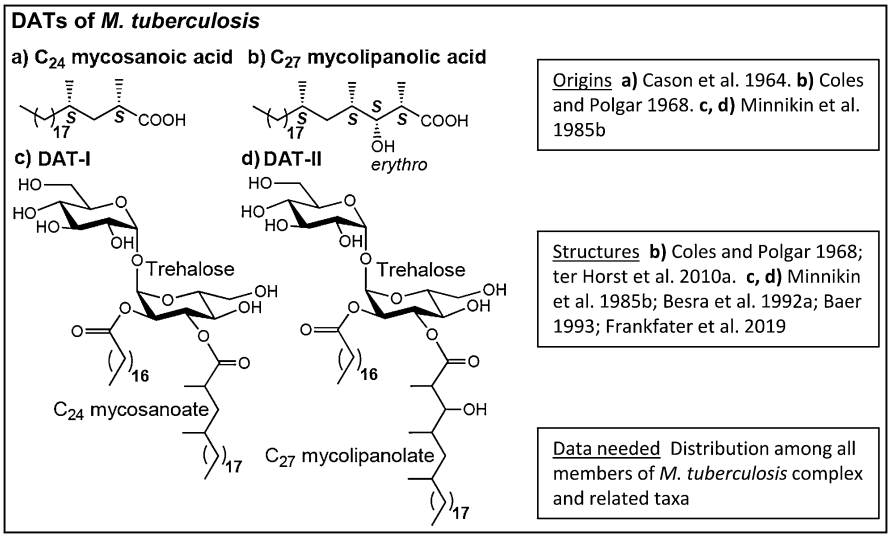

Fig. 7A Diacyl trehaloses (DATs) of *Mycobacterium tuberculosis*

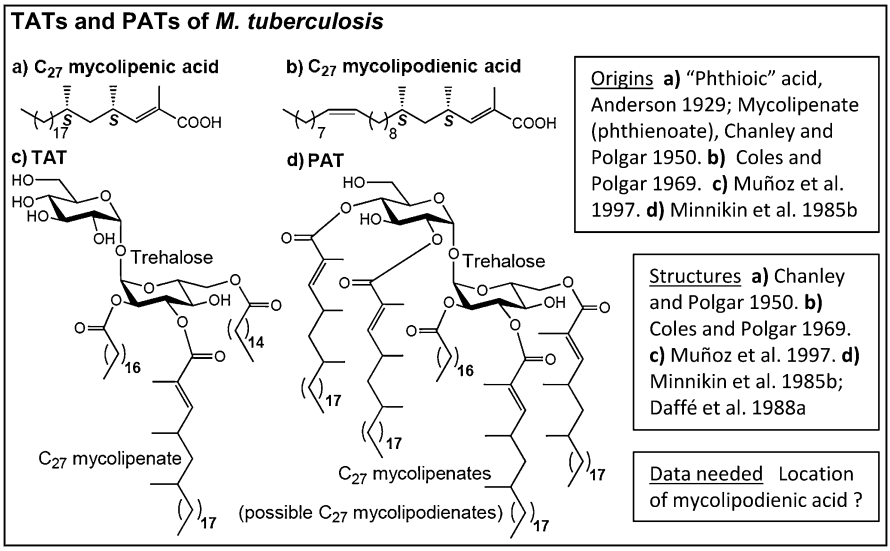

Fig. 7B Tri- and pentacyl trehaloses (TATs, PATs) of *Mycobacterium tuberculosis*

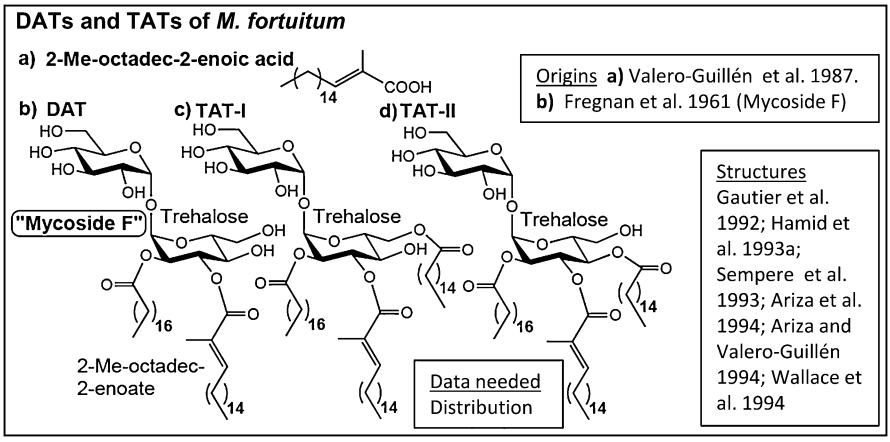

Fig. 8 Di- and triacyl trehaloses (DATs, TATs) of *Mycobacterium fortuitum*

mycoside F, detected by Fregnan et al. (1961). Further detailed studies of the *M. fortuitum* DATs (Ariza et al. 1994; Ariza and Valero-Guillén 1994) showed the presence of four distinct types with additional branched fatty acids, particularly a partially characterized 2-methyl-octadecadienoic acid.

3.2 Sulfoglycolipids (SGLs)

Sulfur-containing lipids were detected by Middlebrook et al. (1959) in extracts of virulent tubercle bacilli and characterized as families of trehalose 2′-sulfates acylated with phthioceranic (PA) and hydroxyphthioceranic acids (HPA) (Fig. 9) (Goren 1970a, b; Goren et al. 1971, 1976; Goren 1984; Goren 1990). The SGLs were assigned to families, labelled SL-I to SL-III (Goren 1984, 1990), with a later addition of SL-IV (Gilleron et al. 2004; Layre et al. 2011a). The trehalose 2′-sulfates are acylated at positions 2, 3, 6, and 6′, the 2-position being invariably occupied by straight-chain C_{16} and C_{18} fatty acids. Early studies recognized an SL-II′ family with acylation at position 4 (Goren 1984; Goren 1990); however, detailed analyses failed to pinpoint this feature (Layre et al. 2011a), so SL-II′ has not been included in Fig. 9. In studies on the SL-IV antigenic diacylated sulfoglycolipids (Gilleron et al. 2004), the nomenclature Ac_2SGL was recommended. It is proposed to make SGL labels more informative and incorporate the original SL nomenclature, as exemplified for Ac_2SGL: SL-IV (Fig. 9h). Layre et al. (2011a) confirmed the SGLs as Ac_4SGL: SL-I′, SL-I, SL-II; Ac_3SGL: SL-III; Ac_2SGL: SL-IV′, SL-IV) (Fig. 9c–h). The illustrative data in Fig. 9 include the common C_{37}-PA and C_{40}-HPA acyl chains, but PA and HPA fatty acids from 25 to 54 carbons are present (Layre et al. 2011a). The structure of Ac_2SGL: SL-IV (Fig. 9h) was confirmed by chemical synthesis, including the absolute stereochemistry of the principal HPA (Fig. 9b) (Geerdink et al. 2013); similarly, the structure of Ac_4SGL: SL-I (Fig. 9d) was validated by synthesis (Geerdink and Minnaard 2014).

Certain MmpL8 lipid transporter mutants of *M. tuberculosis* are perturbed in SGL synthesis (Converse et al. 2003; Domenech et al. 2004; Bertozzi and Schelle 2008; Seeliger et al. 2012), and it was found that Ac_2SGL lipids, in particular, elaborated oxophthioceranic (OPA) acids, analogous to the HPAs (Layre et al. 2011a). It is apparent that Ac_4SGL: SL-II (Fig. 9e), and not Ac_4SGL: SL-I (Fig. 9d), is usually the most abundant SGL family (Layre et al. 2011a; Rhoades et al. 2011). A minor PA-containing component of the Ac_2SGL lipids has been assigned the label Ac_2SGL: SL-IV′ (Fig. 9g). Certain Ac_4SGL fractions apparently contained mycolipanolates, as found in DAT-II glycolipids (Fig. 7A) (Alugupalli et al. 1995).

3.3 Trehalose Polyphleates (TPPs)

"Phleic acids" occur as trehalose esters in the saprophyte *Mycobacterium phlei* (Asselineau et al. 1969b, 1972; Asselineau and Asselineau 1978). Trehalose polyphleates (TPPs) are now found in a range of mycobacteria, including *M. abscessus* and *M. chelonae* (Burbaud et al. 2016). The principal *M. abscessus* heptaacyl trehalose phleate is shown in Fig. 10 (Llorens-Fons et al. 2017). These very nonpolar lipids are structurally analogous to the pentaacyl trehaloses (PATs) from the *M. tuberculosis* complex (Sect. 3.1) (Fig. 7B).

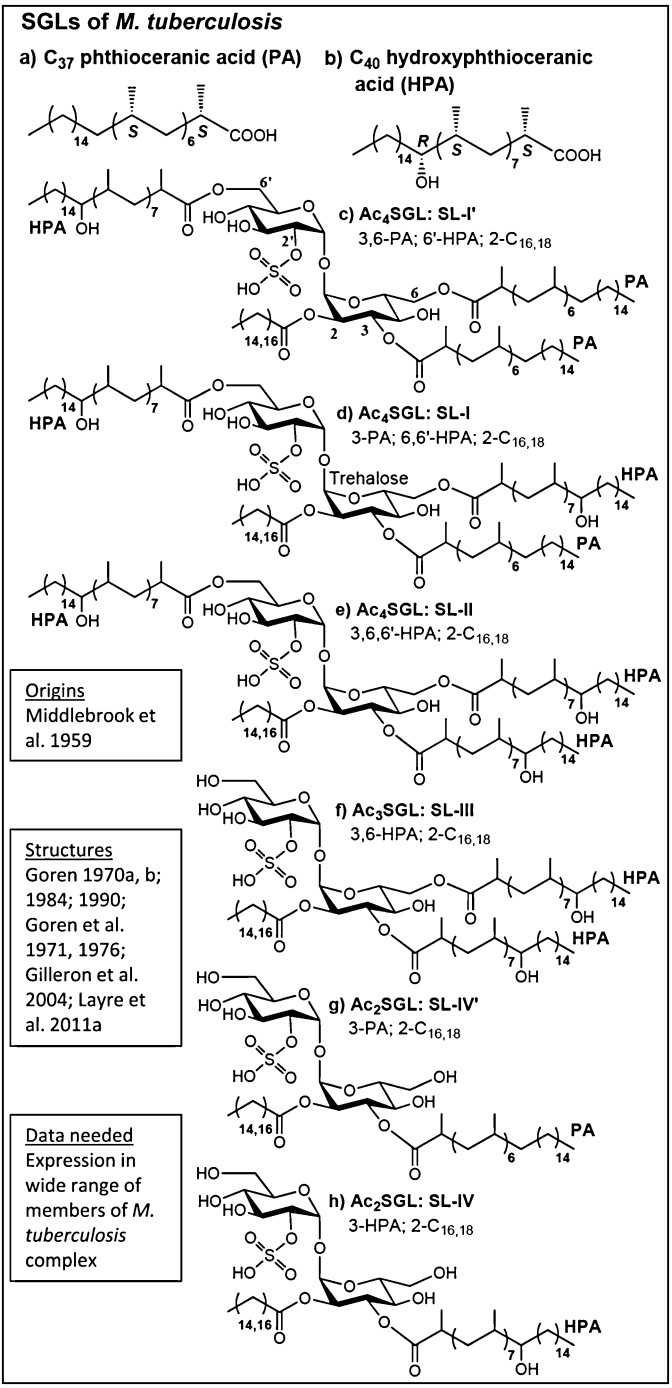

Fig. 9 Sulfoglycolipids (SGLs) of *Mycobacterium tuberculosis*

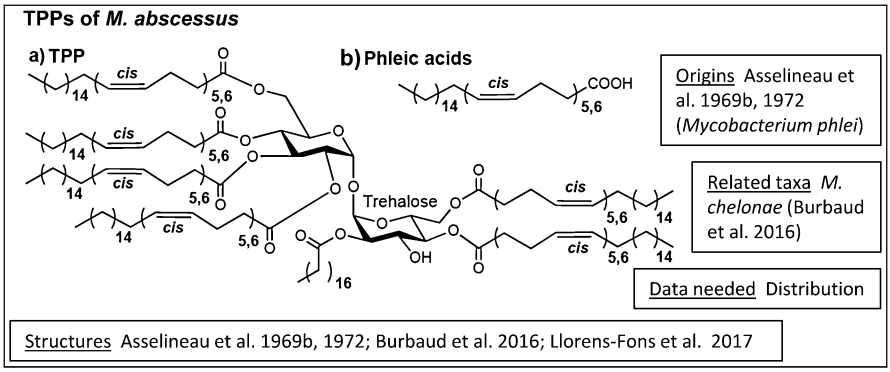

Fig. 10 Trehalose polyphleates (TPPs) of *Mycobacterium abscessus*

3.4 Glycosylated Acylated Trehaloses (Lipooligosaccharides, LOSs)

Characteristic polar lipids were pinpointed by thin-layer chromatography (TLC) in clinically significant NTMs (Jenkins et al. 1972; Jenkins 1981). Brennan et al. (1978) observed that such lipids were alkali labile, unlike the alkali stable glycopeptidolipids (GPLs) (see Sect. 6) from the *M. avium* complex (see Aspinall et al. 1995). These trehalose-based lipids have fatty acyl substituents, and sometimes a methoxy group, on one α-D-glucopyranose (Fig. 11) (Camphausen et al. 1987). The other α-D-glucopyranose residue carries one or more sugars to form distinct oligoglycosyl attachments, responsible for distinct serological activity (Fig. 11) (Hunter et al. 1988).

The nature of alkali-labile "lipooligosaccharides" (LOSs) was revealed for *M. kansasii* (Hunter et al. 1983; Aspinall et al. 1995) (Fig. 11A). Eight such glycolipids (Fig. 11A, LOSs I–VIII) were isolated (Hunter et al. 1984, 1985) and the oligosaccharides were acylated by 2,4-dimethyltetradecanoyl functions. The structure of the *N*-acylamido sugar, labelled *N*-acylkansosamine, was established by Hunter et al. (1984, 1985) (Fig. 11A) and this moiety was the basis of the specific serological identity of *M. kansasii* (Hunter et al. 1985). Subsequent chemical synthesis confirmed the absolute configuration of the sugar (Yoshimura et al. 1987). Four specific polar lipids in eight strains of *M. kansasii* had been previously identified by TLC (Szulga et al. 1966), and they correspond well with four major LOSs displayed later by 2D-TLC in Fig. 10 of Dobson et al. (1985).

M. gastri is closely related to *M. kansasii*, but it has distinct LOSs that are based on essentially common triacyl trehaloses and poly-L-xylose backbones (Fig. 11A) (Gilleron and Puzo 1995). An unusual terminal moiety is found in *M. gastri* LOSs III-IV (see Fig. 11A) (Gilleron et al. 1993, 1994; Longépé et al. 1997).

Mainstream tubercle bacilli do not express LOSs, but the ancestral relative, *M. tuberculosis* Canetti ("*M. canettii*") did express polar antigenic glycolipids resembling LOSs (Papa et al. 1989; Minnikin et al. 1990). This diagnosis was confirmed and the main glycolipids were labelled LOS I and LOS II (Daffé et al.

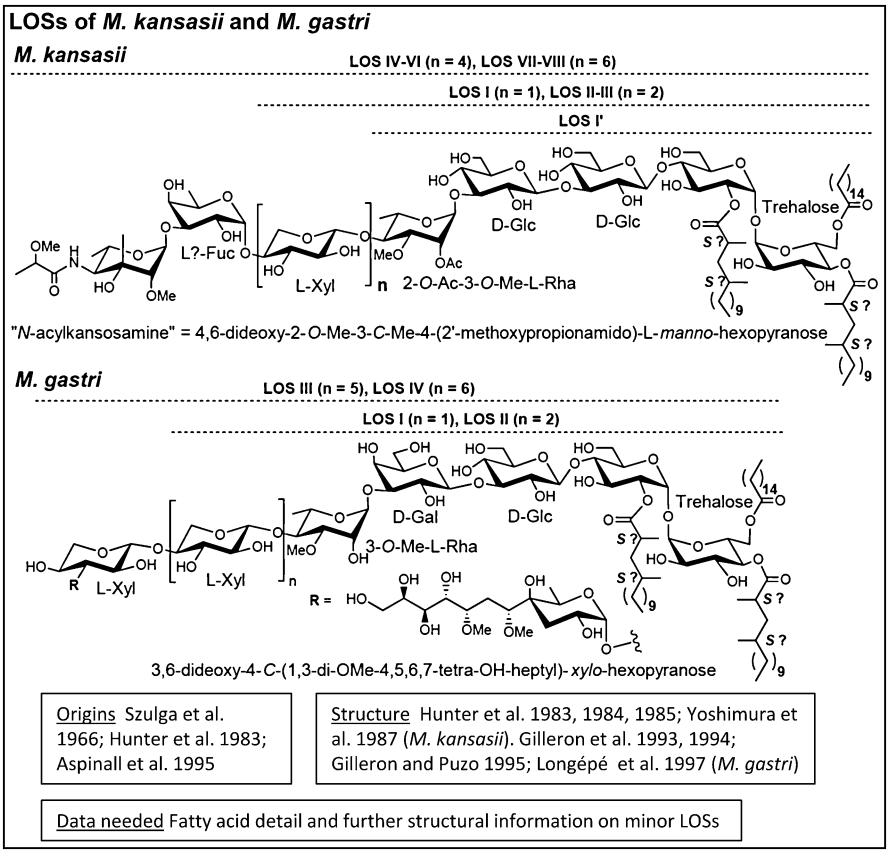

Fig. 11A Lipooligosaccharides (LOSs) of *Mycobacterium kansasii* and *Mycobacterium gastri*

1991b). The specific terminus of LOS II was initially only partially defined, with the presence of 4-amino-4,6-dideoxy-Gal-pyranose being suspected (Daffé et al. 1991b; Gilleron and Puzo 1995). Current opinion indicates 3-amino-3,6-dideoxy-Gal-pyranose and the LOS I, II structures shown in Fig. 11B (Daffé et al. 2014; Angala et al. 2014).

Antigenic LOSs were detected in *M. marinum* by Minnikin et al. (1989) and structural studies showed that the LOSs of *M. marinum* have a core pentasaccharide in LOS-I of a rhamnosyl diglucosyl-acylated trehalose (Fig. 11C) (Burguière et al. 2005; Ren et al. 2007). The heptasaccharide in LOS-II was derived from LOS-I by adding xylose, accompanied by a novel sugar, "caryophyllose" (see Fig. 11C); repeated addition of caryophyllose gave the octasaccharide LOS-III. LOS-IV had a decasaccharide component, including 4-NH-4,6-dideoxy galactose linked to a range of 3-hydroxy-3-methylated-pyrrolidone cycles (Fig. 11C) (Rombouts et al. 2009, 2010, 2011; Sarkar et al. 2011). LOSs in *M. marinum* had been signaled earlier by

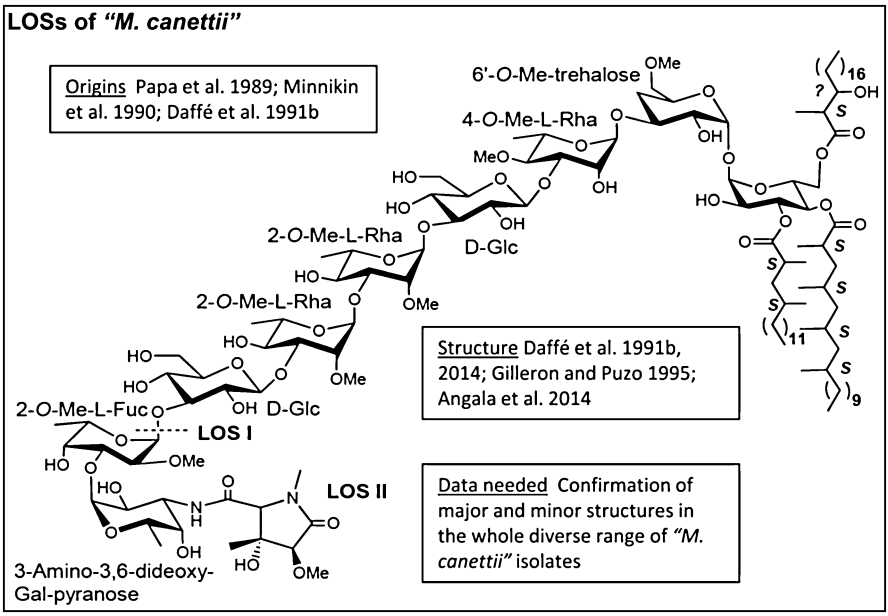

Fig. 11B Lipooligosaccharides (LOSs) of "*Mycobacterium canettii*"

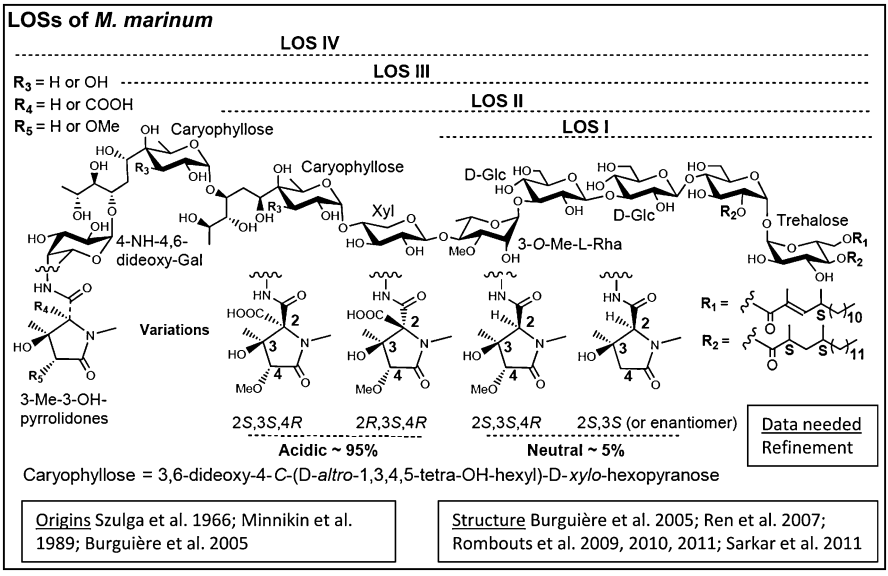

Fig. 11C Lipooligosaccharides (LOSs) of *Mycobacterium marinum*

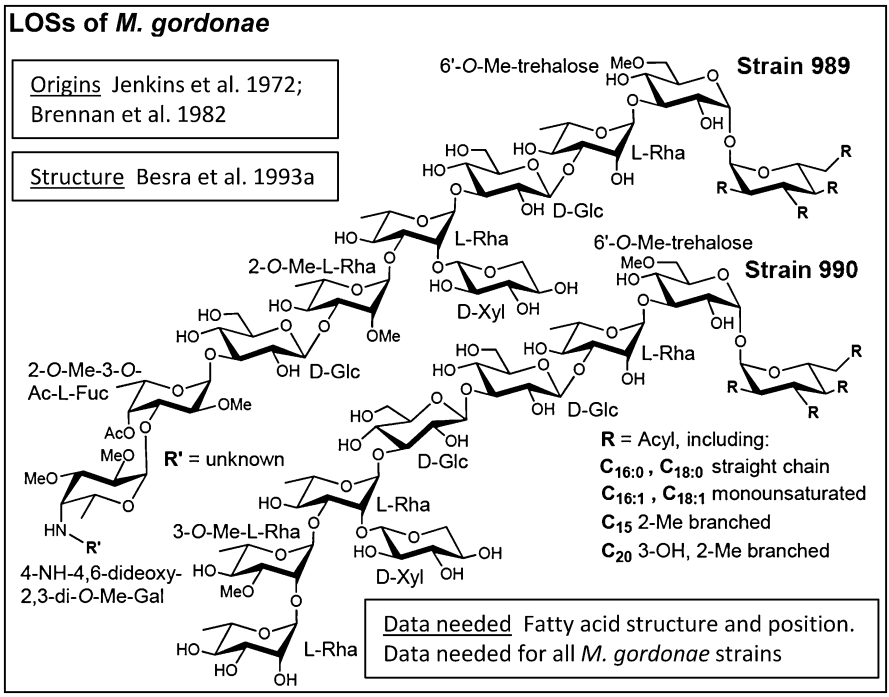

Fig. 11D Lipooligosaccharides (LOSs) of *Mycobacterium gordonae*

Szulga et al. (1966) who recorded the presence of a lipid similar to that revealed by Minnikin et al. (1989).

Characteristic polar lipid patterns were recorded for *M. gordonae* (Jenkins et al. 1972) and seven serovars had distinct profiles that were assigned to the LOS family (Brennan et al. 1982). Structural studies showed that the major LOS-I lipids from *M. gordonae*, strains 989 and 990, are distinct in having methoxylated trehalose bases and branching D-xyloses, with different structures for the two isolates (Fig. 11D) (Besra et al. 1993a). The terminal unit from *M. gordonae* strain 989 remains to be clarified and the full disparate range of LOSs, identified by Brennan et al. (1982), should be structurally characterized.

The family of LOSs from *M. malmoense* are shown in Fig. 11E (McNeil et al. 1987a). LOSs I and II have a terminal dimannoside, but LOS III expresses D-galactofuranose that is uncommon outside mycobacterial arabinogalactans. The original "somewhat tenuous" assignment of a major C_{27} acyl component as 2,4-dimethylpentacosanoate (McNeil et al. 1987a) has been updated by its recognition as 2,4,6-trimethyltetracosanoate (Valero-Guillén et al. 1988; Katila et al. 1991). Again, components that probably correspond to LOSs may have been originally pinpointed in *M. malmoense* by Jenkins (1985).

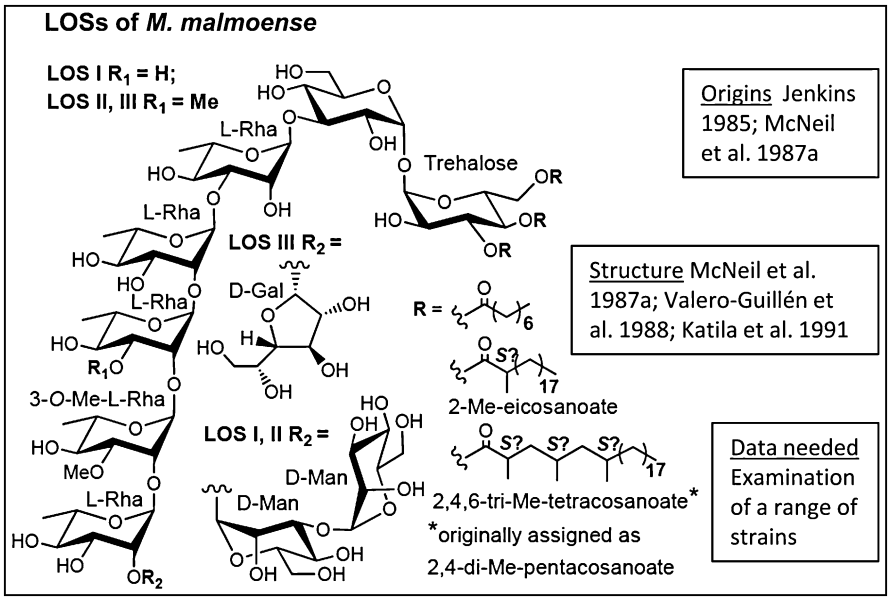

Fig. 11E Lipooligosaccharides (LOSs) of *Mycobacterium malmoense*

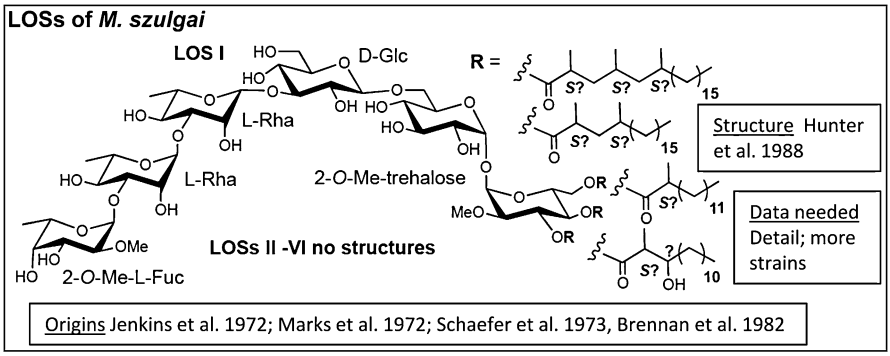

Fig. 11F Lipooligosaccharides (LOSs) of *Mycobacterium szulgai*

Mycobacterium szulgai was proposed as a new species on the basis of distinct polar lipids (Jenkins et al. 1972; Marks et al. 1972; Schaefer et al. 1973). The presence of at least six distinct LOSs in *M. szulgai* has been documented, but only LOS I has been studied (Fig. 11F) (Hunter et al. 1988).

A range of clinical isolates, considered to be "*M. fortuitum* complex," showed a distinct, relatively simple tetraacylated glucosylated trehalose antigen (Fig. 11G: a) (Besra et al. 1992b). However, other studies supported DATs and TATs as the characteristic glycolipid antigens of *M. fortuitum sensu stricto* (Gautier et al. 1992; Hamid et al. 1993; Sempere et al. 1993) (Sect. 3.1). In a study on the "third

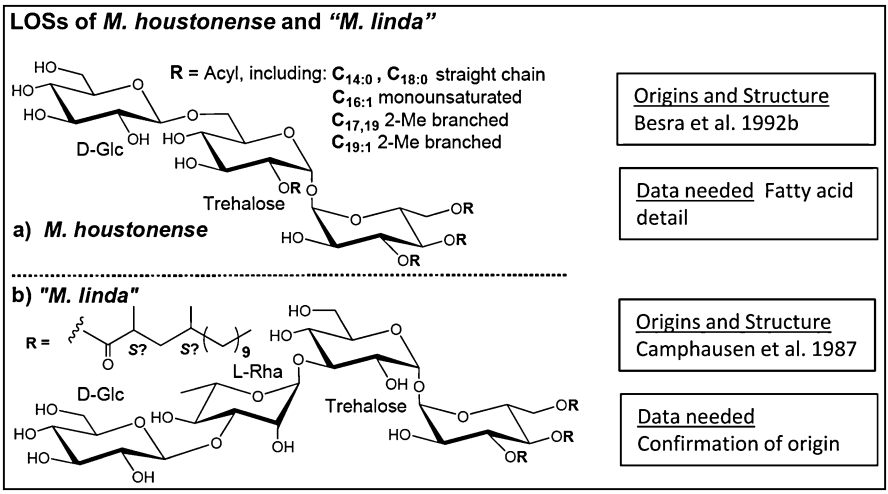

Fig. 11G Lipooligosaccharides (LOSs) of *Mycobacterium houstonense* and "*Mycobacterium linda*"

biovariant" of the "*M. fortuitum* complex" (Lanéelle et al. 1996), *M. fortuitum* produced only the characteristic DATs and TATs, but the third biovariant had the truncated LOS (Fig. 11G: a) (Besra et al. 1992b). The third biovariant was elevated to species status as *Mycobacterium houstonense* (Schinsky et al. 2004). A related simple LOS with an additional L-rhamnose unit inserted between glucose and the acylated trehalose (Fig. 11G: b) has been characterized from mycobacteria, provisionally labeled "*Mycobacterium linda*" and associated with Chrohn's disease (Camphausen et al. 1987). Uncharacterized LOSs have been documented in representatives of "*Mycobacterium mucogenicum*" (Muñoz et al. 1998). Mycobacterial LOSs have been reviewed by Bai et al. (2015).

M. kansasii strains of smooth colony morphology had cell surface LOSs, whereas rough variants were devoid of such surface antigens (Belisle and Brennan 1989). Previous studies (Collins and Cunningham 1981) had shown that the rough forms of *M. kansasii* persist longer than smooth variants in experimentally infected mice. Smooth morphology "*M. canettii*" produces polar LOSs (Fig. 11B) that are not present in rough *M. tuberculosis* (Soto et al. 2000). This correlates with greatly enhanced hydrophobicity in *M. tuberculosis* in comparison with relatively hydrophilic "*M. canettii*" (Minnikin et al. 2015; Jankute et al. 2017). It is probable that rough hydrophobic tubercle bacilli are preferentially suited to aerosol transmission and increased pathogenicity (Jankute et al. 2017). Spontaneous smooth-to-rough "*M. canettii*" variants, which lacked LOSs (van Soolingen et al. 1997), were found to be mutated in the polyketide-synthase-encoding pks5 locus, a phenotype restored by complementation (Boritsch et al. 2016). These rough variants also showed an altered host–pathogen interaction and increased virulence in cellular- and animal-infection models (Boritsch et al. 2016; Supply and Brosch 2017).

4 Mycobacterial Waxes

Waxes are defined as long-chain fatty acid esters of long-chain alcohols, but glycerol esters will also be included here. Saponification-resistant wax fractions from tubercle bacilli produce a range of (-)-laevorotatory fatty acids, originally termed mycocerosic acids (Ginger and Anderson 1945). These acids were found to be multimethyl-branched, using the alternative name, mycoceranic acids (Marks and Polgar 1955; Polgar and Smith 1963). A "methoxyglycol" from the same waxes was designated as phthiocerol (Stodola and Anderson 1936) and these β-diols were found to be diesterified with mycocerosic acids to produce the phthiocerol dimycocerosate (PDIM) waxes (see Asselineau 1966). Waxes composed of long-chain alcohols esterified to mycolic acids (MEWs) have been included in Sect. 2.3.

4.1 Triacylglycerols (TAGs)

Triacylglycerols (TAGs)) are found in most mycobacteria, but the full complexity of such mixtures has not been assessed, other than in the saprophyte, *M. smegmatis* (Purdy et al. 2013). TAGs are likely to be the principal components of internal lipid bodies, expressed under varying culture conditions (Garton et al. 2002). Structurally distinct mycobacterial TAGs have been characterized, with single very long-chain fatty acids analogous to the meromycolate chains of mycolic acids (Kremer et al. 2005; Rafidinarivo et al. 2009) (Fig. 12). Asselineau (1966) named these fatty acids "mycobacteric acids." The mycobacteric acids include functional groups similar to those of the mycolic acids in the same mycobacterial species (Promé et al. 1966). Detailed profiles of C_{30}–C_{56} fatty acids, corresponding to mycobacterates, from *M. tuberculosis* H37Ra have been recorded (Takayama and Qureshi 1978; Qureshi et al. 1980).

The original wax from *M. kansasii* was termed a "monomeromycoloyl diacylglycerol" (MMDAG) (Kremer et al. 2005) but precedence dictates that the

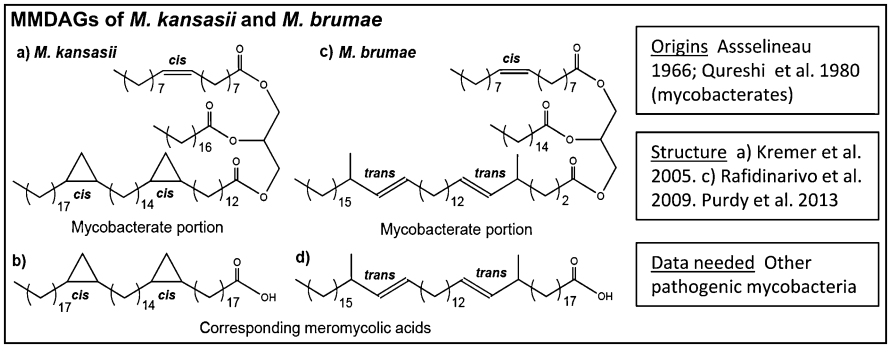

Fig. 12 Monomycobacteroyl diacyl glycerols (MMDAGs) of *Mycobacterium kansasii* and *Mycobacterium brumae*

name should be "monomycobacteroyl diacylglycerol" (Rafidinarivo et al. 2009); the term "meromycolate" should only be used for the designated portion of an intact mycolic acid (Morgan and Polgar 1957). The MMDAGs from *M. smegmatis* had a C_{16} straight-chain acid at the glycerol 2-position (Purdy et al. 2013), so this orientation is suggested for the MMDAGs of *M. kansasii* and *Mycobacterium brumae* (Rafidinarivo et al. 2009) (Fig. 12). Cellular movement of MMDAGs depends on *mmpL11* transporters (Pacheco et al. 2013). Diacylglycerols (DAGs) have been recorded in mycobacteria (Asselineau 1966) but significant structures remain to be determined.

4.2 Phthiocerol Dimycocerosates (PDIMs)

A methoxyglycol, isolated from the chloroform-extracted waxes of M. tuberculosis, was given the name "phthiocerol" by Stodola and Anderson (1936), substantiating the discovery of a "phthioglycol" by Stendal (1934). The C_{34}/C_{36} methoxydiol character of phthiocerol was established in simultaneous investigations (see Drayson et al. 1958; Demarteau-Ginsburg et al. 1959; Ryhage and Stenhagen 1960). The *M. tuberculosis* phthiocerol dimycocerosate (PDIM) waxes (Fig. 13A, B) were characterized in early studies (Noll 1957; Asselineau 1966). Detection of mycobacterial PDIMs was achieved by infra-red spectroscopy (Randall and Smith 1964). The basal methoxylated diols, phthiocerol A, and phthiocerol B (Minnikin and Polgar 1965, 1966b; Maskens et al. 1966) are usually accompanied by a ketone, phthiodiolone A (Fig. 13A) (Minnikin and Polgar 1967d). In some cases, phthiotriol A is the main component (Huet et al. 2009) (Fig. 13A). Closely-related PDIMs are characteristic of *M. bovis*, *M. leprae*, *M. kansasii*, *M. gastri*, and *M. haemophilum* (Fig. 13A) (Minnikin et al. 2002, 2015; Onwueme et al. 2005). PDIM families from *M. marinum* and *M. ulcerans* have alternative absolute stereochemistries for both the acid (Daffé et al. 1984; Daffé and Lanéelle 1988) and diol (Besra et al. 1989, 1990a) components (Fig. 13B).

Nomenclature for the multimethyl-branched fatty acid components of PDIMs is inconsistent and clarification is desirable. The name mycocerosate (Ginger and Anderson 1945) has precedence over mycoceranate, provisionally used for the (-)-laevorotatory multimethyl-branched acids (Marks and Polgar 1955). However, it is inappropriate to assign the label "phthioceranate" to the (+)-dextrorotatory multimethyl-branched acids from the waxes of *M. marinum* and *M. ulcerans* (Daffé et al. 1984; Daffé and Lanéelle 1988). Presumably, it was considered that the phthioceranate components of the sulfoglycolipids (SGLs) (Fig. 9) were the closest relatives of the (+)-dextrorotatory multimethyl-branched acids from the *M. marinum* and *M. ulcerans* waxes, but the dextrorotatory mycosanoates (Cason et al. 1964) from the *M. tuberculosis* diacyl trehaloses (DATs) (Fig. 7A) have precedence. Phthioceranates and mycosanoates are readily released on saponification of low-melting *M. tuberculosis* fats (see Sect. 3.1), but the components of PDIMs require prolonged alkaline hydrolysis (Minnikin et al. 1983). In essence, the *M. marinum* PDIM-derived C_{27}–C_{30} multimethyl-branched acids (Fig. 13B) overlap

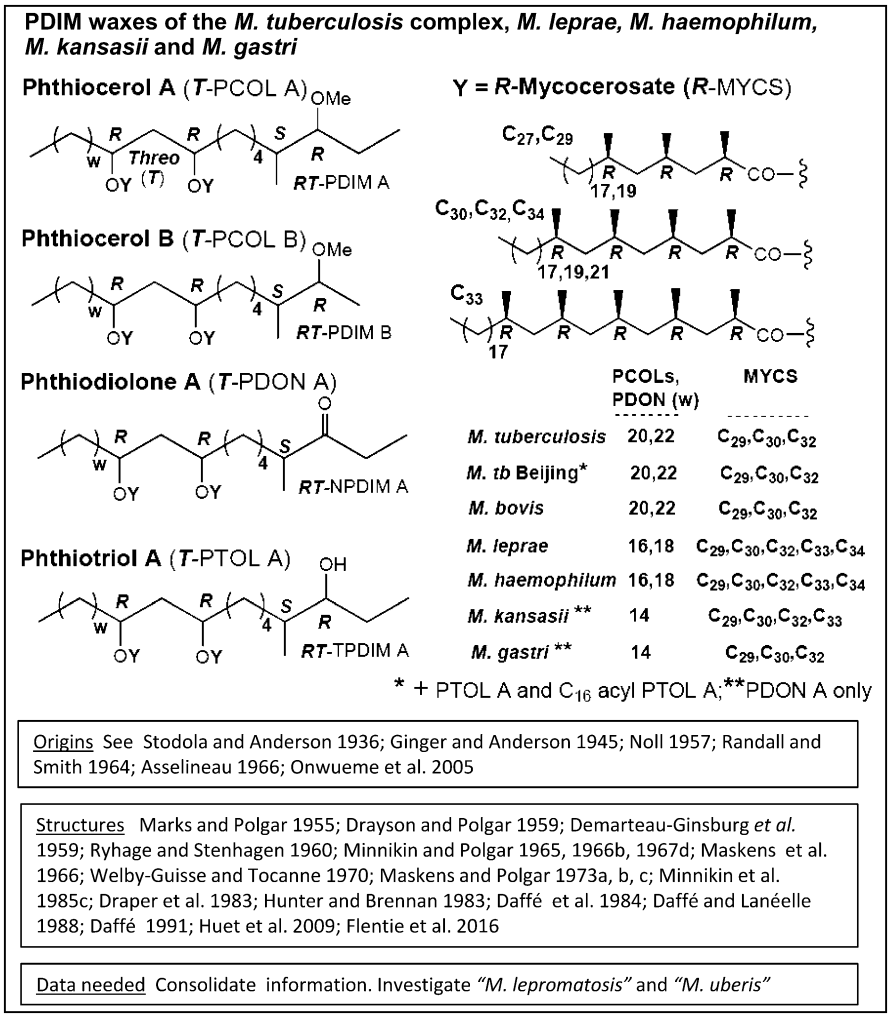

Fig. 13A Phthiocerol dimycocerosate (PDIM) waxes of the *Mycobacterium tuberculosis* complex, *Mycobacterium leprae, Mycobacterium haemophilum, Mycobacterium kansasii* and *Mycobacterium gastri*, based on *threo* phthiocerols and *R*-mycocerosates

in chain length with the established C_{27}–C_{34} mycocerosates (Fig. 13A) and are functionally similar. It is proposed, therefore, to recommend the label "*S*-mycocerosates" for the (+)-dextrorotatory fatty acids from the PDIMs of *M. marinum* and *M. ulcerans* (Fig. 13B) and "*R*-mycocerosates" for the (-)-laevorotatory acids from tubercle bacilli and other mycobacteria. This proposal correlates with a previous rationalization by Onwueme et al. (2005).

The nomenclature of members of the broad phthiocerol family also requires coherence. The main family member was designated phthiocerol A, to distinguish it

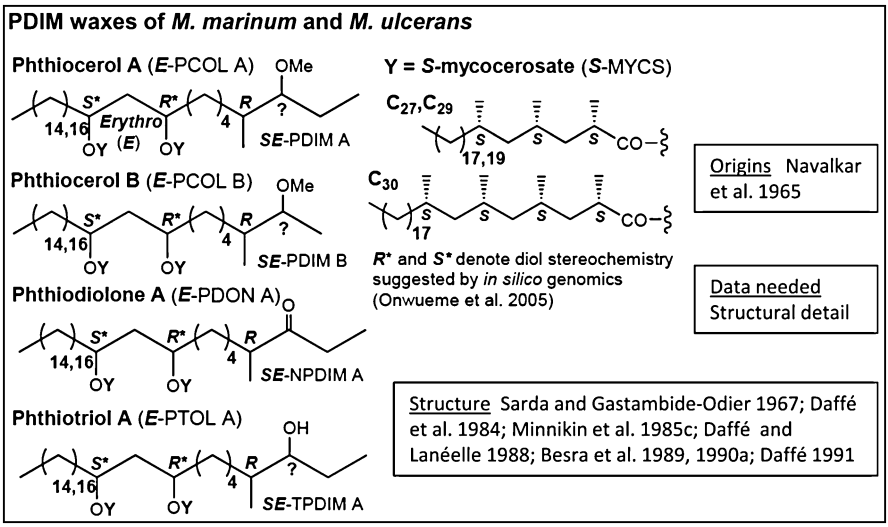

Fig. 13B Phthiocerol dimycocerosate (PDIM) waxes of *Mycobacterium marinum* and *Mycobacterium ulcerans*, based on *erythro* phthiocerols and *S*-mycocerosates

from a closely-related minor component that was appropriately labelled phthiocerol B (Minnikin and Polgar 1965, 1966b). Diols with terminal ethyl or methyl groups constitute the "A" or "B" series, respectively, and the main ethyl-keto component is phthiodiolone A (Fig. 13A) (Minnikin and Polgar 1967d). This logic is followed in most studies, including intact PDIMs (Minnikin et al. 1985c; Daffé and Lanéelle 1988; Daffé 1991; Onwueme et al. 2005). However, some recent studies (Huet et al. 2009; Rens et al. 2018) have disregarded the existence of phthiocerol B and inappropriately used the abbreviation PDIM B for the wax based on phthiodiolone A!

Overall rationalization of the nomenclature of members of the phthiocerol family has been proposed by Onwueme et al. (2005). The term "DIMs" was limited to labelling the entire "dimycocerosate" families of PDIM waxes and phenolic glycolipids (PGLs) (Sect. 5). The convenient abbreviations PCOL, PDON, and PTOL were suggested for phthiocerol, phthiodiolone, and phthiotriol, respectively (Fig. 14). An A or B could be added to signify terminal ethyl or methyl groups, as exemplified for phthiocerol B (PCOL B) or phthiodiolone A (PDON A). Diol stereochemistry could be shown by use of suffix *E* or *T* for *erythro*- (*syn*-) and *threo*- (*anti*-) forms; *E*-PCOL A would represent phthiocerol A from *M. marinum* (Fig. 13B) and *T*-PDON B would be phthiodiolone B from *M. tuberculosis* and related taxa (Fig. 14). The label PDIM should be retained for intact waxes based on phthiocerol A (PCOL A) and phthiocerol B (PCOL B). Waxes based on phthiodiolone (PDON) and phthiotriol (PTOL) could be labelled NPDIMs and TPDIMs, respectively. These basic abbreviations are made additionally informative by providing information about the *R*- or *S*-mycocerosate (MYCS) fatty acid substituents and the *erythro*- or *threo*-diol stereochemistry (Fig. 14). As an example, the main *M. tuberculosis* PDIM wax is described by the label *RT*-PDIM A, signifying

Nomenclature and abbreviations of PDIMs and PGLs

Lipid family	Lipid class	Diol type	Diol	Mycocerosate (MYCS) type	PDIM or PGL	Presence
DIMs	PDIMs	*Threo-* (*Anti-*)	***T***-PCOL A,B ***T***-PDON A,B ***T***-PTOL A,B	***R***-MYCS	***RT***-PDIM A,B ***RT***-NPDIM A,B ***RT***-TPDIM A,B	*M. tuberculosis, "M. canettii", M. bovis, M. leprae, M. kansasii, M. gastri, M. haemophilum*
		Erythro- (*Syn-*)	***E***-PCOL A,B ***E***-PDON A,B ***E***-PTOL A,B	***S***-MYCS	***SE***-PDIM A,B ***SE***-NPDIM A,B ***SE***-TPDIM A,B	*M. marinum, M. ulcerans*
	PGLs	*Threo-* (*Anti-*)	***T***-ϕPCOL A,B ***T***-ϕPDON A,B ***T***-ϕPTOL A,B	***R***-MYCS	***RT***-PGL A,B ***RT***-NPGL A,B ***RT***-TPGL A,B	*"M. canettii", M. bovis, M. leprae, M. kansasii, M. gastri, M. haemophilum*
		Erythro- (*Syn-*)	***E***-ϕPCOL A,B ***E***-ϕPDON A,B ***E***-ϕPTOL A,B	***S***-MYCS	***SE***-PGL A,B ***SE***-NPGL A,B ***SE***-TPGL A,B	*M. marinum, M. ulcerans*

Fig. 14 Proposed scheme for nomenclature and abbreviations of phthiocerol dimycocerosates (PDIMs) and glycosyl phenolphthiocerol dimycocerosates (phenolic glycolipids, PGLs)

R-mycocerosates (*R*- MYCS) esterifying a *threo*-diol (*T*-PCOL A) with a terminal ethyl group (Fig. 14). Nomenclatural labels for the so-called phenolic glycolipids (PGLs) (see next Sect. 5) are also included in Fig. 14.

5 Glycosyl Phenolphthiocerol Dimycocerosates (Phenolic Glycolipids, PGLs)

A study (Noll 1957) of the waxes of *M. bovis* yielded a component with an infra-red spectrum identical to a fraction (G_B) identified by Smith et al. (1954, 1957); this latter investigation also pinpointed a related lipid (G_A) from *M. kansasii*. These glycolipids were called "mycosides" by Smith et al. (1960b) and relabelled mycosides B and A, respectively. Additional infra-red studies revealed mycoside G from *M. marinum* (Navalkar et al. 1965). The essential core phenolphthiocerol dimycocerosate units in mycosides B and A, respectively, were elaborated by Demarteau-Ginsburg and Lederer (1963) and Gastambide-Odier et al. (1965). Further sources of this glycolipid type are *M. leprae* (Hunter and Brennan 1981, 1983; Hunter et al. 1982), *M. haemophilum* (Besra et al. 1990b, 1991), *M. gastri* (Gilleron et al. 1990b), *M. ulcerans* (Daffé et al. 1992), "*M. canettii*" (Daffé et al. 1987), and the Beijing clades of *M. tuberculosis* (Huet et al. 2009). The term "phenolic glycolipid" (PGL) is in general use for this category of lipids (Hunter and Brennan 1981; Hunter et al. 1984; Brennan 1984; Puzo 1990; Onwueme et al. 2005) and representative structures are shown in Fig. 15A–D. Abbreviations for the various components of PGLs are included in Fig. 14 and employed in Fig. 15A–D.

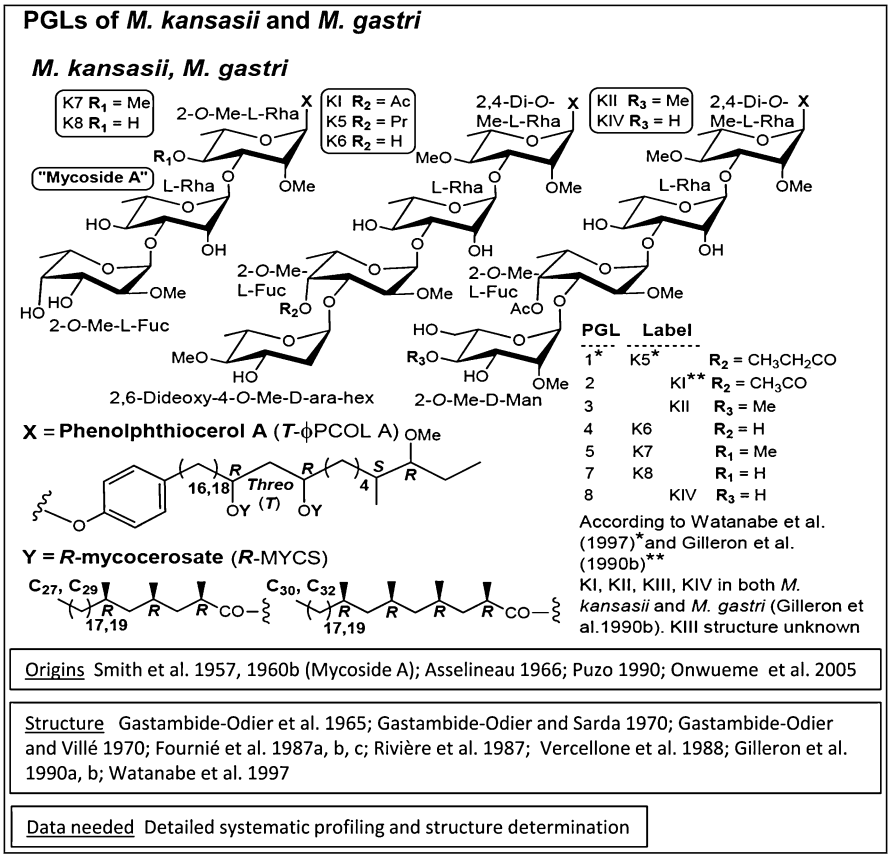

Fig. 15A Glycosyl phenolphthiocerol dimycocerosates (phenolic glycolipids, PGLs) of *Mycobacterium kansasii* and *Mycobacterium gastri*, based on *threo* phenolphthiocerols and *R*-mycocerosates

The essential character of *M. kansasii* mycoside A (Smith et al. 1957, 1960a, b) was elaborated by Gastambide-Odier et al. (1965), Gastambide-Odier and Sarda (1970), and Gastambide-Odier and Villé (1970). Detailed structures were defined by Fournié et al. (1987a, b, c), Rivière et al. (1987), and Gilleron et al. (1990a, b) (Fig. 15A). Similar PGL profiles were produced by *Mycobacterium gastri* (Vercellone et al. 1988; Gilleron et al. 1990b) (Fig. 15A). Gilleron et al. (1990b) and Watanabe et al. (1997) described up to eight well-defined PGLs in *M. kansasii* (Fig. 15A).

The major PGL of *M. bovis* (mycoside B) (Demarteau-Ginsburg and Lederer 1963; Gastambide-Odier and Sarda 1970) was labelled as "*M. bovis* identifying lipid" (MBIL) (Jarnagin et al. 1983). A single 2-*O*-methyl-α-L-rhamnopyranose was linked to the phenolic phthiocerol (Daffé et al. 1988b; Chatterjee et al. 1989), along with minor components based on α-L-rhamnopyranose (Fig. 15B). An additional minor disaccharide-containing PGL has been defined in *M. bovis* BCG (Vercellone

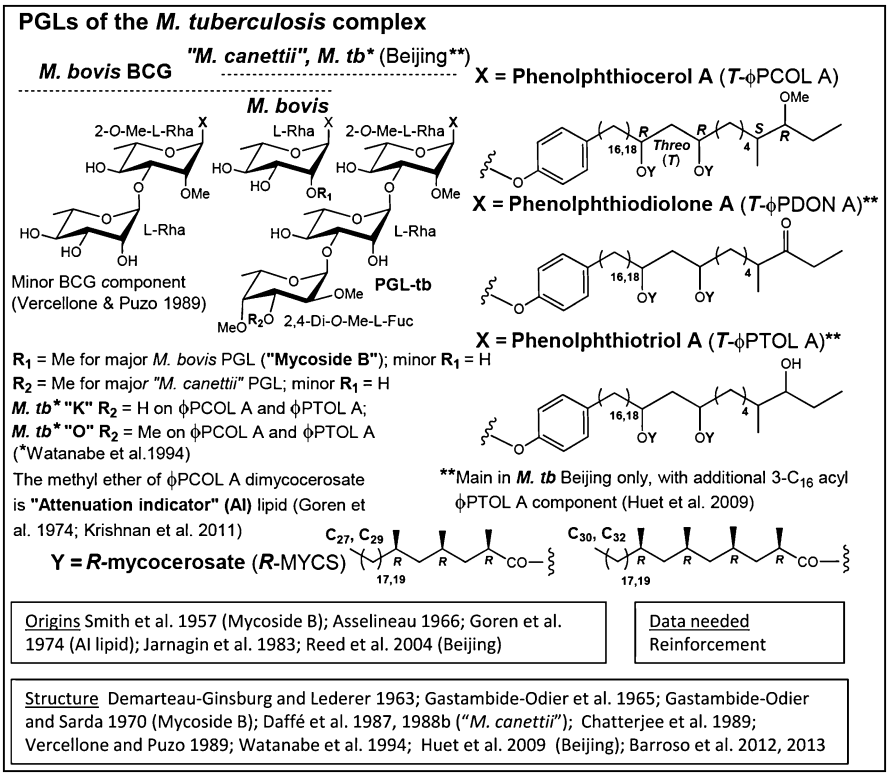

Fig. 15B Glycosyl phenolphthiocerol dimycocerosates (phenolic glycolipids, PGLs) of the *Mycobacterium tuberculosis* complex, based on *threo* phenolphthiocerols and *R*-mycocerosates

and Puzo 1989) (Fig. 15B). *M. tuberculosis* complex Canetti strains, also produce mycoside B, accompanied by a triglycosyl PGL (PGL-tb) (Daffé et al. 1987) (Fig. 15B). Additional components, based on phenolthiotriol, have been found in unusual *M. tuberculosis* strains (Watanabe et al. 1994) and Beijing variants (Huet et al. 2009). The methylated PGL aglycone has been isolated from variants of *M. tuberculosis* and given the name "Attenuation Indicator" (AI) lipid (Fig. 15B) (Goren et al. 1974; Krishnan et al. 2011). Structures of PGL-tb and mycoside B have been confirmed by synthesis (Barroso et al. 2012, 2013).

The specific trisaccharide PGL-I of *M. leprae* (Hunter et al. 1982) (Fig. 15C) and semisynthetic *neo*-antigens have found widespread use for the serodiagnosis of leprosy (Cho et al. 1983; Fujiwara et al. 1984; Spencer and Brennan 2011). Demethylation variations in the PGL-I structure are found in PGL-II (Fujiwara et al. 1984) and PGL-III (Hunter and Brennan 1983) (Fig. 15C). A diglycosyl component, lacking the terminal 3,6-di-*O*-methyl-glucopyranose of PGL-1, was reported by Daffé and Lanéelle (1989) (Fig. 15C). *M. haemophilum* has a distinct trisaccharide PGL (Besra et al. 1991) that shares mono- and di-methyl rhamnoses with the PGLs

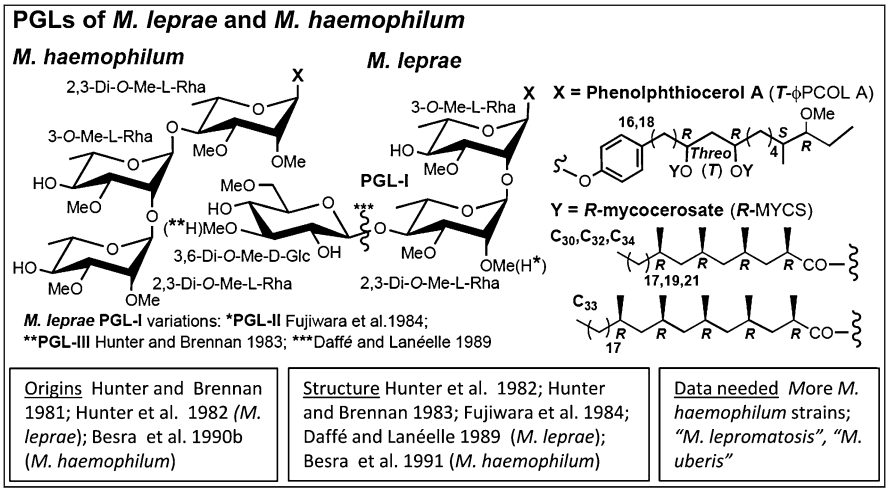

Fig. 15C Glycosyl phenolphthiocerol dimycocerosates (phenolic glycolipids, PGLs) of *Mycobacterium leprae* and *Mycobacterium haemophilum*, based on *threo* phenol-phthiocerols and *R*-mycocerosates

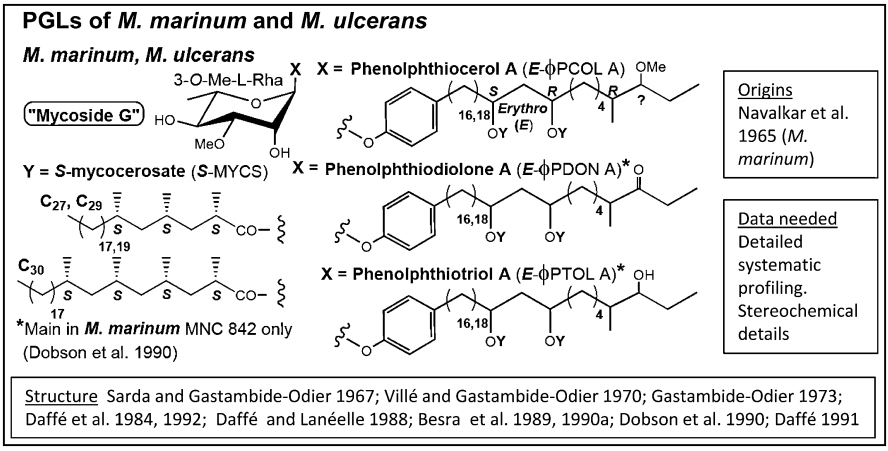

Fig. 15D Glycosyl phenolphthiocerol dimycocerosates (phenolic glycolipids, PGLs) of *Mycobacterium marinum* and *Mycobacterium* ulcerans, based on *erythro* phenolphthiocerols and *S*-mycocerosates

from *M. leprae* and unusual C_{33} and C_{34} mycocerosates were common to both (Fig. 15C), suggesting a phylogenetic relationship.

The mycoside G from *M. marinum*, revealed by Navalkar et al. (1965), was found to be a 3-*O*-methyl-α-L-rhamnosyl diacyl phenolphthiocerol (Fig. 15D) (Sarda and Gastambide-Odier 1967; Gastambide-Odier 1973; Daffé and Lanéelle 1988). Dobson

et al. (1990) have described a series of PGLs from M. marinum, with a predominance of lipids based on phenolphthiodiolone and phenolphthiotriol, in some cases (Fig. 15D). Closely related *M. ulcerans* produces the monoglycosyl PGL found in M. marinum, with enhanced phenolphthiodiolone and phenolphthiotriol components (Daffé et al. 1984, 1992).

In accordance with PDIMs (Figs. 13 and 14), the stereochemistry of *M. marinum* and *M. ulcerans* PGLs is distinct (Fig. 15D). It would be preferable to label the multimethyl-branched acyl chains as *S*-mycocerosates rather than the term "phthioceranates" used by Daffé et al. 1984, 1992; Daffé and Lanéelle 1988, and Daffé 1991 (see Figs. 13B and 14). Again, *erythro*-diols were found in the PGLs of *M. marinum* (Besra et al. 1989) and *M. ulcerans* (Besra et al. 1990a) (Fig. 15D), in contrast to the PGL *threo*-diols in all other taxa (Fig. 15A–C). The stereochemistry at the phenolphthiocerol methyl branch (carbon-4) is considered to be *R* in *M. marinum* and *M. ulcerans* PGLs (Fig. 15D), rather than *S* in other PGLs (Fig. 15A–C) (Onwueme et al. 2005).

For nomenclature of PGLs, the abbreviations φPCOL, φPDON, and φPTOL (Fig. 14) for the methoxy, keto, and hydroxy phenolic diols, respectively, uses suggestions by Onwueme et al. (2005). Similar to the PDIMs, PGLs based on φPDON and φPTOL could be given the abbreviations NPGL and TPGL, respectively (Fig. 14).

6 Glycopeptidolipids (GPLs) and Peptidolipids (PLs)

Infra-red spectroscopy of extracts from the *M. avium* complex (MAC) identified a class of lipids (Smith et al. 1957) eventually labelled "mycosides C" (Smith et al. 1960a, b; Smith and Randall 1965). It was also recognized that MAC lipid extracts contained diagnostic glycolipids, as seen by thin-layer chromatography (TLC) (Marks et al. 1971; Jenkins and Marks 1973; Jenkins 1981). Brennan et al. (1978) applied such TLC procedures to the entire Schaefer (MAC) complex and it was demonstrated that the Marks-Jenkins lipids were synonymous with mycosides C and typing antigens reviewed by Schaefer (1965). These lipids were shown to be "polar" glycopeptidolipids (pGPLs) (Lanéelle 1966; Brennan and Goren 1979; Brennan 1981, 1984; Brennan et al. 1981a, b; Tsang et al. 1983, 1992; Denner et al. 1992). Structures of nonspecific "apolar" aGPLs had been established earlier (Jolles et al. 1961; Lanéelle 1966; Lanéelle and Asselineau 1968; Lederer 1967; Jardine et al. 1989).

All GPL structures (Fig. 16A–F), with the exception of those from *M. xenopi* (Fig. 16G), are based on a tripeptide-amino-alcohol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol), *N*-linked at the D-Phe to a long-chain fatty acyl residue. In the case of the aGPLs, this lipopeptide core is substituted usually by a 6-deoxytalosyl (dTal) unit linked to the *allo*-Thr residue and by an *O*-methylated rhamnosyl unit linked to the terminal alaninol. Further glycosylation, usually on the dTal unit, gave antigenic, serospecific pGPLs (Brennan and Goren 1979). Brennan et al. (1981a, b) showed that these oligoglycosyl haptens were liberated on reductive β-elimination with alkaline sodium borohydride, but *O*-acetyl substituents, important for antigenicity,

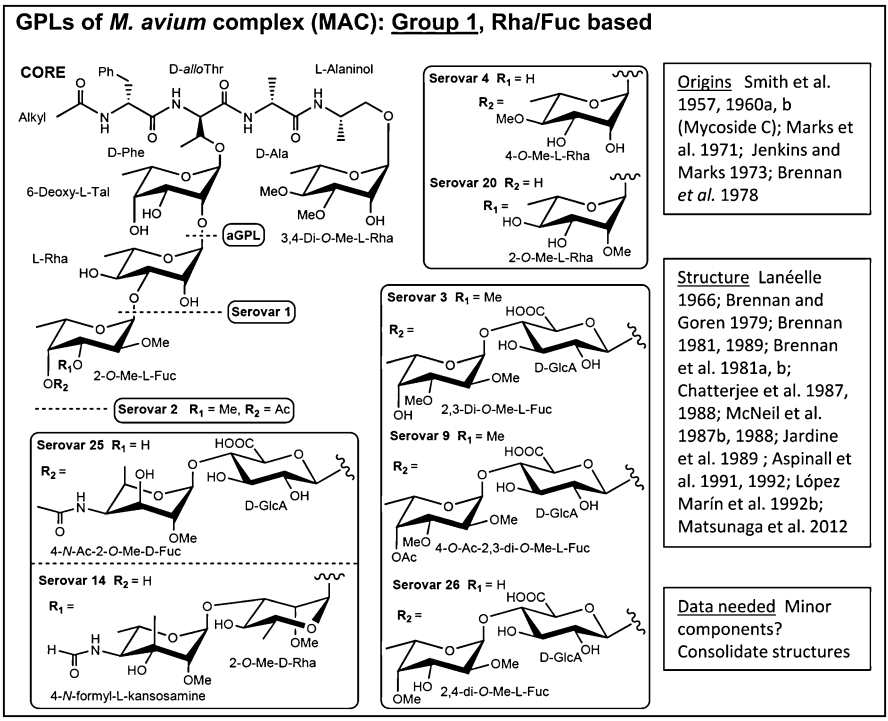

Fig. 16A Glycopeptidolipids (GPLs) of *Mycobacterium avium* complex (MAC): Group 1, rhamnose/fucose based

were lost. Subsequently, the presence of acetates, uronic acids, acetalically linked pyruvic acids, acylaminodideoxyhexoses, and branched-chain sugars was demonstrated (Bozic et al. 1988; Jardine et al. 1989).

Aspinall et al. (1995) classified the oligoglycosyl haptens of 12 of the 31 known serotype/serovar members of the *M. avium* complex (MAC), based on structural similarities. The presence of three principal groups of MAC GPLs is apparent (Fig. 16A–C) (Chatterjee and Khoo 2001). In Group 1 of MAC GPLs (Fig. 16A), a common L-rhamnose-L-fucose discaccharide, linked to the 6-deoxy-L-talose, is further decorated to produce specific polar (pGPLs). The fucose is lacking in serovar 1, but in serovar 2 it is methylated and acetylated (Fig. 16A); this latter GPL was also characterized from supposed *M. paratuberculosis* (Camphausen et al. 1985), but it is suspected that the organism examined was *M. avium* serovar 2 (Chatterjee and Khoo 2001). This correlates with Jenkins (1981) who recorded no specific glycolipid pattern for *M. paratuberculosis*.

Distinct categories of MAC Group 1 pGPLs can be discerned (Fig. 16A). Firstly, serovars 4 and 20 are limited to an additional methylated rhamnose. Secondly, serovars 3, 9, and 26 add simple disaccharides based on D-glucuronic acid (D-GlcA)-L-fucose. Serovar 25 also included D-GlcA but the disaccharide is

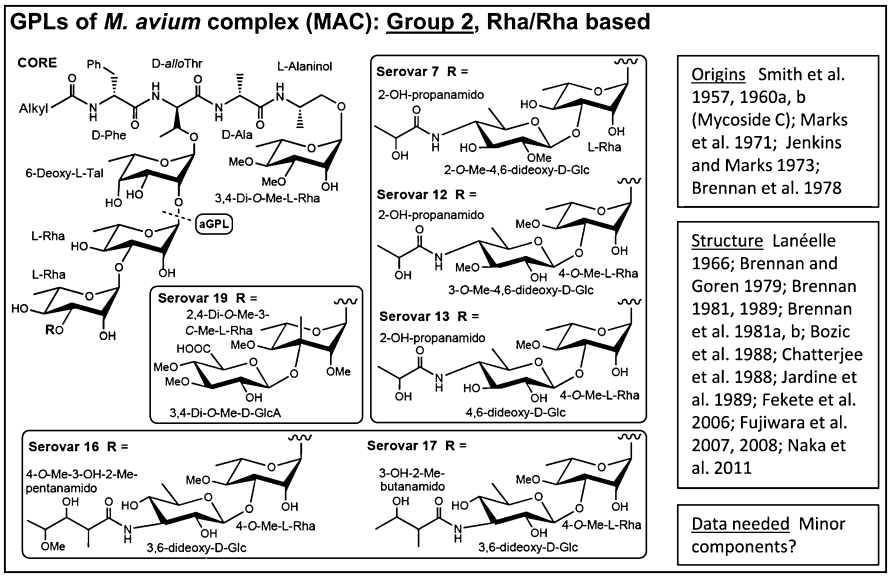

Fig. 16B Glycopeptidolipids (GPLs) of *Mycobacterium avium* complex (MAC): Group 2, rhamnose/rhamnose based

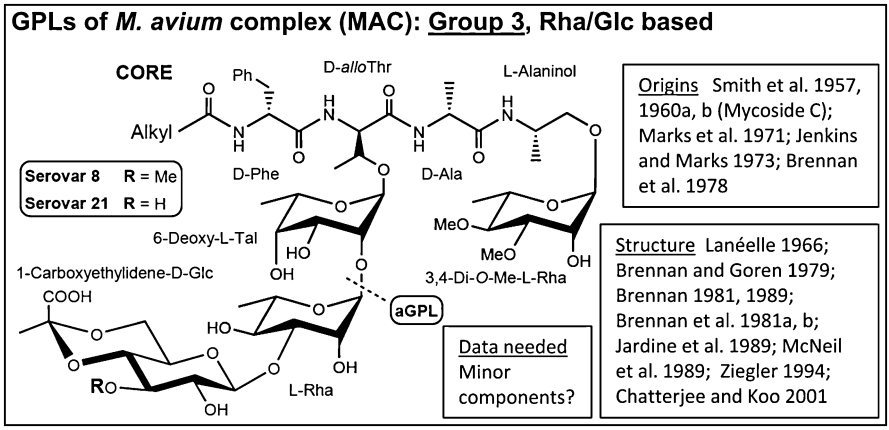

Fig. 16C Glycopeptidolipids (GPLs) of *Mycobacterium avium* complex (MAC): Group 3, rhamnose/glucose based

completed by acetylated amino-D-fucose. The very individual serovar 14 has rhamnose, but as the D-enantiomer, with the rare 4-*N*-formyl-L-kansosamine (Fig. 16A). A variant of serovar 4 had D-valine in place of the core phenylalanine (Matsunaga et al. 2012).

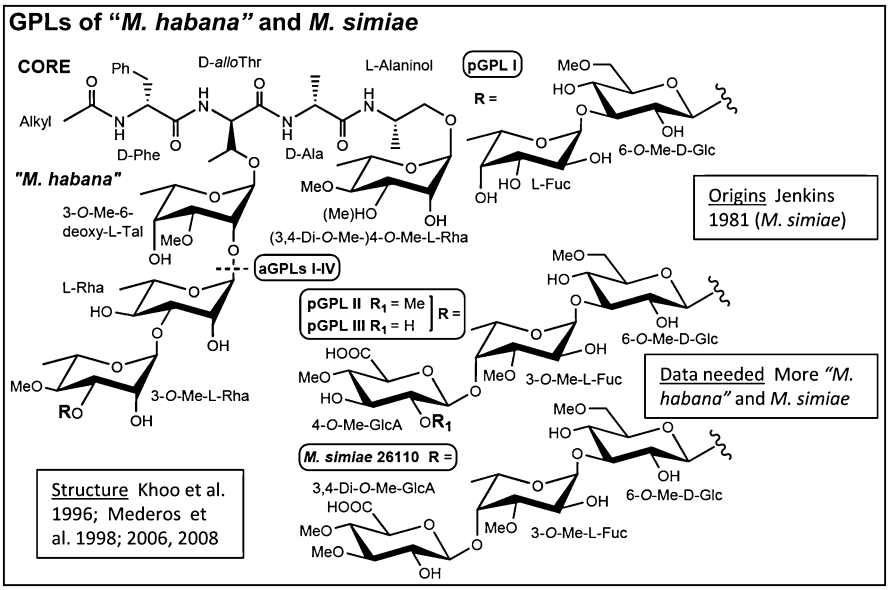

Fig. 16D Glycopeptidolipids (GPLs) of "*Mycobacterium habana*" and *Mycobacterium simiae*

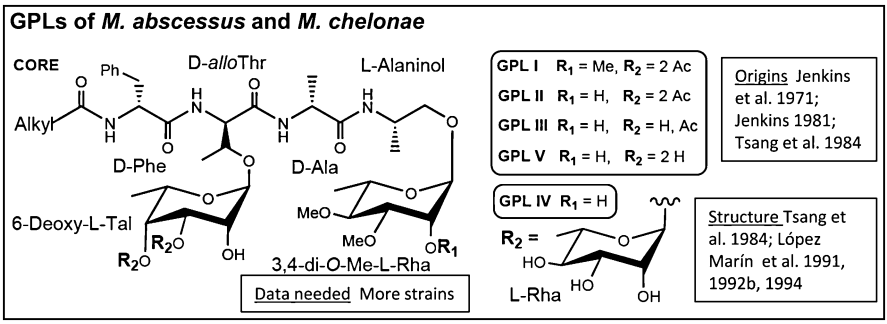

Fig. 16E Glycopeptidolipids (GPLs) of *Mycobacterium abscessus* and *Mycobacterium chelonae*

The basic core extension in Group 2 MAC pGPLs (Fig. 16B) is a di-L-rhamnose disaccharide, but in all cases, the third and fourth sugars are modified L-rhamnoses and D-glucoses; D-Glc-linked amido substituents are a unifying factor. Serovars 7, 12, and 13 have 2-hydroxy-propanamido and serovars 16 and 17 have 4-methoxy-3-hydroxy-2-methyl-pentanamido and 3-hydroxy-2-methyl-butanamido, respectively. The unusual pGPL member in MAC Group 2 is from serovar 19, with a *C*-methylated rhamnose and glucuronic acid (Fig. 16B). The core extension in MAC serovars 8 and 21 Group 3 pGPLs (Fig. 16C) is L-rhamnose-D-glucose, with the latter acetylated with pyruvate.

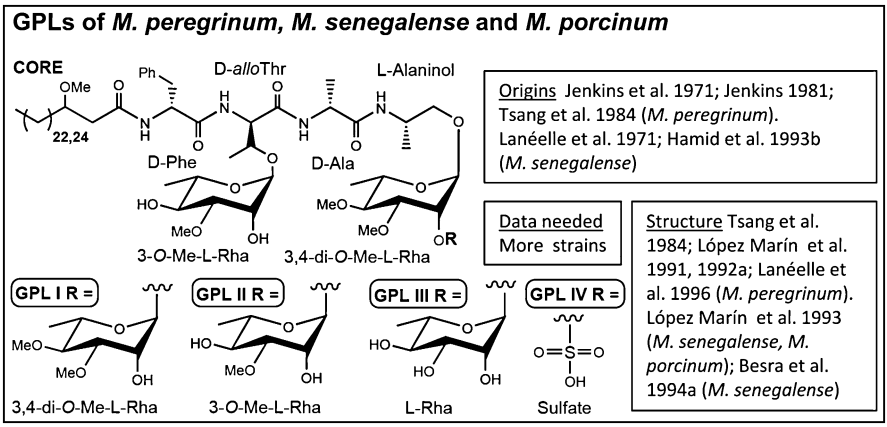

Fig. 16F Glycopeptidolipids (GPLs) of *Mycobacterium peregrinum, Mycobacterium senegalense* and *Mycobacterium porcinum*

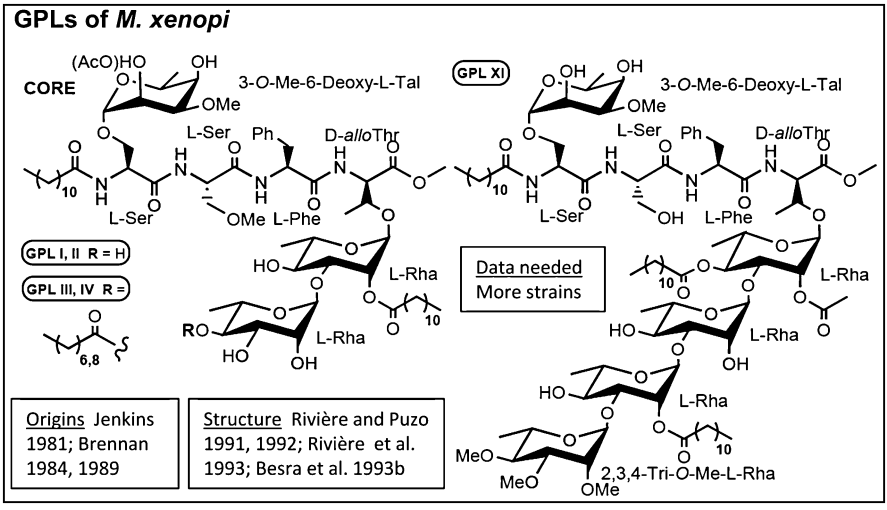

Fig. 16G Glycopeptidolipids (GPLs) of *Mycobacterium xenopi*

The GPLs of "*M. habana*" and *M. simiae* (Fig. 16D) resemble those in MAC Group 2 (Fig. 16B), with a di-L-rhamnose extension (Khoo et al. 1996). The further disaccharide, composed of 6-*O*-methyl-D-glucose and L-fucose, resembles the D-glucuronic acid-L-fucose units in Serovars 3, 9, and 26 in MAC Group 1 GPLs (Fig. 16A). "*M. habana*" is closely affiliated with *M. simiae*, but further detailed investigations revealed that the details of the superficially similar GPLs are able to distinguish the two taxa (Mederos et al. 1998, 2006, 2008).

In the "*M. fortuitum-M. chelonae* complex," *M. fortuitum sensu stricto* lacked GPLs (Tsang et al. 1984) (see Sect. 3.1). *M. fortuitum* biovar *peregrinum* expressed GPLs different to those in *M. chelonae*. The main feature of the simple GPLs from *M. chelonae* and *M. abscessus* is an additional L-rhamnose attached to the core L-rhamnose, rather than on the 6-deoxy-L-talose (Fig. 16E).

The core L-rhamnose in *M. peregrinum* GPLs is also the point of modification and the D-*allo*-threonine carries 3-*O*-methyl-L-rhamnose in place of the usual 6-deoxy-L-talose (Fig. 16F); a sulfated variant is notable (López Marín et al. 1991, 1992a; Lanéelle et al. 1996). Individual GPLs support the rejuvenation of the species *Mycobacterium peregrinum* (Kusunoki and Ezaki 1992). GPLs are similar in *M. peregrinum*, *M. senegalense*, and *M. porcinum* (Fig. 16F) (López Marín et al. 1993; Besra et al. 1994a).

The GPLs of *M. xenopi* (Fig. 16G) have different lipopeptide cores, L-serine providing an anchor point for 3-*O*-methyl-6-deoxy-L-talose (Rivière and Puzo 1991). Up to four decorated L-rhamnose units are linked to the terminal D-*allo*-threonine methyl ester (Rivière et al. 1993; Besra et al. 1993b). Notably, these GPLs have two relatively short acyl chains, one of which is attached to the rhamnosyl oligosaccharides.

Apolar aGPLs probably fulfil a structural role in the mycobacterial outer membrane (MOM), with the antigenic polar pGPLs having specific cell surface activity. Rough *M. avium* variants lack GPLs (Barrow and Brennan 1982), as confirmed by genomic studies (Belisle et al. 1993a, b). GPLs facilitate sliding motility, biofilm formation, and cell wall integrity (see Deshayes et al. 2005; Ripoll et al. 2007; Schorey and Sweet 2008) and possibly mycobacteriophage attachment (Goren et al. 1972). Rough variants of *M. abscessus*, lacking GPLs, are more invasive (Howard et al. 2006; Catherinot et al. 2007; Julián et al. 2010; Mukherjee and Chatterji 2012; Pang et al. 2013; Bernut et al. 2014; Brambilla et al. 2016; Gutiérrez et al. 2018). Rough GPL-free *M. abscessus* strains are relatively hydrophobic and possibly more likely to be spread in aerosols (Minnikin et al. 2015; Jankute et al. 2017; Viljoen et al. 2018). Fregnan et al. (1962) noted that smooth to rough colony changes correlated with GPL loss in a scotochromogenic *Mycobacterium*, "the culture aspect changing from hydrophilic to hydrophobic." Hydrophobicity was a key factor in facilitating aerosol transmission of related MAC organisms (Parker et al. 1983; Falkinham 2003).

Some rough mutants of *M. avium* serovar 2 express peptidolipids (PLs) that represent the core of the usual GPLs (Fig. 16A) (Belisle et al. 1993a). Initial detection of distinct peptidolipids in *M. paratuberculosis* (Lanéelle and Asselineau 1962) was confirmed by subsequent studies (Rivière et al. 1996; Eckstein et al. 2006; Biet et al. 2008). PLs based on a pentapeptide (L5P) and a tripeptide (L3P) were characterized (Fig. 17) (Bannantine et al. 2017), the former in cattle *M. paratuberculosis* strains and the latter in sheep strains. A peptidolipid was isolated from cattle-associated "*Mycobacterium minetti*," a synonym of *M. fortuitum*, and given the name "fortuitine" (Vilkas et al. 1963; Asselineau 1966). Introducing mass spectrometry for peptide sequencing (Barber et al. 1965), a draft structure was assigned for fortuitine (Fig. 17).

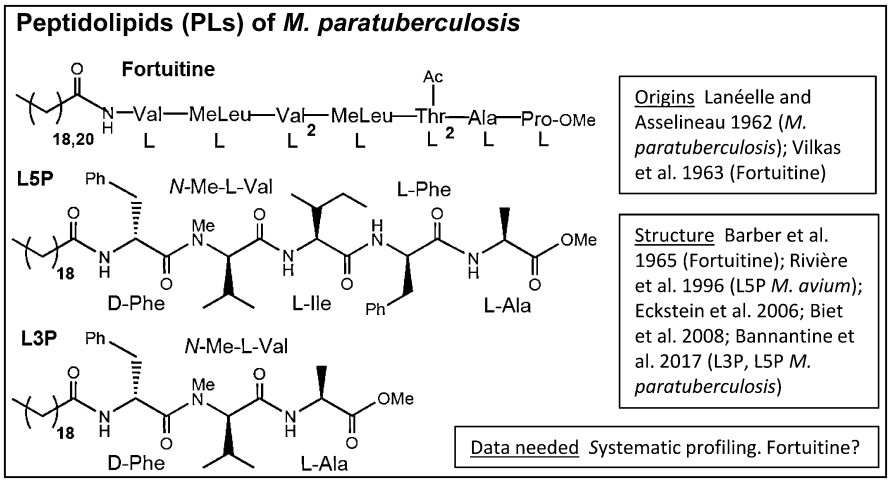

Fig. 17 Peptidolipids (PLs) of *Mycobacterium paratuberculosis* and Fortuitine

7 Isoprenoid Lipids

Isoprenoid quinones are essential in respiratory processes and pigments are characteristic of a number of mycobacterial pathogens. Isoprenoid hydrocarbon chains are favored for a range of carrier molecules and specific halimane diterpenoids, such as 1-tuberculosinyladenosine, are encountered.

7.1 Isoprenoid Quinones

The most common mycobacterial isoprenoid quinone is a dihydrogenated menaquinone with nine isoprene units, abbreviated MK-9(H_2) (Fig. 18) (Minnikin 1982; Collins et al. 1985). Initial isolation from *Mycobacterium phlei* (Gale et al. 1963) was followed by structural characterization (Azerad et al. 1967; Azerad and Cyrot-Pelletier 1973). A related sulfated menaquinone from *M. tuberculosis* has been labelled sulfomenaquinone (Fig. 18) (Holsclaw et al. 2008; Sogi et al. 2016). An unusual non-isoprenoid quinone from *M. avium*, mavioquinone (Fig. 18) (Scherrer et al. 1976), requires substantiation.

7.2 Isoprenoid Pigments

Carotenoid pigments in mycobacteria were established by Chargaff and Lederer (1935). Opportunist pathogens, such as *M. kansasii*, *M. marinum*, *M. gordonae*,

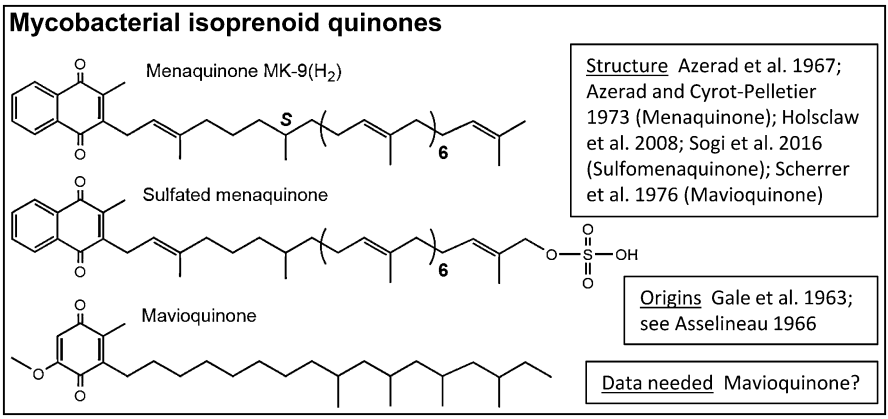

Fig. 18 Mycobacterial isoprenoid quinones

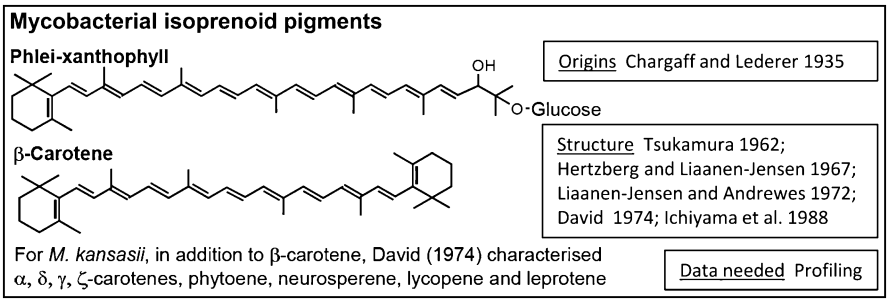

Fig. 19 Mycobacterial isoprenoid pigments

M. scrofulaceum, and *M. szulgae*, express carotenoids (Tsukamura and Mizuno 1969; Goodwin 1972; Liaanen-Jensen and Andrewes 1972; Ratledge 1976; Goren and Brennan 1979; Minnikin 1982; Ichiyama et al. 1988; Robledo et al. 2011). Photochromogenic mycobacteria, such as *M. kansasii* and *M. avium*, are pigmented in light but not in the dark, but scotochromogenic mycobacteria, like *M. gordonae* and *M. scrofulaceum*, are also pigmented in the dark (Goren and Brennan 1979). Ubiquitus β-carotene is often the principal mycobacterial pigment, accompanied by related carotenoids, as illustrated for *M. kansasii* by David (1974), where eight additional compounds were characterized (Fig. 19). Distinct carotenoid groups have been proposed (Ichiyama et al. 1988), including incompletely characterized oxygenated pigments, classed as xanthins or xanthophylls (see Goodwin 1972; Minnikin 1982). A xanthophyll example is the glucoside "phlei-xanthophyll," produced by the saprophyte *Mycobacterium phlei* (Fig. 19) (Hertzberg and Liaaen-Jensen 1967). Unpigmented colonial variants of *M. avium* are less hydrophobic (Stormer and Falkinham 3rd. 1989).

7.3 Isoprenoid Lipid Carriers

A partially saturated C_{35} octahydroheptaprenol and a C_{50} decaprenol ("Dec") (Fig. 20A–J) are the predominant isoprenoid lipids involved in "carrying" carbohydrates to their biosynthetic destinations in mycobacteria. Phosphorylated forms of these two acted as glycosyl acceptors from GDP-mannose (Takayama and Goldman 1970; Takayama et al. 1973; Yokoyama and Ballou 1989) and UDP-glucose (Schultz and Elbein 1974). C_{35}-P-Man and C_{50}-P-Man (Fig. 20a, c) are donors for the synthesis of the higher-order phosphatidylinositol mannoides (PIMs, see Sect. 8) (PIM_4–PIM_6) and also lipomannan (LM) and lipoarabinomannan (LAM) (Scherman et al. 2009); the simpler PIM_1–PIM_3 derive their Man*p* units directly from

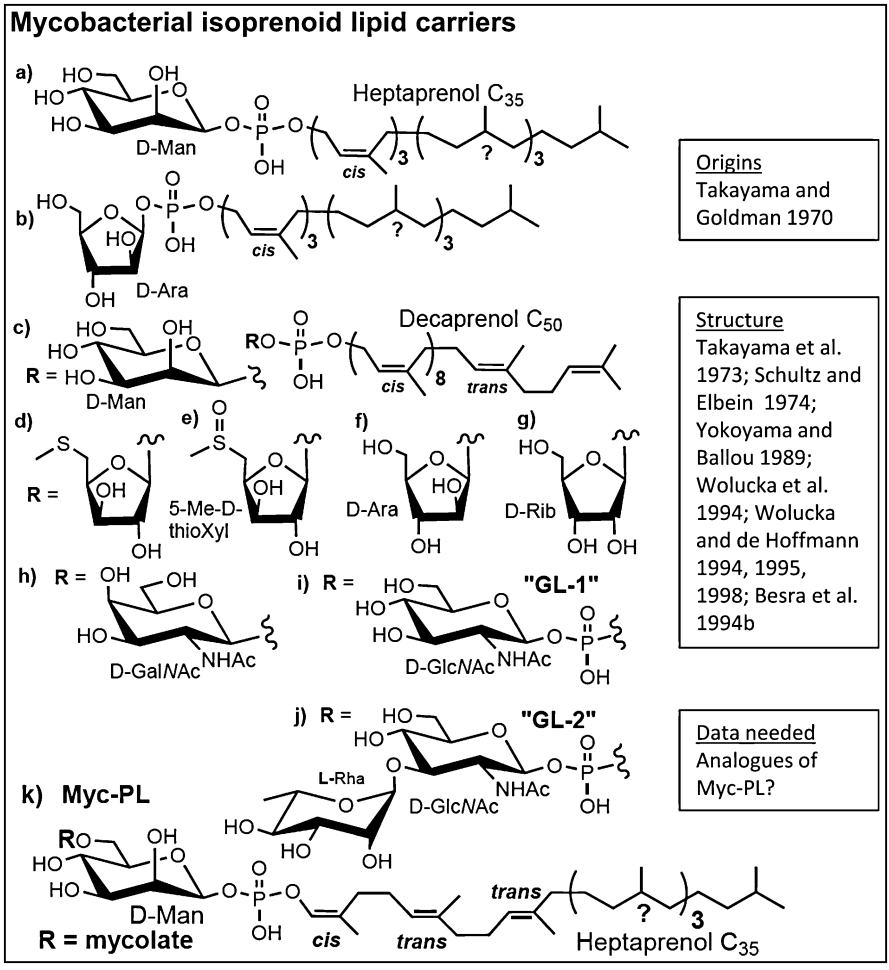

Fig. 20 Mycobacterial isoprenoid lipid carriers

GDP-mannose. The structure of the mycobacterial decaprenol (Fig. 20c–i) is unusual, with mono-*trans*, octa-*cis* olefins (Wolucka et al. 1994; Wolucka and de Hoffmann 1998).

Mikušová et al. (1996) established that C_{50}-P-P-Glc*N*Ac ("GL-1") (Fig. 20i) and C_{50}-P-P-Glc*N*Ac-Rha ("GL-2") (Fig. 20j) are acceptors for the subsequent glycosylation steps leading to the synthesis of the C_{50}-P-P-Glc*N*Ac-Rha-(Gal*f*)x- (Ara*f*)y precursor of mycobacterial cell wall core formation. The precursor of the galactosamine ($GalNH_2$) monomers, attached to the branching Ara units of the mycobacterial cell wall core, is C_{50}-decaprenol-P-GalNAc (Fig. 20j), along with a minor C_{35}-heptaprenol analogue (Škovierová et al. 2010). The 5-methylthioxylose units, substituting the mannoside caps of the LAM from *M. tuberculosis*, originate from a decaprenol-P-linked precursor (Fig. 20d) (Angala et al. 2017), whose oxidized form (Fig. 20e) was also characterized.

A β-arabinofuranosyl-1-monophosphoryldecaprenol (C_{50}-P-Araf) (Fig. 20f) is the precursor of Araf units in mycobacterial cell walls (Wolucka et al. 1994). Mycobacterial β-D-ribosyl-1-monophosphodecaprenol (Fig. 20g) is the probable precursor of C_{50}-P-Araf (Fig. 20f) (Wolucka and de Hoffmann 1995; Scherman et al. 1995, 1996; Mikušová et al. 2005) and a β-arabinofuranosyl-1-monophosphorylheptaprenol (C35-P-Araf) (Fig. 20b) was identified but its role is undefined.

"Myc-PL," is an "apparent carrier in mycolic acid synthesis" (Besra et al. 1994b). This unusual lipid, from *M. smegmatis*, was a mycoloyl-mannosylphosphopolyprenol (Fig. 20k), the C_{35} heptaprenol having similarities to the mannose and arabinose carriers described above (Fig. 20a, b). However, despite indications of this lipid in *M. tuberculosis* (Besra et al. 1994b), the presence of Myc-PL in pathogenic mycobacteria remains to be established.

7.4 Halimane Diterpenoids

In a search for the role of the terpenoid cyclase-associated *M. tuberculosis* gene Rv3377c, a diterpene was characterized and named tuberculosinol (Fig. 21) (Nakano et al. 2005); it is a member of the halimane diterpenoids (Roncero et al. 2018). A distinct bioactive diterpene was recognized in *M. tuberculosis* (Mann et al. 2009), but this material, designated as isotuberculosinols, was shown to be a mixture of enantiomers of halimane diterpenoids (Fig. 21), identical to nosyberkols from sponges (Spangler et al. 2010; Maugel et al. 2010; Hoshino et al. 2011; Mann et al. 2012; Mann and Peters 2012). Scrutiny of the *M. tuberculosis* genome revealed that Rv3378c was responsible for the production of 1-tuberculosinyladenosine (Fig. 21) (Layre et al. 2011b, 2014). Biosynthetic studies pinpointed a distinct isomer, N^6-tuberculosinyladenosine (Fig. 21) (Young et al. 2015; Oldfield 2015); both isomers were confirmed by chemical synthesis (Buter et al. 2016). This family of lipids correspond to uncharacterized *M. tuberculosis* components, labelled X and possibly Z1, Z2 in Fig. 8 of Dobson et al. (1985).

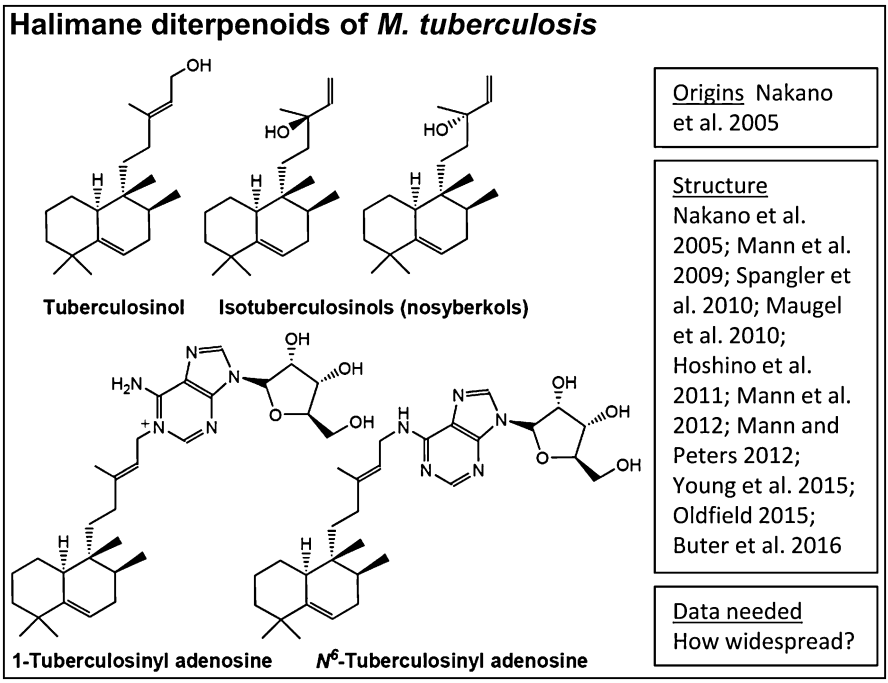

Fig. 21 Halimane diterpenoids of *Mycobacterium tuberculosis*

8 Polar Lipids and Related Lipoglycans

The mycobacterial inner membrane (MIM) (Minnikin et al. 2015) includes universal polar lipids, such a diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). PI is extended to give the glycophospholipid phosphatidylinositol mannosides (PIMs) that are further developed into the lipomannan (LM) and lipoarabinomannan (LAM) water soluble lipoglycans.

8.1 Polar Phospholipids, Glycolipids, and Ornithine Lipids

The principal mycobacterial phospholipids are cardiolipin (diphosphatidylglycerol DPG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (Fig. 22) (Goren and Brennan 1979; Goren 1984). A polar lipid acyl component is "tuberculostearic acid" (TSA) (Fig. 22), first recognized by Anderson and Chargaff (1929), and confirmed as 10-D(*R*)-methyloctadecanoic acid (Spielman 1934; Prout et al. 1948). TSA has been variously synthesized, as reviewed by ter Horst et al. (2010b) who also synthesized regioisomers of mycobacterial PE and proved that

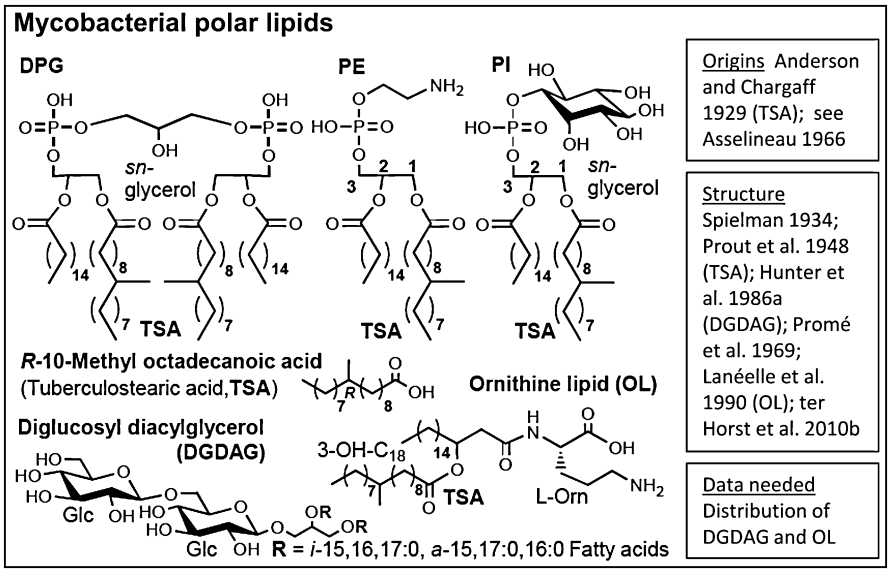

Fig. 22 Mycobacterial polar lipids: diphosphatidylglycerol (DPG), phosphatidyl-ethanolamine (PE), phosphatidylinositol (PI), diglucosyl diacylglycerol (DGDAG) and ornithine lipid (OL)

TSA is located on the *sn*-1 position of glycerol as shown in Fig. 22. By analogy, TSA is also assumed to be on the *sn*-1 positions of glycerol in mycobacterial DPG and PI (Fig. 22).

An ornithine-based polar lipid (OL) (Fig. 22) has been detected in *M. tuberculosis*, *M. bovis*, and possibly *M. marinum* (Promé et al. 1969; Lanéelle et al. 1990). Ornithine lipids replace PE in reduced phosphate cultures in other bacteria (Minnikin and Abdolrahimzadeh 1974). PEs from *M. tuberculosis* have minor 3-OH-$C_{16,\ 18,\ 20}$ straight-chain fatty acids (Alugupalli et al. 1995), similar to that in the ornithine lipids (OL) (Fig. 22). A diglycosyl diacylglycerol was isolated from *M. tuberculosis* (Hunter et al. 1986a) (Fig. 22).

8.2 Phosphatidylinositol Mannosides (PIMs)

A "phosphatide" fraction from tubercle bacilli yielded glycerophosphoric acid, mannose, and the hexahydric alcohol "inositide" (Anderson and Roberts 1930; Anderson 1939, 1941, 1943). Saponification of the phosphatides gave a "phosphorous- containing glycoside," which on dephosphorylation produced a "mannoinositose." The essential structure of phosphatidyl *myo*-inositol dimannoside (PIM_2) (Fig. 23) from *M. tuberculosis* was eventually established (Vilkas and Lederer 1960; Ballou et al. 1963; Lee and Ballou 1964a). Tri-, tetra-, penta-, and hexa-mannosides were recognized and the previously identified pentamannoside (PIM_5) (Nojima 1959; Ballou et al. 1963) was defined by Lee and Ballou (1965)

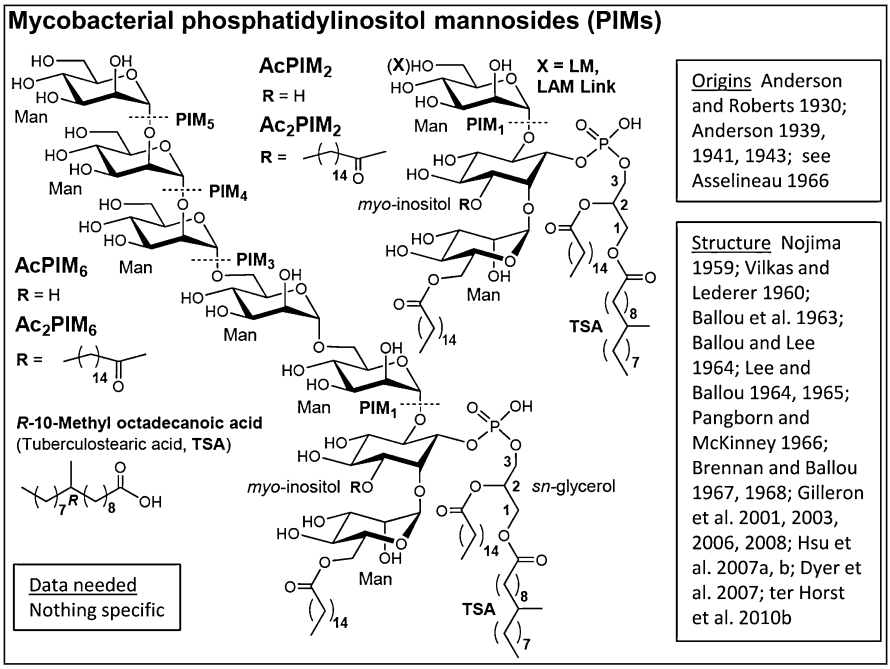

Fig. 23 Mycobacterial polar lipid phosphatidylinositol inositol mannosides (PIMs): monoacyl and diacyl PIMs and point of linkage (X) for formation of lipomannan (LM) and lipoarabinomannan (LAM) lipoglycans

(Fig. 23). PIM_1 was isolated in small amounts, and it was proven that the mannose was attached to the 3-hydroxyl group of the *myo*-inositol ring (Ballou and Lee 1964).

Pangborn and McKinney (1966) isolated from *M. tuberculosis* a series of PIM_2 lipids containing 2, 3, and 4 acyl residues. Brennan and Ballou (1967, 1968) also indicated the presence of di- and mono-acyl PIM_2 and PIM_5. Subsequent publications (Gilleron et al. 2001, 2003, 2006, 2008; Hsu et al. 2007a, b) have described the full extent of acylation in the PIM family in most mycobacteria, the principal components being mono- and di-acyl-PIM_2 and PIM_6 lipids (Fig. 23); *M. leprae* lacks the di-acylated components (Minnikin et al. 1985d). The biosynthesis of PIMs has been reviewed (Guerin et al. 2010; Angala et al. 2014; Sancho-Vaello et al. 2017). The major PIM_2 and PIM_6 lipid classes locate tuberculostearic acid on the *sn*-1 position of glycerol (Fig. 23) (Gilleron et al. 2003, 2006; Dyer et al. 2007; ter Horst et al. 2010b).

The mycobacterial nonlipid, water-soluble, lipomannans (LMs) and lipoarabinomannans (LAMs) incorporate a lipid anchor that is essentially PIM_2 (Fig. 23) (Hunter et al.1986b; Hunter and Brennan 1990). These highly important antigenic lipoglycans have been extensively reviewed (Chatterjee et al. 1992; Chatterjee and Khoo 1998; Nigou et al. 2003; Gilleron et al. 2008; Angala et al. 2014, 2018).

9 Other Lipophilic Molecules

9.1 Mycobactins

Mycobactins are lipophilic siderophores, involved in mycobacterial iron uptake and metabolism (Fig. 24) (Snow 1954a, b, 1965a, b, 1970; Ratledge 1982, 1999, 2004, 2013; Ratledge and Dover 2000; Quadri and Ratledge 2005; Quadri 2008; Horwitz and Horwitz 2014; Patel et al. 2018). Families of closely related mycobactins are found in most mycobacteria, excepting *Mycobacterium paratuberculosis* that requires mycobactin supplementation or added ferric ions (Snow 1970; Ratledge 2004). The original intracellular mycobactins are exemplified by the structure of mycobactin T from *M. tuberculosis* (Fig. 24) (Snow 1965b, 1970). More recently, a more hydrophilic variety of lipophilic iron chelaters has been recognized and termed "carboxymycobactins," as shown for the example from *M. avium* (Fig. 24) (Lane et al. 1995; Ratledge and Ewing 1996); methyl esters of essentially the same compounds were isolated independently by Gobin et al. (1995). These amphipathic mycobacterial mycobactins and carboxymycobactins operate with water-soluble, peptide-based "exochelins" to assimilate essential iron (Ratledge and Dover 2000; Ratledge 2004; Quadri 2008).

9.2 Mycolactones

The causative agent of African Buruli ulcer, *M. ulcerans*, was found to produce a lipid toxin (George et al. 1998) that was characterized as a polyketide and given the name mycolactone (Fig. 26) (George et al. 1999; Gunawardana et al. 1999). Subsequent studies elaborated a family of related mycolactones (Fig. 25) (Stinear and Small 2008; Kishi 2011; Chany et al. 2013; Gehringer and Altmann 2017). The definitive lipids of *M. ulcerans* are interconvertible *E*/*Z* geometric isomers, at

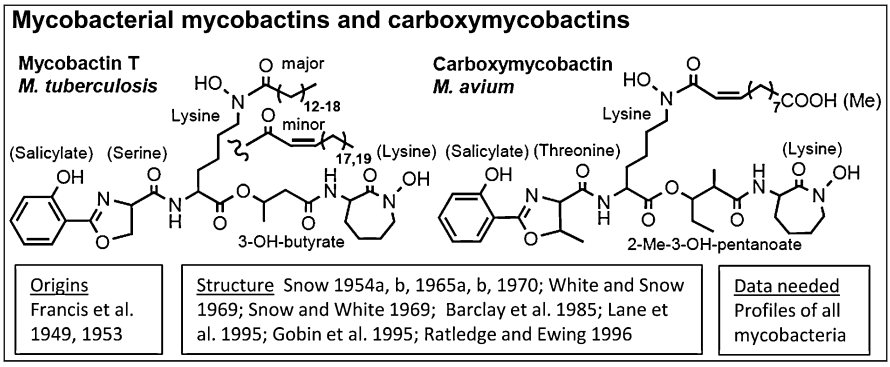

Fig. 24 Mycobacterial mycobactins and carboxymycobactins: lipophilic siderphores for iron assimilation

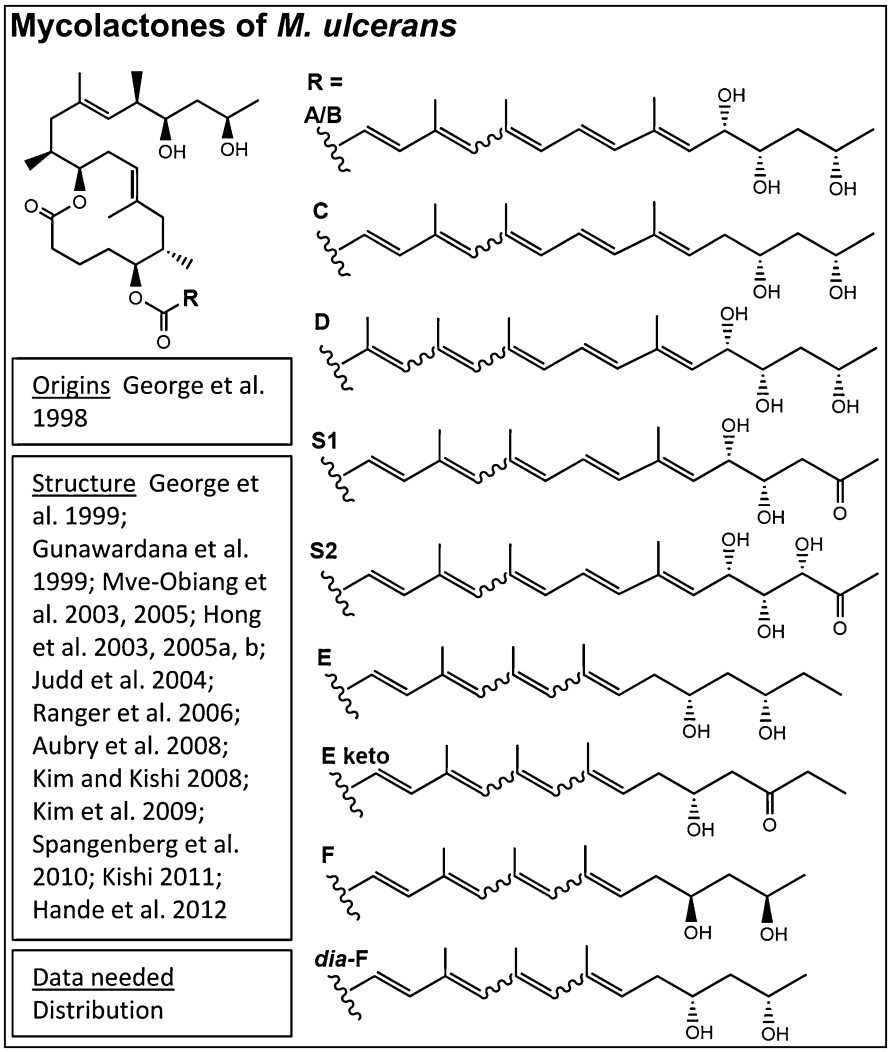

Fig. 25 Mycolactone lipophilic toxins of *Mycobacterium ulcerans* and related taxa

the 4′, 5′ double bond, labelled mycolactones A/B (Fig. 26) (Song et al. 2002). Investigations of reduced virulence Australian and Asian strains (Mve-Obiang et al. 2003) produced mycolactone C (Hong et al. 2003; Judd et al. 2004) and mycolactone D (Hong et al. 2005a), respectively (Fig. 25). The Japanese strain, *M. ulcerans* subsp. *shinshuense* had two keto variants, mycolactones S1 and S2 (Fig. 25) (Hande et al. 2012). Mycolactone E and a keto-analogue (E keto) were provided by "*Mycobacterium liflandii*," a frog pathogen (Fig. 25) (Mve-Obiang et al. 2005; Hong et al. 2005b; Aubry et al. 2008; Spangenberg et al. 2010). *M. marinum*, infecting fresh-water fish, gave mycolactone F (Ranger et al. 2006;

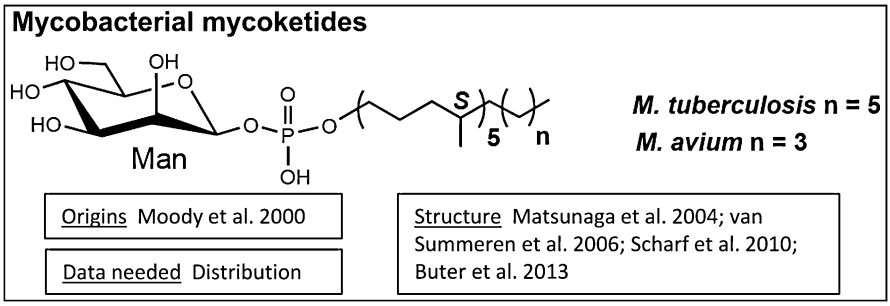

Fig. 26 Mycoketides of *Mycobacterium tuberculosis* and *Mycobacterium avium*

Kim and Kishi 2008), but *M. marinum* strains infecting salt-water fish had diastereomeric mycolactone *dia*-F (Fig. 25) (Kim et al. 2009).

9.3 Mycoketides

A CD1c-restricted, mycobacteria-specific T-cell line recognized previously unknown mannosyl lipids, of apparent isoprenoid nature, from *M. tuberculosis* and *M. avium* (Moody et al. 2000). Detailed studies revealed that these glycolipids were of polyketide origin and they were designated as mycoketides (Fig. 26) (Matsunaga et al. 2004; Quadri 2014). The structures of these mannosyl phosphomycoketides (MPMs) were confirmed by synthesis (van Summeren et al. 2006; Scharf et al. 2010; Buter et al. 2013).

9.4 Polymethylated Polysaccharides (PMPS)

During an investigation of the PIMs of *M. tuberculosis* and *M. smegmatis*, Lee and Ballou (1964b) originally reported on the presence of a 6-*O*-methyl-D-glucose-containing "polysaccharide-like material," later characterized as an extraordinary mycobacterial lipopolysaccharide (Lee 1966). An early structure for these polymethylated polysaccharides (PMPSs) (Saier and Ballou 1968a, b, c) was revised (Forsberg et al. 1982), the key components comprising glucose, 3- and 6-*O*-methylglucoses, and glyceric acid (Fig. 27). Various acylation combinations of PMPSs involve acetate, propionate, isobutyrate, octanoate, and succinate (Keller and Ballou 1968; Gray and Ballou 1972; Smith and Ballou 1973; Narumi et al. 1973; Jackson and Brennan 2009). Acyl function heterogeneity of the PMPS of *M. tuberculosis* was confirmed (De et al. 2018), so only a representative structure is shown in Fig. 27. Succinate content can vary from zero to three, resulting in four main components. PMPSs have been described in a variety of mycobacteria and *Nocardia* (Lee 1966; Smith and Ballou 1973; Tuffal et al. 1995, 1998a, b; Jackson and Brennan 2009).

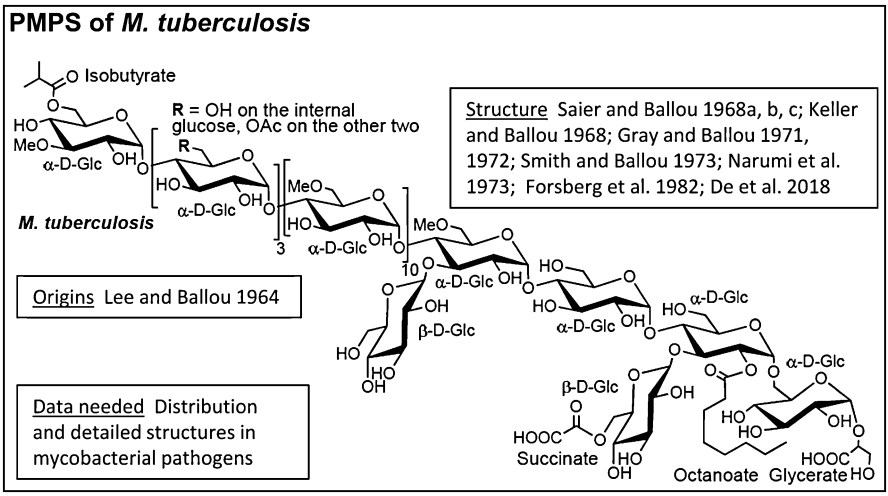

Fig. 27 Polymethylated polysaccharides (PMPS) of *Mycobacterium tuberculosis*

These important minor lipophilic PMPS molecules (0.01% of biomass; De et al. 2018) are possibly involved in the regulation of fatty and mycolic acid synthesis. The PMPSs and the related 3-*O*-methyl-mannose-containing polysaccharides from *M. smegmatis* (MMPs; Gray and Ballou 1971; Maitra and Ballou 1977) are likely fatty-acyl carriers, facilitating processing of long, insoluble fatty-acyl CoAs (Yabusaki and Ballou 1979; Yabusaki et al. 1979).

10 Conclusions

The object of this chapter is to provide, for the first time, a structural database for the lipids of clinically significant mycobacteria, appropriately referenced; however, it is informative to include a summary of the likely cellular location of such lipids. The wide array of lipid types, detailed in Sects. 2 to 9, are principally associated with the two distinct mycobacterial cell envelope membrane bilayers (Fig. 28) (Minnikin et al. 2015). The cytoplasmic mycobacterial inner membrane (MIM) includes conventional polar lipids (Fig. 22); however, phosphatidylinositol dimannosides (PIM_2; Fig. 23) are the main components of the inner leaflet with high proportions of phosphatidylinositol hexamannosides (PIM_6; Fig. 23) in the outer leaflet (Bansal-Mutalik and Nikaido 2014; Minnikin et al. 2015). Non-lipid lipoarabinomannans (LM) and lipoarabinomannans (LAM) are anchored to the MIM outer leaflet by an analogue of PIM_2 (Fig. 23). It is considered that isoprenoid quinones (Fig. 18), carotenoid pigments (Fig. 19), halimane diterpenoids (Fig. 21), and mycolactones (Fig. 25) are MIM-associated. The isoprenoid lipid carriers (Fig. 20) and mycoketides (Fig. 26) probably originate in the MIM domain. Iron sequestering mycobactins (Fig. 24) associate with the MIM outer leaflet; polymethylated

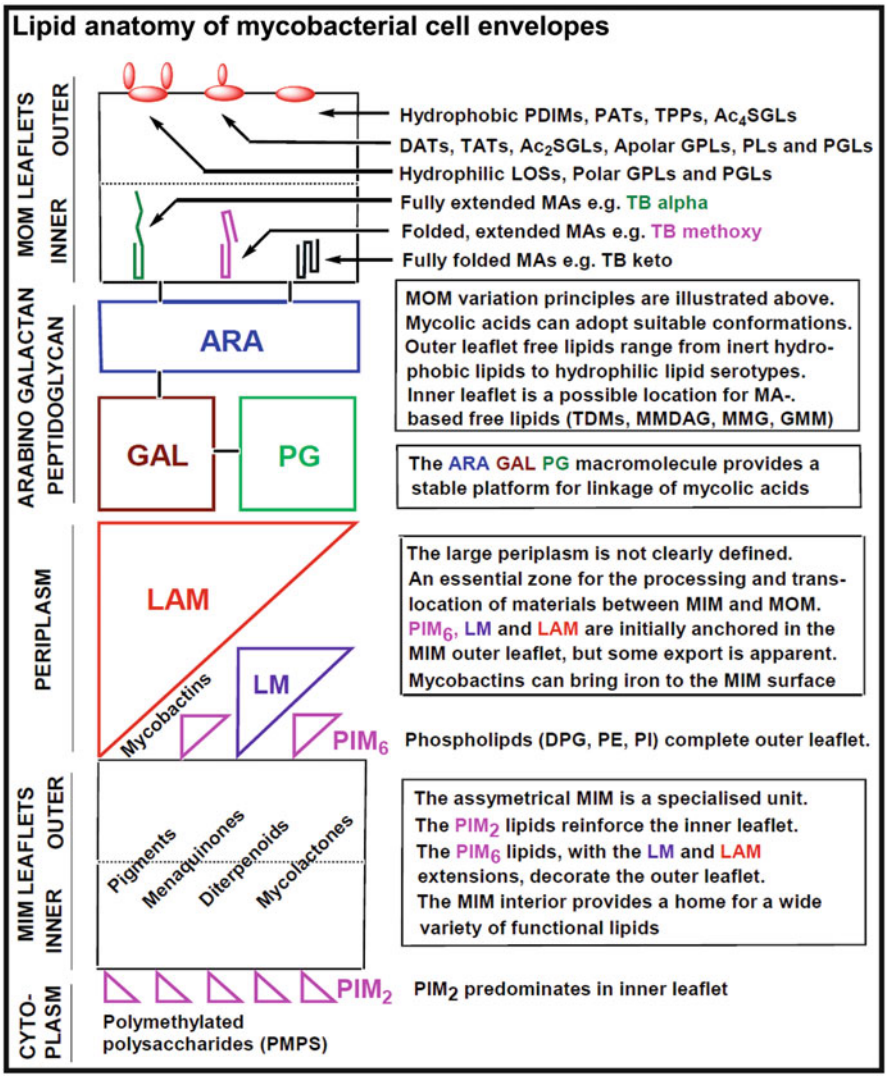

Fig. 28 Essential anatomy and role of mycobacterial lipids in the inner and outer leaflets of mycobacterial cell envelope inner (MIM) and outer (MOM) membranes. Non-lipid lipomannan (LM) and lipoarabinomannan (LAM) lipoglycans are included, as is the arabinogalactan-peptidoglycan (ARA-GAL-PG) macromolecule to which the mycolic acids (MAs) are attached. See text and Figures 1–27 for other abbreviations

polysaccharides (PMPS, Fig. 27) are cytoplasmic but close to the MIM inner leaflet (Fig. 28).

The mycobacterial outer membrane (MOM) is the main location for the majority of the other types of mycobacterial lipids (Fig. 28). The inner leaflet

of the MOM is considered to be a monolayer of long-chain mycolic acids (MAs, Fig. 1A–G), covalently bound to terminal arabinose units presented by an arabinogalactan-peptidoglycan macromolecule (Minnikin 1982; Brennan and Nikaido 1995; Minnikin et al. 2002, 2015). MAs can adopt different conformations to ensure appropriate integrity of the MOM inner leaflet (Villeneuve et al. 2005, 2007, 2010, 2013; Minnikin et al. 2015) (Fig. 28). Essentially, three classes of MOM outer leaflet free lipids, interacting with the bound mycolates to complete the MOM bilayer, can be discerned (Fig. 28). In the first class, phthiocerol dimycocerosates (PDIMs; Fig. 13A, B), pentaacyl trehaloses (PATs; Fig. 7B), trehalose polyphleates (TPPs; Fig. 10), and tetraacylated sulphoglycolipids (Ac_4SGLs; Fig. 9) are relatively inert and probably enhance cell surface hydrophobicity (Minnikin et al. 2015; Jankute et al. 2017). The intermediate second class comprises antigenic di- and triacyl trehaloses (DATs and TATs; Figs. 7A, B and 8), diacylated sulphoglycolipids (Ac_2SGLs; Fig. 9), apolar glycopeptidolipids (aGPLs; Fig. 16A–G), peptidolipids (PLs; Fig. 17), and apolar phenolic glycolipids (PGLs; Fig. 15B, D). Strongly antigenic hydrophilic lipooligosaccharides (LOSs; Fig. 11A–G), polar glycopeptidolipids (GPLs; Fig. 16A–G), and polar phenolic glycolipids (PGLs; Fig. 15A–D) are the third class. Information is required regarding the precise location of mono-mycoloyl glycerols (MMGs; Fig. 4), glucose monomycolates (GMMs; Fig. 3), monomycobacteroyl diacylglycerols (MMDAGs; Fig. 12), and mycolate ester waxes (MEWs; Fig. 6), though MOM association is suspected. Similarly, mono- and dimycoloyl arabinoglycerols (MMAG and DMAGs; Fig. 5), analogues of the terminal unit of the mycoloyl arabinogalactan-peptidoglycan macromolecule, are probably MOM associated. Trehalose monomycolate (TMM) (Fig. 2) is involved with incorporation of MAs into the mycoloyl arabinogalactan-peptidoglycan and trehalose dimycolate (TDM) (Fig. 2) is likely to be associated with the MOM inner leaflet.

11 Research Needs

Specific research needs have been highlighted in the majority of the structural figures, so only general points are listed below.

- Complete the structural details of all lipids from known pathogenic mycobacteria.
- Fully investigate the lipid composition of all related pathogenic mycobacteria.
- Define the physical and conformational behavior of all mycolic acids and various conjugates in order to comprehend the organization of the covalently bound mycolic acid inner leaflet of the mycobacterial outer membrane (MOM).
- Study the interaction of representative mycolic acid monolayers with individual and combinations of MOM outer leaflet free lipids to explore the essential composition and behavior of the lipid domains of the mycobacterial outer membrane.

- Perform a thorough investigation of the characteristics of the special mycobacterial inner membrane (MIM), particularly the role of the phosphatidylinositol mannosides (PIMs).
- In short, assemble a reliable database comprised of information about the structures, cellular location, and function of mycobacterial lipids to inform parallel studies on lipid genomics, biosynthesis, biological activity, and drug targets.

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Mycobacterial Lipid Bodies and the Chemosensitivity and Transmission of Tuberculosis

3

Natalie J. Garton and Michael R. Barer

Contents

N. J. Garton (✉)
Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK
e-mail: njg17@le.ac.uk

M. R. Barer
Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK

Department of Clinical Microbiology, University Hospitals Leicester NHS Trust, Leicester, UK
e-mail: mrb19@le.ac.uk

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Abstract

Just over a quarter of humanity is infected with the tubercle bacillus and risks developing active disease that routinely requires 6-month treatment. The impact of this scourge cannot be underestimated, and reducing the global burden of tuberculosis is the focus of much research. In addition to the need for improved chemotherapy regimens and monitoring thereof, understanding the risk and processes involved in transmission, a critical step in the life cycle of the organism, has even greater potential to impact the burden of disease. Our chance observation that lipid bodies (LBs) were present in *Mycobacterium tuberculosis* in sputum, but not in growing cultures of the lab strain *in vitro,* led us and others to examine this phenomenon further. Transcriptional analysis of the bacilli in sputum identified that upregulation of *tgs1*, a triacylglycerol synthase, was likely responsible for the presence of these LBs. Strikingly, in contrast to the then established view that tubercle bacilli in sputum arose directly from rapidly replicating populations, further transcriptional and cytological analyses led us to link the *M. tuberculosis* sputum phenotype to slow or non-growing persisters. As a result, we and others have directed research to further understanding the biological and clinical significance of LBs and neutral lipids in mycobacteria. There is now greater insight into the biosynthetic pathways and role of neutral lipids during infection, for both growing and dormant *M. tuberculosis*. Links have been made between *tgs1*-related triacylglycerol LB accumulation and growth arrest and with antibiotic tolerance potentially underpinning the need for protracted chemotherapy. The possible clinical significance of this is reflected in the finding that sustained high frequencies of LB-positive *M. tuberculosis* in sputum during treatment are associated with unsatisfactory outcomes. LB-positivity may also support transmission of the organism. Greater understanding of the significance of this "fat and lazy" population will open up new approaches to the combat of this long-standing foe.

Abbreviations

ACSL	Long-chain acyl-CoA synthase
DAG	Diacylglycerol
DC	Differentially culturable
DGAT	Diacylglycerol acyl transferase
FACS	Fatty acyl CoA synthase
FCR	Fatty acyl long-chain CoA reductase
ILI	Intracellular lipophilic inclusion
LB	Lipid body
LCFA	Long-chain fatty acid
LD	Lipid droplet
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cell
RIF	Rifampicin
TAG	Triacylglycerol

TB	Tuberculosis
TCA	Tricarboxylic acid
TGS	Triacylglycerol synthase
THL	Tetrahydrolipstatin
WE	Wax ester
WS	Wax ester synthase

1 Introduction

1.1 Occurrence and Composition of Lipid Bodies

Lipid bodies (LBs) (also known as lipid droplets (LDs) or intracellular lipophilic inclusions (ILIs)) are cytoplasmic accumulations of lipids. LBs are widespread in eukaryotes, where they have generally been viewed as a carbon storage depot but are now recognized as dynamic organelles (Murphy 2012). In contrast, the occurrence of LBs in prokaryotes appears to be more restricted and includes the Actinobacteria *Mycobacterium* and *Rhodococcus*, some *Streptomyces*, and certain hydrocarbon-degrading species of Gram-negative genera including *Acinetobacter*, *Marinobacter*, *Alcanivorax*, and *Thalassolituus* (Alvarez 2016). Bacterial LBs are overwhelmingly viewed as a form of carbon storage, enabling cells to survive "feast or famine" and to colonize and thrive within different niches.

The core of LBs is composed of neutral lipid which is surrounded by a unit phospholipid membrane. Many proteins are associated with the phospholipid membrane and include enzymes involved in neutral lipid synthesis and degradation, but also proteins which stabilize the LB structure or regulate lipid homoeostasis and turnover (Murphy 2012). Eukaryotic LBs are composed of triacylglycerols (TAGs) and cholesterol esters. Bacterial LBs are predominantly composed of TAG, although in certain growth conditions, wax esters (WEs) can form. Representative structures of these are shown in Fig. 1. The acyl composition and distribution of acyl chains on the glycerol backbone of bacterial TAG can vary with bacterial culture conditions and growth state. In general, the *sn*-1,2 positions are esterified with acyl chains of between 16 and 18 carbons which reflects the composition of phospholipids and the 3-position with longer or branched fatty acids (Walker et al. 1970; Alvarez and Steinbüchel 2002). The composition of bacterial LBs can affect their morphology, presumably a reflection of the melting temperature of the components and resulting fluidity. Compared to the relatively circular appearance of TAG-containing LBs, WEs accumulate as more disc-like structures (Alvarez and Steinbüchel 2002; Ishige et al. 2002; Sherratt 2008).

1.2 Mycobacterial Lipid Bodies

Mycobacterial LBs were first reported by Burdon who applied the lipophilic strain, Sudan Black B to smears of *Mycobacterium tuberculosis* (Mtb), *Mycobacterium*

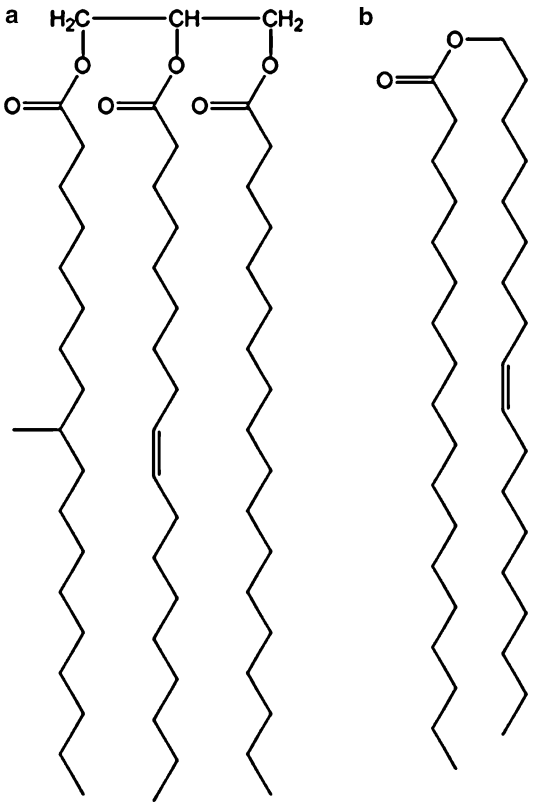

Fig. 1 Representative structures of TAG and WE, which can contain a range of different acyl chains as discussed in the text. (**a**) TAG 1-stearoyl, 2-oleoyl, 3-tuberculostearoylglycerol. (**b**) WE oleyl stearate

leprae, and saprophytic species (Burdon 1946). Since this time, mycobacterial LBs have been recognized by both light and electron microscopy (Garton et al. 2002). During a study of the native organization of envelope lipid domains, we identified LBs in live mycobacteria with application of select fluorescent lipophilic probes (Christensen et al. 1999). This observation was the impetus for our study of the natural history of these structures in the fast-growing saprophytic species *Mycobacterium smegmatis* (Garton et al. 2002). Consistent with a supposed carbon storage role, LBs accumulated in conditions of carbon excess and nutrient limitation. In nitrogen-limited medium LBs formed over a period of several days, whereas supplementation of growing cultures with long-chain fatty acids (LCFAs) resulted in rapid LB accumulation. Transfer to carbon-deficient medium resulted in assimilation of accumulated LBs. TAG was, for many years, considered to be a component of the mycobacterial cell envelope (Minnikin 1982; Ortalo-Magné et al. 1996). We found observation of *M. smegmatis* LBs to be coincident with the presence of TAG and the TAG acyl composition to reflect the culture conditions (Garton et al. 2002). A range of LCFA (C_{14}–C_{24}) were detected in TAG extracted from biomass cultured in Middlebrook broth, with palmitic (hexadecanoic $C_{16:0}$) and oleic (octadecenoic $C_{18:1}$) acyl substituents being most abundant. The chain length profile of acyl

substituents of TAG from nitrogen-limited culture was similar, but was enriched in saturated, particularly stearic (octadecanoic $C_{18:0}$) acyl chains. In contrast, the profile of TAG from oleic acid-supplemented culture was dominated by this acyl substituent (~80%) and also contained palmitoleoyl (hexadecenoic $C_{16:1}$) and trace tuberculostearic (10-methyloctadecanoic $brC_{19:10Me}$) acyl chains. TAG was not observed in cultures in which LBs had been assimilated. Acyl chain lengths of up to C_{28} have been reported in TAG of Mtb treated with nitric oxide (NO), with the most abundant chain length being C_{26} (Daniel et al. 2004).

Our particular interest in LBs of Mtb, the agent of tuberculosis (TB), was driven by the observation that LBs were more readily observed in acid-fast bacilli in TB patient sputum samples, compared with growing *in vitro* culture of the Mtb laboratory strain H37Rv (Garton et al. 2002, 2008). This was in contrast with the abundant presence of LBs in fast-growing *M. smegmatis*. It is interesting to note that Burdon described inconsistent detection of LBs in tubercle bacilli cultured on egg medium, compared with their ready detection in *M. smegmatis* (Burdon 1946). However, we did identify LBs in Mtb sampled from in an *in vitro* hypoxic model of Mtb dormancy and in response to NO, a treatment that results in growth arrest (Garton et al. 2008). Mtb LBs have since been reported in response to other growth arresting stimuli (Sherratt 2008) and in alternative *in vitro* models of dormancy (Deb et al. 2009), including a macrophage models of Mtb persistence (Daniel et al. 2011) and a granuloma model (Kapoor et al. 2013).

Significantly, Mtb growth arrest and dormancy result in a phenotypic state in which the bacilli become tolerant to the action of antibiotics, leading us to propose that Mtb bacilli in TB patient sputa may represent a similarly antibiotic tolerant population. The clinical significance of LBs was highlighted in a recent study linking sputum LB content of Mtb with increased probability of unsatisfactory treatment outcome (Sloan et al. 2015). Understanding the differences between Mtb in sputum and *in vitro* culture, the clinical significance of Mtb LB positivity in sputum, and how this may relate to bacilli in aerosols that transmit infection are major foci of our research.

2 TAG Synthesis, LB Assembly, and Assimilation

There is now much insight into the conditions and biochemical pathways involved in TAG and WE synthesis, organization as LBs, and their breakdown. Here we summarize what is known of these in members of the Mtb complex.

2.1 Synthesis of TAG and WE

Eukaryotic lipid droplets (LDs) contain TAG and cholesterol esters, which are synthesized by distinct acyl transferase enzymes. The biosynthetic (Kennedy) pathway of TAG, similar to that in bacteria shown in Fig. 2, branches from that of phospholipids at the key intermediate phosphatidic acid (diacylglycerol 3-

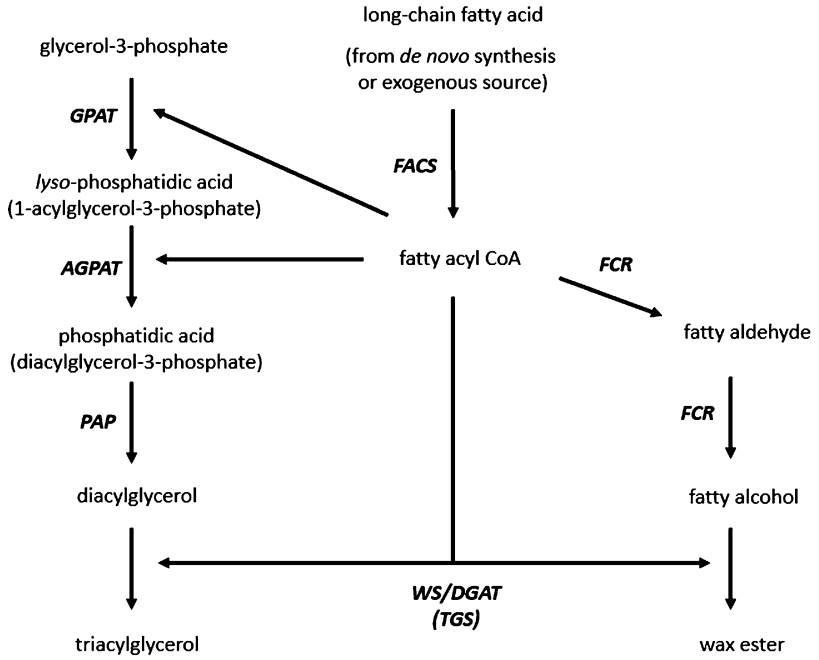

Fig. 2 The biosynthetic pathway of TAG and wax esters in bacteria. Key: GPAT, glycerol 3-phosphate acyltransferase; AGPAT, 1-acylglycerol 3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; FACS, fatty acyl-CoA synthase; FCR, fatty acyl-CoA reductase; WS/DGAT, wax ester synthase/diacylglycerol acyltransferase; TGS, triacylglycerol synthase

phosphate). A specific phosphatidic acid phosphatase hydrolyzes phosphatidic acid to form diacylglycerol (DAG), the substrate for diacylglycerol acyl transferases (DGATs). DGATs catalyze the transfer of long-chain fatty acyl chains, activated as acyl CoA, onto the free hydroxyl of DAG to form TAG. Bacteria contain no homologues of eukaryotic DGATs. The first bacterial enzyme characterized as having DGAT activity was identified in an *Acinetobacter* (*Acinetobacter* sp. ADP1) (Kalscheuer and Steinbüchel 2003) with a screen of transposon mutants to identify those deficient in lipid accumulation. This *Acinetobacter* enzyme was found to possess both DGAT and wax ester synthase (WS) activity and was termed WS/DGAT. These authors identified homologues of this WS/DGAT in mycobacteria.

Daniel and colleagues screened 15 putative Mtb WS/DGAT proteins for these activities with *in vitro* assays. All 15 recombinant proteins were shown to have greater DGAT activity than WS activity, leading to these investigators to term the mycobacterial enzymes TAG synthases (TGS) and those with the greatest TGS activity, as Tgs1 (Rv3130c), Tgs2 (Rv3734c), Tgs3 (Rv3234c), and Tgs4 (Rv3088) (Daniel et al. 2004). Overexpression of *tgs1* in *M. smegmatis* resulted in enhanced TAG LB content (Garton et al. 2008) relating the activity of this enzyme with cytoplasmic LB accumulation. The accumulation in Mtb of TAG in response to hypoxia, or following exposure to NO, correlates with induced transcription of *tgs1*

(Daniel et al. 2004). An Mtb *tgs1* mutant strain fails to accumulate TAG in response to these conditions (Sirakova et al. 2006) or in a multiple stress (low O_2, high CO_2, low nutrient, acidic pH) model (Deb et al. 2009).

Tgs1 is a member of the DosR-regulated dormancy-related regulon comprising ~48 genes (Park et al. 2003). *DosR* expression responds to hypoxia, NO, and carbon monoxide, with the signal transduced by two sensor proteins DosS and DosT (Roberts et al. 2004). It is noteworthy that although *tgs1* shows the greatest induction in response to hypoxia or NO, expression of other *tgs* genes, e.g., *tgs2*, *tgs3*, and *Rv3371*, which are not members of the DosR regulon, is increased in these conditions also (Daniel et al. 2004).

TGS Rv3371 shows the greatest homology with Tgs1 (Daniel et al. 2004) although it was reported to possess weak TGS activity *in vitro*. Over-expression of *Rv3371* in *M. smegmatis* resulted in enhanced TAG content (Rastogi et al. 2017). Colonies of this strain had a smooth appearance, and cells had altered cell surface properties, leading these authors to suggest that Rv3371 is involved in cell wall alterations. However, the intracellular LB content of strains was not examined. In addition to being induced in response to hypoxia and NO (Daniel et al. 2004), *Rv3371* expression is upregulated in conditions of iron -limitation. *Rv3371* expression is repressed by the putative transcriptional regulator Rv1404 (Golby et al. 2008). *Rv1404* has an IdeR (iron-dependent repressor and activator) binding site upstream. Reduced expression of both *ideR* and *Rv1404* in iron-limited conditions led Rastogi and colleagues to suggest that IdeR activates Rv1404, in turn relieving repression of *Rv3371* expression (Rastogi et al. 2017).

In addition to the 15 TGSs, other proteins of the Mtb complex have been identified as having a role in TAG accumulation. Investigation of the protein content of isolated *Mycobacterium bovis* BCG LBs identified homologues of Tgs1 (BCG3153c), Tgs2 (BCG3794c), and two proteins, which, when deleted, reduced the TAG content of the mutants compared with the wild type, a putative 1-acyl glycerol 3-phosphate acyl transferase (BCG1489c) and an uncharacterized protein BCG1169c (Low et al. 2010). Furthermore, homologous over-expression of *BCG1721*, encoding a fifth LB-associated protein which was found to contain both long-chain acyl-CoA synthase (ACSL) and lipase domains, resulted in accumulation of LBs. This suggests that in those conditions, the ACSL activity of this protein was dominant. To date, the activity of the Mtb homologue of this novel bifunctional protein, Rv1109c, has not been investigated.

In 2011, Elamin and colleagues reported that Ag85a, one of three cell envelope mycolyl-transferases, also had TGS activity (Elamin et al. 2011). Over-expression of Ag85a in *M. smegmatis* resulted in the accumulation of TAG LBs and a thickening of the cell envelope. Recently, all three mycolyl-transferases Ag85a, Ag85b, and Ag85c have been reported to possess TGS activity *in vitro*, with Ag85c having the highest TGS activity (Viljoen et al. 2018). Recombinant WS/DGAT of *Acinetobacter* sp. ADP1 has been found to be rather promiscuous in the range of substrates accepted for acylation (Stöveken et al. 2005). It is not known if the Mtb Ag85 proteins have a physiological role in TAG synthesis in the mycobacterial cell, possibly contributing to TAG content of the outer layer of the cell envelope, or

whether this activity simply reflects the ability of these proteins to accept DAG and shorter acyl chains as substrates *in vitro*.

In addition to TAG, WEs have been reported in lipid extracts of Mtb cultures under multiple stress (Deb et al. 2009) or iron -limitation (Bacon et al. 2007). Of the 15 TGS (WS/DGAT) assayed by Daniel and colleagues, TGS2 (Rv3734c) was shown to have the greatest WS activity (Daniel et al. 2004), although contribution of Tgs2 to WE accumulation in bacilli has not been demonstrated. Two long-chain fatty acyl-CoA reductases (FCRs) of Mtb, Fcr1 (Rv3391) and Fcr2 (Rv1543), have been identified as being important for WE synthesis (Sirakova et al. 2012). FCR catalyzes reduction of long-chain fatty acyl-CoA, via an aldehyde intermediate, to long-chain fatty alcohol, a substrate for acylation by WS/DGAT to form WE (Fig. 2). To date, the cellular location of mycobacterial WE is not known. A cell envelope location is implied by the study of Sirakova and colleagues; Mtb Fcr1 and Fcr2 mutants showed faster growth rates and increased ^{14}C-glycerol uptake, suggesting increased cell envelope permeability. However, *M. smegmatis* cultured on hexadecanol accumulates LBs with disc-like morphology (Sherratt 2008) similar to those seen in *Acinetobacter* sp. cultured in conditions which induce WE formation (Singer et al. 1985; Ishige et al. 2002). Intracytoplasmic co-localization of WE and TAG may occur, with the relative concentration of each possibly impacting the LB morphology.

2.2 Proteins with Roles in the Organization of LBs

Eukaryotic LDs contain a high-protein content in the surrounding phospholipid unit membrane (Walther and Farese 2012). Some LD proteins have roles in the formation and stabilization of the LDs for long-term storage by restricting access of lipases; one such example is the oleosins associated with LBs in plant cells. Other LD proteins, such as mammalian perilipin, have roles in regulating lipolytic breakdown. The first protein identified to have a role in bacterial LB organization was a heparin-binding hemagglutinin homologue termed TadA (TAG accumulation deficient) of *Rhodococcus opacus* (MacEachran et al. 2010). A Δ*tadA* mutant showed reduced TAG content and LBs of reduced size and shape. Aggregation of purified LBs on addition of purified TadD led the authors to suggest that TadA has a role in the maturation of LBs, directing aggregation of smaller nascent LBs found in early lipid storage. To date, no similar role has been reported for the Mtb TadA homologue, the heparin-binding hemagglutinin, HbhA.

Two Mtb LB-associated proteins have recently been described which impact LB production or organization. Daniel and colleagues characterized an Mtb protein with weak homology to human perilipin-1 and termed this MPER1 (Daniel et al. 2016). An Mtb Δ*mper1* (*Rv1039c*) mutant did not accumulate LBs and showed reduced incorporation of radiolabeled fatty acid into TAG in multiple stress conditions (Daniel et al. 2016) known to result in TAG LB accumulation in wild-type Mtb (Deb et al. 2009). Armstrong and colleagues investigated PspA (Rv2744c), a phage shock protein which shows similar structural characteristics to Psp proteins in unrelated bacteria such as *E. coli* (Armstrong et al. 2016). Psp proteins are induced

in response to cell envelope stress, whereupon they localize to the inner face of the plasma membrane, assembling to form a scaffold-like complex to maintain membrane integrity and prevent dissipation of the proton-motive force (Armstrong et al. 2016). Although Mtb *pspA* is induced by the cell envelope stressor, SDS, an Mtb *pspA* mutant strain did not show enhanced susceptibility to this stress. However, the authors identified a novel role for the Mtb Psp protein. Compared with wild type, cells of an *M. smegmatis* mutant of the Mtb *pspA* homologue *MS_2695* were found to have a greater number of LBs with a size profile shifted to those of diameter < 50 nm. However, loss or overproduction of *MS_2695*, although influencing the number and the size profile of LBs, did not impact the amount of TAG detected in a purified LB fraction. These authors concluded that PspA is important in regulating LB homeostasis.

2.3 LB Turnover

Mycobacterial TAG LBs are assimilated rapidly in cells transferred to carbon-limited conditions (Garton et al. 2002; Dhouib et al. 2011) or on transfer to growth-permissive conditions following growth arrest, for example, during regrowth from an hypoxic *in vitro* model of Mtb dormancy (Low et al. 2009). LBs are also considered to be a store of carbon and energy to sustain mycobacteria during dormancy (Daniel et al. 2004). Mycobacterial LB turnover is susceptible to inhibition with the pancreatic lipase inhibitor, tetrahydrolipstatin (THL) (Low et al. 2009; Dhouib et al. 2011). Mtb has a Lip family of 24 lipid/ester hydrolases, with a GXSXG consensus motif and annotation as putative esterases or lipases. Assessment of the TAG hydrolytic (lipase) activity of these recombinant proteins revealed LipY (Rv3097c) to be the most active (Deb et al. 2006). Following culture in conditions which induce TAG accumulation, an Mtb Δ*lipY* mutant did not assimilate TAG on transfer to carbon-limited conditions (Deb et al. 2006). LipY is a member of the Mtb PE family of proteins named for the Pro-Glu (PE) motif at their N-terminal, and these proteins have roles in antigenic variation, immune evasion, and virulence (Mishra et al. 2008). Over-expression of *lipY* in mycobacteria reduces cellular TAG content. Interestingly, if the PE domain is lacking in the over-expressed protein, lipase activity is enhanced, suggesting a regulatory role for this domain.

3 Metabolic Significance of TAG LB Accumulation

3.1 TAG Formation During Growth

More so than any other bacteria, mycobacteria with their uniquely lipid-rich cell envelope have a greater requirement to synthesize and manipulate LCFAs to support growth. During infection Mtb utilizes host lipids as a carbon and energy source. Segal and Bloch (1956) first reported that Mtb bacilli recovered from experimentally infected murine lungs showed an enhanced respiratory rate when supplemented with

LCFA, but not with simple sugars; *in vitro* grown bacilli utilized both carbon sources equally well. Subsequent studies have confirmed a requirement for catabolism of both host LCFAs and cholesterol for persistence (McKinney et al. 2000; Pandey and Sassetti 2008). Catabolism of host LCFA requires β-oxidation and the glyoxylate shunt in order to bypass steps of the TCA cycle in which carbon is lost as CO_2 and to retain this for gluconeogenesis (McKinney et al. 2000). In addition to fuelling central metabolism, host LCFA could be incorporated directly into mycobacterial lipids required for cell envelope synthesis during growth.

In axenic culture, LCFAs can be toxic to mycobacteria, probably resulting from the detergent-like activity of these amphiphiles leading to disruption of membranes (Kondo and Kanai 1977). Growing mycobacteria which import LCFA need to balance this uptake with metabolic and biosynthetic requirements. Incorporation of LCFA into TAG would buffer this toxic activity, yet, with hydrolysis by lipases, the LCFA would be readily available for oxidation or incorporation into cell envelope lipid. In growing mycobacteria, LBs may therefore represent a more dynamic metabolic pool, rather than a store of carbon (Fig. 3).

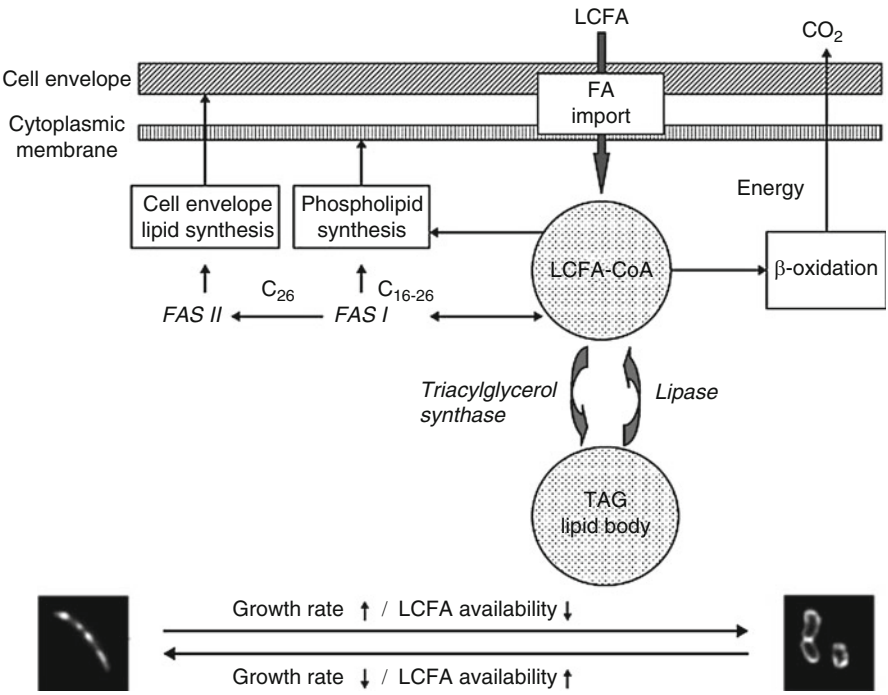

Fig. 3 A proposed dynamic role for the TAG lipid body system. Lipid bodies are formed depending on the environmental balance of availability of LCFA and conditions available for growth. Key: LCFA, long-chain fatty acids; FA, fatty acid; CoA, coenzyme A; FASI and FASII, fatty acid synthase systems 1 and 2; TAG, triacylglycerol. (Originally published in Barer and Garton 2010, published with kind permission of ©Springer Science+Business Media New York, 2003. All rights reserved)

3.2 TAG Accumulation in Slow/Non-growing Mtb

Bacterial accumulation of TAG, rather than alternative carbon storage compounds such as glycogen and polyhydroxybutyrate, provides greater energetic return on oxidation (Alvarez 2016). This is because TAG is the more reduced molecule. Synthesis of LCFA for TAG can act to balance metabolism, preventing accumulation of reduced pyridine nucleotide cofactors which otherwise could inhibit some enzymes of central metabolism. This is particularly the case in hypoxic conditions when Mtb switches to anaerobic respiration.

Baek and colleagues proposed a direct role of TAG accumulation in bringing about growth arrest (Baek et al. 2011). In their *in vitro* experiments, an Mtb H37Rv Δ*tgs1* mutant failed to respond to growth-limiting stresses including hypoxia, low pH, and iron -limitation, with a restriction of growth as observed in the wild-type strain (Baek et al. 2011); failure of the Δ*tgs1* mutant to accumulate TAG under these conditions was also confirmed. These authors proposed that induction of *tgs1*, which results in TAG accumulation, directs acetate (the precursor of fatty acid synthesis by FasI) away from the TCA cycle and into TAG, resulting in growth arrest. They supported this hypothesis by demonstrating that the Δ*tgs1* mutant phenotype could be replicated by increasing acetate flux through the TCA cycle with oxaloacetate supplementation or overexpression of *citA*. Acetate flows into the TCA cycle at citrate synthase (CitA) which catalyzes condensation of this with oxaloacetate to form citrate. Baek and colleagues proposed this would compete for acetyl-CoA that would otherwise be diverted into fatty acid synthesis and ultimately TAG by Tgs1. In growth-permissive conditions, TAG hydrolysis and β-oxidation would make acetyl-CoA available for central metabolism again. Interestingly, an Mtb *Rv3371* mutant also fails to enter non-replicating persistence in response to hypoxia, NO, and iron limitation (Rastogi et al. 2017). This suggests that the activity of both Tgs1 and Rv3371 is required to bring about growth arrest *in vitro*.

Whether this hypothesis holds for Mtb which is supplemented with, or utilizing, LCFA as a carbon source has not been explored. It could be proposed that *tgs1* or *Rv3371* induction in these conditions would direct all imported LCFA into TAG at the expense of fuelling the TCA cycle. Would the likelihood of growth arrest be dependent on availability, or not, of this exogenous resource?

We originally proposed that synthesized TAG is transported to the cell envelope and that, when this became saturated, excess TAG would accumulate as cytoplasmic LBs (Barer and Garton 2010). Recently, Martinot and colleagues characterized two Mtb proteins, lipoprotein LprG (Rv1411c) and Rv1410c, which function in the export of TAG from the cytoplasm and which have a role in regulating intracellular TAG levels (Martinot et al. 2016). Mutation of the LprG-Rv1410c locus in Mtb was shown to result in TAG accumulation, and over-expression led to excess TAG identified in the culture medium. However, the Mtb mutant did not show a growth defect in standard culture medium, even though it is attenuated for growth during murine infection. Therefore, intracellular accumulation of TAG alone is not a sole requirement for growth arrest in vitro. Growth attenuation *in vitro* was observed in conditions which mimic infection, i.e., when cholesterol was used as a sole carbon

source for culture. Furthermore, in these conditions, inhibiting TAG lipolysis through addition of THL enhanced this growth defect. Conversely, supplementation of the cultures with acetate partially relieved the growth arrest of the mutant. These findings lead Martinot and colleagues to propose a model in which during growth, TAG is either incorporated into the cell envelope by the action of Rv1410c and LprG or alternatively hydrolyzed to release LCFA for β-oxidation and anaplerosis of the TCA cycle. Loss of Rv1410c-LprG function resulted in no TAG transport to the cell envelope and accumulation of TAG, which they propose inhibits growth by a currently unknown mechanism.

3.3 Accumulation of TAG Is Not the Sole Factor Resulting in Growth Restriction

There is further evidence that TAG accumulation alone is not the sole contributing factor to growth arrest. TAG accumulation resulting from *tgs1* or *Rv3371* expression in *M. smegmatis*, *M. bovis* BCG, or Mtb is not sufficient to restrict growth (unpublished results). Beijing strains of Mtb accumulate TAG during growth (Reed et al. 2007), and we have confirmed that in contrast with H37Rv, Beijing strains contain high levels of cytoplasmic LBs during growth (unpublished results). The accumulation of TAG by Beijing strains is thought to be a consequence of having *tgs1* constitutively upregulated; it has now been reported that the constitutive expression of the DosR regulon in Beijing strains is as a result of a SNP in the DosR promoter region (within *Rv3134c*), present in all Beijing strains (Domenech et al. 2017). Furthermore, we are now obtaining preliminary results which indicate that recent Mtb clinical isolates contain variable *tgs1* expression and TAG LB content during growth, leading us to believe that H37Rv may be exceptional in showing very low levels (unpublished results). Our observations are supported by a recent report in which LBs were identified in electron micrographs of a small number of Mtb sputum isolates, and not in Mtb H37Rv, growing in liquid culture (Vijay et al. 2017). Therefore, the differing LB content between growth and stasis of some strains does not necessarily reflect the extremes of TAG LB content shown in the images in Fig. 3.

Taken together recent findings indicate that the balance between TAG synthesis, export to the cell envelope, accumulation as cytoplasmic LBs, and turnover by lipases is complex, and the balance between the different fates must be finely tuned in different conditions. If the rate of TAG synthesis does not impact acetyl-CoA flux through the TCA cycle, growth could continue. The tipping point which results in sufficient redirection of acetyl-CoA from the TCA cycle to TAG synthesis to impact growth is not known. Alternatively, factors in addition to TAG accumulation resulting from *tgs1* or *Rv3371* expression must be responsible for growth restriction in conditions which result in Mtb non-replicating persistence. Whether strains which express *tgs*1 during growth respond to growth-limiting stresses to the same extent and via similar mechanisms to those proposed for H37Rv remains unexplored. The importance of investigating these mechanisms in culture

conditions which reflect those during infection and examining strains other than the laboratory-adapted H37Rv must be highlighted.

4 The Significance of LB Accumulation in the Pathogenesis of TB

4.1 Establishment of Infection

Mtb is transmitted via aerosol droplets which are inhaled and engulfed within alveolar macrophages of the lung. It is not known if Mtb bacilli containing LBs have a modified (TAG-laden) envelope which provides an advantage for macrophage uptake. Once within the macrophage, the bacilli resist macrophage bactericidal mechanisms and replicate. The macrophage environment is relatively nutrient limiting, and assimilation of LBs may provide Mtb with an early growth advantage. The potential role of LBs in early infection is a current focus of our research.

4.2 Persistence During Latent Infection

Despite initial bacillary replication, very few infected (~1 in 10) individuals go onto develop active TB. Recruitment of immune cells to the site of infection leads to the production of a granuloma, containing infected macrophages (Russell 2007). Conditions within the granuloma (low O_2, low pH) are not favorable for Mtb growth, and the pathogen is believed to adopt a state of non-replicating persistence (via induction of *dosR*), with low metabolic activity, a switch to anaerobic respiration and accumulation of LBs.

Lipid-laden foamy macrophages are a characteristic feature of the Mtb granuloma (Russell et al. 2009). Infection of macrophages with Mtb leads to remodelling of macrophage lipid metabolism (Lovewell et al. 2016). Transport of oxidized low-density lipoprotein in to the macrophages, and a downregulation of cholesterol efflux results in accumulation of macrophage LBs (hereafter referred to as LDs) and acquisition of a foamy macrophage phenotype. The lipids within the macrophage LDs can be accessed by the Mtb bacilli. In an *in vitro* human PBMC granuloma model in which foamy macrophages are induced following infection with Mtb, phagosomes containing bacilli were observed in close apposition with LDs (Peyron et al. 2008), and ultimately the bacilli were found within LDs. These foamy macrophages were not permissive for Mtb growth, and within the LD the bacilli were found to express *tgs1* and accumulate LBs. Using a model in which foamy macrophages were induced by hypoxia, Daniel and colleagues demonstrated using both radioisotopic labeling and fluorescent LCFA derivatives, the uptake of host LCFA by intracellular Mtb and direct incorporation of this into Mtb TAG (Daniel et al. 2011). The bacilli, which did not grow within these hypoxic foamy macrophages, were also observed to contain LBs.

In addition to a role in bringing about growth arrest, LBs are thought to sustain Mtb throughout dormancy. Armstrong and colleagues reported PspA affects Mtb survival in non-replicating persistence (Armstrong et al. 2016). Both a Δ*pspA* Mtb mutant and an over-producing complemented strain showed reduced survival in a rapid anaerobic dormancy model, suggesting that tightly controlled concentrations of this protein are required. Interestingly, this phenomenon was specific to hypoxia-induced non-replicating persistence and was not reproduced when the same strains were examined for survival in phosphate-buffered saline. Perhaps this reflects requirement for interaction with different lipases.

4.3 Reactivation of Progressive Infection and Active Disease

Individuals with latent Mtb infection have a 10% lifetime chance of reactivation and development of active disease. The resource within TAG LBs may support bacillary regrowth in a newly permissive environment, for example, on aeration of a cavitating lesion. *In vitro* experiments with BCG adapted to non-replicating persistence following hypoxia have revealed a requirement for lipase activity and LB turnover for regrowth on reaeration (Low et al. 2009). An Mtb Δ*lipY* mutant fails to exit dormancy in an *in vitro* granuloma model (Kapoor et al. 2013).

With replication of Mtb within the open lesions, individuals with active disease become infectious and expectorate bacilli in sputum. We reported the presence of LB-positive acid-fast bacilli in TB patient sputa, at variable levels between patients (Garton et al. 2008), an observation supported by recent electron microscopy studies (Vijay et al. 2017). Strikingly, counter to the widely held belief that expectorated bacilli are replicating rapidly, findings of our Mtb sputum transcriptome study suggested quite the opposite (Garton et al. 2008). This transcriptome showed many signatures of slow or non-growth. Large clusters of down-regulated genes showed significant overlap with transcriptional signatures of Mtb in conditions in which the bacilli do not replicate, including during anaerobic non-replicating persistence (Muttucumaru et al. 2004; Voskuil et al. 2004; Garton et al. 2008), non-replicating persistence arising from nutrient deprivation (Betts et al. 2002) and during chronic murine infection (Shi et al. 2005). Groups of genes associated with ribosomal function, ATP synthesis, and aerobic respiration were also down-regulated. The most highly expressed Mtb regulon in sputum was that induced by DosR. These findings led us to conclude that populations of non-replicating persister-like cells are present in sputum, a view consistent with the presence of LB-positive Mtb. The positive correlation between Mtb LB content and "time to positivity" in primary liquid culture of decontaminated sputum samples supported this view (Garton et al. 2008).

Bacterial replication is required for continuous population of sputum; therefore, environmental signals which result in this "persister" phenotype must be experienced by the bacilli as they exit the lung. The sputum environment would not be expected to be hypoxic and induction of the DosR regulon may result from exposure

to NO in the airway. We have preliminary evidence that sputum Mtb LB content correlates with patient-expired NO (unpublished results).

Subsequent studies of the Mtb sputum transcriptome by others have supported our findings and provide further insight into how the environment of the host shapes the Mtb sputum transcriptome (Garcia et al. 2016; Honeyborne et al. 2016; Walter et al. 2016; Sharma et al. 2017). In addition to *tgs1*, the expression of TGS genes, *Rv1425*, *Rv1760*, *Rv3087*, and *Rv3371*, has been identified in Mtb in sputa of untreated patients (Honeyborne et al. 2016). This indicates that LB content of Mtb in sputum may not reflect activity of Tgs1 alone. Garcia and colleagues found that Mtb transcriptional profiles in sputum resembled those in cognate bronchoalveolar lavage samples, with the exception that genes of the DosR regulon showed slightly higher expression levels in lavages (Garcia et al. 2016). An assessment of Mtb transcription in sputum taken from HIV-negative and HIV-positive individuals with active TB revealed that Mtb adapts to the immune status of the host, with lower DosR regulon expression in samples from HIV-positive individuals (Walter et al. 2016). Concomitant host gene expression analysis led the authors to suggest this was a result of alternative pathway activation of macrophages, leading to lower NO production and poorer granuloma formation. A recently published study reporting on differential expression in sputum of Mtb lineage 4 (Euro-American lineage) and Mtb lineage 6 (*Mycobacterium africanum* (Maf) – the cause of ~40% of all TB cases in West Africa) noted that compared with Mtb lineage 4, the Maf lineage 6 shows significantly reduced expression of the DosR regulon (Ofori-Anyinam et al. 2017). Interestingly, preliminary results reveal a higher proportion of LB-positive bacilli of Maf in sputum compared with Mtb (Tientcheu et al. 2016), the converse of what might be expected as a consequence of reduced expression of *dosR* and may reflect activity of additional TGS enzymes.

Expectoration of Mtb is critical for the propagation of infection. The presence of LBs in Mtb in sputum will reflect the bacillary response to the lung environment immediately prior to, or during exit and may reflect adaptation for onward transmission.

5 The Clinical Significance of Mtb LBs

5.1 Implications for the Treatment of TB

At 6 months, chemotherapy for pulmonary TB is prolonged and patients show varying responses. In the majority, the burden of bacilli in sputum, as revealed by smear microscopy and culture, rapidly decreases in the first few days of treatment. The clinical trials underpinning the current standard regimen in the 1970s all showed that while most (~85%) were cured after 3–4 months, 6 months was required to bring the relapse rate below 5%. Despite multiple studies, as yet, no clinical assessments have enabled accurate identification of either those cured, or those requiring more time, and multiple trials to reduce treatment time have yet to

identify a shorter regimen that can be given with confidence. Some patients, although infected with strains demonstrated to be drug sensitive *in vitro*, can still produce positive specimens after many months of treatment. Positive cultures after a 2-month therapy are associated with greater risk of treatment failure and relapse. Shortening the duration of treatment regimens and early identification of patients with high risk of treatment failure or relapse, are major goals of clinical research.

5.1.1 Antibiotic Tolerance Is a Feature of Dormant or Slow-Growing Mtb

In 1979, Mitchison proposed that it is physiological heterogeneity of Mtb bacilli in the tissues that results in persistence of bacilli in the face of chemotherapy (Mitchison 1979). Rapidly replicating bacilli are inactivated very quickly with antimicrobials, resulting in the initial rapid decline in bacilli detected by culture from patient samples. However, other bacilli persist, seemingly resistant to the action of the therapeutics. Mitchison proposed these were non-growing bacilli or those with low metabolic activity. Bacterial populations which show "phenotypic antibiotic tolerance" are characterized by having low metabolic activity, bacteriostasis, or slow growth (Kussell et al. 2005; Lewis 2007); in permissive growth conditions, such bacilli are fully drug sensitive.

The characteristic of "phenotypic antibiotic tolerance" has been observed in Mtb in conditions which lead to growth arrest and in many *in vitro* models of persistence (Wayne and Hayes 1996; Garton et al. 2008; Deb et al. 2009; Daniel et al. 2011; Baek et al. 2011; Kapoor et al. 2013). A common feature of these conditions is *dosR* induction, *tgs1* expression, and LB accumulation. Mtb Δ*tgs1* mutant strains do not develop the antibiotic tolerance observed in the wild type. Furthermore, expression of MPER1 which associates with Mtb LBs is also required for development of antibiotic tolerance (Daniel et al. 2016), suggesting organization of the TAG into LB is required. Although TAG synthesis can bring about growth arrest, it is not known if LBs have a mechanistic role in antibiotic tolerance, or are solely a biomarker for cells with altered physiology. Hammond and colleagues utilized the differential buoyancy of LB-positive mycobacteria (which they refer to as lipid-rich) to physically separate these from lipid-poor bacilli at various points during culture (Hammond et al. 2015). Regardless of the age of the culture from which they were recovered, the subpopulation of "lipid-rich" cells showed greater tolerance of antibiotic action than lipid-poor cells.

The demonstration of differentially culturable (DC or resuscitation promoting factor-dependent Mtb) in sputum samples by Mukamolova and colleagues adds to the body of evidence consistent with a high proportion of bacilli therein being in a slow or non-replicating state (Mukamolova et al. 2010). More recently, multiple populations in sputum samples, including those in a DC state, were shown to be antibiotic tolerant and to lose this phenotype on subculture (Turapov et al. 2016). Work to determine the relationship between DC bacilli and LB positivity is in progress.

5.1.2 The Frequency of LB-Positive Mtb in Sputum Increases During Antibiotic Therapy and Has Relationship with Treatment Success

Mtb in sputum are a sample of those which must be eliminated by chemotherapy. Recognition that LB-positive persister-like bacilli are tolerant to the action of antibiotics has profound implications for the treatment of TB. Two studies now support the clinical significance of Mtb LB content in sputum (Kayigire et al. 2015; Sloan et al. 2015). Kayigire and colleagues determined Mtb LB content in serial sputum samples taken from TB patients in an early bactericidal activity clinical trial of a new compound SQ-109, assessed alone or in combination with rifampicin (RIF). They reported proportions of LB-containing Mtb bacilli patients' sputa increased with antibiotic treatment over the 14 days of assessment (Kayigire et al. 2015). This increase in LB-positive bacilli was more prominent in samples from patients on regimens which contained RIF, reflecting the greater rate of decline in CFU count in those samples than from patients treated with SQ-109 alone. Monitoring sputum Mtb LB content may prove a valuable additional biomarker for assessment of novel antituberculous regimens developed with the aim of shortening treatment to less than 6 months.

Sloan and colleagues also assessed patient sputum Mtb LB content with treatment investigating the relationship between the LB content of patients' sputa with treatment response (Sloan et al. 2015). High Mtb LB positivity in sputum taken after 3–4 weeks of treatment was found to correlate with poor treatment outcome (treatment failure or patient relapse), supporting the hypothesis that such bacilli are antibiotic tolerant persisters. Notably, this correlation was not apparent in sputum samples taken at baseline before the initiation therapy, suggesting that it is LB-positive persisters revealed by therapy that are predictive of treatment response and not the total LB-positive Mtb subpopulation in untreated patients' sputa. This study demonstrates the potential value of monitoring LB-positive Mtb populations in patients' sputa to inform management of patient treatment by providing an early indicator of those patients at risk of treatment failure.

5.2 LB-Positive Mtb and Patient Infectiousness

Transmission between hosts is critical for Mtb; that more than a quarter of humanity are believed to be infected is testament to its success in this regard. The bacilli are under strong selection pressure to maintain and display properties which facilitate transmission at every stage, from facilitating their exit from an infected host, through survival in transit, to establishing infection within a new susceptible host. TB patient infectiousness is currently based on sputum smear acid-fastness, informing infection control and screening of contacts. We proposed that LB-positive Mtb in sputum may reflect a population adapted for onward transmission (Barer and Garton 2010). Slowly replicating or non-replicating bacilli with low metabolic activity are more tolerant to stresses (Kolter et al. 1993; Smeulders et al. 1999). Furthermore, within TAG, LB-positive bacilli have a readily mobilized source of energy and lipid precursors with which to support growth in a new host. Supporting this hypothesis is

a report that Mtb bacilli grown in reduced oxygen conditions, which we have shown to possess LBs (unpublished data), were tenfold more infectious for guinea pigs than aerobically grown controls (Bacon et al. 2004).

A relationship between Mtb of low metabolic activity in sputum and TB patient infectiousness has been reported (Datta et al. 2017). Quantitation of metabolically active Mtb bacilli in sputum was made following staining with fluorescein diacetate (FDA) which requires enzymatic hydrolysis within the bacillus to become fluorescent. FDA-negative Mtb in sputum was associated with greater transmission to household contacts. This supports the view that it is bacilli with low metabolic activity that are adapted for onward transmission. One study examining factors predictive of transmission risk including assessment of index case sputum Mtb LB positivity has been undertaken (Hector et al. 2017). Therein, univariate analysis showed that lower LB% was associated with greater probability of contact tuberculin skin test positivity. However, this was not the case when multivariate analysis was undertaken. Therefore, more extensive studies are required to test the significance of sputum Mtb LB-positivity as a measure of patient infectiousness.

It is important to note, however, that sputum is not the vehicle of transmission; Mtb is transmitted in aerosol droplets. We have preliminary TB patient transcriptional data which provides indication that aerosolized Mtb bacilli are a distinct population to those in sputa (unpublished results). To date, little is known of what promotes Mtb aerosolization. The high cell envelope lipid content and consequent cell surface hydrophobicity of mycobacteria has been linked to the efficacy with which mycobacteria aerosolize from aqueous suspensions (Falkinham 2003). Minnikin has proposed that changes in cell envelope lipid content and consequent increased cell surface hydrophobicity led to the evolution of Mtb strains that were able to enter and thereby via transmit via aerosol (Minnikin et al. 2015). LB-positive Mtb cells, which may have enhanced TAG envelope content and consequently enhanced surface hydrophobicity, may have a greater propensity for aerosolization. Furthermore, enhanced TAG LB content decreases the buoyant density of Mtb (Deb et al. 2009) and could contribute to a concentration of such bacilli at aqueous-air interfaces, i.e., at the surface of respiratory secretions in the alveolus, further increasing the likelihood for aerosolization. These proposals warrant further investigation.

6 Research Needs

Our initial finding that TAG LBs are particularly prominent in sputum samples provides major incentive for both basic and clinical research. Knowledge of the significance of TAG LBs has increased greatly in the last decade. Progress has been made toward understanding the Mtb pathways involved in the synthesis and regulation of neutral lipid accumulation and downstream utilization. Many protein players in these processes have been identified, but roles for all are not understood. Questions such as the functional significance of the 15 different Mtb TGS remain, particularly for those, which in addition to Tgs1, have transcripts identified in

sputum. Neither do we appreciate the relationship between cell envelope composition and LB accumulation. Does LB accumulation reflect saturation of the cell envelope with TAG? There are obvious technical challenges which need to be overcome in order to perform specific and sensitive analyses of Mtb neutral lipid content *ex vivo* and in clinical samples, including patient aerosol samples.

We are now beginning to recognize that unlike Mtb H37Rv, some Mtb isolates exhibit significant LB content during aerobic growth. This fuels the need to understand the role of other TGS enzymes and how expression of *tgs1* and others relates to growth deceleration in different strain backgrounds. Do strains with a high level of TGS activity and LBs during replication have the same capacity to respond to growth arresting stimuli and how does this impact development of antibiotic tolerance? Perhaps the composition of LBs which are present during bacterial replication differs from those which accumulate with growth deceleration; this may reflect the involvement of different TGS enzymes. Different proteins may stabilize and control access of lipases to the lipid reserve in different conditions. LBs which accumulate during growth may be turned over more rapidly than those which accumulate as a result of environmental changes which restrict growth. Different lipases may be involved in their degradation. Furthermore, how lipases are recruited to LBs to initiate TAG turnover remains to be investigated.

There is now support for the clinical significance of Mtb LBs and the value of monitoring these to inform management of patient treatment or assessment of new and potentially treatment-shortening regimens. Further clinical studies are required to validate the role of LBs as a useful biomarker for these assessments. These will be facilitated by developments for improved sputum LB analysis which is currently technically demanding or discovery of alternative biomarkers of the sputum LB population.

We are keen to understand the stimulus, or stimuli, responsible for inducing LBs in Mtb in sputum. This, in combination with transcriptional data, will allow us to develop *in vitro* conditions inducing Mtb cells into a comparable state. Mtb LB content, growth state, and antibiotic tolerance are anticipated to reflect variable characteristics of the both bacilli and host. Furthermore, we must understand the nature of Mtb in aerosol and how this relates to populations we measure in sputum. Being able to influence and study *in vitro* aspects of host-Mtb interplay and factors impacting aerosolization and aerosol survival, will provide new opportunities to explore the persister problem in treatment of TB and also transmission potential, which need to be understood to control dissemination.

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Lipids and *Legionella* Virulence

4

Otto Geiger

Contents

Abstract

The intracellular lung pathogen *Legionella pneumophila* has evolved virulence mechanisms, which allow it to replicate in its natural host protozoa as well as in human macrophages. *L. pneumophila* belongs to the γ-proteobacteria and has several lipid components in its membranes, which are unusual for this bacterial group. Membrane phospholipids are substituted with branched-chain fatty acyl residues, and phosphatidylcholine is a major phospholipid in *Legionella*. *Legionella* phosphatidylcholine is an important virulence determinant and acts through multiple mechanisms. Lipopolysaccharides from *L. pneumophila* show several unusual features, among them the substitution with very long-chain fatty acyl residues. Lipid-containing outer membrane vesicles are important vehicles for the delivery of bacterial effector proteins, and quorum sensing via α-hydroxy ketone signaling molecules is important for virulence. Besides contributing to

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

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virulence with its own lipids, *L. pneumophila* also interferes with the metabolism of host cell membranes and specifically redirects phosphoinositide-controlled signaling pathways, thereby facilitating its replication and spread within the host.

1 Introduction

Legionella pneumophila is a Gram-negative facultative intracellular pathogen, which multiplies in protozoa in its natural environment. The virulence mechanisms evolved by *L. pneumophila* also enable its attachment to as well as survival and replication inside human macrophages, and infection of alveolar macrophages can cause Legionnaires' disease, a severe form of pneumonia in man. In each of the different infection stages of host cells, virulence proteins need to be delivered to their specific place of action, and this is achieved by several specialized secretion machineries (de Buck et al. 2007). Most importantly, a type IV secretion system, which translocates more than 300 bacterial effectors into the host cell, is indispensable for *L. pneumophila*'s intracellular survival (Isaac and Isberg 2014). During this replicative phase, the effectors modulate multiple host cell processes, redirect trafficking of the *L. pneumophila* phagosome, and mediate its conversion into an endoplasmic reticulum-derived organelle, the so-called *Legionella*-containing vacuole that permits intracellular bacterial replication (Shin and Roy 2008). When nutrients are depleted, the bacteria enter the transmissive phase and express virulence proteins, resulting in lysis of host cells and the initiation of a new infection round (Steinert et al. 2007). Characteristically *Legionella* behavior and physiology alternate between a replicative phase and a transmissive phase, and therefore *Legionella* is said to possess a biphasic life cycle (Oliva et al. 2018). To date most of the focus has been on how *Legionella* proteins contribute to bacterial virulence. However, specific *Legionella* lipids are also important determinants for virulence.

2 Membrane-Forming Lipids in *Legionella pneumophila*

In the bacterial model organism, *Escherichia coli*, only the three major membrane lipids phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin occur (Rock 2008). In *Legionella pneumophila*, phosphatidylcholine constitutes another major membrane lipid (Martínez-Morales et al. 2003). The major fatty acyl residues decorating *E. coli* phospholipids are palmitic, stearic, *cis*-vaccenic, and the cyclopropane-containing lactobacillic acid (Rock 2008). In contrast, *Legionella* species contain large amounts (40–90%) of branched-chain fatty acids with a methyl branch mainly at the iso- or anteiso-carbon (Lambert and Moss 1989). Although the biosynthesis of branched-chain fatty acids has been studied in Gram-positive bacteria (see López-Lara and Geiger 2010), their formation in *L. pneumophila* and the relevance of their occurrence in this organism remain unclear. Like other Gram-negative bacteria, *L. pneumophila* has the lipid A-containing lipopolysaccharide in the outer monolayer

of its outer membrane, and remarkably *Legionella* lipid A is modified with very long-chain fatty acyl residues also observed in other, only distantly related, bacteria causing chronic intracellular infections.

2.1 *Legionella* Phosphatidylcholine Contributes to Virulence

A recent estimate suggests that only about 15% of the bacteria contain phosphatidylcholine (PC; lecithin) as a membrane lipid (Geiger et al. 2013). Many of the PC-containing bacteria are known to interact with *Eukarya* establishing symbiotic or pathogenic relationships. Reduction of PC levels in *Bradyrhizobium japonicum* (Minder et al. 2001) or complete absence of PC in *Sinorhizobium meliloti* (Sohlenkamp et al. 2003) strongly affects their symbiotic performance on their respective legume hosts. A PC-deficient mutant of *Agrobacterium tumefaciens* cannot form its type IV secretion system essential to transform the plant host, and therefore no crown gall tumors are induced by such *A. tumefaciens* mutants (Wessel et al. 2006). Clearly, *A. tumefaciens* requires PC for its virulence. Also, *Brucella* mutants unable to synthesize PC have lowered virulence in a mouse model and less efficient replication vacuole formation (Comerci et al. 2006; Conde-Alvarez et al. 2006).

L. pneumophila can form PC via either of two pathways, the phospholipid *N*-methyltransferase (PmtA) or the phosphatidylcholine synthase (Pcs) pathway (Martínez-Morales et al. 2003; Sohlenkamp et al. 2003; Geiger et al. 2013). In *Legionella*, the Pcs pathway is the main route for PC formation (Conover et al. 2008). Notably, the *pcs* gene from *L. pneumophila* has turned out to be a valuable tool for production of PC in *E. coli* (Bogdanov et al. 2010). Double mutants of *L. pneumophila*, deficient in both pathways, are unable to form any PC and are affected in at least three different ways (Conover et al. 2008). First, there is a reduced binding of PC-deficient *Legionella* to macrophages probably due to the loss of PC or a PC derivative on the bacterium that is recognized by the host cell. Initial binding of *Legionella* to macrophages seems to occur via several different receptors; however, the major binding is via the platelet-activating factor receptor (PAF receptor). Usually the PAF receptor detects the platelet-activating factor (PAF) and modulates the immune response of the macrophage receiving the PAF signal (Dubois et al. 1989; Hanahan 1986). PAF harbors the same glycerophosphocholine head group as PC (Fig. 1), but in contrast to PC, PAF is soluble in an aqueous environment. *Legionella* PC is required for efficient binding of *Legionella* to macrophages via the PAF receptor, and therefore PC or a glycerophosphocholine-harboring derivative in *Legionella* might mimic PAF and cause bacterial adhesion to the PAF receptor. Second, a PC-deficient *Legionella* mutant has only a poorly functioning type IVB secretion system (Dot/Icm apparatus) and is severely impaired in delivering virulence protein substrates which are required for bacterial intracellular growth into the cytosol of infected cells. Third, strains lacking PC show lowered cytotoxicity, are non-motile, and have low levels of flagellin protein (Conover et al. 2008).

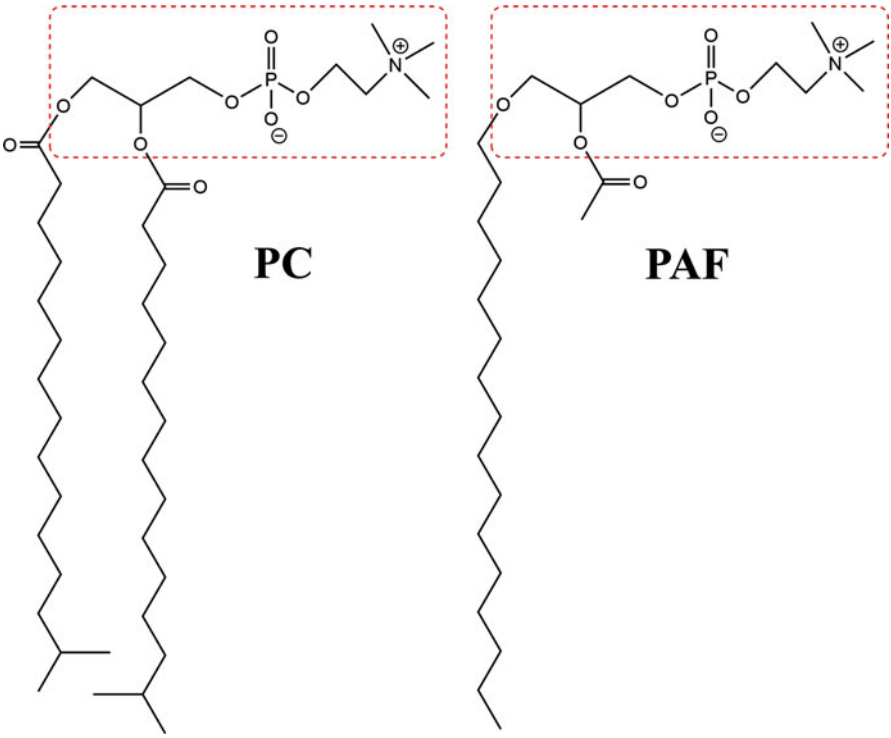

Fig. 1 Molecular mimicry: *Legionella* phosphatidylcholine (PC) might imitate the platelet-activating factor (PAF) when adhering via the PAF receptor to macrophages as both molecules have a glycerophosphocholine head group (circled by interrupted lines)

2.2 Lipopolysaccharide of *Legionella*

Gram-negative bacteria usually have the lipid A-containing lipopolysaccharide (LPS) in the outer monolayer of their outer membrane, and lipid A modification systems have been reviewed (Henderson et al. 2016). LPS of *L. pneumophila* exhibits peculiar chemical features, which may account for its importance as a bacterial virulence factor (Zähringer et al. 1995). The O-chain of this LPS is a homopolymer of the unusual sugar legionaminic acid (Fig. 2a) rendering the *Legionella* surface highly hydrophobic. Legionaminic acid is also found in cell surface glycoconjugates of other bacterial pathogens, such as *Campylobacter jejuni* or *Acinetobacter baumannii* and is thought to be a bacterial sialic acid analog that might interfere with important host cell processes, i.e., cell–cell interactions and the immune response (Hassan et al. 2016). Also, the lipid A part possesses some unique structural features since its backbone is formed by the unusual bisphosphorylated 2,3-diamino-2,3-dideoxy-D-glucosyl-(β1-6)-2,3-diamino-2,3-dideoxy-D-glucose disaccharide (Fig. 2b) with only amide-linked acyl groups (Zähringer et al. 1995). Remarkably, *L. pneumophila* is also unique in harboring more than one copy of certain

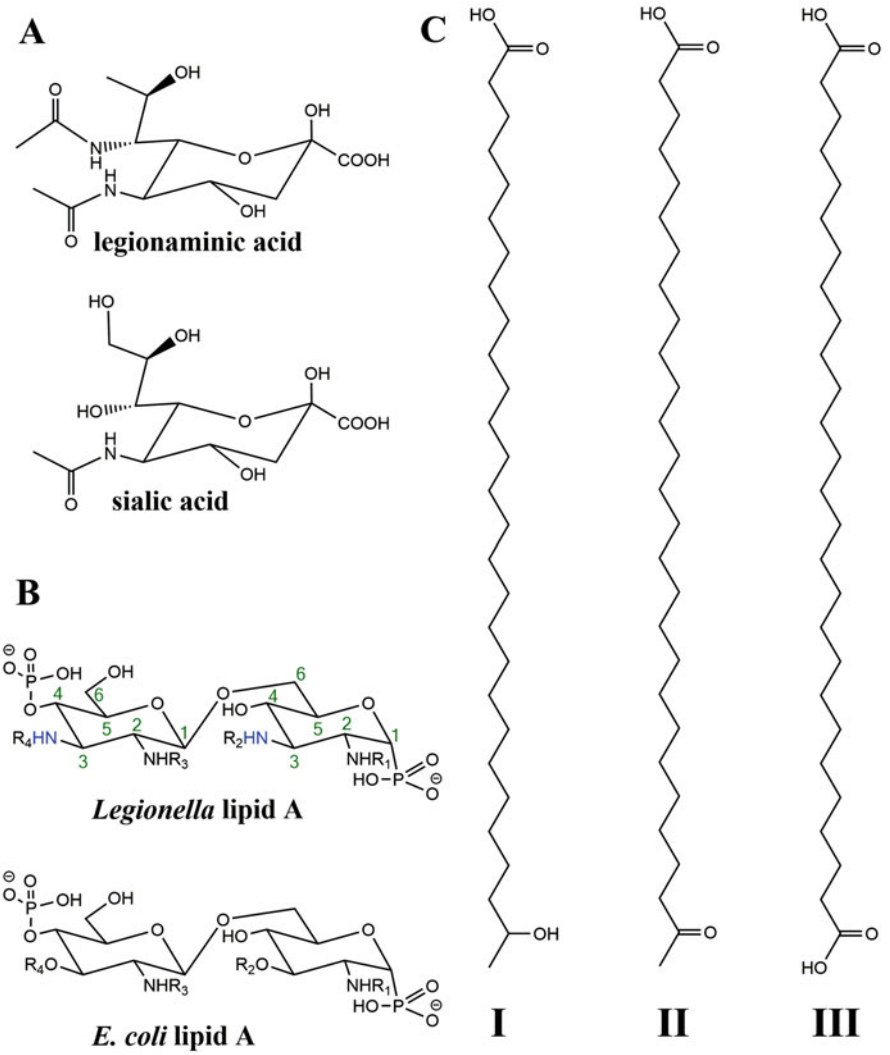

Fig. 2 Special structural features of *Legionella* LPS. (**a**) O-chain component legionaminic acid is a structural analog of sialic acid. (**b**) Monosaccharide residues of the *Legionella* lipid A disaccharide have acylated amino groups in the three positions instead of acylated hydroxyl groups (case of *E. coli*). (**c**) Very long-chain fatty acids decorating lipid A of α-2-proteobacteria (I) or of *Legionella pneumophila* (II, III). Shown are 27-hydroxyoctacosanoic acids, 28:0(27-hydroxy) (I), 28:0 (27-oxo) (II), and 27:0-dioic acid (III)

structural genes required early in lipid A backbone biosynthesis. Its genome contains two paralogues for *lpxA*, two for *lpxB*, and three for *lpxD* (Albers et al. 2007). Differential expression of these paralogues under different growth conditions might cause the formation of chemically distinct lipid A backbones in *L. pneumophila*.

Many proteobacteria of the α-2 group, such as the endosymbiotic rhizobia or facultative intracellular pathogens such as *Brucella* or *Bartonella*, cause chronic infections in their eukaryotic hosts and display the decoration of bacterial lipid A with the very long-chain 27-hydroxyoctacosanoic acid (Fig. 2c). At least for rhizobia, lipid A decorated with this very long-chain fatty acid is important for growth, stress adaptation, and symbiotic performance (Bourassa et al. 2017; Busset et al. 2017), and it might be critical for persistence and function for all these intracellular bacteria within the host cell. Surprisingly, also the lipid A of the γ-proteobacterium *L. pneumophila* is decorated with such very long-chain fatty acids, and clearly, *L. pneumophila* possesses at least five of the genes that are specifically required for the synthesis of very long-chain ω-1-hydroxy fatty acids and their transfer to lipid A (see Fig. 6 in Vedam et al. 2006). However, the very long-chain fatty acids in *Legionella* lipid A are 28:0(27-oxo) and 27:0-dioic acid (Zähringer et al. 1995) (Fig. 2c), and at this point it is not clear how these variations are formed and whether they are functionally important.

3 Secretion and Outer Membrane Vesicles

Secretion of virulence effector proteins is achieved in *L. pneumophila* mainly by the type II Legionella-secretion pathway (Lsp) and by the type IVB Dot/Icm secretion system (De Buck et al. 2007). For example, many of the 19 predicted *Legionella* phospholipases/lysophospholipases are secreted by the Lsp or the Dot/Icm system, act on host membrane phospholipids, and constitute important bacterial pathogenicity factors (Hiller et al. 2018). In addition, *L. pneumophila*-released outer membrane vesicles (OMVs) inhibit fusion of phagosomes with lysosomes (Fernandez-Moreira et al. 2006). Proteomic analysis shows that many OMV-specific proteins are involved in the pathogenesis of Legionnaires' disease (Galka et al. 2008). Orthologues of *Legionella* type IV secretion system components IcmF and DotU are found in a wide range of Gram-negative bacteria, and in many cases they are linked not to other type IV secretion system genes but to genes coding for IcmF-associated homologous proteins (IAHP) which form a type VI secretion system (Filloux et al. 2008). Interestingly, a recent report suggests that upon *L. pneumophila* infection of macrophages, bystander cells are activated differentially, as host cell vesicles activate mainly neighboring epithelial cells whereas bacterial OMVs activate predominantly myeloid cells (Jung et al. 2017).

4 *Legionella* Autoinducer α-Hydroxy Ketone Signaling Molecule

L. pneumophila apparently lacks an *N*-acyl-L-homoserine lactone- or a furanosyl borate diester-based signaling system. However, it harbors a gene cluster homologous to the *Vibrio cholerae cqsAS* quorum sensing system (Higgins et al. 2007), encoding an autoinducer synthase (LqsA) and a sensor kinase (LqsS), which flank a

response regulator (LqsR) (Spirig et al. 2008) (Fig. 3). *Legionella* adopts a biphasic life cycle, which facilitates the switch between extracellular and intracellular niches, and this switch is controlled by a complex regulatory network, including the *Legionella* quorum sensing (Lqs) system (Personnic et al. 2018). Expression of LqsA causes the formation of the α-hydroxy ketone signaling molecule 3-hydroxypentadecan-4-one (*Legionella* autoinducer-1; LAI-1) (Spirig et al. 2008) (Fig. 3), suggesting that it is synthesized in an analogous way as the *Vibrio cholerae* autoinducer-1 (CAI-1) 3-hydroxytridecan-4-one (Wei et al. 2011), i.e., LqsA, couples *S*-adenosylmethionine and dodecanoyl-CoA to produce 3-aminopentadec-2-en-

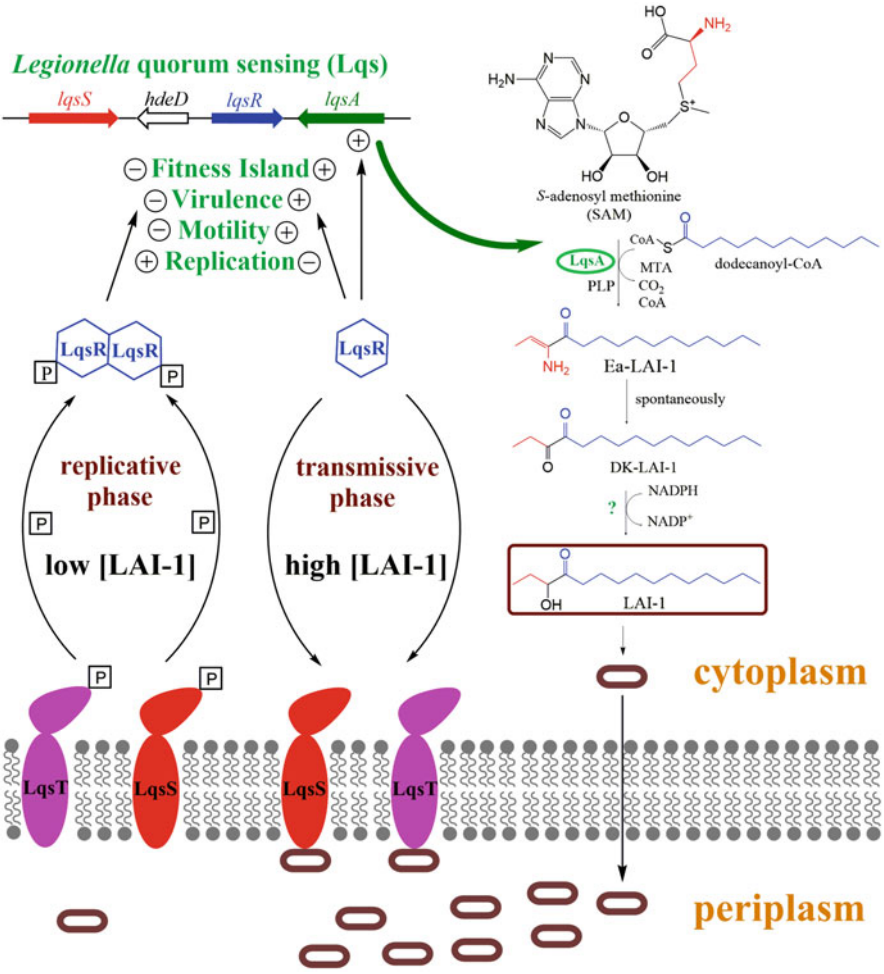

Fig. 3 Quorum sensing via the α-hydroxy ketone signaling molecule 3-hydroxypentadecan-4-one in *Legionella pneumophila* controls biphasic life cycle and virulence. 5′-Methylthioadenosine, MTA

4-one (Ea-LAI-1), which is subsequently converted to LAI-1, presumably through the intermediate pentadecan-3,4-dione (DK-LAI-1) (Fig. 3). LAI-1 is a fatty acid-derived, amphiphilic signal molecule, and in the absence or at low concentrations of the signaling molecule LAI-1, the two sensor kinases LqsS and LqsT are autophosphorylated at a conserved histidine residue (Fig. 3). In turn they transfer the phosphoryl moiety to the response regulator LqsR, which dimerizes upon phosphorylation at its conserved receiver domain aspartate. The phosphorylated dimeric LqsR represses the transcription of numerous operons required for an intracellular lifestyle (virulence, mobility, fitness, etc.) and induces replication. When LAI-1 concentration passes a certain threshold during bacterial growth, it is detected by the sensor kinases LqsS and LqsT; autophosphorylation of either one is inhibited in a dose-dependent manner (Schell et al. 2016), causing subsequently the desphosphorylation of LqsR, dissociation of LqsR dimers, and in consequence elimination of operon repression by LqsR (Fig. 3). Thereby this Lqs quorum sensing system is switched on at the end of the replicative growth phase, and bacterial virulence, motility, and fitness are induced (Personnic et al. 2018). The LqsS and LqsT sensor kinases function partially antagonistic, as most genes that are upregulated in an *lqsS*-deficient mutant are downregulated in the absence of *lqsR*. LqsR is also controlled by RpoS and LetA, and it promotes pathogen-host cell interactions such as phagocytosis, formation of the *Legionella*-containing vacuole, and intracellular replication while inhibiting the entry of *L. pneumophila* into the replicative growth phase. The α-hydroxy ketone-induced quorum sensing seems to be important for the formation of many protein virulence factors secreted via the Icm/Dot type IV secretion system (Tiaden et al. 2008) and therefore acts synergistically to the Icm/Dot system in pathogen–host interactions.

5 Modulation of Host Phospholipid Metabolism by *L. pneumophila*

Secreted and cell-associated phospholipase A and lysophospholipase A activities are important bacterial virulence factors because they promote bacterial survival, spread, as well as host cell modification and damage (Banerji et al. 2008). Some of these enzymes detoxify cytolytic lysophopholipids whereas others show contact-dependent haemolytic activity.

Phosphatidylinositol (PI) and its phosphorylated derivatives (phosphoinositides) control key cellular processes including lipid signaling, vesicular trafficking, and actin polymerization. Intracellular bacterial pathogens, such as species of *Shigella* and *Yersinia*, *Listeria monocytogenes*, *Salmonella enterica*, *Mycobacterium tuberculosis*, and *Legionella pneumophila*, manipulate phosphoinositide metabolism in order to promote their uptake by target cells and to direct in some cases the biogenesis of their replication compartments (Pizarro-Cerda et al. 2015). These intracellular pathogens secrete effector proteins that bind to and modify host cell phosphoinositides thus modulating phagocytosis and intracellular survival of the pathogen. For example, the *Legionella* SidC effector protein, secreted via the

type IVB system, binds directly to phosphatidylinositol-4-phosphate (PI(4)P) and recruits endoplasmic reticulum vesicles to the replication-permissive vacuole (Ragaz et al. 2008) thereby redirecting the program of the host cell.

On one hand, the T4SS-secreted phosphatase SidP hydrolyzes phosphatidylinositol-3-phosphate (PI(3)P) and phosphatidylinositol-3,5-bisphosphate (PI(3,5)P2) (Fig. 4), might deplete PI(3)P levels on the bacteria-containing compartment, inhibits the recruitment of molecules that would foster endosomal fusion, and thereby contributes to bacterial evasion of the endocytic/phagocytic pathway. On the other hand, the T4SS effector SidF displays 3-phosphatase activity toward

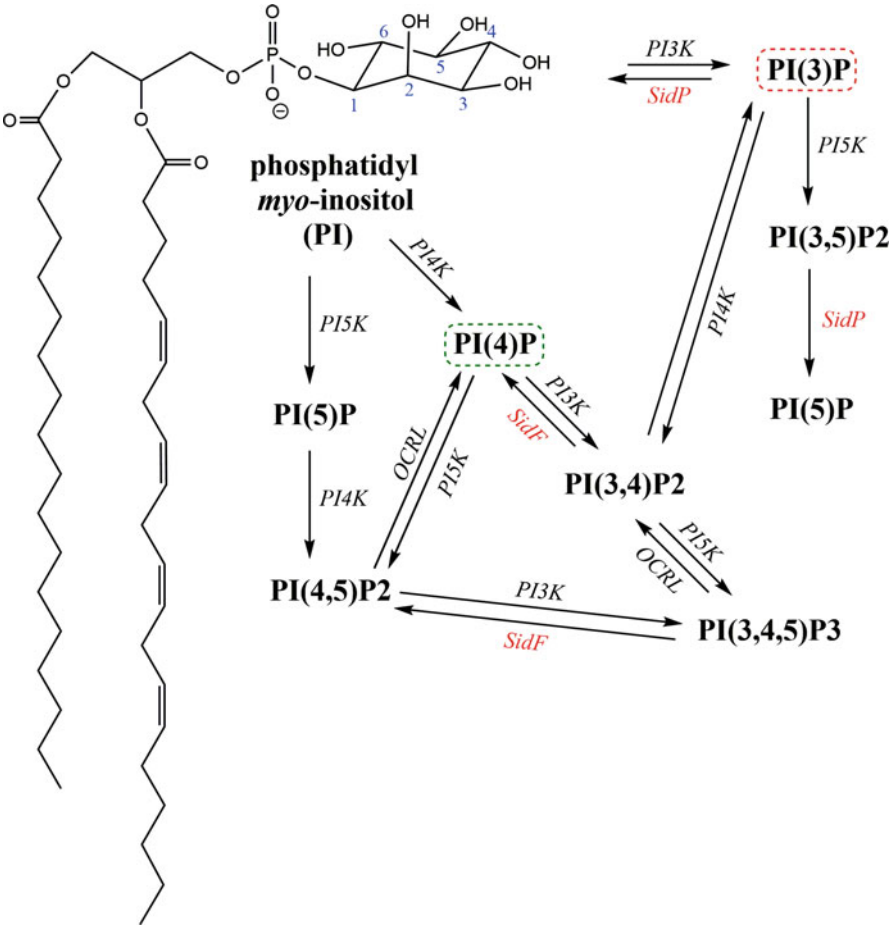

Fig. 4 Phosphoinositide metabolism is modulated by host macrophage or *Legionella pneumophila* enzymes SidF and SidP. SidF contributes to increased levels of PI(4)P, whereas SidP provokes lower levels of PI(3)P. Phosphatidylinositol-5-phosphate (PI(5)P); phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2); PI 3 kinase (*PI3K*); PI 5 kinase (*PI5K*). PI(3)P and PI(4)P circled by interrupted lines. For more details and abbreviations, see text

phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) and, together with a host cell PI 4 kinase (*PI4K*) (Fig. 4), might be responsible for an increase of PI(4)P levels on the *Legionella* vacuole, resulting in the recruitment of SdcA and SidC which promote the recruitment of endoplasmic reticulum vesicles to *Legionella* vacuoles.

Modification of host membrane lipids by *L. pneumophila* enzymes is not limited to glycerol ester-based lipids but may affect other membrane-forming lipids as well. The sphingosine-1-phosphate lyase *Lp*Spl (Lpp2128) targets host sphingolipid metabolism and restrains autophagy (Rolando et al. 2016). The lysoplasmalogenase YhhN (Lpg1991) is able to cleave the vinyl ether bonds of lysoplasmalogens and may protect the bacterium from lysis by lysoplasmalogen derived from plasmalogens of the host (Jurkowitz et al. 2015).

6 Conclusions and Research Needs

Phosphatidylcholine in *Legionella pneumophila* is important for virulence of the bacterium in multiple ways, and although it is clear that phosphatidylcholine is required for attachment to macrophages, delivery of type IV secreted effector proteins, cytotoxicity, and flagella formation, much of the molecular details and mechanisms of its action remain unresolved. The importance of branched-chain fatty acyl residues in phospholipids and of very long-chain fatty acyl residues, as well as altered lipid A and O-chain components in lipopolysaccharides of *Legionella*, is presently not clear, and mutants deficient in making these ingredients are required in order to define the roles of these lipid modifications. Although outer membrane vesicles seem to be important vehicles for the delivery of *Legionella* virulence effector proteins, most of the functional and mechanistic aspects of outer membrane vesicle formation are not understood in any bacterial system (Schwechheimer and Kuehn 2015). The class of α-hydroxy ketone quorum sensing molecule functions in *Vibrio cholerae* and *Legionella pneumophila* and more details on their formation, distribution, as well as their mode of action need to be explored.

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Bacterial Adherence to Plant and Animal Surfaces via Adhesin-Lipid Interactions

5

Claire Rossi, Hélène Cazzola, Nicola J. Holden, and Yannick Rossez

Contents

Abstract

The plasma membrane acts as one of the first lines of defense by establishing a physical barrier against microbes. Nevertheless, bacteria have developed a range of strategies to invade the host tissues efficiently. In this chapter, we focus on this understudied area and describe how bacteria target or redirect host membrane

C. Rossi · H. Cazzola · Y. Rossez (✉)
UMR7025 CNRS Enzyme and Cell Engineering Laboratory, Sorbonne Universités, Université de Technologie de Compiègne, Compiègne, France
e-mail: Claire.Rossi@utc.fr; Helene.Cazzola@utc.fr; Yannick.Rossez@utc.fr

N. J. Holden
Cellular and Molecular Sciences, James Hutton Institute, Dundee, Scotland, UK
e-mail: Nicola.Holden@hutton.ac.uk

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lipids. Domains enriched in sterols and sphingolipids, denominated membrane rafts, in particular, have been reported to be exploited by numerous bacterial pathogens. We will first describe the different strategies employed by bacteria to specifically target the PM via membrane rafts and non-raft counterpart domains; and secondly, we illustrate how development of biomimetic membranes has identified bacterial mechanisms of interaction.

1 Introduction

The cellular membrane is responsible for cell compartmentalization that coordinates the transport of molecules, cell signaling, or pathogen recognition. Most of these mechanisms are dependent on intricate interactions between proteins and lipids. The most abundant lipids are glycerolipids, sphingolipids, and sterols in animals and plants. Glycerolipids and sphingolipids are categorized into different lipid classes based on the chemical structures of their head groups and each class varies in its fatty acid content due to the degree of unsaturation, chain length, and linkage (Harayama and Riezman 2018). To add to this complexity, these molecules are not homogenously distributed across membranes, resulting in a spatial organization of the lipid landscape of the cell plasma membrane (PM). Sphingolipids have a specific physical location within plant and animal membranes. They are mainly located in the outer leaflet of the PM, whereas the glycerophospholipids like phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) occur mainly in the inner leaflet (van Meer and de Kroon 2011; Cacas et al. 2016). Pathogenic bacteria can overcome and even exploit this diversity and complex organization by directly binding interactions to different lipid classes from different cell-surface expressed appendages.

2 Bacterial Adhesion

Bacteria adhere to many surfaces for successful colonization of hosts and as a survival mechanism in the environment. Macromolecules exposed on the bacterial surface involved in adhesion are referred to as adhesins: a generic designation describing various extracellular structures, ranging from outer membrane proteins to a variety of monomeric or polymeric proteinaceous surface appendages (Kline et al. 2009). The best-studied cell appendages, exclusively found in Gram-negative bacteria, are the chaperone–ushered fimbriae (also known as pili). Adhesins typically recognize specific targets thus enabling the bacterium to bind on a specific surface, which results in tropism towards particular tissue types. Many different types of fimbriae have been described but the prototypical fimbriae are Type 1 (Ofek et al. 1977) in *Escherichia coli* and P fimbriae found in uropathogenic *E. coli* (UPEC) (Dodson et al. 2001) which mediate binding to specific receptors enriched in the bladder and upper urinary tract, respectively. Over the years, a large number of

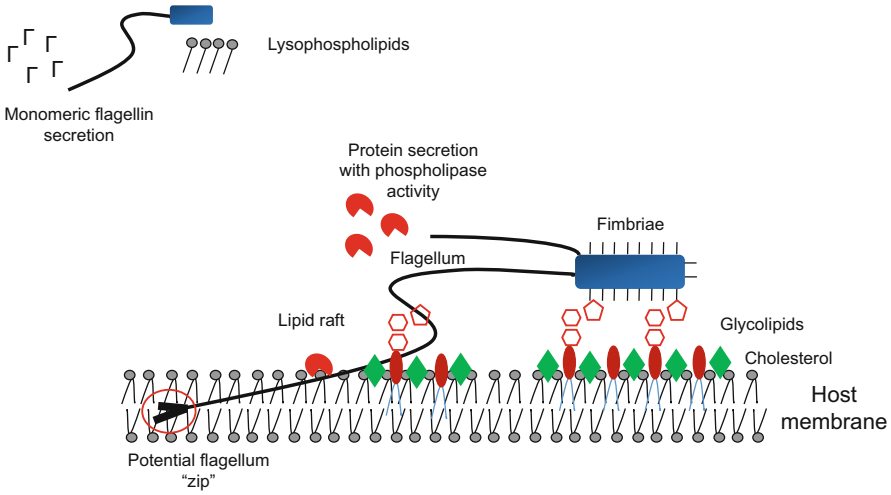

Fig. 1 Bacterial adhesion on host plasma membrane

adhesive proteins have been identified and, for some of them, their affinity for PM lipidic molecules (Fig. 1).

2.1 Fimbriae

Fimbriae are adhesive hair-like organelles expressed on the surface of bacteria, generally with an adhesin at the tip (Pizarro-Cerdá and Cossart 2006). They are exported to the bacterial surface by either a chaperone-usher set of proteins (Costa et al. 2015) or by a type IV or type V pili secretion (T4p or T5p) apparatus consisting each of several proteins (Hospenthal et al. 2017). Although bundle-forming pilus (BPF) of enteropathogenic *E. coli* (EPEC) has been shown to bind directly to PE (Khursigara et al. 2001), most fimbriae have a lectinic adhesin that binds a carbohydrate component of glycolipids. For example, the PapG adhesin of pyelonephritis-associated (P) pilus binds to α-D-galactopyranosyl-(1–4)-β-D-galactopyranoside from a type of glycosphingolipid termed globoside, enriched in upper urinary tract cells (Väisänen et al. 1981; Dodson et al. 2001). Several other bacterial species like *Streptococcus pneumoniae*, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Staphylococcus aureus* have been identified to bind specifically to this carbohydrate harbored by glycosphingolipids ganglioside, fucosylasialo-GM1, asialoGM1, and asialo-GM2 (Krivan et al. 1988). Interestingly, candidate adhesins involved in glycosphingolipid recognition have not yet been identified for these species, implicating potentially uncharacterized fimbriae gene clusters. Until recently, *P. aeruginosa* T4p was described to bind the GalNAcβ1–4Gal moiety of the asialo-GM1 and asialo-GM2 via the adhesin PilA (Lee et al. 1994). However, the interaction was shown to be context dependent since binding to glycosphingolipids only occurred

with sheared T4p organelles, and not when they are expressed from the *P. aeruginosa* bacterial surface (Emam et al. 2006). S-fimbriae found in *E. coli* adheres to galactosyl ceramide, lactosyl ceramide, and sulfated galactocerebroside (or sulfatide) via the SfaA adhesin protein (Prasadarao et al. 1993). Sulfatide can be found on different type of animal tissues and was first isolated from human brain meningeal tissue (Eckhardt 2008), hence these bacteria are often associated with meningitis (Parkkinen et al. 1988). The lipid plays a role in bacterial adhesion for a number of bacterial species like *Actinobacillus pleuropneumoniae*, *Bordetella pertussis*, *and Campylobacter jejuni* (Takahashi and Suzuki 2012). For transmembrane glycoproteins, their surrounding lipidic environment is indirectly involved for adhesion and invasion of target cells. FimH encoded by Type 1 fimbriae binds to mannosylated proteins in the urinary tract and needs lipid rafts for *E. coli* penetration of bladder epithelium (Duncan et al. 2004). In contrast, binding some FimH variants to plant tissue occurs via recognition of hemicellulosic β-(1–4) mannans or *N*-linked glycoproteins in plant cell walls (Marshall et al. 2016).

2.2 Bacterial Flagella

The flagellum is a major organelle present in the majority of bacterial species. This self-assembly protein machinery is mostly used for motility and chemotaxis (Evans et al. 2014). Flagellin, a globular protein forming the flagellum, is recognized by specialized host cell surface receptors such as Toll-like receptor 5 (TLR5) in animals or FLS2 in plants, and because of its virtual ubiquity in bacteria, it is a potent immunogen. Microbe perception occurs on flagellin binding to these receptors, triggering innate immunity activation (Rossez et al. 2015). However, the flagellum has also been identified as an adhesin. Flagella are capable of binding different surfaces including lipids, aided in reaching PM by their length (Rossez et al. 2015). Flagella from *Salmonella enterica* serovar Typhimurium have been shown to have an affinity for cholesterol from studies using cholesterol-coated surfaces. This surprising finding required verification that fimbrial adhesins, such as type 1 fimbriae, were not involved (Crawford et al. 2010). Different *E. coli* flagella serotypes have been demonstrated to bind negatively charged membrane lipids (phospholipids and sulfolipids) *in planta* (Rossez et al. 2014), where it is thought that the length and flagellar rotation help to penetrate the plant cell wall to the underlying PM. *P. aeruginosa* flagellum preferentially adheres on the gangliosides GM1 and aGM1. However, binding is thought to occur as an early step in adhesion before fimbriae adhesion (Feldman et al. 1998), which was supported by the finding that flagellar adhesion to ganglioside is a major factor for TLR activation (Adamo et al. 2004). Cellular stress-induced changes have been shown to influence pathogen interactions with epithelial cells, where stress (e.g., oxidative stress) activates acid sphingomyelinase, which induces PM remodeling, and prevents non-motile bacterial *Shigella flexneri* binding to the cells. In contrast, flagellated bacteria (*S. enterica*) can overcome this lipid remodeling process by accumulating at the remaining sphingolipid rich domains (Tawk et al. 2018). Hydrophobicity has also been

shown to play a role in how bacterial flagella adhere, such that flagella can bind loosely to hydrophilic surfaces, whereas on hydrophobic surfaces, initial attachment is loose but increases progressively. This occurs apparently through conformational changes to the flagella, which may "zip" onto the surface and form stronger bonds with the hydrophobic surface (Friedlander et al. 2015). This work gives some insight on how bacterial flagella could interact with PM but needs to be tempered by the fact that PM are charged and composed by amphiphilic molecules.

Host lipids are not only used by bacteria to adhere on PM but can also serve to indicate the presence of the host cell to the pathogen via intermediate phospholipids like lysophospholipids. For example, in a form of host innate immunity regulation, secretion of *S. enterica* monomeric flagellin is induced by host-produced lysophospholipids to allow pathogen sensing, since only monomeric and polymerized flagellin trigger immune response via TLR-5 binding (Subramanian and Qadri 2006). For some species, secretion of non-flagellin proteins occurs via the flagella apparatus, similar to that of the non-flagellar type three-secretion system (T3SS). For example, in *Yersinia enterocolitica,* YplA, a non-flagellar protein has been shown to be secreted by the flagellar type III secretion system and is associated with a phospholipase activity (Schmiel et al. 1998; Young et al. 1999). In *Campylobacter jejuni,* which lacks non-flagellar T3SS, flagella-mediated secretion has been demonstrated for FlaC, an adhesin that facilitates binding to human cells (Song et al. 2004). This flagellin-like protein is secreted in order to specifically modulate host responses via TLR5 activation (Faber et al. 2016).

2.3 Other Adhesins and Bacterial Toxins

Besides appendages on bacterial surface, some outer membrane proteins can mediate adhesion to host surfaces. The outer membrane protein MAM7 (multivalent adhesion molecule) present in several Gram-negative bacteria has been shown to bind to fibronectin and phosphatidic acid (PA). Their adhesion can occur concurrently, although PA is fundamental to obtain a stable binding to the host and a basic residue (mce2 Lys-166) seems to be essential (Krachler et al. 2011). The rickettsial Outer-membrane protein B (rOmpB) from the obligate intracellular tick-borne pathogen *Rickettsia conorii* facilitates both binding to and invasion of non-phagocytic mammalian cells, requiring complex regulation between several partners for internalization. rOmpB targets a 70 kDa protein, Ku70, found at the host PM and in association with cholesterol-enriched lipid rafts. For internalization, three other proteins are needed: E3 ubiquitin ligase (c-Cbl), clathrin, and caveolin-2 (Martinez et al. 2005; Chan et al. 2009).

There are significant evidences showing bacterial toxins bind to membrane lipid to form pores. Some toxins target specifically membrane lipid raft to penetrate the cell. The best described is the cholera toxin B-subunit (CTxB), the membrane-binding subunit of cholera toxin, which is used as a marker of lipid raft (Day and Kenworthy 2015). This toxin secreted by *Vibrio cholera* has two subunits: The CTxA with an enzymatic activity to G protein which activates adenylate cyclase and a homopentameric CTxB which binds to GM1 gangliosides (van Heyningen

1974). For complete reviews on bacterial toxins and pore formation see Geny and Popoff (2006); Tilley and Saibil (2006); Los et al. (2013).

3 Bacterial Translocons and Effectors

To successfully colonize host tissues, many bacterial pathogens have evolved multiple protein secretion systems. Bacteria use 12 distinct classes of protein secretion systems to export proteins through their multilayered cell envelope (Green and Mecsas 2016). Some of the secretion systems can translocate proteins, termed bacterial effectors, into host cells, which in some cases target PM lipids (Geissler 2012). For this, they need to target host PM to form a pore or to go through after structural contraction (Fig. 2). Of the systems described above that directly interact with host lipids, flagella are ancestrally related to the Type 3 secretion system (T3SS); and OMP are exported via Type 1 secretion systems (T1SS) (Costa et al. 2015). The T3SS, type IV secretion system (T4SS) and type VI secretion system (T6SS), found mainly in Gram-negative bacteria, interact directly with and/or cross the host PM (Costa et al. 2015).

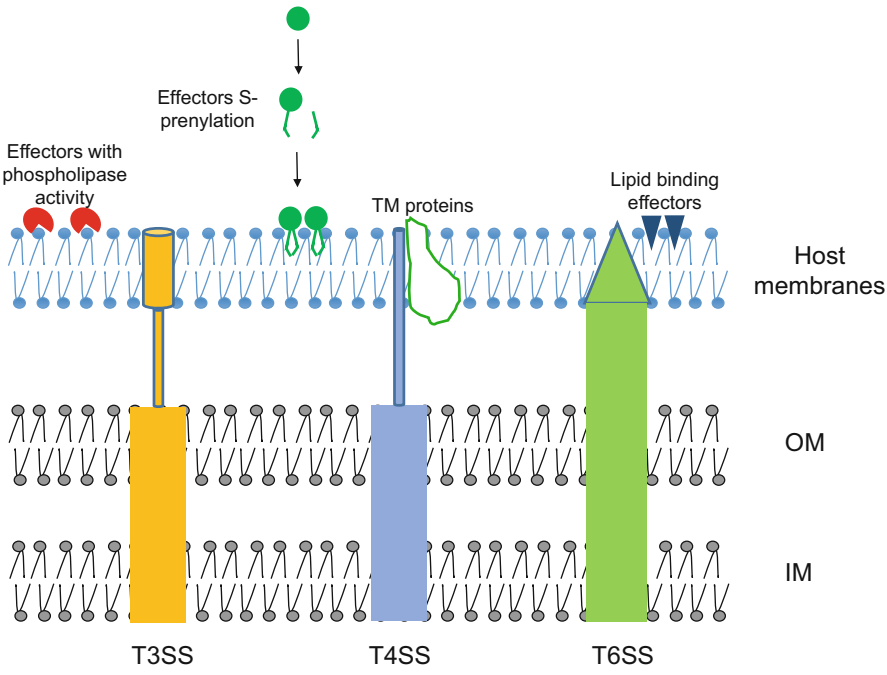

Fig. 2 Secretion systems, plasma membrane, and effectors

3.1 T3SS

The type 3 secretion system (T3SS) is used by both plant and animal pathogens to deliver effector proteins into host cells, via a molecular syringe, to promote host interactions, pathogenic, beneficial, or commensal. The T3SS needle complex encoded by animal pathogens is shorter than that of plant pathogens, with a length of 20–40 nm whereas more than 100 nm is required to penetrate the thick plant cell wall (CW) (Ji and Dong 2015). The morphological and protein subunit similarities between the T3SS and the bacterial flagellar system components indicate that these systems are homologs, arising from a common evolutionary origin (Macnab 1999; Pallen and Gophna 2007). The flagellar apparatus is thought to be the evolutionary precursor of T3SS, which has diversified over a series of gene duplications, losses, and acquisitions and has evolved the ability to deliver effectors to eukaryotic cells (Nguyen et al. 2000; Saier 2004; Abby and Rocha 2012). To inject T3SS effectors, a pore-forming multi-protein organelle, termed a translocon is inserted into host PM upon contact with host cell. Three types of protein tanslocons allow delivery of effectors: two are hydrophobic and one hydrophilic. The hydrophobic translocators are subdivided in two categories, one with only one transmembrane (TM) domain and the other with two TM domains. Due to their TM domains, these proteins insert in the host PM to form the translocon. The hydrophilic protein connects the membrane-inserted translocon and the tip of the T3SS needle (Matteï et al. 2011; Deng et al. 2017). In animals cells, the pore formation process requires transmembrane protein CCR5 (chemokine [C-C motif] receptor 5) in order to modulate cytoskeletal activities and allow hydrophobic translocon assembly (Sheahan and Isberg 2015). In plants, T3SS accessories proteins are needed to degrade the CW and facilitate access for translocon to PM (Charkowski et al. 1998; Kim and Beer 1998). IpaB, in *Shigella*, is one TM domain protein located on the tip of T3SS and binds to the hyaluronan receptor CD44. This interaction is impacted by depletion of cholesterol or sphingolipids from the epithelial cell PM (Lafont 2002; Van Der Goot et al. 2004). Enteropathogenic *E. coli* (EPEC) secrete their own receptor via the T3SS, the translocated intimin receptor (Tir). PM embedded Tir then binds to the EPEC adhesin intimin and induces intimate attachment between the bacterial and host cells (Hartland et al. 1999).

T3SS effectors can target host PM directly by degrading phospholipids like PI, to facilitate host manipulation or colonization. PI lipids are implicated in a multitude of role from actin cytoskeleton arrangements to vesicle trafficking and are the target of pathogenic bacteria; see Hilbi (2006) for review. An effector from *Shigella flexneri*, the inositol phosphate phosphatase IpgD, hydrolyzes phosphatidylinositol-4,5-bisphophate (PIP_2) to produce phosphatidylinositol 5-monophosphate (PI5P). This leads to actin reorganization promoting bacteria entry (Konradt et al. 2011). Others effectors from a number of bacteria (Ham et al. 2011) such as *Mycobacterium tuberculosis* (Beresford et al. 2007), *S. enterica* (Norris et al. 1998; Marcus et al. 2001), and *V. parahaemolyticus* (Broberg et al. 2010) are known to manipulate host membranes in comparable ways.

Effectors from intracellular pathogens like *S. enterica* can modify phagosomes' membranes to prevent fusion with host lysosomes and then to replicate inside host cells. For example, SifA contains Cys-aliphatic-aliphatic-X (CAAX) motif in its last C-terminal residues. When translocated, this motif is isoprenylated and S-acylated by eukaryotic cells. S-prenylation covalently adds isoprene groups, mainly farnesyl (15-carbon) and geranylgeranyl (20-carbon), to cysteine residues. These modifications increase the effector hydrophobicity and anchors them at the membrane (Boucrot et al. 2003; Reinicke et al. 2005).

3.2 T4SS

Like the T3SS, the type 4 secretion system (T4SS) can also reach host PM to transfer material proteins into the cytoplasm of eukaryotic cells and bacteria, although this includes DNA as well as proteins. The prototypical T4SS is from the phytopathogen, *Agrobacterium tumefaciens* (Grohmann et al. 2018). This bacterium uses the T4SS to transfer oncogenic T-DNA and different effectors to plant cells in order to induce crown gall disease (Salmond 1994). The T4SSs of Gram-negative bacteria appears to have evolved through consolidation of four functionally distinct structures. A hexameric ATPase, implicated in DNA/protein coupling and denominated type IV coupling protein (T4CP). Two membrane complexes needed for substrate passage across inner membrane or periplasm and outer membrane. They are named inner membrane complex (IMC) and outer membrane complex (OMC), respectively. The conjugative pilus is an example of the OMC and directly initiates contact with the host cell PM (Christie et al. 2014). Three proteins represent the pilus-assembly components: a transglycosylase (VirB1), a pilin (VirB2), and a pilus-tip (VirB5). To date, only VirB2 and VirB5 have been shown to be responsible for T4SS pilus adhesion on plant cells. Three related plant proteins with putative transmembrane domains, VirB2-interacting protein (BTI)-1, BTI2, and BTI3 were identified together with a membrane-associated GTPase, AtRAB8 to interact with VirB2 (Hwang and Gelvin 2004). Likewise, VirB5 has been described to target the trans-zeatin biosynthetic protein (Tzs) exposed to the plant cell surface (Aly et al. 2008). The human/animal pathogens, the best described T4SS is for *Helicobacter pylori*. The *H. pylori* VirB5 ortholog CagL, which was the first formally recognized bacterial carcinogen, binds specifically to a TM receptor, integrin α5β1 (Kwok et al. 2007). The *H. pylori* cytotoxin-associated gene A (CagA) interacts with PS on the host PM to promote CagA internalization (Murata-Kamiya et al. 2010). Specific host transmembrane proteins act as receptors for the *H pylori* outer membrane protein HopQ. These proteins, called carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), facilitate CagA translocation (Zhao et al. 2018). When translocated, CagA dysregulates the homeostatic signal transduction of gastric epithelial cells by shifting cell behavior. Similar to the T3SS effector SifA in *S. enterica*, the intravacuolar pathogen *Legionella pneumophila* T4SS effectors, AnkB when translocated in to the host are prenylated (Price et al. 2010). To replicate *L. pneumophila* needs to be intracellularly in an ER-associated compartment, the *Legionella*-

containing vacuole (LCV). Approximately 300 effector proteins are translocated; some of the effectors anchor to membrane through PI4P or PI3P to promote the interaction of LCVs with host vesicles and organelles, to affect small GTPases or PI-metabolizing enzymes see Steiner et al. (2018) for review.

3.3 T6SS

This versatile nanomachine was first described in 2006 in *P. aeruginosa* (Mougous et al. 2006). It delivers a wide arsenal of effector proteins directly into prokaryotic and eukaryotic target cells. T6SS share a bacteriophage-related tail structure anchored to the cell envelope by a membrane complex (Cherrak et al. 2000; Leiman et al. 2009). The T6SS tail tube is a structure made of different protein subunits that polymerize to form the contractile sheath. This tube wraps around the hemolysin co-regulated protein (Hcp) and dynamically propels a spike at its tip. This puncturing device is formed by Hcp and valine–glycine repeat protein G (VgrG). Then T6SS effectors are injected across bacterial membranes during this contraction mechanism (Basler et al. 2012). Effectors propelled by T6SS can affect host PM. In *B. thailandensis,* Vgrg-5 induce membrane fusion, either by the recruitment of other factors or by direct participation (Schwarz et al. 2014). In *Vibrio cholerae,* VasX is a secreted, lipid-binding protein, which can disrupt bacterial and host cell signaling. This T6SS protein can both kill and protect bacterial cells (Miyata et al. 2011, 2013). Antibacterial T6SS effectors target bacterial membrane with phospholipase activity in *P. aeruginosa, Burkholderia thailandensis,* or entero-aggregative *E. coli* (Russell et al. 2013; Flaugnatti et al. 2016).

4 Biomimetic Membrane Models

Here we describe application of biomimetic models as tools for the detection and characterization of bacterial proteins or whole bacteria interactions with host cell membranes. Membrane-based biomimetic systems have been developed to reconstitute and investigate biological events occurring at the cell membrane interface under controlled conditions. The complexity of the biological membranes makes direct investigation of their interaction with bacterial cells or compounds at the molecular level very challenging. In an attempt to overcome the limitations, lipid bilayer systems have been introduced as models of biological membranes. These models have been successfully used to study the interaction and the insertion of peptides, proteins, or whole organisms.

The simplest and most commonly used biomimetic models are vesicles and liposomes. These spherical lipid bilayers form an internal aqueous cavity and are classified according to their diameters into: Small Unilamellar Vesicles (SUV), which exhibit a mean diameter inferior to 100 nm; Large Unilamellar Vesicles (LUV) have a diameter between 100 and 1000 nm; whereas Giant Unilamellar Vesicles (GUV) have a size range of 1–200 μm of diameter. Due to their size, GUVs are recognized as the best membrane model for mimicking the cell membrane

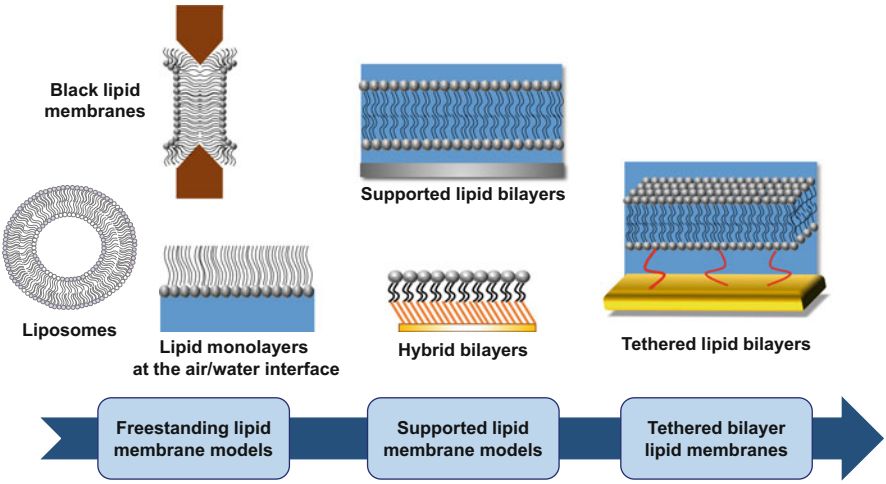

Fig. 3 Evolution of the lipid bilayer model architectures over time

curvature since animal and plant cells have a size ranging from 10 to 30 μm and from 10 to 100 μm, respectively.

A variety of biomimetic membrane systems on solid support have been developed and coupled to surface sensitive techniques such as Surface Plasmon Resonance (SPR), Atomic Force Microscopy (AFM), quartz crystal microbalance, or neutron-reflectometry. Among all the existing models, solid supported membranes provide higher stability than freestanding lipid models (Fig. 3). In 1984, Brian et al. pioneered a simplified method for the construction of supported planar membranes to study biological mechanisms (Brian and McConnell 1984). After this work, a variety of constructions on solid surface were developed including supported lipid bilayers (SLB) directly reconstituted on hydrophilic surfaces and tethered bilayers (tBLM) (Rebaud et al. 2014; Veneziano et al. 2017). Tethered bilayer lipid membranes were designed to overcome the limitations of SLB. They comprise a lipid bilayer spaced out from solid support by the use of molecular spacers or layers that intercalate between the substrate and the bilayer. This enables to study the incorporation or translocation of proteins without interference with the support (Tanaka and Sackmann 2005; Rossi and Chopineau 2007).

4.1 Liposomes and Bacterial Interaction

Liposomes are the simplest model system that mimic cell membranes. They offer the advantage of being very versatile as their lipid composition can be easily tailored to the question. They have been used to characterize the mode of interaction of bacteria with cell membranes and define the functional roles of the host cell membrane and bacterial components. LUVs were used in demonstration of the role of glycan-glycan interactions during the initial binding of *Shigella dysenteriae* to host cells. Outer membrane

vesicles (OMV) containing lipopolysaccharide (LPS) were shown to be spontaneously, secreted by *S. dysenteriae*. In order to characterize the interaction of the O-antigen polysaccharide moiety of LPS, LUV with or without ganglioside GM1 were incubated with the OMVs and showed that the glycosylated part of GM1 from the host PM cells associated directly to the LPS. This phenomenon is involved in *S. dysenteriae* binding to host $CD4^+$ T lymphocytes (Belotserkovsky et al. 2018).

Likewise, GUVs were used to implicate globotriaosylceramide Gb3-dependent invasion of *Pseudomonas aeruginosa.* The authors coined this mechanism "lipid zipper" where the galactophilic lectin (LecA) present in the bacterial outer membrane binds the carbohydrate moiety of Gb3 to mediate the entry of the bacteria. Microscopy analysis showed that the bacterial interaction with GUVs composed of DOPC/Chol/Gb3 was strictly driven by the lectin-Gb3 interaction and lead to the reorganization of membrane lipids into clusters with a negative membrane curvature that accompany the bacterial entry into GUVs (Eierhoff et al. 2014).

The importance of the nature of lipids involved in the bacteria/host cell membrane interaction can also be investigated with such biomimetic systems. Recently, it was shown by co-flotation assays that the signal peptide of the serine-rich repeat adhesin of *Streptococcus gordonii* interacts with anionic lipids of the host cell membrane. The electrostatic interaction was found to be a prerequisite for bacterial preprotein entry across cell membranes and the consecutive bacterial translocation (Spencer et al. 2018).

Liposomes were used in a separate example, to examine the effect of lipoarabinomannan (LAM), a component of the *Mycobacterium tuberculosis* cell wall, on membrane structure. This allowed the mechanistic basis of LAM to be identified for bacterial survival within macrophages. Using fluorescence resonance energy transfer (FRET), it was shown that, after insertion into the liposome bilayer, LAM inhibits the fusion of liposomes having a lipid composition mimicking the phagosomal membrane. Indeed a classic use of FRET is for monitoring lipid exchange or mixing and membrane fusion. Moreover, AFM imaging of lipid domains within a SLB exposed to LAM showed that LAM modifies the lipid distribution and packing, leading to a reorganization of lipid domains. This study neatly described how bacterial lipids can lead to the bacterial persistency within the immature phagosomal compartment of macrophages (Hayakawa et al. 2007).

4.2 Liposomes as Tools for Biosensing

The ability of some proteins such as toxins secreted by bacteria to insert in and then modify the physical properties of the lipid bilayer can be detected by biosensing systems. Zhou and co-authors developed a sensing methodology to detect pathogenic bacteria at an infected wound. The assay was based on the quantification of the release of a fluorescence dye from the lumen of liposomes upon exposure to bacterial lytic agents. Thus, the pathogenic state of the bacteria could be assessed (Zhou et al. 2018). Based on the same principle, the membrane perturbing actions of several bacterial toxins were studied by monitoring the release of a fluorescent dye from liposomes. Recent applications are the vacuolating cytotoxin A (VacA), from

H. pylori (Linn et al. 2018) or the Hemolysin A, the major virulence factor of *Staphylococcus aureus*. The authors have demonstrated that an inhibitor of ATP-gated purinergic receptors (P2XR) interferes with the function of a bacterial pore-forming protein through a direct inhibition of its binding to the membrane and oligomer formation (Schwiering et al. 2017).

4.3 Bacterial Toxin Interaction with Tethered Bilayer Models

Due to their covalent anchoring to the substrate, tethered bilayer lipid membranes (tBLMs) are considerably more robust than freestanding lipid bilayers. Among all the surface sensitive techniques that can be coupled to these models, electrochemical impedance spectroscopy (EIS) can be applied for studying the mode of interaction with lipid membranes of several membranotropic peptides (Atanasov et al. 2006). Indeed, depending on their molecular architecture, the tBLMs could provide a fluid lipid bilayer platform having a high resistivity, highly suited for quantifying the pore-forming ability of toxins produced by bacteria.

In EIS, an alternating voltage is applied over a range of frequencies and the resulting current is measured along with the time delay between a change in the applied potential and the resulting current. The resistance and phase difference between voltage and current changes can be fitted to an equivalent circuit composed of resistance and capacitance describing the electrical properties of the membrane (Fig. 4). For tBLMs, EIS measurements provide information about the quality of the lipid bilayer and charge transport processes across the membrane, for example, by extricated or incorporated channel proteins.

This approach was employed for the characterization of cyanotoxins. The action of a member of the microcystin (MC) family on the lipid membrane was investigated using tethered lipid bilayers (tBLMs). The cyanotoxin class of hepatotoxic MC is responsible for animal and human poisoning cases due to bioaccumulation in the

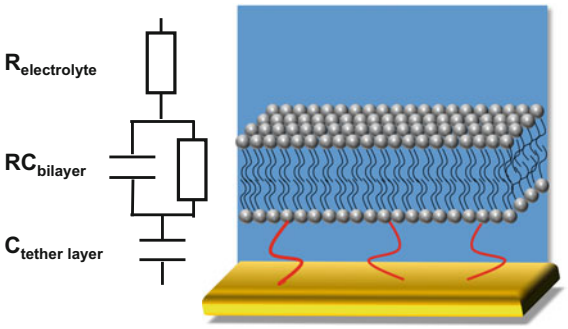

Fig. 4 Scheme of a tBLM (right) and an example of equivalent circuit modeling the resulting impedance data (left). The R(RC)C-circuit consists of a RC element describing the bilayer in series with a capacitor ($C_{tether\ layer}$) corresponding to the tether layer and gold interface and an electrolyte resistance ($R_{electrolyte}$)

liver. This study demonstrated that contrary to the original hypothesis, MC-Leucine Arginine (LR) membrane exposure leads only to small and transient variations in the membrane capacitance, showing that MC-LR is not able to accumulate in cell membranes and modify their integrity (Facey et al. 2019).

The combination of tBLMs and EIS measurements brings a more discriminative detection of the nature of the membrane damages caused by bacterial proteins than the liposome-based assays described above. An assay allowing the detection of toxins and bacterial enzymes was developed by coupling tBLMs with SPR spectroscopy and EIS (Tun et al. 2011). The tBLM used in this study is tethered by a mixture of 2,3-di-O-phytanyl-glycerol-1-tetraethylene glycol-D,L-lipoic ester lipid (DPhyTL) and cholesterol-pentaethyleneglycol. tBLMs integrating DPhyTL are well known to be dense lipid membranes with high electrical sealing properties (Naumann et al. 2003). The combination of these two techniques allows investigators to differentiate between pore formation caused by pore-forming toxins or the lipid loss resulting from lipase action.

The design of tBLMs can be adapted in order to study more complex sequence of events involving bacterial toxins. The binding and the consecutive translocation of the adenylate cyclase toxin (CyaA) were successfully reconstituted and quantified under controlled conditions of calcium concentration and membrane potential (Veneziano et al. 2013). The CyaA toxin is one of the major virulence factors produced by *Bordetella pertussis*. It is a particularly complex protein of 1706-residues and consisting of several active domains. Once translocated across the plasma membrane, the N-terminal catalytic domain (CD) of CyaA binds to calmodulin and catalyzes the production of supraphysiologic level of cAMP (O'Brien et al. 2017). A new tBLM construction was specifically developed to study the in vitro binding of CyaA to lipid membrane and the consecutive translocation of its CD across the lipid bilayer. The bilayer was assembled over a surface derivatized with the protein calmodulin in order to differentiate the external and internal aqueous compartments that are separated by the tethered bilayer. This particular design allows taking into account the underlying cytosolic compartment. It was demonstrated that the bilayer acts as a fluid and protein-impermeable barrier (Rossi et al. 2011). Surface plasmon resonance spectroscopy, application of a controlled membrane potential, and an enzymatic assay were combined in order to monitor the protein binding in real time and to quantify the enzymatic activity resulting from the CD/CaM association. It was found that the translocation of the CyaA catalytic domain is strictly dependent on the presence of calcium and the application of a negative transmembrane potential across the tethered lipid bilayer (Veneziano et al. 2013). Beyond the study done on the CyaA toxin, this biomimetic design provides an unique tool to explore in vitro the molecular mechanisms of protein translocation across biological membranes in precisely defined conditions (Veneziano et al. 2013, 2017)

4.4 Whole-Bacteria Interaction with Membrane Models

While the action on lipid membrane of proteins produced by bacteria have been studied using membrane models, very little attention has been applied for lipid

bilayer systems by studying the interaction of whole bacteria with cell membrane structures.

To our knowledge, the membrane model structures employed for such studies have been exclusively vesicles. Functionalized GUVs (~15–60 μm) have been employed to monitor the interaction of whole bacteria with liposomal nanoparticles by electronic absorption spectroscopy and FRET. It was based on the interaction of the glucose molecules tagged on liposomes with the glycoproteins present on the surface of *E. coli* (Dogra et al. 2012). The same type of interaction was also exploited to investigate the propulsion of lipid vesicles using the strength of *E. coli* bacteria motility as a function of the size of the vesicles (Dogra et al. 2016). The glycolipid-mediated interactions of bacteria with liposomes of different sizes (SUVs, LUVs, and GUVs) were evaluated by FRET microscopy. The authors demonstrated that SUVs and LUVs could be transported by bacteria while increasing their velocity, dependent on the size ratio between the bacteria and vesicle elements. In this study, any bacterial interaction with the liposomes composed of a pure lipid bilayer could be detected.

5 Research Needs

Bacterial adhesion to host tissue is a long-standing area of research, yet the interactions with lipids in this process remain an understudied topic. The major challenges that need to be addressed include:

- *Understanding fatty acids (FA) function for bacterial adhesion and colonization.* A small number of studies have been focused on the role of FA when bacteria invade host tissues although the physical state of the cells is dependent on the FA composition of the PM (Marsh 2010). Published studies are limited: in one study, use of membrane fluidizers like hexanol, benzyl alcohol, and 2-(2-methoxyethoxy)ethyl 8-(*cis*-2-*n*-octylcyclopropyl)octanoate on human epithelial cell line. However, the approach doesn't fully address this question as it cannot discount side effects of the fluidizers on the cell lines (Ismaili et al. 1999). In another approach, cell lines incubated with different fatty acids and *Lactobacillus spp* illustrated the impact of FA on probiotic bacteria adhesion (Kankaanpää et al. 2001). Thus, by combining biomimetic membrane models and cell lines with such approaches, new mechanisms could be highlighted in host-pathogen interactions.
- *Use transmembrane receptors with different plasma membrane lipid composition*. The impact of different PM compositions on transmembrane proteins remains unknown for bacterial adhesion and immune response. The use of biomimetic membranes with controlled lipid composition in presence of reconstituted receptors could be an elegant approach to answer this question.
- *Characterizing molecular recognition*. To develop new molecular-based methods to combat bacterial infection, the scientific community needs to understand precisely which adhesins bind to specific PM lipids. Unfortunately, for most bacteria, this remains to be addressed.

- *Cholesterol implication in bacterial colonization*. Cholesterol-rich microdomains at the host PM have been identified in the internalization steps of several bacteria (Mañes et al. 2003). In most reports, cholesterol was removed with the appropriate drug. An alternative approach has employed mouse embryonic fibroblasts lacking the final enzyme required for cholesterol synthesis to investigate the role of cholesterol in bacterial penetration of host cells (Gilk et al. 2013). However, the precursor of cholesterol, desmosterol, that replaces cholesterol in the cellular PM of these mice may still participate in bacterial internalization (Rodríguez-Acebes et al. 2009). Therefore, additional work is needed to develop new strategies to study the real impact of cholesterol in bacterial adhesion and internalization.

Bacterial adhesion and manipulation of host membrane lipids still need to be better documented. Understanding how bacteria hijack PM organization will underlie new strategies to fight bacterial infection. While encouraging findings are emerging, major steps are still needed to better characterize the various strategies used by bacteria to target host lipids.

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Role of Sphingolipids in Bacterial Infections

6

Katrin Anne Becker, Ryan Boudreau, Aaron Gardner, Aaron P. Seitz, Charles C. Caldwell, Xiang Li, Yang Zhang, Malcolm Brodlie, Michael J. Edwards, and Erich Gulbins

Contents

Katrin Anne Becker, Ryan Boudreau, and Aaron Gardner have contributed equally to this work.

K. A. Becker
Department of Molecular Biology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany
e-mail: katrin.becker-flegler@uni-due.de

R. Boudreau · A. P. Seitz · C. C. Caldwell · M. J. Edwards
Department of Surgery, University of Cincinnati, Cincinnati, OH, USA
e-mail: boudrern@ucmail.uc.edu; seitzap@UCMAIL.UC.EDU; caldwecs@UCMAIL.UC.EDU; edwardm6@ucmail.uc.edu

A. Gardner · M. Brodlie
Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK
e-mail: Aaron.Gardner@newcastle.ac.uk; Malcolm.Brodlie@newcastle.ac.uk

X. Li · Y. Zhang
Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX, USA
e-mail: xli61@central.uh.edu; yzhan219@central.uh.edu

E. Gulbins (✉)
Department of Molecular Biology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Department of Surgery, University of Cincinnati, Cincinnati, OH, USA
e-mail: erich.gulbins@uni-due.de

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Abstract

Lipids play a very important role in the infection of mammalian cells by different pathogens. **Sphingolipids** have been shown by numerous recent studies to possess a particularly essential role in infectious biology. Sphingolipids such as sphingomyelin, **ceramide**, and **sphingosine** organize cell membranes into distinct domains, called rafts that are enriched with sphingolipids and cholesterol. The generation of ceramide within the cell membrane results in the formation of large **ceramide-enriched membrane platforms** that serve the temporal and spatial organization of the cellular signaling machinery. Ceramide-enriched membrane platforms have critical functions for bacterial and viral infections. In addition, ceramide and sphingosine regulate the functions of enzymes, receptors, and organelles such as lysosomes, and therefore contribute to the control of the cellular response to pathogens. Finally, at least sphingosine has a direct antibacterial effect against many pathogens. Here, we present the diverse functions of sphingolipids in **infectious biology** and discuss mechanisms of their actions.

1 Introduction

Many recent studies have revised the original view of the cell membrane suggested by Singer and Nicolson (1972) and indicate that lipids in cell membranes are ordered into domains mediated by interactions of, for instance, sphingolipids and cholesterol (Simons and Ikonen 1997; Brown and London 1998). Membranes of eukaryotic cells are predominantly composed of sphingolipids, (glycerol-) phospholipids, and cholesterol. Sphingolipids contain sphingoid bases, i.e., a 1,3-dihydroxy-2-aminoalkene, as backbone. Sphingosine, or (2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol, is the most prevalent backbone of mammalian sphingolipids. The chain length of the sphingoid bases in sphingolipids is variable, as is the degree of saturation.

In the present chapter, we will focus on **ceramides**: the amide esters of sphingosine and a fatty acid (Kolesnick et al. 2000). The fatty acids in ceramides also vary in saturation and range from 2 to 28 carbon atoms in the acyl chain. The attachment of headgroups to ceramides results in formation of complex sphingolipids, including sphingomyelin, gangliosides, sulfatides, globosides, or cerebrosides. Second, we will discuss the role of sphingosine as an antibacterial molecule.

Sphingomyelin, the most prevalent cellular sphingolipid, comprises a ceramide moiety and a hydrophilic phosphorylcholine headgroup. This headgroup tightly interacts with other complex sphingolipids and cholesterol molecules mostly via the hydrophilic headgroups of glycosphingolipids, while the hydrophobic ceramide

part interacts with the hydrophobic portions of cholesterol via van der Waal forces. These interactions and the high local concentration of sphingolipids and cholesterol result in a lateral association and spontaneous separation of these lipids from other phospholipids in the cell membrane, forming small, distinct membrane domains that were named **rafts** (Simons and Ikonen 1997). Cholesterol and some cholesterol precursors fill voids between bulky sphingolipids and, thus, stabilize the structure of rafts (Brown and London 1998; Kolesnick et al. 2000). This notion is supported by experiments showing that extraction of cholesterol from membranes or interference with cholesterol synthesis using the drugs beta-cyclodextrin, nystatin, or filipin destroys membrane rafts (Xu et al. 2001).

2 Ceramide-Enriched Membrane Domains

Ceramide-enriched membrane domains are special membrane domains that form upon generation of ceramide in membrane. Usually, ceramide formation occurs via the hydrolysis of sphingomyelin by **acid**, **neutral,** and **alkaline sphingomyelinases** at the respective optimum pH for activity (for review, see Hannun and Obeid 2008). In addition, de novo synthesis of ceramide involving the serine-palmitoyl-CoA transferase as the key enzyme, reverse activity of the acid ceramidase catalyzing synthesis of ceramide from sphingosine (Okino et al. 2003), and hydrolysis of complex-glycosylated lipids (Ishibashi et al. 2007) might also result in ceramide-enriched membrane domains, although this has not yet been shown formally.

Acid sphingomyelinase was shown by several studies to release ceramide to the outer leaflet of the cell membrane, leading to large ceramide-enriched membrane domains, also called platforms (Grassmé et al. 2001). Ceramide molecules spontaneously associate, resulting in the formation of small ceramide-enriched membrane domains that spontaneously fuse with other ceramide-enriched membrane domains to form large ceramide-enriched membrane platforms (Nurminen et al. 2002). The generation of ceramide domains alters biophysical characteristics of membranes, which seems to dictate the function of these domains. Ceramide molecules in these domains are tightly packed, stabilizing the domains. These domains are highly hydrophobic. They seem to have a different fluidity than other areas of the cell membrane; it is likely that even the membrane thickness differs from that of other membrane parts (Brown and London 1998; Kolesnick et al. 2000). Thus, ceramide-enriched membrane domains are able to trap and cluster receptor as well as signaling molecules, and they thereby serve the spatial reorganization of the cellular signaling machinery. Receptor clustering may not only bring the receptor and signaling molecules into close vicinity but also may limit lateral diffusion and, thus, stabilize the receptor-ligand interactions.

In addition, ceramide directly regulates intracellular signaling molecules, in particular cathepsin D (Heinrich et al. 1999), phospholipase A_2 (Huwiler et al. 2001), ceramide-activated protein serine-threonine phosphatases (CAPP), (Dobrowsky and Hannun 1993), protein kinase C isoforms (Muller et al. 1995), c-Raf-1 (Yao et al. 1995), and nicotinamide adenine dinucleotide phosphate (NADPH)

oxidases (Zhang et al. 2008). Several, studies also indicate that ceramides regulate the activity of ion channels. In particular the Kv1.3 channel and calcium release-activated calcium (CRAC) channels (Szabo et al. 1996; Gulbins et al. 1997; Lepple-Wienhues et al. 1999) are inhibited by ceramide. In general, ceramide-enriched membrane platforms seem to serve both in the temporal and spatial organization of receptors and signaling molecules and in the direct regulation of enzyme activities.

3 Ceramide, Membrane Domains, Sphingosine, and Infections

3.1 *Pseudomonas aeruginosa*

P. aeruginosa infections are severe and often life-threatening in patients with systemic bacterial infections, ventilator-associated pneumonia, and, in particular, cystic fibrosis. We have previously shown that *P. aeruginosa* infection of epithelial cells triggers a rapid stimulation of the acid sphingomyelinase, which translocates to the extracellular leaflet of the cell membrane to release ceramide from sphingomyelin (Grassmé et al. 2003). This ceramide generation results in formation of ceramide-enriched membrane platforms that are also positive for typical raft markers such as GM1. Ceramide-enriched membranes mediate internalization, cell death, and controlled release of cytokines upon infection of epithelial cells in vitro or bronchial epithelial cells in vivo with *P. aeruginosa* (Fig. 1). At present the exact mechanisms how ceramide-enriched membrane platforms mediate **internalization**, **cell death**, and controlled **cytokine** release are unknown. However, destruction of ceramide-enriched domains or membrane rafts prevents cell death in vitro and in vivo, demonstrating their significance for the cytotoxic effects of *P. aeruginosa*. CD95, which has been previously shown to be required for *P. aeruginosa* mediated cell death (Grassmé et al. 2000), clusters in ceramide-enriched membrane platforms upon infection of epithelial cells (Grassmé et al. 2003). This process might represent a critical event for the induction of host cell death by this pathogen (Becker et al. 2012).

Ceramide-enriched membrane platforms are also critical for a controlled release of cytokines from *P. aeruginosa*-infected cells (Grassmé et al. 2003). This might occur as a direct regulation of the pathways triggering formation and release of cytokines such as interleukin 1 or as an indirect effect on the regulation of cytokine release, for instance, by death and subsequent elimination of infected cells.

Internalization of *P. aeruginosa* might be regulated by ceramide-enriched membrane platforms via clustering of the cystic fibrosis conductance regulator (Cftr) and/or ganglioside asialo-GM1 (Pier et al. 1996; Saiman and Prince 1993). Both molecules were demonstrated to be receptors for *P. aeruginosa* and to be involved in internalization of the bacteria. The observation of clustering of Cftr in ceramide-enriched membrane platforms is consistent with the findings that Cftr is trapped and concentrated within the raft fraction upon infection (Kowalski and Pier 2004). These

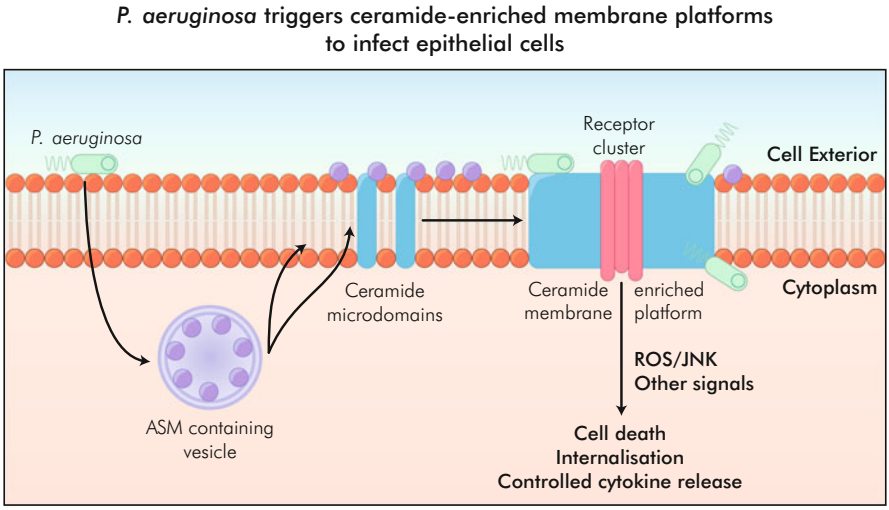

Fig. 1 Membrane platforms in bacterial infections. *Pseudomonas aeruginosa* interacts with the cell membrane to activate the acid sphingomyelinase and to trigger the fusion of vesicles that contain the acid sphingomyelinase with the plasma membrane. *P. aeruginosa*-mediated activation of the acid sphingomyelinase very likely involves the bacterial type III secretion system. Ceramide forms microdomains that fuse to large ceramide-enriched macrodomains. The latter serve to cluster receptor molecules interacting with the bacteria and to recruit intracellular signaling molecules to finally mediate the infection. Ceramide-enriched membrane platforms are required for induction of cell death of the infected cell by *P. aeruginosa* and internalization of the bacteria and to mediate a controlled release of cytokines from the infected cells

studies also evidenced that destruction of rafts or inhibition of the formation of ceramide-enriched membrane platforms prevented internalization of *P. aeruginosa* into pulmonary epithelial cells (Kowalski and Pier 2004). Rafts were disrupted by treatment with drugs that interfere with the cholesterol metabolism (i.e., beta-cyclodextrin, nystatin, or filipin) and, thus, destroy the ensemble of cholesterol and sphingolipids. At present, the molecules that couple rafts and ceramide-enriched membrane platforms to internalization are unknown, but it was suggested that Src-like tyrosine kinases and the major vault protein are involved in this process (Kannan et al. 2008; Kowalski et al. 2007). The role of rafts for internalization of *P. aeruginosa* also applies to other cells, in particular corneal epithelial cells (Yamamoto et al. 2005).

The exceptional role of ceramide-enriched membrane domains for *P. aeruginosa* infections of epithelial cells is best evidenced in experiments employing cells or mice genetically deficient for the acid sphingomyelinase (Grassmé et al. 2003). Cells or mice lacking acid sphingomyelinase were unable to form ceramide-enriched membrane platforms on the cell surface upon infection and failed to internalize the pathogens, to undergo death, and to respond to the infection with an adequate release of cytokines. In vivo, acid sphingomyelinase-deficient mice were unable to eliminate *P. aeruginosa* from the lung after an acute infection, released massive amounts of

pro-inflammatory cytokines, developed **lung failure** and **sepsis,** and finally died. These data demonstrate the importance of rafts and ceramide-enriched membrane platforms for the response of mammalian host cells to *P. aeruginosa* infections.

Several studies have shown that ceramide-enriched membrane platforms regulate the function of several cellular systems that are critically involved in infection processes. For example, ceramide-enriched membrane platforms cluster subunits of **NADPH oxidases** and thereby induce the release of **reactive oxygen species** (ROS) (Zhang et al. 2008), which are required to kill many extracellular pathogens. The failure to generate ROS might explain, at least in part, the high sensitivity of acid sphingomyelinase-deficient mice to pulmonary *P. aeruginosa* infections.

Second, recent studies demonstrated that ceramide-enriched membrane platforms cluster **β1-integrins** in the luminal membrane of CF epithelial cells in the airways, finally resulting in an ectopic expression of β1-integrins (Grassmé et al. 2017). **Cystic fibrosis** is caused by mutations in the ***cystic fibrosis transmembrane conductance regulator*** *(CFTR)* gene with an incidence of ~1 in 2500 births (Ratjen and Döring 2003; Elborn 2016). Mutations in the *CFTR* gene mainly cause pulmonary and gastrointestinal symptoms. Particularly important are pulmonary problems in cystic fibrosis patient, which are characterized by chronic inflammation of the airways, recurrent and chronic infections with *Pseudomonas aeruginosa, Burkholderia cepacia*, *Staphylococcus aureus*, *Haemophilus influenzae*, and other pathogens, bronchiectasis, and fibrosis (Elborn 2016; CF foundation 2010). Several studies have shown that ceramide is increased in epithelial cells and macrophages from mice and patients with cystic fibrosis (Teichgräber et al. 2008; Zhang et al. 2009; Becker et al. 2010, 2012; Ulrich et al. 2010; Brodlie et al. 2010; Bodas et al. 2011; Caretti et al. 2014, 2017; Itokazu et al. 2014; Pewzner-Jung et al. 2014; Quinn et al. 2016). Ceramide accumulates in the lungs in particular in the epithelial cells of large and small bronchi as well as in the trachea. In contrast to the increased ceramide levels, sphingosine in airway epithelial cells and alveolar macrophages of CF mice and humans is dramatically decreased (Pewzner-Jung et al. 2014; Tavakoli Tabazavareh et al. 2016; Grassmé et al. 2017), which will be discussed in detail below.

The increase in ceramide has at least two consequences related to infectious biology: High cellular levels of ceramide induce death of epithelial cells in airways and the intestine, with the deposition of dead cells and DNA into the lumen of the airways and the intestinal tract (Teichgräber et al. 2008). In the airways, the accumulation of DNA reduces the mucociliary clearance and thereby promotes infection (Fig. 2). Secondly, ceramide promotes an ectopic expression of β1-integrins in the luminal membrane of CF epithelial cells (Grassmé et al. 2017). This ectopic expression of β1-integrins mediates a downregulation of **acid ceramidase**, which converts ceramide to **sphingosine**, resulting in a vicious cycle that further increases ceramide levels while decreasing sphingosine expression (Fig. 2).

Degradation of DNA by **inhalation of a DNase** and correction of ceramide and sphingosine levels in the airways have been shown to prevent or eliminate acute and chronic pulmonary infections in cystic fibrosis mice with *Staphylococcus aureus* and mucoid or non-mucoid *Pseudomonas aeruginosa* (Teichgräber et al. 2008; Grassmé et al. 2017).

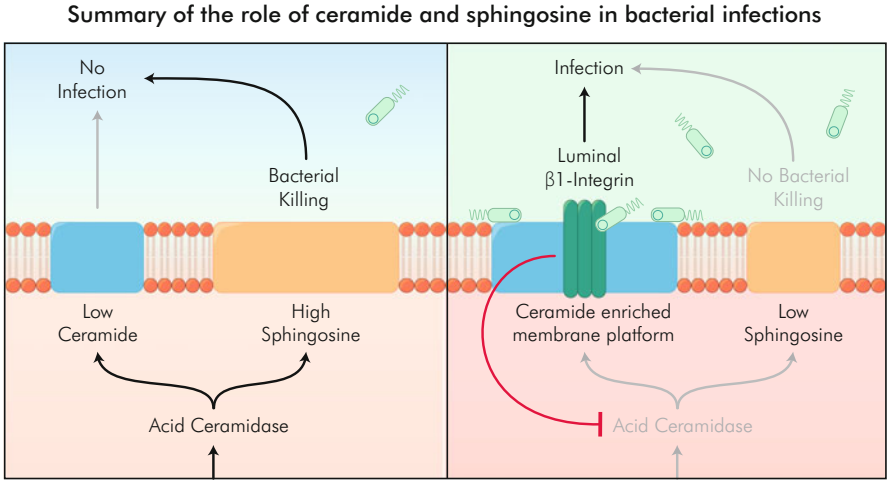

Fig. 2 Summary of the role of ceramide and sphingosine in bacterial infections. An initial accumulation of ceramide in cystic fibrosis cells, which occurs via still unknown mechanisms, clusters and traps β1-integrin in the luminal plasma membrane. Ectopically expressed β1-integrin downregulates acid ceramidase in cystic fibrosis cells, which results in a vicious cycle of increased luminal ceramide and plasma membrane β1-integrin clustering and trapping. The markedly reduced activity of the acid ceramidase in cystic fibrosis cells reduces sphingosine levels, thereby impairing bacterial killing in the airways. Normalization of β1-integrin, ceramide, or sphingosine prevents lung infection in cystic fibrosis

In addition to a role of ceramide in *P. aeruginosa* infections, particularly in cystic fibrosis lungs, an important role of **glucosylceramide** in this process has been also demonstrated (Kovacic et al. 2017). Glucosylceramide was shown to be severely downregulated in cystic fibrosis airway epithelial cells. Restoring normal levels of glucosylceramide in epithelial cells prevented pulmonary *P. aeruginosa* infections. At present, it is unknown how glucosylceramide acts against bacterial infections.

3.2 Neisseria

The first studies that established a role of the **acid sphingomyelinase-ceramide system** in infectious biology studied the infection of epithelial cells with ***Neisseria gonorrhoeae*** (Grassmé et al. 1997; Hauck et al. 2000). *N. gonorrhoeae* is an intracellular pathogen that causes gonorrhoeae. These studies indicated that ceramide mediates internalization of *N. gonorrhoeae* and thereby infection of epithelial cells with this pathogen. Subsequent studies with ***N. meningitidis*** confirmed the role of the acid sphingomyelinase and ceramide for uptake of pathogens and demonstrated a requirement of the acid sphingomyelinase/ceramide system for the infection of endothelial cells by *N. meningitidis* (Simonis et al. 2014).

3.3 *Staphylococcus aureus*

Ceramide also plays an important role in the infection of endothelial cells and epithelial cells with ***Staphylococcus aureus*** (*S. aureus)*. These studies demonstrated an activation of the acid sphingomyelinase with a concomitant release of ceramide upon infection of endothelial cells or macrophages with *S. aureus* (Esen et al. 2001; Li et al. 2017). Ceramide induced internalization of *S. aureus* into endothelial cells and macrophages, death of *S. aureus*-infected endothelial cells, destruction of endothelial tight junctions, and a massive reorganization of the cytoskeleton (Esen et al. 2001; Peng et al. 2015; Li et al. 2017). The destruction of **tight junction proteins** resulted in in vivo in pulmonary edema after systemic infection with *S. aureus*. Inhibition of the acid sphingomyelinase prevented lung edema in septic mice indicating the significance of lipids for the integrity of the endothelial cell layer in the lung (Peng et al. 2015).

The effects of *S. aureus* on mammalian cells are mediated at least partly by toxins. Many toxins bind to membrane domains, including some that are important in the pathogenicity of *S. aureus* infection (Becker et al. 2017a; Ma et al. 2017; Keitsch et al. 2018). This concept was exploited to competitively bind and effectively neutralize *S. aureus* toxins by injection of liposomes that were composed to mimic membrane domains (Henry et al. 2015). These **liposomes** bound toxins in vitro and in vivo, preventing cytotoxic effects in vitro as well as lethality *upon S.* aureus inoculation in vivo.

3.4 *Listeria monocytogenes*

Several studies revealed that the acid sphingomyelinase/ceramide system also plays an important role in cellular infections with ***Listeria monocytogenes*** (*L. monocytogenes*), a gram-positive bacterium causing severe and even lethal foodborne infections. After internalization by macrophages, the survival of the intracellular pathogen is determined by fusion of phagosomes with lysosomes. The fusion of these compartments requires activity of the acid sphingomyelinase and hydrolysis of sphingomyelin to ceramide (Schramm et al. 2008; Utermöhlen et al. 2003). Specifically, *L. monocytogenes* interacts with **β2-integrin** to activate the acid sphingomyelinase, resulting in the release of ceramide, the formation of ceramide-enriched membrane domains within the **phagosome**, clustering and activation of **NAPDH oxidase Nox2**, and the release of ROS (Gluschko et al. 2018). ROS induce a recruitment of **LC3**, a major regulator of **autophagy**, to the phagosomal membrane, which mediates fusion of the phagosomes with lysosomes and thereby kills the pathogen (Gluschko et al. 2018).

3.5 Mycobacteria

Mycobacteria tuberculosis infections are among the most common and serious infectious diseases worldwide. The role of sphingolipids in *M. tuberculosis* infection

is still largely unknown. Tumor necrosis factor-α (TNF-α) is a key factor in the host defense against mycobacteria (Roca and Ramakrishnan 2013). However, excess of **TNF-α** promotes tuberculosis pathogenesis. This effect of high levels of TNF-α is mediated by the induction of ROS and the subsequent modulation of mitochondrial cyclophilin D; this activates the acid sphingomyelinase-ceramide system resulting in necroptosis, release of mycobacteria from infected cells, and, thereby, proliferation and dissemination of the pathogens (Roca and Ramakrishnan 2013).

In addition to activation of the acid sphingomyelinase, ***Mycobacterium bovis* Bacillus Calmette-Guérin (BCG)** also rapidly activates the mammalian neutral sphingomyelinase/ceramide system (Li et al. 2016). Activation of the neutral sphingomyelinase in macrophages triggers a massive release of superoxide, which suppresses autophagy in BCG-infected macrophages in vitro and in vivo. ROS-mediated suppression of autophagy in macrophages allows BCG to survive within macrophages, as evidenced in experiments wherein expression of neutral sphingomyelinase was reduced by genetic knock-down or in which superoxide generation was pharmacologically inhibited. These manipulations restored autophagy in macrophages and allowed killing of intracellular BCG in vitro and in vivo (Li et al. 2016).

3.6 Sphingosine

Sphingosine is formed from ceramide by the activity of acid and neutral ceramidase and has been shown to possess direct antibacterial activity (Bibel et al. 1992; Arikawa et al. 2002; Fischer et al. 2013; Pewzner-Jung et al. 2014; Tavakoli Tabazavareh et al. 2016; Rice et al. 2017; Grassmé et al. 2017; Becker et al. 2017b). As discussed above, several studies have demonstrated that sphingosine levels are severely reduced in nasal, tracheal, and bronchial epithelium of patients and mice with cystic fibrosis compared to levels of this lipid in healthy airways (Pewzner-Jung et al. 2014; Tavakoli Tabazavareh et al. 2016; Grassmé et al. 2017). For a summary of the roles of ceramide and sphingosine in infections please see Fig. 2.

Sphingosine has a remarkable **antibacterial** potency against a variety of pathogens including *P. aeruginosa*, *Acinetobacter baumannii*, *Haemophilus influenzae*, *Burkholderia cepacia*, *Moraxella catarrhalis, Escherichia coli, Fusobacterium nucleatum, Streptococcus sanguinis, Streptococcus mitis, Corynebacterium bovis, Corynebacterium striatum*, or *Corynebacterium jeikeium* (Bibel et al. 1992; Arikawa et al. 2002; Fischer et al. 2013; Pewzner-Jung et al. 2014; Tavakoli Tabazavareh et al. 2016; Grassmé et al. 2017). Sphingosine kills these pathogens very rapidly, even at low micromolar concentrations. Studies that restored the levels of sphingosine in cystic fibrosis epithelial cells suggest that the high susceptibility of cystic fibrosis mice to airway infections with *S. aureus* and *P. aeruginosa* is modulated by the lack of sphingosine in the epithelial cells (Pewzner-Jung et al. 2014; Tavakoli Tabazavareh et al. 2016; Grassmé et al. 2017). The molecular mechanisms by which sphingosine molecules kill such a wide range of gram-positive and gram-negative bacteria are presently unknown (Fig. 2).

4 Research Needs

The central role of ceramide and sphingosine in infectious biology, in particular in the regulation of infection susceptibility in cystic fibrosis mice and patients, suggests it would be useful to modify these lipids in the lungs of cystic fibrosis patients in order to develop novel treatments for this disease. In particular, drugs that decrease ceramide levels and increase sphingosine concentrations in airway epithelial cells might be very useful to develop such treatments. Application of recombinant acid ceramidase that consumes ceramide to sphingosine might be an option. However, small molecules that activate endogenous acid ceramidase might be another option, but need to be developed.

While we understand some of the cellular functions of ceramide and sphingosine, many issues are still open. It remains to be determined how ceramide-enriched membrane domains cluster proteins. In addition, signaling properties of ceramide in other membranes, for instance, in lysosomes or mitochondria, are poorly characterized. Our knowledge about sphingosine is even less developed than for ceramide, and it needs to be determined how sphingosine acts in eukaryotic, but also prokaryotic membranes and whether it interacts with cellular signaling molecules in order to understand the biomedical importance of this membrane lipid.

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Part II

Lipases as Determinants of Pathogenicity

Participation of Bacterial Lipases, Sphingomyelinases, and Phospholipases in Gram-Negative Bacterial Pathogenesis

7

Lauren A. Hinkel and Matthew J. Wargo

Contents

Abstract

Lipid membranes are a ubiquitous property of cellular life. Within the context of infection, Gram-negative bacteria modify and/or destroy host membranes to access intracellular niches or release nutrients. They also modify their own membranes to survive host antimicrobial assault and antibiotic treatment. The key players in the membrane-altering events are secreted sphingomyelinases,

L. A. Hinkel · M. J. Wargo (✉)
Department of Microbiology and Molecular Genetics, Larner College of Medicine, College of Agriculture and Life Sciences, University of Vermont, Burlington, VT, USA
e-mail: lhinkel@uvm.edu; mwargo@uvm.edu; Matthew.Wargo@med.uvm.edu

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lipases, and phospholipases, whose enzymatic activities are important for pathogenesis in a number of Gram-negative bacterial species. Here, we present these lipid-active enzymes based on proposed pathogenic function to emphasize their biological roles during infection.

1 Introduction

Lipids, phospholipids, and sphingolipids are abundant in many environments, and thus it is not surprising that bacteria have evolved diverse enzymes for their metabolism. This chapter focuses on the contributions of these hydrolytic enzymes to Gram-negative bacterial pathogenesis.

The hydrolytic enzymes that attack lipids, phospholipids, and sphingolipids – sphingomyelinases, lipases, and phospholipases (SLPLs) – are categorized based on the bond at which they attack their substrate, summarized in Fig. 1. Lipases cleave lipid tails from glycerolipids, and many also function as broad-specificity esterases. The phospholipases implicated in virulence can be broken down into three categories: Phospholipases A (PLAs) hydrolyze the bond connecting a single acyl chain to a diacylphospholipid and are further classified according to their specificity for the position closest to the phosphate (PLA_2) or that furthest away (PLA_1). Phospholipases C (PLCs) hydrolyze the phospho-linked head group from the diacylglycerol backbone. Phospholipases D (PLDs) cleave the bond linking the head group moiety to the

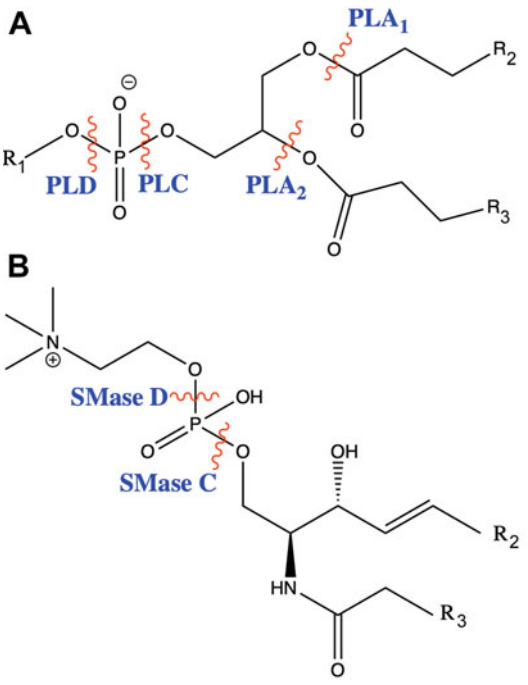

Fig. 1 Reactions catalyzed by the phospholipases and sphingomyelinases involved in Gram-negative bacterial pathogenesis. (**a**) Phospholipase bond hydrolysis (red wavy lines) depicted on a generalized phospholipid. R1 depicts the polar head group (serine, choline, ethanolamine, etc.), while R2 and R3 denote different acyl chains with varying lengths and saturation. (**b**) Sphingomyelinase bond hydrolysis noted on a generalized sphingomyelin. R2 denotes the remaining tail on the sphingosine moiety, while R3 denotes the fatty acid tail, the most common of which is palmitate

phosphate, generating phosphatidic acid. Sphingomyelinases are similar to PLCs and PLDs in their activity (Fig. 1), either removing the phospho-linked head group (SMase Cs) or hydrolyzing the bond linking the polar head group to the phosphate (SMase Ds). Some of these SLPLs show strong specificity for the head group and/or acyl tail, while others show a broader specificity. The enzymes we discuss in this chapter, as well as orthologs in other organisms, are summarized in Table 1.

There are many excellent and encyclopedic reviews of bacterial SLPLs that group these enzymes based on their biochemistry, structure, or substrate preference (we particularly recommend Flores-Diaz et al. 2016). Here, we present these enzymes based on the proposed pathogenic function of these enzymes to emphasize their biological roles in the pathogenic niche. This structure makes it clear that while some SLPLs have strong substrate specificity with defined roles in pathogenesis, many are enzymes evolved for nutrient acquisition that also happen to participate in pathogenesis. This dual function is most apparent when the phylogenetic distribution for many PLCs is examined showing orthologs broadly encoded and expressed in both pathogenic and nonpathogenic species.

We begin this chapter with SLPLs involved in tissue colonization, intracellular invasion and survival, and tissue destruction. From there we continue with those primarily involved in nutrient acquisition and finish up with those that target the bacterium's own membrane or those of competing bacteria in the pathogenic niche. The reader will note that some SLPLs make multiple appearances. When these duplications occur, we have attempted to do so only when in vitro and in vivo evidence directly suggests multiple roles. Finally, in addition to the pathoecological roles of these enzymes, the conditions that control their expression are likely important for their deployment during infection. Therefore, where known, we also discuss regulation of expression.

2 Host Tissue Colonization, Cell Invasion, and Intracellular Survival

For many SLPLs in Gram-negative bacteria, the precise molecular mechanisms leading to the pathogenic phenotype are not known, though likely pathways have been proposed. The notable exceptions to this have been the type III secretion system (T3SS) and type VI secretion system (T6SS) effectors acting on lipids, which have received much attention. We have attempted to organize these SLPLs by separating processes that are often interrelated; therefore some enzymes are likely functional in multiple processes.

2.1 Host Cell Invasion and Intracellular Survival

Legionella pneumophila is the Gram-negative poster child for the role of SLPLs in specific stages of the pathogenic process, all involved in invasion of phagocytes and subsequent intracellular survival (Banerji et al. 2008), and it will come up in other

Table 1 Summary of phospholipases, lipases, and sphingomyelinases produced by pathogenic Gram-negative bacteria

Phospholipases A					
Organism	Gene name	Phospholipase type	Substrate	Known/predicted function	Citations
Acinetobacter baumannii, ATCC 19606	*HMPREF0010_00411*	PLA	PC	PC degradation, growth on palmitate	Stahl et al. 2015
Acinetobacter baumannii	*pldA*	PLA	Membrane phospholipids, lysophospholipids	Bacterial membrane phospholipid recycling, maintaining PL asymmetry in bacterial membranes	Malinverni et al. 2009, May et al. 2018, Powers et al. 2018
Aeromonas salmonicida	Acyltransferase	PLA_2, lysophospholipase	Cholesterol, PC	Cell lysis through alteration of cholesterol/PC ratio in erythrocyte membranes	Buckley et al., 1982
Brucella melitensis	*bveA*	PLA_1	PE	Bacterial membrane alteration for increased intracellular survival	Kerrinnes et al. 2015
Campylobacter coli	*pldA*	PLA	PC	Cell-associated hemolysis	Grant et al. 1997
Escherichia coli	*pldA*	PLA	Membrane phospholipids	Outer membrane homeostasis, detergent stress response	May et al. 2018, Homma et al. 1984
Helicobacter pylori	*pldA1*	PLA	Membrane phospholipids	Associated with colonization of the gastric niche	Dorrell et al. 1999
Klebsiella pneumoniae, strain HS11286	$tle1^{KP}$, $tli1^{KP}$	PLA	Bacterial membrane phospholipids	Intraspecies competition, bacterial killing	Liu et al. 2017, Durand et al. 2014
Legionella pneumophila	*patD*	PLA and LPLA	Poly-3-hydroxybutyrate	Storage lipid metabolism, intracellular survival	Aurass et al. 2009

Legionella pneumophila	*plaB*	PLA_2, LPLA	PC, PG	Degradation of surfactant lipids, hemolysis, tissue colonization, and dissemination	Flieger et al. 2000, Holm et al. 1991, Wang and Notter 1998, Flieger et al. 2004
Legionella pneumophila	*patA/vipD*	PLA/LPLA	Phagosomal membrane phospholipids	Intracellular replication, phagosomal escape	Banerji et al. 2008, Bruggemann et al. 2006
Pseudomonas aeruginosa	*tle1*	PLA_1	Bacterial membrane phospholipids	Intraspecies competition	Sana et al. 2016, Russell et al. 2011, 2013
Pseudomonas aeruginosa	*exoU*	PLA_2	$Pi(4,5)P_2$	Host cell lysis via membrane destabilization/disruption of intracellular signaling, pathogen dissemination, tissue invasion	Sato et al. 2014, Lang et al. 2011, Yahr et al. 2006, Albus et al. 1997, Ramirez et al. 2012
Rickettsia prowazekii	Putative PLA	PLA	Sheep and human erythrocytes	Hemolysis	Winkler and Miller 1980
Salmonella enterica	*sseJ*	PLA	Cholesterol, glycerophospholipids	Intracellular survival and systemic infection	LaRock et al. 2012, Freeman et al. 2003, Ohlson et al. 2005, Lawley et al. 2006
Vibrio anguillarum	*Plp*	PLA_2	PC	Hemolysis, iron acquisition	Rock et al. 2006
Vibrio vulnificus	*plpA*	PLA_2	PC	Induction of necrotic death of epithelial cells contributes to tissue invasion and hemolysis	Jang et al. 2017
Yersinia enterocolitica	*yplA*	PLA	PC	Tissue colonization specifically Peyer's patches and mesenteric lymph nodes	Schmiel and Miller 1999
Yersinia pseudotuberculosis	*pldA*	PLA, acyl hydrolase, Smase	PC, SM	Gastroenteritis	Karlyshev et al. 2001

(continued)

Table 1 (continued)

Phospholipases A					
Organism	Gene name	Phospholipase type	Substrate	Known/predicted function	Citations
Phospholipases C					
Acinetobacter baumannii	*plc1, plc2*	PLC	PC	Hemolysis, iron acquisition	Fiester et al. 2016
Aeromonas hydrophila	*Apl-1*	Lipase, PLC non-hemolytic	Lecithin, NPPC	Host cell membrane damage	Ingham and Pemberton 1995
Burkholderia pseudomallei	*BPSS0067*	Putative PLC	Putatively PC and SM	Virulence, specifically in the liver	Tuanyok et al. 2006
Legionella pneumophila	*plcC*	PLC	PC	Intracellular survival, host cell lysis	Aurass et al. 2013
Legionella pneumophila	*plcA, plcB*	PLC	PG	Intracellular survival, host cell lysis	Aurass et al. 2013
Photobacterium damselae	*Ppp*	PLC	Erythrocyte membrane phospholipids	Hemolysis	Naka et al. 2007
Pseudomonas aeruginosa	*plcH*	PLC, SMase	PC, SM	Phosphate acquisition, host cell lysis	Shortridge et al. 1992, Wargo 2013, Berka et al. 1982, Stonehouse et al. 2002, Son et al. 2007
Pseudomonas aeruginosa	*plcN*	PLC, non-hemolytic	PC, PS (no activity on SM)	Phosphate utilization, secondary carbon source metabolism	Anba et al. 1990, Bleves et al. 2010, Balasubramanian et al. 2013
Pseudomonas aeruginosa	*plcB*	PLC	PE, PC, SM	Phospholipid chemotaxis	Barker et al. 2004
Sinorhizobium meliloti	*SMc00171*	PLC	PC	Phosphate acquisition, membrane remodeling	Zavaleta-Pastor et al. 2010, Krol and Becker 2004, Yuan et al. 2006

Phospholipases D					
Acinetobacter baumannii	*A1S_2989*	PLD	PC	Dissemination and proliferation in blood, heart, and liver	Jacobs et al. 2010
Klebsiella pneumoniae, strain Kp52.14	*pld1 (tle5)*	PLD	PG	Intraspecies competition, bacterial killing, disruption of cardiolipin synthesis, membrane homeostasis	Chen et al. 2018, Lery et al. 2014
Legionella pneumophila	*lpdA*	PLD	PI, IP_3, IP_4, PG	Intraphagosomal survival	Schroeder et al. 2015
Photobacterium damselae	*Dly*	PLD, SMase	SM	Hemolysis	Kreger et al. 1987, Cutter and Kreger 1990
Pseudomonas aeruginosa	*pldA, pldB*	PLD	Major glycerophospholipids	Cell internalization, host cell invasion	Wilderman et al. 2001, Schlam et al. 2015, Bleves et al. 2014, Jiang et al. 2014, Engel et al. 2011, Spencer and Brown 2015
Rickettsia prowazekii	*Pld*	PLD	Phagosomal membrane phospholipids	Phagosomal escape	Driskell et al. 2009, Whitworth et al. 2005
Lipases					
Legionella pneumophila	*lipA, lipB*	Lipase	Acylglycerols	Acylglycerol metabolism, utilization of alternative carbon sources	Aragon et al. 2002
Pseudomonas aeruginosa	*lipA*	Lipase	Fatty acids	Extracellular fatty acid metabolism, utilization of alternative carbon sources	Tielen et al. 2013, Son et al. 2007
Pseudomonas aeruginosa	*tle3, tle4*	Lipolytic, potentially broad-spectrum	Bacterial membrane phospholipids	Intraspecies competition	Sana et al. 2016, Russell et al. 2011, 2013

(continued)

Table 1 (continued)

Phospholipases A					
Organism	Gene name	Phospholipase type	Substrate	Known/predicted function	Citations
Sphingomyelinases					
Photobacterium damselae	*Dly*	PLD, SMase	SM	Hemolysis	Kreger et al. 1987, Cutter and Kreger 1990
Pseudomonas aeruginosa	*plcH*	PLC, SMase	PC, SM	Phosphate acquisition, host cell lysis	Shortridge et al. 1992, Wargo 2013, Berka et al. 1982, Stonehouse et al. 2002, Son et al. 2007
Yersinia pseudotuberculosis	*pldA*	PLA, acyl hydrolase, SMase	PC, SM	Gastroenteritis	Karlyshev et al. 2001

Abbreviations: *PC* phosphatidylcholine, *SM* sphingomyelin, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PI* phosphatidylinositol, *PI(4,5)*P_2 phosphatidylinositol 4,5-bisphosphate, *PI3P* phosphatidylinositol-3-phosphate, *PI4P* phosphatidylinositol-4-phosphoate, and *PS* phosphatidylserine

sections of this chapter. To initiate this process, there is evidence that *Legionella* PLC participates in altering PIP_3 signaling to facilitate host cell entry (Dowling et al. 1992). Once inside the host cell, survival within the phagosome is maintained by the Dot/Icm type IV secretion system (T4SS), which injects ~300 effector proteins into the host cell cytoplasm (Schroeder et al. 2015). One of these injected effectors is the phospholipase D, LpdA. A deletion of *lpdA* resulted in a decreased ability of *Legionella* to replicate in the murine lung (Schroeder et al. 2015). LpdA is localized to host cell membranes, specifically the phagosome in macrophages, and hydrolyzes phosphatidylinositol (PI), phosphatidylinositol-3-phosphate, phosphatidylinositol-4-phosphate, and PG to phosphatidic acid, which alters the membrane composition of endosomal vesicles and impacts vesicle trafficking.

Another *Legionella* phospholipase involved in host intracellular vesicle trafficking is the patatin-like protein PatA/VipD that has PLA and lysophospholipase A (LPLA) activity and impacts vesicular trafficking in yeast (Banerji et al. 2008). Many *L. pneumophila* phospholipases are implicated in the switch from intracellular replication to phagosomal escape, including *patA/vipD*, *patD*, *patE*, and *patI*, all of which are expressed 8 to 11 times higher during the replicative to transmissive phase (Bruggemann et al. 2006). Deletion of PatD and BdhA, a 3-hydroxybutyrate dehydrogenase located in the same operon as PatD, decreases infection ability in macrophages and amoeba. PatD's putative role in metabolism of the important storage lipid 3-hydroxybutyrate, predicted by PatD's localization in a BdhA containing operon, may contribute to its role in virulence (Lang and Flieger 2011).

The *L. pneumophila* phospholipases A are regulated both transcriptionally and posttranslationally. Transcriptional regulation is governed by RpoS and a two-component regulatory system, LetA/S, which positively regulate the PLA/acyltransferase and cell-associated PLA activity while negatively regulating secreted LPLA activity (Banerji et al. 2008). The secreted LPLA, PlaC, also appears to be activated by proteolytic processing by ProA (Banerji et al. 2005).

Other intracellular pathogens also use SLPLs at specific stages of the pathogenic process. The phagosomal escape phase of obligate intracellular pathogen *Rickettsia prowazekii*'s life cycle also involves a phospholipase D, Pld, which has predicted membranolytic activity and is transcribed at elevated levels during phagosomal escape (Whitworth et al. 2005; Driskell et al. 2009). The facultative intracellular pathogen *Brucella melitensis* uses alteration of its cellular envelope by BveA, which displays phospholipase A_1 activity against PE, as an intracellular survival strategy. A lower PE concentration in the cell envelope of *B. melitensis* is correlated with an increase in resistance to membrane disruption, and deletion of *bveA* causes a decrease in survival within macrophages after 4 hours (Kerrinnes et al. 2015). *Salmonella enterica* secretes a PLA, SseJ, via the T3SS whose lipolytic activity is induced after binding to the Rho GTPase RhoA (Freeman et al. 2003; Ohlson et al. 2005). SseJ localizes to the *Salmonella*-containing vacuole and is involved in intracellular survival and altering endosomal vesicle trafficking and is important for systemic infection in the mouse (Lawley et al. 2006).

P. aeruginosa is generally considered an extracellular pathogen but can invade host cells with the help of phospholipase D's PldA and PldB (Jiang et al. 2014). PldA

and PldB impact the phosphoinositol-3-kinase (PI3K)/Akt signaling system of host cells by binding to signaling protein Akt, potentially activating PI3K, which is involved in the uptake of large particles (Bleves et al. 2014; Schlam et al. 2015). Altering this signaling system leads to remodeling of the host apical membrane and enriched rafts of second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and actin, leading to an increase in bacterial uptake (Jiang et al. 2014). Perturbation of the PI3K/Akt system by *P. aeruginosa* contributes to invasion of epithelial, endothelial, and other non-phagocytic cells (Engel and Eran 2011).

2.2 Host Tissue Colonization, Tissue Destruction, and Modification of the Pathogenic Niche

Lysis of host cells leading to danger-associated inflammation and/or direct stimulation of inflammation leads to decreased barrier function, suppression of antimicrobial peptide and protein function, and, under certain conditions, hindrance of phagocytic function, all of which prevent effective clearance of bacterial pathogens and assists tissue colonization and persistence of infecting microbes (Shaver and Hauser 2004).

The *P. aeruginosa* phospholipase A_2, ExoU, causes significant host cell lysis after being injected into the host cell cytoplasm. ExoU, encoded within a pathogenicity island (Kulasekara et al. 2006), acts on phosphatidylinositol 4,5-bisphosphate PI(4,5)P_2, which is located in the inner leaflet of host cell plasma membranes. PI(4,5)P_2 is important in the maintenance of cell membrane integrity and cell-matrix/cell-cell interactions, physically interacting with focal adhesion molecules and adaptor proteins and also serving as a second messenger and for many signaling processes integral to organization of the cytoskeleton, motility, and adhesion. Degradation of PI(4,5)P_2 causes breakdown of signaling processes and physical disruption of cytoskeletal and matrix complexes, leading to destabilization of host cells and ultimately cell blebbing and cytolysis (Sato and Frank 2014). Additionally, cytolysis releases lysophosphatidylcholine (LPC), which increases the cell permeability of surrounding cells making them amenable to lysis or bacterial invasion (Lang and Flieger 2011). Transcription of *exoU* is under the regulation of ExsA, an AraC-family transcriptional activator, whereas ExoU secretion by the T3SS is regulated by low calcium concentrations and host cell contact (Yahr and Wolfgang 2006). The phospholipase domain of ExoU also contributes to pathogen dissemination and tissue invasion by aiding in traversal of epithelial tissues, specifically corneal tissue (Ramirez et al. 2012).

An emerging fish pathogen, *Photobacterium damselae* subsp. *damselae*, possesses a hemolytic phospholipase, damselysin (Dly), with PLD and sphingomyelinase activity against mouse, rat, rabbit, and damselfish erythrocytes and is located on the virulence-associated plasmid pPHDD1 (Kreger et al. 1987; Cutter and Kreger 1990). Dly and the pore-forming toxin PhlyP are both encoded on the pPHDD1 plasmid and are considered major virulence factors in infection of turbot (Terceti et al. 2016). In

addition to Dly, *P. damselae* possesses another hemolytic and extracellular phospholipase, Ppp (Naka et al. 2007).

Other SLPLs that appear to contribute to this mechanistic category that have not been as well studied are encoded by the genus *Aeromonas*. *Aeromonas hydrophila* possesses a non-hemolytic phospholipase C that may cause perturbation of host cells and release of arachidonic acid and activation of protein kinase C, contributing to the inflammatory response (Ingham and Pemberton 1995). A phospholipid/cholesterol acyltransferase with PLA_2 and lysophospholipase activity from *Aeromonas salmonicida* is able to destroy erythrocytes by altering the cholesterol and PC ratios in erythrocyte membranes ultimately leading to cell lysis. The activity of this acyltransferase is increased in the presence of albumin and human apolipoprotein A-1 (Buckley et al. 1982). Destruction of host cells allows for pathogen invasion into host tissues and dissemination of the organisms throughout the body, but persistence of opportunistic pathogens at the site of infection is another pathogenic approach aided by phospholipases.

Pulmonary surfactant, a mixture made up of mostly phospholipids, lowers airway surface tension allowing normal respiration and makes the distal airways a particularly lipid-rich environment. *L. pneumophila* possesses a phospholipase A with the ability to degrade pulmonary surfactant lipids, which may partly explain the increase in surfactant surface tension and resulting lung dysfunction in patients with *L. pneumophila* infection (Flieger et al. 2000). After incubation of concentrated cell culture supernatant from *L. pneumophila* with surfactant phospholipids, concentrations of PG and PC were both markedly decreased, while lysophosphatidylcholine (LPC) was increased, indicating that *L. pneumophila's* PLA was actively degrading the phospholipid content of the surfactant into inflammatory LPC. In addition to the cell-damaging inflammation and type I alveolar cell lysis that is caused by increased levels of LPC, the degradation of surfactant phospholipids caused a significant increase in the measurable surface tension of the surfactant. Similarly, the *P. aeruginosa* hemolytic PLC, PlcH, also degrades pulmonary surfactant lipids increasing surface tension (Wargo et al. 2011), which contributes to decreased lung function, ultimately leading to lowered bacterial clearance and a correlation to antibiotic susceptibility (Lanotte et al. 2003; Nguyen et al. 2007).

3 Acquisition of Host-Derived Nutrients

A key component of pathogenicity, appreciated since the late 1800s, is the ability to use the host as the sole source of nutrients (Pasteur 1878). Lipids represent a readily accessible nutrient source in all infection niches, and acquisition of lipid-associated or cell-sequestered nutrients is one of the most well-studied functions of SLPLs. The hydrophilic heads of phospholipids can be convenient sources of phosphate and small metabolites such as choline, serine, and ethanolamine, while the hydrocarbon tails and glycerol backbones can be effective reservoirs of carbon. This section is structured based on the nutrients directly or indirectly supplied by SLPL action.

3.1 Utilization of Alternative Carbon Sources

A significant source of free phospholipids and sphingolipids in the human host is lung surfactant. The composition of lung surfactant is 90% lipids, including phospholipids, sphingomyelin, and other fatty acids, and 10% protein (Glasser and Mallampalli 2012). *P. aeruginosa* uses the phospholipase *plcH* to exploit PC and sphingomyelin present in lung surfactant, breaking these down into choline-containing compounds, fatty acids, and ceramide (Wargo 2013a; LaBauve and Wargo 2014), for further metabolic degradation by enzymes such as LipA. The lipase *lipA* of *P. aeruginosa* plays a role in the uptake and metabolism of extracellular fatty acids. Kept in reserve in the exopolysaccharide of *P. aeruginosa* biofilms, and secreted in conjugation with alginate, LipA displays high extracellular lipase activity localized near the bacterial cell membrane, making metabolism of fatty acids more efficient (Tielen et al. 2013). When PC is supplied as the sole nutrient source, *lipA* is induced ~37-fold above expression levels observed during growth on citrate, indicating the importance of LipA in utilization of PC as a carbon source in nutrient-limited conditions (Son et al. 2007), partly under control of the CbrA/CbrB two-component system, which is involved in maintaining the carbon-nitrogen balance within bacterial cells when sub-optimal carbon sources are supplied (Abdou et al. 2011).

There have been two similarly named lipases, *lipA* and *lipB,* identified in *Legionella pneumophila* that act on mono-, di-, and tri-acylglycerols (Aragon et al. 2002). In addition to possessing lipases involved in metabolism of acylglycerols, *L. pneumophila* possesses a patatin-like enzyme with phospholipase A and lysophospholipase A activity, *patD*, that is co-transcribed with *bdhA*, a 3-hydroxybutyrate dehydrogenase. The role of these genes in host cell invasion was mentioned previously, but there is also direct evidence of their role in lipid metabolism. A mutant for *bdhA* and *patD* retains high levels of a primary energy storage lipid poly-3-hydroxybutyrate, indicating that these genes are involved in metabolism of this intracellular carbon pool. Loss of the *bdhA-patD* operon also resulted in a decrease in replication within host cells indicating the necessity of poly-3-hydroxybuytrate utilization and *patD*-dependent lipolysis to the intracellular survival of *L. pneumophila* (Aurass et al. 2009).

A highly conserved phospholipase A (PLA) of *A. baumannii* ATCC 19606 is predicted to be involved in growth on PC as a sole carbon source. This hypothesis is supported by the ability of *A. baumannii* to grow efficiently on palmitate, which is a metabolic product of PC degradation by PLAs. The putative *A. baumannii* PLA also has a VasI T6SS sequence, indicating that it may be secreted during infection to break down host PC into the utilizable carbon source of palmitate (Stahl et al. 2015).

Pathogenic Gram-negative bacteria can also take advantage of the nitrogen-containing compounds released from the hydrophilic heads of phospholipids such as choline, due to hydrolysis of SM and PC. In *P. aeruginosa*, *plcH* is both indirectly regulated by choline release, as discussed more fully below via Anr, and directly influenced by choline via the transcriptional regulator GbdR, which induces *plcH* transcription in response to the intermediates of choline metabolism, dimethylglycine and glycine betaine (Wargo et al. 2009).

3.2 Phosphate Acquisition

Phosphate is a much-coveted resource for bacteria, highlighted by a conserved global regulatory network dedicated to phosphate acquisition and maintenance (Santos-Beneit 2015). In *Pseudomonas aeruginosa* the phosphate two-component system *phoB-phoR* positively regulates the transcription of a hemolytic phospholipase C, *plcH,* in phosphate-limited conditions (Shortridge et al. 1992). *plcH* has PLC activity against host phosphatidylcholine (PC) and sphingomyelin (SM), freeing choline phosphate and diacylglycerol for further processing as nitrogen and carbon sources, respectively, while also allowing for liberation of phosphate in conjunction with the phosphorylcholine phosphatase, PchP (Massimelli et al. 2005). There are additional PLCs in *P. aeruginosa* whose roles in pathogenesis are less clear but that are regulated by PhoBR in a similar manner, including PlcN, which acts on host PC and phosphatidylserine (PS) and may prime the betaine-induced positive feedback loop that induces PlcH expression (Ostroff et al. 1990), and PlcB, which enhances chemotaxis up lipid gradients (Barker et al. 2004).

Phosphate-regulated phospholipases have also been identified in marine pathogens belonging to the *Vibrionaceae* family, including *Vibrio anguillarum* and *Vibrio vulnificus* that primarily infect fish but which can also cause severe pathology in immunocompromised humans (Yun and Kim 2018). Extracellular phospholipase activity produced by *V. anguillarum* was decreased in a mutant for *rpoS* (Ma et al. 2009). RpoS is a sigma factor associated with general stress response in bacteria and has been observed in other Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* to be increased in translation and transcription, respectively, under phosphate starvation, which links phospholipase activity in *V. anguillarum* to changes in phosphate concentration. In *V. vulnificus* strain ATCC 29307, phospholipase activity was observed to increase the highest above baseline in phosphate-depleted conditions. Conversely, phospholipase activity was repressed back to baseline activity in phosphate-replete conditions, indicating that the phospholipase(s) responsible are likely under the control of the *phoBR* phosphate-responsive two-component system (Oh et al. 2007).

3.3 Iron Acquisition

Iron is an essential resource that is competed for during infection, but much of the iron in the body is sequestered in cells or by soluble proteins. Bacteria and the host play tug-of-war for iron during infection with both parties employing multiple strategies to keep iron on "their side." Of the 1–2 mg of iron that the human body absorbs per day during digestion, 75% is incorporated into hemoglobin that is stored in red blood cells (RBCs) (Abbaspour et al. 2014). The ability to access the high amounts of iron stored in RBCs confers a significant advantage to infecting pathogens and supports growth and viability within the host. As such, hemolytic PLCs can be instrumental in RBC lysis and iron acquisition.

Lysis of host RBCs and other cells via cleavage of host cell membrane SM and PC is a direct contribution of *P. aeruginosa* PlcH to virulence. PlcH plays a role in

inhibition of angiogenesis and selective host cell death during infection (Vasil et al. 2009), and a mutant in *plcH* greatly decreases *Pseudomonas aeruginosa* strain KU2's growth rate on sheep erythrocytes, implicating *plcH* in erythrocyte lysis and acquisition of nutrients from RBCs (Kida et al. 2011). PlcH was originally discovered as a hemolysin and has selective cytotoxicity to endothelial cells, but is not cytotoxic to many other types of host cells including epithelial cells, macrophages, and HeLa cells (Vasil et al. 2009). Regulation of *plcH* expression occurs in an iron-dependent manner via the interplay between the ferric uptake regulator (Fur) and small noncoding RNAs (snRNAs) *prrF1* and *prrF2.* These snRNAs decrease the expression of many iron-responsive genes in heme-replete conditions, mostly via interaction with complimentary regions of target mRNA transcripts (Oglesby-Sherrouse and Vasil 2010; Reinhart et al. 2015). When iron is depleted, the Fur protein represses *prrF1* and *prrF2* expression, resulting in increased expression of *prrF*-targeted transcripts. *plcH* is among the various metabolism- and virulence-related genes impacted by *prrF1* and *prrF2* (Oglesby et al. 2008), which could be due to a direct or indirect effect on the *plcH* locus. PC cleavage by *plcH* liberates choline into *P. aeruginosa's* cytoplasm, which activates the transcriptional regulator Anr (Jackson et al. 2013), the homolog to *E. coli*'s well-studied Fnr. Although Anr primarily plays a role in bacterial adjustment to different oxygen conditions, a *P. aeruginosa anr* mutant showed decreased gene expression of the iron chelator pyochelin biosynthetic (*pchDCBA)* and transport genes (*pchEFHI*), indicating Anr's role in promoting iron acquisition and transport (Hammond et al. 2015).

Acinetobacter baumannii also possesses iron-regulated PLCs with specificity for PC, *plc1* and *plc2.* Both genes possess putative Fur box binding motifs 100 and 200 nt upstream of their transcriptional start sites, respectively (Fiester et al. 2016). The specificity of Plc1 and Plc2 for PC is demonstrated by a proportional increase in hemolysis of erythrocytes with an increase in the ratio of PC to phosphatidylethanolamine (PE) in the cell membrane, with the least amount of hemolysis seen on sheep erythrocytes, a moderate level on human erythrocytes, and a high level on horse erythrocytes (Fiester et al. 2016). There is an association between clinical hemolytic *Acinetobacter* strains and the *plc* genes, supporting a role of *plc1* and *plc2* in infection. A similar connection of plc genes to virulence was also seen with plant infection *Acinetobacter* strains (Fiester et al. 2016).

Finally, the causative agent of vibriosis in fish, *V. anguillarum*, possesses a phospholipase, Plp, with PLA_2 activity against PC that specifically lyses rainbow trout erythrocytes (Li et al. 2013). *plp* is located in a hemolysin gene cluster that contains other genes regulated by iron concentration (Rock and Nelson 2006).

4 Extracellular Survival within the Infective Niche

4.1 Modifying the Bacterial Outer Membrane

Antimicrobials pose a large threat to Gram-negative bacteria during infection. Their outer membranes serve as the first line of protection against antimicrobial attack, and the maintenance of the outer membrane is important to bacterial

survival within the host. Antimicrobials such as polymyxins and host antimicrobial peptides destabilize and alter the outer membrane, causing a disruption in the asymmetry of membrane phospholipids and lipopolysaccharide (LPS) or lipooligosaccharide (LOS). *Escherichia coli* and *A. baumannii* both possess an outer membrane localized PldA, a lipid-degrading enzyme, that helps to maintain the outer membrane integrity via degradation of misplaced phospholipids at the outer membrane (May and Grabowicz 2018; Powers and Trent 2018). During conditions of membrane stress, mutants of *pldA* are selected which lead to decreased membrane permeability (Thi Khanh Nhu et al. 2016; Powers and Trent 2018). PldA of *E. coli* similarly mediates outer membrane homeostasis and contributes to detergent resistance (Homma et al. 1984; May and Silhavy 2018). Pld1 can also contribute to endogenous membrane homeostasis in *Klebsiella* (Durand et al. 2014; Lery et al. 2014).

4.2 Killing of Co-infecting Bacteria

Polymicrobial infections are very common, especially in immune-compromised patients. To outcompete co-infecting microbes for nutrients and real estate within the host, Gram-negative bacteria have specific strategies focused on targeting and eliminating neighboring bacteria including the T6SS, contact-dependent inhibition, and others. Some of these strategies involve phospholipases. *Klebsiella pneumoniae* strain HS11286 possesses two phospholipases, Tle1 and Tli1, which display phospholipase A activity and are secreted by the T6SS into the periplasm of neighboring bacterial cells (Durand et al. 2014; Liu et al. 2017). T6SS effector lipases that have been characterized in other Gram-negative pathogens such as *P. aeruginosa* and *Vibrio cholera* can be induced by changes in temperature and pH, presence of chitinous substrates, or sub-inhibitory concentrations of antibiotics (Jones et al. 2013; Borgeaud et al. 2015; Liu et al. 2017). The induction of these T6SS effectors in the absence of other bacteria may indicate that these effectors can be triggered to act directly on host cells. Another phospholipase putatively involved in intraspecies killing by *K. pneumoniae,* in hyper virulent strain Kp52.14, is Pld1. Pld1 is active on phosphatidylglycerol (PG) which is abundant in bacterial cell membranes and participates in a synthesis reaction with cytidine diphosphate diacylglycerol to create cardiolipin, which is an important structural component of both inner and outer membranes (Chen et al. 2018). This phospholipase is also positioned in a T6SS operon making it possible for Pld1 to alter the PG concentration of neighboring bacterial cell membranes and cause cell lysis. The lack of a discernible immunity protein paired with Pld1 categorizes it as a T6SS lipase effector (Tle) but not part of an effector-immunity pair (Lery et al. 2014). The activity of Pld1 on the cardiolipin synthesis substrate PG, lack of paired immunity protein, and location in T6SS operon may also suggest that this Tle is directly pathogenic toward host cells, specifically targeting cardiolipin-rich membranes such as those of the mitochondria. *P. aeruginosa* harbors similar effector-immunity pairs injected via the H1-T6SS (Russell et al. 2011). Some of

these T6SS lipase effectors include Tle1, which displays phospholipase A_1 activity, and Tle3 and Tle4 which are both characterized as lipolytic (Sana et al. 2016).

5 Research Needs

The roles of individual SLPLs in Gram-negative bacterial pathogenesis have been relatively well studied, but there exist a few specific areas where additional focus could yield novel insights.

5.1 Molecular Functions of Extracellular SLPLs

Much of the work on extracellular SLPLs secreted by extracellular pathogens has focused on tissue damage and stimulation of inflammation, and rightly so as these have important implications for disease pathogenesis. Tissue damage is certainly at play during times of high bacterial load, whether through experimental infection or later in the natural infective process. However, we question the extrapolation of SLPL function at high concentrations to their role during infection initiation when bacterial cell numbers, and thus local SLPL concentrations, are much lower. We have postulated before (Wargo 2013b), as have others (Kendall and Sperandio 2016), that head groups released by PLC, PLD, or SMase activity might function as signals related to detection of the host. Because many of these enzymes appear to be subject to positive feedback induction stimulated by the head group or an immediate metabolite, head group release by various SLPLs can function as a measure of return on investment for these extracellular enzymes. The contribution of this kind of active host detection to infection progression has not yet been demonstrated to our knowledge.

5.2 Functional and Population Level Redundancies of SLPLs

The best studied SLPLs are those with distinct phenotypes either at a particular time during infection or step of cellular invasion (the latter particularly for *Legionella*). For organisms that encode multiple SLPLs, especially within the same class (e.g., multiple PLCs, SMases, etc.), functional redundancy likely prevents easy detection of phenotypes from mutants in single genes. This tendency to miss redundant enzymes happens at an even more rapid pace now, as transposon sequencing (Tn-Seq), though very powerful, cannot identify enzymatic functions encoded by redundant members. Tn-Seq also misses enzymes whose benefits are provided as a community good, as the abundant cells that are not mutant in the process produce sufficient activity to externally complement the few mutants for that process in the population. It is tempting to speculate that the limited appearance of lipases in this chapter may be due to multiple redundant lipases and lipid-active esterases whose broad and

overlapping specificities prevent easy phenotypic assessment of mutations in individual genes. Examination of this question will require a focused effort on a few pathogens to identify, generate, and characterize strains with all secreted lipases deleted.

5.3 Regulation of SLPLs

Above, we discussed some of the regulation that has been worked out for enzymes in this group, including ExoU, PlcH, and LipA in *P. aeruginosa*, and the SLPLs in *L. pneumophila*. Induction conditions are known for many others described here, but often the particular regulatory pathways are not known. Some, like the phospholipases in *Vibrio* spp., are probably under control of alternative sigma factors. Others, like PlcH of *P. aeruginosa*, have been shown to have expression regulated by their downstream products (e.g., phosphate, choline) or required nutrients liberated by their enzymatic activity (e.g., iron). Many others have simply not received substantial research attention. Even when regulators have been identified, their direct role in regulation has often not been determined.

6 Conclusion

Enzymes in the SLPL group play critical roles in the virulence of many Gram-negative bacteria but are likely more broadly important than we currently appreciate. In addition to the research needs described above, lipids are the physical and chemical interface between the bacterial and eukaryotic kingdoms, and SLPLs are likely to play major roles beyond pathogenesis, particularly in microbiota interactions and symbioses.

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Participation of Bacterial Lipases, Sphingomyelinases, and Phospholipases in Gram-Positive Bacterial Pathogenesis

8

Howard Goldfine

Contents

Abstract

A growing number of both Gram-positive and Gram-negative bacteria are now known to produce lipases, sphingomyelinases, and phospholipases, and many of these enzymes have been shown to be involved in bacterial pathogenesis. In this review, lipases, sphingomyelinases, and phospholipases from Gram-positive bacteria are described and their roles in furthering the entry into and growth within host cells and tissues are discussed. The importance of phospholipases and sphingomyelinases in infections caused by *Clostridium perfringens* and *Listeria monocytogenes* has been demonstrated by many studies on the wild type and mutant forms of these enzymes. The significance

H. Goldfine (✉)
Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
e-mail: goldfinh@pennmedicine.upenn.edu

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of lipases, sphingomyelinases, and phospholipases for other infectious organisms including *Staphylococcus aureus*, *Bacillus anthracis*, and *Mycobacterium tuberculosis* is also discussed.

1 Introduction

Through the work of Macfarlane and Knight, the α-toxin of *Clostridium perfringens* was the first bacterial toxin to be recognized as an enzyme. These investigators showed that a material prepared from the growth supernatant of this organism was capable of hydrolyzing lecithin (phosphatidylcholine) to give rise to phosphocholine and diacylglycerol. This lecithinase activity was inhibited by specific antitoxin (Macfarlane and Knight 1941). The importance of *C. perfringens* (previously *C. welchii*) in frequently fatal gas gangrene infections resulting from war wounds provided impetus to understanding the mechanism of gangrene pathogenesis.

During the 77 years since this pioneering work, a large number of lipases and phospholipases have been identified as secreted bacterial products, and many of these are considered to be involved in pathogenesis. In this chapter, I will provide examples of some of the best studied members of this group, especially those for which there is considerable understanding of their role in disease. A recent thorough review provides information on the structures, activities, and importance of bacterial sphingomyelinases and phospholipases in the pathogenesis of a wide variety of bacteria (Flores-Diaz et al. 2016). Interactions of these enzymes with membranes and the physical effects produced by the products of their activity have also been thoroughly reviewed (Goni et al. 2012).

2 Classification of Bacterial Lipases, Phospholipases, and Sphingomyelinases

Lipases are enzymes that cleave monoacyl-, diacyl-, and triacylglycerols. They cleave at the ester bond between the fatty acyl chains and the glycerol backbone. Although the cleavage is at the same bonds, they are distinct from phospholipases A and B, which are specific for phospholipids. Lipases can be specific for cleavage at a certain position on the glycerol backbone (Rezanka et al. 2017).

Phospholipases are categorized according to the position of action on the phospholipid molecule. Phospholipase A_1 cleaves at the *sn*-1 position of the backbone, phospholipase A_2 cleaves at the *sn*-2 position, phospholipase B cleaves at both the *sn*-1 and *sn*-2 position, phospholipase C (PLC) cleaves between the glycerol backbone and the phosphate, and phospholipase D (PLD) cleaves between the phosphate and the polar head alcohol (Fig. 1). Phospholipases C and D are phosphodiesterases. As indicated in Fig. 1, cleavage by PLC results in release of diacylglycerol and a water-soluble phospo-alcohol ester; cleavage by PLD results in release of phosphatidic acid and the head group alcohol, which may be ethanolamine, choline, serine,

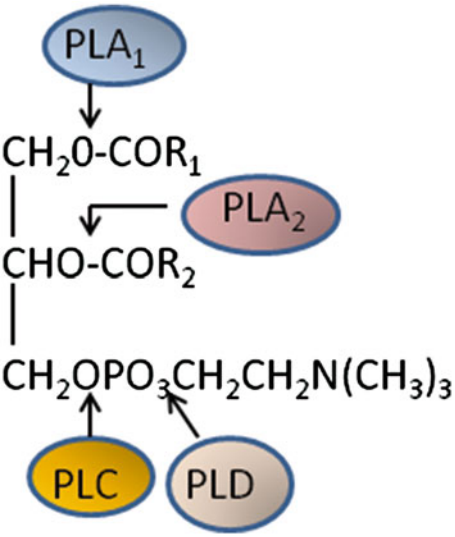

Fig. 1 The site of action of phospholipases on phosphatidylcholine is shown. The two acyl chains are attached at the *sn*-1 and *sn*-2 positions of the glycerol backbone, and phosphocholine is attached at the *sn*-3 position. PLB acts at both the *sn*-1 and *sn*-2 positions

inositol, or other small primary alcohols. Among the bacterial phospholipases, there are zinc-metallophospholipases, which have broad specificity, sphingomyelinases, and enzymes that are specific for phosphatidylinositol, some of which are active on glycosylphosphatidylinositol (GPI)-anchored proteins. The thermodynamics, membrane insertion mechanisms, and significance of hydrophobic and electrostatic interactions of these enzymes with their membrane targets have recently been reviewed (Roberts et al. 2018). These will be discussed in the context of individual bacterial pathogens.

3 Lipases, Sphingomyelinases, and Phospholipases of Gram-Positive Pathogens

3.1 *Clostridium perfringens*

Clostridium perfringens, a Gram-positive, spore-forming, anaerobic bacterium, is widely present in nature including decaying vegetation, soil, and in animal intestinal tracts. It is the cause of a common form of food poisoning, which is caused by an enterotoxin. Infections by *C. perfringens* are characterized by myonecrosis, intravascular leukocyte accumulation, and thrombosis. Among a number of toxins, *C. perfringens* secretes a PLC (α-toxin, CpPLC), which preferentially cleaves phosphatidylcholine and sphingomyelin. It also hydrolyzes phosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol (Urbina et al. 2011). The importance of CpPLC as a virulence factor was demonstrated by mutagenesis of its active site resulting in loss of ability to produce gas gangrene (myonecrosis) (Awad et al. 1995; O'brien and Melville 2004). Immunization with the C-terminal

domain of CpPLC prevented lethal infection and restricted tissue injury (Stevens et al. 2004). In addition to α-toxin, *C. perfringens* secretes a pore-forming toxin, perfringolysin O (PFO). Escape of *C. perfringens* from the phagosomes of macrophage-like J774–33 cells and mouse peritoneal macrophages was mediated by either PFO or CpPLC (O'Brien and Melville 2004).

The multiple roles of CpPLC in the pathogenesis of gas gangrene have been reviewed (Flores-Diaz and Alape-Giron 2003), and the multifarious effects of CpPLC on cells and tissues have been recently summarized (Flores-Diaz et al. 2016). Several of these effects can be attributed to the activation of host PKC through the formation of diacylglycerol, a lipid second messenger, by direct action on host membranes, or through activation of endogenous PLC through a pertussis toxin-sensitive GTP-binding protein, resulting in stimulation of rabbit neutrophil adhesion (Ochi et al. 2002). Alpha-toxin causes cell death in many cell types with enhanced toxicity for cells deficient in gangliosides (Flores-Diaz et al. 2005).

At sublytic concentrations, α-toxin is internalized and activates the MEK1/2, Erk 1/2, and NF-κB pathways resulting in the production of reactive oxygen species (ROS) and cell death (Monturiol-Gross et al. 2014). Gas gangrene is characterized by accumulation of neutrophils of the vascular endothelium at the margins of necrosis. Activation of the ERK1/2/NF-κB and the p38 MAPK pathways induces release of IL-8, which activates migration and binding of neutrophils (Oda et al. 2012b). Recombinant CpPLC induced platelet aggregation, resulting in thrombosis and decreased blood flow (Bryant et al. 2000).

Alpha-toxin was also found to inhibit erythroid differentiation in mouse bone marrow cells. The differentiation of human K562 erythroleukemia cells was negatively affected by CpPLC (Takagishi et al. 2017). In general, these findings are consistent with a model in which the activity of CpPLC is not confined to simple host cell cytolysis. Many of its effects are related to its engagement with various host signaling pathways.

3.2 *Listeria*

3.2.1 *Listeria monocytogenes*

L. monocytogenes (Lm), a Gram-positive, rod-shaped bacterium, is the cause of a potentially fatal food-borne infection which predominantly affects immunocompromised individuals. Among this group, pregnant women and the elderly are frequent targets (Lorber 2007; Radoshevich and Cossart 2018). It enters the blood stream through the intestinal epithelium and lymph nodes and then invades target organs such as the liver, spleen, and the brain stem. In tissues, it can enter non-phagocytic cells using Internalin A (InlA) and Internalin B (InlB), surface proteins which bind to cell membrane receptors E-cadherin and Met, the receptor for hepatocyte growth factor, thus promoting internalization. Lm then enters a phagocytic vacuole from which it is able to escape within minutes (Gaillard et al. 1987; Tilney and Portnoy 1989; Goldfine and Marquis 2007). Three major virulence factors play a role in this process. Listeriolysin O (LLO), a cholesterol-dependent pore-forming hemolysin, is

essential for escape (Gaillard et al. 1986; Kathariou et al. 1987; Portnoy et al. 1988). Two phospholipases, a phosphatidylinositol-specific phospholipase C (PI-PLC, PlcA) (Mengaud et al. 1991a; Leimeister-Wächter et al. 1991; Camilli et al. 1991) and a broad-range Zn^{2+} metallophospholipase C/sphingomyelinase (PC-PLC, PlcB) (Geoffroy et al. 1991; Smith et al. 1995; Zückert et al. 1998), facilitate escape, but their roles are cell-type dependent. In professional macrophages, a deletion in *plcA*, results in a reduction of approximately 40% in the number of bacteria that are released into the cytosol. Deletion of *plcB* did not affect escape in these cells (Camilli et al. 1993; Smith et al. 1995). In Henle 407 human epithelial cells, the broad-range PLC is able to promote escape from the primary vacuole in the absence of LLO (Marquis et al. 1995) (Fig. 2).

The genes for LLO, PlcA, and PlcB are in a single regulon under the positive control of PrfA (Leimeister-Wächter et al. 1990; Mengaud et al. 1991b; Freitag et al. 1993; Miner et al. 2007). Deletion of *plcA* resulted in a small increase in mouse LD_{50}, and deletion of *plcB* led to an approximately 20-fold increase; however, deletion of both phospholipase genes led to an increase of approximately 500-fold increase in mouse LD_{50}, a clear indication of overlapping roles for these

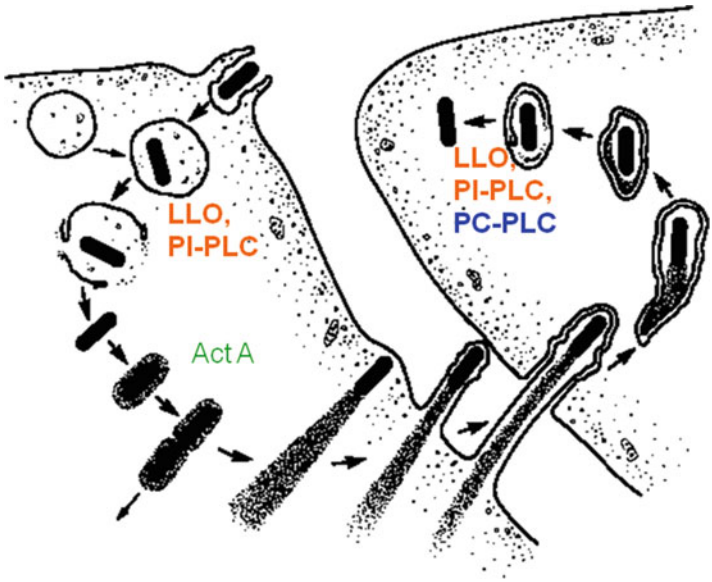

Fig. 2 The intracellular growth of *Listeria monocytogenes* in a macrophage. After uptake, Lm is found in a phagocytic vacuole from which it escapes into the cytosol. Listeriolysin O (LLO) is required and Lm PI-PLC facilitates escape. Once in the cytosol, Lm is propelled through the cytosol, promoted by the Lm protein ActA which is needed for the formation of the polymerized actin tails. Some bacteria produce protrusions that are engulfed by neighboring cells. The bacteria are then surrounded by a double membrane. The one closest to the bacterium will have phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol as the predominant lipids. Both phospholipases and LLO play roles in dissolution of the double-membrane vacuole. (Modified from Tilney and Portnoy 1989 with kind permission from the Journal of Cell Biology)

phospholipases in this murine model of infection (Smith et al. 1995). In a murine cerebral listeriosis model, deletion of *plcB* leads to reduced virulence. There was delayed intracerebral spread of the Δ*plcB* mutant strain, indicating that cell-to-cell spread is an important factor in listerial brain infections (Schlüter et al. 1998). When Lm moves from cell to cell, it becomes encased in a double-membrane vacuole (Fig. 2). It has been proposed that activity of the two phospholipases is needed for the dissolution of the inner membrane of the double-membrane vacuole, and the activity of LLO is required for disruption of the outer membrane (Alberti-Segui et al. 2007).

Immediately downstream from *hly*, the gene for LLO in the Lm chromosome, is *mpl*, encoding a zinc-dependent metalloprotease (Mpl). This protein is needed for processing of the secreted inactive form of PC-PLC to form the mature active form (Poyart et al. 1993). Upon cell-cell spread, decreased vacuolar pH leads to Mpl activation of proPC-PLC, resulting in bacterial release of active PC-PLC (Marquis and Hager 2000). Mpl maturation is similarly pH-sensitive (Forster et al. 2011). pH regulates translocation of PC-PLC across the cell wall, and this process is also pH-sensitive (Yeung et al. 2005, 2007). The activity of recombinant PlcB is also pH-sensitive with optimal activity below pH 7.0 (Huang et al. 2016). Compartmentalization of PlcB activity achieved by making its activation and translocation optimal at reduced pH is critical for Lm virulence suggesting that unregulated activity of this broad-range PLC can be harmful to the host cell (Yeung et al. 2007).

PlcA was identified based on its homology to previously studied PI-PLCs from *Bacillus cereus* and *Bacillus thuringiensis* (Mengaud et al. 1991a; Camilli et al. 1991; Leimeister-WÄchter et al. 1991). The Lm protein, like the classical PI-PLC from *B. cereus*, has a modified TIM barrel structure with a distorted ($\beta_8\alpha_6$) fold (Heinz et al. 1996; Moser et al. 1997; Roberts et al. 2018). It is highly specific for phosphatidylinositol (PI) and does not cleave phosphorylated forms of PI (PI-4-P, PI-4,5-bisphosphate) (Goldfine and Knob 1992). Unlike the previously discovered PI-PLCs of *B. cereus*, *B. thuringiensis*, and *Staphylococcus aureus*, it displays very low activity on glycosyl-PI-anchored membrane proteins (Gandhi et al. 1993; Wei et al. 2005b). LmPI-PLC exerts its effect prior to entry of bacteria into J774 macrophage-like cells. Elevation of intracellular calcium and activation of PKCδ occur before entry, and these consequences of PI-PLC activity decrease the rate of entry of bacteria. LLO is required for PI-PLC-dependent intracellular signaling (Wadsworth and Goldfine 1999, 2002). Once the bacteria are internalized and contained within the primary phagocytic vacuole, LmPI-PLC encounters the lipids of the external leaflet of the plasma membrane, phosphatidylcholine (PC), and sphingomyelin, for which it has little affinity. The formation of pores at reduced pH in the acidified phagosome by LLO presumably permits PlcA to enter the cytosol where it rapidly finds negatively charged lipids in the inner leaflet of the plasma membrane. The high ionic strength of the cytosol activates PlcA for its activity on PI (Chen et al. 2009; Goldfine and Knob 1992).

The minimal activity of LmPI-PLC on GPI-anchored proteins is ascribed to the absence of a small beta-strand (Vb), which is present in *B. cereus* PI-PLC (BcPI-PLC) and is absent in the enzyme from Lm. The Vb beta-strand in BcPI-PLC forms

contacts with the glycan linker of GPI anchors, which presumably increases its activity on GPI-anchored proteins. Deletion of the Vb beta-strand from BcPI-PLC resulted in weakened activity on GPI-anchored proteins and improved the efficacy of BcPI-PLC in promoting escape of Lm from the phagocytic vacuole and increased the virulence of Lm with this modified BcPI-PLC compared to that of Lm expressing intact BcPI-PLC (Wei et al. 2005b).

Cleavage of PI leads to the formation of diacylglycerol (DAG) a lipid second messenger. Human neutrophils are activated by LLO and LmPI-PLC. Phosphoinositide hydrolysis and the respiratory burst were provoked by a recombinant *Listeria innocua* strain producing LmPI-PLC (Sibelius et al. 1999; Lam et al. 2011). In addition to DAG, the action of Lm PlcB on sphingomyelin produces ceramide which acts as a second messenger leading to persistent NF-kappa B activation, increased expression of E-selectin, and increased rolling/adhesion of polymorphonuclear leukocytes (PMN) (Schwarzer et al. 1998). The application of LLO and LmPI-PLC to human endothelial cells resulted in phosphatidylinositol hydrolysis and the generation of diacylglycerol (Sibelius et al. 1996). As noted above, within minutes of addition of wild-type Lm to J774 macrophage-like cells, a series of calcium elevations occurs at the same time as activation of PKC δ and PKC β. These signals are dependent on the presence of LLO and PI-PLC. LLO and PlcB synergize to induce surface expression of FasL on T cells. Calcium elevation appears to be needed for FasL upregulation (Zenewicz et al. 2004).

When mouse bone marrow-derived macrophages with a deletion in PKC β were infected with Lm, the escape of bacteria from the primary phagosome occurs, but it no longer dependent on the action of PlcA (PI-PLC). In these cells, escape is diminished in the absence of PlcB, which has no effect on escape in wild-type bone marrow-derived macrophages (Poussin et al. 2009). These results support the concept that the effects of LmPI-PLC on the ability of Lm to survive and grow in the macrophage are dependent on host PKC β. When PI-PLC from Lm is replaced with that from *B. cereus*, which binds to and cleaves GPI-anchored proteins, escape from the primary phagocytic vacuole of macrophages cells is similar to that shown by Lm with a deletion in *plcA* (Wei et al. 2005b).

Lm spreads from cell to cell by means of host-derived actin-based motility. Actin-based propulsion produces protrusions from the infected cell's plasma membrane which are engulfed by neighboring cells (Tilney and Portnoy 1989) (Fig. 2). In the newly infected cell, Lm is surrounded by a double-membrane vacuole. The inner monolayer of the membrane closest to the bacterium is derived from that of the plasma membrane. It contains phosphatidylethanolamine, phosphatidylserine, and PI. PlcA thus finds its substrate and can release DAG once the double membrane is lysed by PlcB and other players including LLO (Alberti-Segui et al. 2007).

Intracellular bacteria can be targeted by the autophagy machinery while in phagocytic vacuoles or free in the cytosol (Deretic et al. 2013; Rich et al. 2003). Lm PLCs were implicated in autophagy evasion in fibroblasts (Py et al. 2007) After escape from the primary vacuole of macrophages, Lm is targeted by the autophagic machinery, which is present in several forms. There is increasing evidence that the phospholipases C, especially PlcA, along with ActA are involved in avoidance of

autophagy in macrophages. Autophagy requires phosphatidylinositol 3-phosphate (PI3P), and one mechanism by which PlcA assists in resistance of Lm to autophagy is through the reduction of membrane PI, a needed substrate for PI3P formation, thus inhibiting the maturation of preautophagosomal structures (Tattoli et al. 2013; Birmingham et al. 2007, 2008; Mitchell et al. 2015). Avoidance of autophagy mediated by PlcA and ActA is required for the growth of Lm in macrophages. It appears that xenophagy was needed to impede the growth of Lm and that the PLCs were involved in avoidance of this macroautophagic process (Mitchell et al. 2018) (Fig. 3).

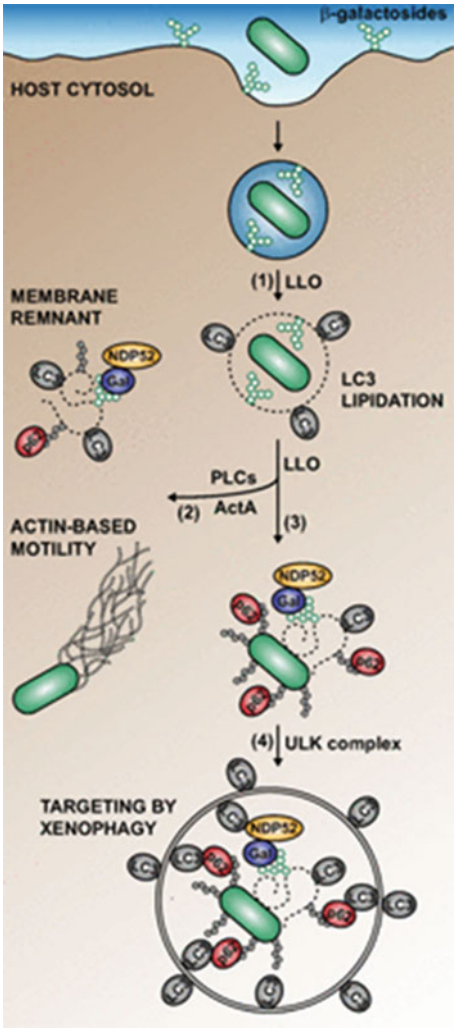

Fig. 3 The interaction of *L. monocytogenes* with the host autophagy machinery during a macrophage infection. Listeriolysin O (LLO) promotes lipidation of LC3 on the phagosome. Bacteria that express ActA and the PLCs interfere with autophagy, permitting replication in the cytosol. The figure depicts the possible association of Lm with fragmented vacuoles which are marked with galectins (Gal) and ubiquitin (UB). Lm is proposed to be targeted to xenophagy with participation of the ULK complex. (Reproduced from Mitchell et al. 2018 with kind permission of the National Academy of Sciences, USA)

3.2.2 *Listeria ivanovii*

In addition to orthologs of LmPI-PLC and the broad-range PLC (PlcB), *Listeria ivanovii*, a ruminant pathogen, produces a specific sphingomyelinase (SmcL) (Gonzalez-Zorn et al. 2000). It is of interest that this organism displays tropism for ruminants and that ruminant red blood cells are characterized by the presence of high concentrations of sphingomyelin. If this is also true of the cell membranes and phagocytic vacuoles of other ruminant cell types, the production of this extra phospholipase with specificity for sphingomyelin may be important for the ability of *L. ivanovii* to infect these animals (Vázquez-Boland et al. 2001). The role of SmcL in virulence was explored by gene disruption. These experiments confirmed a need for sphingomyelinase in disruption of the primary phagosome in the bovine epithelial-like MDBK and J774 macrophage-like cell lines. Furthermore, the LD_{50} of the mutant strain was four times higher than that of wild-type *L. ivanovii* in a murine model of infection (Gonzalez-Zorn et al. 2000).

3.3 *Bacillus anthracis*

Three species of Bacillus, *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*, are closely related. Each has a PI-specific PLC, which is active on PI- and GPI-anchored proteins, a PC-PLC with activities on a broad spectrum of glycerol-phospholipids, and a sphingomyelinase. The PI-PLCs of these three species have >94% amino acid identity (Read et al. 2003; Volwerk et al. 1989). The ability of *B. cereus* PI-PLC (BcPI-PLC) to release alkaline phosphatase from tissues was central to the discovery of GPI-anchored proteins (Ikezawa et al. 1976; Low and Finean 1977).

B. cereus is an opportunistic pathogen, and secretion of PI-PLC appears to be important in its ability to cause disease (Callegan et al. 2002a, b). *B. cereus* SMase also contributes to its virulence. When the gene for BcSMase was inserted into a strain without this gene, the transformant grew in vivo in a mouse infection, whereas a transformant carrying a gene for an inactive enzyme did not grow in mice. When treated with BcSMase, mouse macrophages produced less H_2O_2 in response to activation by peptidoglycan (Oda et al. 2012a, 2014).

B. anthracis, the causative agent of anthrax, can infect through the skin or by inhalation of spores from infected animals. The resulting septicemia leads to production of a lethal toxin. *B. anthracis* secretes a PI-PLC, a PC-PLC, and a sphingomyelinase, which are induced under anaerobic conditions (Klichko et al. 2003). The importance these phospholipases for *B. anthracis* infectivity was tested in a murine model of inhalation anthrax. Deletion of any one of the three phospholipases had essentially no effect on infectivity. Deletion of all three resulted in attenuation of virulence for mice. The PLC-null strain was unable to grow in association with macrophages (Heffernan et al. 2006).

B. anthracis expresses a pore-forming cholesterol-dependent cytolysin, anthrolysin O (ALO) (Shannon et al. 2003). Deletion of ALO alone had no effect on the virulence of *B. anthracis* for mice. However, combinations of ALO deletion

with deletions of one or more of the PLCs resulted in reduced virulence in both tissue cultures and in mice, indicating that these toxins have overlapping roles in the pathogenesis of *B. anthracis* (Heffernan et al. 2007). The ability of *B. anthracis* PI-PLC to cleave GPI-anchored proteins has been implicated in down-modulation of the immune response, thus allowing this pathogen to survive in the mammalian host. Treatment of murine dendritic cells with *Bacillus*, but not *Listeria* PI-PLC, which has very low activity on GPI-anchored proteins, inhibited the activation of dendritic cells by Toll-like receptor (TLR) ligands. In addition, infection of mice with Lm expressing *B. anthracis* PI-PLC resulted in reduced antigen-specific CD4 T cell response. These data indicate that *B. anthracis* PI-PLC down-modulates DC function and T cell responses, mediated by cleavage of GPI-anchored proteins which are important for DC activation through TLR (Zenewicz et al. 2005). Since many immune receptors are GPI-anchored proteins, there are likely to be other examples of such downregulation of innate immunity (Roberts et al. 2018).

When *B. anthracis* PI-PLC was expressed in *L. monocytogenes* in place of LmPI-PLC, the ability of the hybrid strain to escape from the primary vacuole was greatly reduced compared to wild-type *L. monocytogenes*, and the ability to spread from cell to cell, which requires escape from the secondary double-membrane vacuole, was also diminished. The cytotoxicity of a *L. monocytogenes* strain expressing both ALO and BaPI-PLC was less than that of the strain expressing both ALO and LmPI-PLC. These toxins appear to have coevolved to protect the host cell while promoting growth of each pathogen (Wei et al. 2005a).

3.4 *Staphylococcus aureus*

The β-toxin of *Staphylococcus aureus*, encoded by *hlb*, is a sphingomyelinase toxic to human keratinocytes (Huseby et al. 2007), which inhibits interleukin-8 expression, thus decreasing the transendothelial migration of neutrophils (Tajima et al. 2009) and contributing to escape from the phagosome of human epithelial and endothelial cells (Giese et al. 2011). It strongly stimulates biofilm formation in a rabbit model of endocarditis presumably through forming covalent cross-links in the presence of DNA independent of its sphingomyelinase activity (Huseby et al. 2010).

4 Phospholipases of *Mycobacterium tuberculosis* and Related Pathogens

Tuberculosis is one of the leading causes of disease caused by infectious organisms resulting in approximately 1.7 million deaths annually. The World Health Organization estimates that 558,000 people are infected with multidrug resistant *Mycobacterium tuberculosis* (Mtb). It is a leading cause of death among those infected with HIV.

The lipases of Mtb belong to the alpha/beta hydrolase fold superfamily (Johnson 2017). Of these homologs, the Mtb genome contains at least two genes encoding

PLA_2. One of these, encoded by *cut4*, is cytotoxic to macrophages (Schue et al. 2010; Dedieu et al. 2013). Orthologous genes are found in several virulent mycobacteria (Dedieu et al. 2013). These findings suggest, but do not conclusively show, that Cut4 plays a role in Mtb infectivity.

Remarkably some *M. tuberculosis* isolates can carry as many as four genes (*plcABCD*) encoding significantly homologous proteins with PLC/SM activity. All these genes encode orthologs of the PlcH and PlcN of *P. aeruginosa* (Stonehouse et al. 2002), despite the fact that *M. tuberculosis* is very distantly related to *P. aeruginosa* which is a gram-negative bacterium. Phospholipase C (PLC) and sphingomyelinase (SM) activities were found to be associated with the most virulent species of mycobacteria (Johansen et al. 1996). Moreover, triple and quadruple mutants of *M. tuberculosis* examined in a mouse infection model revealed that both mutants were attenuated compared to the wild-type parent in the late phase of infection (Raynaud et al. 2002).

Finally, with regard to the functional similarities between the PLC/SM of *M. tuberculosis* and *P. aeruginosa*, recent studies showed that the expression of PlcABCDs of *M. tuberculosis* and the expression of PlcH and PlcN of *P. aeruginosa* are strongly upregulated under phosphate limitation and that the PLC/SM-proficient *M. tuberculosis* strains survived better than the PLC/SM deletion mutants under conditions where phosphatidylcholine served as a sole phosphate source (Le Chevalier et al. 2015). This is of possible importance since there are significant amounts of phosphatidylcholine in lung surfactant. These observations open new perspectives for the further role of the PlcABCDs in the lifecycle of *M. tuberculosis*.

5 Research Needs

The last four decades have produced a remarkable body of work describing the pathogenesis of bacterial infections and the virulence factors that promote and participate in the infectious process. Exploration of bacterial genomes aided by improved methods for sequencing DNA has greatly expanded the identification and understanding of the roles of lipases, phospholipases, and sphingomyelinases in promoting the ability of bacteria to colonize and damage host tissues. As described in this chapter, many of these actions do not arise from simple destruction of host cell membranes and organelles. Rather, deeper explorations show that downstream effects are often caused by the production of lipid second messengers such as diacylglycerol and ceramide. Diacylglycerol activates many members of the PKC family. However, the trail often ends when the targets of these kinases remain unknown. The study of bacterial infections has revealed many previously unknown details of eukaryotic cell biology. For example, description of the cellular participants and mechanisms of autophagy have been elaborated through the study of intracellular bacteria.

As discussed above, there is a clear distinction between PI-PLCs that interact with the outer surface of cells and cleave GPI-anchored proteins (*B. cereus*, *B. anthracis*, *B. thuringiensis*, and *S. aureus*) and that from *L. monocytogenes*, which has little or

no activity on GPI-anchored proteins. Its sole substrate appears to be PI, since it does not cleave phosphorylated forms of PI. Many of the GPI-anchored protein targets of the enzymes of *B. cereus* such as alkaline phosphatase, erythrocyte acetylcholinesterase, 5′-nucleotidase, scrapie prion protein, CD59, and Thy-1 are well known (Paulick and Bertozzi 2008), but the link between cleavage of these and the many other GPI-anchored proteins of mammalian cells and downregulation of innate immunity needs much more study. Continued study of bacterial lipases, phospholipases, and sphingomyelinases should prove rewarding in deciphering the intricate networks of lipid messengers, proteins, and protein complexes that either protect cells from infection or promote the infectious process.

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Part III

Body Surfaces: Lipids, Infections, Personal Hygiene, and Personal Care

Skin: *Cutibacterium* (formerly *Propionibacterium*) *acnes* and Acne Vulgaris

9

Holger Brüggemann

Contents

Abstract

Acne vulgaris is an extremely common condition, experienced by approximately 80% of adolescents. The initiation of this disease and the chain of events in acne formation are poorly understood. The Gram-positive omnipresent skin commensal *Cutibacterium* (formerly *Propionibacterium*) *acnes* has been identified as a

H. Brüggemann (✉)
Department of Biomedicine, Aarhus University, Aarhus, Denmark
e-mail: brueggemann@biomed.au.dk

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contributing factor in acne, but the importance and the precise role of this bacterium in disease formation are still not understood in full detail.

Since the decipherment of the first genome of *C. acnes* in 2004, many new discoveries have been made that shed light on the dual role of this bacterium, i.e., its host-beneficial and host-detrimental properties. This chapter summarizes recent findings in *C. acnes* microbiology and host-interacting properties and discusses these in the context of the pathophysiology of acne vulgaris. Based on these new discoveries, novel treatment strategies are being developed that aim to supersede the use of antibiotics.

1 Introduction

The Gram-positive bacterium *Cutibacterium* (formerly *Propionibacterium*) *acnes* is ubiquitously found on human skin. The determination of the skin microbiome has highlighted the predominance of *C. acnes* at several skin sites (Oh et al. 2016; Byrd et al. 2018). The microorganism is very prevalent on oily skin sites such as the face and upper back, but is also detected at dry skin sites. It was shown that sebaceous follicles of healthy individuals are colonized almost exclusively with *C. acnes* (Bek-Thomsen et al. 2008; Jahns and Alexeyev 2016). Furthermore, healthy human skin is colonized by several different strains of *C. acnes* that usually belong to different phylotypes (McDowell et al. 2013; Oh et al. 2016; Petersen et al. 2017).

Despite its presence on normal skin, the bacterium is associated with the formation of inflammatory acne and other skin disorders such as progressive macular hypomelanosis (PMH). A disease-causing or disease-supporting role of *C. acnes* is corroborated by the observation that antibacterial treatment strategies are usually successful against acne vulgaris and PMH (Williams et al. 2012; Nast et al. 2016; Petersen et al. 2017). Moreover, *C. acnes* is frequently isolated from postoperative and medical device-related infections (Perry and Lambert 2011). This has often been described as contamination, due to possible skin contact during the sampling process; however, more recently, the bacterium is increasingly regarded as an etiological agent of such infections (Achermann et al. 2014).

Recent advances in understanding the dual role of this bacterium in human health and disease have been made, for instance, regarding the interaction with the innate and adaptive immune system. This chapter gives an overview of the current status of *C. acnes* research, including the population structure, the association of specific *C. acnes* phylotypes with disease, bacterial host-interacting factors, and new insights into host responses to *C. acnes*.

2 *C. acnes*' Pan-genome, Its Population Structure, and New Nomenclature

In 2016, a new species name was introduced: *Propionibacterium acnes* was renamed to *Cutibacterium acnes* (Scholz and Kilian 2016). This was proposed based on substantial discrepancies between the results of traditional phylogenetic analyses

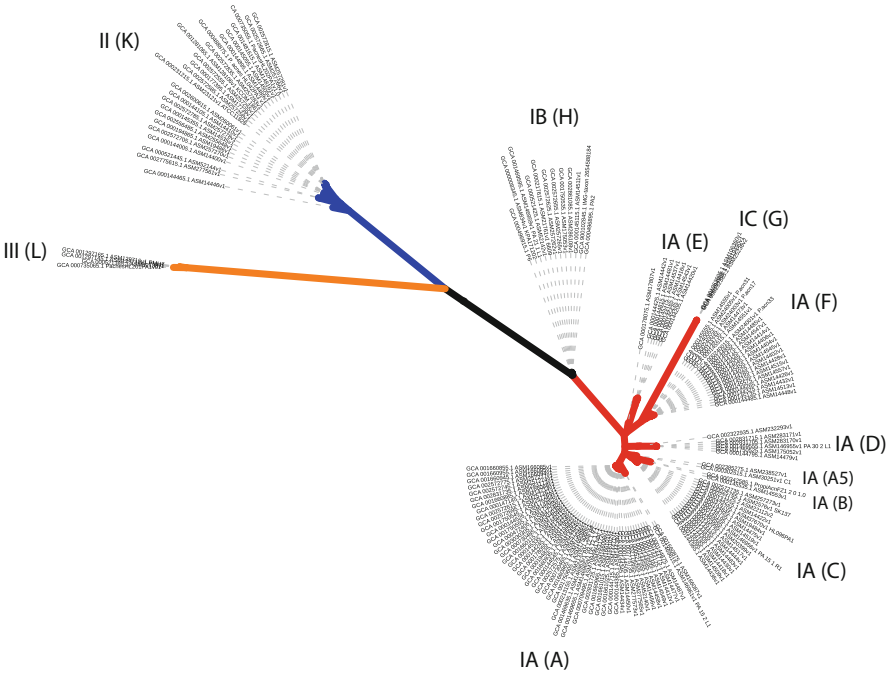

Fig. 1 Population structure of *C. acnes.* The figure shows a phylogenomic comparison of all publicly available genome sequences of *C. acnes* (May 2018). The tree is based on the aligned core genome and the analysis of core genome-located single nucleotide polymorphisms. Clearly separated are the major phylotypes I, II, and III. There are several clades among type I strains, including the subtypes IA, IB, and IC. The letters in brackets correspond to the SLST scheme nomenclature (see also http://medbac.dk/slst/pacnes)

of 16S rRNA gene sequences and core genome analyses of all available propionibacterial genomes. The latter revealed the non-monophyletic nature of the genus *Propionibacterium*; it became apparent that the cutaneous propionibacteria form a distinct and coherent clade that is separate from the dairy propionibacteria (Scholz and Kilian 2016).

In 2004, the first closed genome of *C. acnes* was sequenced (Brüggemann et al. 2004). Since then, many more *C. acnes* strains were genome-sequenced which helped to elucidate the pan-genome. Currently, 16 complete genome sequences and 131 draft genomes have been deposited in the GenBank database (April 2018). Based on the available genomes, a phylogenomic analysis of the core genome results in a detailed picture of the population structure of *C. acnes* (Fig. 1). The population is divided into the main phylotypes I, II, and III, with a further subdivision of type I strains into IA, IB, and IC. The phylogenomic analysis of the *C. acnes* core genome was recently further exploited to create a single-locus sequence typing (SLST) scheme: a single fragment of the core genome was identified that can be used for an amplicon sequencing approach similar to 16S rRNA phylogenetic analysis (Scholz et al. 2014). The SLST typing scheme has a high-resolution power and can distinguish all major and also minor *C. acnes* types. This allows the determination of

all *C. acnes* types in a given sample without the need for cultivation. The SLST scheme uses the letters A–L (type III, L; type II, K; type IB, H; type IC, G); it further subdivides type IA strains into A, B, C, D, E, and F strains, based on core genome differences (Fig. 1).

In addition, it was recently suggested to rename to main phylotypes of *C. acnes*, based on geno- and phenotypical differences (Dekio et al. 2015; McDowell et al. 2016):

- Type I: *C. acnes* subsp. *acnes*; most isolates sequenced to date belong to this type.
- Type II: *C. acnes* subsp. *defendans*; the name refers to the unique presence of a CRISPR/cas system in this type.
- Type III: *C. acnes* subsp. *elongatum*; the name refers to the long, filamentous cell shape of strains of this type.

2.1 Accessory Genome of *C. acnes*

Apart from the core genome, the accessory genome encodes a variety of functions involved in fitness, environmental adaption, bacterial competition, phage and phage defense, and host interactions (Brüggemann et al. 2012a; Scholz et al. 2016). The extent of the accessory genome varies considerably among *C. acnes* strains; the accessory genome is often phylotype-specific, i.e., all strains of a given phylotype carry the same accessory genome (Scholz et al. 2016). However, there are a number of exceptions, i.e., genomic loci, that are shared by more than one phylotype or, more often, loci that are only present in a subfraction of strains of a given phylotype. Together, 66 genomic loci that represent the accessory genome have been identified in the whole *C. acnes* pan-genome (Scholz et al. 2016). Several islands bear signatures of their acquisition by horizontal DNA transfer; they are directly flanked by, or are inserted in, tRNA genes. As an example, one island that is present in type IB and some type IA strains contains genes for a nonribosomal peptide synthase/polyketides synthetase, putatively involved in the production of a cyclic lipopeptide. This class of substances has usually either antibiotic (e.g., surfactin), or antifungal (e.g., candicidin), or immunosuppressive (e.g., cyclosporin) effects. Another island, present in type IB and type III strains, contains thiopeptide biosynthesis genes. This genomic island was recently investigated: thiopeptide-positive strains have antimicrobial as well as host cell cycle-modulating activities; it was suggested that the bacterial berninamycin-like thiopeptide can inhibit FOXM1, a master regulator of human cell cycle progression (Sayanjali et al. 2016).

Interestingly, some strains of *C. acnes* contain a linear plasmid, approx. 53 kb in size (Brüggemann et al. 2012b; Kasimatis et al. 2013; Davidsson et al. 2017). This plasmid was identified in a small subfraction of acne-associated type IA strains and was also found in some type II strains, in particular in prostatic isolates. The plasmid contains genes for its conjugative transfer as well as for the synthesis of tight adherence (*tad*) pili, also called Flp (fimbrial low-molecular-weight protein) pili. The putative pilin subunit was identified in the surface-exposed protein fraction of

plasmid-positive *C. acnes* type II strains, and cell appendages could be detected in *tad* locus-positive type II strains (Davidsson et al. 2017). Such pili may support colonization and possibly persistent infection of human tissue by *C. acnes*.

3 Disease Associations of *C. acnes*

3.1 Skin Disorders

C. acnes is considered to be one of the pathophysiological factors responsible for the formation of acne vulgaris (Bojar and Holland 2004). Acne is a multifactorial skin disorder related to the formation of comedones in sebaceous follicles and triggered or influenced by hormonal stimulation, follicular hyperkeratinization, bacterial colonization, and host inflammatory responses (Williams et al. 2012). The precise interdependence and the choreography of pathogenic events in acne are still unclear; some studies regarding host inflammatory responses to *C. acnes* have led to the assumption that the bacterium is actually a key factor in the formation of inflammatory acne (Kim et al. 2002; Nagy et al. 2006; Agak et al. 2014; Kistowska et al. 2015). One of the difficulties in assigning a role of *C. acnes* in acne is the presence of this bacterium also on normal skin, where it does not harm the host. Recent research showed, however, a dysbiosis of the *C. acnes* type distribution in acne compared to normal skin: whereas healthy skin is usually colonized by multiple types of *C. acnes*, a predominance of certain type IA strains is seen on acne-affected skin (Lomholt and Kilian 2010; McDowell et al. 2011; Fitz-Gibbon et al. 2013; Dagnelie et al. 2018) (Fig. 2). In particular, the SLST type A (also called clonal complex CC18) of type IA is predominant in acne. It remains to be seen, if the prevalence of such type IA strains is the cause or the consequence of disease. It might be that such strains have a growth advantage in acne-affected skin sites, and/or other types are outcompeted or eliminated.

Another skin disease that is associated with *C. acnes* is PMH; it is a disorder that is characterized by hypopigmented macules on the skin of the upper body and usually seen in younger adults. PMH lesions contain more *C. acnes* bacterial cells than normal skin, and antibiotic treatment is usually efficient against PMH. Recent typing efforts have shown in two independent studies that type III strains (*C. acnes* subsp. *elongatum*) are highly dominating in PMH-affected skin lesions (Barnard et al. 2016; Petersen et al. 2017). A causative role of type III *C. acnes* in PMH has not yet been experimentally proven.

3.2 *C. acnes*-Associated Infections Beyond the Skin

In addition to skin manifestations, the bacterium has often been isolated from a variety of sites of infection and inflammation, such as postoperative infections and medical device-related infections (Perry and Lambert 2011). In the last years, it has become increasingly accepted that *C. acnes* and the closely related *Cutibacterium*

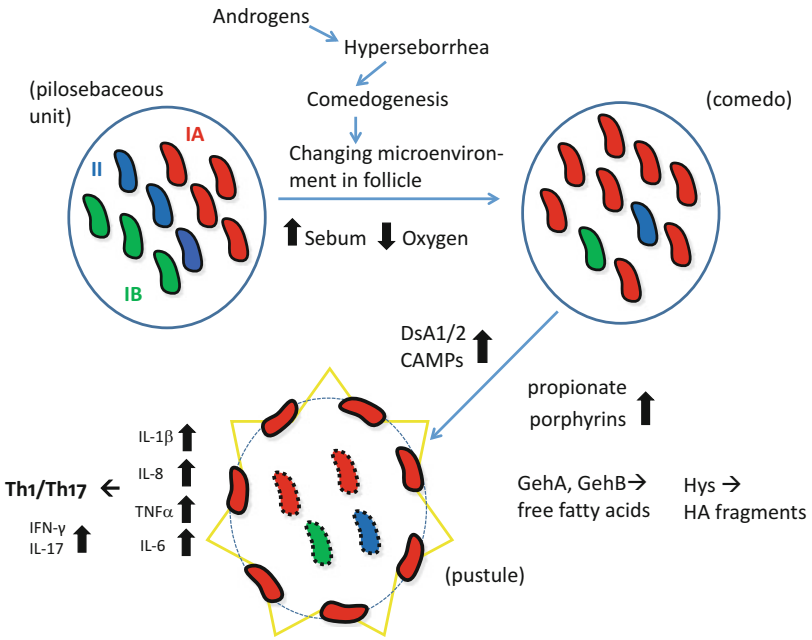

Fig. 2 Tentative model of the implication of *C. acnes* in inducing or supporting acne vulgaris. The healthy sebaceous follicle is colonized with a mixture of different *C. acnes* types. Androgen levels in the puberty are increased; these in turn activate sebaceous glands to produce more sebum. Exceeding sebum and hyperkeratinization lead to the clogging of the sebaceous duct and thus comedo formation. The microenvironment of the comedo is anaerobic with high lipid content, providing an advantage and/or disadvantage for type IA and type IB/II strains of *C. acnes*, respectively, resulting in the predominance of certain type IA clones in the comedo. The metabolic activity of such type IA strains results in the release of propionate and porphyrins as well as the secretion of tissue component-interacting/-degrading factors, including lipases (GehA, GehB) and hyaluronidase (Hys) that lead to the accumulation of free fatty acids and hyaluronic acid fragments, respectively, in the comedo. This paves the way for a closer contact of the bacterium with the cellular microenvironment of the follicle, including keratinocytes, sebocytes, and skin-resident dendritic cells (DCs)/Langerhans cells. In addition, type IA strains produce CAMP factors (CAMP1 and 2) as secreted and cell surface-attached proteins and the adhesive surface glycoproteins dermatan-sulfate adhesins/fibrinogen-binding proteins (DsA1/DsA2). The bacterial contact with keratinocytes, sebocytes, and DCs activates a local innate immune response in a TLR2-dependent manner, resulting in the release of chemokines/cytokines such as IL-8 and TNF-α. Skin-resident DCs/macrophages sense the irritated tissue site, infiltrate at the site, and/or interact with CD4+ T cells to induce mixed Th1/Th17 responses, resulting in IFN-γ and IL-17 secretion. Together, this results in the formation of pustules as seen in inflammatory acne. In the course of disease, numbers of *C. acnes* cells in such inflamed skin regions usually decrease due to activated host defense systems

avidum are causative agents of implant-associated infections (Achermann et al. 2014; Aubin et al. 2014). In such conditions, there seems to be an overrepresentation of *C. acnes* strains belonging to the phylotypes IB and II (McDowell et al. 2013). There are also reports that *C. acnes* can lead to bona fide joint and bone infections,

such as the SAPHO syndrome (synovitis-acne-pustulosis-hyperostosis-osteitis syndrome), rheumatoid arthritis, and osteomyelitis (Perry and Lambert 2011). In the last years, *C. acnes* has been associated with chronic lower back pain, based on the identification of *C. acnes* biofilms in intervertebral disc tissue obtained from patients suffering from the degenerative disc disease (Albert et al. 2013; Capoor et al. 2017). Current research efforts focus on the identification of disease mechanisms that might explain a causative role of *C. acnes* in the formation of degenerative disc disease (Slaby et al. 2018).

C. acnes has also been described as a etiological agent of sarcoidosis, based on the presence of the bacterium in sarcoid granulomas and the detection of *C. acnes*-derived insoluble immune complexes in sinus macrophages in sarcoid lymph nodes (Eishi 2013; Suzuki et al. 2018).

Several studies have found that *C. acnes* is also a common isolate of the prostate, especially in patients with prostate inflammation or prostate cancer (Cohen et al. 2005; Alexeyev et al. 2007; Fassi Fehri et al. 2011). A recent large cohort study showed the presence of *C. acnes* in 60% of cancerous prostate specimens versus only 26% in healthy prostates (Davidsson et al. 2016). Most prostatic isolates belonged to the phylotype II (Davidsson et al. 2017). If *C. acnes* plays a role in prostate cancer, formation has so far not been clarified.

Taken together, the *C. acnes* type distribution in disease largely differs from the health-associated type distribution. There is evidence that different *C. acnes* types have distinguishable bacterial and host-interacting properties (see below). This might resolve, at least partially, previous ambiguous evaluations regarding *C. acnes*' pathogenic potential.

4 Host-Interacting Factors of *C. acnes*

The genome sequence of *C. acnes* gave clues about the lifestyle of the bacterium with regard to its ability to colonize and reside in the harsh environment of human skin and other lipid-rich tissue sites. It revealed several unexpected traits such as an extended set of proteins with predicted host tissue-degrading activities (Table 1) (Brüggemann 2005). Cell envelope and cell wall-associated surface proteins with predicted host-interactive properties were found; their presence or activity could trigger host inflammation (Lodes et al. 2006; Grange et al. 2017). Candidates conferring hemolytic and cytotoxic activities were also identified (Valanne et al. 2005). In the last decade, only a few of the predicted factors were analyzed in more detail by molecular techniques. This is, in part, due to a lack of an efficient mutagenesis approach for *C. acnes*. So far, mutant generation was only reported in one single strain of *C. acnes*: the type IB strain KPA171202 that has been genome-sequenced in 2004 (Sörensen et al. 2010). In the following, only those factors of *C. acnes* are presented, for which experimental data, in particular regarding their involvement in the acne pathogenesis, has been obtained in the last decade (Fig. 2).

Table 1 Studied host-interacting factors of *C. acnes*

Factor	Gene name (or gene locus in strain KPA171202)	Function/comment	Key references
Christie–Atkins–Munch-Petersen (CAMP) factor	CAMP1–CAMP5	Cytotoxic, inflammation-inducing, TLR-2 agonist (CAMP1), co-hemolytic (CAMP2)	Valanne et al. (2005), Sörensen et al. (2010), Lo et al. (2011), and Lheure et al. (2016)
Dermatan-sulfate adhesins	DsA1, DsA1	Binding to dermatan sulfate, immunoreactive	Lodes et al. (2006), McDowell et al. (2011), and Grange et al. (2017)
		DsA1: fibrinogen-binding glycoprotein	
Hyaluronate lyase/ hyaluronidase	Hys-IA, Hys-IB/II	Degradation of hyaluronic acid, two variants, high and low activity in type IB/II and type IA strains, respectively	Nazipi et al. (2017)
Thiopeptide	PPA0866	Antimicrobial compound, host cell cycle-modulating properties	Sayanjali et al. (2016)
Sialidase	PPA1560	Cleaving sialoglycoconjugates, increases cytotoxicity	Nakatsuji et al. (2008)
Lipase	GehA, GehB	Degradation of lipids within sebum	Miskin et al. (1997) and Bek-Thomsen et al. (2014)
Radical oxygenase	RoxP	Antioxidant, essential for growth of *C. acnes* in oxic conditions	Allhorn et al. (2016)
Porphyrin	Many genes	Proinflammatory compound	Schaller et al. (2005) and Kang et al. (2015)
Propionate	Many genes	Proinflammatory via HDAC inhibition in TLR-activated keratinocytes	Sanford et al. (2016)

4.1 Christie–Atkins–Munch-Petersen (CAMP) Factors

All *C. acnes* types encode five different CAMP factors, designated from CAMP1 to CAMP5. The name refers to the scientists Christie, Atkins, and Munch-Petersen who have established the so-called CAMP reaction, a synergistic hemolysis of sheep erythrocytes by the CAMP factor and the β-toxin (sphingomyelinase C) from

Staphylococcus aureus. CAMP factors have been found only in a few other species, such as in Group B *streptococcus.* They were characterized as pathogenic determinants that exerted lethal effects when administered to rabbits and mice and have pore-forming cytotoxic activity (Lang and Palmer 2003).

Most strains of *C. acnes* produce high amounts of at least two CAMP factors, i.e., CAMP1 and CAMP2, although expression levels differ between type IA, IB, and II *C. acnes* strains (Valanne et al. 2005; Holland et al. 2010; McDowell et al. 2013). These CAMP factors are either secreted or surface-attached. CAMP1 and CAMP2 were also identified in vivo, in human sebaceous follicle casts (Bek-Thomsen et al. 2014). At least one CAMP factor, CAMP2, was shown to be responsible for the co-hemolytic reaction of *C. acnes* with erythrocytes, since a knockout mutant of CAMP2 showed reduced co-hemolytic activity in the CAMP reaction (Sörensen et al. 2010). In addition, it was shown that CAMP2 can act as an exotoxin, exhibiting cytotoxic activity on host cells (Lo et al. 2011). Interestingly, neutralizing CAMP2, e.g., by anti-CAMP2 antibodies, can efficiently attenuate *C. acnes*-induced inflammation in the mouse ear model (Liu et al. 2011). Recently, it was shown that at least one CAMP factor, CAMP1, is a ligand of toll-like receptor 2 (TLR-2) (Lheure et al. 2016). This shows that CAMP factors can interact with the innate immune system and are likely to induce inflammation.

4.2 Dermatan-Sulfate Adhesins/Fibrinogen-Binding Proteins

C. acnes has adhesive properties. It attaches to eukaryotic cells and forms biofilm on human tissue sites as well as on various medical devices in vitro and in vivo. Also, plasma proteins are bound to *C. acnes*, including fibrinogen, IgG, and complement factors, which is associated with aggregation (Petersson et al. 2018). The key factor of adhesion and aggregation, the cell surface of *C. acnes*, with its lipoglycan moiety and its embedded surface-associated proteins, is also a major immunogenic factor (Lodes et al. 2006).

Several cell surface or surface-exposed proteins with cell wall anchor signatures (LPxTG motifs) have been determined from the genome sequences (Brüggemann et al. 2004). Some of these proteins harbor characteristic multiple repeats of the dipeptide proline–threonine (PT repeat) and, to a lesser extent, of proline–lysine. Such multiple repeats of amino acid patterns have also been detected in antigenic proteins of *Mycobacterium tuberculosis* that are expressed in vivo after aerosol infection but prior to the development of clinical tuberculosis (Singh et al. 2001). A study was able to identify two of these proteins, designated dermatan-sulfate adhesins DsA1 and DsA2, as major immunoreactive surface proteins of *C. acnes* (Lodes et al. 2006). These two proteins share some homology with an M-like protein of *Streptococcus equi* and have dermatan-sulfate-binding activity and thus are likely to be MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Dermatan sulfate is expressed in many mammalian tissues and is the predominant glycan present in the skin. A recent study highlighted the role of DsA1 as an MSCRAMM: it also binds human fibrinogen (Grange et al. 2017). Moreover, it

was found that DsA1 is highly glycosylated. DsA1 and DsA2 are variably expressed in the different *C. acnes* types, possibly due to phase variation signatures in the promoter region (Lodes et al. 2006; Holland et al. 2010). There is also some evidence for within-strain phase variation in expression, at least in type IA strains (McDowell et al. 2011). Besides variable expression, there is antigenic variation due to the variable length of the PT repeat in different types and strain. Thus, several lines of evidence suggest that these proteins contribute to host-interaction and inflammation.

4.3 Hyaluronate Lyase/Hyaluronidase

A hyaluronate lyase/hyaluronidase (Hys) of *C. acnes* has also been investigated (Nazipi et al. 2017). This enzyme catalyzes the degradation of hyaluronic acid (HA), a nonsulfated glycosaminoglycan, which is the main polysaccharide component of the extracellular matrix of connective tissues. HA chains (polymeric D-glucuronate-beta-1,3-*N*-acetyl-D-glucosamine connected by beta-1,4-glycosidic linkages) are ultimately broken down by Hys to 3-(4-deoxy-beta-D-gluc-4-enuronosyl)-*N*-acetyl-D-glucosamine. Two different variants of Hys have been identified in the *C. acnes* population: one variant is present in type IB and type II strains; it is highly active, resulting in complete HA degradation (Nazipi et al. 2017). The other variant is present in type IA strains; it has low activity, resulting in incomplete degradation. This might explain why type IA strains are primarily found on the skin surface, while type IB/II strains are more often associated with soft and deep tissue infections, which would require elaborate tissue invasion strategies, possibly accomplished by a highly active Hys. Moreover, the different HA fragment sizes produced by type IB/II and type IA strains, respectively, could be of importance, since it is known that the size of the HA fragments determines the impact on host tissue. For instance, low-molecular-weight HA fragments, i.e., oligosaccharides of various sizes, can serve as danger signals and are involved in cell signaling events leading to the induction of inflammation, angiogenesis, and cancer-promoting processes (Scheibner et al. 2006; Petrey and de la Motte 2014). It needs to be investigated in the future, if HA fragments produced by type IA strains could be involved in the induction or amplification of proinflammatory responses as seen in acne.

4.4 Lipases

C. acnes has saprophytic properties; it acquires nutrients for growth from within sebaceous follicles, such as lipids of the sebum. Sebum, secreted by the sebaceous glands of the skin, is composed mainly of triglyceride (~40%) and other lipids, such as wax monoesters, free fatty acids, and squalene. Sebum is usually overproduced during puberty due to the influence of androgens on the sebaceous gland activity (Williams et al. 2012).

A lipolytic activity of *C. acnes* has been shown, and a secreted lipase, the triacylglycerol lipase GehA, has been identified (Miskin et al. 1997). The products of GehA activity, free fatty acids, might amplify the inflammatory process in the sebaceous follicles during acne. Several other lipases are encoded in the *C. acnes* genome. Interestingly, in human sebaceous follicles, another lipase of *C. acnes* was detected in larger amounts than GehA; this lipase, designated GehB, shares similarity with GehA (Bek-Thomsen et al. 2014). So far, more detailed experiments of these lipases are lacking; for instance, it is not known if GehA and GehB use different substrates.

4.5 Sialidase

A sialidase/neuraminidase of *C. acnes* was investigated (Nakatsuji et al. 2008). Sialidases can cleave sialoglycoconjugates to obtain sialic acids for use as carbon and energy sources. In the mentioned study, the involvement of *C. acnes*' sialidase to disease pathogenesis was shown by treatment of a human sebocyte cell line with recombinant sialidase, which increased susceptibility to *C. acnes* cytotoxicity and adhesion. Moreover, the authors demonstrated that a recombinant sialidase-based vaccine provided protective effects against *C. acnes*-induced ear inflammation in mice. Based on these results, the authors proposed a sialidase-based vaccine, which may be an efficient modality for the prevention of early *C. acnes* infection.

4.6 Porphyrins

C. acnes can produce porphyrins. This is linked with the biosynthesis of the essential cofactor cyanocobalamin (vitamin B_{12}), since biosynthesis pathways of vitamin B_{12} and porphyrins have the same precursor, 5-aminolevulinic acid (and uroporphyrinogen III). The biosynthesis of porphyrins in propionibacteria is inversely correlated with the biosynthesis of vitamin B_{12}; an excess of (external) vitamin B_{12} can repress cobalamin biosynthesis in *C. acnes*, resulting in increased porphyrin production and secretion into the follicle (Kang et al. 2015). It is not known if secretion of porphyrins plays an active role in *C. acnes* biology or if this is a bypass to get rid of an excess of cobalamin precursor molecules. HPLC analysis of the in vivo porphyrin pattern secreted by *C. acnes* revealed a predominance of coproporphyrin III in acne lesions (Schaller et al. 2005). This porphyrin fraction also modestly induced IL-8 expression in exposed keratinocytes, and thus, it can be speculated that released porphyrins contributes to the immunostimulatory activity of *C. acnes*. Moreover, strain differences in producing porphyrins have been reported: acne-associated type IA strains seem to produce higher levels of porphyrins compared with health-associated type II strains (Johnson et al. 2016).

4.7 Radical Oxygenase RoxP

One of the most abundantly secreted proteins of *C. acnes* is RoxP (PPA1939), a small protein of approx. 16 kDa (Holland et al. 2010). It was shown that RoxP is essential for growth of *C. acnes*: a *roxP*-deletion mutant did only grow under anaerobic, but not aerobic conditions (Allhorn et al. 2016). Moreover, experiments with a skin model showed that the *roxP*-deletion mutant was not able to colonize skin for an extended time, in contrast to the wild-type strain. Biochemical tests have shown that RoxP is able to reduce free radicals and can protect molecules from oxidation (Allhorn et al. 2016). RoxP was detected in human sebaceous follicles in vivo and in skin swabs (Bek-Thomsen et al. 2014; Ertürk et al. 2018). Thus, RoxP is a crucial factor, allowing *C. acnes* to colonize human skin, in particular in oxic conditions. It was furthermore suggested that the protein acts as an antioxidant that is involved in the maintenance of the redox homeostasis of the skin (Allhorn et al. 2016). This could indicate a host-beneficial role of RoxP, which needs to be further elucidated.

4.8 Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) are secreted as metabolic end products of many fermentative bacteria. SCFAs produced by gut bacteria have been shown to have host cell modulating and immunomodulatory effects (Tan et al. 2014). Besides being energy sources, SCFAs can interfere in host cell signaling via G protein-coupled receptors and inhibition of histone deacetylases (HDACs); this impacts on host cell gene expression with diverse consequences for the cellular fate and differentiation. For instance, SCFAs can modulate the differentiation and activity of colonic regulatory T cells that control intestinal inflammation.

C. acnes produces mainly propionate in the course of its fermentative metabolism. It was recently shown that *C. acnes*-produced propionate impacts on keratinocytes; it induces inflammation via HDAC inhibition in TLR-activated cells (Sanford et al. 2016). The relevance of this finding remains to be investigated in more detail. It can be speculated that environmental conditions that favor propionate production of *C. acnes*, e.g., anaerobic conditions in the presence of sufficient nutrients, would result in increased inflammation in the presence of *C. acnes*-exposed or *C. acnes*-secreted TLR agonists, e.g., CAMP factors of *C. acnes*. This would require, however, close contact of *C. acnes* with cells of the follicle, e.g., with keratinocytes and sebocytes.

5 Host Immune System Interaction with, and Response to, *C. acnes*

5.1 Innate Immune Responses

C. acnes is known for its extensive immunostimulatory activity. Many studies were done with different cell culture models, including keratinocytes, sebocytes, and monocytes/macrophages that show variation regarding the (innate) response to

C. acnes. *C. acnes* has been shown to stimulate the production of the antimicrobial peptide human β-defensin-2 (hBD2), the chemokine CXCL8 (IL-8), and the cytokine TNF-α in skin cells such as sebocytes (Nagy et al. 2006). In keratinocytes, the activation of hBD2, TNF-α, GM-CSF, and IL-1α, IL-1β, and IL-8 has been observed upon contact with the bacterium, and in primary monocytes, *C. acnes* can induce the production of proinflammatory cytokines IL-1β, IL-8, IL-12, and TNF-α (Chen et al. 2002; Nagy et al. 2005; Yu et al. 2016). Innate immune responses as well as complement activation are triggered by *C. acnes* at higher levels than by other skin commensals such as *Staphylococcus epidermidis*.

In groundbreaking studies, innate host cell receptors were identified such as pattern-recognition receptors (PRRs). They can sense conserved bacterial components (pathogen-associated molecular patterns, PAMPs; microbe-associated molecular patterns, MAMPs), either extra- or intracellularly, in order to initiate and conduct a cellular response. PRRs that sense *C. acnes* components have been identified: a crucial role was assigned to TLR-2, since it could be shown that TLR-2 was sufficient for NF-κB activation in response to *C. acnes* (Kim et al. 2002). Interleukin(IL)-12 and IL-8 production, triggered by *C. acnes* in primary human monocytes, was inhibited by anti-TLR2-blocking antibody. TLR2-dependent NF-κB activation has been identified as an underlying mechanism of cytokine activation also in other cells such as primary keratinocytes. Recently, the involvement of human TLR-2 heterodimers (TLR-2/1 and TLR-2/6) in sensing *C. acnes* was shown (Su et al. 2017). A TLR-2 agonist, the CAMP1 protein of *C. acnes*, was recently identified as a possible PAMP (Lheure et al. 2016). Other *C. acnes* ligands/PAMPs that are involved in TLR-2-dependent activation are not known. Other studies reported the involvement of TLR-9 in the immunostimulatory process, which seems to differ among different cell lines (Kalis et al. 2005). Moreover, other receptors such as members of the intracellular NOD-like receptor (NLR) family might be involved in sensing intracellular *C. acnes* (Tanabe et al. 2006).

More recently, *C. acnes* has been shown to activate the NLRP3 inflammasome, a system responsible for the activation of inflammatory processes via IL-1β maturation (Kistowska et al. 2014; Qin et al. 2014). In acne lesions, mature caspase-1 and NLRP3 were detected around the pilosebaceous follicles. The studies suggested that the encounter of *C. acnes* with macrophages could locally result in the release of IL-1β and therefore exacerbate inflammation.

5.2 Adaptive Immune Responses

C. acnes exerts different effects on CD4+ T cells. First, it was found that the bacterium has T cell mitogenic activity (Jappe et al. 2002). More recently, several studies focused on the impact of *C. acnes* on T cell responses. Besides the fact that *C. acnes* is a potent inducer of T helper type 1(Th1)-type cytokines (e.g., IL-12, IFN-γ, and TNF-α), it can also trigger a T helper type 17 (Th17) response (produced cytokines, e.g., IL-17 and IL-22), at least in human peripheral blood mononuclear cells (PBMCs) (Agak et al. 2014). *C. acnes*-induced IL-17 production was observed, and both Th1 and Th17 effector cytokines are strongly upregulated in acne lesions.

Another study showed that *C. acnes* can promote Th17 responses as well as mixed Th1/Th17 responses by inducing the concomitant secretion of IL-17A and IFN-γ from specific CD4+ T cells (Kistowska et al. 2015). *C. acnes*-specific Th17 and Th1/Th17 cells can also be found in the peripheral blood of patients suffering from acne. Thus, the impact of *C. acnes* on T cells seems to be relevant in the immunopathogenesis of acne. Interestingly, different *C. acnes* strains have distinguishable abilities to induce Th1/Th17 cells, resulting in variable secretion of IFN-γ and IL-17; thus, depending on the *C. acnes* strain, CD4+ T cell responses may differ, which could contribute to either acne pathogenesis or homeostasis (Agak et al. 2018).

Taken together, the immune responses to *C. acnes* are complex. In acne, early and late stages can be differentiated. In the early stage, androgen-dependent changes of the sebaceous follicle microenvironment seem to favor *C. acnes* types/strains with elevated proinflammatory activity that elicit innate immune responses in exposed keratinocytes and/or sebocytes (Fig. 2). The second phase includes skin-resident macrophages/dendritic cells (DCs; Langerhans cells) that infiltrate into the cytokine-releasing skin sites; activated macrophages/DCs can then further induce inflammation, either directly (inflammasome activation) or indirectly (collaboration with CD4 + T cells to induce Th1/Th17 responses).

6 Outlook and Research Needs

Here, an overview of the current status on *C. acnes* research in the postgenomic era was given. The research on *C. acnes* has increased in recent years. It is more accepted now that *C. acnes* is an opportunistic pathogen, e.g., causing medical device-related infections. In addition, previously unrecognized disease associations of *C. acnes* are currently investigated, such as chronic lower back pain and sarcoidosis. Nevertheless, a critical evaluation regarding the importance of this bacterium in the mentioned diseases is important. Since every human being harbors *C. acnes* on the skin, usually without signs of disease, it is thus difficult to differentiate between infection-causing organism and (skin-derived) contaminant. Recently improved and applied bacterial typing approaches, supported by pan-genome analyses, could reveal and distinguish disease- from health-associated *C. acnes* types and strains, in a disease-specific manner.

In order to prove a causative role of (certain types of) *C. acnes*, insights in disease-causing mechanisms are crucial. This includes the elucidation of bacterial host-interacting properties. One obstacle for further in-depth studies on bacterial virulence factors is the lack of an efficient genetic knockout system for *C. acnes*. One approach was described so far that, however, works apparently only for one type of *C. acnes* (type IB) (Sörensen et al. 2010). Thus, a better system is needed.

Another research need is a better understanding of the metabolism of *C. acnes* and the (secreted) primary and secondary metabolites, such as SCFAs, porphyrins, and other non-proteinaceous molecules, as well as host-derived compounds such as

lipids and HA that are processed by *C. acnes* activities in vivo in sebaceous follicles. With advances in metabolomics research, this could be addressed in the near future.

The interactions of *C. acnes* (and other skin microorganisms) with components of the human immune system and their consequences are currently intensively investigated. These interactions are very complex and involve different cell types, including also skin-resident DCs/Langerhans cells and macrophages and different layers of the host immune system, including innate and adaptive functions (Belkaid and Tamoutounour 2016; Chen et al. 2018). We start to understand how *C. acnes* is sensed on normal skin and how this recognition is controlled to guarantee skin homeostasis; this involves different mechanisms such as the restriction of bacterial proliferation, the host selection of *C. acnes* types with relatively low inflammatory activity, and the interaction with skin-resident DCs/Langerhans cells, macrophages, and CD4+ T cells. In this respect, more research is also needed regarding the timing of events, including the first exposure to *C. acnes* in life and its consequences, i.e., the acquisition of tolerance.

In addition, other skin microorganisms such as (specific strains of) *Staphylococcus epidermidis* might impact directly or indirectly on *C. acnes* and dampen or control *C. acnes*-induced inflammation. More research is needed to study the skin microbiome as a whole superorganism as well as the different interactions of the individual skin microorganisms.

New therapeutic strategies are being developed. In the light of the threat of ineffective antibiotics, as well as the severe side effects of excessive antibiotic therapy and other anti-acne therapeutics, alternative strategies are preferred. Probiotics for the skin are currently being developed. Several companies already offer bacteria-based formulations that can be applied to the skin. Many challenges need to be addressed, such as long-term colonization efficiencies of topically applied formulations as well as personalized probiotic compositions. If skin probiotics have health-beneficial effects and could be successfully used to treat skin disorders such as atopic dermatitis or acne needs to be seen in the future.

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Methanotrophy, Methylotrophy, the Human Body, and Disease

10

Rich Boden

Contents

Abstract

Methylotrophic *Bacteria* use one-carbon (C_1) compounds as their carbon source. They have been known to be associated with the human body for almost 20 years as part of the normal flora and were identified as pathogens in the early 1990s in end-stage HIV patients and chemotherapy patients. In this

R. Boden (✉)
School of Biological and Marine Sciences, Sustainable Earth Institute, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK
e-mail: rich.boden@plymouth.ac.uk

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chapter, I look at C_1 compounds in the human body and exposure from the environment and then consider *Methylobacterium* spp. and *Methylorubrum* spp. in terms of infections and its role in breast and bowel cancers, *Methylococcus capsulatus* and its role in inflammatory bowel disease, and *Brevibacterium casei* and *Hyphomicrobium sulfonivorans* as part of the normal human flora. I also consider the abundance of methylotrophs from the *Actinobacteria* being identified in human studies and the potential bias of the ionic strength of culture media and the needs for future work. Within the scope of future work, I consider the need for the urgent assessment of the pathogenic, oncogenic, mutagenic, and teratogenic potential of *Methylobacterium* spp. and *Methylorubrum* spp. and the need to handle them at higher containment levels until more data are available.

1 Introduction

The methylotrophs are a functional guild of the *Bacteria* that can grow on organic one-carbon (C_1) compounds as their sole source of carbon and energy. C_1 compounds do not contain carbon-carbon bonds, and thus every carbon-carbon bond must be made de novo. Historically, methylotrophy was considered to be a restricted and specialist metabolic mode that was only found in distinct taxonomic groups, but in the last decade (e.g., Boden et al. 2008), the range of "generalist" organisms displaying methylotrophic growth has increased. That said, on examination of any recent taxonomy articles of generalist heterotrophs being isolated, the reader will note a paucity of C_1 compounds on the list of substrates examined – thus we probably have an underestimation of the diversity of methylotrophic organisms. The full gamut C_1 compounds that occur in Nature or have been synthesized is vast, and not all of them have been examined for growth by the *Bacteria* – while the following list is exhaustive, it is no doubt incomplete: methane, methanol (MeOH), dimethyl ether (DME), formaldehyde, formate, methanethiol (MeSH), methaneselenol (MeSeH), methanetellurol (MeTeH), thioformaldehyde, dimethyl sulfide (DMS), dimethyl sulfoxide (DMSO), dimethyl sulfone ($DMSO_2$), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), dimethyl tetrasulfide (DMQS), methanesulfonate (MSA), hydroxymethanesulfinate (Rongalite), monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), trimethylamine *N*-oxide (TMAO), methyl chloride, methyl bromide, methyl fluoride, formamide, *N,N*-dimethylformamide, cyanamide, methanimine, diazomethane, carbon tetrachloride, carbon tetrabromide, carbon tetraiodide, iodoform, bromoform, chloroform, fluoroform, methylene fluoride, methylene chloride, methylene bromide, methylene iodide, various mixed halomethanes (e.g., dichlorodifluoromethane), methanesulfonyl chloride, hexamethylphosphoramide (HMPA), 1,3,5-trinitro-1,3,5-triazinane (RDX or hexogen), *N*,*N*′-bis-(1*H*-tetrazol-5-yl)-hydrazine (HBT), 1,3,5,7-tetranitro-1,3,5,7-tetrazocane (HMX or octogen), 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride or TCT), 6-chloro-*N*,*N*-dimethyl-1,3,5-triazine-2,4-diamine,

1,3,5-triazine-2,4,6-triamine (melamine), 1,3,5,-triazine-2,4,6-triol (*sym*-triazinetriol), 1,3,5-triazine-2,4,6-trione (*sym*-triazinetrione), melamine-cyanurate (1:1 mix of melamine and *sym*-triazinetrione), 2-cyanoguanidine (DCD), isocyanic acid (carbamide), 1,3,5-triazine (vedita), 1,3,5-trioxane (trioxin), 1,3,5-trithiane (thioform), 1,3-diazetidine, 1*H*-tetrazole, and 1,3,5-trithiane-1,1,3,3,5,5-hexaoxide. Methyl iodide hydrolyzes spontaneously in water, as does methyl formate; thus these probably do not contribute directly to methylotrophic growth. Thioformaldehyde trimerizes into 1,3,5-trithiane spontaneously, and thus the former is probably not a growth substrate itself. Phosgene, urea, carbonyl sulfide, and carbon disulfide are also one-carbon compounds but are inorganic or are metabolized via carbon dioxide; thus while they support growth, it is by autotrophs (e.g., *Thiobacillus thioparus*). While not all of the C_1 compounds listed above are found in Nature, their use as building blocks in organic chemistry, solvents, explosives, plastics, pesticides, etc., results in their exposure to humans and to the environment. Some are also formed within the human body from the breakdown of medications or poisons – such as those based on 1*H*-tetrazole.

Methanotrophs are subset of methylotrophs that can grow on methane as a sole carbon source – for some reason this metabolic trait is relatively restricted – but methanotrophs range from being very restricted, apparently "only" growing on methane, methanol, and sometimes MMA (but they have seldom been tested on a truly wide range of substrates), to more generalist methanotrophs that grow on fatty acids and intermediates of Krebs' cycle.

The diversity of methylotrophs and methanotrophs has now been shown to include a diverse range of genera, including members of the classes *Alphaproteobacteria* (e.g., *Paracoccus*, *Methylosinus*, *Methylobacterium*, *Methylorubrum*, *Xanthobacter*), *Betaproteobacteria* (e.g., *Methylophilus*), *Gammaproteobacteria* (e.g., *Methylococcus*, *Crenothrix*, *Klebsiella*, *Pseudomonas*, *Sphingomonas*), *Actinobacteria* (e.g., *Leifsonia*, *Mycobacterium*, *Gordonia*, *Rhodococcus*), and *classis incertae sedis* in the phylum "*Verrucomicrobia*" (*Candidatus* Methylacidiphilum). In light of this, the potential for human-associated methylotroph diversity to be much higher than previously considered is vast. It is worth noting that the catabolism of the full range of one-carbon compounds is not fully understood – many enzymes have not been purified and pathways are only putative – this makes predictions of methylotrophic potential from human- or animal-associated metagenomic datasets very difficult even in 2018, and considerable biochemical and physiological studies are needed to understand the full gamut of methylotrophic catabolism.

2 C_1 Compounds and the Human Body

The human body produces C_1 compounds directly or as a consequence of the action of normal microbial flora or pathogenic flora. Some genetic pathologies lead to an increase in the production of C_1 compounds. Additionally, topical application of some C_1 compounds in the form of "dietary supplements" or "alternative medicines"

(e.g., DMSO, $DMSO_2$) occurs, as well as occupational exposure to C_1 compounds from the diet and from exposure to, e.g., chemicals by laboratory researchers.

While not all C_1 compounds are odorous, many are foul-smelling and can be detected at low levels, and thus have significant interest in terms of hygiene and the cosmetics industry, which has led to a significant number of studies determining various C_1 compounds in body fluids, etc. The odor thresholds for C_1 compounds include (ppb by volume) DMS (3.0), DMDS (2.2), MeSH (0.07), MMA (35.0), DMA (33.0), and TMA (0.032). For comparison (ppb by volume), hydrogen sulfide (0.5), acetic acid (6.0), and butyric acid (0.19) are in similar orders of magnitude (data from those curated by Wood and Kelly (2010) and Leonardos et al. (1969)).

2.1 Exogenous Exposure of the Human Body to C_1 Compounds

In this section I have outlined some of the major routes by which the human body can be exposed to exogenous C_1 compounds or from consumption of precursors such as pectin. It is not intended to be exhaustive but to give the reader a flavor of the myriad routes to exposure.

2.1.1 C_1 Exposure from the Diet

Many foods are known to contain C_1 compounds, including TMA and TMAO in fish and seafoods (the former produced from the latter by bacterial action, Bystedt et al. (1959)) and DMS in *Allium* spp. L. (garlic, leek, onions, etc., Amiri (2007) and Lawson et al. (1991)), *Brassica* spp. L. (cabbage, cauliflower, turnip, etc., Engel et al. (2002)), *Solanum lycopersicum* L. (tomato, Williams (1973)), *Camellia sinensis* L. (tea, Smet et al. (1998)), *Zea mays* var. *rugosa* L. (sweetcorn, Bills and Keenan (1968)), and *Tuber* spp. P.Micheli *ex* F.H.Wigg (truffles, Bellesia et al. (1996) and Talou et al. (1990)). Methanol is produced from pectin (a heteropolysaccharide from plant cell walls and used as a gelling agent in jams, etc. – found at particularly high concentrations in citrus peels, carrots, apples, and apricots) breakdown in the gut at a rate of about 10–20 mmol per day (Lindinger et al. 1997) and is also taken up from alcoholic drinks – for example, a 50 mL measure of brandy contains 2.5 mmol methanol (Lindinger et al. 1997).

2.1.2 C_1 Exposure from "Dietary Supplements" and "Alternative Medicines"

Two C_1 sulfur compounds have become marketed as "dietary supplements" ($DMSO_2$) or "alternative medicines" (DMSO) in the last 20 years. The former is sold as "methylsulfonylmethane" or MSM and is produced from waste from the Kraft paper process, as is DMSO, which is sold as an embrocation for use on stiff joints, etc. Thus far, peer-reviewed clinical trials have shown minor beneficial effect in some conditions (Usha and Naidu 2004), and in vitro studies have shown antioxidant potential in $DMSO_2$. It has been shown as safe for consumption of >2.5 g per day with no ill effects. $DMSO_2$-containing products currently marketed include toothpastes, mouthwashes, shampoos, etc., all of which could have

implications for perturbing the methylotrophic body flora. It is worth noting that sports supplements containing choline are converted to TMA in the gut, and thus can be an indirect source of TMA from such products.

2.1.3 C_1 Exposure from the Environment and Occupational Exposure

Formate (as formic acid) is produced by ants of the genus *Formica* L. (wood ants, Hoffman (2010)) and in the stinging hairs of *Urtica dioica* L. (the stinging nettle, Klaessen (2013)). It is also produced from soils in deciduous forests (Sanhueza and Andreae 1991) and probably from most soils, as is methane. Methylated amines and sulfur compounds are produced in various plants and in decaying animal tissue. DMS, DMSO, and $DSMO_2$ are abundant in marine environments, in both the water and the atmosphere, as is MSA.

Occupational exposure is of course largely for individuals working in the laboratory sciences, where MeSH, DMS, DMSO, etc., can readily be absorbed through the skin (Ljunggren and Norberg (1948) and from the author's own experience: 5 mL of a 10 mM DMS solution [totaling 50 μmol DMS or 3.1 mg] spilt on the hand could be tasted for 16–24 h and was detectable in the odor of urine for 48 h!). TMA and DMA emitted during the making of solutions of their hydrochloride salts cling on the hair and skin for several hours. Solvents such as dichloromethane and carbon tetrachloride are used in the chemical industry and can be inhaled resulting in their transfer to the bloodstream or can be exposed to the skin. DMS and DMSO are produced in the Kraft paper process and workers can be exposed (Leach and Chung 1982). In addition to laboratory and chemical industry workers, there is exposure to DMS and methane at significant concentrations in swine and fowl farm workers (Kim et al. 2007; Tymczyna et al. 2007). In animal rendering plants, Maillard reactions in slaughterhouse waste heated to >105 °C for tallow extraction result in production of high concentrations of DMS that workers may be exposed to (van Langenhove et al. 1982). Formaldehyde is used in embalming in mortuaries and in the preservation of medical and zoological specimens in spirit collections, and so occupational exposure through these routes is likely even with extraction systems.

2.2 Endogenous Exposure of the Human Body to C_1 Compounds

Per Sect. 2.1, in this section I have outlined some of the major routes by which the human body can be exposed to endogenous C_1 compounds. Per my comments on Sect. 2.1, it is not intended to be exhaustive.

2.2.1 Oral Cavity

The odor of the human oral cavity changes with diet, medication, sexual intercourse, and age. Oral malodor (halitosis) typically results from the production of DMS, MeSH, carbon disulfide, MMA, DMA, TMA, and other volatiles (including methanol and some methane), particularly from *Bacteria* located in the periodontal pockets and on the rear of the tongue, as well as in the tonsillar crypts (Tonzetich 1973; Turner et al. 2006). Methylated selenium species (MeSeH and

dimethylselenide) may potentially be present in the oral odor profile of persons consuming high-selenium diets or taking "selenium yeast" as a dietary supplement, but the examination of methylated selenium and tellurium species in the human body thus far has not been undertaken.

2.2.2 Skin

Human skin odor again, like that of the mouth, is subject to change and can contain methylated amines and methylated sulfur species. It also varies across the body, with those of the axillae and inguinal regions being particularly distinctive and varied by biological sex, age, hormone profile, hormone cycle position, sexual intercourse, exercise, adrenaline levels, medication, and diet. Full details of the odor of the skin and hair are given in Wood and Kelly (2010) in a previous edition of this handbook.

Bromhidrosis (offensive body odor) can be caused by overgrowth of *Corynebacterium* spp. and *Micrococcus* spp. The feet are also a cause of malodor, and, like all body odor, this is largely mediated by clothing (Callwewaert et al. 2014).

Various clinical conditions are associated with altered body or skin odor such as phenylketonuria (mouse or musty odor) or 2-oxoglutarate dehydrogenase complex deficiency (maple syrup or caramel odor). Some of these conditions result in C_1 compound production such as hypermethioninemia or type I tyrosinemia (carbon disulfide and/or MeSH) and trimethylaminuria, which results in the inability of the body to oxidize TMA into TMAO, resulting in TMA secretion and a strong odor that may be fish-like or may just be unpleasant. This is present across the whole body, including the mouth and urine, but the skin is the largest source (Mitchell and Smith 2001). Similar effects are observed in dimethylglycinuria. The impacts of trimethylaminuria and dimethylglycinuria have been discussed by Wood et al. (2010) in a previous edition of this handbook. Gangrene and skin ulcers emit strong odors containing methylated amines, DMS, etc., also, but this has not been investigated in detail with respect to C_1 compounds – DMTS has been found in some cancer-associated wounds (Shirasu et al. 2009). While not odorous, $DMSO_2$ can be detected on the backs but not the arms of humans (Gallagher et al. 2008), which is interesting since $DMSO_2$ is found in cerebrospinal fluid, particularly in the spine (Engelke et al. 2005).

2.2.3 Genitalia

The odor of the healthy female genital tract is dominated by fatty acids, which increases during arousal and following sexual intercourse. The odor of TMA may be present post-intercourse or after ovulation owing to bacterial activity, but is very strong during bacterial vaginosis (Wood et al. 2010) and sexually transmitted infections (Preti et al. 1978). TMA is not present in the odor profile of most healthy vaginae, *pace* immediately following menstruation in some individuals, as mentioned above (Wolrath et al. 2001). The odor profile of the neovagina of trans women is lower and subject to less discharge, and the flora largely resembles those of the

anal canal or bacterial vaginosis flora (Weyers et al. 2009). Penile skin flora, particularly in uncircumcised males, produces TMA around the base of the glans, particularly if copious smegma is evident (Cold and Taylor 1999). There is a paucity of data on the odor of the neopenis in trans men.

2.2.4 Gastrointestinal Tract

The large intestine produces methane and a range of sulfur-containing gases such as DMS, MeSH, and hydrogen sulfide (Suarez et al. 1999). This varies with diet (Florin et al. 1991), with sulfur-rich and selenium-rich foods contributing MeSH, DMS, DMTS and DMDS, or MeSeH and dimethylselenol, respectively (Moore et al. 1987). The processes of their formation by anaerobic *Bacteria* and *Archaea* acting ultimately on cysteine, cystine, and methionine from digested proteins are analogous to the putrefaction of proteins in decaying animal tissue, in landfill, or in anoxic soils, which are outlined in Schäfer et al. (2010).

3 Methylotrophs Known to Be Associated with the Human Body

The first reports of methylotrophs associated with the human body as part of the normal flora were published by Dr. Ann P Wood's group (formerly of King's College London, UK) in the early part of this century and made use of both isolation and molecular methods. Since this pioneering work almost 20 years ago, there have been a considerable number of reports of human-associated methylotrophic *Bacteria*. As I discuss herein, there has been a considerable volume of work as a direct result of these studies at King's College London, which have now begun to lead to an understanding of the role of methylotrophs and methanotrophs from the normal flora in hollow organ and breast cancers and in various pathologies – it is of no doubt that those early studies regarding "*Methylobacterium podarium*" (now *Methylorubrum podarium*) will, in the fullness of time, directly lead to work that save lives.

Isolation of strains for characterization has been a core part of microbiology for over 100 years, and, while it is not as fashionable as metagenomics, etc., it is entirely necessary for obtaining evidence on enzyme biochemistry, evolution, etc., and some of the human-associated methylotrophs in pure culture are very well characterized, but we do not have genome sequences yet for any *Methylorubrum podarium* strains, for example. It is worth noting that there are a lot of studies in the last decade that produce a large number of isolates without any identification or characterization (e.g., feet and oral isolates from Waturangi et al. (2011) that were isolated on 24 mM methanol), and while these libraries of organisms might be very useful to the community, it is really critical that proper identifications are made and deposits into international service collections are made before strains are lost.

In this section I have covered three particularly interesting taxa in some detail and summarized the remainder of the work done, including some recent molecular ecological studies that have detected methylotroph presence.

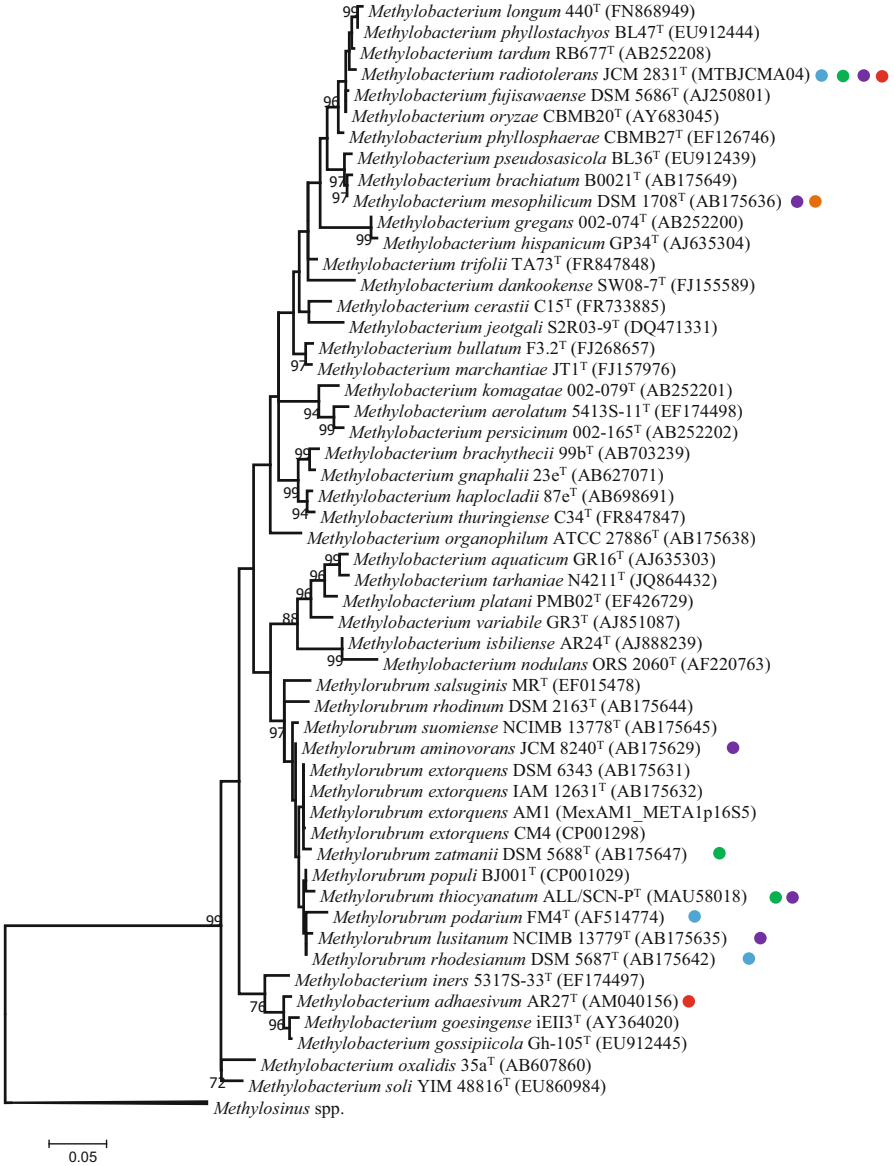

Fig. 1 Phylogenetic tree on the basis of the 16S rRNA (*rrs*) gene, showing the positions of *Methylobacterium* spp. and *Methylorubrum* spp., using the sequences from *Methylosinus* spp. as the outgroup. Nucleotide sequences were aligned using MUSCLE (Edgar 2004) without use of any presets for speed that reduce accuracy. Aligned sequences were tested for best fit to models on the basis of the Bayesian information coefficient (BIC) in MEGA 7.0.26 (Kumar et al. 2016) and the trees reconstructed accordingly, using the Tamura-Nei model (Tamura and Nei 1993) with a discreet gamma distribution to model rate differences across sites (gamma parameter, 0.1439) and allowance for some sites to be evolutionarily invariable (67.04% of sites). The tree shown is the optimal tree

4 *Methylobacterium* and *Methylorubrum* spp.

Methylobacterium spp. (*Mbt.*) are the archetypal "pink-pigmented facultative methylotroph" (PPFM) that one can easily isolate in the classical experiment of placing leaves on mineral salts agar for a few hours and then removing the leaves and incubating in an atmosphere of methanol vapor – they appear as salmon, pink, or orange colonies that fit the shape of the original leaf. Recently, Green and Ardley (2018) have proposed the reclassification of a large group of *Methylobacterium* spp. to *Methylorubrum* (*Mrb.*), a newly created genus (as shown in Fig. 1). Members of these genera are found in a myriad of environments including plants, bryophytes, and soils to more nutrient-poor environments such as drinking water (*Methylobacterium adhaesivum* (Gallego et al. 2006), *Methylobacterium hispanicum* and *Methylobacterium aquaticum* (Gallego et al. 2005a), and *Methylobacterium isbiliense* (Gallego et al. 2005b)), the air (*Methylobacterium aerolatum* and *Methylobacterium iners*, Weon et al. 2008), and fermented seafoods (*Methylobacterium jeotgali* Aslam et al. 2007) – all potential routes through which the human body could make contact and become colonized – but it is interesting to note that these taxa are not among those known to cause infection.

Methylobacterium spp. and *Methylorubrum* spp. are found in many bathrooms as pink or orange biofilms growing on tiling grout or on shower curtains and taking up C_1 compounds found near to toilets. It has recently been proposed that the presence of *Methylobacterium* spp. and *Methylorubrum* spp. in bathroom biofilms corresponds to a lack of potentially pathogenic *Mycobacterium* spp. in said biofilms and that this could be a useful indicator (Falkinham et al. 2016).

4.1 Role in Normal Human Flora

From the feet of healthy volunteers, various strains of *Methylorubrum podarium* (basonym, *Methylobacterium podarium*) have been isolated. The type strain ($FM4^T$ = DSM 15083^T = ATCC BAA-547^T) was isolated from a toe cleft. The

Fig. 1 (continued) with the highest log-likelihood after 5,000 replications (−18,617.15), with numbers at nodes indicating the percentage of 5,000 bootstrap replications in which the topology was preserved (values <70% omitted for clarity). All positions at which there was less than 95% coverage were omitted from the final analysis, in which 1,345 nt was used. Branch lengths are to scale and indicate the number of substitutions per site – bar represents 0.05 substitutions per site. Accession numbers are given in parentheses and refer to the GenBank database, unless they contain an underscore ("_"), which designates that they are from the Integrated Microbial Genomes and Metagenomes (IMG/M) database. Type strains are indicated by a superscript "T" – multiple strains of *Mbt. extorquens* are included, viz., the "workhorse" strain AM1 and the various strains that grow on methyl halides, and were reclassified into this species. Colored dots indicate *cyan*, skin isolate; *green*, mouth isolate; *orange*, vaginal isolate; *violet*, opportunistic pathogen; *red*, associated with cancer

cleft was swabbed with a sterile cotton swap which was used to inoculate an enrichment culture using 20 mM MMA as the sole carbon source, from which the isolate was obtained on MMA agar. This pink-pigmented species grows on MMA, DMA, TMA, methanol, MeSH, DMS, DMSO, $DMSO_2$, formate, and a wide range of organic acids, alcohols, and sugars (Anesti et al. 2004).

Methylorubrum zatmanii (basonym, *Methylobacterium zatmanii*) PI-UM was isolated from the mouth of a patient with significant dental caries and periodontal disease by enrichment culture on 12 mM methanol (Carvajal et al. 2011). *Methylorubrum rhodesianum* (basonym, *Methylobacterium rhodesianum*) was isolated from the front of the human scalp (Uy et al. 2013), and a further *Methylobacterium* or *Methylorubrum* sp. was isolated from the nasal vestibule (Uy et al. 2013). A strain of *Methylobacterium radiotolerans* was isolated from the naval (David and Diongzon 2013) – some properties of this strain are reviewed in Carvajal and Galvez (2015). *Methylorubrum thiocyanatum* (basonym, *Methylobacterium thiocyanatum*) and *M. radiotolerans* strains have been isolated from the human mouth (Anesti et al. 2005; Hung et al. 2011), as well as strains from the urinary tract (Lee et al. 2004). Lauder et al. (2016) detected *Methylobacterium* or *Methylorubrum* spp. on placental samples.

4.2 Role as Human Pathogens

In spite of *Methylobacterium* spp. being "workhorse" organisms in laboratories worldwide for many decades, they have recently been identified as opportunistic pathogens, before we fully understood that they were part of the normal flora in the seminal work of Wood, though we did have an understanding of their prevalence in human-associated environments, viz., their presence as 20% of the cultivable flora in dental unit waterlines (Barbeau et al. 1996). The first reports in the 1980s were of *Methylobacterium mesophilicum* in nosocomial infections (Gilardi and Faur 1984; Gilchrist et al. 1986; Rutherford et al. 1988; Smith et al. 1985) and later came reports of a patient given contaminated bone marrow (Brown et al. 1996) and in HIV patients with bacteremia (Truant et al. 1998). It is interesting to note that the type strain *Mbt. mesophilicum* ATCC 29983^T was isolated from a leaf (Austin and Goodfellow 1979). The ability of this genus to form robust biofilms no doubt contributes to its prevalence in the environment and its pathogenicity (Kovaleva et al. 2014). *Methylobacterium radiotolerans*, *Methylorubrum thiocyanatum*, *Methylorubrum aminovorans* (basonym, *Methylobacterium aminovorans*), and *Methylorubrum lusitanum* strains have all been isolated from patients with hospital-acquired bacteremia (Lai et al. 2011). It is worth noting that *Mrb. lusitanium* is likely a heterotypic synonym of *Mrb. rhodesianum* (Kato et al. 2005).

Methylobacterium mesophilicum was included in the Vaginal Flora Microbial DNA qPCR Array (Qiagen 2013), indicating that the importance of this opportunistic pathogen and accurate diagnosis thereof has been accepted. Molecular ecological methods have identified methylotrophs in the vagina during bacterial vaginosis (Wood et al. 2010) and in sex workers (Schellenberg et al. 2011).

Transcripts from *Methylobacterium* spp. and *Methylorubrum* spp. were found albeit in relatively small numbers in both women with bacterial vaginosis about to undergo treatment with 200 mg lactoferrin applied *per vagina*, and in women after treatment with 100 mg lactoferrin, which restored this genus that was not easily detected in the pretreatment state (Pino et al. 2017).

4.3 Potential Roles in Breast Cancer

Recently several links between *Methylobacterium* spp. or *Methylorubrum* spp. and breast cancers have been published. A decreased level of these genera in breast tissue and breast cancer was reported (Wang et al. 2017), suggesting that some compound they would typically degrade on/in the breast could reach the blood at higher levels when the organism is present in lower numbers, with potentially carcinogenic effects. It is hard to determine what that compound is since it may not be a C_1 compound, owing to their facultative nature, but it is entirely plausible that, e.g., dimethyl sulfone taken orally could act as a probiotic, encouraging growth of *Methylobacterium* spp. or, more likely, *Methylorubrum* spp. on the skin and, potentially, in some way reducing cancer risk. That said, the Wang et al. (2017) study is one study: significantly more work needs to be undertaken before we can be certain of any role of these genera in cancer prevention, and given we know *Methylobacterium* spp. and *Methylorubrum* spp. are pathogenic, any proposed therapies need to be implemented with great caution and not used in immunocompromised individuals.

While the above Wang et al. (2017) study suggests *Methylobacterium* being depleting in number may be associated with breast cancer, Xuan et al. (2016) found the opposite. In their study, *Methylobacterium radiotolerans* was highly enriched in breast tissue, along with *Sphingomonas yanoikuyae* (we know that some *Sphingomonas* spp. grow methylotrophically, cf. Boden et al. 2008). Yadzi et al. (2016) evaluated the presence of these two species in sentinel lymph nodes removed at mastectomy from breast cancer patients, finding their presence to be much higher in nodes adjacent to stage 1 tumors than in controls (e.g., 97% of stage 1 tumor samples contained *Mbt. radiotolerans* or *S. yanoikuyae* DNA), and in stage 2 and stage 3 tumors, the levels of these two organisms were even higher.

The type strain of *Methylobacterium radiotolerans* (ATCC 27329^T = NCIMB 10815^T) was isolated from rice grains and tolerates 0.18 Mrad (= 1.8 kGy) doses of gamma radiation (Ito and Iizuka 1971) – if indeed this organism is involved in cancer causation, this could have implications in gamma radiotherapy, where local doses are in the region of 0.002 kGy during breast cancer treatment (Hong et al. 2018), and would not kill the organism. The radiation tolerance mechanisms are probably an evolutionary adaptation to dehydration, in common with other radiation tolerant organisms and in keeping with its source of isolation in dehydrated rice (Musilova et al. 2015). Interestingly, the type strain of *S. yanoikuyae* (ATCC 51230^T = DSM 7462^T) was originally isolated from an unspecified clinical specimen, suggesting it may be prevalent on the human body (Yabuuchi et al. 1990).

Of course it is obvious that breast tumors – like many cancers – often become necrotic and emit MMA, DMA, TMA, DMS, MeSH, DMDS, DMTS, and other pungent volatiles (Warnke et al. 2003; Shirasu et al. 2009); the *Methylobacterium* spp. present may simply be "mopping up" these compounds and may have absolutely no role in cancer.

4.4 Potential Roles of *Methylobacterium* spp. and *Methylorubrum* spp. in Other Cancers

Methylobacterium spp. or *Methylorubrum* spp. were found to be enriched in number in cancerous tissue of colorectal cancer patients versus controls (Chen et al. 2012) and in gastric cancer patients – specifically *Mbt. adhaesivum* in the latter case (Castaño-Rodriguez et al. 2017). It is worth noting that the type strain (CCM 7305^{T} = CECT 7069^{T}) of *Mbt. adhaesivum* has been isolated from drinking water supplies (Gallego et al. 2006); thus the route of exposure to the stomach is easy to determine.

4.5 Perspectives

It is interesting to note that the pathogenic *Methylobacterium* spp. and *Methylorubrum* spp. are seemingly limited to a relatively small number of species. Figure 1 shows a maximum likelihood tree based on the 16S rRNA (*rrs*) gene of *Methylobacterium* spp. and *Methylorubrum* spp. using *Methylosinus* spp. as the outgroup. Full details of the tree algorithms, etc., are given in the legend to Fig. 1. It can be seen from Fig. 1 that the cancer-associated organisms are found in both genera but that the nonpathogenic skin, vagina, and mouth isolates are mostly relatively closely related – though some of these are agents of infection – and are members of *Methylorubrum*.

While findings on *Methylobacterium* and *Methylorubrum* and their associations with human cancers are interesting, there is, however, a major caveat that must always be upheld: findings from molecular-based studies probably need to be reinforced with isolation-based work. This is owing to the presence of contaminating biomass/DNA from these genera – and from other methylotrophs – being commonplace in DNA and RNA extraction and purification kits, as well as in other molecular ecology sundry items. Salter et al. (2014) gives a good overview of the relevant literature on these kits. Contamination of so-called "ultrapure" (filtered) water supplies with the same organisms is also well understood with the evidence base going back over 25 years now (e.g., Kulakov et al. 2002; McFeters et al. 1993) – obviously glass-distilled water that has then been autoclaved may provide a suitable alternative as DNA/RNA would not be anticipated to distill over! It is critical that all molecular-only demonstrations of the presence of these organisms in the human

body, particularly where their presence is then correlated with some pathology or other, are validated to show the presence of viable and/or isolable cells.

It is clear that a proper appraisal of (1) infectious agent status and (2) oncogenesis is needed in *Methylobacterium* spp. and *Methylorubrum* spp. as a priority – these species are handled worldwide in research laboratories and teaching laboratories, occur in domestic bathrooms, are found on the skin of probably most humans on the planet, and are potentially causative agents of infectious disease and cancer, but are not currently handled as such.

5 *Hyphomicrobium sulfonivorans*

Hyphomicrobium sulfonivorans S1^T (=ATCC BAA-113^T = DSM 13863^T) was originally isolated from garden soil using $DMSO_2$ as the sole carbon source; it also grows on other C_1 sulfur compounds and amines and is very well characterized (Borodina et al. 2000, 2002; Boden et al. 2011). *Hyphomicrobium* spp. are prosthecate organisms that have holdfasts and form rosettes and flocks in culture and can adhere strongly to surfaces in biofilms. They are well known to grow in low-nutrient environments, and workers in most C_1 research laboratories will be familiar with *Hyphomicrobium* spp. being found in the distilled water aspirator! *H. sulfonivorans* CT and DTg were isolated from the teeth and tongues of healthy volunteers (Anesti et al. 2005) using 2 mM DMS as the sole carbon source. These strains can grow on DMS, DMSO, $DMSO_2$, MeSH, methanol, MMA, TMA, formaldehyde, and formate but not DMA. They also grow on a range of organic acids and sugars – it is thus difficult to know if they are actually "methylotrophing" when in the human mouth, though in all likelihood, they are probably assimilating a whole range of carbon sources at the same time when growing in situ versus our in vitro cultures on single substrates.

6 *Brevibacterium casei*

Brevibacterium casei 3Tg and 3S(a) were isolated from dental hospital patients with periodontal disease using 2 mM DMS as the sole carbon source (Anesti et al. 2005). They grow on DMS, DMSO, MeSH, methanol, MMA, TMA, and formate but not DMA and grow on a range of organic acids, sugars, and complex media. *Brevibacterium* is not considered a "classical" methylotrophic genus; however, the first report was in *Brevibacterium methylicum* (Nešvera et al. 1991). Interestingly, the type strain *B. casei* DSM 20657^T will grow on methanol, MMA, MeSH, and DMS (Anesti et al. 2005), demonstrating that methylotrophy is probably widespread in this species, if not this genus. Hung et al. (2011) isolated *Brevibacterium* spp. from the human mouth also.

7 Immune Modulation by *Methylococcus capsulatus*

Methylococcus capsulatus (*Mcc.*) is probably the best studied obligate methanotroph. The type strain Texas^T was originally isolated from a sewer but has not been studied in much depth – the Bath strain has become the international workhorse methanotroph. It contains intracellular stacked membranes of the "type I" variety (bundles of disc-shaped vesicles in the middle of the cytoplasm); the dominant fatty acids are palmitic ($C_{16:0}$) and palmitoleic ($C_{16:1}$) acids. It uses 18-methylene-ubiquinone-8 as the major respiratory quinone, which is rather unusual in the *Gammaproteobacteria* but common in the *Methylococcales*. An excellent review of *Methylococcus capsulatus* general physiology and metabolic potential is that of Kelly et al. (2005).

While not a human isolate, freeze-dried, lysed methane-grown cells of the Bath strain (=NCIMB 11132) of *Methylococcus capsulatus* (albeit incorrectly described as a "soil bacterium" when it came from sulfidic groundwater!) have been shown to prevent inflammatory bowel disease in mouse models (Indrelid et al. 2017) and adhere to human dendritic cells, influencing their ability to produce cytokines, activate T cells, etc. Previous work by Christoffersen et al. (2015) showed that *Mcc. capsulatus* Bath reduced intestinal inflammation in animal models, and work by Kleiveland et al. (2013) showed that *Mcc. capsulatus* Bath reduced ulcerative colitis in animal models.

8 Other Human Isolates and Isolation Work Biases

In the oral work of Anesti et al. (2005), *Micrococcus luteus* and *Variovorax paradoxus* strains were isolated on C_1 compounds. Further oral work by Hung et al. (2011) isolated *Gordonia*, *Leifsonia*, *Microbacterium*, *Micrococcus*, *Rhodococcus*, *Achromobacter*, *Klebsiella*, *Pseudomonas*, and *Ralstonia* strains on C_1 compounds, growing variously on MSA, MMA, and $DMSO_2$. All of these organisms are canonically "atypical" methylotrophs, but it is worth noting that the author has isolated DMA-using *Mycobacterium fluoranthenivorans* DSQ3 and *Rhodococcus erythropolis* DSQ4, TMA-using *Pseudomonas mendocina*, and MMA-using *Brevibacterium casei* MSQ5 (Boden et al. 2008) from river sediment, and there are many reports of Gram-positive methylotrophs.

These "noncanonical" methylotrophs are relatively easy to isolate, and it is worth noting that the media used for the studies of Boden et al. (2008), Hung et al. (2011), Borodina et al. (2000, 2002), and Anesti et al. (2004, 2005) were all using basal salts of ionic strength 1.12 M (determined according to Debye and Hückel (1923) using data from the above studies minus the carbon source) – this is a comparatively very high ionic strength relative to media used for *Methylobacterium* spp. or methanotrophs, for example, nitrate mineral salts (NMS) of Dalton and Whittenbury (1976) have an ionic strength of 0.11 M (using data from Smith and Murrell 2011), and "dilute" variations used for *Methylocella* spp. could be as low as 0.01 M. It is possible that using higher ionic strength media in future work could reveal the diversity of Gram-positive methylotrophs (and potentially methanotrophs), but

similarly, use of lower ionic strength media in future human studies may help isolate "canonical" methylotrophs and methanotrophs.

9 Research Needs

1. The *urgent* assessment of pathogenicity, oncogenesis, mutagenesis, and teratogenesis in all *Methylobacterium* spp. and *Methylorubrum* spp. is held in the international service collections and potentially the rest of the *Methylobacteriaceae*, if not all of the *Rhizobiales*. This is necessary to fully understand the gamut of potential infections and cancers that these organisms are associated with, and to aid current and future patients with *Methylobacterium* or *Methylorubrum* infections and cancers but is also critical for the protection of laboratory workers worldwide. In the meantime, I strongly recommend all *Methylobacterium* and *Methylorubrum* spp. be treated as Containment Level 2 (CL2) organisms rather than their usual Containment Level 1 (CL1) handling until we know more about the potential hazards and risks. It would seem prudent to remove them from the teaching laboratory unless at CL2 level.
2. Reevaluation of molecular-only studies that associate *Methylobacterium* and *Methylorubrum* spp. with human cancers, demonstrating the presence of viable cells and ruling out kit/reagent contamination.
3. Reappraisal of methylotrophy in known human-associated *Bacteria*, particularly Gram-positive organisms from the class *Actinobacteria*. Isolation with higher ionic strength media may achieve this.
4. The normalization of workers assessing growth on methanol, MMA, etc., in all new bacterial isolates to help see the true diversity of methylotrophy.
5. An appraisal of methylotrophs and methanotrophs in the anal canal and perianal area since methane (and DMS, MeSH, etc.) and oxygen are present, and this environment should support growth.
6. Greater understanding of the role of *Methylobacterium* and *Methylorubrum* in breast cancer prevention – is it cause or effect? Does it only apply to the breast? What happens in other cancers? Does $DMSO_2$ consumption reduce the risk or elevate of these types of cancer in model organisms?

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Part IV

Pathogens in Oil

Hydrocarbon Degraders as Pathogens

11

Fernando Rojo and José Luis Martínez

Contents

Abstract

Among the microorganisms isolated from soil and aquatic environments that are able to degrade hydrocarbons, it is not infrequent to find species that can be pathogenic for humans, animals, or plants. In most cases, these microorganisms are opportunistic pathogens, that is, species that can infect only debilitated individuals who have a previous disease or are immunocompromised. Several opportunistic pathogens can thrive in many different habitats, a eukaryotic host being just one of them. Certain specialized pathogens, for which the main way of life is to infect a host, can degrade hydrocarbons as well. In addition to the scientific interest of these findings, the success of pathogenic strains in oil-contaminated environments poses some concerns when it comes to implementing bioremediation strategies for treating oil spills or polluted sites. This chapter analyzes the possible reasons as to why several pathogenic bacteria are efficient hydrocarbon degraders.

F. Rojo (✉) · J. L. Martínez
Centro Nacional de Biotecnología (CNB), CSIC, Madrid, Spain
e-mail: frojo@cnb.csic.es; jlmtnez@cnb.csic.es

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1 Introduction

A quick look at published data on bacterial strains able to degrade aromatic or aliphatic hydrocarbons immediately allows identifying several bacterial species that are well-known opportunistic pathogens for several eukaryotic hosts, such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, or *Xylella fastidiosa* (Cerniglia 1992; Lee et al. 1996; Alonso et al. 1999; Yuste et al. 2000; Marín et al. 2001; van Hamme et al. 2003; Wentzel et al. 2007). A survey of gene banks also shows that key enzymes for oxidation of hydrocarbons are present in many opportunistic or obligate pathogenic bacteria. For example, genes for alkane degradation belonging to the AlkB family of alkane hydroxylases are found in many strains of pathogens such as *P. aeruginosa*, *Pseudomonas syringae*, or several species of the *B. cepacia* complex (van Beilen et al. 2003; our own unpublished results). AlkB-related alkane hydroxylases are also present in the genomes of several *Acinetobacter baumannii* isolates that are known to create serious outbreaks in hospitals due to the occasional emergence of antibiotic-resistant strains (Vallenet et al. 2008) or in the genome of several *Legionella pneumophila* strains, which can infect humans (Steinert et al. 2007). Most notably, AlkB-related alkane hydroxylases are also present in several pathogenic *Mycobacterium* species, such as *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium*, or *Mycobacterium ulcerans*.

Stimulation of microbial growth in oil-contaminated sites to facilitate the degradation of hydrocarbons and attenuate pollution can lead to a significant increase in bacterial population numbers. Although population numbers will eventually decrease and return to standard levels upon consumption of the available nutrients, and by the action of predators and bacteriophages, these changes in the structure of the microbial populations can favor the enrichment of potentially pathogenic bacterial species. This may raise some concerns regarding the implementation of bioremediation strategies. In the following sections, we discuss some ideas that may explain why pathogenic bacterial species can in some circumstances be so successful in contaminated sites.

2 Lifestyles of Pathogenic Hydrocarbon-Degrading Bacteria

Most of the hydrocarbon-degrading opportunistic pathogens mentioned above are free-living saprophytes that have a very versatile metabolism and can thrive in many different environments using a wide array of carbon sources. There are several possible reasons as to why several of these opportunistic pathogens are isolated so frequently from sites contaminated with hydrocarbons. One explanation is merely technical: in many cases, hydrocarbon-degrading strains are obtained by classical enrichment procedures, which leads to the isolation of those strains that grow faster in laboratory growth media. Bacteria belonging to the *Pseudomonas*, *Burkholderia*, or *Acinetobacter* genera meet this criterion. However, this does not necessarily mean

that they play a major role in the bioremediation of polluted sites. In fact, recent data show the opposite. After exposure to a load of petroleum hydrocarbons, the microbial communities of the affected sites change both taxonomically and functionally. This process is in turn dynamic and characterized by the successional appearance of different bacterial groups (Acosta-González et al. 2013; Gutiérrez et al. 2013; Brakstad et al. 2015; Rodríguez-R et al. 2015; Handley et al. 2017). The species that become predominant at each time vary depending on the characteristics of the polluted site (temperature, salinity, oxygen concentration, carbon sources available, etc.). Several analyses using in situ techniques to unravel which strains metabolize hydrocarbons in contaminated sites have shown that degradation of these compounds can occur either by the combined action of several, often many, different bacterial species or by growth of a limited set of hydrocarbon-degrading species, often specialized hydrocarbonoclastic bacteria (Kasai et al. 2002; Head et al. 2006; McKew et al. 2007; Yakimov et al. 2007). At least in some cases, and particularly in soils, strains belonging to the genera *Pseudomonas*, *Burkholderia*, and *Acinetobacter* have been shown to participate – among several other bacterial species – in the biodegradation process, although they rarely play a major role (Jeon et al. 2003; Kaplan and Kitts 2004; Margesin et al. 2003; Redmond et al. 2010; Hu et al. 2017).

Therefore, these opportunistic pathogens seem to be truly adapted to degrade hydrocarbons in contaminated sites. However, they can assimilate many other carbon sources as well and are not restricted to live on hydrocarbons. When looking to the structure of bacterial populations, three different categories can be foreseen (Martínez 2018). One is formed by specialists, such as *M. tuberculosis*, which can only colonize a restricted range of habitats. A second group is formed by generalists, such as different members of the *Pseudomonas* genus, which have large genomes that allow them to colonize several habitats. The third one is formed by multispecialists, a group in which the species as a whole can colonize several ecosystems, although different members of the species can present distinct properties. An example of the later can be *Escherichia coli,* which presents a small core genome and a large pan-genome that allows the specialization of individual members of the species, leading to both commensal and pathogenic strains. The question arises as to whether the strains that can degrade hydrocarbons and infect eukaryotic hosts are specialized strains that have acquired a distinct set of genes that make them particularly infective or rather the pathogenic abilities of these strains result from traits that are present in most strains from any environment. This has been studied with some detail in the case of *P. aeruginosa*, *B. cepacia*, and *S. maltophilia*.

P. aeruginosa can be found in many different environments such as soils and water or associated to plants or animals. It is an important opportunistic pathogen for humans, animals, and plants that can produce severe infections in debilitated hosts, and it is a major factor of mortality in cystic fibrosis patients (D'Argenio 2004). *P. aeruginosa* populations tend to be particularly successful in environments contaminated by human activities (Pirnay et al. 2005). Several independent analyses using different approaches have indicated that *P. aeruginosa* strains isolated from soil or water environments, termed environmental strains, are functionally

indistinguishable from strains isolated from infected humans (termed clinical isolates). Most *P. aeruginosa* strains isolated from either clinical or environmental habitats can degrade hydrocarbons such as alkanes and contain AlkB-related alkane hydroxylases (van Beilen et al. 1998; Alonso et al. 1999; Belhaj et al. 2002; Marín et al. 2003; Smits et al. 2003). Detailed genotypic and taxonomical studies of the structure of *P. aeruginosa* populations analyzing large collections of strains isolated from diverse sources found no significant differences between clinical and environmental strains (Römling et al. 1994, 1997; Foght et al. 1996; Kiewitz and Tümmler 2000; Pirnay et al. 2002, 2005; Cabrol et al. 2003; Wolfgang et al. 2003; Morales et al. 2004; Wiehlmann et al. 2007). Virulence determinants such as toxins, secreted proteases and lipases, type III secretion apparatus, or quorum-sensing regulation are found in both clinical and environmental strains. In other words, most strains, regardless of the environment from which they were isolated, have all traits necessary to cause a wide variety of human infections, indicating that clinical isolates are not specialized strains. This does not mean, however, that all strains are identical. For instance, the presence of ExoU, an effector of the type III secretion system in some (not all) *P. aeruginosa* isolates, correlates with increased virulence (Sawa et al. 2014).

Comparison of the genome sequence of five different *P. aeruginosa* strains showed that they all share a highly conserved common core genome, interrupted in each strain by combinations of specific blocks of genes (Mathee et al. 2008). The core genome included about 90% of the genes in the five strains compared. The additional strain-specific DNA regions correspond to DNA segments arranged in genomic islands and islets that can move from one strain to another by horizontal transfer and then be conserved or discarded (Tümmler 2006; Mathee et al. 2008). One of these DNA segments, found in one of the sequenced strains, includes a large cluster of genes involved in diterpenoid metabolism. This genomic plasticity greatly facilitates niche adaptation, and, since acquisition of a new trait does not necessarily eliminate others, the bacterial populations retain their ability to survive in a wide range of environments (Mathee et al. 2008). Traits common to *P. aeruginosa* strains such as secretion of proteases or lipases can be useful both for living on dead plant material and for infecting an immunocompromised human individual. Recent work has shown however the expansion of specific clones (termed high-risk clones) at hospitals worldwide (Woodford et al. 2011). It is possible that these clones have incorporated virulence determinants through horizontal transfer, making them more prone for causing infections and hence being in the route for specialization (Martínez 2013). However, analysis of their virulence using a *C. elegans* model has shown that this is not fully true, since virulence varied broadly depending on the high-risk clone considered (Sanchez-Diener et al. 2017). Since these widespread clones are usually highly resistant to antibiotics, it is possible that their resistance phenotype may enrich for their presence at hospitals and patients under antibiotic therapy (Treepong et al. 2018). Whether or not this enrichment could modify the overall population structure of *P. aeruginosa* remains to be established.

The cases of *B. cepacia* and *S. maltophilia* are rather similar to that of *P. aeruginosa*. The *B. cepacia* complex is a group of nine closely related species

that have a versatile metabolism that allows them to grow in many different environments; they also behave as opportunistic pathogens for humans, occasionally infecting debilitated or immunocompromised individuals (Mahenthiralingam et al. 2005; Chiarini et al. 2006). They can colonize the lungs of patients suffering from cystic fibrosis, a process that is normally secondary to *P. aeruginosa* infection of the same individuals but that severely increases lung deterioration. On the other hand, *B. cepacia* strains have useful properties as plant pest antagonists and can promote plant growth, but their potential pathogenicity has hampered their use in biotechnology (Chiarini et al. 2006). Several strains belonging to the *B. cepacia* complex can assimilate hydrocarbons such as toluene, naphthalene, fluorene, or alkanes (Grifoll et al. 1995; McClay et al. 1996; Smits et al. 1999; Yuste et al. 2000; Marín et al. 2001; Jeon et al. 2003; Chiarini et al. 2006). Not all members of the *B. cepacia* complex are equally infective, and not all are recovered with equal frequency from distinct environments such as plant rhizosphere or surface waters. However, it seems that *Burkholderia cenocepacia* is particularly well adapted both to the human lung and to the plant rhizosphere, a characteristic that has been ascribed to its higher metabolic versatility (Alisi et al. 2005; Chiarini et al. 2006). Inspection of gene banks shows that several *B. cenocepacia* strains contain AlkB-related alkane hydroxylases, so that they can presumably assimilate alkanes. Interestingly, a genomic island was recently identified in a *B. cenocepacia* strain that includes both virulence- and metabolism-associated genes (Baldwin et al. 2004). Some genomic islands in *P. aeruginosa* contain genes that are highly homologous to genes identified in *Burkholderia* sp. This has led to propose that there may be frequent exchange of genetic material between the two microorganisms, a likely process considering that the two species can inhabit the same environmental niches and can form mixed biofilms in the lungs of cystic fibrosis patients (Eberl and Tümmler 2004, and references therein). *B. cepacia* virulence factors include secreted proteases and lipases and production of efficient siderophores for iron uptake, among others (Mahenthiralingam et al. 2005). These traits confer a wide metabolic versatility and facilitate the colonization of many different habitats, including human lungs. Therefore, as it occurs with *P. aeruginosa*, it is likely that the same traits that facilitate the successful colonization of many different habitats to *Burkholderia* sp. also allow for the invasion of eukaryotic hosts.

S. maltophilia, on its hand, is a free-living organism that can colonize diverse ecosystems. Several isolates have been described that can assimilate different hydrocarbons (Hawle-Ambrosch et al. 2007; Hassanshahian et al. 2013; Arulazhagan et al. 2017; Kumari et al. 2018). At the same time, *S. maltophilia* can cause infections in patients with underlying diseases. Recent work has shown that this species presents a large core genome and that environmental and clinical isolates do not form independent evolutionary branches (Lira et al. 2017).

The observations summarized above do not mean however that environmental hydrocarbon degraders cannot evolve toward virulence. Indeed, recent work suggests that some clinical isolates belonging to the *Pseudomonas putida/Pseudomonas fluorescens* complex have acquired a set of virulence determinants that allow them to colonize the infected patient (Molina et al. 2016a, b). Nevertheless, infections

by these organisms are still rare, and knowing whether or not they will constitute a problem in the future remains to be established.

The finding that certain bacterial species considered to be specialized pathogens can also degrade hydrocarbons is perhaps more surprising. Several mycobacteria can degrade polycyclic aromatic hydrocarbons (Kim et al. 2005; López et al. 2006, 2008) or *n*-alkanes (van Beilen et al. 2002; Kotani et al. 2006; Funhoff et al. 2006). These strains are frequent in soils and probably use, among other carbon sources, the hydrocarbons present at low concentrations produced by plants or other organisms or derived from the combustion of plants. However, the genome of the obligate parasite *Mycobacterium tuberculosis* contains an AlkB-related alkane hydroxylase that was shown to be functional in heterologous expression assays (Smits et al. 2002; van Beilen et al. 2002). Since the natural habitat of this bacterium is the human body, it is not clear what the use of this enzyme system could be, although it is worth noting that lipid metabolism is very important in *M. tuberculosis* (Cole et al. 1998; Niederweis 2008) and alkane hydroxylases can facilitate the assimilation of fatty acids through omega hydroxylation (Watkinson and Morgan 1990).

3 Characteristics That Can Be Useful for Both Oil Biodegradation and Pathogenesis

The genes that allow a microbe to infect a given host are considered as virulence factors. These factors can be, for example, toxins, proteases, lipases, or cell surface components such as adhesins or glycoproteins. Although not considered as virulence factors, the genes conferring resistance to antibiotics are also important because they allow the infection of patients under antibiotic treatment. Several traits that can be useful to degrade hydrocarbons are also important for pathogenesis or for antibiotic resistance. The relevance of metabolic versatility has been stressed above. Some additional examples are discussed in detail below.

3.1 Efflux Pumps: Suited to Extrude Both Toxic Hydrocarbons and Antibiotics

Many bacterial species contain efflux pumps in their envelopes that extrude toxic compounds out of the cell. These pumps have been named multidrug resistance (MDR) efflux pumps and have attracted much interest because some of them can confer simultaneous resistance to multiple antibiotics (Lubelski et al. 2007). However, efflux pumps can extrude many different toxic compounds, not just antibiotics. The expression of genes coding for efflux pumps is usually regulated by activators or repressors that respond to the presence of the extruded compounds. The substrate range of the pumps is rather broad, so that they extrude a number of often dissimilar compounds, including molecules that do not act as inducers. These pumps probably contribute to the survival of the bacteria in habitats containing toxic molecules. For example, several bacteria that infect plants or colonize the plant rhizosphere have

MDR pumps that extrude toxic compounds secreted by the plants (Palumbo et al. 1998; Hearn et al. 2003; Maggiorani Valecillos et al. 2006; Stoitsova et al. 2008).

Opportunistic pathogens such as *P. aeruginosa*, *B. cenocepacia*, or *S. maltophilia* contain several MDR pumps that contribute to the intrinsic low susceptibility to antibiotics of these bacterial species (Alonso and Martínez 2000; Morita et al. 2001; Guglierame et al. 2006). However, some of these pumps also extrude toxic compounds that are present in crude oil and that pose a problem for the bacteria, thereby facilitating the use of oil hydrocarbons as carbon source. For example, the *P. aeruginosa* efflux pump MexAB-OprM, which contributes to intrinsic antibiotic resistance (Morita et al. 2001) and to virulence (Hirakata et al. 2002), is involved as well in the tolerance to oil hydrocarbons such as hexane, xylene, or toluene (Li et al. 1998). Another *P. aeruginosa* efflux pump, MexGHI-OpmD, confers resistance to vanadium (Aendekerk et al. 2002), a toxic heavy metal that is abundant in crude oil. At the same time, efflux pumps as MexAB-OprM and MexEF-OprN (Olivares et al. 2012) modulate the *P. aeruginosa* quorum-sensing response (Aendekerk et al. 2005), an important regulatory network both for colonizing nonclinical ecosystems and for triggering the expression of virulence determinants in the infected host (Rutherford and Bassler 2012).

Tolerance to organic solvents such as those present in crude oil is a multifactorial response that involves several physiological changes, mainly modifications in the composition of membranes to compensate for the alterations in membrane fluidity caused by the solvents, the induction of a stress response that increases the production of chaperones that assist in the correct folding of solvent-affected proteins, and the induction of efflux pumps able to extrude the solvent and reduce its concentration inside the cell (Segura et al. 2012; Ramos et al. 2015). The ability of efflux pumps to extrude hydrocarbons and other toxic compounds has been documented for several bacterial strains that are generally not pathogenic but that illustrate well the broad use that bacteria can make of detoxification systems. *Pseudomonas fluorescens* cLP6a, which was isolated from a petroleum-contaminated soil and degrades naphthalene, phenanthrene, and anthracene, has an efflux pump named EmhABC that can extrude phenanthrene, anthracene, and fluoranthene, but not naphthalene or toluene, and that is active as well with the antibiotics chloramphenicol and nalidixic acid (Hearn et al. 2003). Another particularly interesting example is that of *Pseudomonas putida* strain DOT-T1E, which is highly resistant to solvents and can grow in a medium containing high amounts of toluene, which in addition is used as carbon source (Ramos et al. 1995). Efficient tolerance to toluene in this strain requires the activity of three efflux pumps named TtgABC, TtgDEF, and TtgGHI, which extrude toluene and reduce its toxic effect on the cell membrane (Rojas et al. 2001). Degradation of the solvent and its use as carbon source is not determinant for solvent tolerance, since mutants in genes of the catabolic pathway were as tolerant to toluene as the parental strain (Mosqueda et al. 1999). A similar MDR pump-dependent solvent tolerance mechanism has been observed in another *P. putida* strain, named S12 (Kieboom et al. 1998; Kieboom and de Bont 2001). Functioning of these efflux pumps is energy-expensive, which may at least in part explain why solvent tolerance requires an increase in energy consumption. The *P. putida* DOT-T1E TtgABC pump confers resistance not only to toluene but to antibiotics such as carbenicillin, ampicillin,

tetracycline, nalidixic acid, or chloramphenicol (Rojas et al. 2001). This efflux pump is also involved in the resistance of *P. putida* to the toxic effects of several flavonoids produced by plants as a defense mechanism (Teran et al. 2006). Therefore, efflux pumps may be a critical element in the competitive colonization of plant roots by *P. putida* strains. This situation has been described as well in the case of the SmeDEF *S. maltophilia* efflux pump that, besides being the main determinant of quinolone resistance, is required for the colonization of the plant roots by this opportunistic pathogen (Alonso and Martínez 2001; García-León et al. 2014). This suggests that relevant elements to resist the toxic effects of solvents or antimicrobials might have ecological functions going beyond this detoxification role (Martínez et al. 2009).

In summary, the examples described above show that MDR efflux pumps can be useful in several situations. In the case of bacteria that have a versatile metabolism and can assimilate the hydrocarbons, the efflux pumps can protect them and confer a competitive advantage in environments that are contaminated with crude oil and that contain high concentrations of toxic hydrocarbons. On the other hand, the efflux pumps can also be beneficial for the infection of a eukaryotic host such as a plant or a human, by extruding compounds that act as antibiotics (Fig. 1).

3.2 Surfactants: Useful for Oil Emulsification and for Adhesion to Surfaces

Many bacteria produce and excrete diverse compounds that have surface-active properties and facilitate the interaction of the microorganism with surfaces or interfaces (Neu 1996; Ron and Rosenberg 2001). These compounds, named surfactants, are amphipathic (contain both hydrophobic and polar groups) and concentrate at interfaces. Surfactants that stabilize dispersions of one liquid into another, such as an oil-water emulsion, are called emulsifiers. Low-molecular-weight biosurfactants are generally glycolipids or lipopeptides, while high-molecular-weight surfactants can be polysaccharides or lipoproteins. Hydrocarbon-degrading bacteria frequently produce biosurfactants that can emulsify the oil phase, facilitating the uptake of hydrocarbons and therefore improving their degradation (Hommel 1990; Ron and Rosenberg 2002). However, surfactants probably have many other roles, and distinct surfactants may be useful under different situations. Some opportunistic pathogens produce surfactants. A well-studied example is that of *P. aeruginosa*, which produces rhamnolipid surfactant that can stimulate the uptake of hydrocarbons such as hexadecane, facilitating its assimilation (Beal and Betts 2000; Noordman and Janssen 2002). However, these rhamnolipids participate in several other biological processes that are unrelated to hydrocarbon metabolism but that have in common the interaction of the bacteria with a surface or an interface. Rhamnolipids facilitate the adherence of *P. aeruginosa* cells to the human airway epithelia, facilitating the infection process (Zulianello et al. 2006). Therefore, they can be considered as a virulence factor. Rhamnolipids also modulate the swarming motility patterns of *P. aeruginosa* on semisolid surfaces (Kohler et al. 2000; Caiazza et al. 2005) and mediate detachment of cells from biofilms (Neu 1996; Boles et al. 2005).

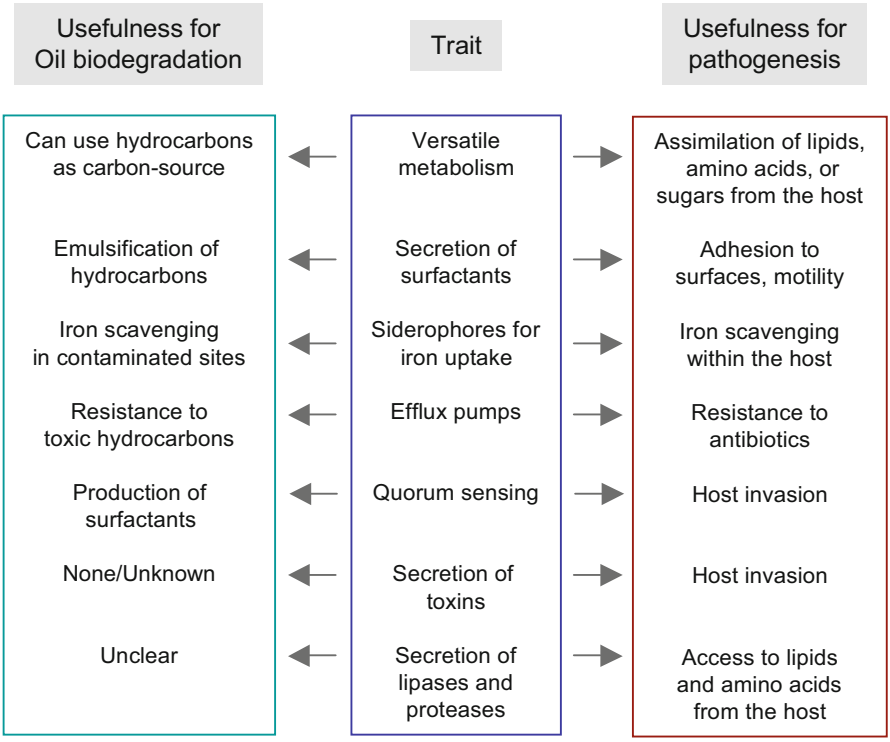

Fig. 1 Bacterial traits present in many opportunistic pathogens and that can be useful for oil degradation and/or for infection of a eukaryotic host

Therefore, the biosurfactant produced by an opportunistic pathogen such as *P. aeruginosa*, which is also an efficient hydrocarbon degrader, can serve different purposes in distinct environmental conditions, the uptake of hydrocarbons being just one of the processes where the properties of surfactants can be useful. It should be noted that production of rhamnolipids is an energy costly process and attaining concentrations being high enough to generate oil emulsification can be better achieved when bacterial population numbers are high. Accordingly, production of rhamnolipids by *P. aeruginosa* occurs during the stationary phase of growth and is controlled by quorum sensing (reviewed in Ron and Rosenberg 2001).

4 Research Needs

Although hydrocarbon-degrading opportunistic pathogens can adapt to many environments, they are not equally successful in all cases so that they will not always be of concern. Bioremediation strategies that facilitate the growth of nonpathogenic

strains can be a good solution in many cases, but this requires a deep knowledge of microbial population dynamics and of the growth requirements of each bacterial species in distinct environments. These issues have only been investigated for some bacterial species and environments and should be extended to other cases. In addition, there is not enough evidence as to the real risks associated to the increase in population numbers of most opportunistic pathogens in oil-contaminated sites. Opportunistic pathogens such as *P. aeruginosa* are rather frequent in many habitats, so that it is not clear that a transient increase in their populations during a bioremediation process will generate a health problem. In this sense, it is important to determine for each bacterial species whether pathogenicity results from traits that are present in most strains from most environments or responds to the acquisition of specific genes by a given strain. This is relatively well known for *P. aeruginosa* and perhaps *S. maltophilia* and the *B. cepacia* complex, but not for other opportunistic pathogens that appear in oil-contaminated sites. The study of these issues, together with the genetic structure of the populations of relevant bacterial species involved in oil biodegradation, is of clear interest and deserves future research efforts.

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Part V

Lipid-Containing Molecules for Disease Prevention and Therapy

Infection Prevention: Oil- and Lipid-Containing Products in Vaccinology

12

Thomas Ebensen, Barbara Fuchs, Kai Schulze, and Carlos Alberto Guzmán

Contents

Abstract

Vaccination remains the most valuable tool for preventing infectious diseases. However, the performance of many existing vaccines should be improved, and there are diseases for which vaccines are still not available. The use of well-defined antigens for the generation of subunit vaccines has led to products with an

[$] Thomas Ebensen and Kai Schulze have equally contributed to this work.

T. Ebensen · K. Schulze · C. A. Guzmán (✉)
Department of Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Braunschweig, Germany
e-mail: Thomas.Ebensen@helmholtz-hzi.de; Kai.Schulze@helmholtz-hzi.de; CarlosAlberto.Guzman@helmholtz-hzi.de

B. Fuchs
Global Research Immunology, In vivo Biology, ALK, Hørsholm, Denmark
e-mail: Barbara.Fuchs@alk.net

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improved safety profile. However, purified antigens are usually poorly immunogenic, making essential the use of sophisticated delivery systems or, more frequently, adjuvants. Despite the fact that adjuvants have been used to increase the immunogenicity of vaccines for more than 70 years, only a handful has been licensed for human use (e.g., aluminum salts, the micro-fluidized squalene-in-water emulsion MF59, monophosphoryl lipid A (MPL A), AS03, AF03, LT, and virosomes). Thus, the development of new delivery systems, combinations of already known adjuvants and novel adjuvants, which are able to promote broad and sustained immune responses at systemic and mucosal levels, still remains a major challenge in vaccinology. Recent advances in our understanding of the immune system have facilitated the identification of new biological targets for screening programs aimed at the discovery of novel immune stimulators. This resulted in the identification of new candidate adjuvants, which made possible the modulation of the immune responses elicited according to specific needs. A number of promising delivery systems and adjuvants based on lipids, which are currently under preclinical or clinical development, will be described in this chapter.

1 Introduction

Vaccines are the most cost-efficient tool to combat the global threat represented by infectious diseases to human health. According to the World Health Organization, vaccination averts about 1.5 million deaths per year, reducing also the economic burden resulting from both the direct and indirect costs associated to infectious diseases. Nevertheless, an estimated 19.5 million infants worldwide are still missing out on basic vaccines (World Health Organization 2018). The successful implementation of a vaccination program will not only protect the immunized subjects but will also reduce the horizontal transmission to susceptible hosts, thereby minimizing pathogen circulation in the community. Although highly effective vaccines against a variety of infectious agents are currently available, there are still many diseases for which vaccines are not available or the available vaccines are suboptimal in terms of immunogenicity, safety, and/or associated costs. Moreover, many vaccine formulations need to be improved and customized for application in specific groups of individuals (e.g., children or the elderly). On the other hand, despite the fact that the advent of subunit vaccines has resulted in formulations with improved safety profiles, purified antigens are poorly immunogenic, making critical their co-administration with adjuvants. Up to now, only six adjuvants have been used in licensed human vaccines: Alum, MF59, AS03, AF03, virosomes, and heat-labile enterotoxin (LT) (Tregoning et al. 2018). Thus, the development of efficient and safe antigen delivery systems and adjuvants for human use remains a critical need and a challenge. In these two specific areas, oil- and lipid-containing products represent critical tools for the generation of innovative products, which are being translated into the vaccine development pipeline.

2 Oil- and Lipid-Based Delivery Systems

2.1 General Properties

New-generation subunit vaccines, based on either recombinant proteins or DNA, are often less immunogenic than traditional whole cell vaccines. Thus, delivery systems are needed, which are able to efficiently present the antigens in a proper context to antigen-presenting cells (APC) and/or to bring the gene expression cassettes into the cells responsible for the biosynthesis of the selected antigens. It is also a considerable asset if the selected systems deliver the antigens in the context of a danger signal, which in turn promotes the activation and maturation of APC. For this purpose, customized vaccine delivery systems have been developed. Most of them are particulate or vesicle-like (e.g., microparticles, nanoparticles, viruslike particles, virosomes, and liposomes) and have similar dimensions to the pathogens, which the immune system has specifically evolved to combat. More sophisticated delivery systems have also been developed, in which immune-stimulatory compounds have been incorporated together with the antigens. The rationale for this approach is to ensure that both the antigen and adjuvant are co-delivered and presented to the same population of APC, thereby creating a microenvironment which is conductive toward antigen processing and presentation (Riedmann et al. 2007; Singh et al. 2007). Especially, the ability to mount an effective immune response (e.g., against cancer or infections) requires a coordinated control of CD4+ T-cell and CD8+ T-cell function by APC (Bandola-Simon and Roche 2018), whereby CD4+ T-cells are able to promote antitumor immunity by numerous mechanisms including enhancing antigen presentation, co-stimulation, T-cell homing, T-cell activation, and effector function (Melssen and Slingluff 2017). By the use of these delivery systems, the stability of the antigens has been improved, together with their availability to APC and their capacity to promote strong adaptive responses. This has in turn increased the overall efficacy of the specific vaccine formulations. Moreover, some technology platforms bear the unique possibility to specifically target vaccine antigens to the cells in which adaptive immune responses are initiated (i.e., dendritic cells; DC) (Tanaka et al. 2008). Delivery systems not only allow the reduction of required antigens dose but also improve the pharmacological properties of the formulation (e.g., increased half-life or stability of the antigen, protracted antigen release), thereby ensuring a long-lasting stimulation of the immune system. Finally, the biocompatibility of many of these systems minimizes the risk for side effects when compared to conventional formulations. There are currently several vaccines based on lipid-containing delivery systems either in the market or in advanced development phases.

2.2 Liposomes

Liposomes are particulate vesicular lipid structures in which antigens, adjuvants, and targeting molecules can be incorporated and which are able to modify pharmacokinetic and bio-distribution of drugs (Altin and Parish 2006; Naik et al. 2013).

Liposomes are concentric bilayer membrane vesicles composed of phospholipids and cholesterol, which show high biocompatibility and degradability and that per se are non-immunogenic. Among all potential carrier systems, liposomes drew attention due to their high versatility. Liposomes have a long history as vehicles for antigen delivery (Kersten and Hirschberg 2004). Their physical properties are highly variable, depending on the composition and the manufacturing method (Khatri et al. 2008; Krishnan and Sprott 2008). Liposomes can carry both membrane-associated antigens and water-soluble molecules. This allows the optimization of the design for specific tasks (e.g., targeting of organs or cell subsets, co-delivery of adjuvants or drug-in-liposome systems) (Alving and Rao 2007; Chen et al. 2014). More recently, the so-called virosomes have been developed, which consist of the hemagglutinin from influenza virus incorporated into liposomes. This glycoprotein guides virosomes to APC and promotes their fusion with the endosomal membrane at low pH. This in turn leads to the cytoplasmic release of the antigens, thereby providing optimal processing and presentation in the context of MHC class I molecules (Felnerova et al. 2004; Gluck et al. 2005; Moser et al. 2003). This technology platform has been exploited for the generation of vaccines against seasonal flu and hepatitis A, which are currently in the market (e.g., Inflexal® V, Epaxal®). The human skin is a remarkably efficient barrier. Using modified liposomes, the so-called ethosomes, first steps have been made toward transcutaneous immunization against hepatitis B using HBsAg as antigen (Carita et al. 2018; Dubey et al. 2007; Filskov et al. 2017; Jain et al. 2008; Mishra et al. 2008). Moreover, for the reduction of the worldwide disease burden, effective therapeutic drugs for the treatment of patients with chronic hepatitis C virus (HCV) infection are under development (Filskov et al. 2017). In this context, uptake by DC was shown, which in turn led to T-cell activation and subsequent stimulation of robust systemic and mucosal immune responses (Dubey et al. 2007; Mishra et al. 2008).

Liposomes can be also exploited as a delivery system for anticancer agents and, more specifically, as drug nanocarriers in cancer chemotherapy (e.g., to deliver encapsulated anthracycline molecules for cancer treatment) (Al-Jamal and Kostarelos 2011; Lee et al. 2017; Minko et al. 2006). The main advantages of liposomal drugs over the non-encapsulated drugs include (i) improved pharmacokinetics and drug release, (ii) enhanced intracellular penetration, (iii) tumor targeting, (iv) reduction in side effects, and (v) capacity to accommodate several active ingredients in a single-drug delivery platform. This lipid-based strategy offers the possibility for prophylactic or therapeutic options against recurrent, drug-resistant, or chronic infections (Lee et al. 2017; Nisini et al. 2018). There are also other applications for which liposomes are exploited in which proof of concept has been already provided in phase I clinical trials (e.g., induction of long-lasting antibody responses against a synthetic malaria peptide) (Cech et al. 2011; Felnerova et al. 2004; Gluck et al. 2005, 2004; Moser et al. 2007, 2011; Peduzzi et al. 2008). AS01, a liposome-based adjuvant comprising of two immunostimulants, has recently been approved in a recombinant protein vaccine for older adults, which showed very high efficacy against herpes zoster infection in clinical trials (Weinberger 2018).

2.3 Other Delivery Systems

There is increasing interest for improved drug delivery systems with greater versatility. Surface-modified lipid nanocontainers or solid lipid nanodispersions (SLN) are formulated with a homolipid template with Phospholipon 90 G as surface modifier (Attama and Muller-Goymann 2007; Attama et al. 2008, 2007). The SLN possessed good properties and could prove to be efficient drug or vaccine delivery systems for parenteral or ocular administration (Nasr et al. 2015). Formulation of antigenic epitopes in lipid-based nanocarriers, either vesicular such as liposomes, transfersomes, and ethosomes or non-vesicular such as cubosomes, solid lipid nanoparticles, nanostructured lipid carriers, solid-in-oil nanodispersions, lipoplexes, and hybrid polymeric-lipidic systems, improves protein stability and prevents proteolytic degradation, thereby resulting in a long-lasting release of the incorporated antigens. When used as particulate delivery system, the exploitation of lipid nanoparticles can influence both bio-distribution and cellular uptake of immune stimulators (e.g., CpG motifs) by APC (Esposito et al. 2008). This is due to the striking capacity of APC to take up particulate antigens, even at very low concentrations (Wilson et al. 2007).

Lipid core particles (LCP) also represent an attractive alternative delivery system (Moyle and Toth 2008). LCP are synthetic derivatives with a hydrophobic core and branching lysines, in which one or more peptides can be hooked. This system elegantly combines disease-related antigenic epitopes with a lipid moiety exhibiting adjuvant properties. Moreover, multiple peptides encompassing epitopes belonging to one or more pathogens can be combined into one single vaccine. For example, efficient protection against challenge with *Streptococcus pyogenes* was obtained following vaccination with self-adjuvanting LCP including T- and B-cell epitopes administered by subcutaneous route. The efficacy of the vaccine was dependent on the insertion point of the lipid moiety, being the best results observed with a C-terminal lipid moiety (Abdel-Aal et al. 2008). Additionally, studies showed that full protection against a lethal mucosal challenge with a heterologous *S. pyogenes* strain can be obtained following intranasal immunization with a LCP-based formulation containing the Toll-like receptor (TLR) 2/6 agonist MALP-2 (i.e., macrophage-activating lipopeptide of 2 kDa) (Olive et al. 2007; Schulze et al. 2017). Targeted delivery of LCP to DC has been also achieved by inserting D-mannose. By using this approach, a tri-epitopic group A streptococcal lipopeptide vaccine was generated (Moyle et al. 2006a, b, 2007).

Another delivery system is represented by viruslike particles consisting of the viral capsid missing the viral DNA. For example, the human papillomavirus type 16/18 (HPV-16/18) AS04-adjuvanted cervical cancer vaccine Cervarix® consists of the L1 protein and is licensed for females aged 10 years and above. Another example is the hepatitis B vaccine Engerix® consisting of the HBV surface antigen. Moreover, a phase III trial combining both vaccines revealed that co-administration of both vaccines was well tolerated and did not affect their efficacy (Leroux-Roels et al. 2011).

3 Oil- and Lipid-Based Adjuvants

3.1 General Properties

Adjuvants generate a local microenvironment conducive toward antigen processing and presentation, thereby promoting the elicitation of robust adaptive immune responses at both local and systemic levels. The chemical nature of the adjuvants and their modes of action are extremely variable. Potential mechanisms by which they exert their biological activity are the "depot" effect, antigen targeting to APC, improvement of antigen processing and presentation, and immune activation or modulation via the upregulated expression of cellular mediators. Thus, they allow the induction of strong immune responses, even against poorly immunogenic antigens, thereby reducing the amount of antigen needed. They also enable to modulate the obtained responses, according to specific needs. In fact, adjuvants exhibit also immune-modulatory properties. This allows fine-tuning and customizing immune responses to match the effector mechanisms required to achieve microbial clearance, since it is essential to promote an adequate type of response to promote effective protection. The elicitation of a suboptimal response pattern may reduce the overall efficacy or even lead to side effects, as a result of immune pathological reactions. Thus, the identification of adjuvants able to promote predictable responses is a major issue in vaccinology. In the past, the screening for new adjuvants has been largely empiric. However, our current knowledge on the structure and functional properties of the innate and adaptive immune systems has allowed the identification of well-defined molecular targets for intervention. In the following sections, we will discuss lipid moieties exhibiting adjuvant properties.

3.2 Toll-Like Receptor (TLR) Agonists

After a human homologue to the drosophila Toll receptor was first described by Medzhitov et al. (Medzhitov et al. 1997), several members of the TLR family have been discovered (for review, see Baxevanis et al. 2013; Brodsky and Medzhitov 2007; Medzhitov 2007; Vidya et al. 2018). Initially, Toll/TLRs were discovered in a developmental context; however, recent studies have revealed that Toll/TLRs carry out previously unanticipated functions not only in development but also in regulating cell fate and cell number (Anthoney et al. 2018). Moreover, TLR play an important role in innate and adaptive immunity, as well as in the achievement of a balance between productive immune responses and tolerance. TLR have a central role in the host recognition process of dangerous entities, such as pathogenic microorganisms. This process is based on the specific recognition by different TLR of pathogen-associated molecular patterns (PAMP). For example, the lipopolysaccharides of gram-negative bacteria are detected by TLR4 (Nahori et al. 2005; Reed et al. 2016) and bacterial lipopeptides and lipoproteins by TLR2 heterodimers (Henneke et al. 2008). These heterodimers recognize different structures: diacylated lipopeptides, e.g., MALP-2, require TLR2/6 (Wilde et al. 2007), whereas triacylated

lipopeptides are mainly recognized by TLR2/1 (Soboll et al. 2006a, b) and lipoproteins by TLR2/4 (Revets et al. 2005). Several vaccines composed of antigen and TLR ligand in single molecules (TLR ligand-antigen conjugates) using Pam3/2Cys, lipid A analogues, recombinant flagellin, imidazoquinoline analogues, and un-methylated CpG motifs to activate immune systems through TLR2, TLR4, TLR5, TLR7/8, and TLR9 are under development (for review, see Fujita and Taguchi 2012).

It is generally accepted that TLR agonists alert the host immune system through a mechanism similar to what is observed during the course of natural infections. This involves the initial interaction with a TLR, followed by the delivery of a "danger signal" to the immune system. The underlying molecular event encompasses the activation of signal transduction cascades within different immune cells, which leads in turn to the activation of master transcriptional regulators. This results in the activation, maturation, and migration of different cell subsets, as well as the release of soluble mediators. The form in which this global activation of the innate immune system is taking place also shapes the strength, breath, and quality of adaptive responses (Jiang and Koganty 2003). Combinations of TLR agonists have been shown to have a synergistic effect on individual cytokine secretion (Fischetti et al. 2017). Thereby, the pattern of inflammatory mediators released can alter the quality and quantity of the adaptive immune response to vaccination.

Each TLR is expressed in a variety of immune cells (e.g., macrophages, DC, and B-cells), as well as in other cell types (e.g., endothelial cells, epithelial cells) (Delneste et al. 2007; Medvedev et al. 2006; Pegu et al. 2008). Different TLR can exert distinct but to some extent also overlapping sets of biological effects (Hemmi et al. 2002; Ito et al. 2002; Yamamoto et al. 2002). Thus, the exploitation of well-defined TLR agonists for the establishment of immune prophylactic or therapeutic interventions seems to be an extremely promising field. With the advent of well-defined synthetic small molecules, which are designed to structurally mimic natural occurring PAMP, it is now possible to standardize conditions, reaching a degree of reproducibility which enables drawing meaningful conclusions. In the last years, several TLR molecules reach the clinical research level against different disease-related infections, such as a synthetic TLR4 agonist for the mosquito-transmitted West Nile virus (WNV), a member of the *Flaviviridae* family (Van Hoeven et al. 2016); a combination of rintatolimod (TLR3 agonist) and FluMist was well-tolerated and generated cross-protection against influenza virus (Overton et al. 2014) as well as the TLR-5 agonist, flagellin, which induced a potent immune response against influenza virus in the elderly (Taylor et al. 2011).

3.2.1 Bacterial Lipoproteins (BLP)

Bacterial lipoproteins and their synthetic analogues are strong immune modulators during infection, signaling through TLR2/1 heterodimers. Synthetic analogues of bacterial lipoproteins were described more than a decade ago, but their capacity to act as adjuvants has only recently been dissected more closely. These low molecular weight non-immunogenic molecules can be reproducibly synthesized and are safe and of easy handling and administration. Synthetic derivatives, such as Pam3Cys, have been broadly exploited in the field of vaccinology. Lipids are the most abundant

component of membranes, and bacteria possess a unique set of lipids that can initiate or modify the host innate immune response (Chandler and Ernst 2017). For example, in *Candida albicans* infections and bovine tuberculosis, they act as adjuvants and modulators of CD4+ effector T-cell responses (Schwarz et al. 2003; Sutmuller et al. 2006a, b; Wedlock et al. 2008), although stimulation of TLR2 failed to increase CTL responses (Schwarz et al. 2003).

3.2.2 Macrophage-Activating Lipopeptide (MALP-2)

MALP-2 was detected in supernatant fluids from *Mycoplasma fermentans* and showed activity at 10^{-11} M concentrations. Subsequent studies determined that this moiety acts as agonist of the heterodimer formed by TLR2/6. MALP-2 also exerts its immune-modulatory activities on DC, promoting their activation and maturation, which in turn increase their ability to process antigens and present them to both naïve and antigen-specific T-cells (Link et al. 2004), as shown in Fig. 1. Interestingly, it seems that CD36 is also involved in MALP-2-binding, suggesting the potential usefulness of this molecule as APC targeting moiety (Hoebe et al. 2005).

Furthermore, MALP-2 exhibits potent adjuvant activity when co-administered with antigens by either systemic or mucosal route (Borsutzky et al. 2005; Rharbaoui et al. 2002). Preclinical studies demonstrated that MALP-2 can be exploited as mucosal adjuvant for antigens from bacteria, viruses, and parasites. MALP-2 or its synthetic derivative BPPcysMPEG can be co-administered with purified antigens or given in combination with other delivery systems (Borsutzky et al. 2005; Rharbaoui et al. 2002, 2004; Schulze et al. 2006, 2017). Promising results were also obtained when MALP-2-based formulations were incorporated in prime-boost protocols (Becker et al. 2006; Borsutzky et al. 2003, 2006). Interestingly, the immunogenicity of a live attenuated measles vaccine was considerably improved by MALP-2 (Luhrmann et al. 2005). On the other hand, intranasal co-administration of MALP-2 with cruzipain resulted in decreased parasite burden and tissue injury in an experimental *Trypanosoma cruzi* infection model (Cazorla et al. 2008). This compound, which was also proposed for immune therapies, has been recently tested in a phase I/II trial in pancreatic cancer patients. MALP-2 was well tolerated following intra-tumor injection. By and large, the patients showed no concerning side effects and a remarkably high mean survival following therapy (Schmidt et al. 2007). The use of MALP-2 in the fields of allergy and wound healing has also raised considerable interest, and promising results were obtained by different groups (Deiters et al. 2004; Weigt et al. 2003, 2004). The use of the TLR2/6 agonist is a promising therapeutic approach in diseases with an imbalance in T-cell responses, such as asthma (Fuchs et al. 2010). The bacterial lipopeptide MALP-2 and its synthetic derivative BPPcysMPEG are well tolerated with low pyrogenicity; thus these molecules are of interest for infections or chronic airway diseases, such as COPD (Switalla et al. 2010).

3.2.3 Lipid A Derivatives

Lipid A, the component of the TLR4-ligating LPS complex which is responsible for toxic effects, is also a potent adjuvant. The incorporation of lipid A into liposomes

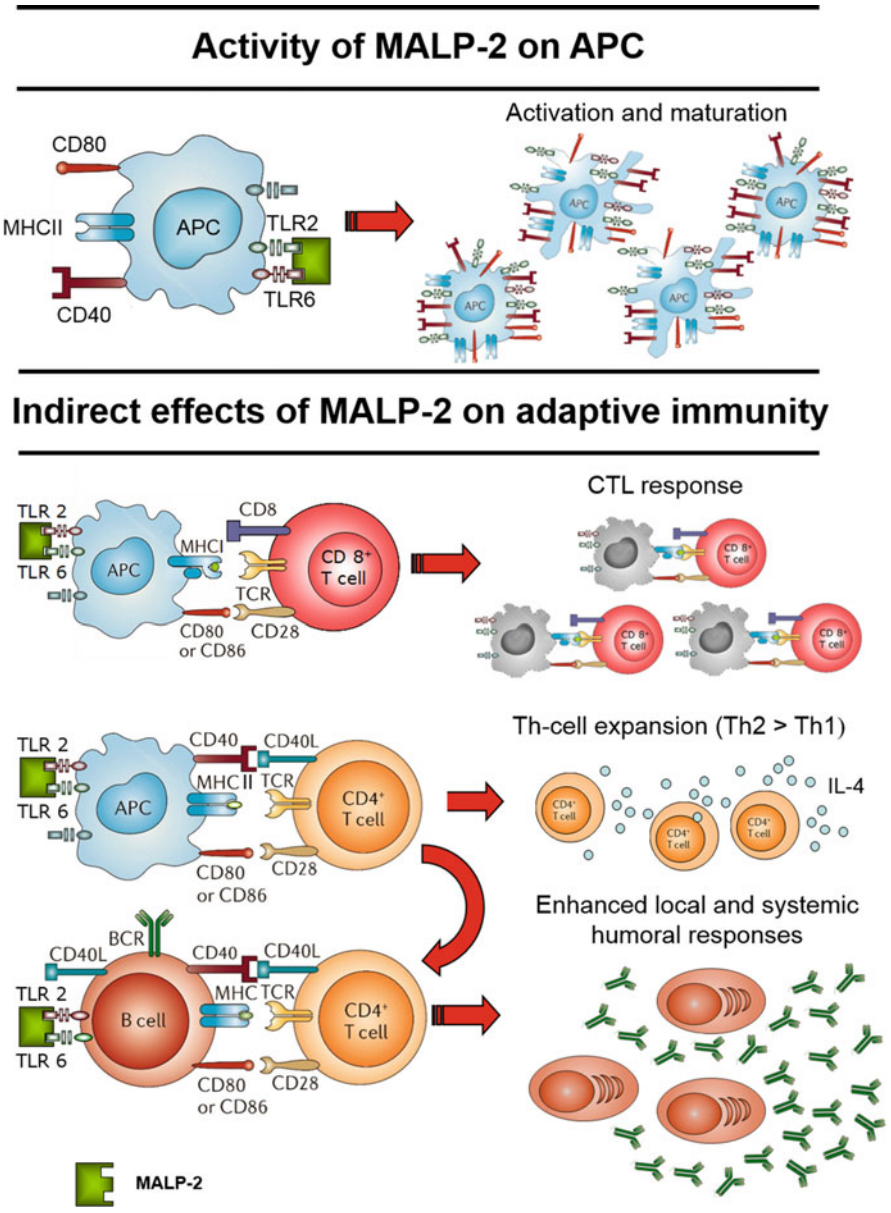

Fig. 1 MALP-2 promotes a TLR 2/6-dependent activation and maturation of APC, which induces strong humoral and cellular immune responses against co-administrated antigens

was exploited to induce neutralizing antibodies able to neutralize primary isolates of two HIV-1 clades in mice (Alving and Rao 2007). However, this molecule is far too reactogenic for human use. Therefore, nontoxic synthetic lipid A derivatives have

been generated, which still induce maturation of DC and secretion of pro-inflammatory cytokines (Hamdy et al. 2007). These lipid A derivatives have been frequently combined with other technology platforms to increase their performance. For example, combination of MPL A, a TLR4 agonist, with hepatitis C viruslike particles resulted in a modest enhancement of both humoral and cellular immune responses (Jeong et al. 2004). The use of cationic liposomes containing MPL A and a *Mycobacterium tuberculosis* subunit vaccine resulted in controlled bacteria replication and protection in a *M. tuberculosis* infection model (Agger et al. 2008). The protective response was characterized by an accelerated recruitment of IL-17- and IFN-γ-producing lymphocytes leading to an early formation of granulomas, which are pivotal in controlling bacterial growth. Moreover, the mannose-PEG-cholesterol conjugate (MPC), which was synthesized as a lectin-binding molecule and anchored onto liposomes entrapping MPL A, is a potent adjuvant-delivery system of cold chain-free oral mucosal vaccines (Wang et al. 2014). MPL A is also used in allergen-specific immunotherapy (AIT) which is the only available treatment aimed to tackle the underlying causes of allergy (for review, see (Chesne et al. 2016; De Souza Reboucas et al. 2012; Klimek et al. 2017; Pfaar et al. 2012)). MPL A has recently been approved for use in humans (Persing et al. 2002). MPL A is safe and well tolerated in clinic; thus it could be considered as a new approach in prevention or even treatment of cerebral ischemic insult consequences (Hosseini et al. 2018).

3.3 CD1d Agonists (αGalCer, αGalCerMPEG)

TCR+ NKT-cells represent a unique subset of immune regulatory T-cells, which are able to recognize glycolipids presented by APC via the MHC class I-like molecule CD1d. They have a central role in the stimulation of innate immune responses, via the release of different cytokines. Thus, NKT-cells are attractive targets for the development of immune therapies. One compound addressing this pathway is α-galactosylceramide (αGalCer), originally isolated from a marine sponge (Fig. 2). Rapid activation of NKT-cells, which is characterized by cytokine secretion, surface receptor downregulation, expansion, and secondary activation of a variety of bystander cells from the innate and adaptive immune systems, was demonstrated after αGalCer administration (Ogura et al. 2007). Different studies have shown that the immune-modulatory properties of αGalCer can be exploited to combat cancer, chronic inflammatory diseases, infections, and autoimmunity (Table 1).

The response of NKT-cells to distinct αGalCer analogues seems to differ both at quantitative and qualitative levels. These findings indicate that specific glycolipids could be exploited to fine-tune NKT-cell responses, thereby shaping the elicited immune responses in vivo. This approach will certainly facilitate the development of effective and safe NKT-cell-based immune therapies (Motohashi and Nakayama 2008). Different studies have also demonstrated that αGalCer could act as an effective adjuvant when co-administered with antigens (e.g., Shiga toxin, inactivated influenza virus) (Adotevi et al. 2007; Youn et al. 2007), probably due to its direct effect on APC. In the context of other diseases relevant for vaccination, such as HIV, αGalCer has also demonstrated its efficacy (Huang et al. 2008).

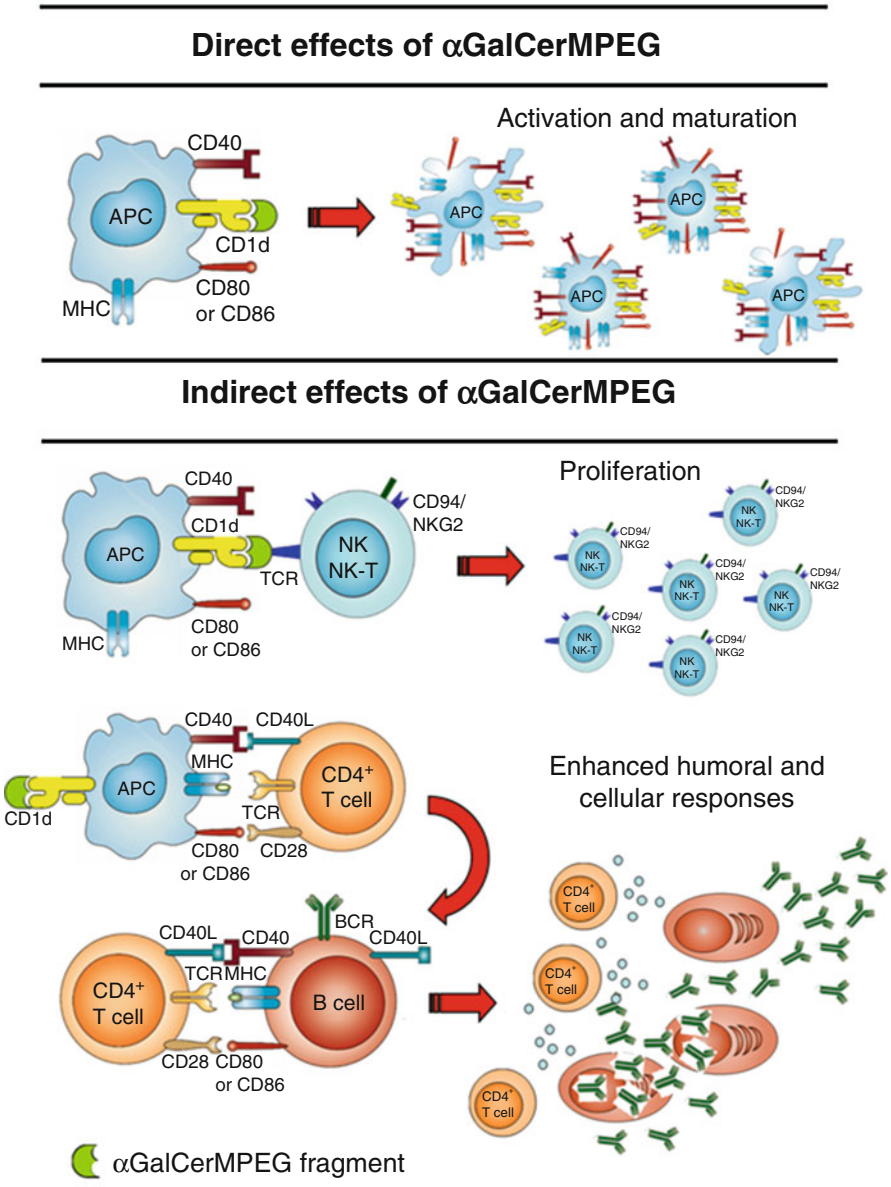

Fig. 2 αGalCerMPEG activates and maturates APC and is presented on CD1d, a MHCI-like receptor. This CD1d presentation promotes NKT-cell proliferation and the induction of adaptive immune responses against co-administrated antigens

Additional work revealed that αGalCer is also a potent mucosal vaccine adjuvant, which promotes humoral and cellular immune responses able to confer protection against viral infections and tumors. In fact, intranasal co-administration of a formalin-inactivated influenza virus A/PR/8/34 (PR8) with αGalCer-induced enhanced

Table 1 αGalCer and derivatives thereof and their application

Compound	Application field	Literature
αGalCer	Cancer immune therapy	Antitumor immunity and cell-based tumor immunotherapy (Motohashi 2007; Motohashi and Nakayama 2008); combination therapy of IL-18 with αGalCer (Nishio et al. 2008); alpha-GalCer-pulsed DC therapy in a randomized phase II study (Saka et al. 2017)
	Allergy	Suppression of allergen-induced eosinophilic airway inflammation (Knothe et al. 2011; Morishima et al. 2005) and asthma (Fujii et al. 2006; Iwamura and Nakayama 2007)
	Autoimmune diseases	Th1 autoimmunity, autoimmune myocarditis, and myasthenia (Liu et al. 2011; Takagi et al. 2004; Wang and Chen 2013; Wang et al. 2015)
	Infectious diseases	Prophylactic antibacterial effect on *E. coli*, *P. aeruginosa*, and methicillin-resistant *S. aureus* (Minagawa et al. 2005); influenza infection (Youn et al. 2007); *T. cruzi* infections (Duthie and Kahn 2006, 2002; Minagawa et al. 2005)
	Vaccinology	Shiga toxin or inactivated influenza virus, DNA vaccines (Adotevi et al. 2007; Huang et al. 2008; Youn et al. 2007); influenza vaccination in the elderly (Akmatov et al. 2017)
	αGalCer analogues	6″-O-carbamate and galacturonamide analogues (Guillaume et al. 2015; Tashiro et al. 2010); 2″-deoxy-beta-GalCer (Thakur et al. 2014); galactosylsphingamides (Guillaume et al. 2017)
	7DW8-5	Adjuvant effect of 7DW8-5 in a phase I clinical trial by administering a single fixed dose of NMRC-M3V-Ad-PfCA (malaria vaccine candidate) (Mazzuca et al. 2017; Padte et al. 2011)
αGalCerMPEG	Mucosal vaccination	PEGylated derivative exploited as mucosal adjuvant (Ebensen et al. 2007)
	Human in vitro allergy model	αGalCerMPEG suppresses allergic airway inflammation (Knothe et al. 2011)
	Intranasal vaccination	NKT-cells control the cytokine microenvironment (Zygmunt et al. 2012)

levels of PR8-specific IgG and IgA antibodies in serum and lung lavages, as well as cytotoxic T-lymphocyte (CTL) responses. Vaccinated animals were protected against challenge with live PR8. Because it did not redirect antigens to the brain, αGalCer would likely pose no risk if administered as a nasal adjuvant (Youn et al. 2007). Interestingly, it was shown that DC, but not B-cells, initiated mucosal immune responses in mediastinal lymph nodes. The CD8α-B220-CD11c + subset seem to play the most prominent role in both direct presentation and cross-presentation of protein antigens to naïve T-cells and in triggering naïve T-cells to differentiate into effector T-cells (Ogura et al. 2007). αGalCer binds more stably to DC and acts as a very effective link between innate and adaptive immunity in vivo (Fujii et al. 2006).

Despite the excellent results obtained using αGalCer, there are major drawbacks preventing the efficient transfer into the clinic, such as its poor solubility. To provide soluble formulations, nonorganic solvents or detergents are needed, which represent a safety concern and might affect the immunological properties of some antigens. An efficient method to improve the solubility of chemical compounds in aqueous solutions is their conjugation with polyethylene glycol (PEG). The process of PEGylation can also improve their half-life by shielding, as well as by reduction of both metabolic degradation and receptor-mediated endocytosis (Chang et al. 2005; Hinds et al. 2005). Of particular interest for applications in the vaccinology field is the fact that PEG is per se very poorly immunogenic (Guiotto et al. 2003; Molineux 2003). A PEGylated derivative of αGalCer was previously described, αGalCerMPEG, which is water-soluble and retains the specificity for the CD1d receptor of αGalCer. The in vitro stimulatory properties on immune cells (e.g., DC and lymphocytes) are maintained intact, even when tested at a 33-fold lower concentration of the active moiety than αGalCer (Ebensen et al. 2007). NKT-cell stimulation by αGalCerMPEG induced the generation of highly functional educated and uneducated NK cells (Riese et al. 2015). NK cells isolated from mice treated with αGalCerMPEG have also stronger cytotoxic activity on YAC-1 cells than those obtained from animals receiving either αGalCer or CpG.

Vaccination studies showed that this new compound exhibits stronger adjuvant activity than αGalCer when tested at 0.35 versus 11.7 nmol/dose. αGalCerMPEG is able to stimulate directly APC and through indirect effects induces high titers of antigen-specific antibodies in serum, as well as strong Th2 and sIgA responses, suggesting that this synthetic derivative is a promising adjuvant for clinical development (Ebensen et al. 2007). The immunomodulatory potential of alphaGalCerMPEG was evaluated in a human in vitro allergy model resulting in the abrogation of allergic airway inflammation (Knothe et al. 2011).

Another interesting glycolipid analogous to αGalCer was identified, 7DW8-5, that exhibits a superior adjuvant effect than the parenteral compound αGalCer on HIV and malaria vaccines (Li et al. 2010). The interaction of 7DW8-5 with CD1d molecules induces activation of NKT-cells, thereby activating various immune-competent cells including DCs to provide a significant adjuvant effect for several vaccines, such as malaria vaccine candidate (NMRC-M3V-Ad-PfCA) (Padte et al. 2011). Moreover, the combination of 7DW8-5 and MPL A resulted in the induction of a vaccine-induced CD8+ T-cell response and protective immunity against malaria (Coelho-Dos-Reis et al. 2016).

3.4 Emulsions

Water-in-oil (W/O) emulsions are routinely used in many veterinary vaccines. They strongly activate antibody production and are regarded as a depot from which antigens are slowly released, resulting in prolonged antigen stimulation. Earlier studies concluded that the activity of W/O emulsions is in part due to the immune-stimulatory activities of the oil phase (Jansen et al. 2007). Therefore,

antigens are added after emulsification, so that conformational epitopes are not lost by denaturation, as well as to facilitate manufacturing (Hisert et al. 2005; O'Hagan 1998). However, the original compounds were too reactogenic to enable their use in humans. Thus, recent work led to the development of new candidate adjuvants with considerable improved safety and efficacy profiles. These new emulsions have probed their usefulness both in preclinical and clinical studies. For example, clinical trials of several MF59-adjuvanted vaccines (see below), which were performed in different age groups, have demonstrated their safety and immunogenicity (O'Hagan 1998, 2001; O'Hagan and Lavelle 2002; O'Hagan et al. 2001, 1997; O'Hagan and Singh 2003). Thus, immunosenescence contributes to increased incidence and severity of many infections in aged individuals and is responsible for impaired immunogenicity and efficacy of vaccines. The oil-in-water emulsions, such as MF59 and AS03, have been licensed in seasonal or pandemic influenza vaccines and were used successfully in the elderly (see review (Weinberger 2018)). These vaccines were able to stimulate a more robust and persistent antibody response for both homologous and heterologous influenza strains when compared with non-adjuvanted vaccines (Deng et al. 2014; Stassijns et al. 2016; Wilkins et al. 2017). Nevertheless, well-designed phase IV studies are mandatory, since preclinical studies performed in animal experimental models suggested that the use of oil-based adjuvants may be associated with a higher risk for autoimmunity (Kuroda et al. 2004). This seems to be related to the ability of hydrocarbons to induce IL-12, IL-6, and TNF-α. Whether this is of relevance for human vaccination is a matter of discussion, since immune toxicity depends on many factors, such as the species, genetic makeup, route of administration, dosage, and duration of the administration. In fact, up to now, clinical studies do not support a high risk for autoimmune disease. Safety and effectiveness data from clinical trials and observation studies (e.g., >30,000 individuals with more than 160 million doses of licensed vaccine have been administered) attest the MF-59-adjuvanted influenza vaccine safety and enhanced effectiveness in children and the elderly (Black 2015). However, the mechanism of action of MF59 and AS03, respectively, remains still unclear. These adjuvants induce pro-inflammatory cytokines and chemokines, including CXCL10, but independently of type 1 interferon, which is associated with improved recruitment, activation, and maturation of APC at the injection site (see also Sect. 3.4.3) (Wilkins et al. 2017). There are other candidate adjuvants in advanced stages of preclinical and clinical development, such as a new generation of W/O emulsions (e.g., Montanide ISA51, OW-14, CSA 720, or natural immune-enhancing delivery system (NIDS)), which demonstrated to be able to trigger efficient immune responses in several animal species and humans (Aucouturier et al. 2002; Dupuis et al. 2006; Galliher-Beckley et al. 2015; Kirkley et al. 1996; Patel et al. 2017).

Yet, safety and effectiveness of Montanide ISA51 was analyzed and showed that the mixing procedure of antigen and adjuvant might influence the occurrence of adverse events (AEs), such as injection site reaction, injection site pain, myalgia, headache, fatigue, and fever (review in Van Doorn et al. 2016).

3.4.1 Freund's Complete Adjuvant (FCA)

Freund's complete adjuvant (FCA) was first described in 1935. Since then, it has been frequently used in animal studies, remaining as one of the most potent tested adjuvants. The water droplets containing antigen are emulsified in mineral oil containing killed mycobacteria or their cell wall fragments. FCA can lead to profound toxic effects, including granuloma formation at the injection site. In addition, the use of FCA results in tuberculin sensitivity. Therefore, its use in humans has been precluded. Efforts to reproduce the adjuvant effect of FCA in a preparation with less reactogenicity has led to the development and testing of several prototypes containing subcellular fractions of mycobacteria, such as the cell wall from *Mycobacterium bovis* BCG and trehalose dimycolates (TDM) (Azuma and Seya 2001). Subsequent work allowed the isolation of the active components and a better understanding of the underlying mechanisms of adjuvanticity. This resulted in the identification of molecules exhibiting similar immune-modulatory properties and an adequate safety profile.

The minimal component responsible for the adjuvant activity of FCA is N-acetylmuramyl-L-alanyl-D-isoglutamine (i.e., muramyl dipeptide, MDP) (Ellouz et al. 1974). This molecule is a synthetic derivative of a component present in the bacterial peptidoglycan. Animal studies revealed that MDP exhibits a broad array of immunological effects, including (i) enhancement or suppression of antibody levels dependent on the time of administration relative to antigen; (ii) improvement of cell-mediated immunity; (iii) enhancement of non-specific immunity to bacteria, viruses, fungi, and parasites; (iv) stimulation of natural resistance to tumors; (v) promotion of cytokine release; and (vi) pyrogenicity. MDP is recognized by the nucleotide-binding and oligomerization domain 2 (NOD 2), but not by TLR2 or heterodimers formed by TLR2 with TLR1 or TLR6 (Behr and Divangahi 2015; Inohara et al. 2003). In contrast to intact and diacylated MDP, derivatives with a single octanoyl or stearoyl fatty acid chain were found to activate TLR2 and TLR4 and exert their activities through the MyD88-dependent pathway on APC (Uehori et al. 2005). Derivatives of MDP, such as murabutide, have gained considerable attention in the past decades, because of their potent adjuvant effects. MDP derivatives interact with cells of the innate and adaptive immune system being effective for clinical use in the treatment of cancer and other diseases (Jakopin 2013; Melief 2011; Ogawa et al. 2011). Studies performed using a *Mycobacterium tuberculosis* experimental animal model also showed that NOD2 and TLR are two non-redundant recognition mechanisms, which synergize for the induction of pro-inflammatory cytokines (Ferwerda et al. 2005). Interestingly, MDP also exerts pronounced activities at the level of the nervous system, probably through the interaction with 5-hydroxytryptamine receptors. However, some of these biological activities are undesirable for clinical use (Sevcik and Masek 1999). Thus, structurally well-defined synthetic derivatives from the MDP were generated, which exhibit improved pharmacological properties (Azuma and Seya 2001; Hui 1994; Jiang and Koganty 2003; Kaliuzhin 1998; O'Reilly and Zak 1992). Among these derivatives can be mentioned the adamantylamide dipeptide (AdDP), MDP-Lys (L18), and murabutide. The AdDP

is a synthetic compound in which the dipeptide was combined with the antiviral compound amantadine. AdDP exerts its adjuvant properties when administered by either parenteral or mucosal route, leading to the elicitation of strong humoral and cellular responses at both systemic and mucosal levels (Becker et al. 2001, 2007a, b; Bertot et al. 2004). Moreover, in the case of the well-studied NOD2 agonist murabutide, recent studies showed that novel amphiphilic desmuramyl peptides were able to trigger the secretion of higher levels of pro-inflammatory cytokines, such as TNF-α, when compared with murabutide (Khan et al. 2017).

3.4.2 Freund's Incomplete Adjuvant (FIA)

Freund's incomplete adjuvant (FIA) is a W/O emulsion without mycobacterial products, which has been successfully used in a number of veterinary vaccines. FIA was also exploited to enhance the immunogenicity of influenza and inactivated polio vaccines in humans (Aucouturier et al. 2006). Moreover, FIA can be used in several vaccines to treat various maladies such as cancer, melanoma, HIV, and malaria, while its mechanism of action remains poorly understood (Hailemichael and Overwijk 2014). However, antigens may be partially denatured during the production of the emulsion, which could in turn account for its failure when formulated with certain immunogens (e.g., antigens from HSV, adenovirus, and trachoma). FIA is not licensed for use in the USA because of reported side effects, which include local sterile abscesses and cysts, granulomas, and carcinogenicity in mice. Furthermore, it was shown that adjuvant hydrocarbon oils, such as Bayol F, are able to induce autoimmunity. This observation has deep implications for the use of oil-based adjuvants in human and veterinary vaccines (Kuroda et al. 2004). Nonetheless, FIA-adjuvanted vaccines had been administered in hundreds of thousands doses before this issue was raised in the context of murine studies, and long-term follow-up of the recipients from these FIA-adjuvanted vaccines has not demonstrated an increase in mortality, tumor development, or autoimmune diseases (Kuroda et al. 2004). Thus, FIA-adjuvanted vaccines may have intrinsic properties that limit their efficacy, resulting in only rare therapeutic benefit with weak inflammatory reactions at vaccine injection sites (Hailemichael et al. 2013).

3.4.3 Squalene- and Saponin-Based Adjuvants

Squalene or squalane emulsions are efficient adjuvants, which can be stabilized by microfluidization, so that the emulsions can be frozen or kept for years at room temperature, allowing also their sterilization by terminal filtration (Ott et al. 1995). The squalene-in-water emulsion MF59 has been recently approved for human use (Ott et al. 1995; Podda and Del Giudice 2003). MF59 promotes both humoral and cellular immune responses (Mesa and Fernandez 2004; O'Hagan 2007; O'Hagan et al. 1997, 2007; Ott et al. 1995; Podda and Del Giudice 2003), and it is included in commercially available vaccines against influenza (De Bruijn et al. 2007). MF59-containing formulations against other pathogens have been also tested in clinical trials with promising results (Harro et al. 2001; Maek et al. 2003; Mitchell et al. 2002).

MF59 consists of 5% squalene with the surfactants polysorbate 80 (Tween 80, polyoxyethylene sorbitan mono-oleate) and sorbitan tri-oleate (Span 85). Emulsification by high-pressure homogenization results in a stable droplet size of 200–300 nm. The small droplet size and the stability of the emulsion account for its immunological activity. MF59 in combination with antigens has elicited antibody responses up to 50-fold higher titers than those obtained using alum as adjuvant (Wack et al. 2008). Higher levels of T-helper responses and in some cases MHC class I CTL responses have also been demonstrated (O'Hagan et al. 2000). Preclinical studies have shown that MF59 can be successfully combined with CpG in order to induce strong and long-lasting antibody and Th1 responses against influenza (Wack et al. 2008). Moreover, it was shown that MF59-adjuvanted inactivated vaccine was more efficacious than non-adjuvanted vaccine in preventing influenza infection in young children and in reducing hospitalization due to the influenza infection in the elderly (Del Giudice and Rappuoli 2015; Tetsutani and Ishii 2012; Wilkins et al. 2017).

AS03 is an adjuvant system composed of alpha-tocopherol, squalene, and polysorbate 80 in an oil-in-water emulsion (Garcon et al. 2012). The AS03 has been used as adjuvants in both seasonal adjuvanted trivalent influenza vaccines (ATIVs) and pandemic monovalent influenza vaccines in infants and young children (Wilkins et al. 2017). However, despite the high benefit-to-risk ratio of adjuvanted vaccines, the fear of negative side effects (e.g., narcolepsy associated with the European AS03-adjuvanted pandemic influenza vaccine) has discouraged many people from getting vaccinated even when there is an attributable risk of 0.59 cases per 100,000 doses (Ahmed et al. 2016; Stowe et al. 2016). Another interesting compound is AF03, a squalene-based emulsion adjuvant that is present in the adjuvanted pandemic influenza vaccine Humenza (Caillet et al. 2010; Klucker et al. 2012).

Through cyclase-catalyzed pathways, saponins are synthesized from squalenes. Saponins are a chemically heterogeneous group of sterol glycosides and triterpene glycosides, which are common constituents of plants. They are known since the 1920s to cause substantial enhancement of immune responses. Naturally occurring saponins from *Quillaja saponaria* stimulate humoral responses against T-cell-dependent and T-cell-independent antigens and CTL responses (Kensil 1996; Kensil et al. 1996). Furthermore, saponins are able to modify the activities of T-cells and DCs and can induce either pro-inflammatory Th1/Th2 or anti-inflammatory Th2 immunities via receptor-mediated and non-receptor-mediated mechanisms (reviewed in (Marciani 2018)). Despite their use in animal vaccines, the development of saponin-based formulations for humans has been impeded by their complexity and concerns about toxicity (Cox et al. 1998; Giudice and Campbell 2006). On the other hand, QuilA, which results from partial purification from crude food-grade extracts, is contained in several veterinary vaccines. Further purification provided concentrated saponin fractions, such as QS-21, which is currently under clinical investigation in humans (Kensil et al. 1995; Kirk et al. 2004; Mccluskie and Weeratna 2001). In the last decade, QS-21 has been highlighted in the development of a variety of prophylactic and therapeutic vaccines with a favorable ratio efficacy/toxicity. Over 120 clinical trials for around 20 vaccine indications in infectious

diseases, cancer, and degenerative disorders with over 50,000 patients have been reported (Lacaille-Dubois and Wagner 2017). Combinations of immunostimulatory molecules, such as the liposome-based vaccine adjuvant system AS01, containing two immunostimulants, MPLA and the saponin QS-21, have opened the way to the development of new or improved vaccines. AS01 is efficient at promoting $CD4^+$ T-cell-mediated immune responses and is an appropriate candidate adjuvant for inclusion in vaccines targeting viruses or intracellular pathogens (Didierlaurent et al. 2017).

Interestingly, purified saponins seem to exhibit also adjuvant activity when delivered by the oral route (Kirk et al. 2004; Rajput et al. 2007). Saponins have been combined with cholesterol and other lipids to generate immune-stimulating complexes (ISCOMs), which are open cage-like structures with built-in adjuvant activity that promote antibody, T-helper, and CTL responses. ISCOMs seem to enhance antigen targeting to APC, as well as their subsequent uptake, processing, and presentation. The use of ISCOMs results in the production of pro-inflammatory cytokines, such as IL-1, IL-6, and IL-12 (Morein et al. 1984, 1998, 1999). ISCOMs and ISCOMATRIX™ adjuvants are versatile and flexible systems with various phospholipids and saponin components for the development of vaccines against either infectious diseases or cancer (Garcia and Lema 2016; Lovgren Bengtsson et al. 2011). Both compounds induce high and long-lasting levels of broadly reacting antibodies supported by a balanced Th1/Th2 response including multifunctional T-cells and CTL. Moreover, these molecules showed improved quality of immune responses combined with dose-sparing capacity and acceptable safety profile (Lovgren Bengtsson et al. 2011).

4 Conclusions and Outlook

There is still a high demand for adjuvants able to stimulate cellular immunity, which is essential for combating intracellular pathogens and tumors. In this context, there are several candidate adjuvants in the pipeline, which are based on well-characterized moieties able to exert their biological activity through stimulation of defined cellular targets and/or signaling cascades. An in-depth understanding of the underlying mechanisms of action of these compounds, together with the availability of defined synthetic derivatives, will certainly facilitate rational vaccine design and improve the safety profiles of the resulting candidates. In fact, a number of promising oil- and lipid-based antigen delivery systems and adjuvants are currently under clinical development (Table 2).

Nevertheless, the identification of novel agonists of pattern recognition receptors present on APC, which provide a link between the innate and adaptive immune system, is still a high priority in vaccinology. Depending on the nature of the adjuvant, it might be or not possible to exploit it for different vaccines (e.g., preexisting immunity might affect the activity of protein adjuvants). On the other hand, depending on the effector mechanisms required for clearance, very specific types of immune responses might be desirable following vaccination.

Table 2 Example of oil-based formulations under clinical development

Compound	Field of application	Literature
Liposomes	Interferon-β gene therapy for high-grade glioma Muramyl tripeptide phosphatidylethanolamine against osteosarcoma pulmonary metastases Encapsulation of doxorubicin and cyclophosphamide as therapy in metastatic breast cancer, liposomes as delivery system for cancer therapy Safety evaluation of the recombinant factor VII for severe hemophilia A therapy Theranostic nanoscale delivery systems combining simultaneous therapeutic and imaging functions Noninvasive transdermal drug delivery system	Wakabayashi et al. (2008) Anderson (2006), Mori et al. (2008) Giotta et al. (2007), Vorobiof et al. (2004) Powell et al. (2008) Corvo et al. (2016), De Barros et al. (2013), Zhu et al. (2014) Al-Jamal and Kostarelos (2011), De Jesus Valle et al. (2016) Chen et al. (2014), Kajimoto et al. (2011), Yamazaki et al. (2017)
MALP-2	Intra-tumor injection of MALP-2 in patients with pancreatic carcinoma Local immunostimulation with MALP-2 (a) in influenza virus-infected mice improved pulmonary bacterial elimination or (b) effect on melanoma metastasis to the lung	Schmidt et al. (2007), Schneider et al. (2004) Reppe et al. (2015)
Lipid A molecules	Safety and immunogenicity of a new adjuvanted hepatitis B vaccine Mucosal vaccine adjuvant-delivery system Neuroprotective effect of monophosphoryl lipid A	Bernstein (2005), Dupont et al. (2006) Wang et al. (2014) Hosseini et al. (2018)
αGalCer	NKT-cell-based immunotherapy for solid cancer αGalCer-pulsed DC therapy lung cancer patients Combination of αGalCer and MPLA induces CD8 (+) T-cell responses and protective immunity αGalCer induce protective mucosal immune responses against influenza virus infection	Giaccone et al. (2002) Saka et al. (2017) Coelho-Dos-Reis et al. (2016) Lee et al. (2011)
Virosomes	Influenza vaccine optimized for the immunization of children under antiretroviral therapy Immunogenicity and safety of a pediatric dose of a virosomal hepatitis A vaccine in healthy children Adjuvanted virosomes elicited stronger humoral and cellular responses than did adjuvanted subunit vaccine	Tanzi et al. (2006) Jain et al. (2014) Liu et al. (2014)
ISCOM's (saponin)	Induction of IgM and IgG in high-risk breast cancer patients after immunization to blood group-related carbohydrate antigens Malaria vaccines in different populations Virosomal vaccine supplemented with GPI-	Gilewski et al. (2007) Ragupathi et al. (2011) Ockenhouse et al. (2006), Polhemus et al. (2007), Stoute et al. (2007)

(continued)

Table 2 (continued)

Compound	Field of application	Literature
	0100, a semisynthetic saponin derivative, allows the use of very low antigen doses Mucosal administration of influenza vaccines	Liu et al. (2014) Liu et al. (2012)
MF59	MF59 in the context of influenza vaccines: Virosomal MF59-adjuvanted influenza vaccine optimized for the elderly, trivalent inactivated influenza vaccine, subunit influenza vaccine in HIV-seropositive adults Recombinant envelope protein gB vaccine in cytomegalovirus-infected toddlers Papillomavirus vaccine in adult volunteers HIV/AIDS vaccine trials The individual components of MF59 showed no direct immunostimulatory activity Delivery of TLR4 agonist within MF59 emulsion oil droplets leads to a more potent response against a *Meningococcus* vaccine	De Bruijn et al. (2007) Harro et al. (2001), Maek et al. (2003), Mitchell et al. (2002) Calabro et al. (2013) Singh et al. (2012)

Therefore, the availability of a broad panel of adjuvants exhibiting different immune-modulatory properties would make feasible to choose the most appropriate molecule, according to the specific clinical needs. Thus, basic research in immunology will continue being the main driving force for innovation in this field.

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Tuning Activity of Antimicrobial Peptides by Lipidation

13

Bauke Albada

Contents

Abstract

Antimicrobial peptides (AMPs) are amino acid-based bioactive molecules that specifically target microbes. As such, they are a potent class of antibiotics, especially against bacterial infections. Naturally occurring AMPs are usually too long to be considered for therapeutic applications. To solve this, short sequences that mimic the activity of AMPs are designed. However, such endeavors are often accompanied with a reduction in antibacterial activity. To counter this, lipophilic molecules can be attached that function as a lipid anchor and target the short sequence to the bacterial membrane. For a range of short AMPs, this strategy has proven to lead to more active constructs. Although these lipidated short AMPs often work as complex target specific surfactants, more delicate modes of action that do not deviate too much from the nonlipidated counterparts are also known. This is readily observed by the large differences in activities that are detected when

B. Albada (✉)
Laboratory for Organic Chemistry, Wageningen University and Research, Wageningen, The Netherlands
e-mail: bauke.albada@wur.nl

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alterations in the lipid chain length and chirality of the amino acids residues are implemented. It is not uncommon to see that inactive or poorly active short AMPs can be turned into potent antibacterial agents. Importantly, selectivity of the short lipidated AMPs (lipoAMPs) for the bacterial membrane can be enhanced by alteration of the amino acid chirality. This strategy has led to lipoAMPs with submicromolar activities; in fact, activities that rival that of vancomycin have been observed for several short AMPs. Future research needs to determine (i) the effect of lipidation on the formation of lipid rafts in the bacterial membrane, (ii) if structural complications like branched lipids or chiral substituents on the lipid chain can be used to further increase the activity and selectivity of the conjugates, and (iii) if additional functionalities other than a membrane-anchoring ability can be bestowed on the lipid chain, e.g., redox activity or scavenger for small molecular components that traverse the lipid membrane. The interplay between degree of lipophilicity and the chirality of the amino acids of the AMP also needs further exploration, especially to see if more potent and selective (lipo)AMPs can be obtained that can be applied systemically. It may also be advisable to measure the most potent lipoAMPs in a centralized facility in order to obtain objective and comparable antibacterial activities.

1 Introduction

Although the problem of increasing resistance of bacteria and fungi toward existing antibiotics has been signaled already decades ago (Hancock and Sahl 2006), the enormous efforts to develop novel antibiotics has not produced the desired number of medicinal compounds that are needed to keep pathogenic invasions at bay. Numbers of resistant bacteria are still steadily increasing across the globe, and news items in which outbreaks of resistant bacteria in our houses of care occur more frequently. Perhaps, inspired by the ability of multicellular organisms to resist bacterial infections using short cationic amphiphilic peptides with direct acting antimicrobial and/or indirect acting immunomodulatory activities as part of their defense system (Boman 1995; Zasloff 2002; Selsted and Ouellette 2005; Hancock et al. 2006), scientists have turned to this class of biomolecules as potential entry points to new antibiotics. Unfortunately, however, most direct-acting, naturally occurring antimicrobial peptides (AMPs) are limited to topical applications. This is especially unfortunate in view of their broad spectrum of activity. Since there is a demand for novel antibacterial agents that can be applied systemically, it is clear that these naturally occurring AMPs may best serve as a source of inspiration to develop smaller derivatives that allow such applications. In order to do this efficiently, their mode of action needs to be elucidated in great detail. Lastly, from a production point of view, peptide-based antibacterial agents need to be prepared on large scale and in a cost-effective manner. Also, this aspect calls for size-reduction measures.

The direct-acting, naturally occurring AMPs that inspired many endeavors to make smaller derivatives are assembled in the Antimicrobial Peptide Database (APD) (Wang et al. 2016). As of the end of 2017, the APD had 2927 peptides

deposited in the database. The average length of the deposited AMPs is 32.8 amino acid residues, and the average net positive charge is 3.27. Assuming the average AMP is also a promising drug candidate, its costs for treatment might well approach that of Fuzeon (€52/day, 2003) (McKerrow 2003), which is a therapeutically applied peptide of comparable length. Although from a cost-effective point of view a therapeutic AMP can in principle be obtained – provided sufficient effort is performed in optimizing its synthesis – the pharmacological drawbacks of natural peptides are not solved. With these considerations in mind, it is clear that most AMPs depicted in the synoptic chronological overview below are poor therapeutic candidates (Fig. 1).

However, from the secondary structure of some of these AMPs, a common pattern emerges that inspired most synthetic efforts to develop small AMPs. Many antimicrobial peptides target the lipid bilayer of bacteria. Since many AMPs are cationic and amphipathic (Nguyen et al. 2011), it is not surprising that a major step in

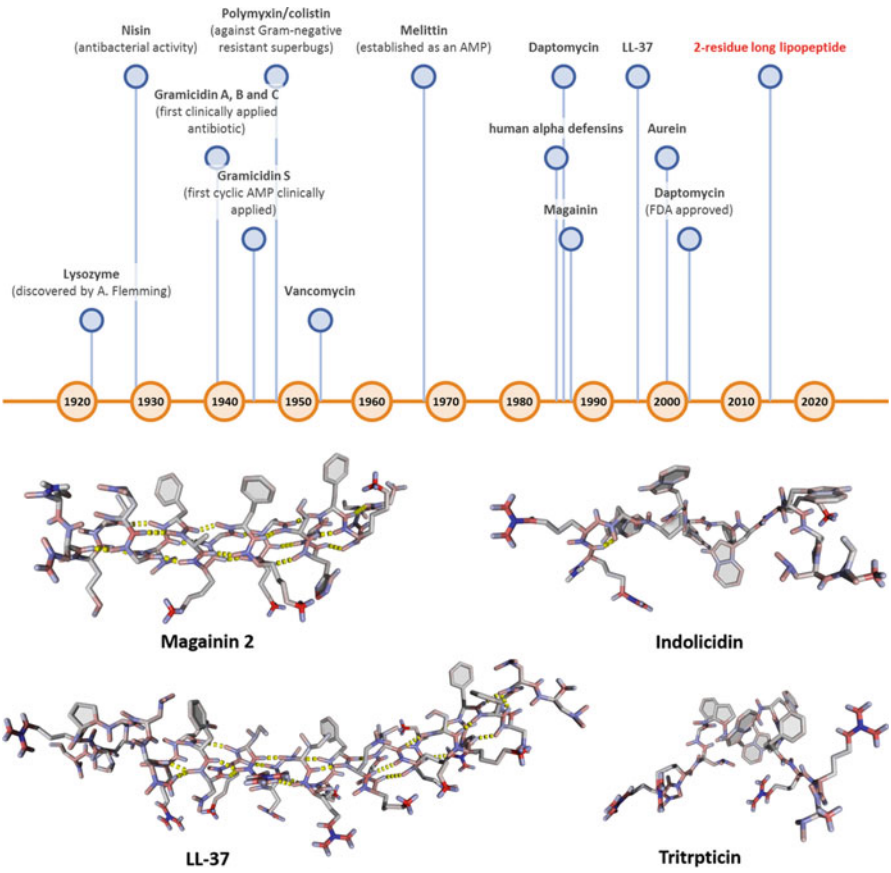

Fig. 1 *Left*: synoptic chronology of AMPs (lysozyme is included as the start of antibacterial polypeptides). *Right*: secondary structures of four typical amphipathic naturally occurring AMPs; the positive charges are positioned at the bottom, and the hydrophobic side on the top

the activity of cationic AMPs is their initial binding to the negatively charged outer membrane of bacteria. It has been suggested for many short amphipathic AMPs that they interact with the bacterial membrane via the so-called carpet mechanism: the peptide accumulates on the surface of the membrane (Shai 2002). Such a mechanism is supported by the frequently made observation that minimal inhibitory concentrations (MICs) of AMPs are usually not lower than 1 μM. The amphiphilic nature of the bilayer has enticed scientists to attach lipids to peptides in order to increase the affinity of the peptides for the bilayer. This is mostly driven by a desire to reduce the length of the AMP in order to arrive at a still very potent but more attractive candidate for therapeutic applications (Mangoni and Shai 2011). Another driving force that can be identified is the elegance by which nature uses lipidation and clusters of cationic amino acids to position bioactive proteins in the membranes of the different cellular compartments (e.g., the Ras protein (Brunsveld 2009)). A last reason that drives scientists to reduce the size of peptide-based drugs, especially those that target a fluidic membrane, is that their structure largely depends on the environment. Even for a relatively short sequence like that of aurein 2.2, it was shown that the structure of its derivatives is affected by the membrane composition (Cheng et al. 2010). Since the bioactive structure might differ from the preferred structure in model membranes, unraveling of its mode of action based on structural features is severely hampered. This does not mean that its MOA cannot be determined (Wenzel et al. 2015), but it just became much more complicated.

Although the attachment of linear natural lipids has been quite fruitful when it comes to increasing the activity of otherwise poorly active AMPs, the realm of AMP-based bioconjugates based on unnatural lipophilic moieties has not been extensively explored. Examples of lipophilic moieties that remain underexplored so far include: (i) branched lipids, (ii) oligoaryl lipids, and (iii) lipophilic organometallic moieties. In this overview, I limit myself to lipoAMPs that are, in principle, not able to span the bacterial membrane due to their limited size (i.e., those that have up to 10 amino acid residues).

2 Lipidated AMPs: Synthesis, Lipid Types, and Peptide Complexity

2.1 Synthesis of Lipidated AMPs

For the synthesis of lipidated AMPs, scientists mostly rely on the attachment of a fatty acid to an amino group of the AMP, leading to an amide-bond-linked lipidated AMP. Although other ligation methods are in principle possible, these amide-bond-linked conjugates dominate the field. Naturally occurring AMPs, when they contain one reactive amino group, can be reacted with an activated ester (e.g., a (sulfated) *N*-hydroxysuccinimide ester) of a fatty acid (Fig. 2a). When a sulfated active ester is used, the reaction can be performed in water; if an ester is used that is not soluble in water, then an organic cosolvent (like dimethylsulfoxide, acetonitrile, or dioxane) needs to be used. The inverse reaction is also possible, i.e., where the AMP has

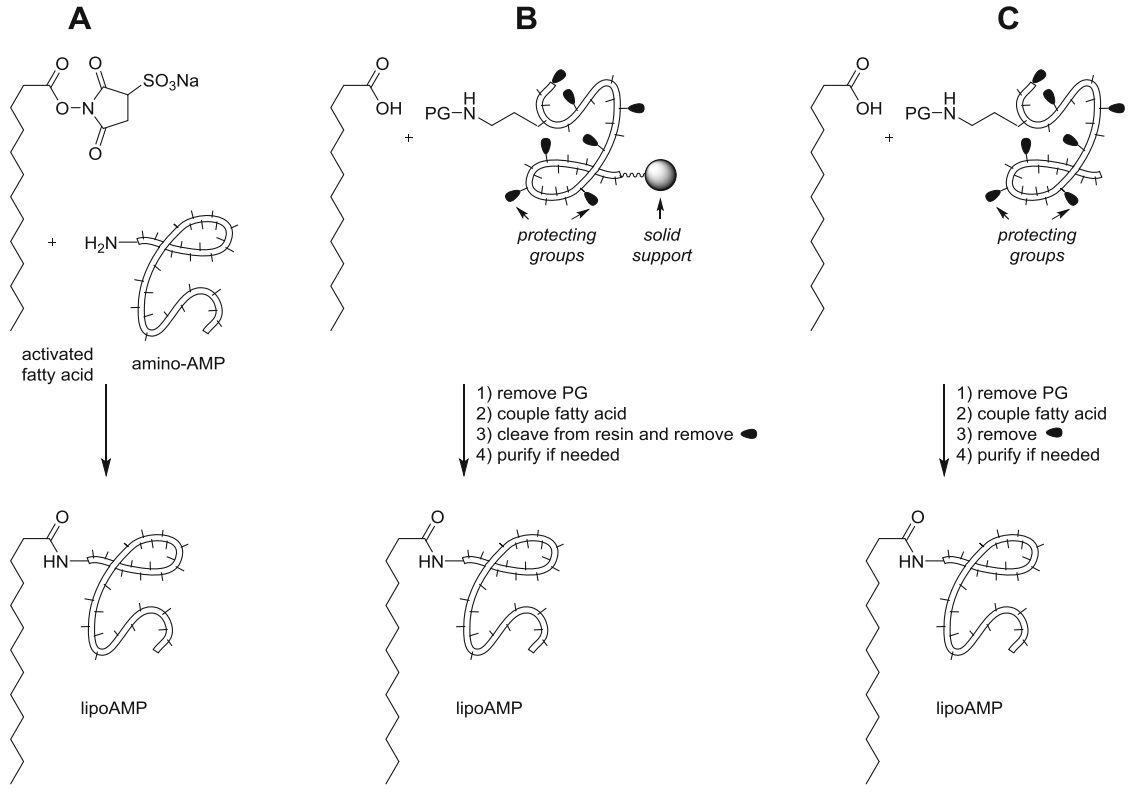

Fig. 2 Three commonly applied routes for the synthesis of lipoAMPs. (**a**) Lipidation of a peptide with a uniquely reactive amino group. (**b**) Lipidation of a resin-bound, side chain protected peptide that has one orthogonally protected amino group that can be lipidated selectively. After acidic removal of the resin-bound protected lipoAMP, which also removes the other protecting groups, the target lipoAMP is obtained. (**c**) Lipidation of a side chain protected peptide that has one orthogonally protected amino group that can be lipidated selectively. After acidic removal of the other protecting groups, the target lipoAMP is obtained

a carboxylic acid and the lipid contains an amino group (see for example later). For unnatural peptides, which are prepared using synthetic methods that rely on solution-phase (Fig. 2b) or solid-phase (Fig. 2c), a specifically protected amino group can be introduced. This specific protecting group allows for a selective deprotection of the targeted amino group, followed by its conjugation to the fatty acid. After successful conjugation of the fatty acid to the synthetic AMP, the protecting groups that were still present on the other functional groups can be removed and the lipidated AMP can be obtained by reversed phase chromatography (HPLC). Once these lipidated AMPs have been obtained, usually after (repeated) lyophilization from aqueous solutions, they can directly be applied in biological studies.

Here, I note that solid-phase synthesis is beneficial in the discovery-phase of a project, as it facilitates the rapid synthesis of a library of lipoAMP derivatives that quickly provide insight in the most active compounds (vide infra). Thus, identified lead compounds can, in principle, be prepared by solution phase methods in order to obtain larger amounts of the lipoAMPs, which are needed for more in-depth activity studies.

2.2 Types of Lipids

A large variety of lipophilic molecules are available for conjugation to AMPs in order to tune their activity by means of lipidation. However, most methods described focused on commercially available lipophilic molecules like fatty acids. The chart below gives an overview of the some of the lipophilic molecular fragments that have been applied; as is custom to the chemical community, the point where the lipid is attached to the peptide is indicated by the wavy bond next to the carbonyl moiety (C=O) by which the lipid is attached (Fig. 3).

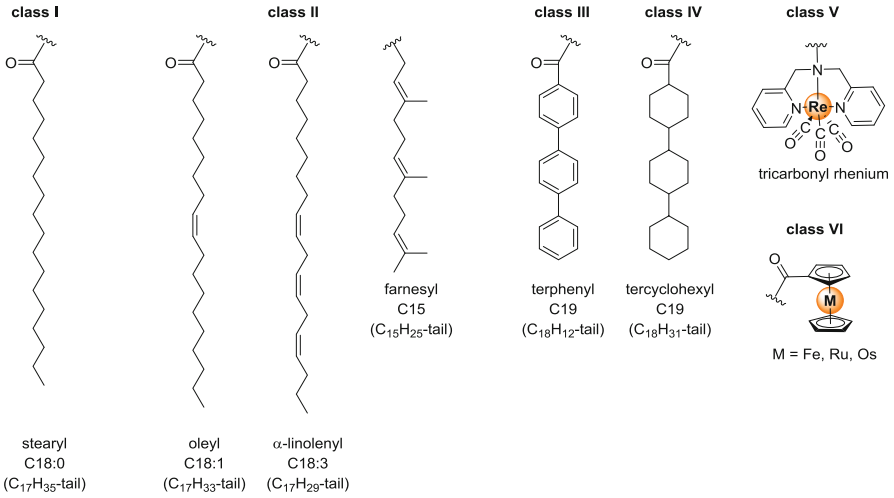

Fig. 3 Overview of some of the naturally occurring (class I and II) and unnatural (class III–VI) lipophilic moieties that have been used in antibacterial agents

As mentioned, most lipophilic moieties that have been used so far to construct lipoAMPs were simple saturated fatty acids (class I). Even commercially available unsaturated lipids like oleic acid, linoleic acid, linolenic acid, and arachidonic acid (class II) have barely been utilized to crank up the lipophilicity of AMPs. Still within the hydrocarbon family, but now in cyclic form, aromatic moieties have sometimes been used (class III); examples of saturated cyclic hydrocarbons (class IV) are rarer. Lastly, more exotic variants of the previously mentioned classes, i.e., metal-containing lipophilic complexes (class V), are even rarer. Lastly, a special class of metal-containing lipophilic anchors, namely the organometallic complexes (class VI), has been studied with special emphasis on group 8 metallocenes (i.e., with Fe, Ru, and Os) (Albada et al. 2012a).

That bacterial membranes are not composed of the same lipids is a well-known fact (Vance and Vance 2002; Sohlenkamp and Geiger 2016). The charts show, however, only which type of polar head group is present; it does not contain information on the even larger variation in the types of fatty acids that are found in these bacterial lipids. A nonexhausted survey of the literature indicates that >20 different types of fatty acids are easily found in bacterial membranes (see for the case of *P. aeruginosa*, and its highly surface-dependent composition: Benamara et al. 2014). In view of this huge complexity, and since many lipoAMPs target the bacterial membrane, it is important to understand the correlation between AMP activity and the lipid composition of the bacterium (Teixeira et al. 2012). This is hardly studied at the moment, even though the study of lipid composition of bacterial membranes has been ongoing for many decades. Of course, such endeavors are complicated by the dynamic nature and variation in composition of the bacterial membrane (Fig. 4).

2.3 Types of AMP

Since most studies of the effect of lipidation on the activities of AMPs are on synthetic AMPs (synAMPs), I will first present an overview of various synAMPs before I shortly treat the effects on natural AMPs.

2.3.1 Ultrashort synAMPs (≤4 Amino Acids)

In a seminal paper by the group of Shai, it was shown that even ultrashort synAMPs that were otherwise completely inactive against bacteria were turned into potent antibacterial agents by means of lipidation (Makovitzki et al. 2006). Their antibacterial activities could rival that of many native AMPs. Following the observation that lipopeptides that contain longer peptide sequences (six to seven amino acids) were non-cell-selective membrane destabilizers, the authors realized that a threshold of hydrophobicity was required to obtain selective antimicrobial activity. By combining cationic D- and L-amino acids with fatty acids of different lengths, several lipoAMPs were identified with very potent and selective activities (Chart 1). Specifically, peptide sequences of the type KXXK-NH_2 (with X = L, A, G, K, or E) were lipidated on the N-terminus with a C12, C14, or C16 lipid.

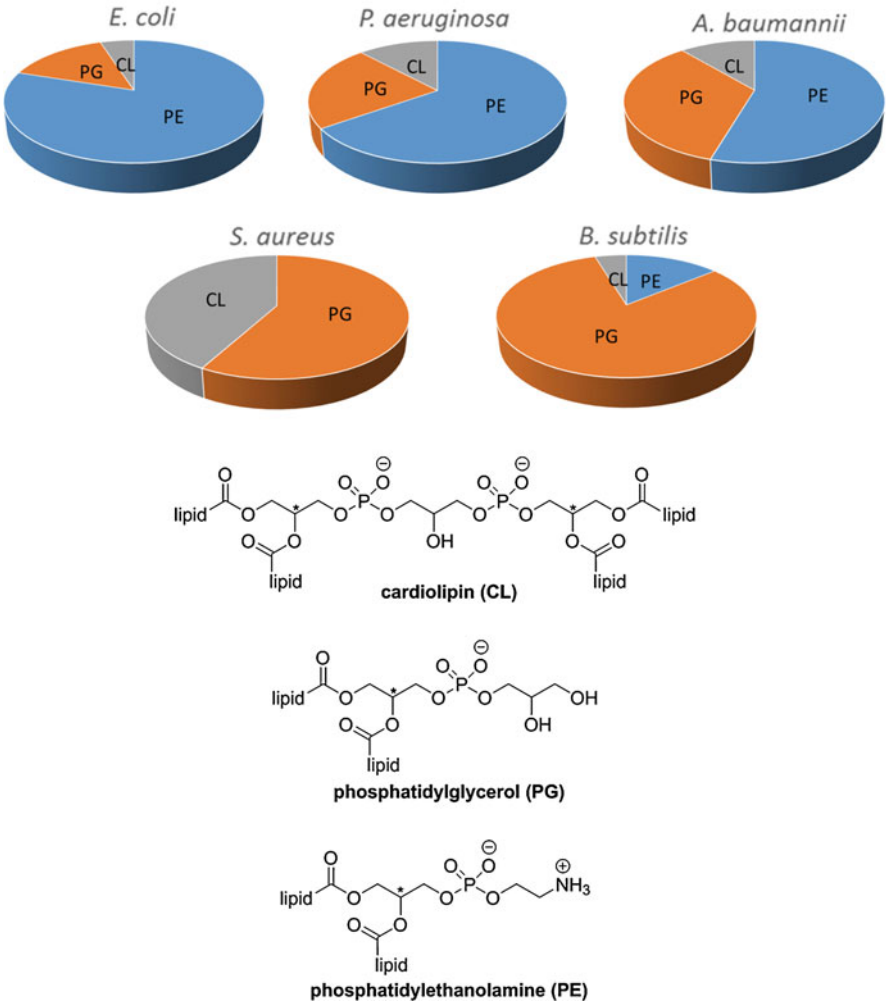

Fig. 4 Pie charts that indicate different types of head groups that are present in the lipids that are found in the membranes of the various bacteria. The structures of the head groups are given on the right (the *position* of the chiral carbon atoms are indicated by the asterisk). *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine, *CL* cardiolipin. Note: this is an oversimplified representation of the actual composition (Goldfine 1982; Shaw 1974)

When the MIC values of the C_{16}-lipidated tetrapeptides against the three Gram-negative bacteria are compared, it is striking that the AMP with the least hydrophobic amino acid (i.e., Gly, G) is more active than that with the most hydrophobic residue (i.e., Leu, L). It should be noted, however, that the comparison is unfair since two differences were included in these sequences: not only was L replaced with G, also the N-terminal Lys (or K) residue was switched from L (in the KLLK sequence)

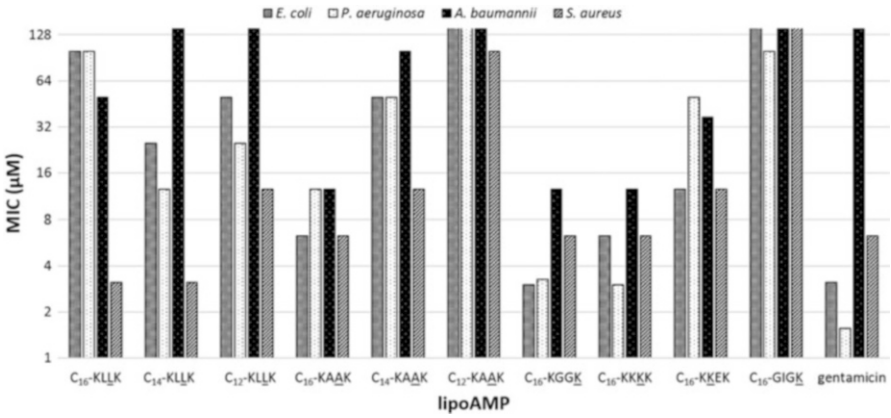

Chart 1 Correlation between antibacterial activities of ultrashort lipidated AMPs (Makovitzki et al. 2006)

to D (in the KGGK) sequence. It could well be that the observed difference in activity is due to this L to D substitution (vide infra).

High hemolytic activity of the most active C_{16}-KGGK sequence points to a general surfactant-type mechanism in which biological membranes are dissolved irrespective of their origin. This is also seen by the broad spectrum of pathogens against which most of these lipoAMPs are active. Nevertheless, the study showed that both the size of the aliphatic chain and the length and composition of the peptide sequence are important for the specificity of the lipoAMPs toward the different cell types. This notion is further supported by the large differences in antibacterial activities that were observed for lipidated peptoid (lipopeptoid) derivatives (peptoids are peptide mimics in which the amino acid side chain is shifted from the Cα-atom to the amino group) (Chongsiriwatana et al. 2011). As expected, the major target for these ultrashort lipoAMPs was the membrane of the pathogen. Since the membrane is the main target for these constructs, it can be considered as no surprise that lipo-β-peptides mostly retained the antibacterial activity of their α-peptide counterparts (Serrano et al. 2009). Applications of these lipoAMPs were, so far, limited to plants (Makovitzki et al. 2007; Brotman et al. 2009).

Although this chapter mainly deals with antibacterial agents, a few words should be addressed to antifungal agents as well, especially in view of the antifungal potency of ultrashort lipopeptides. In order to deviate not too much from the antibacterial story, the focus is on the opportunistic fungal pathogen *Aspergillus fumigatus* that invades pulmonary tracts. The group of Yechiel Shai revealed that palmitoylated Lys-Ala-D-Ala-Lys was able to efficiently clear the lungs of mice that were infected with *Aspergillus* (Vallon-Eberhard et al. 2008). Interestingly, the antimicrobial activity of ultrashort lipopeptides can be increased by their incorporation into a multivalent construct. Whereas the His-His-C_{14} lipopeptide was inactive

against fungi, its trivalent construct proved to be a potent pH-dependent antifungal agent that was able to clear the lungs of 80% of the treated mice from their fungal burden (Arnusch et al. 2012).

2.3.2 Short synAMPs (5–10 Amino Acids)

Whereas lipidated ultrashort peptides could still be considered as rather complex surfactants, the analogy is less suitable for longer peptides. The longer the peptide sequence is, the more features are present in the amino acid code, and thus the more subtle the balance between lipid tail and peptide composition. However, as was clear from the antibacterial activities of the ultrashort lipoAMPs presented in the previous section, it should also be expected to observe similar subtle influences of both amino acid composition and lipid length for longer sequences.

In view of the amphipathic nature of many naturally occurring AMPs, chemists have sought to identify the minimal pharmacophore of membrane-targeting AMPs. It turned out that short peptides that contain alternating tryptophan and arginine residues were already potent AMPs (Strøm et al. 2007). Interestingly, tryptophan could be replaced with the slightly less hydrophobic phenylalanine (π_R Trp = 2.25 vs. π_R Phe = 1.79, cf. π_R Gly = 0) (Fauchère and Pliska 1983) or arginine could be replaced with the less basic lysine (pK_a Arg = 12.5 vs. pK_a Lys = 10.5), albeit with a reduction in activity. However, the Arg-Trp tandem proved a valuable playground to study in great details the structure–activity relationship of lipoAMPs. Already, very short sequences of these residues, due to their potential pharmaceutical applications (Svenson et al. 2007; Haug et al. 2008), were subjected to approaches that aimed at the increase of their hydrophobic character (Haug et al. 2004). However, such approaches that depend on the chemical modification of amino acid residues are outside of the scope of this review. For studies of the effect of lipidation, the H-RWRWRW-NH_2 sequence was used. By itself, it had limited antibacterial activities and therefore was the candidate of choice to study activity-enhancing modifications.

Figure 5 shows the two lead structures that were used to study the effect of lipidation on the H-RWRWRW-NH_2 sequence. Whereas the lipophilic moiety is usually attached to the N-terminus of the peptide, we wanted to study the effect of

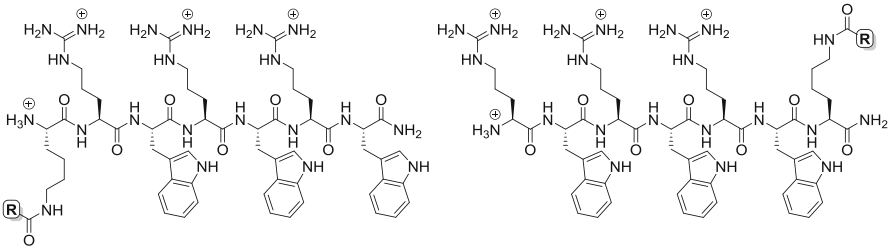

Fig. 5 Chemical structures of the short lipoAMPs with the sequence RWRWRW that were lipidated on the N-terminus (left) or C-terminus (right) with a lysine-lipidated residue

the positioning of the lipid, i.e., C- versus N-terminal. In order to not alter the charge of the AMP, but also to facilitate incorporation of the lipid on the C-terminus of the AMP sequence, we used a lysine residue. Facilitated by the ability to incorporate a lysine residue that is protected with a trityl group, it was possible to selectively make the side chain amino group of lysine available for lipidation with various fatty acids. To illustrate the time benefit of SPPS, it took only 2 days to prepare a set of 16 lipoAMPs of sufficient purity to test for their antibacterial activity. (Here I note that we went through the trouble of purifying each peptide by preparative HPLC, which doubled the time for our studies; however, we also tested the crude peptides and very similar results were obtained.) With this set of lipoAMPs, we were able to determine the MIC values against a panel of Gram-positive and Gram-negative bacteria (Albada et al. 2012b) (Chart 2).

From this study, we found that the antibacterial activity of lysine-lipidated amino acid residues increased with increasing length of the carbon-tail of the lipid, with optimal activity at C_8 and C_{10}. Although the lipoAMPs with C-terminally positioned lipids were on average a bit more active, the difference with N-terminally positioned counterparts was not significant in most cases. Notably, the lipophilicity of the C- and N-terminally lipidated was almost identical. Even though the N-terminally positioned lipid was closer to the N-terminal amino group that is mostly charged under physiological pH, this did not lead to a significant effect on the lipophilicity or antibacterial activity. Interestingly however, a difference was observed in the hemolytic activity: whereas the N-terminally positioned C_8-lipidated lysine residue led to >50% hemolysis when erythrocytes were exposed to 250 μg/mL (142 μM) of the lipoAMP, "only" 10–20% hemolysis was observed for its C-terminal counterpart.

Apart from tuning the lipophilicity of AMPs by means of lipidation, i.e., the straightforward route, it is also possible to alter the lipophilicity of peptides by combining L- and D-amino acids. In a succeeding study, we explored the effect of an L-to-D substitution scan (in analogy to a more commonly applied alanine scan) in order to probe for the effect of chirality of the amino acid residues on their antibacterial activity (Albada et al. 2013). Despite the fact that the retention time of 32 diastereomeric lipoAMPs with the sequence H-RWRWRW-Lys(C(O)C_7H_{16}) in a C_{18} RP-HPLC columns ranged from 18.4 to 19.5 min, the MIC values were very comparable over the range. This showed that for these lipoAMPs the different lipophilicity caused by incorporation of both L- and D-amino acids did not result in more active constructs. Interestingly though, large differences were observed in their hemolytic activity: 0–17.2% hemolysis with 250 μg/mL (142 μM) was observed, with multiple lipoAMPs exhibiting almost no hemolysis. This is important since the applicability of lipoAMPs that are active at biological membranes still prevents them from finding widespread therapeutic applications (Wenzel et al. 2016a and 2016b).

That this finding was not generally applicable was discovered a bit later when the same team studied the effect of an L-to-D substitution scan on a ferrocenoylated (FcC(O)-) or ruthenocenoylated (RcC(O)-) WRWRW-NH_2 peptide (Albada et al. 2014). For this construct, the retention time on the same column as before ranged from 19.4 to 20.1 min. However, whereas the hemolytic activity ranged from 2% to 6% (with 121 μM peptide), the antibacterial activity increased from 5.8 to

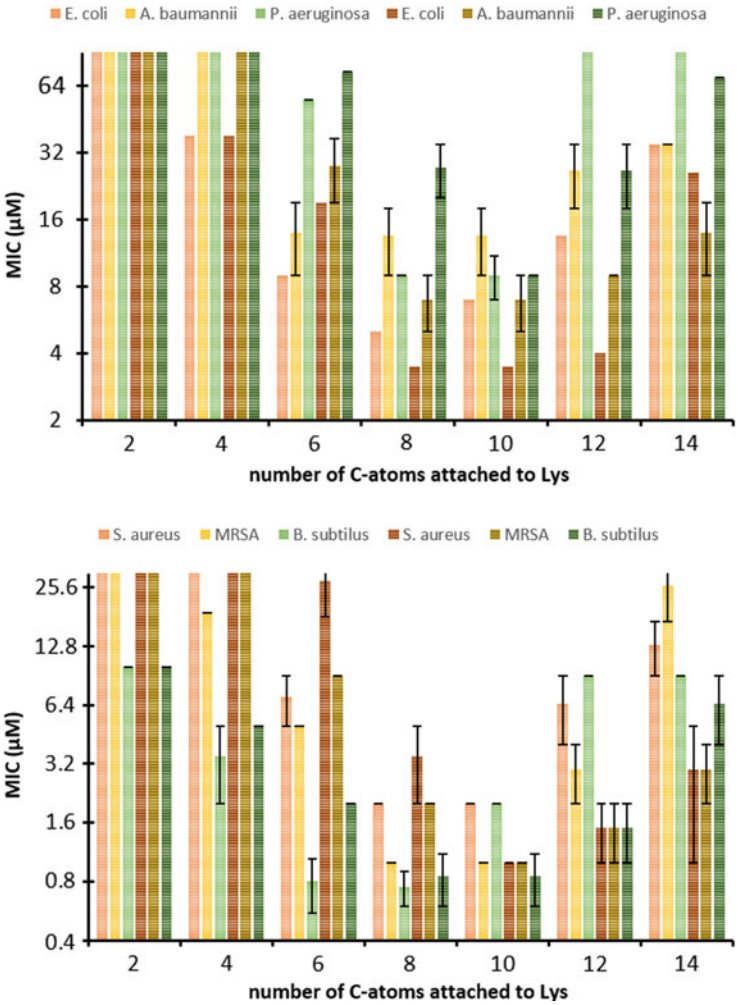

Chart 2 Effect of lipid length on the antibacterial activity (in μM) of lysine-lipidated H-K(lipid) RWRWRW-NH_2 (the three light bars on the left side of each group of six) or H-RWRWRWK(lipid)-NH_2 (the three dark bars on the right side of each group of six) lipoAMPs against Gram-negative (left chart) and Gram-positive (right chart) bacteria. Values were determined in duplicates; error-bars are indicated where different values were obtained

1.1 ± 0.4 μM (against MRSA). Since both the FcC(O)- and RcC(O)-derivative can be considered as a $C(O)C_6H_{14}$ lipid, it is safe to conclude that we do not understand all the details that lead to higher antibacterial activity of lipophilic AMPs. What we do know, however, is that the mode of action of the lipidated AMPs was very similar to that of the nonlipidated counterparts (Wenzel et al. 2014; Wenzel et al. 2016). Nondisruptive membrane integration was determined for both the C- and N-terminally lipidated derivatives, as well as for the nonlipidated versions (Wenzel et al.

2014). Synergistic activity of one lipoAMP from a prior study, i.e., H-RWRWRWK (C_{10})-NH_2, with colistin or tobramycin against *P. aeruginosa* that was isolated from cystic fibrosis patients was shown (De Gier et al. 2016).

As a special class, which shows the structural possibilities that can be prepared by synthetic means, α-AApeptides (these are special unnatural peptides that contain both secondary and tertiary amide bonds) were turned into potent broad-spectrum antibacterial agents by means of lipidation (Hu et al. 2012). Equipped with C16 fatty acids and various hydrophobic and cationic side chain groups, MIC values as low as 2 mg/mL (1.4–1.9 μM) were obtained. Evidence for a surfactant type of mechanism was provided.

2.4 Balancing the Interplay Between Lipidation and AMP Complexity

In a recent study, the complexity of lipoAMPs was increased by adding lipidated cationic peptides to the C-terminus of vancomycin (Blaskovich et al. 2018). Importantly, the nonligated lipophilic peptides, which were called electrostatic effector peptide sequences (EEPSs), themselves were not active (MIC >8 μg/mL). This proves that just having a peptide-based surfactant is not sufficient to obtain an antibacterial compound. Although it was observed that making the lipid anchor too short or too long decreased the potency of the lipoAMP, the length of the EEPS could be reduced from a 16 Lys-rich sequence to a 3 Lys-only sequence. The initially applied disulfide linkage between lipid and vancomycin proved to be too instable for in vivo applications. This was solved by replacing it with a more resilient amide bond, leading to the identification of constructs that showed potent in vivo activity and did not elicit a high propensity to cause resistance. Although the optimized compounds were inactive against Gram-negative *E. coli*, they were 20-fold to over 100-fold more active than vancomycin against the Gram-positive strains MRSA, and significantly more active against Vancomycin-insensitive *S. aureus* (VISA), Vancomycin-resistant *S. aureus* (VRSA), and Vancomycin-resistant enterococci (VRE). The simultaneous targeting of the membrane and the D-Ala-D-Ala-OH cell wall precursor resulted in an enhanced ability to inhibit peptidoglycan biosynthesis. It also led to membrane disruption, although permeabilization was not unequivocally proven.

3 Conclusions

Lipidation has proven itself as a valuable method to increase the activity of AMPs, or to make otherwise inactive peptides more potent. However, as it generally increases the affinity of the peptide for any biological membrane, the specificity for bacterial membranes might be lost. To counter this, a detailed analysis of the tedious interplay between the peptide component and the lipid is required in order to identify a "sweet spot" in a library of lipoAMPs in which both components are systematically altered. Although a lot is known about the effect of simple lipids on rather simple peptides,

much less is clear when it comes to lipoAMPs that are more complex in either or both components. Since large differences in activity can be observed when more complex peptides are tethered to a variety of lipids, as was recently shown for a nisin fragment (Koopmans et al. 2015), much can likely be gained by attaching lipids with higher complexity to more complex peptides. Along similar lines, it would be very interesting to find out what happens if lipids that have chemical functionalities like redox properties or conjugated unsaturated carbon atoms are applied. It is quite clear that, aside from the basic fundamental observation that lipidation increases membrane affinity and allows for selective targeting of the bacterial membrane, we barely scratched the surface of what is possible with the interplay between lipid and peptide. Lastly, when it comes to the expected lower likelihood for the development of resistance against membrane-targeting antibiotics, it became already clear in the last decade that bacteria – when treated with membrane-targeting tetracycline and polymyxin – developed alterations in the fatty acid composition of their membrane (Dunnick and O'Leary 1970). In one of our studies, we also found that the application of AMPs resulted in the upregulated synthesis of branched lipophilic amino acid Val, which suggested alteration of the membrane lipids with more branched fatty acids, which would result in more rigid membranes.

4 Research Needs

Despite the efforts that have been undertaken so far, it is safe to say that the potential of lipidation to boost the antibacterial properties of AMPs has not been fully explored. In order to provide some guidelines for future studies, the following three points deserve attention in future endeavors.

- We need to obtain a better understanding of the effect of lipidation on the formation of lipid domains (also called lipid rafts) in the bacterial membrane. Evidence suggests that negatively charged domains can be formed by clustering of negatively charged lipids around polycationic substances (Epand and Epand 2009). What the effect of this clustering is on the other sections of the membrane (where a more positive charge would be found), and on the localization of the embedded biomolecules remains to be determined. For example, do the embedded biomolecule travel with the lipid rafts, or are they left behind? And if that is the case, what is the effect on the biological function on those biomolecules. Lastly, and more importantly, can this effect be used to develop more effective antibacterial agents?
- Apart from the functionalization of AMPs with passive lipid anchors, it is also possible to attach functional lipid anchors. For example, the lipophilic ferrocenoyl moiety (an organometallic complex) can, in principle, release an electron in the bacterial membrane. Since this is the place where respiration resides, injecting electrons in the membrane-bound electron transfer chain might well result in dysfunctional respiration. However, such approaches are rarely studied. Even more, for the mentioned ferrocenoyl derivative, it was shown that its

enhanced antibacterial activity was only due to its lipophilic character, and not associated in any way with its redox chemistry. This does not mean, however, that inserting functional lipid anchors into the bacterial membrane does not have a place for future antibacterial agents.

- We need to establish correlation between lipidation, AMPs length, and chirality of the amino acids (especially for linear AMPs). We have shown that the combination of lipidation and enantiomeric peptides leads to surprising activities. In one study, we found significant reduction of the hemolytic properties of lipoAMPs (Albada et al. 2013), whereas in another study we found significant increase in the antibacterial properties (Albada et al. 2014). At the moment, we do not understand why. Although the lipids that constitute the membrane are often regarded as achiral moieties, they in fact are not. Not only does each lipid contain at least one chiral carbon atom, they also contain at least one chiral phosphorous atom (see Fig. 4). It could very well be possible that this chirality can be utilized to obtain more active lipoAMPs. However, for this to occur, a more focused study on the structure–activity relation of lipidation and the chirality of the amino acids of the lipoAMP is required to gain this valuable information that might well lead to more potent antibacterial agents that breach the MIC = 1 μM activity that is now associated with the most active AMPs.

Another point that can be considered is the following. Maybe, it would be beneficial to establish an international facility where antimicrobial activity can be tested under identical conditions. Now, antibacterial compounds are tested in different labs; the inclusion of known compounds with known activities is used as external standards. However, the results so far indicate that this might not be one-on-one translatable to AMPs, as their mode of action relies on membrane interaction and not with a specific biological target. Therefore, it is very important to use the appropriate controls, and to specify in great detail the conditions of the activity assay (Jaskiewicz et al. 2017). Alternatively, an international facility where the activities of lipoAMPs are tested under identical and standardized conditions may be needed.

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Antimicrobial Activity of Essential Oils

14

Marta Ribeiro and Manuel Simões

Contents

Abstract

The rise of multidrug-resistant (MDR) pathogens subsequent to antibiotic use, associated with increased mortality and a significant economic problem, has prompted researchers to exploit novel compounds that are simultaneously effective and safe and with the capacity to control the emergence of MDR microorganisms. The study of natural plant-derived products has been a successful approach for the discovery of new therapeutics. Essential oils (EOs) are a complex mixture of hydrocarbons and oxygenated compounds, in which the active compounds of these EOs can be terpenes (mainly monoterpenes and sesquiterpenes), terpenoids, or phenylpropenes. EOs are recognized for their

M. Ribeiro · M. Simões (✉)
LEPABE – Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, Faculdade de Engenharia da Universidade do Porto, Porto, Portugal
e-mail: ribeiro_marta88@hotmail.com; mvs@fe.up.pt

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bioactive properties, including their antimicrobial abilities. In this chapter, EOs are described from their chemical properties, extraction techniques from plants, and applications. Mechanisms of action and biochemical and molecular targets of EOs in bacteria, fungi, and virus are also discussed. Besides the well-documented antimicrobial activity of EOs, other significant biological properties of these compounds including anti-inflammatory, antioxidant, and anticancer, which make them promising agents in the treatment of different diseases, are described.

1 Introduction

The emergence of multidrug-resistant (MDR) pathogens is one of the most serious threats to successful treatment of microbial infections caused by these MDR microorganisms. They are associated with increased mortality compared to those caused by susceptible microorganisms, carrying an important economic burden (Hemaiswarya et al. 2008; Munita and Arias 2016; Rudramurthy et al. 2016). In fact, the antibiotic resistance issue has been identified by the World Health Organization as one of the three most important public health treats of the twenty-first century (Munita and Arias 2016). Besides that, during the past 50 years, only a few new classes of antibiotics have been developed, showing that the world's capacity for antibiotic discovery is already falling behind the rate of emergence of bacterial resistance (Lewis 2013). The identification of new chemical compounds that are simultaneously effective and nontoxic is one of the reasons behind this decline (Kalan and Wright 2011). However, the use of synthetic chemicals for the control of pathogenic microorganisms is continuously associated with carcinogenic effects, acute toxicity, and environmental hazard potential (Akthar et al. 2014). In this regard, the interest in natural therapies and increasing demand for the use of effective and safe products has prompted researchers to exploit new natural compounds to control and overcome the prevalence and spread of MDR pathogenics.

Medicinal and aromatic plants constitute a major source of natural organic compounds. The medicinal properties of plants have received a great interest because of their low toxicity, pharmacological activities, and economic viability (Auddy et al. 2003). Most plants produce antimicrobial secondary metabolites, also referred to as natural products or phytochemicals, either as part of their normal growth and development or in response to pathogen attack or stress conditions (Bourgaud et al. 2001; Kliebenstein and Osbourn 2012; Malheiro et al. 2016). The proven effectiveness of numerous phytochemicals against microorganisms has been reported, making them a rich source of antimicrobial agents (Simões et al. 2009; Abreu et al. 2012; Malheiro et al. 2016; Ribeiro et al. 2018). Besides that, an important benefit of using plant-derived antimicrobials is that they do not exhibit the side effects associated with the use of synthetic chemicals (Upadhyay et al. 2014). These natural products are structurally diverse, and many are distributed

among a very limited number of species within the plant kingdom, many of which accumulate in surprisingly high concentrations in some species (Bourgaud et al. 2001; Kliebenstein and Osbourn 2012; Gyawali and Ibrahim 2014).

As natural products, essential oils (EOs) are among the most valuable products used in medicine and complementary treatment approaches. The exploration of EOs and their constituents has gained a renewed interest presenting broad well-documented antimicrobial activity (antibacterial, antifungal, antiviral) (Tullio et al. 2007; Solorzano-Santos and Miranda-Novales 2012; Gilling et al. 2014) and other biological properties including anti-inflammatory, antioxidant, and anticancer activities (Sharma et al. 2009; Miguel 2010; Serrano et al. 2011). This chapter starts with a briefly description on chemical aspects of EOs and their constituents, methods for their extraction, and lastly their antimicrobial action as well as other bioactive properties.

2 Essential Oils and Their Constituents

EOs are complex mixtures of natural, volatile, and aromatic compounds obtained from plant material such as buds, flowers, leaves, seeds, twigs, bark, herbs, wood, fruit, and roots. The chemistry of EOs is affected by many factors such as plant ecotype or variety, genetic variation, plant nutrition, geographic location of the plants, surrounding climate, seasonal variations, stress during growth or maturity, application of fertilizers, and the post-harvest drying and storage. Additionally, the type of plant material used and the extraction method determine the yield and composition (constituents) of an essential oil (Bakkali et al. 2008; Nerio et al. 2010; Raut and Karuppayil 2014). Due to their hydrophobic nature and their density frequently lower than that of water, they are generally lipophilic, soluble in organic solvents, and immiscible with water (Asbahani et al. 2015). Around 3000 EOs have been produced by using at least 2000 plant species, out of which 300 are commercially available in the food, agronomic, cosmetic, perfume, and pharmaceutical industries (Bakkali et al. 2008; Nerio et al. 2010; Raut and Karuppayil 2014). EOs have been also used against nosocomial infections, as disinfectants of medical equipment and surfaces (Warnke et al. 2009; Asbahani et al. 2015) or as an aerosol in operating suites and waiting rooms for air disinfection (Asbahani et al. 2015). Therefore, most EOs are recognized as safe by the US Food and Drug Administration (Nerio et al. 2010).

EOs are constituted by hydrocarbons including terpenes and oxygenated compounds such as alcohols, aldehydes, ethers, esters, ketones, lactones, and phenols (Cowan 1999; Bakkali et al. 2008; Nerio et al. 2010; Barbieri et al. 2017). Then, the active compounds of EOs can be divided into different groups according to theirchemical structure: terpenes, terpenoids, and phenylpropenes (Table 1) (Hyldgaard et al. 2012; Asbahani et al. 2015). Terpenes are hydrocarbons produced from the combination of several isoprene units. Terpenes contain a hydrocarbon backbone that can be rearranged into cyclic structures by cyclases, thus forming monocyclic or bicyclic structures (Hyldgaard et al. 2012; Nazzaro et al. 2013).

Table 1 EOs groups according to their chemical structure and the most well-known compounds of each group

EOs group		Compounds	References
Terpenes	Monoterpenes ($C_{10}H_{16}$)	Sabinene, limonene, *p*-cymene, α-pinene, *y*-terpinene	Hyldgaard et al. 2012; Nazzaro et al. 2013; Pandey et al. 2016
	Sesquiterpenes ($C_{15}H_{24}$)	β-Caryophyllene, α-bisabolol, valerenic acid	Asbahani et al. 2015; Pandey et al. 2016
Terpenoids		Carvacrol, thymol, linalool, menthol, geraniol, linalyl acetate, citronellal, piperitone	Hyldgaard et al. 2012; Nazzaro et al. 2013; Pandey et al. 2016
Phenylpropenes		Eugenol, isoeugenol, vanillin, safrole, cinnamaldehyde	Hyldgaard et al. 2012; Nazzaro et al. 2013; Pandey et al. 2016

The main terpenes are the monoterpenes and sesquiterpenes, but hemiterpenes, diterpenes, triterpenes, and tetraterpenes also exist in EOs at low concentration. Monoterpenes consist of two isoprene units, being the most representative molecules constituting 90% of EOs extracted from many plants (Bakkali et al. 2008; Hyldgaard et al. 2012; Nazzaro et al. 2013; Tongnuanchan and Benjakul 2014). Although sesquiterpenes are larger molecules, their structure and functional properties are similar to monoterpenes (Ruberto and Baratta 2000). The most well-known terpenes are *p*-cymene, limonene, terpinene, sabinene, and pinene (Hyldgaard et al. 2012; Nazzaro et al. 2013).

Terpenoids are terpenes with added oxygen molecules or that have had their methyl groups moved or removed by specific enzymes. They are derived from five-carbon isoprene units, and most terpenoids have multi-cyclic structures that differ from one another in their functional groups and basic carbon skeletons (Hyldgaard et al. 2012; Nazzaro et al. 2013). Terpenoids are a large group of antimicrobials active against a broad spectrum of microorganisms, with carvacrol, thymol, linalool, menthol, geraniol, linalyl acetate, citronellal, and piperitone being the most common and well-known terpenoids (Hyldgaard et al. 2012; Nazzaro et al. 2013). The most active monoterpenoids identified so far have been carvacrol and thymol (Dorman and Deans 2000; Hyldgaard et al. 2012).

Phenylpropenes contain a six-carbon aromatic phenol group and a three-carbon propene tail from cinnamic acid, which is produced in the first step of phenylpropanoid biosynthesis. These compounds constitute a relatively small part of EOs, and those that have been most thoroughly studied are eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde (Hyldgaard et al. 2012; Nazzaro et al. 2013).

3 Extraction Techniques

Essential oils can be extracted from numerous plants using several methods, particularly distillation, solvent, and solvent-free microwave extraction. Among all methods steam distillation has been the most commonly used method, particularly

for commercial scale production (Cassel and Vargas 2006; Di Leo Lira et al. 2009; Solorzano-Santos and Miranda-Novales 2012; Tongnuanchan and Benjakul 2014). The extraction method is one of main factors determining the quality of EOs, in which an inappropriate extraction process can lead to the damage or alteration of the chemical signature of EOs (Tongnuanchan and Benjakul 2014). Besides that, the extraction time, temperature, number of repeated extractions of the sample, and the choice of extraction solvents are crucial factors influencing the extraction yield (Brglez Mojzer et al. 2016).

3.1 Distillation

Steam distillation. Steam distillation is the most widely used method for plant EOs extraction based on the scheme of Fig. 1 (Tongnuanchan and Benjakul 2014). In this technique heat is applied, and steam is forced over the plant material, being the main cause of burst and breakdown of plant material structure. The hot steam helps to release the aromatic compounds or EOs from plant material since the steam forces open the pockets in which the oils are kept in the plant material. The compounds of these volatile oils are then released from the plant material, evaporated into steam, and passed through a cooling system to condense the steam, producing liquid from which the essential oil and water are separated (Helena and Aleksovski 2006; Roldan-Gutierrez et al. 2008; Tongnuanchan and Benjakul 2014; El Asbahani et al. 2015). In this method the steam from boiling water is passed through the raw material for times ranging from 60 minutes to many hours, which drives out most of its volatile fragrant compounds (Roldan-Gutierrez et al. 2008).

Hydrodistillation. Hydrodistillation method is based on the same principles as steam distillation, and it is often used to isolate nonwater-soluble compounds with high boiling point. In this process the difference is that the plant material is immersed directly in boiling water (Helena and Aleksovski 2006; Tongnuanchan and Benjakul 2014; Asbahani et al. 2015). This method protects the oils extracted to a certain degree since the surrounding water acts as a barrier to prevent it from overheating (Helena and Aleksovski 2006; Tongnuanchan and Benjakul 2014).

Hydrodiffusion. Hydrodiffusion method is a type of steam distillation, which is only different in the inlet way of steam, which occurs downward (Tongnuanchan and Benjakul 2014; Asbahani et al. 2015). This technique is particularly used when the plant material has been dried and it is not damaged at boiling temperature (Tongnuanchan and Benjakul 2014).

3.2 Solvent Extraction

Organic solvent extraction. This method has been applied mainly for fragile or delicate plant materials, which are not tolerant to heat. Several solvents have been used for extraction such as ethanol, methanol, acetone, or hexane (Durling et al. 2007; Tongnuanchan and Benjakul 2014; Brglez Mojzer et al. 2016). During this process, the solvent is mixed with the plant material and then heated to extract the

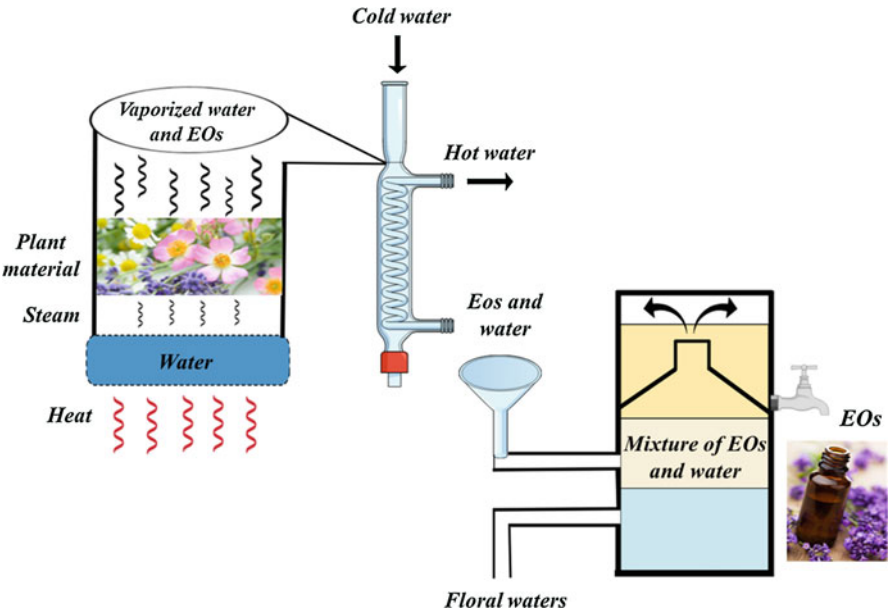

Fig. 1 Schematic illustration of steam distillation method. In this process heat is applied, and steam is forced over the plant material, which is the main cause of burst and breakdown of plant material structure. Then, the hot steam helps to release the aromatic compounds or EOs from plant material, which are evaporated into steam and pass through a cooling system to condense the steam, producing liquid from which the essential oil and water are separated (Roldan-Gutierrez et al. 2008; Tongnuanchan and Benjakul 2014; El Asbahani et al. 2015)

EOs, followed by filtration. Afterwards, the filtrate is concentrated by solvent evaporation, which it is then mixed with pure alcohol to extract the essential oil and distilled at low temperatures. Subsequently, the alcohol with the fragrance is evaporated and the aromatic oil remains (Tongnuanchan and Benjakul 2014; Brglez Mojzer et al. 2016).

Supercritical carbon dioxide. Supercritical fluid extraction is based on the use of supercritical fluids, which above their critical point present liquid-like as well as gas-like properties (Capuzzo et al. 2013; Asbahani et al. 2015). Carbon dioxide (CO_2) is the most used supercritical fluid since it is odorless, highly pure, safe, cost-effective, nontoxic, non-flammable, and recyclable gas allowing supercritical operation at relatively low pressures and near room temperature. Consequently, under high-pressure conditions, CO_2 turns into liquid being used as very inert and safe medium to extract the compounds from plant material (Glišić et al. 2007; Chan and Ismail 2009; Capuzzo et al. 2013; Tongnuanchan and Benjakul 2014). During the extraction procedure, the low viscosity of CO_2 enables it to penetrate the matrix to reach the plant material being extracted. Subsequently, the low latent heat of evaporation and high volatility of CO_2 allow its easy removal without leaving a solvent residue in the final product (Khajeh et al. 2005; da Silva et al. 2016).

3.3 Solvent-Free Microwave Extraction

Conventional methods of extraction such as steam distillation, hydrodistillation, or solvent extraction have several drawbacks. Long extraction time, chemical changes in the essential oil compounds due to the high temperatures, losses of the volatile compounds, and toxic solvent residues in the final product are some of the disadvantages of conventional methods (Roldan-Gutierrez et al. 2008; Zhang et al. 2012; Mohamadi et al. 2013; Tongnuanchan and Benjakul 2014; Asbahani et al. 2015; Brglez Mojzer et al. 2016).

Solvent-free microwave extraction is a rapid extraction technique of essential oils from aromatic herbs, spices, and dry seeds presenting numerous advantages, including short extraction time, higher yield, and selectivity, and is environmentally friendly (Farhat et al. 2010; Périno-Issartier et al. 2013; Tongnuanchan and Benjakul 2014). This technique combines microwave heating with dry distillation, performed at atmospheric pressure, for the isolation and concentration of EOs in fresh plant materials (Bayramoglu et al. 2008; Périno-Issartier et al. 2013; Tongnuanchan and Benjakul 2014; Asbahani et al. 2015). As shown in Fig. 2, in this method the plant material is placed in a microwave reactor, without water or any organic solvent.

The advantages and disadvantages of EO extraction methods are presented in Table 2.

4 Essential Oils as Antimicrobials

The research about antimicrobial potential of EOs and their mode of action has received prominence in recent decades in parallel with advances in traditional approaches for protecting the health of humans and food against pathogenic and spoilage microorganisms (Calo et al. 2015). Several in vitro studies have reported the antimicrobial properties of EOs against bacteria (Burt 2004; Lv et al. 2011; Solorzano-Santos and Miranda-Novales 2012; Zhang et al. 2016), fungi (Pinto et al. 2007; Tullio et al. 2007; Tian et al. 2011), yeasts (Souza et al. 2007; Tserennadmid et al. 2011), and viruses (Romeilah et al. 2010; Gilling et al. 2014).

It is important to keep in mind that EOs are composed of numerous compounds, and, therefore, their antimicrobial activity is dependent on the amount of each single compound as well as on the chemical diversity (Nazzaro et al. 2013). Different amounts of specific compounds can affect the antimicrobial activity of EOs since their antimicrobial effect could be the result of a synergism of all compounds or due only those of the main compounds present at higher amount (Bakkali et al. 2008; Nazzaro et al. 2013; Calo et al. 2015). Furthermore, because of the great number of constituents, EOs seem to have no specific cellular targets (Bakkali et al. 2008; Nazzaro et al. 2013).

Several researchers have proposed that, in most of the cases, EOs confer antibacterial activity by disrupting the cell wall and cytoplasmic membrane, leading to lysis and leakage of intracellular compounds (Burt 2004; Trombetta et al. 2005;

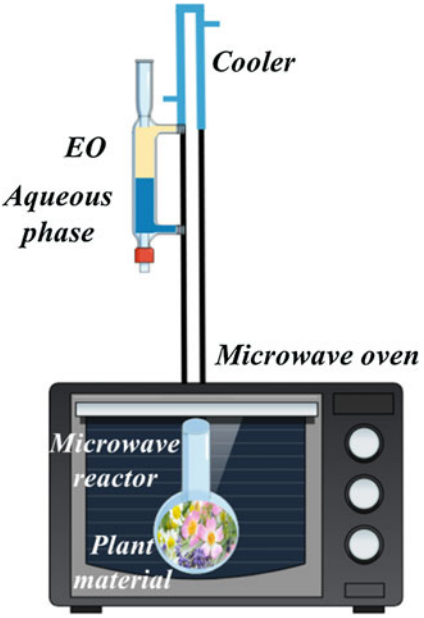

Fig. 2 Schematic illustration of solvent-free microwave extraction. This method is based on the combination of microwave heating and dry distillation at atmospheric pressure of a fresh plant material, which is placed in a microwave reactor, without water or any organic solvent. The internal heating of the in situ water content of plant material distends the plant cells and leads to rupture of the glands and oleiferous receptacles. This process releases EOs, which are evaporated by in situ water of the plant material. A cooling system outside the microwave oven condenses the distillate continuously. The excess of water is refluxed to the extraction vessel in order to restore in situ water to the plant material (Bayramoglu et al. 2008; Li et al. 2013; Périno-Issartier et al. 2013; Tongnuanchan and Benjakul 2014; Asbahani et al. 2015; Kusuma et al. 2016)

Bakkali et al. 2008). For example, Lambert et al. (2001) showed that oregano essential oil inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* by altering cell membrane permeability with consequent leakage of protons, potassium, and phosphates (Lambert et al. 2001). Likewise, citronellol, citronellal, carveol, and carvone essential oils were shown to modify hydrophobicity and disrupt membrane integrity of *S. aureus* and *E. coli*, leading to the leakage of potassium ions (Lopez-Romero et al. 2015). Additionally, some studies have reported that the chemical structure, such as the presence and location of the functional groups in the compound, and the aromaticity can affect its antimicrobial behavior (Dorman and Deans 2000; Nazzaro et al. 2013). Generally, phenolic compounds, having the hydroxyl group attached to a phenyl ring, have the greatest activity among the phytochemicals found in EOs (Dorman and Deans 2000; Lambert et al. 2001). In a work performed by Ultee et al. (2002), they tested the antibacterial activity of different compounds against *Bacillus cereus*, all with structures comparable to that of carvacrol (e.g., thymol, cymene, menthol, and carvacrol methyl ester), and showed that the hydroxyl group of carvacrol was important for the

Table 2 Different extraction techniques of EOs and the advantages and disadvantages of each extraction method

Extraction method	Advantages	Disadvantages	References
Steam distillation	Simplicity of installations, not requiring expensive equipment Large-scale oil production Absence of toxic residues in the extracted oils	Long extraction time High temperatures used during the long extraction periods can cause chemical modification of the oil compounds	Roldan-Gutierrez et al. 2008; Mohamadi et al. 2013; Tongnuanchan and Benjakul 2014; Asbahani et al. 2015
Hydrodistillation	Simplicity of installations, not requiring expensive equipment Selectivity Absence of toxic residues in the extracted oils	Long extraction time High temperatures used during the long extraction periods can cause chemical modification of the oil compounds Loss of some polar molecules in the water extraction	Mohamadi et al. 2013; Tongnuanchan and Benjakul 2014; Asbahani et al. 2015
Hydrodiffusion	Short extraction time Absence of toxic residues in the extracted oils	Loss of some volatile compounds Low extraction efficiency	Tongnuanchan and Benjakul 2014; El Asbahani et al. 2015
Organic solvent extraction	Useful for heat-sensitive compounds	Long extraction time Loss of some volatile compounds Low extraction efficiency Toxic solvent residues in the extracted oils High cost	Tongnuanchan and Benjakul 2014; Brglez Mojzer et al. 2016
Supercritical carbon dioxide	Short extraction time High extraction efficiency Absence of toxic residues in the extracted oils Low working temperatures avoid the loss and degradation of some volatile compounds	High cost Less effective in the extraction of polar compounds due to the low polarity of CO_2	Bhattacharjee et al. 2007; Glišić et al. 2007; Capuzzo et al. 2013; Asbahani et al. 2015; da Silva et al. 2016
Solvent-free microwave	Short extraction time Higher yield and selectivity Environmental friendly Cost-effective	Thermal degradation of some compounds	Farhat et al. 2010; Périno-Issartier et al. 2013; Tongnuanchan and Benjakul 2014

antibacterial action of this compound. For instance, the methyl ester, containing a methyl ester instead of a hydroxyl group, and cymene, without a hydroxyl group, did not inhibit the growth of *B. cereus*. On the contrary, the compound thymol possessing a hydroxyl group presented an antibacterial activity comparable to that of carvacrol (Ultee et al. 2002). Antibacterial activity of different EOs against multidrug-resistant (MDR) bacterial strains has also been reported for *S. aureus* (Espina et al. 2015), *S. epidermidis* (Nostro et al. 2004), *E. coli* (Si et al. 2008), *P. aeruginosa* (El-Shouny et al. 2018), *Acinetobacter baumannii* (Alanís-Garza et al. 2018), and *Klebsiella pneumoniae* (Khan et al. 2009).

The antifungal action of EOs is quite similar to that found for bacteria. EOs have the capacity to penetrate and disrupt fungal cell wall and cytoplasmic membranes through permeabilization and, consequently, disintegration of the mitochondrial membranes (Aleksic and Knezevic 2014). In fact, in recent years, there has been a significant increase in the incidence of invasive fungal infections. *Candida* and *Aspergillus* are fungal genera that have a major role in human pathology and the most common pathogens related with these invasive infections, presenting high mortality rates and, consequently, assuming an essential role in invasive fungal infections worldwide (De Pascale and Tumbarello 2015; Wójtowicz and Bochud 2015; Goncalves et al. 2016; Sanglard 2016). In addition, the emergence of *Candida* and *Aspergillus* resistance to antifungal agents has been reported in the literature (Ben-Ami et al. 2011; Arendrup 2014; Maubon et al. 2014; Fuhren et al. 2015; Sanguinetti et al. 2015; Verweij et al. 2016; Garcia-Rubio et al. 2017). Therefore, EOs could have an important role for the development and implementation of therapeutic antimicrobial approaches, even against pathogens that have developed resistance to conventional antimicrobial agents. In a work performed by Pinto et al. (2009), they evaluated the antifungal activity of clove essential oil and its main component, eugenol, against *Aspergillus*, *Candida*, and dermatophyte clinical and ATCC strains. They observed a large spectrum of action of clove oil and eugenol against all the tested strains, including *Aspergillus*, *Candida*, and dermatophyte, and an impairment in ergosterol biosynthesis, a specific fungal cell membrane component that has an essential role in preserving the integrity and function of the cell membrane. As a consequence of the inhibition of ergosterol biosynthesis, the cytoplasmic membrane was disrupted (Pinto et al. 2009). In another study Ahmad et al. (2011) investigated the antifungal effect of the *Coriaria nepalensis* essential oil against various fluconazole-sensitive and fluconazole-resistant *Candida* isolates. They found a strong antifungal activity of this essential oil against all the *Candida* isolates including the resistant strains, attributed to the inhibition of ergosterol biosynthesis and consequent disruption of the membrane integrity (Ahmad et al. 2011). Numerous other studies have shown the antifungal activity of different EOs against several fungi (Jantan et al. 2008; Dambolena et al. 2010; Khan and Ahmad 2011; Lang and Buchbauer 2012; Stević et al. 2014; Souza et al. 2016).

In the case of viruses, antiviral mechanism can be either through inhibition of specific processes in the viral replication cycle, so that little or no viral progeny is produced, or interference with viral components, which are necessary for adsorption or entry into host cells and cell-to-cell virus diffusion (Astani et al. 2010, 2011;

Aleksic and Knezevic 2014). In a study performed by Reichling et al. (2009), they found that EOs interfered with the virus envelope by masking viral components, which are necessary for adsorption or entry into host cells. For instance, eugenol, the main component of clove oil, was shown to be very effective in vitro against HSV-1 and HSV-2, two antigenic types of herpes simplex virus (HSV) (Tragoolpua and Jatisatienr 2007). This virus is a crucial pathogen for humans, infecting and replicating in cells at the site of entry, the mucocutaneous surface. After the acute ganglionic infection subsides, HSV establishes latency and persists in the neurons for the lifetime (Astani et al. 2010, 2011). In another work, Gilling et al. (2014) evaluated the antiviral efficacy of oregano oil and its primary active compound, carvacrol, against the nonenveloped murine norovirus, a human norovirus surrogate (Gilling et al. 2014). Human noroviruses (NoVs) are recognized as the major global cause of viral gastroenteritis and a main contributor to foodborne illness, spreading easily and by multiple routes including by water, food, and airborne routes, as well as incidental hand contact with contaminated surfaces and through person-to-person contact (Said et al. 2008; Belliot et al. 2014). Furthermore, NoVs have a protein capsid that is very resistant to lipophilic disinfectants, such as quaternary ammonium compounds and solvents, such as alcohols, protecting the integrity of the viral RNA and initiating the infection by adsorbing to the host cell, and are thus quite resistant to these antimicrobials (Said et al. 2008; Cliver 2009). Gilling et al. (2014) observed that carvacrol was effective in inactivating nonenveloped murine norovirus by acting directly on the viral capsid and subsequently the RNA, showing the potential of a natural essential oil to control human NoVs.

5 Essential Oils and Other Biological Properties

EOs have been also reported to possess a wide variety of effects on health and, consequently, an increased interest in looking other biological properties of EOs including anti-inflammatory (Miguel 2010), antioxidant (Miguel 2010; Serrano et al. 2011), and anticancer properties (Edris 2009), making them promising agents for different therapeutic purposes.

5.1 Anti-inflammatory Properties

Inflammation is a response induced by tissue injury or infection to fight aggressors in the body such as microorganisms and non-self-cells and to eradicate damaged or dead host cells (Miguel 2010; Azab et al. 2016). Chronic inflammation is viewed as an essential baseline reaction responsible for manifestations of several chronic human disorders like atherosclerosis, arthritis, diabetes, autoimmune, Alzheimer's, and cancer diseases, being a key factor associated with tumor progression (Medzhitov 2008; Sun et al. 2014; Hossen et al. 2017). Several anti-inflammatory drugs are currently available to prevent or diminish the progression of inflammation

like nonsteroidal anti-inflammatory drugs including ibuprofen, acetyl salicylic acid and diclofenac, and corticosteroids (Pérez et al. 2011). However, associated with their use, there is a number of side effects such as development of ulcers in the stomach, inhibition of uterine motility, hypersensitive reaction, breathing problems, hyperglycemia, suppression of the function of pituitary-adrenal, and increased susceptibility to infections, among others (Perrone et al. 2003; Hippisley-Cox and Coupland 2005; Pérez et al. 2011; de Santana Souza et al. 2014). EOs derived from numerous plants and their constituents have been reported to possess anti-inflammatory activities (Chao et al. 2008; Koh et al. 2013; De Lima et al. 2014; Gholamnezhad et al. 2015). In an in vivo study, Boukhatem et al. (2014) evaluated the topical and anti-inflammatory effects, assessed using croton oil-induced ear edema and carrageenan-induced paw edema in mice, respectively, of lemongrass essential oil. Their oral administration presented dose-dependent anti-inflammatory action, while in the topical application, the ear edema was significantly reduced by prior topical treatment with this essential oil compared to the control, showing the inhibition of the skin inflammatory response in animal models (Boukhatem et al. 2014).

5.2 Antioxidant Properties

Antioxidant activity has also been intensively studied in essential oil research because oxidation damages several biological substances and, subsequently, causes many diseases including cancer (Paz-Elizur et al. 2008), liver disease (Li et al. 2015), Alzheimer's disease (Moreira et al. 2005), diabetes (Jain 2006), Parkinson's disease (Beal 2003), and AIDS (Sepulveda and Watson 2002). Therefore, various diseases have been treated with antioxidants to prevent oxidative damage (Moon and Shibamoto 2009). Various researchers have been investigating the antioxidant activity of different EOs and their constituents as safe natural antioxidants for the replacement of synthetic ones. Consequently, several studies have shown that EOs are ideal natural sources of antioxidants (Erkan et al. 2008; Fasseas et al. 2008; Viuda-Martos et al. 2010; Özcan and Arslan 2011; Teixeira et al. 2013; Bączek et al. 2017). In a recent study developed by Takayama et al. (2016), they evaluated the antioxidant activity of the EOs obtained from *Rosmarinus officinalis* in a gastric ulcer model in vivo, using absolute ethanol to produce severe hemorrhagic lesions in the stomach. They observed that rats pretreated with *R. officinalis* presented a significant inhibition of mucosal injury, probably by the modulation of the activity of the enzymes superoxide dismutase and glutathione peroxidase and the increase or maintenance of the glutathione levels (Takayama et al. 2016). In another study performed by Arigesavan and Sudhandiran (2015), the antioxidant activity of carvacrol on colitis-associated colon cancer induced by 1,2 dimethylhydrazine (DHM) and dextran sodium sulfate (DSS) in a rat model was evaluated. In carvacrol treated rats, they observed an increased antioxidant status and restoration of histological lesions in the inflamed colonic mucosa, which was confirmed by the decrease of free-radical accumulation and suppression of expression of pro-

inflammatory mediators. They also observed an increase of antioxidant enzyme levels including catalase, superoxide dismutase, and glutathione and a decrease of lipid peroxides, nitric oxide, and myeloperoxidase, by carvacrol compared to DMH/DSS induced rats (Arigesavan and Sudhandiran 2015).

5.3 Anticancer Properties

Cancer is characterized by uncontrolled growth of cells, invasion and, in the worst case, metastasis, and it belongs to a huge class of diseases causing more than 10% of all human deaths (Adorjan and Buchbauer 2010). Existing cancer chemotherapy treatments are based mostly on highly cytotoxic drugs that target proliferating cell populations, which due to their lack of selectivity against cancer cells lead to critical side effects in normal cells (Khazir et al. 2014). EOs from different plants have been reported to possess important anticancer properties when tested on a number of cell lines (de Sousa et al. 2004; Cragg and Newman 2005; Manosroi et al. 2006; Edris 2009; Zu et al. 2010; Kathirvel and Ravi 2012; Jayaprakasha et al. 2013), being expected to induce lower side effects compared to synthetic drugs. Also, EOs are composed of several constituents, and, in some cases, their activity is dependent of their individual compounds. For instance, an in vivo work developed by Jayakumar et al. (2012) evaluated the chemopreventive nature of carvacrol during diethylnitrosamine (DEN)-induced liver cancer in rats, and they observed a potent anticancer effect in liver cancer by this compound. The outcomes of this work suggested the prevention of lipid peroxidation, hepatic cell damage, and protection of the antioxidant system in DEN-induced hepatocellular carcinogenesis by carvacrol (Jayakumar et al. 2012). The efficacy of carvacrol has also been demonstrated on a number of cancer cell lines including oral squamous cell carcinoma (Dai et al. 2016), human cervical cancer cells (Mehdi et al. 2011), gastric cancer cells (Günes-Bayir et al. 2017), human colon cancer cells (Fan et al. 2015), human prostate cancer cells (Khan et al. 2017), and others. Another plant-derived compound, thymoquinone, which is the bioactive compound isolated from *Nigella sativa* oil, has indicated that it is a potential therapeutic anticancer agent (Khader and Eckl 2014). Its anticancer activity has been demonstrated against several cancer cell lines such as human ovarian cancer cells (Wilson-Simpson et al. 2007), human cervical squamous carcinoma cells (Ng et al. 2011), human colon cancer cells (Hsu et al. 2017), human breast cancer cells (Dastjerdi et al. 2016), and human prostate cancer cells (Kou et al. 2017). The anticancer potential of thymoquinone has also been showed in various in vivo animal models including ovarian cancer mice (Wilson et al. 2015), breast cancer mice (Woo et al. 2013), leukemic mice (Salim et al. 2014), gastric cancer mice (Zhu et al. 2016), and colorectal cancer mice (Gali-Muhtasib et al. 2008). Numerous other individual compounds with anticancer properties have been described in the literature like thymol (Kang et al. 2016; Aydin et al. 2017; De La Chapa et al. 2018), linalool (Ravizza et al. 2008; Gu et al. 2010), limonene (Vandresen et al. 2014; Yu et al. 2018), α-pinene (Chen et al. 2015), and eugenol (Ma et al. 2017), among others.

6 Conclusion

EOs extracted from aromatic plants worldwide have been considered an appealing source of bioactive compounds presenting antimicrobial activity, as well as other significant properties including anti-inflammatory, antioxidant, and anticancer. From the wide body of scientific reports reviewed in this chapter, EOs and their constituents seem to have great potential as new and innovative therapeutic agents in the attempt to overcome the spread of multidrug resistance, as well as significant side effects associated with the synthetic drugs currently used. Nevertheless, research on EOs as antimicrobial, anti-inflammatory, antioxidant, and anticancer therapeutic agents is still in early growth phase.

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Part VI

Gastrointestinal Tract

Gastrointestinal Tract: Fat Metabolism in the Colon

15

Lesley Hoyles and R. John Wallace

Contents

Abstract

Lipids in colonic digesta are derived from the undigested residue of dietary fat and partly from endogenous secretions and shedding of colonocytes. Generally, only a small proportion of dietary fat consumed by man reaches the large intestine. Studies in mice have shown that high-fat diets alter the composition and function of the gastrointestinal (GI) microbiota, though nonequivalent control diets confound these findings. While a western-style diet, high in fats and refined carbohydrates, is associated with obesity and detrimental health effects, the explicit effects of high-fat diets on the human gut microbiota are poorly understood. Some clinical conditions result in increased fluxes of lipids to the large intestine, and, increasingly, slimming drugs that inhibit pancreatic lipases or adsorbents, including fatty acid-adsorbing *Lactobacillus* spp., that enable fat to

L. Hoyles
Department of Bioscience, Nottingham Trent University, Nottingham, UK
e-mail: lesley.hoyles@ntu.ac.uk

R. J. Wallace (✉)
Rowett Institute, University of Aberdeen, Aberdeen, UK
e-mail: john.wallace@abdn.ac.uk

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bypass the small intestine also result in large quantities of dietary lipids reaching the colon. GI bacterial lipases and phospholipases release fatty acids and various glycerides that may then be metabolized further to form products that have implications for GI health. The bacterial species responsible for lipase activity in the human colon are poorly characterized.

1 Introduction

Lipids appearing in the colon and feces arise partly from dietary lipid that escapes digestion in the small intestine, partly from bacterial synthesis, and partly from the host. Of the fat that is ingested, the vast majority is known to be absorbed in the small intestine in healthy humans. More fats may reach the intestine in individuals who consume a high-fat diet, and certain disease conditions can increase the flow of undigested lipid to the intestine. Furthermore, some slimming drugs or additives effectively prevent absorption of fat from the small intestine, leaving more to be metabolized by the colonic microbiota. This chapter describes the metabolism of fats in the intestine and explores the health implications of increased fat metabolism in the intestine.

2 Dietary Fats in the Intestine

Thomson et al. (1997) reported that the average so-called Western adult consumes about 100 g of triacylglycerols (TAG) and 4–8 g of phospholipid each day. The efficiency of absorption in the small intestine is generally high (76–99%, depending on the TAG ingested; Mu and Posgaard 2005), but lack of lipase and absence of bile salts can reduce absorption. A study in mice demonstrated the small intestinal microbiota is required for murine lipid digestion and absorption to occur in the small intestine (Martinez-Guryn et al. 2018). The role of the small intestinal/proximal microbiota in human lipid digestion and absorption – beyond bile salt modifications – remains to be determined. Dietary medium-chain fatty acids (FAs) are absorbed completely from the upper gastrointestinal (GI) tract and do not appear in significant quantities in the large intestine (Newmark and Lupton 1990). Unsaturated FAs are much more readily absorbed than saturated FAs (Hashim and Babayan 1978). In addition, the specific distribution of FAs on TAG structures also influences their absorption (Bracco 1994). Therefore, the nature of the lipid entering the small intestine, and its absorption through the stomach wall, directly affects the amount and type of lipids entering the large intestine.

Much higher GI lipid loads (up to 134 g) are observed in individuals with certain clinical conditions, most notably those suffering from cystic fibrosis, or a pancreatic deficiency, or those who have undergone GI resectioning (Wiggins et al. 1969; Juste 2005). Orlistat is an anti-obesity drug that acts by inhibiting GI lipases, decreasing fat absorption by approximately 30%, and leading to increased fecal fat (Zhi et al.

2003). In vitro and in vivo, Orlistat has no significant effect on the composition of the human fecal microbiota (Hoyles 2009; Morales et al. 2016). An in vitro study looking at the effect of Orlistat on the human gut microbiota showed huge variation in the lipase potential of individuals' microbiotas and the effect of Orlistat on lipase activity (Hoyles 2009). Other treatments for obesity include the adsorption of fats onto matrixes that decrease absorption, such as chitosan (Pittler and Ernst 2005) and *Lactobacillus* spp. that lower fat absorption from the small intestine. The anti-obesity effect of a probiotic strain of *Lactobacillus gasseri* was suggested to be mediated by changes in the droplet size of fats in the intestine (Ogawa et al. 2014). High-throughput screening of GI microbes led to the isolation of a strain of *Lactobacillus reuteri* that adsorbed FAs and thus prevented their absorption; *L. reuteri* was successful in effecting weight loss in a human trial (Chung et al. 2016). Different species of *Lactobacillus* have different effects on body weight in rodents and man (Drissi et al. 2017). The obesity epidemic will inevitably lead to more use of these and similar treatments.

Commonly occurring side effects of increased fat entering the colon include bloating, GI discomfort, fecal incontinence and urgency, increased steatorrhea, oily spotting and increased defecation (Chanoine et al. 2005), and losses of fat-soluble vitamins (Rössner et al. 2000). The increased flow of dietary lipids entering the large intestine may also lead to an increase in fecal bile acid excretion, with its own health implications (▶ Chap. 17, "Gastrointestinal Tract: Microbial Metabolism of Steroids"). FAs released by lipolysis may also have a detergent effect that damages the mucosa, predisposing to tumor development (Newmark et al. 1984).

3 Endogenous Fat Sources

The host itself and its commensal bacteria contribute to the fats present in the GI tract. Host-derived FAs originate from bile, intestinal secretions, and desquamation of the GI epithelium (Chen et al. 1998). These lipids are rich in cholesterol and phospholipids. Phospholipids, predominantly phosphatidylcholine, from bile are absorbed in the small intestine after hydrolysis by pancreatic phospholipase A_2 (Phan and Tso 2001). However, phospholipids and sphingolipids from the dead cells of prokaryotes, short-chain FAs, and branched-chain FAs produced as metabolic by-products of fermentation by bacteria and cholesterol of endogenous or dietary origin make a substantial contribution to the amount and types of lipids and FAs present in the large intestine (Juste 2005). Chen et al. (1998) estimated between one quarter and a third of all the lipids excreted in human feces are of bacterial origin (Table 1). In contrast to eukaryotes, bacteria do not have reserves of TAG. Depending on their taxonomic group, bacteria have cell membranes and walls containing a mixture of C_{12} to C_{24} FAs: these contain a mixture of saturated FAs and unsaturated FAs that are *cis* or *trans*, cyclopropanes, hydroxy, or branched chain (Mackie et al. 1991). In addition to the even-numbered FAs, bacteria also possess many odd-numbered FAs. Therefore, the GI microbiota could be a substantial source of FAs, common and rare (Juste 2005).

Table 1 Calculation of the contribution of bacterial FAs to total fecal FAs

Diet	Weight of bacterial fraction (g)	Amount of bacterial fraction that is fat (g)[a]	Crude fat (g/day)	Contribution of bacterial fat to fecal fat (%)
Control	12.3 ± 1.8	1.2	4.1 ± 0.7	29.3
Wheat bran	14.4 ± 2.3	1.4	5.2 ± 0.6	26.9
Control	14.9 ± 2.6	1.5	4.1 ± 1.3	36.6
Oat bran	20.3 ± 3.4	2.0	7.5 ± 1.0	26.7

Data are from Chen et al. (1998); $n = 5$ men. Control data were collected during constant low-fiber diets, and other data were collected during the same diet into which wheat bran or oat bran was incorporated. It should also be noted that these authors demonstrated that consumption of wheat bran and, to a lesser extent, oat bran increased bacterial activity in the large intestine (and, thus, fecal bulking), which explains why higher values are given compared with the controls for the two diet groups

[a]Chen et al. (1998) assumed bacteria are 10–20% fat, taking the higher value for their calculations based on data presented by Czerkawski (1976). We have chosen to use 10% to calculate amounts of fecal fat contributed by bacteria as this is closer to values given by Neidhardt (1996) for fat content (dry weight) of *Escherichia coli*

4 Breakdown of Fats by the GI Microbiota: Lipase

The first step in the metabolism of TAG and its constituent FAs, glycerol, and glycerides is lipolysis. Lipase activity is recorded in the anaerobes handbook (Holdeman et al. 1977), where many species are derived from human feces. Very few are recorded as lipase positive. The same is true of ruminal bacteria, except for studies that have been carried out with the most lipolytic species yet isolated, *Anaerovibrio lipolytica* (Henderson 1971), which indicated that *A. lipolytica* had sufficient activity to be a major player in lipase activity of the mixed ruminal microbiota (Prins et al. 1975). No culture of *A. lipolytica* has been isolated from the human colon, and no 16S rRNA gene sequence derived from man corresponding to this species appears in databases, although it should be noted that less numerous species are often overlooked by random cloning methods. Approximately 4400 isolates were recovered from 15 healthy adult donors and screened on tributyrin agar for lipolytic activity: 596 isolates were esterase/lipase positive, representing between 0.3% and 23.4% of the total microbiota of the different donors (Hoyles 2009). Screening of isolates from five of the donors on rhodamine B/olive oil agar identified *Bifidobacterium*, *Collinsella*, *Bacteroides*, and *Blautia* spp. as having lipase activity (Hoyles 2009). Thorasin et al. (2015) confirmed that human *Collinsella* spp. had lipase activity, along with *Eggerthella lenta*. Available genome sequences of representatives of the aforementioned genera encode lipases, but none has been characterized biochemically. Whether the lipase activity encoded by these bacteria is active in vivo and/or contributes to host lipid metabolism is unknown, but results from the in vitro Orlistat study of Hoyles (2009) suggest that, at least in some donors, lipase activity may occur in the human large intestine.

We would identify the lack of knowledge about GI bacterial lipases as being an important deficiency in our understanding of the human colon, especially with fat passage to the intestine likely to be of increasing importance in coming years.

5 Breakdown of Fats by the GI Microbiota: Phospholipase

Intestinal bacteria possess phospholipase C activity, which produces 1,2-*sn*-diacylglycerols (DAGs) from phospholipid (Morotomi et al. 1990). DAGs are significant because they can act as a secondary messenger that specifically stimulates protein kinase C, an enzyme that plays a key role in signal transduction and growth control. Using synthetic DAG at concentrations similar to those found in feces, Friedman et al. (1989) were able to induce mitogenesis in colon adenoma and colon carcinoma cell cultures, consistent with the link between high-fat diets, the risk of colorectal cancer, and the suspicion that GI bacteria are somehow involved in the etiology of colorectal cancer.

Morotomi et al. (1990) investigated the production of DAG (and monoacylglycerol and free FAs) by fecal bacteria. They found that GI bacteria produced DAG and that this activity was markedly enhanced in the presence of the bile acids, cholic, chenodeoxycholic, and deoxycholic acids. Increasing the pH of the environment from 5 to 8 also increased the degradation of phosphatidylcholine. Bacteria capable of degrading phosphatidylcholine to DAG were subsequently identified and characterized, at least in terms of sequence data and DAG production, by Vulevic et al. (2004). Twelve strains that produced DAG at high levels were isolated from fermenters that had been inoculated with human feces. Eight were identified as *Paraclostridium* (formerly *Clostridium*) *bifermentans*, two were *Escherichia coli*, one was *Bifidobacterium infantis*, and one was an isolate that was closely related to an uncultured ruminal bacterium. Ten of the strains originated from vessels run at pH 8.5, while one of the *Paraclostridium bifermentans* and one of the *Escherichia coli* strains came from vessels run at pH 7.5, with strains originating from four of six donors. Those strains originating from the pH 7.5 vessels produced less DAG than the pH 8.5-derived strains. Phospholipase C activity in the 12 strains examined was not determined, so no conclusions can be drawn with regard to the mechanism of DAG production by these strains. In addition, only 12 of the 159 strains shown to produce DAG were characterized; consequently, we do not have a full picture of the bacteria involved in DAG production in vitro.

DAG concentrations in ten fecal samples from healthy donors were examined by Morotomi et al. (1990). These authors found that the DAG-producing activity of fecal bacteria and the absolute amount of fecal DAG varied considerably (>27-fold) among the individuals but that there was little variation (<fourfold) in samples taken from one donor over a period of 115 days. In addition, there was no correlation between an individual's DAG-producing activity and the absolute level of 1,2-*sn*-DAG in their feces, reflecting the fact that multiple factors (e.g., intake of specific dietary lipids, lipid-metabolizing activity of specific members of the GI microbiota, and levels of specific bile acids) may influence the steady-state levels of DAG. The

study of Vulevic et al. (2004) using batch culture fermentations (at pH 6.8, 7.5, and 8.5) to monitor DAG production by fecal bacteria also demonstrated inter- and intraindividual variation ($n = 6$ fecal donors; 2 male, 4 female) in DAG production.

6 Influence of Dietary Fats on the Gut Microbiome

It is known that the nature of the fat that reaches the large intestine will have a large influence on the effects that the fat has on the microbiome. Many of the effects probably derive directly from the physicochemical nature of fats and FAs, including changes in intestinal permeability, gut barrier function, and inflammation (Matsunaga et al. 2009; Bibbò et al. 2016), but others undoubtedly are mediated via the microbiota. Western-style diets high in saturated fats have the effect of lowering the diversity of the gut microbiota, which in turn may expose the host to increased risks of infection and metabolic disorders (Ley et al. 2005; Turnbaugh et al. 2006, 2009).

Much experimental evidence for health claims associated with obesity and the gut microbiota comes from rodent studies. Obesity in mice has been linked to changes in the gut microbiota, with a lower ratio of Bacteroidetes/Firmicutes proposed to be representative of an obese-type microbiota (Ley et al. 2005). In such animal studies, mice are made obese by being fed high-fat diets, and control groups are fed commonly used chow, introducing dietary confounders that will influence the gut microbiome (Dalby et al. 2017). Examining the effects of feeding a refined high-fat diet and two control diets (refined low-fat diet with nutritional composition matching the refined high-fat diet and typical chow diet) in mice demonstrated large changes in microbiota composition, GI fermentation, and gut morphology compared with the chow diet, with body weight and fat, and glucose intolerance only increasing in the mice fed the refined high-fat diet. Therefore, choice of control diet in rodent studies has a profound effect on interpretation of results, and the gut microbiota can be uncoupled from obesity and glucose tolerance in mice (Dalby et al. 2017). The relevance of the Bacteroidetes/Firmicutes ratio to obesity in humans cannot be generalized, and neither the ratio nor the abundance of Firmicutes is significantly associated with obesity in human studies (Sze and Schloss 2017). Obese individuals do have significantly less diverse microbiotas than nonobese individuals, but the biological significance of this difference is questionable (Sze and Schloss 2017). To what extent different dietary choices, and thus flows of fats to the colon, contribute to this difference remains unclear.

In mice, Li et al. (2017) found that fish oil, with a high content of unsaturated FAs, promoted a gut microbiota structure that was substantially different from those resulting from comparable soybean oil and lard supplementation in both in vitro and in vivo studies. The relative abundances of the phylum Proteobacteria and the genus *Desulfovibrio* in the cecal and colonic contents were the highest in the fish oil group. It is well established that polyunsaturated FAs have a stronger inhibitory effect on gut bacteria than saturated FAs (Lourenço et al. 2010), and it may be assumed that the same is true for human intestinal bacteria.

High-fat diets tend to promote a pro-inflammatory gut microbiota (Bibbò et al. 2016), yet fats reaching the intestine can be beneficial if certain FAs are formed (O'Shea et al. 2012). Health issues are described in more detail in chapter 29.

7 Research Needs

Our knowledge of bacterial fat metabolism in the human GI tract remains remarkably limited. However, interests in links between dietary fat, the gut microbiome, and obesity research may be expected to prompt interest in the processes involved. Fundamental research needs to be done to characterize the bacteria that carry out lipolysis in the GI tract. In practical terms, we need to know how to minimize the unhealthy and/or unpleasant consequences of drugs/additives that promote lipid bypass in the small intestine.

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Gastrointestinal Tract: Intestinal Fatty Acid Metabolism and Implications for Health

16

Lesley Hoyles and R. John Wallace

Contents

Abstract

Short-chain fatty acids (SCFA) are formed from the fermentation of sugars and complex carbohydrates by gastrointestinal (GI) bacteria in man. Acetate is the most abundant SCFA, with lower amounts of propionate and butyrate formed. Propionate and butyrate are also formed from the products of carbohydrate fermentation by other bacteria, for example, from lactate, succinate, and acetate. SCFA play a role in regulating transit of digesta through the GI tract, and in health by, for example, decreasing the risk of colon cancer (butyrate), and promoting satiety and reducing cholesterol load (propionate). Major butyrate-producing (*Roseburia* and *Faecalibacterium* spp.) and propionate-producing (*Negativicutes* and *Bacteroides* spp.) bacteria are among the most abundant microbes present in the large intestine. Metabolism of longer-chain fatty acids occurs mainly by hydration or hydrogenation of unsaturated fatty acids, the pathway depending

L. Hoyles (✉)
Department of Bioscience, Nottingham Trent University, Nottingham, UK
e-mail: lesley.hoyles@ntu.ac.uk

R. John Wallace
Rowett Institute, University of Aberdeen, Aberdeen, UK
e-mail: john.wallace@abdn.ac.uk

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on the individual. Hydroxystearic acids are formed in the intestine, particularly under disease conditions. Metabolism of linoleic acid results in the formation of conjugated linoleic acids (CLA) by several species, including *Roseburia hominis* and *Roseburia inulinivorans*. Enhancement of GI CLA formation, possibly using probiotics, may be useful in preventing or treating inflammatory bowel disease and be protective of key health-promoting bacteria such as *Faecalibacterium prausnitzii*.

1 Introduction

The human large intestine harbors 10^{10}–10^{11} bacteria per gram of wet weight contents (Macfarlane et al. 1998). In this milieu, short-chain fatty acids (SCFAs) are formed from dietary fiber, resistant starches, and other poly- and oligosaccharides that escape digestion in the stomach and small intestine (Flint et al. 2008). This metabolism is considered to be generally beneficial to the health of the host, for reasons that will be described below. Longer-chain fatty acids (FAs) are also released from the various dietary, host, and bacterial fats that enter the gastrointestinal (GI) tract ▶ Chap. 14, "Antimicrobial Activity of Essential Oils". The metabolism of these FA is restricted, as far as we know, mainly to the more reactive mono- and polyunsaturated FA (MUFA and PUFA, respectively), which undergo hydration or hydrogenation or perhaps both. Once again, this FA metabolism has important implications for health. This chapter summarizes what is known about FA metabolism by GI bacteria, particularly where the metabolism may impinge upon human health.

2 SCFA Production

Fermentation of polysaccharides predominates in the ascending colon, where bacterial numbers are highest, and the microbial by-products of carbohydrate fermentation include CO_2, CH_4, H_2, SCFA, bacterial cell mass, and some heat. Polysaccharides entering the large intestine comprise insoluble plant fiber, in the form of plant cell wall polysaccharides, oligosaccharides, storage polysaccharides (e.g., inulin), and resistant starch. Approximately 10% of the human energy balance is derived from fermentation of polysaccharides by bacteria resident in the large intestine (Bergman 1990). These polysaccharides are fermented to SCFA, predominantly acetate, propionate, and butyrate. SCFA concentrations are highest in the cecum (131 mmol/kg digesta). Concentrations fall during passage through the intestine due to absorption of the SCFA across the gut wall (Cummings et al. 1987).

Acetate is the SCFA produced in the greatest quantity, followed by propionate and butyrate (57:22:21 molar ratio; Cummings et al. 1987). Other organic acids, such as lactate, succinate, formate, valerate and caproate, or branched-chain fatty acids (BCFA) generated from amino acids, are found in much smaller amounts in the

intestine (Cummings et al. 1987). Ethanol can also be produced in minor amounts as a by-product of carbohydrate fermentation. The overall pathways of SCFA production are summarized in Fig. 1. Only a few features of SCFA production, mainly those most relevant to health, can be discussed here.

Absorbed acetate is the principal route by which the body obtains energy from carbohydrates not absorbed in the small intestine (Salminen et al. 1998). Approximately 60% of the acetate produced by GI bacteria is retained in the liver (Pouteau et al. 2003). Animal studies have shown that acetate is secreted by the liver when portal blood concentrations fall below a critical level (Salminen et al. 1998). Acetate is formed by nearly all heterotrophic anaerobic gut bacteria, but up to one-third of all acetate in the large intestine can come from reductive acetogenesis (Flint 2006), whereby species such as *Blautia producta* form acetate from H_2 and CO_2 (Ohashi et al. 2007; Rey et al. 2010) (Fig. 1). Acetate formation benefits bacteria because it results in energy generation by substrate-level phosphorylation of ADP to ATP (Macfarlane and Gibson 1997).

Propionate can be formed by several routes in the GI microbiota – directly from sugars by single species or indirectly by cross-feeding from succinate and lactate producers (Fig. 1). There are three metabolic pathways for propionate formation: the propanediol pathway, the succinate pathway, and the acrylate pathway (Reichardt et al. 2014; Louis and Flint 2017). Belenguer et al. (2007) showed the acrylate pathway was the main pathway by which lactate was converted to propionate. Succinate producers are fairly common. *Bacteroides cellulosilyticus*, *Bacteroides fragilis*, *Bacteroides ovatus*, and *Bacteroides thetaiotaomicron* have been shown to produce acetate and succinate from sugars in pure culture (Robert et al. 2007). Most cultured members of *Clostridium* cluster IX (e.g., *Selenomonas*, *Megasphaera*, and *Veillonella* spp.) produce propionate via decarboxylation of succinate (Flint 2006). Propionate formation achieves two needs of bacteria, one for the disposal of reducing equivalents, especially NADH, and the other for ATP synthesis. The presence of electron transport chain components such as cytochromes is widespread in these bacteria (Macfarlane and Gibson 1997), and it can be assumed that they produce ATP by electron transport-linked as well as substrate-level phosphorylation.

Butyrate is presently thought to be the most significant of the SCFA in terms of its influence on human health. Like propionate, butyrate can be formed by more than one route. In terms of substrate, butyrate is formed from sugars by many members of *Clostridium* clusters XIVa and IV, including *Eubacterium*, *Fusobacterium*, *Roseburia*, and *Butyrivibrio* (Walker et al. 2005; Duncan et al. 2006; Flint 2006). It may also be formed from lactate, probably by the same bacteria. The fermenter studies of Belenguer et al. (2007) suggested that *Eubacterium hallii* played a major role in the conversion of lactate to butyrate. Bacteria also employ two metabolic routes to form butyrate, one in which the final step is butyrate kinase and the other where butyrate is released from butyryl-CoA by an acyl transferase. The latter route seems to be more common, used by the predominant *Roseburia* and *Faecalibacterium* species (Duncan et al. 2002; Louis et al. 2004). The enzymic mechanism facilitates the formation of butyrate from exogenous acetate, an

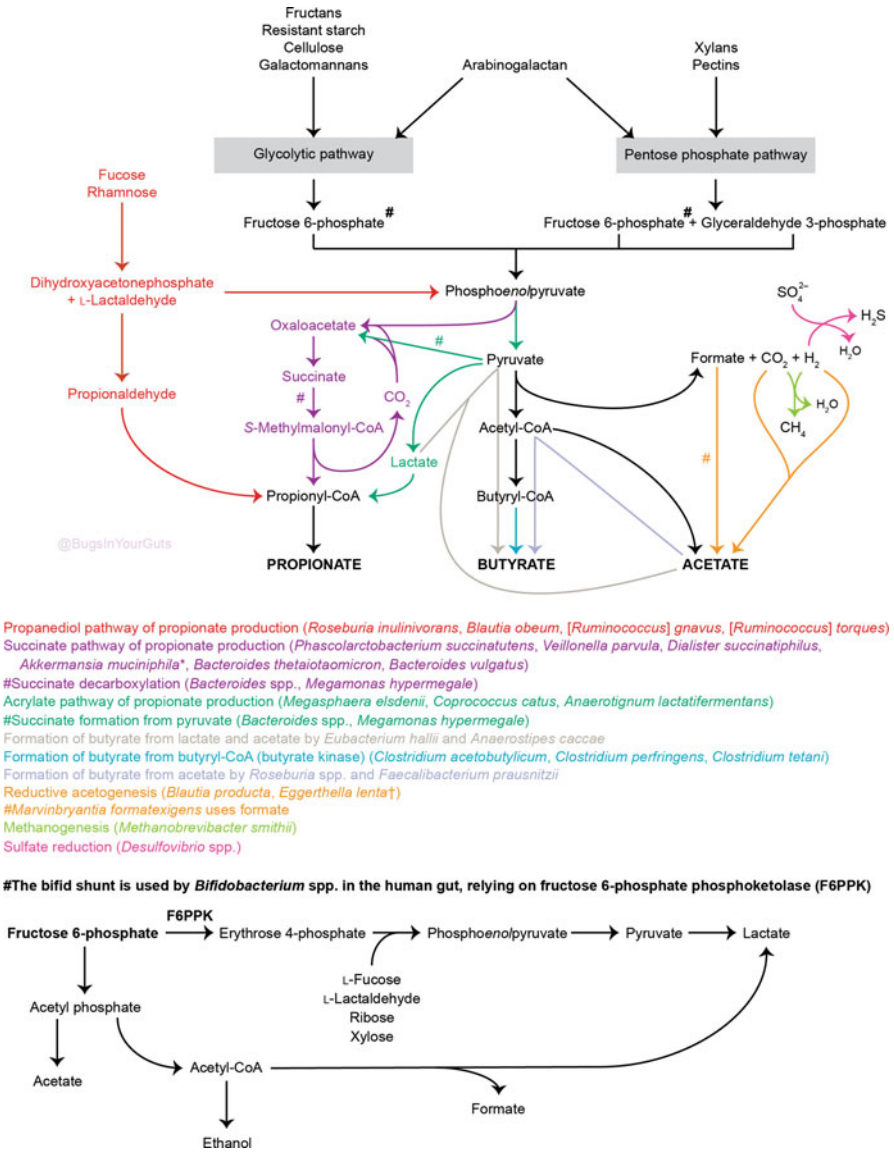

Fig. 1 Prokaryotes involved in the formation and conversion of SCFAs in the human large intestine. A simplified diagram of polysaccharide breakdown and the main routes of carbohydrate fermentation in the large intestine are shown. Two distinct cross-feeding mechanisms operate in the GI tract: one due to the consumption of fermentation end products (lactate, acetate, succinate) and the other due to cross-feeding of partial breakdown products from complex substrates (Falony et al. 2006; Belenguer et al. 2007; Reichardt et al. 2014). Both mechanisms contribute to the production of butyrate and propionate. Updated from Hoyles and Wallace (2010) to include propionate formation (Reichardt et al. 2014) and the bifid shunt, which is restricted to *Bifidobacterium* spp. in the human gut via the action of fructose 6-phosphate phosphoketolase (Pokusaeva et al. 2011).

important route of butyrate synthesis (Duncan et al. 2002; Duncan et al. 2004a; Falony et al. 2006).

Lactate seldom accumulates in the colon to a significant level, despite many GI bacterial species producing lactate in pure culture (Macfarlane and Gibson 1997). Interspecies cross-feeding on lactate undoubtedly contributes to the low concentrations in the colon, but it is also likely that the specific growth rate of bacteria in situ is low, such that bacteria that form lactate at maximum growth rates form different products at lower growth rates. This behavior is well documented in ruminal bacteria such as *Streptococcus bovis* and *Selenomonas ruminantium* (Russell and Wallace 1997).

Nothing is known about the main members of the microbiota responsible for converting amino acids to BCFA (Flint 2006). However, it is known that 2-methylbutyrate, isobutyrate, and isovalerate are formed as products of isoleucine, valine, and leucine fermentation, respectively, by the fecal microbiota (Macfarlane and Gibson 1995). The proportion of BCFA versus total SCFA is higher in the descending colon compared with other FAs, reflecting that protein fermentation exceeds carbohydrate fermentation in this region due to substrate availability and increased pH (Cummings et al. 1987).

3 Implications of SCFA Production for Health

The total concentration of SCFA in the GI tract and concentrations of individual SCFA have implications for human health. High total SCFA concentrations lead to a lowering of pH. A low pH in feces has been associated with a decreased incidence of colorectal cancer in various populations (Malhotra 1982; Walker et al. 1986). In addition, SCFA contribute to the normal function of the large bowel by stimulating colonic blood flow and water and salt uptake (Roediger 1980; Salminen et al. 1998). SCFA also affect gut motility. Via their remote effects, SCFA are involved in the regulation of upper gut motility in a dose-dependent manner (Cherbut 2003). There are two types of contraction involved in gut motility: tonic contractions, which decrease the volume of the GI tract, and peristaltic contractions, which are propagated over long or short distances and are associated with the backward and forward movement of GI contents. Transit time is dependent upon the pattern of occurrence of these two types of contraction (Cherbut 2003). It is well known that nutrients in the proximal and distal small intestinal lumen, particularly SCFA, participate in regulating gastric motility and emptying. SCFA also influence appetite, via effects on leptin, glucagon-like peptide-1 (GLP-1), and peptide YY (PYY), which act as

Fig. 1 (continued) **Akkermansia muciniphila* is thought to be produce propionate via the succinate pathway. †Species predicted from sequence analyses to be capable of reductive acetogenesis (Ohashi et al. 2007; Hylemon et al. 2018). (Figure reproduced with permission of Lesley Hoyles (original available from http://bugs-in-your-guts.com/?p=309))

signals of satiety to the human body (Canfora et al. 2015). Failures in leptin signaling are associated with severe obesity, hyperphagia, infertility, and immunological defects. Studies in mice have suggested a role for SCFA (C_2–C_6 SCFA and C_4–C_6 branched-chain FA) in leptin production (Xiong et al. 2004), with SCFA (particularly C_3 and C_5) activating FFAR3 (GPR41). In addition, in vitro and in vivo studies in mice and humans have shown roles for FFAR3 and FFAR2 (GPR43) in GLP-1 and PYY secretion (Canfora et al. 2015).

Among specific SCFA, acetate is an energy source that is not only absorbed from the intestine but also acts as a precursor for butyrate formation in the intestine. Absorbed propionate is largely cleared from the system by the liver (Salminen et al. 1998). A study in which humans were fed propionate supplements has suggested a role for propionate in improving glucose tolerance and insulin sensitivity, mediated by its effect on hepatic carbohydrate metabolism (Venter et al. 1990). Another feeding study, in which subjects consumed propionate-enriched bread, showed that propionate ingestion reduced high-density lipoprotein and increased serum triglyceride concentrations in addition to improving carbohydrate tolerance (Todesco et al. 1991). In addition, the activity of salivary amylase was inhibited by propionate. It was suggested that this was an additional mechanism by which propionate acted on the host, in addition to its proposed inhibitory effect on HMG-CoA, one of the key regulatory enzymes of cholesteryl ester synthesis from cholesterol.

Of the SCFA produced in the large intestine, butyrate has received most attention due to its role in protection from colorectal cancer and colitis (Roediger 1990; Williams et al. 2003). Butyrate is the principal energy source for colonocytes, is essential in maintaining tissue homeostasis in the colonic epithelium, plays roles in suppressing GI inflammation and in lipid metabolism, and exhibits a range of antitumorigenic effects on many cancer cell lines (Hamer et al. 2008). It is estimated that butyrate provides between 40% and 70% of the energy required by the colonic mucosa (Roediger 1980). Using microarray analysis of butyrate-regulated genes in colonic epithelial cells, 221 potentially butyrate-responsive genes specifically associated with the processes of proliferation, differentiation, and apoptosis have been identified (Daly and Shirazi-Beechey 2006).

Lactate only accumulates in significant amounts (up to 100 mM) in the feces of individuals suffering from severe malabsorption, or who have undergone gut resections or are suffering from inflammatory bowel disease and proctitis or Crohn's colitis (Hove et al. 1994; Hove and Mortensen 1995). The accumulation of D-lactate in short bowel syndrome can lead to neurotoxicity and cardiac arrhythmia (Duncan et al. 2004b). Under normal conditions, the amount of lactate present in the feces is low (<5 mM; Duncan et al. 2004b). Secretion of lactate by the mucosa is minimal in healthy individuals but is increased in patients with colonic inflammation: increased fecal lactate concentrations in these patients are due to mucosal secretions and not to bacterial activity (Hove et al. 1995).

Higher (25%) concentrations of isobutyrate and isovalerate formation have been observed in vitro with systems inoculated with feces from individuals with inflammatory bowel disease (five with Crohn's disease; three with ulcerative colitis) compared with systems inoculated with feces from healthy individuals (van Nuenen

et al. 2004). This is undoubtedly linked to the detrimental effects of other products of proteolytic fermentation in the etiology of inflammatory bowel disease (Gibson et al. 1989; Martin and Rhodes 2000), but much more work is required to establish if there is a direct effect of BCFA.

Upon their uptake into systemic circulation, the three main SCFA are potent bioactive molecules and are found at micromolar concentrations in the peripheral blood of healthy individuals (acetate, 22–42 μM; propionate, 0.9–1.2 μM; butyrate, 0.3–1.5 μM) (Hoyles et al. 2018). It is becoming clear SCFA contribute to the gut–brain axis [i.e., gut-microbiota-generated metabolites influence the central nervous system (CNS)]. All three SCFAs activate members of the free fatty acid receptor (FFAR) family of G protein-coupled receptors; acetate, propionate, and butyrate have affinity in the low millimolar to high micromolar range for FFAR2; propionate and butyrate have mid- to low micromolar affinity for FFAR3. FFAR3 is expressed in human and animal brain cells (Hoyles et al. 2018). Propionate stimulates intestinal gluconeogenesis via a gut–brain neural circuit involving FFAR3 in mice (De Vadder et al. 2014), and increased GI propionate has been associated with reward pathway activity in humans (Byrne et al. 2016). In vitro, both propionate and butyrate are protective to the blood–brain barrier, while this is not the case for acetate, suggesting FFAR3 links SCFA to regulation of brain barrier function (Hoyles et al. 2018). Gut-microbiota-generated acetate can be transported across the blood–brain barrier to the hypothalamus, decreasing food intake through appetite suppression (Frost et al. 2014). The link between SCFA and the gut–brain axis warrants further study. Prebiotics may be one way of promoting SCFA production by GI bacteria to influence the gut–brain axis.

FFAR2 and FFAR3 are expressed in the human white adipose tissue, skeletal muscle, and liver. Acetate and propionate activate FFAR2 in vitro to reduce intracellular lipolytic activity of murine adipocytes (Ge et al. 2008). Hydroxycarboxylic acid receptor 2 HCAR2 (GPR109a), which responds to butyrate but not acetate or propionate, is expressed in gut epithelial cells, adipocytes, and immune cells and contributes to intestinal barrier function. Olfactory receptor Olfr78 (Or51e2), expressed in kidney and vascular smooth muscle cells of mice, can modulate blood pressure when stimulated by butyrate and propionate (Canfora et al. 2015; Kim 2018). The interaction of this small number of host receptors with SCFA and our limited understanding of their effects in humans suggest there may be many more host–microbiome receptor interactions to be discovered. Readers are directed to Canfora et al. (2015) and Kim (2018) for reviews of our current knowledge on the interactions between host receptors and SCFA and implications for host health.

4 Metabolism of FA by GI Bacteria: Mechanisms

Our knowledge of lipid metabolism in the human colon has for many decades been rudimentary in comparison with the rumen (Harfoot and Hazlewood 1997). Nevertheless, the knowledge gained from rumen studies has helped advances to be made recently in FA metabolism by human GI bacteria. There is no evidence of oxidation

or elongation of FA originally from the diet, although such reactions must occur within bacteria for their endogenous FA synthesis. Metabolism of FA in the large intestine occurs predominantly with MUFA and PUFA, via hydration and biohydrogenation of the unsaturated bonds in the aliphatic chain.

It has been known for many years that the composition of FA in human feces differs greatly from the FA composition of foods (James et al. 1961). For the most part, it was unclear to what extent the different composition was due to differential absorption of FA from the GI tract and how much was due to the metabolic activity of bacteria in the intestine. Some hydroxy FAs were present that did not appear in the diet (James et al. 1961), however, indicating a likely involvement of bacteria. James et al. (1961) postulated that hydroxystearic acids (HSA) arose as an intermediary of the oxidation of stearic acid by GI bacteria. Evidence that HSA were produced by GI bacteria was obtained using dogs that had steatorrhea (fecal fat excretion) secondary to experimentally produced GI blind loops. Dogs treated with tetracycline or which had the blind loop excluded overcame the steatorrhea and had lower concentrations of HSA in the feces, with both outcomes apparently resulting from the elimination of bacterial overgrowth in the intestine (Kim and Spritz 1968). This work showed that HSA could be produced in significant amounts only from oleic and linoleic acids when incubated with the feces of a human and a dog with steatorrhea. Stearic acid was not converted to a hydroxy derivative in significant amounts in either of these in vitro systems (Kim and Spritz 1968). Thus, HSA were formed by hydration of the Δ^9 double bond in unsaturated FAs rather than by the oxidation of the saturated acid. Thomas (1972) showed that many anaerobic bacteria, including some colonic species, carried out hydration of oleic acid to HSA. *Clostridium perfringens* was the most active species. Pearson (1973) incubated 228 strains of GI bacteria from 5 genera with oleic acid and found that 103 strains formed HSA. Thus, HSA formation from unsaturated FA is a widespread function among GI bacteria.

Other hydroxy acids may be formed as intermediates in the metabolism of PUFA. Pearson (1973) did not detect unsaturated hydroxy FAs being produced from linoleic acid by 14 strains of fecal bacteria. In contrast, Devillard et al. (2007) found that some *Roseburia* strains formed a hydroxy FA identified as a 10-hydroxy,*cis*-12-18:1. Strains of *Lactobacillus*, *Lactococcus*, *Eubacterium*, *Propionibacterium*, *Bifidobacterium*, and *Faecalibacterium* produced the same hydroxy FA, although to a lower extent than most *Roseburia* strains. The 10-hydroxy,*cis*-12-18:1 was converted by the mixed GI microbiota transiently to *cis*-9,*trans*-11-18:2 and then to *trans*-11-18:1 (Fig. 2, Devillard et al. 2007). A different metabolic route can be found in some lactic acid bacteria, such as *Lactobacillus plantarum*, resulting in the formation of conjugated linoleic acid (CLA) via 10-hydroxy,*cis*-12-18:1 and also 10-oxo,*cis*-12-18:1 (Kishino et al. 2013); however, although *L. plantarum* is described as a "representative gut bacterium," its abundance is many-fold lower than the typical obligate anaerobes (Qin et al. 2010), and the predominant metabolic fate of linoleic acid in the human colon is biohydrogenation (Devillard et al. 2009). Nevertheless, if they are formed, 10-hydroxy,*cis*-12-18:1 could be beneficial in terms of lowering inflammation in gut tissues (Miyamoto et al. 2015), while 10-oxo,*cis*-12-18:1 has a variety of anti-obesity effects (Kim et al. 2017).

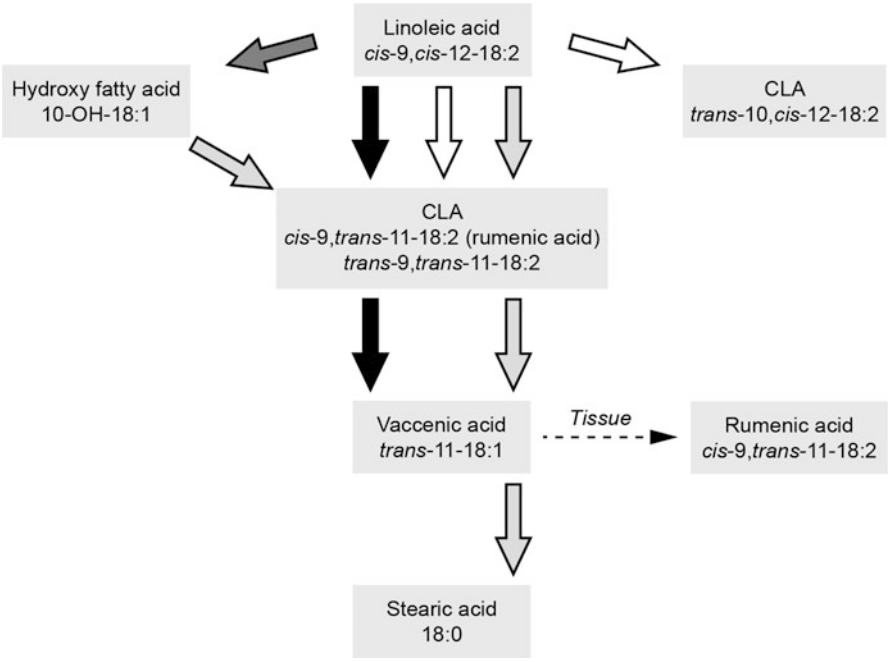

Fig. 2 LA metabolism by human fecal bacteria (Adapted from Devillard et al. 2007). Different fill colors of arrows indicate that the reaction is carried out by bacterial different species. CLA, conjugated linoleic acid, of which rumenic acid is one geometric isomer

The main route of FA metabolism, biohydrogenation, was found in mixed GI bacteria from rats (Eyssen and Parmentier 1974) but was not confirmed in man until the work of Howard and Henderson (1999). The same discovery had been made in the rumen 35 years earlier (Polan et al. 1964). Most attention has been paid to the biohydrogenation of linoleic acid (LA, *cis*-9,*cis*-12-18:2), which is metabolized mainly by conversion to the conjugated dienoic acid, rumenic acid (RA, *cis*-9, *trans*-11-18:1), which is then hydrogenated to vaccenic acid (VA, *trans*-11-18:1) and then to stearic acid (18:0) (Fig. 2). α-Linolenic acid (LNA, *cis*-9,*cis*-12,*cis*-15-18:3) is also metabolized rapidly by the fecal microbiota, forming a mixture of 18:3 and 18:2 isomers (Howard and Henderson 1999). Since the route of metabolism of LA by fecal bacteria is similar to that of the ruminal microbiota, one might expect that the pattern by which LNA is metabolized, via firstly *cis*-9,*trans*-11,*cis*-15-18:3, to be similar as well (Harfoot and Hazlewood 1997). *Butyrivibrio fibrisolvens*, *Roseburia inulinivorans*, and *Roseburia hominis* produced VA rapidly from LA, presumably via RA. The bacteria responsible for the conversion of vaccenic acid to stearic acid in the human colon are unknown. Identification of these bacteria may be difficult. From work done with ruminal bacteria (Maia et al. 2007; Paillard et al. 2007), it is known that the bacteria responsible [related to *Butyrivibrio proteoclasticus* (formerly *Clostridium proteoclasticum*)] are extremely sensitive to the

toxic effects of unsaturated FAs. Growth of the bacteria was necessary for stearate formation to occur, but, as LA was toxic at concentrations as low as 5 μg/ml, growth was inhibited by the substrate. The same may be true of human GI bacteria (Devillard et al. 2007). qPCR based on 16S rRNA gene sequences indicated that *B. proteoclasticus* was present only at very low numbers in human feces (Devillard et al. 2009), indicating that, as with the earlier steps in the pathway, the species responsible for stearate formation in the two gut ecosystems might be different.

To establish the mechanism by which LA was metabolized by human GI bacteria, mixed and pure cultures were incubated with deuterium oxide, and the fate of the deuterium was analyzed (McIntosh et al. 2009). Fecal bacteria from four human donors and six species of human GI bacteria were incubated with LA in deuterium oxide-enriched medium. The FA products were derivatized, separated by GC, and identified by mass spectrometry. The main CLA products in fecal suspensions, RA and *trans*-9,*trans*-11-18:2, were labeled at C-13, as were other 9,11 geometric isomers. Traces of *trans*-10,*cis*-12-18:2 formed were labeled to a much lower extent. In pure culture, *Bifidobacterium breve* formed labeled RA and *trans*-9,*trans*-11-18:2, while *Butyrivibrio fibrisolvens*, *Roseburia hominis*, *Roseburia inulinivorans*, and *Blautia obeum*-like strain A2-162 converted LA to VA, labeled in a manner indicating VA was formed via C-13-labelled RA. *Propionibacterium freudenreichii* subsp. *shermanii*, a possible probiotic, formed mainly RA with smaller amounts of *trans*-10,*cis*-12-18:2 and *trans*-9,*trans*-11-18:2, labeled the same as in the mixed microbiota. Ricinoleic acid (12-OH-*cis*-9-18:1) did not form CLA in the mixed microbiota, in contrast to CLA formation described for *Lactobacillus plantarum* (Ogawa et al. 2005). Results were similar to those reported for the mixed microbiota of the rumen. Thus, though the bacterial genera and species responsible for biohydrogenation in the rumen and the human intestine differ, and a second route of RA formation via a 10-OH-18:1 is present in the intestine, the overall labeling patterns of different CLA isomers formation are common to both gut ecosystems. A hydrogen-abstraction enzymic mechanism was proposed that may explain the role of a 10-OH-18:1 intermediate in 9,11-CLA formation in pure and mixed cultures (McIntosh et al. 2009).

5 Influence of Fats on, and Metabolism of FA by, GI Bacteria: Health Implications

In general, the gut microbiota has important implications for health. The composition of the diet, including fats, has a profound influence on the GI microbiota (Scott et al. 2013). Humans consuming a high-fat diet possess a different gut microbiota from those consuming a diet with a lower fat content (Turnbaugh et al. 2009), which has prompted proposals that there is a gut microbiome that is associated with, and by implication causes, obesity (Ley et al. 2005; Turnbaugh et al. 2006). Arguments and experiments for and against such a mechanism have been well rehearsed elsewhere ▶ Chap. 15, "Gastrointestinal Tract: Fat Metabolism in the Colon". In particular, observations such as those by Hildebrandt et al. (2009), who found the same changes

in the microbiota of mice (the high-fat diet increased Firmicutes and Proteobacteria and decreased Bacteroidetes) in both lean and obese animals, question the cause-effect relationships involving dietary fat, the microbiota, and obesity.

Different fats and their constituent FA also have different effects on gut tissues depending on their degree of unsaturation: fish oil had a greater inflammatory effect than lard, for example, in mice (Li et al. 2017).

HSA concentrations in feces increase as a consequence of various clinical conditions (Wiggins et al. 1974). Patients with ileal disease, ileal resections, or small intestinal bacterial colonization all had more than 5% HSA in their feces. The explanation given for the seemingly high levels of HSA in samples from the ileal disease and colonization patients was the simultaneous occurrence of relatively high concentrations of fat, bacteria, and bile salts in the small intestine. High levels of HSA in the ileal resection patients were not due solely to the resections as patients with comparable ileal resections and steatorrhea but who had undergone removal of most of the colon, thus reducing the time in contact with bile salts, fat and bacteria, had normal levels of HSA. Therefore, the authors concluded that the main site of formation of HSA in ileal resection patients was the colon. This was also the case for patients suffering from pancreatic insufficiency. They suggested that finding normal (<6%) concentrations of HSA in feces could not be taken to exclude any diagnosis; however, the finding of more than 5% HSA in an individual consuming a normal diet with mild steatorrhea and without previous surgery would suggest the presence of ileal disease or a stagnant loop syndrome.

Whether HSA has any implications for health other than a consequence of disease or abnormality is less clear. HSA is chemically similar to ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid), the major FA in castor oil, a known cathartic. The presence of HSA in human feces led to the suggestion that it contributes to the diarrhea frequently associated with steatorrhea (James et al. 1961). In a study examining 87 patients and 12 controls, Wiggins et al. (1974) demonstrated that, in general, the percentage of HSA in feces increased as the fecal fat output rose. In individuals without steatorrhea and excreting 20 g fat/day, less than 5% of the fecal fat comprised HSA, while in individuals with steatorrhea (and, consequently, excreting more fat), between 6% and 23% of the fecal fat comprised HSA. However, no correlation was found between HSA levels and steatorrhea in the majority of cases. A positive correlation was found between fecal weight and HSA excretion expressed as an absolute value, and the authors suggested that HSA may have a direct effect on water absorption. However, they found a correlation of similar magnitude between fecal weight and steatorrhea and stated that it was impossible to conclude that there was a specific action of HSA in the GI tract. Tiruppathi et al. (1983), when working with samples from tropical sprue patients, found no correlation between HSA and the diarrhea associated with the condition, instead suggesting that unsaturated FAs may play a role in diarrhea via their inhibition of colonocyte basolateral membrane ATPases (thereby interfering with colonic Na^+ and water absorption).

In addition to converting VA to *cis*-9,*trans*-11-CLA, Δ^9-desaturase in host tissues converts stearic acid to oleic acid ($C_{18:1}$). Oleic and stearic acids are known to decrease plasma cholesterol concentrations (Bonanome and Grundy 1988), so

biohydrogenating C-18 PUFA and MUFA might be considered in some ways beneficial to health. However, it is the MUFA and PUFA themselves that many consider to be more beneficial to health.

CLA and VA, on the other hand, are considered to have possibly potent effects on human health. In vitro and in vivo animal studies have suggested that the usually most abundant CLA, RA (*cis*-9,*trans*-11-18:2), has anticarcinogenic, anti-atherosclerotic, and immune-modulating effects, as well as favorable influences on body composition, blood lipids, liver metabolism, and insulin sensitivity (Belury 2002; Wahle et al. 2004; Tricon and Yaqoob 2006). Whether such benefits can be obtained in humans consuming CLA-rich foods remains uncertain, however, partly because the doses used in rodent trials cannot be replicated in man (Fuke and Nornberg 2017). VA may arguably be considered to be functionally equivalent to RA. VA is converted to RA via the host's Δ^9-desaturase, an enzyme present in the intestine and liver (Rhee et al. 1997; Turpeinen et al. 2002; Mosley et al. 2006). VA has been shown to suppress the growth and affect cellular responses of human mammary and colon cancer cell lines through its conversion to RA by Δ^9-desaturase in these cells (Miller et al. 2003). Therefore, increasing CLA and VA intake may have potential benefits on health.

A different sort of benefit deriving from biohydrogenation involves the protection of beneficial bacteria. De Weirdt et al. (2013) noted that *Lactobacillus reuteri*, a probiotic species, was highly sensitive to toxic effects of linoleic acid and that its survival in a gut-simulating fermenter was enhanced by biohydrogenating bacteria colonizing the mucosal layer. Even more directly, De Weirdt et al. (2017) observed a protective effect of biohydrogenating species *Roseburia* and *Pseudobutyrivibrio* toward the highly beneficial anaerobe *Faecalibacterium prausnitzii.*

One possibility to deliver more CLA/VA to the host was thought to be to use the biohydrogenating ability of GI bacteria. CLA formed in the intestine might be absorbed and contribute to systemic CLA. However, experiments with germ-free rats inoculated with a human fecal microbiota and fed a diet enriched with sunflower-seed oil indicated that no benefit accrued in terms of tissue concentrations of CLA (Kamlage et al. 1999). Kamlage et al. (2000) found that glucose inhibited CLA formation by mixed fecal microorganisms and speculated that this may be the reason for the earlier result. It now seems more likely that CLA is not absorbed from the intestine. Druart et al. (2014) found that CLA, and the corresponding CL*n*A (*cis*-9, *trans*-11, *cis*-15-18:3) formed from linolenic acid (*cis*-9, *cis*-12, *cis*-15-18:3), accumulated in the cecum and colon, but not in the jejunum and ileum. No increase was observed in plasma, suggesting that the conjugated FA were not absorbed. In contrast, Neyrinck et al. (2011) suggested that increased RA content in white adipose tissue was a response to the fermentation of arabinoxylan in the large intestine of obese mice, which would only occur if RA, or perhaps VA – a precursor of rumenic acid in mammalian tissues as described above – were absorbed from the intestine. CLA-producing lactic acid bacteria used as probiotics result in increased tissue levels of CLA in rodents (O'Shea et al. 2012); however, it might be argued that the CLA formation could occur in the small intestine, from which it is known that FA, including CLA, are absorbed.

Nevertheless, even if CLA absorption from the intestine is minimal, there may be in situ benefits from GI CLA production. In mouse models of inflammatory bowel disease, CLA were shown to exhibit anti-inflammatory properties via endoplasmic and nuclear mechanisms (Bassaganya-Riera et al. 2002; Bassaganya-Riera et al. 2004). Further studies demonstrated that CLA exerted anticarcinogenic activity in the rat colon (Nichenametla et al. 2004) and exhibited antiproliferative properties on the growth of human colon cancer cells in vitro (Kemp et al. 2003). Increased abundance of Bacteroidetes/*Prevotella* and *Akkermansia muciniphila* occurred in the cecum of mice receiving dietary CLA, which was interpreted as a beneficial effect (Chaplin et al. 2015). Therefore, mechanisms by which CLA might be delivered to and formed in the intestine have important implications for long-term human gut health. The limited evidence we have suggests that different individuals may have different types of gut microbial FA metabolism (Devillard et al. 2009; Hoyles 2009).

The precise isomer(s) of CLA that is formed from LA is significant, given the very different biological effects of RA and other isomers, particularly *trans*-10,*cis*-12-CLA (Pariza 2004; Bauman et al. 2005). Such information could have particular relevance to patients using the slimming drug tetrahydrolipstatin (Orlistat; Hauptman et al. 2000), which prevents lipid absorption in the human small intestine. Large amounts of lipid reach the large intestine, sometimes with deleterious consequences (Chanoine et al. 2005). An in vitro study conducted in a gut fermentation model (Macfarlane et al. 1998) inoculated with human feces plus olive oil in the presence and absence of Orlistat showed nonsignificant changes in *cis* CLA levels over time in the presence of Orlistat, but did not examine other CLA isomers (Hoyles 2009). Orlistat did, however, inhibit microbial lipases in a donor-dependent manner, and this is likely to influence isomers of CLA and other FA produced in the large intestine in the presence of the drug (Hoyles 2009). Conversion of LA to RA, the *cis*-9,*trans*-12 CLA isomer, under such circumstances might be beneficial, while the formation of 10,12 isomers could be considered potentially detrimental (Bauman et al. 2005; Pariza 2004). Although the formation of the latter was minimal in our in vitro experiments with a limited number of individuals (McIntosh et al. 2009), it is possible, by analogy with the rumen, that the population may flip over from the production of 9,11 CLA isomers and VA to the production of 10,12 CLA isomers and *trans*-10-18:1. This type of population shift occurs in dairy cows, causing milk fat depression.

In an effort to increase the amount of RA available to humans, probiotic bacteria have been suggested as a possible method for increasing CLA in the human intestine (Coakley et al. 2003; O'Shea et al. 2012). They may also serve to ensure that the correct isomer of CLA is formed. The rationale for this approach is that ingested bacteria could use dietary LA to produce CLA. *Lactobacillus*, *Propionibacterium*, and *Bifidobacterium* species are known to be involved in the formation of CLA from LA (Devillard et al. 2007). In the study of Coakley et al. (2003), strains of *Bifidobacterium breve* and *Bifidobacterium lactis* were identified that were able to convert LA to CLA at high levels. Bifidobacteria have long been used as probiotics in human foods, and they have been shown to elicit specific health benefits upon the

host, for example, production of vitamins [folate, cobalamin (B_{12}), menaquinone (K_2), riboflavin (B_2), and thiamine (B_1)] and bacteriocins, and prevention of diarrhea (O'Connor et al. 2005; Khedkar and Ouwehand 2006). Therefore, the identification of probiotic bifidobacteria with the ability to synthesize CLA may offer novel opportunities in the rational design of improved health-promoting functional foods (Coakley et al. 2003).

6 Research Needs

Our detailed understanding of gut bacteria responsible for producing SCFA in humans needs to be underpinned with detailed understanding of the mechanisms by which SCFA influence host cells, organs, and systemic health. We still have no knowledge as to the efficiency of electron transport-linked ATP synthesis during propionate and butyrate production, and the species that are most active in BCFA formation. With the long-chain FAs, our knowledge is limited mainly to the metabolism of LA. We need to know more about the metabolism of other PUFA and MUFA. For example, Howard and Henderson (1999) found that arachidonic acid was not metabolized – is this true for other PUFA such as the fish oil FA? In microbial ecology terms, the glaring unknown is that we still do not yet know species that form stearate from LA or VA, nor do we have a true picture of the lipolytic potential of the human gut microbiota.

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Gastrointestinal Tract: Microbial Metabolism of Steroids

17

Philippe Gérard

Contents

Abstract

The human gastrointestinal tract hosts a complex and diverse microbial community, whose microbiome encodes biochemical pathways that humans have not evolved. As a consequence, the gut microbiota produces metabolites from a large range of molecules that host's enzymes are not able to convert. This is of first importance as these bacterial metabolites may have beneficial or deleterious effects on human health. In particular, cholesterol and bile acids are exposed to the gut microbiota and undergo bacterial metabolism: cholesterol is mainly converted into coprostanol, a nonabsorbable sterol which is excreted in the feces. Conversely, over 20 different secondary bile acid metabolites are produced by the gut microbiota from the primary bile acids: cholic and chenodeoxycholic

P. Gérard (✉)
Micalis Institute, INRA, AgroParisTech, University Paris-Saclay, Jouy-en-Josas, France
e-mail: philippe.gerard@inra.fr

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acids. The main bile salt conversions in the human gut include deconjugation; oxidation and epimerization of hydroxyl groups at C3, C7, and C12; 7-dehydroxylation; esterification; and desulfation. Through these transformations, the gut bacteria produce a variety of bile acids that, via receptors expressed on many cell types throughout the host body, regulate glucose and energy metabolism, immune system, and other physiological functions.

1 Introduction

Steroids are a family of organic compounds consisting of a four-ring perhydrocyclopentanophenanthrene nucleus. Two main classes of steroids are exposed to the gut microbiota and subsequently undergo microbial metabolism leading to various metabolites. These classes are cholesterol originating from the diet or synthesized de novo in the liver and other tissues and bile acids synthesized from cholesterol in the liver and excreted via the biliary tract.

2 Metabolism of Cholesterol by the Gut Microbiota

Every day, up to 1 g of cholesterol enters the colon. Indeed, despite huge inter-individual variations, only half of the dietary cholesterol is absorbed on average, primarily in the duodenum and proximal jejunum. This unabsorbed dietary cholesterol represents around 200 mg/day and is added to the biliary cholesterol secretion, the main source of cholesterol in the lumen, and to cholesterol of cells sloughed from the intestinal epithelium. More recently, direct non-biliary excretion of plasma-derived cholesterol into the intestinal lumen via a pathway termed transintestinal cholesterol efflux (TICE) has been described (Van der Velde et al. 2010). Although its contribution to cholesterol excretion is unclear in humans, TICE accounts for up to 70% of fecal neutral sterol excretion in mice. Intestinal cholesterol conversion was established as far back as the 1930s (Schoenheimer 1931), and it was subsequently shown that intestinal microbiota was responsible as germfree rats only excreted unmodified cholesterol (Kellogg and Wostmann 1969). Therefore, it was revealed that cholesterol is reduced to coprostanol and minor amounts of coprostanone by the intestinal microbiota. In humans, this microbial conversion of cholesterol starts during the second half of the first year of life (Midvedt and Midvedt 1993). Several studies have also reported that the rate of microbial cholesterol-to-coprostanol conversion in human populations was bimodal, with a majority of high converters (almost complete cholesterol conversion) and a minority of low or inefficient converters (coprostanol content representing less than one-third of the fecal neutral sterols content) (Wilkins and Hackman 1974; Veiga et al. 2005). These conversion patterns were found equally distributed with respect to sex and were independent of age. Only recently has it been demonstrated that the efficiency of cholesterol conversion in the human gut results mainly from the

abundance of cholesterol-reducing bacteria (Gérard et al. 2004; Veiga et al. 2005). In a study including 15 human volunteers, it was established that the level of cholesterol-reducing bacteria must be at least 10^6 cells/g (wet weight) of stool to efficiently convert cholesterol in the human gut, while a population containing more than 10^8 cells/g (wet weight) of stool leads to nearly complete conversion (Veiga et al. 2005). Moreover, a correlation was found between the overall structure of the fecal microbial community and the cholesterol-reducing activity in the human gut. Recently, high coprostanol levels in feces have been associated with the levels of bacteria belonging to the *Lachnospiraceae* and *Ruminococcaceae* families of the order *Clostridiales* (Antharam et al. 2016). Conversely, higher amounts of lactobacilli and bifidobacteria were observed in samples with higher levels of coprostanol in vegetarians and omnivorous subjects (Olejnikova et al. 2017) suggesting that this microbial conversion could be performed by distant phylogenetic bacteria.

Two major pathways have been proposed for the conversion of cholesterol to coprostanol (Fig. 1). The first pathway involves direct reduction of the 5-6 double bond. The second pathway starts with the oxidation of the 3β-hydroxy group and isomerization of the double bond to yield 4-cholesten-3-one, which undergoes two reductions to form coprostanone and then coprostanol. This second pathway is supported by the presence of coprostanone in human feces and by the reduction of intermediate products to coprostanol by fecal samples. Nevertheless, both pathways may coexist in the human gut (Macdonald et al. 1983).

Numerous attempts have been made in order to isolate cholesterol-reducing bacteria. A first cholesterol-reducing strain has been isolated from rat cecal contents, using a cholesterol-rich calf brain powder medium (Eyssen et al. 1973). Later on, several strains have been isolated from feces and intestinal contents of baboons. All these strains have been assigned to the genus *Eubacterium*. They require strict

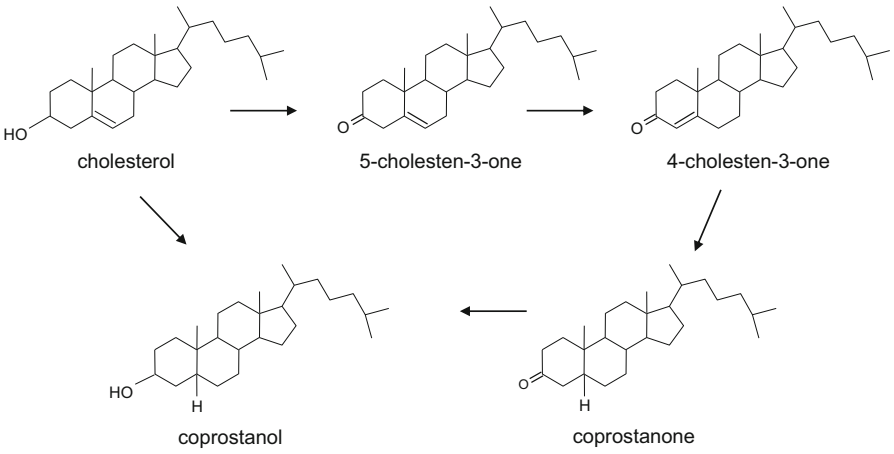

Fig. 1 Direct and indirect pathways for the conversion of cholesterol to coprostanol by the gut microbiota

anaerobic conditions for growth, and all but two strains require a plasmalogen (plasmenylethanolamine) to reduce cholesterol to coprostanol (Mott and Brinkley 1979). More recently, a small, anaerobic, gram-positive coccobacillus that reduces cholesterol to coprostanol was isolated from a hog sewage lagoon. This isolate, which does not require cholesterol or plasmalogen for growth, was named *Eubacterium coprostanoligenes* ATCC 51222^T (Freier et al. 1994). The mechanism of cholesterol reduction by *Eubacterium coprostanoligenes* was deciphered, showing that isomerization of the 5-6 double bond to a 4-5 double bond occurred via a mechanism involving the transfer of C-4 H to the C-6 position during the cholesterol-to-coprostanol conversion (Ren et al. 1996). This indicated an indirect pathway involving the formation of 4-cholesten-3-one. More recently, the first cholesterol-reducing bacterium from human origin has been isolated and characterized (Gérard et al. 2007). Unlike all other cholesterol-reducing strains isolated so far, phenotypic analyses revealed that this isolate belongs to the *Bacteroidetes* phylum. This bacterium was then named *Bacteroides* sp. strain D8 (Fig. 2). Phylogenetic tree construction showed that this strain clustered in an independent clade with the two isolates of the *Bacteroides dorei* species. Nevertheless, no cholesterol-reducing activity was detected in *B. dorei*-type strain cultures. Finally, strains of *Lactobacillus* have also been reported to remove cholesterol in vitro through its conversion to coprostanol (Lye et al. 2010).

As observed with *E. coprostanoligenes*, *Bacteroides* sp. strain D8 started to reduce cholesterol to coprostanol on the third day of growth in vitro, and 7 days were necessary to achieve complete cholesterol conversion. 4-Cholesten-3-one and coprostanone were detected during cholesterol conversion by *Bacteroides* sp. strain D8. Moreover, it was observed that this strain was able to convert 4-cholesten-3-one and coprostanone to coprostanol in vitro, suggesting an indirect pathway for coprostanol production by *Bacteroides* sp. strain D8. The cholesterol-to-coprostanol reduction efficiency of resting cells of *Bacteroides* sp. strain D8 was found to be 0.57 mg (1.5 μmol) cholesterol reduced/mg bacterial protein/h (Gérard et al.

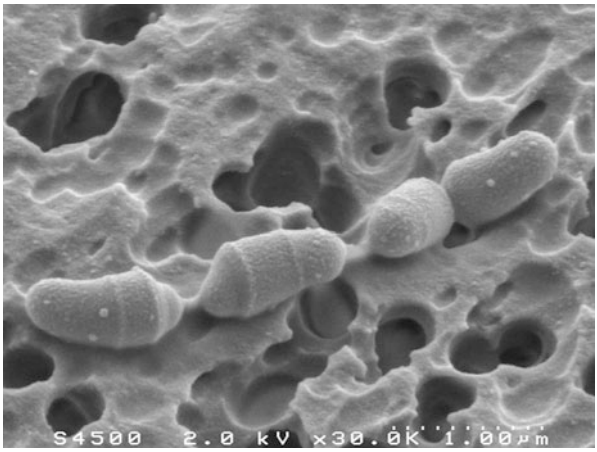

Fig. 2 Scanning electron micrograph of *Bacteroides* sp. strain D8, the first cholesterol-reducing bacterium isolated from human feces

2007), which is higher than the maximum yields previously obtained with *E. coprostanoligenes* ATCC 51222^T.

Coprostanol, unlike cholesterol, is poorly absorbed by the human intestine (Lichtenstein 1990). Hence, conversion of cholesterol to coprostanol might be a way of lowering serum cholesterol in humans and thus reducing the risk of cardiovascular disease. Indeed, our data suggest a relationship between intestinal microbiota and host cholesterol metabolism (Rabot et al. 2010), and it has been demonstrated that germfree conditions accelerates the atherosclerosis in ApoE-deficient mice (Stepankova et al. 2010). In humans, modulation of the gut microbiota by neomycin impacts serum cholesterol and fecal sterols in hypercholesterolemic patients (Miettinen 1979) and an inverse relationship have been observed between serum cholesterol levels and the coprostanol to cholesterol ratio in feces (Sekimoto et al. 1983). Therefore, several studies with animal models were designed to investigate the effect of microbial cholesterol metabolism in the gut on serum cholesterol concentration, through feeding of *E. coprostanoligenes*. In the first study, it was shown that oral administration of *E. coprostanoligenes* resulted in a significant decrease of plasma cholesterol concentration in dietary-induced hypercholesterolemic rabbits. Moreover, this hypocholesterolemic effect lasted for at least 34 days after the last bacterial feeding (Li et al. 1995). Concurrently, coprostanol-to-cholesterol ratio was found significantly greater in the digestive contents of treated rabbits. Nevertheless, the impact of cholesterol-to-coprostanol conversion on cholesterolemia is still unclear as oral administration of *E. coprostanoligenes* to laying hens and mice failed to affect plasma cholesterol (Li et al. 1996, 1998).

3 Metabolism of Bile Acids by the Gut Microbiota

Bile acids are saturated, hydroxylated C24 cyclopentanophenanthrene sterols. Primary bile acids (in humans, cholic and chenodeoxycholic acids are the two primary bile acids while rodents produce cholic and muricholic acids) are synthesized from cholesterol in the liver and conjugated to either taurine or glycine via an amide linkage at the C24 carboxyl. They are then excreted through the canaliculi to the biliary system. More than 95% of the bile acids secreted in bile are reabsorbed in the distal ileum and return to the liver. This processed is named enterohepatic circulation and 4 to 12 cycles occur each day. The main function of bile acids is to assist the absorption of dietary lipids and lipid-soluble nutrients. However, they are now recognized as signaling molecules through activation of receptors like Farnesoid X receptor (FXR) or Takeda G-protein-coupled receptor (TGR5). Therefore, they may modulate lipid, glucose, energy, and drug metabolisms as well as their own biosynthesis (Wahlström et al. 2016). The remaining bile acids (200–800 mg in humans) pass into the colon where they undergo bacterial metabolism. These modifications lead to the presence of over 20 different secondary bile acids in human feces. These bacterial conversions appear very early in life as 16 different bile acids were identified in meconium. The main bile salt conversions in the human gut include deconjugation; oxidation and epimerization of hydroxyl

Table 1 Bacterial genera of the gut microbiota involved in bile acid metabolism

Reactions	Bacterial genera
Deconjugation	*Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus*, *Listeria*, *Enterococcus*
Oxidation and epimerization	*Bacteroides*, *Clostridium*, *Escherichia*, *Eggerthella*, *Eubacterium*, *Peptostreptococcus*, *Ruminococcus*
7-Dehydroxylation	*Clostridium*, *Eubacterium*
Esterification	*Bacteroides*, *Eubacterium*, *Lactobacillus*
Desulfation	*Clostridium*, *Fusobacterium*, *Peptococcus*, *Pseudomonas*

groups at C3, C7, and C12; 7-dehydroxylation; esterification; and desulfation (Table 1) (Macdonald et al. 1983; Baron and Hylemon 1996; Ridlon et al. 2006). Microbial metabolism of bile acids leads to a more hydrophobic pool which favors elimination of bile acids in the feces.

3.1 Deconjugation

The hydrolysis of the C24 N-acyl amide bond of conjugated bile acids is catalyzed by constitutive, intracellular enzymes bile salt hydrolases (BSHs) (E.C.3.5.1.24). Most BSHs hydrolyze both glycine- and taurine-conjugated bile acids, whereas a few display strong specificity. Using a functional metagenomics approach, *bsh* coding sequences have been identified across both domains of life (bacterial and archaeal) and in all the major bacterial phyla in the gut (Jones et al. 2008). The enzyme has been purified from *Bacteroides fragilis*, *B. vulgatus*, *Clostridium perfringens*, *Listeria monocytogenes*, and several species of *Lactobacillus* and *Bifidobacterium*. Presence and arrangement of genes encoding BSH are highly variable, and up to four different genes have been identified in *Lactobacillus plantarum* (Ren et al. 2011). The crystal structure of BSH has been obtained for three bacterial species: *C. perfringens*, *B. longum*, and *L. salivarius*. They all display a αββα fold with differences in amino acid sequence within their binding pocket which could explain different substrate specificity (Xu et al. 2016). It has been suggested that BSH activity could be a way for bacterial species to detoxify bile acids. Besides, deconjugation was shown to improve the bacterial colonization of the gastrointestinal tract of mammals, and deletion of the *bsh* gene significantly reduced infectivity of *L. monocytogenes* in vivo (Dussurget et al. 2002). Lastly, some bacterial species may obtain carbon, nitrogen, and sulfur from bile acid deconjugation.

3.2 Oxidation and Epimerization of 3-, 7-, and 12-Hydroxy Groups

Bile acid hydroxysteroid dehydrogenases (HSDHs) from intestinal bacteria catalyze the reversible oxidation of hydroxy to oxo groups. HSDHs have been identified in

Actinobacteria, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Kisiela et al. 2012). Epimerization of hydroxyl groups occurs via stereospecific oxidation followed by stereospecific reduction of the resulting oxo group. Epimerization requires the actions of two stereochemically distinct HSDHs and can be performed by a single species containing both α- and β-HSDHs or by two species, one possessing an α-HSDH and the other a β-HSDH (Lepercq et al. 2004a).

3α- and 3β-HSDHs have been detected in the gut microbiota in several bacteria belonging to the *Firmicutes* phylum, whereas intraspecies 3-hydroxy epimerization has been observed only in *Blautia producta*, *C. perfringens*, and *Eggerthella lenta*.

7α-HSDHs are widespread among members of the genera *Clostridium*, *Eubacterium*, *Bacteroides*, or *Escherichia*, and 7β-HSDHs have been detected only in *Firmicutes*. Bacteria capable of intraspecies 7-epimerization include species of the genera *Clostridium*, *Eubacterium*, and *Ruminococcus* (Lepercq et al. 2004a, b). The genes encoding 7α-HSDHs have been cloned from several species, and the crystal structure of the *E. coli* 7α-HSDH has been solved.

12-Oxo bile acids are present at very low levels in the feces of healthy humans. 12α- or 12β-HSDHs have been detected in different members of the *Firmicutes*, but to date, no isolate has been found to possess both 12α- and 12β-HSDHs. Interestingly, comparative genomic analyses also identified a 12α-HSDH homolog in *Methanobrevibacter*, an archaea commonly found in human gut (Jones et al. 2008).

3.3 7-Dehydroxylation

The most quantitatively important and the most physiologically significant conversion of bile acids in humans is the 7α-dehydroxylation of the primary bile acids (cholic and chenodeoxycholic acids), leading to deoxycholic and lithocholic acids. Deoxycholic acid may therefore account for up to 25% of the total bile acid pool. 7α-Dehydroxylation increases the pKa and hydrophobicity of bile acids, favoring their passive absorption in the intestine. Most of the bacterial species which possess 7α-dehydroxylation activity are members of the order *Clostridiales*, including *Clostridium sordellii*, *C. hiranonis*, *C. bifermentans*, *C. hylemonae*, and *C. scindens*. Unlike bile acid oxidation and epimerization, 7α-dehydroxylation is restricted to free bile acids. The bile acid-inducible (bai) enzyme system which dehydroxylates 7α-hydroxy bile acids has been identified in *C. sordellii*, *C. hiranonis*, *C. hylemonae*, and *C. scindens* where it has been extensively characterized. It was first noticed that the induction of 7α-dehydroxylation activity in *C. scindens* by primary bile acids led to the production of new proteins (Paone and Hylemon 1984). Accumulation of multiple bile acid intermediates in cell extracts of *C. scindens* induced by cholic acid was then observed suggesting a multistep pathway for this metabolism (Hylemon et al. 1991). Later on, a *bai* regulon encoding at least ten open reading frames has been found. Finally, a second associated bai operon which catalyzes 7β reactions was recently discovered in

C. scindens. The bai operons involve genes for bile acid import, conversion, and export from bacterial cells (Ridlon et al. 2006).

3.4 Esterification and Desulfation

Saponifiable derivatives (esters) of bile acids account for 10–30% of the total bile acid content in human feces. Moreover, large amounts of deoxycholic acid oligomers, formed by esterification of the C-24 carboxyl group of one molecule with the 3α-hydroxy group of the next one, are detected in human feces (Macdonald et al. 1983). These esters are not present in bile suggesting their production by intestinal bacteria. Mixed fecal cultures were therefore found to convert bile acids to their C-24 ethyl esters, and this activity was detected in a few intestinal isolates belonging to the genera *Bacteroides*, *Eubacterium*, and *Lactobacillus* (Baron and Hylemon 1996).

Bile acid sulfatase activity has been detected in intestinal isolates belonging to the genera *Clostridium*, *Peptococcus*, *Fusobacterium*, and *Pseudomonas*. This activity requires a 3α- or 3β-sulfo group (bile acids sulfated at other positions are not desulfated) and a free C24 or C26 carboxyl group (Baron and Hylemon 1996). Up to this date, enzymes catalyzing the reaction have only been purified from *Pseudomonas testosteroni* (Tazuke et al. 1994).

3.5 Gut Microbiota-Bile Acid Interactions: Impact on Health and Disease

Recent studies revealed that bile acids exert a much wider range of biological activities than initially recognized (Zwicker and Agellon 2013). Moreover, it was established that secondary bile acids produced by the gut microbiota are present in peripheral tissues, including the liver, kidney, and heart, emphasizing their possible broad influence on mammalian homeostasis. Therefore gut microbiota dysbiosis and associated bile acid alterations may contribute to diverse diseases that manifest outside of the GI tract. As an example, it was recently demonstrated that the dysbiosis observed in inflammatory bowel disease leads to decreased bile acid deconjugation and desulfation activities and then to a modification in the luminal bile acid pool composition which may contribute to chronic inflammation. As bile acids are ligands for host receptors (including the Farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR5) important for host metabolism), they may play a central role in metabolic diseases including obesity, diabetes, or liver diseases. Importantly, these receptors have different affinities for individual bile acids: the stronger natural FXR agonists are CDCA>DCA>CA>LCA, while Tα- and TβMCA and UDCA are antagonists. Similarly, bile acids activate TGR5 with different potencies (LCA>DCA>CDCA>CA). Activation of these receptors by their respective

agonists has been shown to protect from inflammation in the gut and the liver. However, it was also shown that the gut microbiota can induce obesity through FXR signaling (Parseus et al. 2017). Overall levels of BSH activity in the gut may also affect host metabolism including weight gain or cholesterolemia. Indeed, deconjugation leads to increased fecal excretion of bile acids and consequently to higher consumption of cholesterol for de novo synthesis of bile salts in the liver which can conduce to reduced serum cholesterol. Accordingly, a few clinical studies have demonstrated a potential for BSH-expressing microbes to treat mild hypercholesterolemia. Besides, the dehydroxylation of chenodeoxycholic acid leads to lithocholic acid which is toxic to liver cells and has been linked to colon carcinogenesis. Similarly, high levels of deoxycholic acid (known to cause DNA damage) in blood and feces are associated with increased risks of cholesterol gallstone disease and colon and liver cancer (Yoshimoto et al. 2013). Conversely, ursodeoxycholic acid, produced by the epimerization of the 7α-hydroxyl group of chenodeoxycholic acid, is thought to be chemopreventive and is used to treat cholesterol gallstones. Finally, because only a limited fraction of the gut bacteria possesses the capacity to produce secondary bile acids, any alteration of these species may have important health consequences to the host.

4 Research Needs

Mechanisms of cholesterol metabolism by the gut microbiota have been well examined. Nevertheless, only a few cholesterol-reducing bacteria have been isolated, and the genes or enzymes involved in this metabolism are still unknown. Identification of these genes would be a prerequisite to a possible application for medical purpose. Concurrently, more studies on animal models, specially using gnotobiotic animals, are needed to determine the effect of this bacterial metabolism on plasma cholesterol. Interestingly, it was recently shown that human microbiota retained its level of cholesterol-reducing bacterial population and cholesterol-reducing activity in gnotobiotic rats (Gérard et al. 2004). Rats harboring a human gut microbiota could therefore be used as a model to explore the impact on health of microbial cholesterol metabolism.

Bile acid metabolism is recognized as a microbiota-associated characteristic and is widespread among the main genera constituting the human gut microbiota. Nevertheless, our knowledge of bacterial bile acid metabolism has been mainly obtained from classical culturing techniques. Molecular techniques, particularly sequencing of bacterial genomes and of the human gut microbiome, should allow the discovery of novel genes involved in bile acid metabolism and to understand the real diversity of bile acid-converting bacteria. This may help to define the relationship between these bacterial populations and disease risks, including cholesterol gallstone, metabolic and liver diseases, and colon cancer, and could lead to new therapies targeting the gut microbiota.

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Microbial Oils as Nutraceuticals and Animal Feeds

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Beatriz Galán, María Santos-Merino, Juan Nogales, Fernando de la Cruz, and José L. García

Contents

B. Galán
Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas (CIB) (CSIC), Madrid, Spain
e-mail: bgalan@cib.csic.es

M. Santos-Merino · F. de la Cruz
Intergenomic Group, Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) (UC-CSIC), Santander, Spain
e-mail: carmen.santos@unican.es; fernando.cruz@unican.es

J. Nogales
Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas (CIB) (CSIC), Madrid, Spain

Department of Systems Biology, Centro Nacional de Biotecnología (CNB) (CSIC), Madrid, Spain
e-mail: jnogales@cnb.csic.es

J. L. García (✉)
Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas (CIB) (CSIC), Madrid, Spain

Department of Applied Systems Biology and Synthetic Biology, Instituto de Biología Integrativa de Sistemas (I2Sysbio) (CSIC-UV), Valencia, Spain
e-mail: jlgarcia@cib.csic.es

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Abstract

Lipids and oils are produced by all single-cell organisms for essential structural and functional roles; however, the term single cell oils (SCOs) is mainly restricted to describe the lipids produced by a limited number of oleaginous microorganisms (archaea, bacteria, yeast, fungi, and microalgae) with oil contents higher than 20% of biomass weigh. SCOs have different fatty acid compositions from those of plant seed or fish oils and are nowadays considered as new sources of nutraceuticals and animal feeds. In spite of the current commercial success of some SCOs, the development of more efficient microbial fermentation processes, and the possibility of manipulating by systems metabolic engineering, the lipid composition of cells requires new biotechnological strategies to obtain high yields of the desired SCOs. Understanding the synthesis and regulatory mechanisms involved in the production of SCOs is fundamental to eliminate the metabolic bottlenecks that impair achieving high oil yields.

1 Introduction

The term, single cell oils (SCOs), also named microbial lipids or microbial oils, was created by Ratledge and Wynn (1974) to identify those lipids of single-cell organisms – microorganisms – that would be suitable for human and animal consumption as an alternative to plant and animal oils and fats. SCO was initially created to designate the triacylglycerol (TAG) fraction of the total cell lipids to be equivalent to the commercial plant and animal oils. However, SCO is now used to include all types of fatty acid (FA) containing lipids, and therefore, SCO includes not only free fatty acids and TAGs but also other complex lipids such as glycosylated and sulfur-containing lipids.

Lipids and oils are produced by all single-cell organisms for essential structural and functional roles; however, the term SCO is nowadays mainly restricted to describe the lipids produced by a limited number of organisms, named oleaginous

microorganisms (see below), which are those species able to accumulate 20–80% of lipids per dry biomass as a reserve storage material.

Although the prospects of obtaining useful and cheap SCO have been considered more than 60 years ago (Ratledge 2013), it was only in the past three decades that they have begun to be produced commercially at large scale for food and feed markets (Béligon et al. 2016). More recently, SCO has been considered as possible source of biofuels opening new perspectives for the energy and transport industrial sectors (Hu et al. 2008).

One of the main advantages of SCOs is that their production processes are independent from season, climate, and location, and moreover, they can be synthesized using a wide range of carbon sources including organic waste and renewable carbon sources (see below).

On the other hand, because SCOs have different FA compositions from those of plant seed or fish oils, they are considered as new sources of nutraceuticals highly valuable for human life. Nutraceuticals can be defined as foodstuffs, such as a fortified food or dietary supplement that provides health benefits in addition to its basic nutritional value. Nevertheless, this term and its applications have not attained a common regulatory definition in all countries so far.

In spite of the current commercial success of some SCOs, the development of more efficient microbial fermentation processes and the possibility of manipulating the lipid composition of cells require new biotechnological strategies to obtain high yields of the desired SCOs. Although recombinant oleaginous microorganisms can be engineered and accepted by industry and society, nonetheless, several approaches have been also employed to convert other model GRAS (generally regarded as safe) microorganisms into oleaginous cells by genetic engineering.

Therefore, taking into account all these considerations, there is a growing interest to know how the microbial oils can be produced in a cost-effective manner and how they can be tailor-made to meet the demands of the two most important branches of the oil industries: oils for human and animal consumption and oils for biofuels. However, only the food and feed uses of microbial oils will be reviewed in this chapter. In this sense, other recent journal reviews can provide complementary information on this matter (Béligon et al. 2016; Bellou et al. 2016; Lee et al. 2016; Ochsenreither et al. 2016; Yoshida et al. 2016; Bharathiraja et al. 2017).

2 Oleaginous Microorganisms

As mentioned above oleaginous microorganisms are defined as cells with oil contents higher than 20% of biomass weigh. Single cell oils are produced by different microorganism groups such as archaea, bacteria, algae, yeasts, and fungi. When growing on limiting concentrations of a key nutrient (typically nitrogen) and enough or excess of carbon source, these microorganisms will utilize the available carbon to synthetize and store it as reduced lipids like TAG or sterol esters (SE). Table 1 summarized the most important oleaginous microorganisms described so far. While the eukaryotic microorganisms synthesize TAGs with similar compositions to

Table 1 Most relevant oleaginous microorganisms and marketed products

Organism	Product	Company
Bacteria	Lipids	
Rhodococcus opacus	Lipids	
Rhodococcus jostii	Lipids	
Yeast		
Yarrowia lipolytica	EPA. EPA-SCO	E.I. du Pont (USA)
Rhodosporidium toruloides	Lipids	
Rhodotorula glutinis	Lipids	
Lipomyces starkeyi	Lipids	
Cryptococcus albidus	Lipids	
Candida curvata	Lipids	
Fungi		
Mucor (javanicus) circinelloides	Oil of Javanicus (GLA-rich oil)	J&E Sturge (UK)
Cunninghamella echinulata	GLA. DHA-SCO	Martek/DSM (The Netherlands)
Mortierella isabellina	ALA. GLA	Sigma (USA)
Mortierella alpina	ARA. EPA. ALA. ARA-SCO. CABIO-oil	DSM (The Netherlands). Cargill Alking Bioengineering Co. Ltd. (Hubei, China). Suntory Co. (Japan)
Pythium ultimum	Lipids	
Rhizopus arrhizus	Lipids	
Microalgae		
Nannochloropsis sp.	EPA	Aurora. Cellana. Qualitas Health. Necton. Reed Mariculture. Fitoplanton Marino. Yantai. Astaxa. Proviron. Archimede
Schizochytrium sp.	DHA. DHA-SCO-S. DHA for plus. DHAgold. NeoGreen. AlgaPrime DHA. EPA	Martek/DSM (The Netherlands). Alltech (USA). DSM Nutritional Products (USA). Coppens International BV (The Netherlands). TerraVia Holdings Inc. (formerly Solazyme, USA) and Bunge Ltd. (USA). Veramaris (DSM-Evonick)
Phaeodactylum tricornutum	DHA. EPA	Micoperi Blue Growth (Italy)

(continued)

Table 1 (continued)

Organism	Product	Company
Porphyridium cruentum	EPA	Asta Technologies Ltd. (The Netherlands)
Chaetoceros sp.	DHA	
Isochrysis galbana	DHA	Symrise AG (Germany)
Crypthecodinium cohnii	EPA	Martek/DSM (The Netherlands)
Chlorella sp.	DHA	
Thraustochytriidae sp.	DHA	
Aurantiochytrium sp.	DHA DHA-natur	Lubrizol Corp (USA) ADM Animal Nutrition (USA)
Ulkenia sp.	DHA. DHA-CL. DHA-*Aid*	Lonza (Switzerland)
Algae	Onavita DHA	ADM (USA)
Algae	Onavita ALA	ADM (USA)

vegetable oils, prokaryotic cells also synthesize other neutral lipids (e.g., polyhydroxyalkanoates (PHAs) and wax esters (WEs)), and thus the peculiarities of these organisms are described below case by case.

2.1 Archaea

Archaea do not synthesize fatty acyl esters, instead, their lipids are based on isoprenoid chains. Archaeal membrane lipids are very different from those of bacteria and eukaryotes as they are made up of saturated chains containing methyl branches, attached to glycerol by ether linkages with a stereochemistry in the two positions of the glycerol opposite that of conventional mesophilic lipids. So far, no accumulation of TAGs has been reported yet in archaea. However, as in bacteria, archaea produce PHA under conditions of nutrient limitation when carbon is available in excess.

2.2 Bacteria

Generally, bacteria produce SCO with a composition quite different from other microorganisms. Bacteria can produce a large variety of complex neutral and polar lipids, but only few of them can accumulate large amounts of TAGs. Oleaginous bacteria have the advantage of showing high cell growth rates under simple

cultivation methods. In bacteria, the most abundant class of neutral lipids used as intracellular carbon and energy storage compounds are PHAs (bioplastics), such as polyhydroxybutyrate (PHB) or polyhydroxyvalerate (PHV), TAG, WE, and, to a lesser extent, SE.

Unlike eukaryotes only a minority of prokaryotes can accumulate TAG or WE. The highest level of TAG accumulations has been reported in the actinomycetes group, such as the genera *Mycobacterium*, *Streptomyces*, *Rhodococcus, Micromonospora, Dietzia, Gordonia*, and *Nocardia* (Kosa and Ragauskas 2011). TAGs in cells of *Rhodococcus opacus* cells accounted up to 87% of the cellular dry weight. Some bacteria such as *Rhodococcus ruber* are capable of accumulating both PHAs and TAGs (Garay et al. 2014). Accumulation of WE of about 200 nm diameter has been reported in some *Acinetobacter* spp. (Wältermann and Steinbüchel 2005). Some marine-related *λ-Proteobacteria* like *Alcanivorax borkumensis* accumulate while growing in petroleum hydrocarbons' lipid droplet (LD) reserves that consist of mixtures of TAGs and WE (Kalscheuer et al. 2007). Numerous bacterial species of marine origin have now been shown to produce very long-chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Such isolates have been found to be particularly prevalent in high-pressure, low-temperature deep-sea habitats and permanently cold marine environments (DeLong and Yayanos 1986; Yano et al. 1997).

2.3 Yeasts

Most oleaginous yeast can accumulate lipids at levels of more than 40% of their dry weight and as much as 70% under nutrient-limiting conditions. However, the lipid content differs, and fatty acid profiles differ between species. The oily yeast genera include *Candida, Cryptococcus, Lipomyces, Rhodosporidium, Rhodotorula, Rhizpus, Trichosporon*, and *Yarrowia*. Yeasts exhibit advantages for lipid production due mainly to their low duplication times and metabolic versatility. They can utilize many different carbon sources (e.g., glucose, xylose, glycerol, starch, cellulose hydrolysates, and industrial and municipal organic wastes) for the production of different lipids like TAG, surfactants, or PUFAs. The yeast *Lipomyces starkeyi* is unique because it does not reutilize their own lipids accumulating high amounts of lipids (Holdsworth et al. 1988).

2.4 Fungi

Oleaginous fungi are reported to accumulate intracellular lipid up to 50–70% in the form of cytosolic lipid bodies mainly composed of TAGs (Sancholle and Lösel 1995). The distribution and diameters of their lipid bodies vary with the organism, growth phase, and environmental conditions. Glucose, lactose, starches, oils, corn steep liquor, and agricultural waste have been used as carbon sources for lipid production in fungi (Thevenieau and Nicaud 2013).

The diversity of fungal species has facilitated the selection of oleaginous strains as they can compete at commercial scale with the traditional lipid production from plant and animal sources on the basis of several reasons: (i) high growth rates, (ii) the ability to grow on cheap waste materials as substrate for oil production, (iii) the controlled environment that is not affected by seasonal and climatic conditions, and (iv) the possibility to engineer the key steps of lipid synthesizing enzymes to end up with the formation of lipid and other valuable products such as PUFAs. Fungi species, such as *Aspergillus terreus*, *Claviceps purpurea*, *Tolyposporium*, *Mortierella alpina*, or *Mortierella isabellina*, are reported to accumulate lipids (Bellou et al. 2012). These fungi are explored mainly for the production of special lipids such as PUFAs. The oleaginous fungus, *Mucor rouxii*, is known to accumulate high amounts of intracellular lipids and γ-linolenic acid (GLA). *Mucor alpine* and *Mortierella alliacea* have a high productivity of arachidonic acid (ARA) (Eroshin et al. 2000; Aki et al. 2001). The production of PUFAs is related to the age of the mycelia, since the production decreases as the cells grew older (Fakas et al. 2009).

2.5 Microalgae (Cyanobacteria and Algae)

The term microalgae, in applied phycology, usually includes the microscopic algae sensu stricto and the photosynthetic bacteria (i.e., cyanobacteria), formerly known as *Cyanophyceae*. The cell structure is eukaryotic in microalgae and prokaryotic in cyanobacteria. Several microalgae species are able to produce large amounts of neutral lipids, typically in the form of TAGs as storage products for carbon and energy under specific environmental stress conditions, such as nitrogen or phosphate limitations. The lipid content can reach up to 80% in dry biomass, and therefore these microorganisms are referred to as oleaginous microalgae (Chisti 2007; Hu et al. 2008). The interest in algae lipids arises mainly from the fact that these organisms are able to synthesize considerable quantities of PUFAs that reach humans or animals via the food chain or are used as food supplements (Bellou et al. 2014).

In some species belonging to *Porphyridium*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Tetraselmis*, *Phaeodactylum*, *Chlorella*, and *Schizochytrium*, lipid content varies between 30 and 50%. Higher productivities can be reached by varying the culture conditions, like, for example, temperature, irradiance, or nutrient availability. The indisputable advantage of microalgae is that they can use carbon dioxide and sunlight as carbon and energy sources, respectively, for photoautotrophic growth. In addition, they can use organic carbon under heterotrophic or mixotrophic culture conditions.

Microalgae applications range from human and animal nutrition to cosmetics and the production of high-value molecules. The majority of applications concern biomass production destined for animal or human consumption. There is also an increasing interest in the use of microalgae lipids in numerous commercial applications, such as food, chemical, and pharmaceutical industries (Bellou et al. 2014). However, there are still some limitations in using microalgae to produce PUFAs in large scale due to the low biomass density in the reactors under industrial conditions.

There are few strains with special fatty acids biosynthetic capacity and therefore of industrial interest. The most prominent DHA producer among microalgae is the heterotrophic dinoflagellate *Crypthecodinium cohnii* containing more than 50% (w/w) DHA of total fatty acids (Jiang and Chen 2000; Ratledge et al. 2001; de Swaaf et al. 2003a,b). Other significant DHA producers are microalgae of the genus *Schizochytrium*, e.g., *Schizochytrium* sp. S31 (Wu et al. 2005), *Schizochytrium* G13/2S (Ganuza and Izquierdo 2007), and *Schizochytrium limacinum* (Chi et al. 2007). *Amphidinium* sp. and *Prorocentrum triestinum* are also known as efficient DHA producers (Makri et al. 2011). *Porphyridium cruentum* and *Nannochloropsis salina* are able to synthetize EPA with content of 25% of total lipids (Bellou and Aggelis 2013).

3 Microbial Oil Biosynthesis

3.1 Pathways

There are two sequential steps in the synthesis of microbial oils: FA and TAG syntheses (Ratledge 2004; Garay et al. 2014). TAGs are also known as "neutral lipids."

The pathway for saturated FA synthesis is conserved among microbial species. It starts from acetyl-CoA. In yeast, fungi, and bacteria, acetyl-CoA is produced from organic carbon sources (glucose, acetate, etc.). Microalgae, on the other hand, may use either inorganic carbon (CO_2) or organic carbon sources (Liang and Jiang. 2013). Carboxylation of acetyl-CoA forms malonyl-CoA. This molecule serves as a building block for several steps of condensation and reduction, resulting in FAs of different chain length.

The synthesis of saturated FAs is catalyzed by the enzyme fatty acid synthase (FAS). There are two groups of FAS, based on the organization of their catalytic units. Type I FAS, present in fungi and yeast genomes, carries out all steps of fatty acid biosynthesis as a multimeric protein complex (Schweizer and Hofmann 2004). Type II FAS is composed of independent polypeptides and is found in bacteria and microalgae (White et al. 2005). Regardless of their classification, both types of FAS include the same seven enzymatic components: a malonyl/acetyltransferase, an acyl carrier protein, a ketoacyl synthase, a ketoacyl reductase, a dehydrase, an enoyl reductase, and a thioesterase.

The first committed step of FA synthesis is catalyzed by acetyl-CoA carboxylase (ACC) and consists of an ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA (Fig. 1a). This reaction takes place in the cytosol in heterotrophs or both, cytosol and plastids, in autotrophs (Bellou et al. 2016). As in the case of FAS, there are two different forms of ACC. The first one, present in prokaryotes (AccABCD) and archaea, consists of three functional enzymes: biotin carboxylase (BC, AccC), biotin carboxyl carrier protein (BCCP, AccB), and carboxyltransferase (CT, AccA, and AccD) (Guchhait et al. 1974; Lombard and Moreira 2011). The eukaryotic form

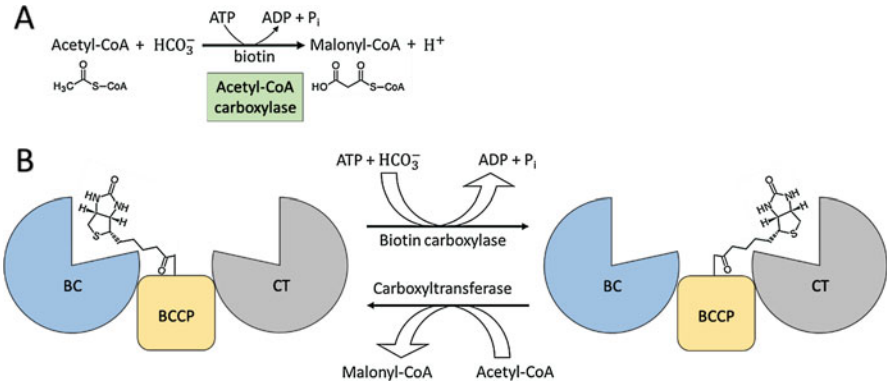

Fig. 1 Reaction catalyzed by the ACC. (**a**) Overall reaction catalyzed by ACC. (**b**) The two steps of the reaction catalyzed by ACC. In the first step, BC catalyzes the carboxylation of biotin, a cofactor covalently linked to BCCP. In the second step, the carboxyl group is transferred from carboxyl-BCCP to acetyl-CoA by CT

(Acc1) consists of three functional domains, homologous to the bacterial subunits, on a single polypeptide (Konishi and Sasaki 1994). The reaction proceeds in two steps and is biotin-dependent, as shown in Fig. 1b (Tran et al. 2015).

Malonyl-CoA is subsequently transformed into malonyl-ACP by the action of malonyl-CoA: ACP transacylase (MAT). In type I FAS, this reaction is catalyzed by FabD. The next step is carried out by the β-ketoacyl-ACP synthases (KS). These enzymes are involved in FA chain extension via Claisen condensation of fatty acyl-thioesters and malonyl-ACP to form a β-ketoacyl-ACP intermediate elongated by two carbon atoms.

Typically, there are two or three KSs involved in type II FAS: FabH (KS III), FabF (KS II), and FabB (KS I). In many cases, the *fabB* gene is absent (Kuo and Khosla 2014; Wang and Cronan 2004). The initial cycle of elongation is catalyzed by FabH, involving condensation of malonyl-ACP and acetyl-CoA, while subsequent cycles of elongation are performed by FabB or FabF. On the other hand, type I FAS contains only one KS. Following the condensation reaction, the β-ketoacyl-ACP is reduced to β-hydroxyacyl-ACP using NADPH and H^+. This reaction is catalyzed by a β-ketoacyl-ACP reductase (KR). The same enzyme is denominated FabG in type II FAS. After that, β-hydroxyacyl-ACP is dehydrated to trans-2-enoyl-ACP by water elimination. The enzyme involved in this reaction is a β-hydroxyacyl-ACP dehydrase (DH). In type II FAS, this reaction may be catalyzed by FabA or FabZ. However, in some organisms in which desaturation step is not dependent of type II FAS, such as cyanobacteria or gram-positive bacteria, FabA is absent. The last step in each elongation cycle is carried out by an enoyl-ACP reductase (ER). This enzyme catalyzes the reduction of 2-enoyl-ACP to fatty acyl-ACP at the expense of H^+ and NADPH/NADH. In type II FAS, this enzyme is denominated FabI. Finally, acyl-ACP-thioesterase (FAT) cleaves the acyl chain and liberates the FA. The pathway is represented in Fig. 2.

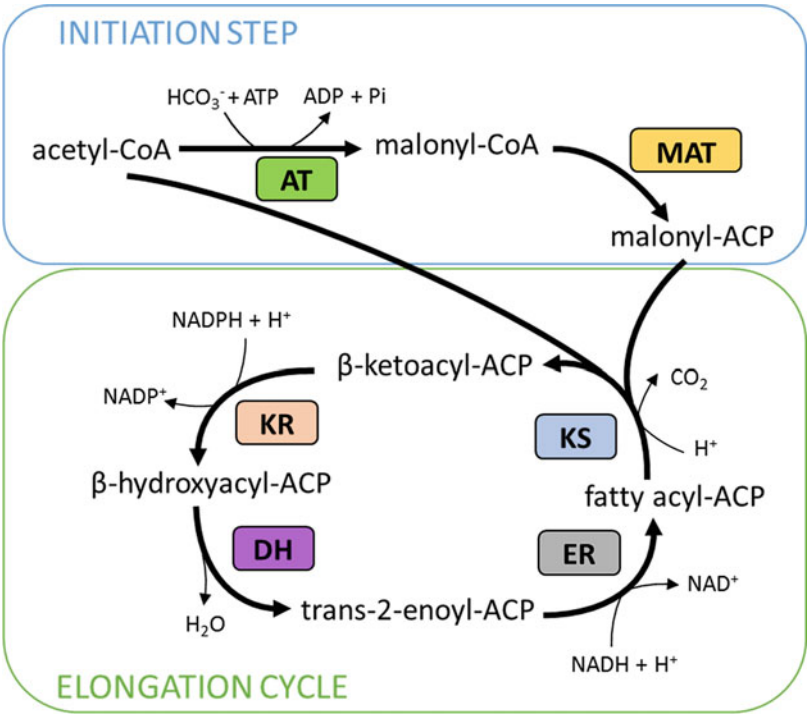

Fig. 2 Fatty acid synthesis pathway

There is considerable diversity in the mechanisms used by bacteria to generate unsaturated FAs (uFAs). In most bacteria, the bifunctional FabA is involved in the synthesis of uFAs (Heath and Rock 1996) (Fig. 3a). In *Streptococcus pneumoniae*, FabM is the enzyme responsible for introducing the double bond, which is unrelated to FabA, but it is a member of the hydratase/isomerase superfamily (Marrakchi et al. 2002). In some bacteria, there is an alternative route for the generation of uFAs after the elongation cycle. *Bacillus subtilis* expresses a desaturase, Des, which insert a double bond in FA chains (Altabe et al. 2003). *Pseudomonas aeruginosa* produces uFAs using FabA/B pathway. However, it possesses two aerobic desaturases: DesA, introduces the double bond into acyl chains attached to phospholipids and has a similar structure to the *B. subtilis* Des enzyme, and DesB, an inducible acyl-CoA Δ9-desaturase (Zhu et al. 2006).

One pathway for PUFA synthesis involves the concerted action of desaturases and elongases (Uttaro 2006) (Fig. 3b). It is found in lower eukaryotes and is denominated the aerobic pathway, because desaturation is an aerobic process that uses molecular oxygen. Some yeast species, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, synthesize FAs up to oleic acid (C18:1n-9). These species only possess a Δ9-desaturase (OLE1). Other yeasts, such as *Saccharomyces kluyveri* and *Kluyveromyces lactis*, are able to produce up to ALA (alpha-linolenic

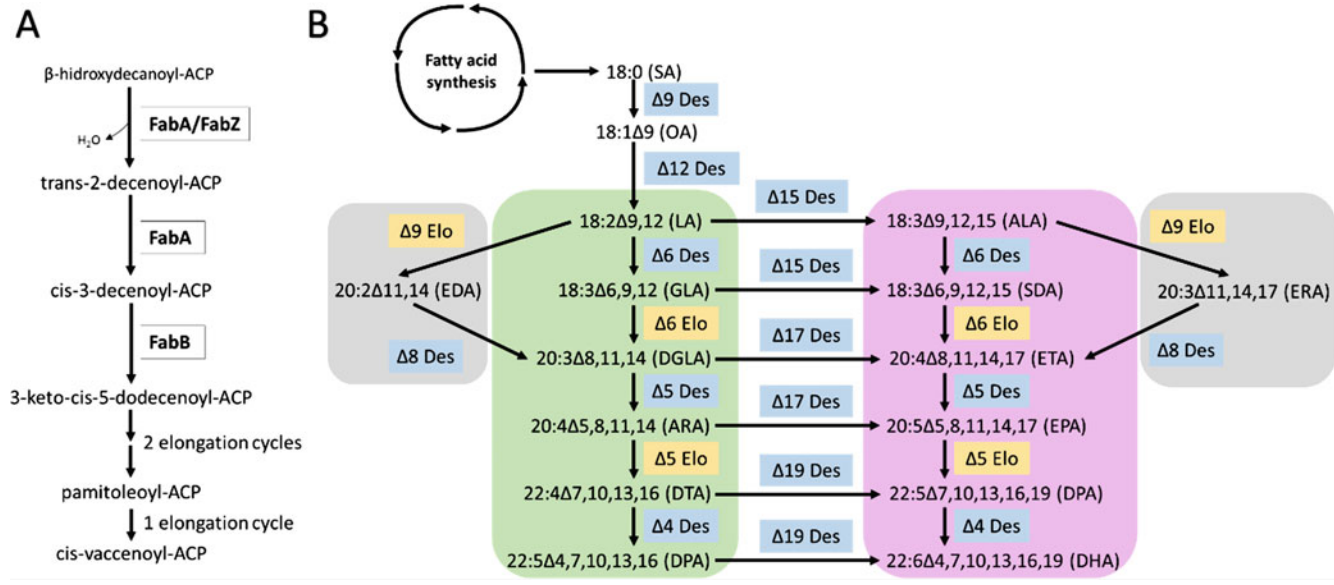

Fig. 3 Unsaturated fatty acid synthesis. (**a**) Synthesis of unsaturated fatty acids via FAS II in most bacteria. FabA carries out the dehydration of β-hydrodecanoyl-ACP to trans-2-decenoyl-ACP. After that, it performs the isomerization of trans-2-decenoyl-ACP to cis-3-decenoyl-ACP. The product of FabA isomerization reaction is used by FabB, skipping the reductase step and initiating the elongation of a monounsaturated FA. The product of the first two elongation cycles is palmitoleoyl-ACP, which may be the substrate for the next elongation cycle to produce cis-vaccenoyl-ACP. (**b**) Synthesis of PUFAs via aerobic pathway. The green-shaded and the pink-shaded areas represent omega-6 and omega-3 biosynthesis pathway (conventional Δ6-pathway), respectively. The gray-shaded area represents the alternative Δ8-pathway. Desaturases and elongases are represented by blue and yellow rectangles, respectively. Fatty acids are OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, di-homo-γ-linolenic acid; ARA, arachidonic acid; ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid

acid; C18:3n-3). Both possess a Δ9-desaturase, as well as Δ12-desaturase and Δ15-desaturase (also known as ω3-desaturase) (Uemura 2012).

A number of lower eukaryotes such as fungi and microalgae produce large amounts of PUFAs with chain lengths of C20 and greater (Uttaro 2006), following the aerobic pathway. It is predicted that these organisms contain the complete set of enzymes involved in the transformation of stearic acid to EPA (20:5n-3) and DHA (C22:6n-3). Other lower eukaryotes also hold a Δ8-desaturase involved in an alternate aerobic pathway to produce C20 PUFAs (Arao and Yamada 1994; Qi et al. 2002; Wallis and Browse 1999). Most of the higher producers of EPA and DHA are microalgae and fungi, inhabitants of cold marine environments.

The anaerobic pathway occurs in eukaryotic microalgae and some bacteria (marine bacteria and terrestrial myxobacteria). In these organisms, PUFA synthases are huge enzyme complexes with multiple catalytic domains, denominated polyketide synthases (PKSs). PKSs carry out the same reactions as FAS and use the same small protein, an acyl carrier protein (ACP), as a covalent attachment site for the growing carbon chain (Metz et al. 2001; Hayashi et al. 2016; Cronan and Thomas 2009).

The most important anaerobic pathway to produce TAGs is the *sn*-glycerol-3-phosphate (G3P) or Kennedy pathway (Fig. 4). In the first step, G3P is acylated with an acyl-CoA to form lysophosphatidate (LPA), which is catalyzed by G3P O-acyltransferase (GPAT). LPA is further condensed by LPA acyltransferase (LPAT), with another acyl-CoA to produce phosphatidate (PA). Afterward, PA can be dephosphorylated by phosphatidic acid phosphatase (PAP) to produce diacylglycerol (DAG). Finally, synthesis of TAG is catalyzed by acyl-CoA:

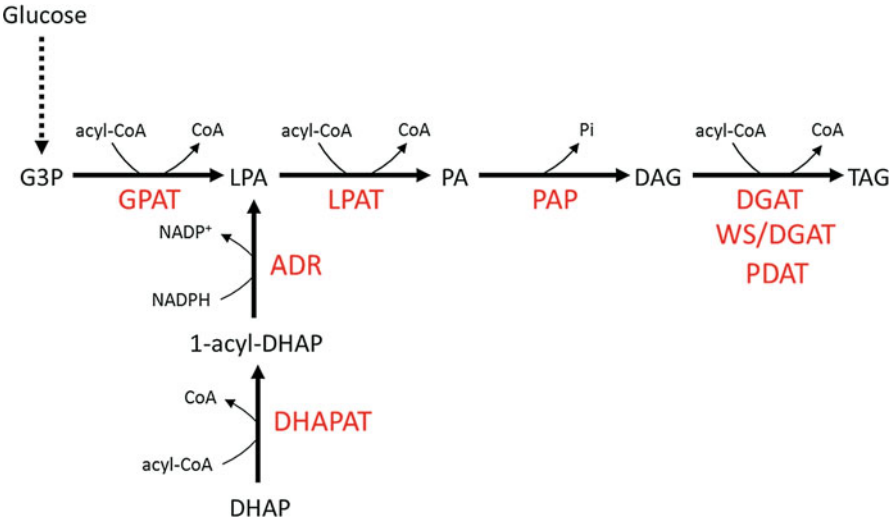

Fig. 4 Schematic diagram of the main lipid classes and biochemical pathways involved in the production of TAG

diacylglycerol acyltransferase (DGAT), which incorporates the third acyl-CoA into DAG (Amara et al. 2016). In yeast, in addition to DGAT-catalyzed TAG formation, there is an acyl-CoA-independent biosynthesis of TAG. The enzyme involved in this process, phospholipid:diacylglycerol acyltransferase (PDAT), transfers an acyl group from the *sn*-2 position of phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine) to *sn*-3 position of diacylglycerol, yielding TAG and sn-1-lysophospholipid (Banas et al. 2013). It was also found that *Acinetobacter calcoaceticus* ADP1, a bacteria able to accumulate both WE and TAG, has a bifunctional WE synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) that exhibits both WE synthase and DGAT activities (Stöveken et al. 2005).

In yeast, LPA is synthetized from two precursors: G3P and dihydroxyacetone phosphate (DHAP) (Athenstaedt et al. 1999). Synthesis of LPA from DHAP occurs in two acylation steps. The first is catalyzed by a DHAP acyltransferase (DHAPAT), which transform DHAP into 1-acyldihydroxyacetone phosphate (1-acyl-DHAP). In the second step, 1-acyl-DHAP is reduced in an NADPH-dependent reaction catalyzed by 1-acyldihydroxyacetone phosphate reductase (ADR), yielding LPA (Carman and Han 2009). The reactions sequence is also represented in Fig. 4.

3.2 Regulation

There is little information about the regulation of TAG synthesis in oleaginous microorganisms. In the most widely studied and engineered oleaginous yeast, *Yarrowia lipolytica*, the regulatory network of TAG biosynthesis is starting to be elucidated (Zhu and Jackson 2015). *Y. lipolytica* is a promising microbial cell factory for the production of TAGs, because it can accumulate a large amount of fatty acids in the form of the storage lipid TAG in the cell (Seip et al. 2013; Kerkhoven et al. 2016) reconstructed a genome-scale metabolic model of this yeast and used this for integrative analysis of multilevel omics data. Metabolite profiling and lipidomics were used to quantify the cellular physiology, while regulatory changes were measured using RNAseq. Analysis of the data showed that lipid accumulation in *Y. lipolytica* does not involve transcriptional regulation of lipid metabolism but is associated with regulation of amino acid biosynthesis, resulting in redirection of carbon flux during nitrogen limitation from amino acids to lipids.

Limitation of nitrogen during continued growth of *Y. lipolytica* provokes a dramatic response in the biomass composition and an increase in virtually all lipids. *Y. lipolytica* under nitrogen limitation attempts to minimize the usage of nitrogen. In these conditions, transcripts related to amino acid metabolism are downregulated. Nitrogen restriction triggers nitrogen catabolite repression, which is regulated by interaction of four GATA transcription factors: Gln3, Gat1, Gzf3, and Dal80 (Kerkhoven et al. 2016). These transcription factors are characterized by their ability to bind to the DNA sequence "GATA." As an alternative route to recycle nitrogen, *Y. lipolytica* has an ortholog of the *Aspergillus nidulans* xanthine dehydrogenase (Cultrone et al. 2005), which is absent in *S. cerevisiae*. By means of this pathway, purines can be degraded via allantoin to release ammonia. Several genes in this

pathway are under control by Gat1 and Gln3, whereas this whole pathway is upregulated during nitrogen restriction. A more central role in nitrogen sensing and signalling is played by the TOR complex (Zhang et al. 2011). High-quality nitrogen sources such as ammonium or glutamine stimulated the activation of TOR complex (Stracka et al. 2014), whereas nitrogen starvation inhibits this complex (Zaman et al. 2008). Based on a study of differential gene expression profile, Kerkhoven et al. determined the upregulation of autophagy, proteasome, peptidases, and ubiquitylation mediated by TOR complex during nitrogen starvation (Kerkhoven et al. 2016). Another key regulator of metabolism is Snf1, a protein kinase that is involved in many signalling pathways. In *S. cerevisiae* Snf1 inhibits the activity of the acetyl-CoA carboxylase Acc1 by phosphorylation (Shi et al. 2014), and these phosphorylation sites on Acc1 are conserved in *Y. lipolytica* Acc1. Moreover, Snf1 increases expression of β-oxidation. Nonetheless, it is likely that additional regulation takes place such as posttranslational modifications, as it is known that Acc1 activity is repressed by phosphorylation by Snf1 (Kerkhoven et al. 2016). This regulatory pathway is represented in Fig. 5.

In microalgae, the mechanism of nitrogen starvation induced neutral lipid accumulation. When the nitrogen levels required for the protein synthesis of growing cells are insufficient, the excess of carbon from photosynthesis is diverted into storage molecules, such as TAGs (Scott et al. 2010). The phenomenon of nitrogen starvation in microalgae has been analyzed using diverse omics approaches (Blaby et al. 2013; Dong et al. 2013; Schmollinger et al. 2014; Park et al. 2015). These

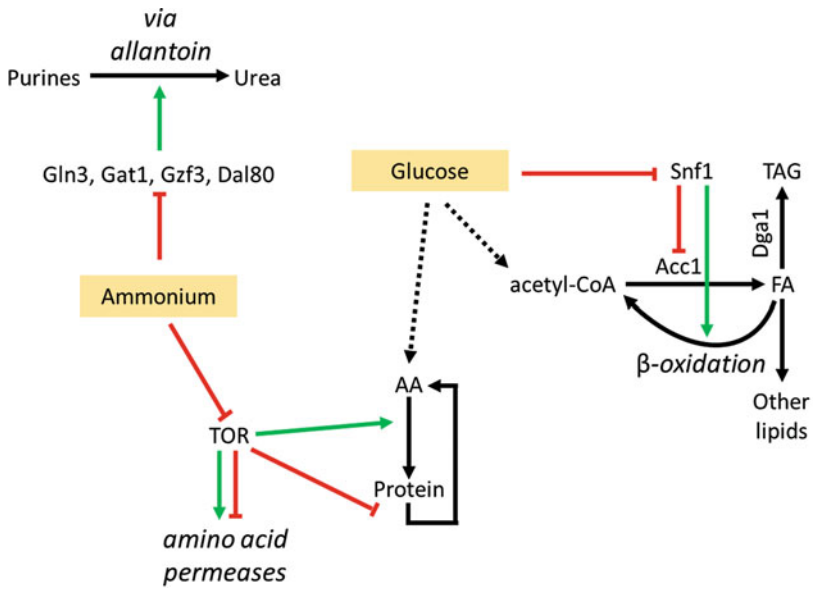

Fig. 5 Schematic overview of regulation that occurs during nitrogen limitation in a high lipid-producing strain. (Figure adapted from Fig. 5 in Kerkhoven et al. 2016)

studies have notably contributed to the elucidation of TAG biosynthetic pathway in microalgae; however, limited putative regulators of this metabolic response have been pointed out. Nitrogen response regulator 1 was identified in *Chlamydomonas* as a putative transcription factor with a regulatory role in nitrogen assimilation and TAG accumulation in nitrogen-depleted conditions (Boyle et al. 2012). An insertional mutation of nitrogen response regulator 1 led to a 50% reduction in TAG accumulation; it is possible that there are other key regulators controlling lipid synthesis under nitrogen stress. Another study identified TAG accumulation regulator 1, a tyrosine kinase involved in the control of TAG accumulation upon both nitrogen and sulfur deficiencies (Kajikawa et al. 2015). The changes in TAGs cannot only be explained by modulation in intracellular nitrogen or carbon/nitrogen availability. These changes appear to be controlled by sensing mechanisms involving transcription factors (Park et al. 2015). An early study showed that silicon starvation not only induces TAG synthesis but also modulates FA profile, with increasing proportions of saturated and monounsaturated fatty acids (MUFAs) being observed under silicon-starvation conditions (Roessler 1988). An increase up to 50% in lipid content was observed under silicon-starvation conditions in several microalgae (Griffiths et al. 2012).

In bacteria, a few studies suggest that TAG is synthesized during times of stress and resource depletion. Moreover, TAGs are used to generate precursors that will be converted to phospholipids or other products when food supplies improve and growth resumes (Alvarez et al. 2000, 2001, 2013; Olukoshi and Packter 1994). For example, in *Rhodococcus* members and other actinomycetes, the biosynthesis and accumulation of TAGs seems to be a process linked to the stationary growth phase or as a response to stress. Detailed research of TAG metabolism in these microorganisms started only a few years ago. Thus, the fundamental understanding of this process and its regulation remain to be clarified. Recently, a regulatory protein (NlpR: Nitrogen lipid Regulator), which contributes to the modulation of nitrogen metabolism and TGA accumulation in oleaginous rhodococci was identified (Hernandez et al. 2017). NlpR acts as a pleiotropic transcriptional regulator by activating of nitrate/nitrite assimilation genes and others genes involved in fatty acid and TAG biosynthesis, in response to nitrogen deprivation. Moreover, this regulator contributes to the distribution of carbon into the different lipid fractions in response to nitrogen levels, increasing the rate of carbon flux into lipid metabolism (Hernandez et al. 2017).

3.3 Oil Accumulation (Subcellular Structures)

TAG needs to be stored within the cell in a way that allows FA mobilization when needed. This function is fulfilled by cytosolic organelles called lipid bodies. Lipid bodies consist primarily of TAGs and cholesterol esters surrounded by a phospholipid monolayer rich in characteristic proteins and are present in the cytoplasm as a form of energy storage (Ryckebosch et al. 2014a). These lipid-rich compartments are formed in all eukaryotic organisms (Zweytick et al. 2000), including fungi and

yeasts, as well as in a few prokaryote genera such as *Rhodococcus* and *Streptomyces* (Wältermann et al. 2005). All oleaginous microorganisms contain lipid bodies in the cells, where most lipids are concentrated as neutral lipids (Garay et al. 2014).

Most yeasts produce small numbers of cytosolic lipid bodies, but oleaginous yeasts accumulate up to 25% (w/w) storage lipid in response to a high carbon: nitrogen ratio (Holdsworth and Ratledge 1991). In *S. cerevisiae*, lipid bodies contain equal amounts of TAGs and sterol esters (Leber et al. 1994). LD biogenesis takes place between two membrane leaflets of the endoplasmic reticulum (Choudhary et al. 2011). DGATs synthesize TAGs in the inner and outer leaflet of the endoplasmic reticulum, which begin to accumulate, generating lenslike protrusions and promoting the recruitment of structural proteins. When there is enough accumulation of TAG between the leaflets, the outer buds off and the LD formed (Adeyo et al. 2011; Bozaquel-Morais et al. 2010).

In microalgae, lipid bodies emerge from plastidial membranes (Fan et al. 2011; Goodson et al. 2011). They grow facing the cytosol or toward the inside of the plastid, facing the stroma, which is the major aqueous fluid surrounding the thylakoids inside the chloroplast (Liu and Benning 2013). The mechanisms underlying the orientation of LD growth in plastid membranes are not well understood.

Many prokaryotes accumulate lipophilic compounds as lipid bodies in the cytoplasm. Members of most genera synthesize polymeric lipids such as PHB or other PHAs (Steinbüchel 2001), whereas accumulation of TAGs and WEs in lipid bodies is a property of only a few prokaryotes. Like the formation of PHA, TAG and WE biosynthesis is also promoted in response to stress imposed on the cells during imbalanced growth, for example by nitrogen limitation, if an abundant carbon source is present. Lipids act as storage compounds for energy and carbon, needed for maintenance of metabolism and synthesis of cellular metabolites during starvation and in particular when growth resumes.

Large amounts of TAGs have been reported mainly in nocardioforms such as *Mycobacterium* sp., *Nocardia* sp., *Rhodococcus* sp., *Micromonospora* sp., *Dietzia* sp., *Gordonia* sp., and *Streptomyces* sp., which accumulate LDs in cells and mycelia (Akao and Kusaka 1976; Alvarez et al. 1996; Alvarez and Steinbuchel 2002; Barksdale and Kim 1977; Hoskisson et al. 2001; Olukoshi et al. 1994), as well as in *Alcanivorax* sp. and other hydrocarbonoclastic marine bacteria (Kalscheuer et al. 2007). Moreover, TAGs frequently accumulate in members of the gram-negative genus *Acinetobacter*, though the amounts are small in comparison to accumulated WEs (Stöveken et al. 2005). In general, TAGs are stored in spherical lipid bodies, with quantities and diameters depending on the respective species, growth stage, and cultivation conditions. One interesting example is *R. opacus* PD630, where lipids can exceed 70% of the dry weight (Alvarez et al. 1996).

The first reports on WE biosynthesis in gram-negative bacteria were published more than 30 years ago, mainly involving the genus *Acinetobacter* (Fixter and Fewson 1974; Fixter and McCormack 1976; Gallagher 1971; Scott and Finnerty 1976). Meanwhile, accumulation of WEs was also described for *Moraxella*, *Micrococcus*, and *Fundibacter* (Bredemeier et al. 2003; Bryn et al. 1977; Russell and Volkman 1980). WE biosynthesis has also been reported in actinomycetes: for

example, in *Corynebacterium*, *Mycobacterium tuberculosis*, and *Nocardia* (Bacchin et al. 1974; Raymond and Davis 1960; Wang et al. 1972). In *Acinetobacter calcoaceticus*, WEs can reach 25% of the dry weight, indicating that WEs act as main storage compound (Fixter and Fewson 1974; Wältermann et al. 2005), but only one or a few WE bodies are observed per cell (Scott and Finnerty 1976; Wältermann et al. 2005). WEs are not exclusively produced as intracellular bodies. Some strains of *Acinetobacter* sp. and the marine bacterium *Fundibacterium jadensis* also produce extracellular WEs from alkanes (Bredemeier et al. 2003; Dewitt et al. 1982; Makula et al. 1975; Singer et al. 1985). The function of extracellular WEs and the mechanisms of export are not known yet.

Few prokaryotes accumulate TAGs as energy stores. They tend instead to sequester glycogen, polyphosphates, and PHAs. Biosynthesis of PHAs is characteristic among prokaryotes (Koutinas et al. 2014), and, as in the case of TAGs, PHAs are stored as insoluble cytosolic inclusions (Poirier et al. 1995). The species that are able to produce PHAs accumulate these compounds in lipid bodies, which are also called carbonosomes (Bartz et al. 2007; Jendrossek 2009). The PHA bodies are surrounded by amphipathic, small surface proteins called phasins (Grage et al. 2009; Jendrossek 2009). They contain PHB, polyhydroxyvalerate, or copolymers such as poly (3-hydroxybutyrate-co-3-hydroxyvalerate). PHAs have thermoplastic and elastomeric properties and are recyclable materials that can be easily degraded into carbon dioxide and water (Philip et al. 2007).

Accumulation of PHA was first reported in *Bacillus megaterium* (Lemoigne 1926). Since then, many reports on the occurrence of PHAs have been published, including reports on members of the halobacteria, whereas PHA was not detected in lactobacilli, *Enterobacteriaceae*, and methanogens (Garay et al. 2014). *Ralstonia eutropha* is the best characterized bacterium related to PHA metabolism. These bacteria accumulate 10 to 20 intracytoplasmic inclusions of PHB per cell and amounting up to 90% of the cell dry weight (Eggers and Steinbuchel 2014). A few actinomycetes, for example, *R. ruber*, simultaneously synthesize and accumulate similar amounts of TAGs and the copolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from unrelated substrates such as gluconate, glucose, and acetate (Alvarez et al. 2000).

4 Commercial Oils, Applications, and Market

4.1 The Diversity of Fatty Acids

FAs differ by the length of the aliphatic chain, the degree of unsaturation, the location, and the *cis* or *trans* conformation of double bonds. In general, the FAs are classified as saturated fatty acids (e.g., palmitic, stearic), MUFAS (e.g., palmitoleic, oleic), and PUFAs. Moreover, PUFAs can be classified in several families (groups or classes) such as omega-3 (ω-3, v-3, or n-3), omega-6 (ω-6, v-6, n-6), and other groups. The ω-3 family includes ALA (C18:3n-3), EPA (C20:5n-3),

and DHA (C22:6n-3), while the ω-6 family gathers linoleic acid (LA (C18:2n-6)), GLA (C18:3n-6), ARA (C20:4n-6), and conjugated linoleic acid (CLA).

PUFAs of the ω-3 and ω-6 families are essential for maintaining many functions in mammalians including humans. ALA and LA are the precursors for the synthesis of more highly unsaturated and longer-chained fatty acids of ω-3 and ω-6 families, respectively. Because mammals lack the ability to synthesize LA and ALA, they must be supplied by the diet from different foods sources.

LA is practically found in all foods and is the predominant PUFA in land-based meats, dairy, vegetables, vegetable oils, cereals, fruits, nuts, legumes, seeds, and breads. GLA can be found in some plant oils such as evening primrose and borage oils. DHA and ARA are found in mother's milk which provides their requirements for neural development and visual acuity to newborns. However, since DHA and ARA are absent from cow's milk, when it is used in place of mother's milk, these PUFAs should be added to the diet of babies to ensure a normal development.

Although fish oil is the best source of PUFAs, the inclusion of fish oil into the infant milk formulas is not recommended due to the presence of environmental pollutants in fishes. Moreover, the production of fish oil is now reaching their limits (see below). Therefore, oleaginous microorganisms can provide an alternative and economically feasible source of PUFAs, provided that most of the PUFAs occur in TGAs which is the preferred form to take lipids within the diet. For instance, it has been shown that microalgae oils contain sufficient ω-3 PUFA to serve as an alternative for fish oil (Ryckebosch et al. 2014b). Thus, this topic will be focused in the applications and market of PUFAs and in particular PUFA-containing microbial oils.

4.2 PUFAs for Human Nutrition and Their Nutraceutical Properties

As mentioned above, strictly, EPA and DHA are nonessential ω-3 FAs as the human body can convert essential ALA into EPA and DHA. However, in humans, this conversion is not efficient enough to meet the EPA and DHA demand to impart beneficial health effects; thus, it is expected to obtain these fatty acids from dietary sources (Lee et al. 2016; Béligon et al. 2016).

DHA is an essential component of cell membranes in some human tissues. For instance, it accounts for over 60% of the total fatty acids in the rod outer segment in the retina. Moreover, DHA is an essential nutrient during early human development (Sijtsma and de Swaaf 2004). DHA also can act as breast and colon cancer chemopreventive agent (Hou et al. 2016).

DHA and EPA are involved in early neural and retinal development being essential for the proper visual and neurological development of infants. As preterm and young infants are unable to synthesize DHA at a rate fast enough to keep up with the demand from the rapidly growing brain, they should obtain these compounds from their diet. As mentioned, breastfeeding serves as a good source of PUFAs. In general, while total fat levels in the typical Western diet are too high, the intake of long-chain ω-3 PUFA is too low. DHA supplementation at either 50 mg/day or

100 mg/day for 6 weeks was effective in increasing plasma phospholipid DHA contents of children.

The nutritional benefits of EPA are less clear than those for DHA. Nevertheless, EPA has been advocated as a highly desirable PUFA that can exert beneficial effects on hypertension, thrombosis, arteriosclerosis, arthritis, and various inflammatory responses (Lenihan-Geels et al. 2013). The baseline blood levels of EPA are inversely related to the risk of sudden death due to cardiovascular disease. It has been used to prevent arteriosclerosis and coronary heart disease, and a low dose of 20 mg/kg/day appears to protect against cardiac arrhythmia. It has also been used for the alleviation of some neuropsychiatric disorders, including manic depression (bipolar disorder), depression, schizophrenia, and also attention deficit hyperactivity disorder in children (Ratledge 2013).

Interestingly, DHA, when administered as a single PUFA, either to young children or to adults, can be retro-converted into EPA by simple loss of a C2 unit. EPA was, however, contraindicated in babies, and thus, DHA was not as effective as originally hoped. However, when DHA was given to infants along with ARA, ARA prevents the retro-conversion of DHA to EPA by blocking the degradative pathway. A combination of two volumes of ARA and one volume of DHA is the most effective ratio for providing these PUFAs to newly born infants, including premature babies (Sinclair and Jayasooriya 2010).

GLA has been also incorporated into infant formula and used for treatments of atopic eczema, rheumatoid arthritis, multiple sclerosis, and premenstrual tension.

Combinations of EPA and DHA can be satisfied by consumption of fish oils particularly those from the so-called oily fish. But not every person and particularly some people that do not eat animals are willing to have fish products in their diet.

ARA is the most abundant PUFA in humans and has a major role as a structural lipid associated predominantly with phospholipids and is a direct precursor of a number of eicosanoids regulating lipoprotein metabolism, blood rheology, leukocyte function, and platelet activation.

The supplementation with ARA and DHA appears to be beneficial in reducing the risk of HIV-1 transmission, particularly during the period of breastfeeding.

Antibacterial activity of PUFAs on *Propionibacterium acnes* and *Staphylococcus aureus* to treat acne and superficial infections has been also investigated (Desbois and Lawlor 2013).

The combination of multifunctional factors with synergic effect including PUFAs is now considered as a possibility to develop more effective multifunctional foods (Yamada 2017).

4.3 Animal Feed

Microalgae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to farm animals. The main applications of microalgae in aquaculture are associated with nutrition as a source of proteins, vitamins, and PUFAs. Fish meal and fish oil were used to complement pig and poultry diets in

the last century, but with the growth of aquaculture from the 1960s onward, these products have been diverted toward feeding fish. Today diets complemented with fish oil are used for companion animals.

Fisheries are currently providing fish not only for human consumption but also for fish feed, and they are supplying fish at a so large mass scale that it appears no longer sustainable. In fact, the Food and Agriculture Organization (FAO) of the United Nations suggests that fish oil demand will be soon over the production capacity.

Currently, aqua feed relies on the inclusion of marine ingredients, fish meal, and fish oil since, as with humans, farmed carnivorous marine fish species are inefficient at converting ALA into significant levels of EPA and DHA. Therefore, these PUFAs must be supplied in the fish diet usually from marine ingredients and fish oil derived from pelagic fisheries (Sprague et al. 2016).

Microalgae are required for larval nutrition during a brief period, either for direct consumption in the case of mollusks or directly as food for the live prey fed to small fish larvae. The most frequently used species in aquaculture are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema,* and *Thalassiosira*. Microalgae such as *Dunaliella salina*, *Haematococcus pluvialis,* and *Spirulina* are also used as a source of natural pigments for the culture of prawns, salmonid fish, and ornamental fish. Mainly the microalgae *Arthrospira* and *Chlorella* are used also to feed many types of animals: cats, dogs, aquarium fish, ornamental birds, horses, poultry, cows, and breeding bulls (Spolaore et al. 2006).

However, the aquaculture industry is experiencing so rapid increases in fish oil price due to flat supply and increased global demand for this commodity that these finite marine ingredients have gradually been replaced with alternatives of terrestrial agricultural origin. Rapeseed oil is the most commonly utilized fish oil alternative in Europe. Nevertheless, terrestrial plants are not so efficient since they only contain shorter-chain PUFAs such as ALA and are completely devoid of any EPA and DHA. Because of that, PUFA-containing microalgae biomass has been used directly as an alternative feed additive to plant oils (Chauton et al. 2015; Sprague et al. 2016, 2017).

4.4 Market

As mentioned, fish oil is the main source of PUFAs, but the world supply of fish oil is currently stabilized at around one million t per year (Finco et al. 2017). Because approximately 70% of the available fish oil is used for fish feed production for salmonids, only a small portion of the captured fish is now used to produce functional foods for humans.

The global PUFA market has been estimated to be valued at US$ 4212 M by 2016 end and is expected to witness a compound annual growth rate (CAGR) of 10.7% over the period 2016–2026 (FMI 2016). Growth of the global PUFA market is mainly driven by increasing prevalence of chronic diseases such as cardiovascular

diseases, stroke, cancer, and diabetes. On the basis of product type, the market is categorized into ω-3 and ω-6 PUFAs.

The ω-3 market segment is further segmented into ALA, DHA, and EPA. According to FAO (2014) and Grand View Research (2014), the global demand for ω-3 PUFAs was 21,900 t in 2012, and it is expected to increase to more than 135,500 t in 2025. Other studies estimate a market of $9.94 billion for ω-3 PUFAs in 2015 with a projected grow at a CAGR of 13.8% from 2015 to 2020 (Marketsandmarkets 2016). The particular DHA segment is expected to register a high CAGR of 11.7% during the 2016–2026 periods (FMI 2016).

The ω-6 market segment is further segmented into LA and ARA. The ARA segment is expected to dominate over LA segment and is estimated to account for the highest market share of 73.2% by 2016 (FMI 2016). The global market for ARA was $176.48 million in 2016 and is expected to grow at a CAGR of 6.8% during 2017–2022 (Mordor Intelligence 2017).

The emerging problem is to provide enough food resources containing PUFAs to an increasing population. Thus, the potential demand for ω-3 PUFAs is 1.274 million t based on a supply of 500 mg/day. However, the supply by oil fish is only 0.84 million t, so the gap is 0.434 million t.

Some of the major company players in this market are Koninklijke DSM N.V., Enzymotec Ltd., Aker BioMarine AS, Croda International PLC, GlaxoSmithKline plc, FMC Corporation, Omega Protein Corporation, BASF SE, Cargill Incorporated, Polaris Nutritional Lipids, Zymes LLC, Denomega Nutritional Oils, Barlean's Organic Oils, Vega Nutritionals Ltd., Arista Industries, Copeinca ASA, Horizon Organic, Pharma Marine USA LLC, Cabio Bioengineering, Cayman Chemicals, Guangdong Runke, Kingdomway, Suntory, Zhejiang Weiss (Wecan), Lonza Group AG, GC Rieber Oils AS, Kerry Group, Cellana Inc., Clover Corporation, Archer Daniels Midland Company, Nordic Naturals Inc., Smit & Zoon, Solutex GC, Stepan Company, Neptune Wellness Solutions, FrieslandCampina, and others. The manufacturers, marketers, and supporters of EPA and DHA omega-3 fatty acids have created the GEOED association (http://www.goedomega3.com/).

Several sources have been evaluated as alternative market sources of PUFAs, but due to their wide availability and ability to accumulate large amounts of lipids in a short time, oleaginous microorganisms have been used in the production of oils rich in PUFAs, mainly ω-3 acids, from the mid-1990s. In this sense, while oleaginous plants can reach 500–5000 kg/ha/yr. of lipids, microorganisms can achieve up to 2000 kg/m^3/yr. of lipids (Finco et al. 2017).

Commercial production of microbial oils is mainly restricted to yeasts, filamentous fungi, and algae. The first microbial oil that was produced at commercial scale was the GLA-rich oil, named Oil of Javanicus, from *Mucor circinelloides*. This oil was produced by J&E Sturge (UK) from 1985 to 1990 competing with the GLA from the seed oil of *Oenothera biennis*. This oil is mainly sold in the UK as a dietary supplement for the alleviation of premenstrual tension in women and due to price competitions only low volumes of the microbial oil have been produced (i.e., 5–10 t) (Ratledge 2006).

After the initial approach of microbial oil to the market in the 1990s, David Kyle at Martek Inc. (USA) company began to explore the possibility of producing long-chain microbial PUFAs. The key targets were initially EPA and DHA (Boswell et al. 1992). The company exploited *Crypthecodinium cohnii* as DHA producer.

Currently DHA is also produced commercially by other microorganisms such as *Schizochytrium*, *Aurantiochytrium*, and *Ulkenia* (also known as *Labyranathula*). The company that has originally developed this process was OmegaTech (USA) led and pioneered by Bill Barclay that was acquired by Martek in 2002. This oil is now also produced by other companies including Lonza Group (Switzerland), Jiangsu Tiankai Biotechnology Co. Ltd. (China), and several smaller companies in the USA and the UK. The FDA gave approval and GRAS status to DHA as DHASCO oil in 2002. About 20 countries with 150 companies were producing DHA in 2015 (Finco 2017).

In addition, in the 1980s, a process for the microbial ARA production was identified in Japan using *Mortierella alpina* (Totani et al. 1987). However, the production of ARA by fermentation was developed by the company, Gist-brocades (Netherlands). An agreement was subsequently reached between Martek Inc. and Gist-brocades for the latter company to produce the ARA-rich oil for exclusive sale to Martek. The ARA-rich oil is now also produced by Suntory in Japan, as SUNTGA40S, and by Cargill, as CABIO oil, together with Wuhan Alking Bioengineering Co. Ltd. in China, where it is used for infant nutrition. In 2012, the EU gave approval to allow for sales of the ARA-rich oil for infant nutrition in Europe.

As commented above, the combination of ARA and DHA oils was found to be most appropriate for providing these key PUFAs to newly born infants. Safety trials have shown the oil to have an unimpeachable safety record (Ryan et al. 2010). Sales of DHA and ARA oils have steadily increased since they were launched to the market and are now commercialized into over 70 countries for more than 20 companies.

DSM (Dutch State Mines) (Netherlands) has the global leadership on the production of ω-3. The company is the main supplier of DHA to the US market (with 80% of the market share) and to European and Asian markets (excluding China). The position of DSM is because the company Martek one of the largest suppliers of these oils was taken over by DSM, who had previously acquired Gist-brocades in 2011. For its last year of trading as an individual company, Martek Biosciences recorded a revenue of US $ 317 million for sales of the oil for infant nutrition. Although the selling price of the oil is commercially sensitive, it is considered that at least 2000 t of microbial DHA are sold annually.

Alltech, a global animal nutrition company, produces DHA from heterotrophic algae in closed stainless steel fermenters in one of the largest algae production sites in the world in Winchester (Kentucky, USA) aimed at the animal feed market.

Microbial EPA is mainly produced using microalgae. The EPA producer companies include Qualitas Health Ltd. (Israel), Photonz (New Zealand), and Algisys LLC (USA). The oil produced by Qualitas Health is sold as EicoOil, being a mixture of EPA with other PUFAs. DuPont (USA) has developed an alternative to microalgae producing EPA using a genetically modified *Y. lipolytica* (Xue et al. 2013). However,

by using naturally occurring oleaginous yeast, DuPont has been also able to produce EPA (Damude et al. 2011), and now this EPA-rich oil has received the GRAS status from the FDA.

Concerning the patents on microbial oils, it is possible to classify them according to different general subjects: (i) downstream processes for the isolation of microbial oils, (ii) microbial oil production and uses, and (iii) engineering microorganisms for oil production (Table 2).

Table 2 Examples of patents concerning to microbial oils

Subject	Organism	Patent	Proprietary
Downstream processes	Algae, fungi	WO2015095688A1	Dsm Ip Assets B.V.
	All	EP2419520A4	Solazyme, Inc.
	All	US6166231	Martek Biosciences Corp
	All	US4905761	IIT Research Institute
	Algae, fungi	US6255505	DSM Gist BV
	All	WO2008151149	Solazyme, Inc.
	All	WO2006046943A2	Martek Biosciences Corp
	All	WO2003049832A1	Martek Biosciences Corp
	C. cohnii	WO1991011918A1	Martek Biosciences Corp
	All	WO2011153246A2	Kanegafuchi Kagaku Kogyo KK
	Algae, fungi	EP0207475A2	DSM Gist BV
	Algae, fungi	US6255505	
Microbial oil production and uses	*C. cohnii*	US20040072330	University of Hull
	Algae, fungi	US6428832	Koninklijke DSM NV
	M. schmucker	US5882703	OmegaTech Inc.
	Fungi	EP0269351A3	Lion Corp
	Schizochytrium	US5130242	Phycotech Inc.
	All	US5374657	Martek Biosciences Corp
	M. schmucker	US7666657	Martek Biosciences Corp
	Mortierella	US4783408	Agency Ind Sci & Technol
	Mortierella	EP0276541A2	Suntory Ltd.
	Ulkenia	US6509178	Nagase and Co. Ltd., Suntory Ltd.
	Dinoflagellates	US5397591	Martek Biosciences Corp
	Marine	WO1989000606A1	Maricultura, Incorporated
	S. limacinum	US8232090	ABL Biotechnologies Ltd.
	S. limacinum	WO2013010090	Allthech Inc.

(continued)

Table 2 (continued)

Subject	Organism	Patent	Proprietary
Engineering microorganisms	Yeasts	US8951776	MIT USA
	All	US8765404	MIT USA
	Y. lipolytica	US20130344548	MIT USA
	S. cerevisiae	US7736884	Fluxome Sciences AS
	Yeasts	US7198937	E I du Pont de Nemours and Co.
	Yeasts	US20060160193	E I du Pont de Nemours and Co.
	Cyanobacteria	US20100081178	Targeted Growth Inc.
	Y. lipolytica	US8435758	E I du Pont de Nemours and Co.
	All	US20110223641	MIT USA
	S. cerevisiae	WO2005118814	Fluxome Sciences As & Authors
	Cyanobacteria	WO2012087963	Targeted Growth, Inc.
	Cyanobacteria	WO2012087982	Matrix Genetics, Llc
	Pseudomonas	US6207441	Seong Gu Ryu
	YS-180	US7550286	E I du Pont de Nemours and Co.
	Y. lipolytica		

5 Industrial Production Processes

A crucial point for the establishment of microbial lipids utilization is the cost-effective production and purification of fuels or products of higher value. This topic will analyze the state of the art on the production and extraction of microbial oils.

5.1 Culture Conditions

The development of a microbial PUFA production process requires the selection of the proper microorganism and optimized cultivation techniques. Bacteria, algae, fungi, and yeasts are able to accumulate lipids, but in fact, there are only few studies on PUFAs produced by bacteria. Moreover, the potential for lipid production is a species-specific characteristic, as well as the ability to produce PUFAs.

In order to select appropriate lipid producer strains, the most important parameters to be considered include the specific growth rate, the biomass production under optimal culture conditions, the total lipid content, and their PUFA proportion. Therefore, to control, to validate, and to optimize the processes, one of the most important parameters for lipid production is the lipid content of the cell mass. The yield and the composition of the microbial lipids depend on the type of fermentation and the particular operational conditions (e.g., culture medium, nitrogen source, pH, temperature, aeration, etc.).

In general, the production of microbial oils can be realized by submerged (SmF) or solid-state fermentations (SSF). In SmF conditions oleaginous microorganisms can be cultivated as batch, fed-batch, or continuous cultures, using baffled and unbaffled flasks as well as stirred tank reactors (Ochsenreither et al. 2016). On the other hand, SSF reproduces the natural microbiological processes such as food production, composting, and ensiling. In general, the advantages of SSF are a higher productivity, the possibility to use low-cost media and to reduce energy and waste water costs. The disadvantages of SSF are, for example, the difficulties in scale-up, in the control of process parameters, and an increasing cost for product recovery (Ochsenreither et al. 2016).

One advantage of using microorganisms to produce PUFAs is that they can be cultivated on various types of carbon substrates, even on different organic industrial waste. Since carbon sources constitute over 60% of the total production cost in typical fermentation processes, the use of low-cost carbon sources, such as organic waste, should therefore be considered as an important factor to make the process economically feasible. As well organic and inorganic nitrogen sources are used individually or in combination including yeast extract, urea, peptone, glycine, KNO_3, NH_4NO_3, and $(NH_4)_2SO_4$ (Ochsenreither et al. 2016).

In addition to oleaginous yeast and fungi, microalgae are currently produced at large scale as a microbial oil source. In fact, microalgae are currently cultivated at large scale to produce lipids for biofuels as well as food and feed products. The cost of microalgae production is a challenge for the commercial utilization of this biomass as biofuels. However, as the target price level of microalgae as food and feed ingredients is higher, the economic feasibility appears to be closer for nutrition purposes than that of biofuel uses.

Two main approaches are taken to improve the production economics of phototrophic microalgae, that is, increasing the productivity of the cultivation systems and reducing both capital and operational production costs. In this sense, large-scale industrial cultivation of phototrophic microalgae can be conducted in open pond systems or in closed photobioreactor systems (CPS). Open ponds are often cheap raceway constructions which provide large volumes, but these systems have a low productivity and high energy costs required to harvest the cells at low densities of 0.1–0.2 g dry cell weight/l. CPS are more expensive to build, but have much higher productivity, since they are designed to maximize the utilization of light energy and to achieve efficient uptake of nutrients and CO_2 and have higher cell densities at 2–4 g dry weight/l, which lower the harvesting costs.

The economics of microalgae production depend on the photosynthetic productivity, and there are ongoing efforts to increase the microalgae productivity following different strategies (Chauton et al. 2015). The first strategy is to exploit the cultivation conditions to direct the metabolism toward lipid production. The second is to improve biomass productivity or lipid yield by mutagenesis and selective breeding, and the third strategy is to improve strains by genetic modifications to optimize light absorption and increase the biosynthesis of EPA and DHA (see below).

Lipids serve as a carbon and energy storage in microalgae under limiting growth conditions when photosynthesis exceeds the limitations for growth. To control the

balance between biomass production and lipid accumulation, physiological variables can be adjusted to induce lipid accumulation and also shift the lipid composition toward the desired lipids: growth restriction due to limited illumination and deprivation of nitrogen, sulfur, or phosphate is used to induce lipid accumulation and composition changes.

On the other hand, many efforts are focused on how to achieve maximum biomass productivity by increasing the photosynthetic efficiency (PE), which is the percentage of the solar irradiation that is converted to biomass. The overall PE of a cultivation system will depend both on the technological efficiency of the cultivation system and the biological efficiency of the production strain to convert the irradiation into chemical energy. Then photosynthetic productivity is directly depending on the intensity of the solar irradiation. Only the light within the wavelength range of 400 to 700 nm, called photosynthetically active radiation (PAR), can be utilized by plants and algae, which in practice means that only 40–45% of total solar energy can be utilized for photosynthesis.

5.2 Downstream Processes

Downstream processing cost is one of the major obstacles to be solved for full economic efficiency of microbial lipids (Béligon et al. 2016; Ochsenreither et al. 2016). Because microbial oils are intracellular for storage purposes, they have to be extracted upon further applications. Natural oleaginous strains have not been engineered so far to excrete TAGs or free fatty acids in order to simplify downstream processing. However, metabolic engineering efforts have been conducted for secretion of fatty acids in *Escherichia coli* (Liu et al. 2012; Meng et al. 2013) and *S. cerevisiae* (Michinaka et al. 2003; Leber et al. 2015).

Furthermore, for downstream processing, it is important to know if PUFAs are present as part of the membrane structure, e.g., in phospholipids, or as part of TAGs in the cytosol, as well as to know the type of PUFAs present in the cells.

The extraction method and the lipid quantification must be fast, efficient, and applicable to an industrial scale-up. Therefore, the extraction methods, that may be highly suitable for analytical purposes, might not be applicable in industrial large-scale operations due to high costs or simply a non-scalable extraction setup. The optimal extraction method should also enable a reproducible, quantitative, cost-effective, and nontoxic removal of lipids under mild conditions to prevent oxidative damage to PUFAs. The final processing of oils involves purification or refining and modification in order to stabilize the crude oil. Extraction methods, using solvents, developed to extract lipids from fishes or vegetables have been modified and adapted for microbial lipids extraction. Microbial lipids applied in food industry cannot be extracted with toxic solvents or should in the best case avoid any solvents to prevent solvent residues in food or contaminations with heavy metals (Uematsu et al. 2002). Additionally, the optimal method in terms of oil recovery has to be elucidated for each strain.

Extraction methods include Soxhlet extraction, Folch extraction, pressurized liquid extraction, and extraction with supercritical fluids (Ochsenreither et al.

2016). Several automated Soxhlet extraction systems have been developed and are commercially available. The Folch method, similar to the method developed by Bligh and Dyer, is the most reliable extraction method for total lipids and is often used as a standard technique in extracting microbial lipids. The pressurized liquid extraction is similar to Soxhlet extraction but uses liquid solvents at elevated temperatures and pressures. For extracting lipids supercritical CO_2 is a good solvent (Sahena et al. 2009).

For the extraction of lipids from microbial biomass, cell disruption is very important, because efficiency of cell disruption directly influences subsequent downstream operation and overall extraction efficiency (Senanayake and Fichtali 2006). A multitude of cell disruption and lipid extraction methods are available which can be roughly divided in mechanical and nonmechanical methods. Nevertheless, depending on microorganism, scale, economics, and lipid application, the method spectrum is narrowed to a few.

Cell disruption by mechanical methods is achieved by bead milling, high-pressure homogenization, and ultrasound (Ochsenreither et al. 2016). Cell disruption by nonmechanical methods is achieved by physical or chemical disruption methods.

Physical methods for disruption include decompression, osmotic shock, microwave treatment, pulsed electrical fields, and (freeze)-drying, but, their scale-up is limited for most of them.

Disruption by chemical methods includes the permeabilization by a variety of chemicals such as antibiotics, chelating agents, chaotropes, detergents, solvents, alkalis, and acids. Acid-catalyzed in situ transesterification which combines cell disruption, lipid extraction, and transesterification to fatty acid methyl (FAME) or ethyl (FAEE) esters has been used for biodiesel production. Cell disruption with lytic enzymes, a process that can be carried out at mild reaction conditions, has been used especially for oleaginous yeasts. The enzymatic treatment in combination with solvent extraction, pressing, or ultrasound has been demonstrated for the lipid extraction of microalgae. However, some chemical treatments are excluded in food applications or require intensive downstream processing to eliminate them.

A direct application of solvents offers the possibility of combining cell disruption and lipid extraction without further pretreatment, but the use of large amounts of organic hazardous solvents, like chloroform and methanol or even less toxic one, like hexane/isopropanol, is not always efficient since the cell walls of most microorganisms are usually impermeable to most solvents (Ochsenreither et al. 2016).

6 Metabolic Engineering Strategies for Producing Microbial Oils

6.1 Genetic Engineering (Mutagenesis)

Improvement in the production of microbial oils involved the use of classical genetic techniques, such as mutagenesis, together with genetic engineering (Liang and Jiang 2013; Gong et al. 2014; Ledesma-Amaro 2015; Shi and Zhao 2017). Despite the low

cost of genome sequencing, only few oleaginous yeasts genomes have been sequenced. Therefore, genetic tools remain scarce or under development for this kind of yeast. Among them, only *Y. lipolytica* was used as a model organism (Fickers et al. 2005), so its genome was made public a few years ago, and many genetic tools for its modification are available (Bredeweg et al. 2017; Dujon et al. 2004).

The first attempts at enhancing lipid accumulation were performed by modification of the expression of key enzymes situated in the crossroads of metabolic routes. In *Y. lipolytica* the increase of G3P pools by modifying gene expression of the enzymes leading to its production and/or its degradation resulted in threefold increase in lipid accumulation compared to the wild-type strain. The simultaneous abolishment of the ß-oxidation gave rise to an obese yeast capable of accumulating more than 80% of its cell dry weight in lipids (Beopoulos et al. 2008; Dulermo and Nicaud 2011). In this organism, overexpression of the DGAT resulted in a great increase of TAG content of the cells (Beopoulos et al. 2012; Silverman et al. 2016).

PUFAs are highly susceptible to oxygen radical attack, and the resulting oxidative species are detrimental to cell metabolism and limit lipid productivity. Xu et al. (2017) investigated cellular oxidative stress defense pathways in *Y. lipolytica* to improve the lipid titer, yield, and productivity.

The oleaginous fungus *M. alpina* has been engineering for the production of PUFAs (Sakuradani et al. 2013; Okuda et al. 2015; Shi et al. 2016; Kikukawa et al. 2016).

In microalgae, most of the studies of genetic engineering have targeted lipid biosynthesis in order to enhance TAG accumulation. In several cases endogenous or heterologous (from *S. cerevisiae* or *C. reinhardtii*) DGATs have been overexpressed in different *Nannochloropsis* species (Beacham and Ali 2016; Iwai et al. 2015; Li et al. 2016; Xin et al. 2017; Zienkiewicz et al. 2017; Wei et al. 2017a). Overexpression of the endogenous DGAT1a-encoding gene in *Nannochloropsis oceanica* resulted in a 39% increase in TAG content per cell, and RNAi repression resulted in a 20% decrease in TAG content per cell following N deprivation (Wei et al. 2017a). Overexpression of the endogenous DGAT number 5 in *N. oceanica* resulted in a 3.5-fold increase in TAG (Zienkiewicz et al. 2017). Furthermore, the DGAT number 7 has also been overexpressed in *N. oceanica* IMET1, resulting in 69 and 129% increase in dry weight (DW) of TAG content under N-replete and N-deprived conditions (Li et al. 2016). The overexpression of malonyl-CoA ACP transacylase (MCAT) of *N. oceanica* IMET1 has been resulted in a 36% DW increase in lipids (Chen et al. 2017).

Overexpression of malic enzyme in transgenic *P. tricornutum* markedly increased the total lipid content (Xue et al. 2015). A similar approach has been followed with the green microalga *Chlorella pyrenoidosa* (Xue et al. 2016). *P. tricornutum* has been engineered to accumulate DHA using the Δ5-elongase from the picoalga *Ostreococcus tauri* (Hamilton et al. 2014, 2016). EPA was also produced by using a similar method (Peng et al. 2014; Wang et al. 2017) that used a sequential metabolic engineering strategy to overcome the metabolic bottlenecks in *P. tricornutum* in order to overproduce ARA and DHA. For this purpose, the malonyl-CoA acyl carrier protein transacylase and desaturase 5b were cloned and

coordinately expressed, increasing the production of ARA and DHA. Niu et al. (2013) have improved the neutral lipid and PUFAs biosynthesis by overexpressing a type II diacylglycerol acyltransferase in *P. tricornutum*.

Escherichia coli could be an alternative fatty acid producer, since it has been successfully used to generate many valuable platform chemicals and biofuels; however, it is not an oleaginous microorganism, so its low yield of intracellular lipids restricts its utility for lipid production. Nevertheless, *E. coli* has been genetically engineered to increase its fatty acid biosynthesis (Lennen and Pfleger 2012; Meng et al. 2011; Lee et al. 2014; Wu and San 2014; Cao et al. 2016; Xiao et al. 2018).

Some experiments have been performed in yeast to improve TAGs accumulation by random mutagenesis using ultraviolet irradiation. One of these experiments was carried out in *Lipomyces starkeyi*-selecting mutants by cerulenin, a compound displaying inhibitory effects on lipid biosynthesis. This strategy resulted in an increase in lipid productivity up to 31% (Tapia et al. 2012). The same procedure was done in the oleaginous yeast *Rhodosporidium toruloides,* and the lipid productivity in this yeast was successfully improved (Yamada et al. 2017). UV mutagenesis was also used to improve TAGs accumulation in the green microalgae *Scenedesmus obliquus*. For this aim, starchless mutants were obtained in which TAG content reached up to 49.4% DW (de Jaeger et al. 2014; Breuer et al. 2014).

6.2 Systems and Synthetic Biology

Increasingly, there are a number of studies that use various systems biology tools to understand the metabolic switches in oleaginous microorganisms. However, their applications to metabolic engineering of oleaginous microorganisms are still in their infancy. Actually, studies on metabolic engineering of oleaginous microorganisms often involve target screening processes which require extensive experimental works (Silverman et al. 2016; Friedlander et al. 2016). Systems biology provides easier and more efficient ways to guide metabolic engineering of oleaginous microorganisms.

In systems biology, both bottom-up and top-down approaches are central to assemble information from all levels of biological pathways that must coordinate physiological processes (de Lorenzo and Galperin 2009). The top-down approaches, which are based on "omics" tools for high-throughput measurement of cellular components, enable data-driven discovery of key players controlling the systems and their interactions. Meanwhile, the bottom-up approaches employ predictive mechanistic models developed based on existing knowledge to perform systematic analysis of the cellular processes (Lee et al. 2005; Park and Lee 2008).

Studies using top-down approaches reveal that lipid accumulation in most oleaginous fungi is a consequence of reduced fluxes through central carbon metabolism caused by nitrogen limitation. In contrast, for oleaginous bacteria, central carbon metabolism is upregulated during nitrogen limitation together with activation of the Kennedy pathway. The difference between oleaginous fungi and bacteria implies that engineering of central carbon metabolism in oleaginous fungi to simulate the

regulatory patterns in oleaginous bacteria would be an effective strategy for lipid overproduction (Park et al. 2017).

Related to bottom-up approaches, genome-scale models of metabolism (GEMs) and constraint-based modeling (CBM) methods are used to understand and engineer microbial oil synthesis (Kavscek et al. 2015; Kerkhoven et al. 2016). A GEM is a structured collection of all metabolic reactions that exist in the cell, which can be built systematically using genome annotations and biochemical knowledge (O'Brien et al. 2015).

Recently, reflecting the growing interests on oleaginous microorganisms, several GEMs for oleaginous microorganisms have been published (Table 3). In general, special attention to lipid-related pathways was made during the reconstruction. Modeling results highlight that the pentose phosphate pathway is preferred for the generation of NADPH in *Y. lipolytica* (Kavscek et al. 2015) and *Candida tropicalis* (Mishra et al. 2016), while the malic enzyme is identified as a key node in the regulation of NADPH regeneration in *Mortierella alpina* (Ye et al. 2015).

Although oleaGEMs have been used for understanding mechanisms of lipid production and optimizing fermentation conditions, currently there is no report on the use of oleaGEMs for metabolic engineering. During the past decade, various computational strain design algorithms (CSOMs) have been developed, and majority of them are looking for a design, which enables growth-coupled production of target compounds (Maia et al. 2016). However, as oleaginous microbes produce lipids in the nutrient-limited nongrowing phases, it is hard to apply existing CSOMs to oleaGEMs for designing improved lipid producers. Furthermore, until a recent date, there was no objective function suitable for predicting metabolic fluxes in the nutrient-limited conditions (Park et al. 2017).

Synthetic biology emerged around the year 2000 as a new biological discipline, and many different definitions have been applied to this field. However, one commonly used way to describe synthetic biology is as the design and construction of new biological functions that are not found in nature (Serrano 2007).

Y. lipolytica was modified to secrete FAs by considering two synthetic approaches, firstly where FAs are produced by enhancing the flux through neutral lipid formation, as typically occurs in eukaryotic systems, and secondly by mimicking the bacterial system to produce free FAs (Ledesma-Amaro et al. 2016).

TALEN (transcription activator-like effector nucleases)-based genome-editing technology was applied to *Y. lipolytica* inducing targeted genome modifications. This is an illustration of how a combination of molecular modeling and genome-editing technology can offer novel opportunities to rationally engineer complex systems for synthetic biology in order to obtain a significant increase of myristic acid (C14) production (Rigouin et al. 2017).

Hayashi et al. (2016) have investigated in *E. coli* the biological function of the tandem ACP domains in PUFA synthases to construct PUFA synthase derivatives with less and more active ACP domains than the native enzyme and examined the

Table 3 Metabolic maps at genomic scale of some oleaginous fungi, bacteria, and microalgae

Organism	GSM model	Reference
Fungi		
Yarrowia lipolytica	*i*NL895 2002 reactions 1847 metabolites 895 genes	Loira et al. 2012
Yarrowia lipolytica	*i*YL619_PCP 1142 reactions 843 metabolites 619 genes	Pan and Hua 2012
Yarrowia lipolytica	*i*MK735 1336 reactions 1111 metabolites 735 genes	Kavscek et al. 2015
Yarrowia lipolytica W29	*i*YALI4 1942 reactions 1691 metabolites 847 genes	Kerkhoven et al. 2016
Yarrowia lipolytica	*i*YLI647 1347 reactions 1119 metabolites 647 genes	Mishra et al. 2018
Yarrowia lipolytica	iYL_2.0 1471 reactions 1083 metabolites 645 genes	Wei et al. 2017b
Candida tropicalis	iCT646 945 reactions 712 metabolites 646 genes	Mishra et al. 2016
Mucor circinelloides	*i*WV1213 1326 reactions 1413 metabolites 1213 genes	Vongsangnak et al. 2016
Mortierella alpina	iCY1106 1854 reactions 1732 metabolites 1106 genes	Ye et al. 2015
Gram-positive bacteria		
Rhodococcus jostii RHA1	*i*MT1174 1935 reactions 1243 metabolites 1174 genes	Tajparast and Frigon 2015
Cyanobacteria		
Synechocystis sp. *PCC*	*iJN678* *863 reactions* *795 metabolites* *678 genes*	Nogales et al. 2012

(continued)

Table 3 (continued)

Organism	GSM model	Reference
Synechocystis sp. PCC 6803	*i*Syn731 1156 reactions 996 metabolites 731 genes	Saha et al. 2012
Cyanothece sp. ATCC 51142	*i*Cce806 667 reactions 587 metabolites 806 genes	Vu et al. 2012
Cyanothece sp.	*i*Cyt773 946 reactions 811 metabolites 773 genes	Saha et al. 2012
Chlorella vulgaris UTEX 395	*i*CZ843 2294 reactions 1770 metabolites 843 genes	Zuñiga et al. 2016
Synechococcus sp. PCC 7002	*i*Syp728 742 reactions 754 metabolites 728 genes	Hendry et al. 2016
Synechococcus elongatus PCC7942	*i*Syf715 851 reactions 838 metabolites 715 genes	Triana et al. 2014
Synechococcus 2973	*i*Syu683 1178 reactions 1028 metabolites 683 genes	Mueller et al. 2017
Synechococcus sp. PCC 7002	*i*Syp611 552 reactions 542 metabolites 611 genes	Hamilton and Reed 2012
Synechococcus sp. PCC 7002	*i*Syp708 746 reactions 581 metabolites 702 genes	Vu et al. 2013
Microalgae		
Chlamydomonas reinhardtii	*i*RC1080 2190 reactions 1068 metabolites 1080 genes	Chang et al. 2011
AlgaGEM *Chlamydomonas reinhardtii*	AlgaGEM 1725 reactions 1862 metabolites 866 genes	Dal'Molin et al. 2011

(continued)

Table 3 (continued)

Organism	GSM model	Reference
Chlamydomonas reinhardtii	*i*Cre1355 2394 reactions 1133 metabolites 1355 genes	Imam et al. 2015
Chlorella variabilis	*i*AJ526 1455 reactions 1236 metabolites 526 genes	Juneja et al. 2016
Phaeodactylum tricornutum	iLB1027 4456 reactions 2172 metabolites 1027 genes	Levering et al. 2016
Nannochloropsis salina	*i*NS934 2345 reactions 1985 metabolites 934 genes	Loira et al. 2016

effects on PUFA productivity. Tee et al. (2014) developed in *E. coli* a rational strain design process in systems biology, an integrated computational and experimental approach for carboxylic acid production, as an alternative method.

7 Research Needs

PUFAs have many health benefits and are essential for supporting the development of children. Although several microorganisms produce PUFAs naturally, native microorganisms frequently have low growth rates and produce low yields of these FAs, which are usually far below the desirable level to be commercially industrialized. Therefore, it is required to increase the research efforts to modify these microorganisms through metabolic engineering to accumulate higher amounts of lipids enriched in the desired FAs demanded by the industry. Additionally, these microorganisms should be further engineered to release the synthetized FAs to the culture medium, to facilitate the downstream processing. Metabolic engineering has the potential not only to improve yields but also to generate novel sources of PUFAs from food-grade microorganisms.

The SCO production cost depends mainly upon the species chosen for cultivation, lipid concentration within cells, and the concentration of cells produced. On the first sense, more screening efforts should be done to increase the number of available oleaginous microorganisms. Concerning lipid concentration, the overall yield and productivity of SCOs is normally constrained by different metabolic and regulatory bottlenecks. Thus, systems biology should contribute to proposing metabolic

alternatives that can be further implemented by using novel synthetic biological tools. Different strategies such as limiting the acyl exchange of intermediates, increasing the metabolic flux toward the products, enhancing precursors supply, and reducing the use of precursors and end products by competing pathways should be the targets to alleviate some of the bottlenecks. The use of heterologous enzymes such as acyltransferases, desaturases, elongases, and others will allow to better control the fluxes. Synthetic biology will contribute to redesigning gene clusters and pathways by chemically synthesizing them with optimized codons, promoters, or intergenic sequences, to facilitate their heterologous expression in the most productive microbial hosts. In fact, the integration of classical genetic, metabolic and protein engineering, system biology, synthetic biology, and evolutionary engineering, recognized as the new field of systems metabolic engineering, has been suggested some years ago as a global approach to increase the FA production in the oleaginous microorganism (Tee et al. 2014).

From commercial and industrial standpoints, it is important to reduce operating costs. In this sense, developing fermentation procedures using low-cost media and efficient product separation processes can lower operating costs. Even with a high level of productivity, SCOs are too expensive to compete with chemical (lubricants) or commodity (biofuels) products. This explains why industrial developments are focused on the high-value products as PUFAs for dietary supplements and for infant nutrition (Thevenieau and Nicaud 2013). Nevertheless, the use of renewable substrates must also be considered as an ecological added value. Organic waste can be used to grow oleaginous microorganisms to be converted into PUFAs decreasing the final price of the product, in a biorefinery concept (Huang et al. 2013; Béligon et al. 2016). The endogenous production of FAs can reduce cell viability due to the loss of inner membrane integrity (Lennen et al. 2011), and thus the secretion of endogenous FAs could moderate the toxicity effect while reducing product extraction costs (Ledesma-Amaro et al. 2016).

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Microbiome Metabolic Potency Towards Plant Bioactives and Consequences for Health Effects

19

Charlotte Grootaert and Tom Van de Wiele

Contents

Abstract

Plant-based bioactive molecules containing a (modified) hydrocarbon backbone may have diverse health-promoting effects, but because of large interindividual variability the cause-consequence relationship is difficult to establish. In this chapter, we use the case of polyphenols to explain the main determinants causing this variability, i.e., the complex interplay between dietary, host, and especially microbial factors. Finally, we focus on the potential of microbiome-driven population stratification as a tool to improve the understanding of mechanisms of bioavailability and bioactivity.

C. Grootaert
Laboratory of Food Chemistry and Human Nutrition, Ghent University, Ghent, Belgium
e-mail: charlotte.grootaert@ugent.be

T. Van de Wiele (✉)
Center for Microbial Ecology and Technology, Ghent University, Ghent, Belgium
e-mail: tom.vandewiele@ugent.be

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1 Introduction

Plant-based foods are sources of bioactive molecules with impact on health. Plant bioactives have been defined as "inherent non-nutritive constituents in food plants with anticipated health promoting/beneficial effects, and/or toxic effects, when ingested" (Gry et al. 2007). Major classes include (i) flavonoids and compounds such as tannins, (ii) carotenoids, (iii) phytosterols, (iv) glucosinolates, and (v) sulfur containing compounds. The first three classes can be considered as plant bioactives with a (minimally modified) hydrocarbon backbone (Fig. 1). The basic flavonoid structure consists of two aromatic rings linked through three carbons forming an oxygenated heterocycle. Phytosterols have a backbone that is closely related to cholesterol, and the majority of the carotenoids are derived from a 40 carbon-polyene chain.

Many observational studies have reported on a positive correlation between plant bioactives consumption and reduction of cardiovascular diseases and cancer. However, meta-analysis of these studies seldom gives strong conclusive evidence (He et al. 2006). Even in well-designed intervention studies with isolated bioactives, a variable response in health effects is often observed, thereby restricting the significance of the results. Among other many determinants, this may be partially explained by the interindividual variability in intestinal microbial composition and activity, as many bioactives escape upper gastrointestinal degradation and reach the

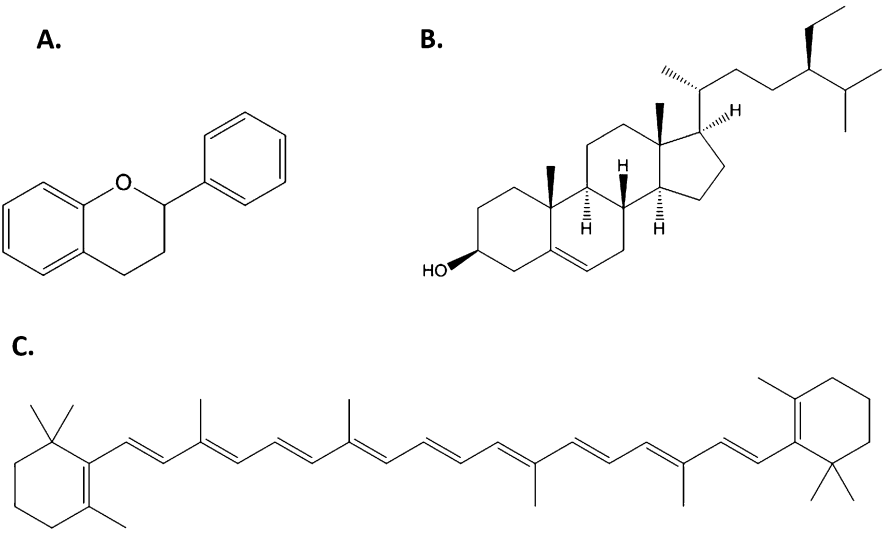

Fig. 1 Examples of common plant bioactives with (minimally modified) hydrocarbon backbones, including polyphenols (flavonoid backbone) (**a**), phytosterols (backbone) (**b**), and carotenoids (β-carotene) (**c**)

colon microbial community intact, where they are excessively metabolized. As more than 95% of dietary polyphenols escape digestion in the upper gastrointestinal tract and reaches the colon microbiota intact, the contribution of colonic metabolites to the bioactivity is expected to be high (Cassidy and Minihane 2017).

This section will focus on flavonoids, the most prevalent food polyphenol group of which the average daily intake is estimated to range between 201.2 and 581.3 mg (Zamora-Ros et al. 2016; Molina-Montes et al. 2016). The bioavailability and bioactivity of flavonoids is largely dependent on microbiome characteristics and we will elaborate on how this can contribute to the large interindividual variability in physiological response. Finally, we explain how population stratification based on microbiome may improve the identification of cause-consequence relationships between dietary intervention and biological effects, as required for health claim approval by the European Food Safety Authority (EFSA).

Bioavailability As a Key Towards Bioactivity

"*Bioavailability*" is defined as "the fraction of an ingested compound that reaches the systemic circulation and the specific sites where it can exert its biological action" (Bolca et al. 2007). Bioavailability is on the one hand determined by "*bioaccessibility*," which is defined as "the fraction that is released from its food matrix and is available for absorption in the stomach or intestine," and on the other hand, "*ADME*" characteristics, i.e., (i) absorption of the compound into the lymph and blood stream, (ii) its distribution by the circulatory system to different tissues and organs, (iii) compound metabolism by host cells, and (iv) excretion of remaining bioactives from the body (through bile or urine). Therefore, in vivo bioavailability studies often include blood (plasma) sampling at different hours after ingestion, and total collection of feces and urine up to a period of 3 days after ingestion. Important plasma descriptors are the "*peak plasma concentration*" and the "*area under the curve (AUC)*." However, plasma concentration can only be used as a predictive indication of tissue uptake, because compound affinity is tissue specific, interactions between compounds may occur, and plasma half-life is compound specific. As food bioactives (such as flavonoids) are excessively metabolized by the human body and the endogenous microbial community, the bioavailable fraction comprises both the bioactive (such as flavonoids) as such, as well as its metabolites (such as phenolic acids). For sufficient characterization, multiple samples need to be taken over time, as the ADME characteristics are strongly dependent on the individual. Because of the complexity of the end product, and the sample matrix (especially plasma and feces), determination of bioactives bioavailability is still a strong analytical challenge. Furthermore, as the time-dependent plasma concentrations of the cocktail of metabolites is variable, it may (partially) explain the variable health response to bioactives intake.

2 Determinants of Flavonoid Bioavailability

2.1 Diet

According to the composition of their side chains, flavonoids are classified as isoflavones, flavanones, flavones, flavonols, anthocyanins, and flavan-3-ols such as catechins and pro(antho)cyanidins. Flavonoids mainly occur as glycosidic conjugates with one or more sugars linked to their hydroxyl groups. Besides, association with carboxylic and organic acids, amines, lipids, and other polyphenols are common in the plant and food matrix (Cassidy and Minihane 2017). Flavonoid interaction with other biomolecules depends on Van der Waals interactions or hydrogen bonding as a consequence of the basic phenol structure that combines the hydrophobic planar aromatic ring with a hydrophilic polar hydroxy substituent (Aura 2008). As such, the food matrix may thus strongly affect flavonoid bioaccessibility (reviewed by Bohn 2014).

Besides mechanical, acidic, and enzymatic degradation of the food matrix by the host, a particular role for the microbiome in flavonoid bioaccessibility has been described for those glycosylated with a rhamnose group, as the human body lacks enzymes with rhamnosidase activity. This is the case for hesperidin, the major flavonoid in citrus fruits (Van Rymenant et al. 2018b). Furthermore, microbial enzymatic activity is involved in the release of monomers from strongly polymerized procyanidins. As an example, Wu et al. (2017) demonstrated that epicatechin release and absorption from Aronia berry juice procyanidins gradually increases along the different colon compartments of the simulator of the human intestinal microbial ecosystem (SHIME®). Finally, the microbiome may also indirectly improve flavonoid bioaccessibility by fermentation of the fibers to which polyphenols are attached (Gonzalez-Aguilar et al. 2017).

2.2 Host

Host-related determinants of flavonoid bioavailability and bioactivity include the (epi)genetic background of the host, as well as the health status of the individual. The genetic background may have a direct or indirect impact on flavonoid bioavailability. Examples of direct impact are the differential expression of cellular transporters and enzymes affecting flavonoid metabolism, and the motility of the gut thereby influencing contact time of the bioactive with the intestinal epithelia. An indirect impact is, for instance, the capacity of host secreted products, such as IgA, into the gut lumen to modulate the mucosal environment and (thereby) microbial colonization, composition, and flavonoid degrading activity, such as illustrated by a rat study performed by Taira et al. (2015). They found increased fecal mucin and immunoglobulin A levels and less gut microbial dysbiosis, caused by a high fat diet, after consumption of Aronia, haskap and bilberry polyphenols. Finally, polyphenols as such have also been identified as epigenetic modulators in the host and possible targets of polyphenols include microRNA expression, DNA methylation, and

histone modifications (Link et al. 2010). Therefore, this epigenetic crosstalk between bioactives and host is an emerging field in nutrition research in general (Tammen et al. 2013). With respect to health status, it is a trend that individuals at risk of a disease, in general have a higher response to the flavonoid. Some mechanisms include the expression of ß-glucuronidases as a response to cellular stress, thereby suggesting a release of the often more potent polyphenol aglycones at local sites of inflammation (Terao et al. 2011). Recent findings also show that cells exposed to stress molecules, such as valinomycin and lipopolysaccharides from microbial origin, but not TNF-α, accumulate polyphenols (Gonzales et al. 2016). The mechanisms need to be further elucidated but may possibly be due to differential regulation of drug transporters during inflammation.

2.3 Microbiome

The direct impact of the microbiome on flavonoid bioactivity can be divided into three major mechanisms, i.e., their antimicrobial effects, flavonoid-degrading capacity, and their prebiotic potency.

2.3.1 Antimicrobial Effect

The antimicrobial effect of polyphenols has been extensively studied and reviewed (Daglia 2012). Flavonoids and tannins in general have a higher and more diverse antimicrobial effect compared to other polyphenols. Besides inducing microbial aggregation, they have an impact on diverse microbial virulence factors, such as inhibition of biofilm formation and quorum sensing (Huber et al. 2003) and neutralization of bacterial toxins (Choi et al. 2007). Finally, they have a synergistic effect with antibiotics (Sanhueza et al. 2017). (Pathogenic) microorganisms vulnerable to the antimicrobial effect of flavonoids are *Vibrio cholerae*, *Streptococcus mutans*, *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Bacillus cereus*, *Helicobacter pylori*, *Staphylococcus aureus*, *Lactobacillus acidophilus*, *Actinomyces naeslundii*, *Prevotella oralis*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Chlamydia pneumonia* (Daglia 2012).

2.3.2 Flavonoid Degradation

Because of the revolution in the technology and methodology for chemical analysis in complex matrices such as blood and feces, our current understanding of polyphenol metabolism has dramatically increased. A wide number of human studies now describe not only host-related polyphenol metabolites, such as those produced by phase II metabolism, but also the even more diverse set of gut microbial metabolites, which in turn can undergo host metabolism (reviewed by Teng and Chen 2018). Degradation of flavonoid aglycones by colonic microbiota involves C-ring cleavage, as well as dehydroxylation, demethylation, or decarboxylation of the functional groups (Aura 2008). Various hydroxylated aromatic compounds derived from the A-ring (e.g., phloroglucinol, 3,4-dihydroxybenzaldehyde, or 3,4-dihydroxytoluene)

and phenolic acids derived from the B-ring have been reported as relevant products of the colonic transformation of flavonoids (Monagas et al. 2010). Microbial metabolism, either or not followed by phase II metabolism, may either increase or decrease its bioactivity, as illustrated by Van Rymenant et al. (2018a), who investigated the vasorelaxing effect of twenty-one physiologically relevant polyphenol metabolites on isolated mice arteries. It remains a challenge to clarify the mechanisms behind these complex degradation kinetics, in order to steer the microbial community to generate a pool of metabolites with improved health benefits. To this end, multiple in vitro digestion models have been applied, although many of them only consider bioaccessibility, and not in vitro bioavailability, of these metabolites. In a recent study by Van Rymenant et al. (2018b), a combined SHIME®-Caco-2 cell absorption model was applied to investigate hesperidin metabolism and absorption, and the pool of in vitro bioavailable phenolics was compared with a human bioavailability study with the same product. It was observed that the type of phenolics generated in vitro, and in the plasma and urine of the volunteers, was largely similar. However, the combined in vitro setup gave an overestimation of bioavailable small phenolics and a strong underestimation of hesperitin (the aglycon) absorption, whereas the latter was the major circulating metabolite that was directly linked with hesperidin consumption. Therefore, we may conclude that in vitro technology, combining (microbial) digestion, absorption, and bioactivity, needs further optimization.

2.3.3 Prebiotic Potency

The bioactivity of flavonoids is not only determined by their extensive metabolism but also by their impact on the microbiome (Cassidy and Minihane 2017; Dueñas et al. 2015). For this reason, experts in microbiology assigned by the International Scientific Association for Probiotics and Prebiotics now also consider polyphenols as prebiotics (Gibson et al. 2017). Depending on the experimental setup and the chemical structure, flavonoids have shown to increase, for instance, *Lactobacillus-Enterococcus* spp., and butyrate producers such as those belonging to the *Clostridium coccoides-Eubacterium* rectale group, *Akkermansia* spp., and *Faecalibacterium prausnitzii* (reviewed by Dueñas et al. 2015). Their effect on *Bifidobacterium* spp. is variable, with higher prevalence in in vivo studies, and lower in digestion simulators such as the SHIME®. Interestingly, the presence of short chain fatty acids, common microbial fermentation products either or not generated from the phenolic structure, may have an impact on (poly)phenolic transporter expression and phase II metabolism, and therefore possibly their bioactivity. This was illustrated by Van Rymenant et al. (2017) and Ziegler et al. (2016), who showed that the degree of hesperetin and ferulic acid transport and transformation was mainly increased by propionate and butyrate, and decreased by acetate and lactate. Besides the stimulation of probiotic bacteria and short chain fatty acid production, other prebiotic activities were reported upon polyphenol consumption. Degradation products of flavonoids such as ferulic acid stimulate adhesion and proteome changes in the probiotic *Lactobacillus acidophilus* NCFM. Polyphenols, such as flavonoids, have an impact on Toll-like receptors by modulating the gene expression in the host as well as by alteration of the microbiome (reviewed by

Pérez-Cano et al. 2014). Also the potential of polyphenols to act as a prebiotic treatment for cancer has been reviewed by Thilakarathna et al. (2018). An interesting observation in this case is that the composition of the microbiome is crucial for the chemopreventive effects of especially oligomeric polyphenols, such as proanthocyanidins. Finally, the interplay between flavonoids and intestinal microbiota has a strong impact on intestinal barrier function and inflammation, such as reviewed by Gil-Cardoso et al. (2016).

3 Stratification of the Population as Explanatory Tool for Health Effects of Phenolics

Because of the complex interplay between gut microbial composition and activity, diet, and the host's (epi)genetic background, strong interindividual responses upon bioactives consumption exist (Thilakarathna et al. 2018). One approach to simplify the interpretation of the cause-consequence relationship between polyphenol consumption and health effects is the stratification of the test population according to the individual's microbiome fingerprint, which is not only based on microbial composition but also on its unique metabolic phenotype, also called metabotype (Tomas-Barberan 2017). Based on their ability to convert a certain type of polyphenols, individuals can be subdivided into different metabotypes as low, middle, or high metabolite producers. One of the most studied examples is the conversion of isoflavones, such as daidzein present in soy, to the active phytoestrogen equol (Decroos et al. 2006). Bolca et al. (2007) demonstrated that stratification of a population of 100 menopausal women, based on the daidzein converting potential of the fecal microbiota, is strongly predictive for in vivo equol production. Tomas-Navarro et al. (2014) showed that volunteer stratification in converters/nonconverters based on fecal incubations is more predictive for flavanone bioavailability than orange juice processing. Another example is the conversion of ellagic acid (not a flavonoid, but a polyphenol released from tannins) to several types of urolithins, where a distinction can be made based on nonconverters, urolithin A, and urolithin A/B converters (Garcia-Villalba et al. 2017). This stratification based on metabotype was not dependent on health status, age, gender, body mass index, or the amount or type of ellagitannin food source ingested (Tomas-Barberan et al. 2014).

Although there is evidence that the stratification, based on in vitro polyphenol-converting microbiome activity, may be predictive for the in vivo bioavailability of the polyphenol, only few studies have demonstrated that this stratification is also predictive for its bioactivity. One interesting exception is the study of Gonzalez-Sarrias et al. (2017), which was a double-blind, crossover, dose–response, randomized, placebo-controlled human trial with 49 either healthy, overweight, or obese individuals, consuming pomegranate ellagitannin extract for 6 weeks. It was demonstrated that the urolithin B-producing metabotype was positively correlated with the highest baseline for cardiovascular risk, and that the lipid-lowering effect of ellagic acid administration was the highest in this metabotype. We therefore

recommend that in the future, more emphasis is put on microbiome stratification of target populations in order to better predict and investigate the beneficial health effects of polyphenols.

4 Research Needs

We may conclude that the research needs in the field of plant bioactives are situated in the understanding of the complex interplay between dietary-, host-, and microbiome-related factors determining the final health effects. To this end, a personal fingerprint of the individual's microbiome and its metabolic potential towards the plant bioactive may help in the improved design of human intervention trials, and the consequent stratification strategy. In addition, there is an urgent need to cross the gap between in vivo trials and conventional digestion, absorption, and bioactivity models, by the development of more realistic in vitro models. In drug research, cell and tissue engineering is an emerging field for screening of bioactivity. However, their compatibility with foods, digestive fluids and microbiota is still poor and needs further investigation.

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Poly-Beta-Hydroxybutyrate (PHB) and Infection Reduction in Farmed Aquatic Animals

20

Joseph Leopoldo Q. Laranja and Peter Bossier

Contents

Abstract

There is a continuous effort in finding effective and sustainable strategies to control diseases in farmed animals, and in recent years, the application of the bacterial storage compound poly-β-hydroxybutyrate (PHB) was identified as a new disease control agent for aquaculture. The idea of using PHB as a biocontrol agent was conceived based on the knowledge that this biopolymer can be degraded into short-chain fatty acids (SCFAs), and SCFAs are known compounds with antimicrobial properties. At the beginning of this chapter, an overview about the PHB granule, its detection, quantification, production, and recovery

J. L. Q. Laranja
Aquaculture Department, Southeast Asian Fisheries Development Center, Iloilo, Philippines

Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Ghent, Belgium
e-mail: jllaranja@seafdec.org.ph; jlqlaranja@gmail.com

P. Bossier (✉)
Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Ghent, Belgium
e-mail: peter.bossier@ugent.be

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in microorganisms is presented. The main topic focuses on the application and beneficial effects of PHB in farmed aquatic animals. The mechanisms by which PHB provides beneficial effects to the host are discussed.

1 Introduction

Disease outbreaks are being considered as a significant constraint on animal production and trade. These problems have brought major socioeconomic losses in the food animal industry as well as threats to food availability/security and the livelihood of vulnerable sectors in the society. For example, in aquaculture, approximately 40% (approx. >3 billion US$) of the total tropical shrimp production is lost annually due to diseases (Lundin 1996; Stentiford et al. 2012). Conversely, the application of antibiotics to control diseases and to improve growth performance in farmed animals has, however, brought limited success in the past because of the development of antibiotic resistance in pathogens. Thus, making these antibiotics ineffective as well as posing a direct threat to the human health and the environment (Cabello 2006). Therefore, finding alternative strategies that are equally effective and sustainable to mitigate diseases for farmed animals have been the primary focus for research in the food animal industry. To date, several alternative strategies have been developed to mitigate diseases and these include the application of PHB. PHB is an important member of the family of polyhydroxyalkanoates (PHAs), a class of microbially produced polyesters that have similarities in its physical properties with synthetic polymers (i.e., polypropylene), except that these biopolymers are biodegradable, biocompatible, recyclable, and nontoxic (Brandl et al. 1990). Due to these properties, there has been considerable interest in the commercial use of these biopolymers for several practical applications such as in daily life, biomedicine, and agriculture (Brandl et al. 1990; Philip et al. 2007). Recently, the application of PHB as a novel biocontrol agent for aquaculture was explored. While the PHB strategy is very promising, there are still areas that require further research in order to explore the full potential of PHB as a (new) biocontrol agent for farmed animals.

2 The PHB Granule

PHB, a linear polyester of D(-)-3-hydroxybutyric acid, is the simplest and the first PHA discovered by a French scientist Maurice Lemoigne in 1923 in an aerobic spore-forming *Bacillus* "M" strain (Lemoigne 1923). Accumulation of these biopolymers is a natural phenomenon in a large variety of bacteria that include the genera *Alcaligenes*, *Rhizobium*, *Pseudomonas*, and *Bacillus* to store carbon and energy, when nutrient supplies are imbalanced such as the depletion of nitrogen, phosphorus, or oxygen while the carbon that is present is in excess (Anderson and Dawes 1990). As carbon and energy reserve, these storage compounds are utilized by bacteria to enhance their survival during periods of starvation. In spore-forming

bacteria, observations show that the levels of PHB decrease with the concomitant appearance of mature spores suggesting that PHB might be utilized as an energy source to fuel their sporulation process (Kominek and Halvorson 1965).

PHB or PHAs in general are formed and deposited intracellularly in the form of inclusion bodies (PHB/PHA granules). Inside the bacterial cell, the diameter of PHB/PHA granules ranges from 200 to 500 nm in wild type strains, but the size mostly depends on the species and culture conditions used (Jendrossek and Pfeiffer 2014). Electron microscopical studies suggest that PHB granules in vivo are covered by a surface layer that is distinct from the polymer core. According to the findings of Griebel et al. (1968), native PHB granule consisted of ~97.7% of PHB, 1.87% protein, and trace amounts of lipids (phosphatidic acid and unidentified acetone soluble compound). The report suggests that PHB granules in vivo are surrounded by a lipid (mono)layer into which proteins are embedded. However, the in vivo presence of phospholipids in the PHB granule is still being debated in some papers and suggests the need to investigate its presence or absence in the PHB granules (Jendrossek and Pfeiffer 2014). Nevertheless, the model that was mostly used in describing the PHB granule structure in several review papers was based on the report of Griebel et al. (1968) wherein a coil of amorphous polymer chains are surrounded by a layer of phospholipids and proteins (Fig. 1). Both in vitro and in vivo studies show the presence of a considerable number of different PHB specific proteins in the surface layer of the polyester granules. In *Bacillus cereus* and *Bacillus megaterium*, a dense membrane of about 15–20 nm was observed at the surface of its PHB granule (Lundgren et al. 1964), while the PHB granules from *Caryophanon latum* cells revealed a densely packed, paracrystalline-like layer of particles with a diameter of ~8 nm (Jendrossek et al. 2007). It was concluded that these membrane particles represent PHB granule-associated proteins (PGAPs) and were thought to mostly or completely cover the granule surface (Fig. 2). These PGAPs were reported to play a major role in the synthesis and degradation of PHB as well as in the formation of the PHB granule (Pötter and Steinbüchel 2005; Jendrossek 2009). Four different types of granule-associated proteins were identified and reported

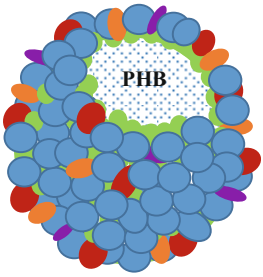

Fig. 1 Scheme of a native PHB granule. The core consists of PHB polymer that is covered by a lipid monolayer (green) with integrated proteins consist of PHB synthase (red), PHB depolymerase (orange), phasins (blue), and transcriptional repressor of phasin (violet). (Redrawn from Pötter and Steinbüchel 2006)

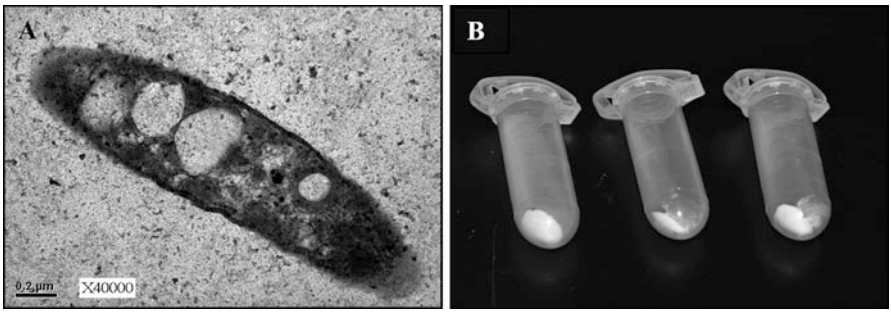

Fig. 2 (**a**) Transmission electron micrograph of a *Bacillus* sp. JL47 strain containing intracellular amorphous PHB (**b**) Crystalline PHB extracted from the *Bacillus* sp. JL47 using chloroform extraction method (Laranja 2017)

in PHB/PHA producing bacteria showing prominent function to PHB/PHA metabolism: These are: (1) PHB/PHA synthase, a key enzyme for PHB/PHA synthesis; (2) PHB/PHA depolymerases, enzymes that degrade PHB/PHA; (3) phasins, suggested to be the major structural proteins of the membrane surrounding the inclusion; and (4) regulator of phasin expression (PhaR) (Pötter and Steinbüchel 2005; Jendrossek 2009).

PHB granules can vary in terms of physical states: the intracellular native, amorphous state wherein the granules are still present in vivo in bacterial cells and the (partially) crystalline (55–80%) state wherein the granule is already extracted outside the bacterial cell (Fig. 2). Once the PHB granules are exposed to chemical (e.g., treatment with alkali or solvents), physical (freezing, pelleting by centrifugation), or biochemical (treatment with enzymes, bioactive compound) stresses, the granules rapidly denature and become resistant to the attack of intracellular PHB depolymerases (Merrick et al. 1965).

3 PHB Detection, Quantification, Production, and Recovery

PHB granules inside the bacterial cells can be rapidly detected by staining with Sudan Black B (Wei et al. 2011). Other methods like fluorescent staining using acridine orange (Kumar and Prabakaran 2006), Nile blue A (Ostle and Holt 1982), or Nile red (Spiekermann et al. 1999) were also being used for detecting PHB and/or rapid screening of PHB-accumulating bacteria. The most common method to quantify PHB is through spectrophotometric method wherein PHB is extracted from the bacterial cells through chloroform and the extracted PHB is converted to crotonic acid using concentrated sulfuric acid, and PHB is estimated spectrophotometrically at 235 nm (Law and Slepecky 1961). Gas chromatography (Braunegg et al. 1978), high performance liquid chromatography (Karr et al. 1983), ionic chromatography, and enzymatic determination (Hesselmann et al. 1999) are also some of the methods used for PHB quantification.

With the considerable interest in the commercial application of PHB, selection of microorganisms for PHB production should consider its high PHB productivity (i.e., can grow efficiently to high cell densities with a high PHB content in a relatively short period of time). Likewise, the PHB yield (defined as gram PHB produced per gram of carbon substrate consumed) should also be high so as not to waste the utilization of substrate to non-PHB materials (Lee 1996). While these factors are important, the carbon source that will be used should also be low-cost, considering its major contribution in the overall cost of the PHB production. Hence, alternative carbon sources like from waste materials and other by-products have been tested (Koller et al. 2010). A variety of taxonomical different groups of microorganisms were identified to be producing PHA (Brandl et al. 1990). Some PHA-producing microorganisms require the limitation of an essential nutrient (i.e., N, P, Mg, K, O, or S) to stimulate the synthesis of the biopolymer from an excess carbon source (e.g., *Alcaligenes eutrophus, Pseudomonas oleverans, Protomonas extorquens,* etc.), while some bacteria do not require nutrient limitation (e.g., *Alcaligenes latus*, mutant strain of *Azotobacter vinelandii*, and recombinant *E. coli* harboring the *A. eutrophus* PHA biosynthesis operon) (Lee 1996). Different fermentation strategies were being employed in these microorganisms to attain maximum PHA production. For example, fed-batch culture with two-step cultivation method is often employed in those bacteria requiring a nutrient limitation wherein cells are first grown to desired concentration without nutrient limitation, after which an essential nutrient is limited to allow efficient PHA synthesis while those bacteria that do not require nutrient limitation were strategized on the nutrient feeding itself (i.e., carbon and nitrogen source used). Most bacterial species used for the industrial scale production of PHA are Gram-negative bacteria such as *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii, Pseudomonas oleovorans*, recombinant *Escherichia coli,* among others. The utilization of Gram-positive bacteria (i.e., *Bacillus* species) as source of PHAs has also been suggested (Valappil et al. 2007). The lack of lipopolysaccharides (LPS) in Gram-positive bacteria was suggested to be beneficial considering that LPS may co-purify the PHAs and may have some immunogenic reactions. This aspect is important especially in PHAs that are used for medical applications. The application of recombinant DNA technique has allowed the development of various recombinant bacteria that can produce PHA more efficiently, synthesize PHA from inexpensive carbon sources, and/or synthesize unusual and industrially important PHAs. These advances will lower the PHA production cost.

Furthermore, efficient PHB/PHA isolation is considered an important economic factor in the overall PHB/PHA production and thus industries put more effort in developing efficient methods for PHA recovery. Based on the data of Mudliar et al. (2008), it can be calculated that the chemicals used for the extraction of PHB represent 30% of the total operation cost. In general, cells containing the PHA are separated from fermentation broth by centrifugation to concentrate cells. Subsequently, the harvested cells are broken up and various chemicals are added to digest non-PHA materials. Cell lysis by hypochlorite has been used as an excellent method for efficient and cheap release of PHA (Berger et al. 1989). Solvent-based

extraction methods using chloroform, methylene chloride, propylene carbonate, dichloroethane are also being employed. After removing all the cellular components, the polymer is precipitated in cold ethanol or methanol. The method was suggested to attain high PHA purity (>98%) (Zinn et al. 2001).

4 PHB as a Biocontrol Agent for Aquatic Farmed Animals

The application of PHB as a biocontrol agent for farmed animals was first demonstrated in a model crustacean species gnotobiotic *Artemia* by Defoirdt et al. (2007b) based on the knowledge that this biopolymer can be degraded into short chain fatty acids (SCFAs) (i.e., β-hydroxybutyric acid). Generally, SCFAs are known compounds with antimicrobial properties, i.e., capable of exhibiting bacteriostatic and bactericidal effects towards pathogenic bacteria (Ricke 2003). Previous studies demonstrated that these compounds can inhibit or decrease the growth of pathogenic bacteria such as *Salmonella typhimurium*, *Salmonella enteritidis, Escherichia coli,* and *Staphylococcus aureus* (McHan and Shotts 1993; Shin et al. 2002; Van Immerseel et al. 2003; Alva-Murillo et al. 2012). In fact, SCFAs are already used commercially as feed additives to control *Salmonella* in meat and poultry products (Mani-Lopez et al. 2012). In aquaculture, SCFAs were also demonstrated to inhibit the growth of fish and shrimp pathogens such as *Vibrio anguillarum, Vibrio pelagius, Vibrio alginolyticus, and Vibrio campbellii* (Vázquez et al. 2005; Defoirdt et al. 2006). However, the only limitation of using SCFAs in aquaculture settings is that they are water-soluble substances, and hence, the practical application of these compounds for aquaculture could be a challenge in terms of its effective delivery to the target sites. The addition of SCFAs in formulated feed could be lost in great amounts in the water due to leaching and if it is added directly in the water will require a considerable dose to be effective. Likewise, a higher level of SCFAs present in the water could give rise to bacterial growth that could result to oxygen depletion in the water and/or potential proliferation of bacterial pathogens. Hence with these concerns, the application of PHB as a source of SCFAs suggests a more promising approach. PHB is a water-insoluble compound, and it is stable at relatively high temperatures (melting point: 171–182 °C; Brandl et al. 1990), and therefore the compound can be easily incorporated in formulated feed or can be easily introduced via bioencapsulation method. With these methods, PHB as a precursor of SCFAs can easily be delivered in the gut for further microbial degradation where SCFAs are released as degradation products. Indeed, the findings of Defoirdt et al. (2007b) in gnotobiotic *Artemia* demonstrated that the application of PHB was about 100 times more efficient than its monomer form β-hydroxybutyrate. It was shown that only 100 mg L^{-1} was needed to obtain a similar effect with using 10 g L^{-1} β-hydroxybutyrate. These results provided the first indication on the potential and efficient application of PHB as a biocontrol agent for aquaculture. To date, a number of studies were already conducted exploring the beneficial effects of PHB in various aquatic farmed animals (Table 1). From these studies, PHB is applied either in crystalline form (i.e., PHB is extracted from bacteria) or in amorphous state

Table 1 Overview of published studies on the application of crystalline or amorphous PHB in various aquatic farmed animals

Species name	Developmental stage	PHB form	Effects observed	Reference
Artemia franciscana	Nauplii (Instar II)	Crystalline PHB	Prolonged the survival of starved *Artemia* Preventive and curative effect in *Artemia* against *Vibrio* infection	Defoirdt et al. (2007b)
A. franciscana	Nauplii (Instar II)	Amorphous PHB-containing bacterial strains	Enhanced the survival of *Vibrio*-challenged *Artemia*	Halet et al. (2007)
A. franciscana	Nauplii (Instar II)	Crystalline PHB	Enhanced the survival of PHB-fed *Artemia* during *Vibrio* challenge Enhanced the Hsp70 and phenoloxidase protein production Stimulated the prophenoloxidase and transglutaminase genes in PHB-fed *Vibrio*-challenged *Artemia*	Sheridan (1988)
A. franciscana		Crystalline PHB	Degradation intermediates of PHB inhibited the biofilm formation, luminescence, motility behavior, hemolysin production, and *N*-acyl-homoserine lactone (AHL)-mediated quorum sensing pathway of pathogenic *Vibrio* PUGSK8 strain Enhanced the survival of PHB-fed *Artemia* challenged with *Vibrio* PUGSK8 strain	Kiran et al. (2016)
A. franciscana	Nauplii (Instar II)	*Bacillus* strain containing different levels of amorphous PHB	Protected the *Artemia* against *Vibrio* infection Superior protective effect of the *Bacillus* strain containing higher amorphous PHB level	Laranja et al. (2018)
Penaeus monodon	Postlarvae	Different amorphous PHB-containing *Bacillus* strains supplemented in the diet	Enhanced the growth Enhanced the survival of nonchallenged and *Vibrio*-challenged shrimps	Laranja et al. (2014)

(*continued*)

Table 1 (continued)

Species name	Developmental stage	PHB form	Effects observed	Reference
P. monodon	Postlarvae	Amorphous PHB-containing *Bacillus* strain supplemented in the diet	Stimulated the prophenoloxidase and transglutaminase genes of the shrimp before and after the *Vibrio* challenge	Laranja et al. (2017)
P. monodon	10–15 g	Crystalline PHB supplemented in the diet at varying levels	Enhanced the survival rate of WSSV-challenged *P. monodon* Enhanced the immunity of the shrimp	Monica et al. (2017)
P. monodon	Postlarvae	Crystalline PHB enriched in *Artemia*	Enhanced the survival of the shrimp exposed to ammonia stress and pathogenic *Vibrio* challenge No significant effects on growth	Ludevese-Pascual et al. (2017)
Litopenaeus vannamei	Juvenile	Crystalline PHB supplemented in the diet at varying levels	Improved the growth performance of the shrimp Lowered the feed conversion ratio Higher activity of amylase, lipase, and trypsin in PHB-fed shrimps Modulated the intestinal digestive and immune function of the shrimp Increased the intestinal SCFA and body composition (i.e., protein and lipid content)	Duan et al. (2017b)
L. vannamei	Juvenile	Crystalline PHB supplemented in the diet at varying levels	Composition and diversity of intestine microbiota was altered in PHB-fed shrimps The expression of mammalian target of the rapamycin (mTOR) signaling-related genes were increased in PHB-fed shrimps	Duan et al. (2017a)

L. vannamei	Postlarvae	PHB-based biodegradable plastic as artificial substratum	Enhanced growth and survival in shrimps provided with PHB-based artificial substratum as compared with shrimps provided with polyvinylchloride (PVC)-based artificial substratum Observed higher trend of visit of the shrimp in the PHB-based substratum Water quality was improved (i.e., higher total ammonia-nitrogen conversion) in the rearing water provided with PHB-based artificial substratum	Ludevese-Pascual et al. (2018)
Macrobrachium rosenbergii	Larvae	Crystalline PHB or in combination with HUFA-rich lipid emulsion enriched in *Artemia* nauplii	Increased the survival and development of the prawn Low total bacteria and *Vibrio* counts in PHB-fed prawn larvae	Nhan et al. (2010)
M. rosenbergii	Larvae	Lyophilized *Alcaligenes eutrophus* H16 containing different levels of amorphous PHB enriched in *Artemia*	Increased larval development Enhanced survival of nonchallenged and challenged prawn larvae Better efficiency in *A. eutrophus* containing higher amorphous PHB level	Thai et al. (2014)
Eriocheir sinensis	Zoea 3 larvae	Crystalline PHB enriched in rotifer and *Artemia*	Enhanced the growth Enhanced the survival of nonchallenged and challenged PHB-fed larvae	Sui et al. (2012)
E. sinensis	Zoea 2 to megalopa	Crystalline PHB enriched in rotifer and *Artemia*	Enhanced the larval development, survival and tolerance to osmotic stress in PHB-fed larvae	Sui et al. (2014)

(*continued*)

Table 1 (continued)

Species name	Developmental stage	PHB form	Effects observed	Reference
Dicentrarchus labrax	Postlarvae	Lyophilized amorphous PHB contained in *A. eutrophus*	Stimulated the insulin-like growth factor 1 (indicator of relative growth), antimicrobial peptides dicentracin and hepcidin (innate immunity), and cell surface molecules MHC class IIa and IIb (adaptive immunity) No effects on growth and survival	Franke et al. (2017a)
D. labrax	Larvae	Lyophilized amorphous PHB contained in *A. eutrophus*	PHB affected the expression of the antimicrobial peptides ferritin and dicentracin Best larval survival in PHB-fed fish No effects on growth No effects on microbial community	Franke et al. (2017b)
D. labrax	Juvenile	Crystalline PHB supplemented in the diet at varying levels	Enhanced the survival at 2%, 5% and 10% PHB supplementation Highest weight gain and lowest FCR at 5% supplementation Decrease the intestinal pH in all PHB-fed fish Highest bacterial range-weighted richness in the intestine of all PHB-fed fish	De Schryver et al. (2010)
Acipenser baerii	Fingerlings	Crystalline PHB supplemented in diets at varying levels	Improved weight gain, specific growth rate and survival of fish fed the 2% PHB level Affected the microbial species richness and diversity in the gut PHB stimulated the abundance of *Bacillus* and *Ruminococcaceae* in the fish	Najdegerami et al. (2012)

A. baerii	Larvae	Crystalline PHB or in combination with HUFA-riched lipid emulsion enriched in *Artemia*	Increased the whole body lipid content High pepsin activity in both PHB and PHB+ HUFA-fed fish Suppressed the amylase activity Changed the microbial community in the distal intestine of the larvae Decreased the growth of both the PHB and PHB +HUFA-fed fish Decreased the survival of PHB-fed larvae exposed to salinity and ammonia stresses	Najdegerami et al. (2015b)
Oncorhynchus mykiss	Fry	Crystalline PHB supplemented in diet at different levels	Decreased the weight in the first 2 weeks but increased the growth after 6 weeks High total protease, pepsin, and pancreatic enzyme secretion in fish fed the 0.5% PHB supplementation Enhanced survival in PHB-fed fry challenged with *Yersinia ruckeri*	Najdegerami et al. (2015a)
Oncorhynchus niloticus	Juveniles	Crystalline PHB supplemented in diet at different levels	Increased lipase activity and lipid deposition Increased resistance against pathogenic infection No significant increase in weight gain	Situmorang et al. (2016)
O. niloticus	All male juveniles (25 g)	Crystalline PHB-hydroxyvalerate (HV) extracted from *B. thuringiensis* and supplemented in the diet at varying levels	All doses of PHB-HV used stimulated the specific and nonspecific immune response of the fish Enhanced survival of the fish at 5% PHB level	Suguna et al. (2014)
Mytilus edulis	Larvae	Crystalline and amorphous PHB	Amorphous PHB increased the survival of the mussel larvae No significant effect on growth or development Gut microbiota was not significantly modulated	Van Hung et al. (2015)

(i.e., PHB is still inside the bacterial cell) and is either supplemented in formulated feed as a feed additive or introduced in larvae via bioencapsulation method. Based on these studies reported, the application of PHB has demonstrated several beneficial effects to the cultured animals, and these include improved growth, survival, and robustness (i.e., the animal has higher resistance against biotic and abiotic stresses).

4.1 Effects of PHB on the Survival and Robustness of Farmed Aquatic Animals

The survival and robustness enhancing effect of PHB to the cultured animal was observed in several studies (Table 1). We suggest that PHB through its degradation product β-hydroxybutyrate can provide survival and robustness enhancing effect to the animal in three possible ways: (1) by inhibiting the growth or decreasing the virulence of the pathogen, (2) by enhancing the immune response of the animal, and (3) by serving as energy source to the animal.

4.1.1 Antipathogenic Effects of PHB

PHB that is supplemented in formulated feed or introduced via bioencapsulation method can be available for degradation in the gut of the animal either by PHB-degrading bacteria which are known to be present in the gut of cultured aquatic animals (Liu et al. 2010) or could also be (partially) degraded by the animal through its digestive enzymes without the aid of any PHB degraders (Defoirdt et al. 2007b). The combination of these two degradation processes inside the animal can also be possible. One strong indication on the release of the SCFA β-hydroxybutyrate from PHB degradation is the lowering of the gut pH, and this was clearly observed by De Schryver et al. (2010) in the gut of juvenile seabass after feeding the fish with PHB-supplemented diet. Indeed, in *L. vannamei*, feeding the shrimp with a shrimp diet supplemented with PHB resulted in a significantly higher intestinal SCFA content specifically acetic, propionic, and butyric acids (Duan et al. 2017b). It has been suggested that the SCFAs produced from the degradation of PHB can acidify the cytoplasm of the pathogen, and as a consequence, the pathogen has to redirect its cellular energy to maintain homeostasis, resulting to either lower cell growth, decrease in virulence, or even cell death (Defoirdt et al. 2009). Indeed, SCFAs in general have been described to be capable of exhibiting antibacterial properties depending on the physiological status of the organisms and the physicochemical characteristics of the external environment (Ricke 2003). The main bacteriostatic and/or bactericidal effect of SCFAs is believed to be due to the undissociated form of the acid, which can easily penetrate the lipid membrane of the bacterial cell through a diffusion process. Once inside the cytoplasm, the acid encounters a near neutral pH environment and dissociates into anions and protons (H^+) resulting to the lowering of the pH in the cytoplasm (Eklund 1983). This scenario leads to a potential problem to bacteria that needs to maintain a more or less constant pH in the cytoplasm to sustain its functional macromolecules. Since the cytoplasmic membrane is impermeable to protons (according to the chemiosmotic theory), the removal of these

protons requires active transport; hence, the bacterium needs to redirect its efforts towards the efflux of excess protons using its metabolic energy in the form of adenosine triphosphate (ATP), thereby exhausting its cellular energy and leading the organism to lower cell growth or even cell death (Davidson et al. 2013). This mechanism has been considered to be the major mechanism of growth inhibition caused by SCFAs in pathogens. The other suggested cytotoxic effects of SCFAs were also described wherein SCFAs may interfere with cytoplasmic membrane structure and membrane proteins in such a way that electron transport is uncoupled and subsequent ATP production is reduced; or that the acids serves as uncouplers and generally dissipate pH and electrical gradients across cell membranes (Sheu et al. 1972; Ricke 2003). Moreover, other less direct growth interfering effects associated with SCFAs also include interference with nutrient transport, cytoplasmic membrane damage resulting to leakage, disruption of outer membrane permeability, and influencing macromolecular synthesis (Ricke 2003).

In aquaculture applications, Defoirdt et al. (2006) investigated the effects of different concentrations of SCFAs, namely, formic, acetic, butyric, propionic, and valeric acids on the growth of pathogenic *V. campbellii* in Luria Bertani (LB) medium. The study showed that at pH 6, all the fatty acids completely inhibited the growth of the pathogen at a concentration of 100 mM. The results further showed that the growth-inhibitory effect of the fatty acids was pH dependent and that the effect decreases with increasing pH, a similar phenomenon observed in a previous study (McHan and Shotts 1993). Finally, adding 20 mM of the SCFAs to the culture water of the *Vibrio* challenged-gnotobiotic *Artemia* has resulted to a significantly higher survival of the infected nauplii. Interestingly, the antimicrobial activity of β-hydroxybutyrate was also investigated by Defoirdt et al. (2007b). The same with their findings on other SCFAs, the fatty acid was also effective in controlling the growth of the pathogenic *Vibrio campbellii* in LB medium at 100 mM dose at pH 6. Adding the β-hydroxybutyrate in the culture water of the *Vibrio*-challenged gnotobiotic *Artemia* resulted in a significantly increased survival of the infected nauplii. Furthermore, in the same study, the application of PHB was also investigated to determine if the biopolymer has similar protective effect with its monomer form β-hydroxybutyrate. The results showed that adding PHB to the culture water of the gnotobiotic *Artemia* at 100 mg L^{-1} or more offered a preventive and curative protection to the *Artemia* against the pathogenic *Vibrio campbellii*. In fact, a complete protection (mortality was not significantly different from unchallenged *Artemia*) was even observed in the challenged *Artemia* when PHB was added in the water at 1000 mg L^{-1}. Worth mentioning, the PHB was even 100 times more efficient as compared to its monomer form β-hydroxybutyrate in terms of dosage used. Such results could be due to the efficient and/or consistent delivery of the SCFA β-hydroxybutyrate in the target sites through PHB degradation.

Furthermore, a recent study also showed that PHB through its degradation intermediate β-hydroxybutyrate, effectively shut down the phenotypic expression of virulence factors of the *Vibrio* PUGSK8 specifically its biofilm formation, luminescence, motility behavior, hemolysin production, and the *N*-acyl-homoserine lactone (AHL)-mediated quorum sensing (Kiran et al. 2016). Also, in another

study, the antiadhesive activity of PHB was also demonstrated using a microtiter plate assay wherein the biofilm formation of pathogenic *Vibrio*s spp. was inhibited after coating the wells with PHB (Kiran et al. 2014). These studies demonstrated that PHB can also affect the virulence expression factors of pathogens and the possible explanation can be due to the changes in the cellular status of the pathogen (e.g., changes in cytoplasmic pH) due to its exposure to β-hydroxy-SCFAs from PHB. It has been described that the in vivo virulence expression such as in luminescent *Vibrios* are regulated by quorum sensing signal molecules (a form of bacterial cell-to-cell communication) (Defoirdt et al. 2008), and the production of these signal molecules (e.g., *N*-acyl homoserine lactones) has an intermediate metabolic cost in these signaling bacteria (Keller and Surette 2006). The possibility that the energy that is supposed to be used for virulence expression is redirected to other more important functions such as maintaining cellular homeostasis to sustain the pathogen's survival is inevitable.

It is also worth mentioning that the antipathogenic effects of PHB may also influence the microbial community of the animal in the gut, thereby favoring the growth of beneficial bacteria while inhibiting the growth of the potential pathogens. Indeed, feeding the *M. rosenbergii* larvae with *Artemia* enriched with PHB resulted in a significantly lower *Vibrio* spp. counts as well as total bacterial counts in the gut of the freshwater prawn, suggesting that PHB (through the mechanisms described above) may have inhibited the growth of these potentially pathogenic microorganisms. The work of Duan et al. (2017a) on *L. vannamei* showed an increased in abundance of beneficial bacteria such as *Bacillus*, *Lactobacillus*, *Clostridium*, and *Bdellovibrio* while decreased the abundance of *Alpha-proteobacteria* after feeding PHB in the shrimp. In Siberian sturgeon fingerlings, PHB feeding stimulated the abundance of *Bacillus* and *Ruminococcaceae* species in the fish gut (Najdegerami et al. 2012).

4.1.2 Enhancing the Immune Response

Enhancing the immune response in the host has been suggested as a strategy to protect the cultured animals from diseases. The first study that demonstrated the immune enhancing effect of PHB was observed by Suguna et al. (2014) in tilapia *Oreochromis mossambicus* wherein the fish showed a significant immunostimulatory effect on both specific (i.e., increased antibody response) and nonspecific (i.e., increased lysozyme, total peroxidases, and antiprotease activity) immunity of the fish after feeding with diet containing PHB-hydroxyvalerate (PHB-HV) extracted from *Bacillus thuringiensis.* Their study further showed that when the fish were challenged with a virulent *Aeromonas hydrophila*, the survival of the tilapia was significantly higher in those fish fed the highest PHB dose (5%) as compared to fish fed the 1% and 3% PHB. The study discussed that the immune-enhancing effect of PHB-HV in the fish has proved the efficacy of the compound to protect the animal from disease infection. The immune-enhancing effect of PHB applied in amorphous state was also investigated in previous studies. The work of Laranja et al. (2017) demonstrated that *Penaeus monodon* postlarvae fed the shrimp diet supplemented with amorphous PHB contained in a *Bacillus* strain

(i.e., 55% amorphous PHB on cell dry weight) stimulated the prophenoloxidase (proPO) and transglutaminase (Tgase) genes of the shrimp before and after the *Vibrio campbellii* challenge. The authors discussed that the observed increased survival of the shrimp from their previous experiment (Laranja et al. 2014) was associated to the enhanced immunity of the shrimp due to amorphous PHB. However, it can also be argued that since the amorphous PHB was delivered inside a bacterial cell, the possibility that other components of the bacterial cell aside from the amorphous PHB might have elicited an immune response. However, their further experimentation in gnotobiotic *Artemia* revealed that the amorphous PHB contained in the *Bacillus* cell appears to be the main determinant in the observed protective effects in the *Vibrio*-challenged gnotobiotic *Artemia* (Laranja et al. 2018). The work of Franke et al. (2017a) in European seabass larvae also showed that amorphous PHB (contained in *Alcaligenes eutrophous* at 75% PHB on cell dry weight) stimulated the expression of insulin-like growth-factor 1, an indicator of relative growth as well as the expression of the antimicrobial peptides dicentracin and hepcidin genes (innate immunity) and the major histocompatibility complex (MHC) class IIa and MHC class IIb (adaptive immunity). In *L. vannamei* juveniles, PHB supplementation resulted in the significant expression of Hsp70, Toll, and immune deficiency genes as well as increased the activity of the immune enzymes lysozyme, total antioxidant capacity, inducible nitric oxide synthase, and nitric oxide content of the shrimp (Duan et al. 2017b). In another study, PHB also significantly activated the mammalian target of the rapamycin (mTOR) signaling pathway of *L. vannamei* (Duan et al. 2017a), where such pathway plays a crucial role in intestine inflammation and epithelial morphogenesis.

The mechanism by which PHB and/or its degradation products stimulates the immune response of the animal is not clear to date. However, Baruah et al. (2015) proposed that PHB enhances the immune system of the animal through Hsp70 biosynthesis. In their study on gnotobiotic *Artemia*, PHB significantly stimulated the expression of heat shock protein 70 (Hsp70) and proPO genes as well as increased the phenoloxidase protein activity in *Vibrio*-challenged gnotobiotic *Artemia*. The authors suggest that the degraded PHB might have caused cellular acidification in the animal and this might have created a (mild) stress conditions that could lead to the Hsp70 biosynthesis. The induction of this stress protein in *Artemia* might have conferred the immunity of the animal by possibly mediating the expression of proPO genes which eventually resulted to increased phenoloxidase protein production and activity in the challenged *Artemia* larvae.

Conversely, it can also be hypothesized that the β-hydroxybutyrate produced from PHB degradation might have stimulated the immune system of the cultured (aquatic) animal in a similar manner with mammalian system wherein SCFAs binds to free fatty acid receptors such as G protein-coupled receptors (GPRs) to activate immune cells. It was described previously that SCFAs regulate the mammalian immune system by binding to SCFA receptors such as the GPR43, a G protein-coupled receptor that recognizes endogenous SCFA ligands, such as acetate, propionate, and butyrate (Brown et al. 2003). This free fatty acid receptor is observed to be highly expressed in neutrophils, macrophages, and monocytes (Le Poul et al.

2003). The immune-modulation of SCFAs through fatty acid receptors were highlighted in previous studies wherein the SCFAs derived from commensal bacteria promote neutrophil chemotaxis in mice through the GPR43 receptor (Sina et al. 2009; Vinolo et al. 2011). Hence, the idea that the SCFA β-hydroxybutyrate stimulates the immune system of cultured aquatic animals (e.g., fish) in the same manner as with mammalian system is interesting to investigate considering that these immune cell types are also known to be present in fish (Rombout et al. 2005). Conversely, since β-hydroxybutyrate and butyrate are two relatively different compounds, it cannot be simply assumed that it will induce similar immune modulation as described above, something that would need further investigation.

4.1.3 PHB as a Source of Energy

PHB as a fatty acid is considered as a typical source of energy (Azain 2004). Previous study shows that the β-hydroxybutyrate in the form of ketone bodies was used as energy source in developing *Artemia* (Weltzien et al. 2000) while in another study, PHB prolonged the survival of starved *Artemia* (Defoirdt et al. 2007b). Furthermore, in a more recent finding, the lipid-saving effects of dietary PHB (either in the amorphous or crystalline form) was also demonstrated in gnotobiotic *Artemia* wherein the whole body lipid content of the starved *Artemia* was increased (Ludevese 2016). The research of Situmorang (2015) demonstrated in detail the compartment distribution of PHB in Nile tilapia and suggested the PHB's metabolic fate in the fish. Their results showed that there was a faster and higher incorporation of (^{13}C-labelled) PHB in the intestine, liver, spleen, and kidney as compared to the heart, blood, brain, and muscle of the fish. The author suggested that PHB is degraded and absorbed in the intestines resulting in the transport of free fatty acids (FAs), consisting of long- and short-chain fatty acids (LCFAs and SCFAs) in the blood. It has been described that LCFAs are esterified to triglycerides in enterocytes, incorporated into chylomicrons, and then enter the lymphatic system, and ultimately enter the blood vascular system, while SCFAs from the intestinal tract enter the portal vein as free acids (Sheridan 1988; Schönfeld and Wojtczak 2016). SCFAs that are absorbed via the portal circulation are not directly esterified to form chylomicrons, as is the case with LCFAs (Knittle and Hirsch 1965). This portal mechanism of absorption permits elongation and oxidation of acids in the liver before they enter the systemic circulation and presented in the adipose tissue. SCFAs that are transported to the liver tend to remain in their unesterified state, while those SCFAs that are not elongated are probably oxidized such as into ketone bodies. These ketone bodies are further circulated in the blood and are utilized by the heart, brain, and muscle as their energy source. Therefore, the capability of PHB and/or its degradation intermediate β-hydroxybutyrate to serve as additional energy source for the animal could be considered beneficial especially during stressful conditions such as molting, reproduction, handling of pathogens, or exposures to adverse environmental conditions. These conditions are considered energy-demanding and therefore with PHB as additional energy source, the animal may have a better chance of surviving from such stressful circumstances. Indeed, the work of Ludevese-Pascual et al. (2017) demonstrated that PHB supplementation increased the survival of the

P. monodon postlarvae after exposure to a lethal dose of ammonia. Their study also confirmed that PHB enhanced the survival of the animal after exposure to a sublethal dose of ammonia and subsequent exposure to pathogenic *Vibrio campbellii*. A similar finding was also observed in the work of Laranja et al. (2014) when *P. monodon* postlarvae was fed with artificial diet supplemented with amorphous PHB. From these studies, it can be suggested that PHB as additional energy source could increase the quality of the (post)larval *P. monodon* and their chances of surviving under adverse environmental conditions caused by both biotic and/or abiotic stress.

4.2 Growth-Promoting Effects of PHB

It can be hypothesized that the growth-enhancing effects of PHB can be related to its ability to influence the microbial community in the gut. The work of De Schryver et al. (2010) in juvenile seabass and of Najdegerami et al. (2012) in juvenile Siberian sturgeon showed that fish fed with PHB led to a change in the microbial composition in the gut, and that this change in the microbial community composition seemed to be closely associated with the observed increased fish growth. The shift towards more beneficial microbes in the gut which have the capability to help in the digestion and absorption of nutrients may have enhanced the growth of the animal, considering that commensal microbiota were shown to be affecting a wide range of biological process in the gut including nutrient processing and absorption (Rawls et al. 2004). The work of Duan et al. (2017b) on *L. vannamie* showed that PHB-fed shrimps resulted to a higher activity of amylase, lipase, and trypsin in the intestine and that it improved the growth performance as well as decreased the feed conversion ratio in the shrimp. In their other experiment on *L. vannamei*, the PHB-fed shrimps resulted to an altered composition and diversity of the shrimp's intestinal microbiota (Duan et al. 2017a). Furthermore, the growth promoting effect of PHB could also be related to its ability to improve the intestinal health status of the animal. The work of Silva et al. (2018) demonstrated that feeding the Pacific white shrimp *L. vannamei* with shrimp diet supplemented with PHB or sodium butyrate increases the intestinal length and villi width of the animal, suggesting that PHB could improve the integrity and absorptive capacity of the mucosal membrane. It is also worth mentioning that the lowering of the intestinal pH due to PHB degradation might also enhance the activity of the digestive enzymes thereby leading to a better nutrient digestibility and absorption (Luckstadt 2008).

Indeed, the growth promoting effects of PHB was demonstrated in several cultured species including juvenile European seabass *Dicentrarchus labrax* (De Schryver et al. 2010), Siberian sturgeon *Acipenser baerii* fingerlings (Najdegerami et al. 2012), giant tiger prawn *Penaeus monodon* postlarvae (Laranja et al. 2014), Pacific white shrimp *Litopenaeus vannamei* juveniles (Duan et al. 2017b), giant freshwater prawn *Macrobrachium rosenbergii* larvae (Nhan et al. 2010), and Chinese mitten crab *Eriocheir sinensis* zoea to megalopa (Sui et al. 2012, 2014). However, application of PHB in some cultured species or on the same species but

with different developmental stages such as on European seabass *Dicentrarchus labrax* larvae and postlarvae (Franke et al. 2017a, b), Siberian sturgeon *Acipenser baerii* larvae (Najdegerami et al. 2015b), tilapia *Oncorhynchus mykiss* juveniles (Situmorang et al. 2016), mussel larvae (Van Hung et al. 2015), and *P. monodon* mysis to postlarvae (Ludevese-Pascual et al. 2017) did not show significant growth effects. Findings from all of these studies seemed to show that the growth-promoting effects of PHB differs between PHB doses, developmental stages of the animal, and probably between species. For example, the work of Ludevese-Pascual et al. (2017) showed no significant growth effect of applying crystalline PHB in *P. monodon* mysis to postlarvae, while in the work of Laranja et al. (2014), feeding the *P. monodon* postlarvae (PL30) with diet supplemented with amorphous PHB contained in bacilli showed a significant growth effect. The difference in the results of the two researches using the same shrimp species could possibly be attributed to difference in the developmental stage of the shrimp used wherein different degradation capacities (enzymatic (i.e., presence of appropriate digestive enzymes) and/or microbial activity (i.e., presence of PHB-degrading bacteria)) towards PHB may have occurred in these two different shrimp stages or it could also simply be due to the degradation efficiency of the amorphous PHB as compared with crystalline PHB. In these studies, it can be observed that the earlier stage *P. monodon* (mysis) were fed with crystalline PHB as enrichment to *Artemia,* and the PHB in this form is sometimes difficult to degrade by the animal due to its level of crystallinity while the *P. monodon* postlarvae were fed with PHB in amorphous (native) form where the compound is expected to be easily depolymerized (Yu et al. 2005).

5 Efficiency of Amorphous PHB as Compared with Crystalline PHB

While it has already been demonstrated that crystalline PHB (i.e., extracted form) can be an effective biocontrol agent for aquaculture, previous findings (Halet et al. 2007; Thai et al. 2014; Laranja et al. 2018) suggested that the application of amorphous PHB contained in a whole bacterial cell can be considered more efficient than using crystalline PHB. The higher efficiency of amorphous PHB required only a smaller amount to be used to obtain a similar significant effect with crystalline PHB. In the previous results in gnotobiotic *Artemia* experiment (Laranja et al. 2018), the amorphous PHB used was ~82–410 times lower than the suggested effective level of crystalline PHB for *Artemia* (Defoirdt et al. 2007a), in the previous shrimp culture experiment (Laranja et al. 2014), the amount of amorphous PHB supplemented in the artificial feed was ~73–88 times lower than the crystalline PHB level used in shrimp (Duan et al. 2017b), using amorphous PHB as enrichment in *Artemia* as food for *P. monodon* postlarvae (Laranja et al. 2017) was ~3.6 and 18.2 times lower compared to the enrichment of crystalline PHB in *Artemia* for *P. monodon* (Ludevese-Pascual et al. 2017) and *M. rosenbergii* (Nhan et al. 2010) (post)larvae, respectively. We suggested that the higher efficiency of using amorphous PHB compared with crystalline PHB could be due to two factors: (1) the more

efficient degradation of amorphous PHB than with crystalline PHB and (2) the possible added beneficial effects of the bacterial strain containing the amorphous PHB. The efficient degradation of amorphous PHB can be due to (1) the (bio) physical state of PHB and (2) the smaller particle size of the amorphous PHB inside the bacterial cell as compared with crystalline PHB. It was suggested that crystallinity of the compound affects its degradability such that an increasing crystallinity reduces the degradability of the biopolymer (Tokiwa et al. 2009). Indeed, it has been demonstrated that amorphous PHB can be hydrolyzed 30 times faster than crystalline PHB because the hydrolytic enzymes can diffuse faster in the amorphous phase than in crystalline phase (Yu et al. 2005). Furthermore, the smaller particle size of amorphous PHB inside the bacterial cell (<0.5 μm in diameter (Anderson and Dawes 1990)) as compared to crystalline PHB (~30 μm (Defoirdt et al. 2007a)) could also have an effect on the depolymerization efficiency of the compound such that the smaller particle amorphous PHB can be more susceptible to enzymatic and microbial degradation as compared to crystalline PHB. Furthermore, it could also be hypothesized that the higher efficiency of amorphous PHB could also be due to the bacterial carrier used, wherein some bacterial components (aside from amorphous PHB) might have some additional beneficial effects to the cultured animal (Fig. 3). In previous studies, it was demonstrated that aside from the amorphous PHB level, the bacterial cell density (used as live or lyophilized) also significantly influenced the survival of the gnotobiotic *Artemia* (Laranja et al. 2018) and giant fresh water prawn larvae (Thai et al. 2014) during pathogenic bacterial challenge. This does not exclude the possibility that the whole bacterial cell could contribute as an additional nutrient source for the animal, considering that bacterial cells contains 25–49% protein, 2.5–11% carbohydrate, 2.5–9% lipid, and 4.7–14% ash (Brown et al. 1996). Furthermore, other bacterial components (e.g., expressed proteins) associated

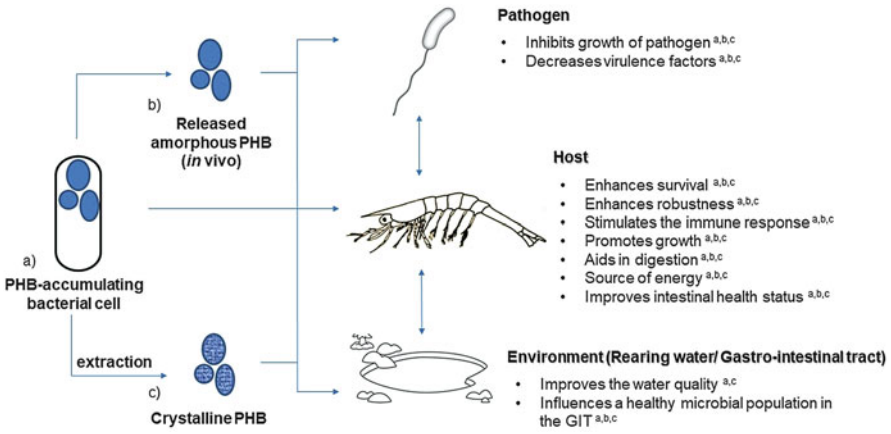

Fig. 3 Schematic diagram of the (possible) settings by which (a) PHB-accumulating bacterial cell, (b) amorphous PHB, or (c) extracted crystalline PHB acts on the pathogen, host, and/or on the environment/GIT

with amorphous PHB might have some immunostimulatory effects to the animal. In a recent study, analyzing the protein fingerprint of the *Bacillus* sp. JL47 containing low and high amorphous PHB content showed a higher number of peaks in higher PHB content *Bacillus* cells, and these additional peaks might represent expressed proteins with immunostimulatory effects or might be associated with cellular changes resulting in immunostimulation (Laranja et al. 2018). Likewise, the presence of non-self molecules (i.e., microbe-associated molecular patterns) in bacterial cells might also trigger an immune-response in the host.

Nevertheless, the effectiveness of crystalline PHB can be improved through the addition of PHB-degrading bacteria where PHB can be depolymerized by extracellular enzymes secreted by the PHB-degrading bacteria. A previous study showed that the protective effect of feeding the gnotobiotic *Artemia* with PHB and a PHB-degrading bacterium resulted in an improved survival than when feeding the *Artemia* with crystalline PHB alone (Liu et al. 2010). The advantage, however, of using amorphous PHB is that it does not require anymore the aid of PHB degraders to effectively depolymerize the compound. It is also worth mentioning that supplementation of crystalline PHB in the feed at a higher dose can be economically unattractive considering that the prevailing price of PHB (~4 € kg^{-1}) is about four times higher than a kg of fish feed (Johnson et al. 2010). It can be calculated that the chemicals used for the extraction process for producing pure PHB requires 30% of the total operation cost while with amorphous PHB, the extraction process is already omitted.

6 Research Needs

The beneficial effects of PHB (used either in amorphous form, crystalline form or is still inside a bacterial cell) (Fig. 3) and the possible mechanisms by which PHB provides beneficial effects to the cultured animals are suggested (see above), yet further research is needed to elucidate its exact mode of action. For instance, how PHB and/or its degradation products stimulate the immunity of fish or crustacean species (e.g., if it involves binding to receptors that will trigger immune cells such as in mammalian system) or how PHB and/or its degradation products enhance the growth of these animals requires further investigation. In these studies, the utilization of (appropriate) gnotobiotic animal models to unravel such mechanisms is highly important. Furthermore, the PHB degradation involved (enzymatic and/or microbial) and the degradation capacities of each of these animals in relation to developmental stage, its microbial community present and the physico-chemical properties of PHB applied (i.e., crystallinity or amorphous, chain length, type of co-polymer used) will be of specific importance.

To our knowledge, no experiments yet are conducted on the application of PHB in terrestrial animals such as those used in poultry and livestock. It might also be interesting to evaluate the beneficial effects of PHB in these food-producing terrestrial animals to broaden its application as a biocontrol agent.

The application of amorphous PHB-containing probiotic *Bacillus* species was investigated in the previous work (Laranja 2017). Other probiotic bacteria such as *Lactobacillus* species that are able to accumulate high amount of PHB can also be explored knowing that these microorganisms can accumulate PHB (Aslım et al. 1998) and are already known probiotics in aquaculture and in humans.

In aquaculture, integration of amorphous PHB-containing probiotic bacteria to the biofloc system to enhance the added value of biofloc can be an interesting strategy to investigate. It has been described that ~2–20% of the organic fraction in the bioflocs is believed to be living microbial cells while ~70% of the total floc weight is total organic matter (Wilén et al. 2003). Hence, the presence of heterotrophic bacteria in biofloc can be an interesting avenue to incorporate the PHB strategy such that these microorganisms could be "trained" to accumulate significant amounts of PHB through C/N manipulation. With such concept, we suggest that PHB can be used as a parameter to determine the quality of the biofloc, considering the beneficial effects of PHB.

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