Rani Gupta
Namita Gupta

Fundamentals of Bacterial Physiology and Metabolism

Springer

Fundamentals of Bacterial Physiology and Metabolism

Rani Gupta · Namita Gupta

Fundamentals of Bacterial Physiology and Metabolism

Springer

Rani Gupta
Department of Microbiology
University of Delhi
New Delhi, Delhi, India

Namita Gupta
Foundation for Innovation and Technology
Transfer (FITT)
Indian Institute of Technology Delhi
New Delhi, Delhi, India

ISBN 978-981-16-0722-6 ISBN 978-981-16-0723-3 (eBook)
https://doi.org/10.1007/978-981-16-0723-3

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Microbiology is an important discipline of science encompassing various aspects of microorganisms that directly or indirectly affect human life. Everyone knows that microbes are ubiquitously present even in extremes of environments where life can hardly exist. They are thought to be the earliest progenitors of life, and surprisingly, these tiny cells represent all details of various physiological, biochemical and genetic functions. The advent of modern molecular techniques has unraveled several novel aspects of microbial life which reflect complex and diverse metabolic pathways employed during survival under extreme conditions.

Be it bacteriology, virology, pathology, industrial microbiology, biotechnology, genetics or recombinant DNA technology, all these disciplines need understanding of microbial physiology and metabolism. The best approach for clear understanding of a subject is a proper amalgam of basic and applied aspects in an integrated approach. Both exploitation of the wide range of microorganisms present in the environment for finding their industrial utility as well as understanding of pathogenesis to counter infection and resistance development using genetics or recombinant DNA technology require deep understanding of physiology and metabolism.

These subjects are generally taught in isolation, and students are hardly able to develop understanding so as to exploit the knowledge acquired in each class. Further, during interaction with students over the years, we realized that even though students are interested in this subject and know that a strong foundation of physiology and metabolism can make them good researchers they are scared to take this up. In the present book, we have tried to present each chapter in a simplified manner while incorporating the latest findings in the field. Efforts have been made to interconnect each chapter with bacterial pathogenesis by discussing perspective drug targets wherever possible. Clues have also been provided for pathway engineering so that a reader can well appreciate the importance of the subject of physiology and metabolism. To make the content clearer, the book also provides case studies, as well as interesting facts and techniques that help showcase the interdisciplinary nature and bridge the gap between various aspects of applied microbiology.

The book is divided into ten parts, and each part is further divided into multiple chapters.

Part I is *Prokaryotes: Bacterial and Archaeal Diversity and Cell Structure* and is divided into three chapters. Chapter 1 deals with **diversity of prokaryotes** wherein detailed diversity of both archaea and bacteria is described, and various phyla of archaea are described comprehensively taking key genera into account. The selected diverse genera of bacterial groups of biotechnological and medical importance have also been discussed. Chapter 2 describes **prokaryotic cell structure** in detail, and specific differences in their archaeal counterparts have also been discussed. Chapter 3 is about **bacterial cell wall** biosynthesis and inhibitors. It starts with description of gram-positive and gram-negative bacterial cell walls followed by various biochemical steps for synthesis of bacterial cell wall. Along with this, the outer membrane and lipopolysaccharide of gram-negative bacteria is also discussed. A special focus has been kept on various cell wall synthesis inhibitors which are used as antimicrobials.

Part II is *Bacterial Cell Division and Growth* which includes Chaps. 4 and 5. Chapter 4 describes **bacterial cell division** wherein bacterial cell cycle, cytokinesis, regulation of septum and divisome proteins as antimicrobial targets have been dealt with. Chapter 5 deals with **growth physiology and kinetics** with detailed account on bacterial growth curve, diauxic growth measurement of microbial growth and growth kinetics in batch, fed-batch and CSTR operations along with the effect on growth physiology during recombinant protein production.

Part III is *Transport across Cell Membrane* which includes Chaps. 6–8. Chapter 6 discusses **general account of solute transport** across cell membrane along with different types of transporters and related mechanisms. Chapter 7 deals with **specialized transporters**: ABC transporters and group translocation. Bacterial multidrug efflux pumps have also been discussed. Chapter 8 deals with **protein secretion** wherein different secretory systems have been described for folded and unfolded proteins in both gram-negative and gram-positive bacteria.

Part IV describes *Central Metabolic Pathways* used for glucose metabolism. It comprises Chaps. 9–13, dealing with **Glycolysis and Gluconeogenesis** (Chap. 9), **Pentose Phosphate Pathway** (Chap. 10), **Entner–Doudoroff Pathway** (Chap. 11), **Tricarboxylic Acid Cycle** (Chap. 12) and **Alternate Tricarboxylic Acid Cycle** (Chap. 13). All the pathways have been dealt extensively. Apart from basic pathway historical account, modifications of pathway during various physiological conditions of oxygen and oxidative stress have been discussed dealing with specific bacterial examples. Toward the end of each chapter, engineering of carbon metabolic pathways to achieve biotechnologically relevant strains have been described as case studies for citric acid overproduction and lignocellulose utilization to produce ethanol and succinic acid in relevant chapters. Along with this, the regulation of pathways and reductant regeneration has also been discussed.

Part V discusses *Electron Transport and Energy Generation* during aerobic and anaerobic respirations. It includes Chap. 14**Electron Transport and Energy Generation** that covers topics encompassing process of energy generation by coupled flow

of electrons in membrane. Various electron donors and carriers involved in electron transport chain have been explained very well along with their redox potentials. Chapter presents a detailed note on bacterial electron transport chain, proton motif force, ATP synthase and oxidative phosphorylation. Toward the end, a note on uncouplers and inhibitors of electron transport is presented.

Part VI discusses *Metabolic Diversity of Carbon and Energy Source* which includes Chap. 15 dealing with nutritional diversity among bacteria wherein **chemolithotrophy and phototrophy** have been discussed.

Part VII *Nitrogen Metabolism* consists of Chaps. 16 and 17. Of these, Chap. 16**Nitrogen Assimilation and Dissimilation** gives an overview on nitrogen cycle and its assimilation encompassing topics such as nitrogen fixation, ammonification, nitrification, denitrification, anammox and GS-GOGAT pathway. Chapter 17**Glutathione and Polyamines in Bacteria** provides an overview on a non-ribosomal redox regulating tri-peptide called glutathione. Its role as redox regulator during stress has also been discussed followed by detailed note on polyamine biosynthesis, their role in cellular physiology and their industrial applications.

Part VIII describes *Lipid Metabolism and Nucleotide Biosynthesis* and their regulation which includes Chaps. 18 and 19. Of these, Chap. 18**Lipid Biosynthesis and Degradation** gives a detailed note on bacterial and archaeal lipids and covers lipid and fatty acid biosynthesis. Toward the end, a note on fatty acid biosynthesis enzymes as drug targets is also given followed by a detailed note on fatty acid degradation and its regulation. Chapter 19**Nucleotide Biosynthesis and Regulation** covers both *de-novo* and salvage pathways of nucleotide synthesis with featured examples of drug targets at every possible step.

Part IX *Response to Environmental Signalling* comprises of four Chaps. 20–23. Chapter 20 deals with **two-component system** of signal transduction. It comprises diversity of these systems in three domains of life. The types of two-component systems along with detailed account of sensor kinases and response regulators are presented. Chapter 21 deals with **bacterial response to availability of oxygen** and various oxygen sensors. Arc AB and FNR sensors are described in detail. Chapter 22 describes how **anaerobic respiration in bacteria** is regulated. Nitrate, nitrite, fumarate, DMSO and TMAO respiration are dealt with. Chapter 23 deals with **Pho regulon** in phosphate starvation and response to **osmotic stress** in bacteria**.**

Part X *Cell to Cell Signalling* has two Chaps. 24 and 25. Chapter 24 deals with the phenomenon of **quorum sensing** which encompasses accounts on its discovery quorum sensing molecules in gram-negative and gram-positive bacteria. The chapter also includes the role of quorum sensing in some important physiological phenomenon. A detailed account on regulation of **bioluminescence** in two different species of *Vibrio*, *V. fischeri* and *V. harveyi* is presented along with **chemotaxis** in *E. coli*. Chapter 25 describes in detail **the competence and sporulation in bacillus subtilis**.

We have added a summary at the end of each chapter to highlight the most significant points covered. A list of references for each chapter will help the reader in

accessing the source materials, research articles, reviews, books, etc., that we have used. We have also included some questions for the students to test their grasp on the concepts explained in the chapters.

New Delhi, India

Rani Gupta
Namita Gupta

The original version of the book was revised: The authors and co-authors have been cited within the chapters. The correction to this book is available at https://doi.org/10.1007/978-981-16-0723-3_26.

Acknowledgements

We express our extreme gratitude toward our publisher Springer Nature who have been together with us in this long journey. We truly appreciate their patience and time to time support by understanding delays from our side.

We are also very grateful to the publishers of various journals and books who graciously extended permissions to use some of their copyrighted figures and images in the book. These figures and images have been very helpful in explaining clearly the concepts and overall subject. We have duly acknowledged the publishers for each of these figures and images, wherever incorporated in the book.

We are truly indebted to the present and ex-Ph.D. students of Prof. Rani Gupta, namely Meenu Saini, Amuliya Kashyap and Shruti Bindal for their untiring help in several ways such as contributing content/drawings/figures, editing, typing, formatting, etc . They deserve a special appreciation for being there whenever required.

We shall remain indebted to all who have contributed toward this book in any way.

Contents

Part I Prokaryotes: Bacterial and Archaeal Diversity and Cell Structure

1 Diversity of Prokaryotes 3
Rani Gupta, Namita Gupta, and Meenu Saini
1 Three-Domain Classification 3
2 Classification and Discovery of Archaea 3
3 Phylum Euryarchaeota 4
4 Phylum Crenarchaeota: Hyperthermophiles 8
5 Newly Discovered Archaea 11
6 Diversity of Bacteria 12
7 The Deeply Branching Bacteria 13
8 The Proteobacteria 14
9 The Non-proteobacteria Gram-Negatives 24
10 Firmicutes: The Low G + C Gram-Positive Bacteria 29
11 Actinobacteria: The High G + C Gram-Positive Bacteria 33
References 39

2 Prokaryotic Cell Structure and Function 43
Rani Gupta and Namita Gupta
1 Morphology of Bacterial Cell 43
2 Cell Organization 44
3 Bacterial Membranes 45
4 Cell Wall 52
5 Bacterial Capsule 56
6 Bacterial Cytoplasm 58
7 Extracellular Appendages 64
References 77

3 Bacterial Cell Wall Biosynthesis and Inhibitors 81
Rani Gupta, Namita Gupta, and Shruti Bindal
1 Components of Bacterial Cell Wall 81
2 Proteins Present in Outer Membrane 87

3 Biosynthesis of Bacterial Cell Wall 87
4 Lipopolysaccharide Synthesis 93
References 97

Part II Bacterial Cell division and Growth

4 Cell Division 101
Rani Gupta, Namita Gupta, and Amuliya Kashyap
1 Bacterial Cell Cycle 102
2 Chromosomal Replication and Segregation 103
3 Cytokinesis/Cell Division 111
4 Membrane Constriction and Septum Progression 120
5 Regulation of Septum/Z-Ring Formation 124
6 Divisome Proteins as Antimicrobial Targets 131
References 135

5 Growth Physiology and Kinetics 137
Yogender Pal Khasa and Shilpa Mohanty
1 Growth Curve in Bacteria 137
2 Effect of Environmental Factors on Microbial Growth 140
3 Diauxic Growth 144
4 Effect of Recombinant Protein Production on Bacterial Growth 146
5 Measurement of Microbial Growth 147
6 Growth Kinetics of Batch Fermentation 156
7 Continuous Stirred Tank Reactor (CSTR) 167
8 Fed-Batch Culture 170
9 Applications of High-Cell-Density Cultivation Strategies 174
References 177

Part III Transport Across Cell Membrane

6 Solute Transport 183
Rani Gupta and Namita Gupta
1 Biological Membranes as Transport Barrier: A General Account 183
2 Transport Across Membranes: Passive and Active Transport 183
3 Membrane Transport Proteins 190
4 Specialized Channel Proteins 196
5 Membrane Transport Proteins: Carriers 198
6 Special/Unique Transport Proteins: Ionophores 200
7 Iron Transport in Bacteria: Concerted Action of Primary and Secondary Active Transport 203
References 207

7 ABC Transporters and Group Translocation 209
Rani Gupta, Namita Gupta, and Richa Sharma
1 Historical Background of ABC Transporters 209
2 ABC Transporters: Substrate Specificity, Domain Organization and Mechanism of Transport 210
3 Bacterial Multidrug Efflux Pumps 215
4 Group Translocation: PEP-PTS System 221
References 231

8 Protein Secretion 235
Rani Gupta and Namita Gupta
1 Sec System for Transport of Unfolded Proteins 235
2 Tat System for Transport of Folded Proteins 237
3 Protein Secretion in Gram-Negative Bacteria 239
4 Sec-dependent Secretion Systems of Gram-Negative Bacteria 240
5 Sec-independent Secretion Systems of Gram-Negative Bacteria 248
6 Protein Secretion in Gram-Positive Bacteria 256
References 262

Part IV Central Metabolic Pathways

9 Glycolysis and Gluconeogenesis 267
Rani Gupta and Namita Gupta
1 Glycolysis: Embden–Meyerhoff–Parnas Pathway 267
2 Regulation of Glycolysis 273
3 Modification of Glycolysis in *Pyrococcus Furiosus* 274
4 Atypical Glycolysis in *Clostridium Thermocellum* 274
5 Methylglyoxal Pathway: An Off-Shoot of Glycolysis 276
6 Gluconeogenesis 277
7 Key Scientists and Their Contribution to EMP Pathway 283
References 287

10 Pentose Phosphate Pathway 289
Rani Gupta and Namita Gupta
1 Reactions of Pentose Phosphate Pathway 289
2 Pentose Phosphate Pathway During Oxidative Stress Response 293
3 Pentose Phosphate Pathway in Heterofermentative Lactate Fermentation 297
4 Pentose Degradation in Archaea 298
5 Improving Regeneration of NADPH 298
6 Engineering *E. Coli* for Cometabolism of Glucose and Xylose to Yield Ethanol: A Case Study (Chiang et al. 2013) 300
References 304

11 Entner–Doudoroff Pathway 307
Rani Gupta and Namita Gupta
1 Discovery of Entner–Doudoroff (ED) Pathway 307
2 Comparison Between EMP and ED Pathways 308
3 Key Reactions of ED Pathway 309
4 Modes of ED Pathway 311
5 Regulation of ED Pathway in *E. Coli* 317
6 Carbohydrate Metabolism in *Zymomonas Mobilis*, an Alternative Ethanol Producer, Using ED Pathway 319
7 Metabolic Fate of Pyruvate 321
References 326

12 Tricarboxylic Acid Cycle 327
Rani Gupta and Namita Gupta
1 Historical Background of TCA Cycle 327
2 Bridge Between Glycolysis and TCA Cycle—Pyruvate Dehydrogenase Complex 328
3 Tricarboxylic Acid Pathway/Krebs's Cycle 331
4 Citric Acid Production by *Aspergillus Niger*: An Overview 342
References 346

13 Alternate Tricarboxylic Acid Cycle 347
Rani Gupta and Namita Gupta
1 Distribution of TCA Cycle Among Bacteria Under Microaerophilic and Anaerobic Conditions 347
2 Branched TCA 348
3 Glyoxylate Cycle 351
4 Role of Glyoxylate Cycle in Gluconeogenesis 355
5 Glyoxylate Operon and Its Regulation 356
6 Metabolic Engineering of *E. Coli* for Over-production of Succinic Acid: An Overview 358
References 364

Part V Electron Transport and Energy Generation

14 Electron Transport and Energy Generation 367
Rani Gupta, Namita Gupta, and Sudha Choudhary
1 Primary Electron Donors 367
2 Terminal Electron Acceptors 372
3 Electron Transport Complex 374
4 Bacterial Electron Transport: A General Account 381
5 Branched Electron Transport Chain in *E. coli* 382
6 Oxidative Phosphorylation 385
7 Uncouplers and Inhibitors of Electron Transport and Oxidative Phosphorylation 386
References 389

Part VI Metabolic Diversity of Carbon and Energy Source

15 Nutritional Diversity Amongst Bacteria: Chemolithotrophy and Phototrophy 393
Rani Gupta, Namita Gupta, and Sunita Aggarwal
1 Nutritional Diversity Amongst Bacteria 393
2 Chemolithotrophy 394
3 Hydrogen Oxidizers 396
4 Carbon Monoxide Oxidizers (Carboxydobacteria) 397
5 Ammonia Oxidizers 398
6 Nitrite Oxidizers 400
7 Sulphur Bacteria 401
8 Iron Bacteria or Iron Oxidisers 403
9 Economic Importance of Chemolithotrophs 405
10 Phototrophy 405
11 Diversity of Photosynthetic Bacteria 406
12 Classification of Phototrophic Bacteria 408
13 Photosynthetic Pigments 409
14 Oxygenic Photosynthesis 413
15 Anoxygenic Photosynthesis 414
16 C1 Metabolism 418
References 427

Part VII Nitrogen Metabolism

16 Nitrogen Assimilation and Dissimilation 431
Rani Gupta and Namita Gupta
1 Nitrogen Cycle and Nitrogen Assimilation: An Overview 431
2 Assimilation/Utilization of Inorganic Nitrogen NO_3^- and NH_4^+ 435
3 De Novo Biosynthesis of Amino Acids 438
4 Amino Acid Utilisation 459
References 463

17 Glutathione and Polyamines in Bacteria 465
Rani Gupta and Namita Gupta
1 Diversity of Low Molecular Weight Thiols in Bacteria 465
2 Structure of Glutathione 465
3 Glutathione Biosynthesis and Transmembrane Cycling 466
4 Glutathione Homeostasis 468
5 Glutathione for Reduction and Glutathionylation of Proteins 470
6 Role of Glutathione During Environmental Stress and Chemical Toxicity 470
7 Distribution of Common and Unusual Polyamines in Bacteria 472
8 Putrescine and Cadaverine Biosynthesis 474
9 Spermidine and Spermine Biosynthesis 476

10 Polyamines Transport and Their Role in Cell Physiology 477
11 Polyamine Inhibitors and Disease Prevention 480
12 Industrial Application of Polyamines and Overproduction Strategies for Cadaverin Synthesis 481
References 485

Part VIII Lipid Metabolism and Nucleotide Biosynthesis

18 Lipid Biosynthesis and Degradation 491
Rani Gupta and Namita Gupta
1 Distribution of Lipids in Microbes and Their Biological Significance 491
2 Bacterial Lipids 492
3 Archaeal Lipids 494
4 Biosynthesis of Lipids 495
5 Lipid Accumulation in Yeasts 507
6 Fatty Acid Biosynthesis Pathway Enzymes as Drug Targets 509
7 Lipid Degradation 511
8 Regulation of Fatty Acid Degradation in *E. coli* 516
References 521

19 Nucleotide Biosynthesis and Regulation 525
Rani Gupta and Namita Gupta
1 Purines and Pyrimidines 525
2 Synthesis of Purine Ribonucleotides 527
3 Regulation of Purine De Novo Synthesis 535
4 Synthesis of Pyrimidine Ribonucleotides 535
5 Regulation of Pyrimidine Synthesis in *E. coli* 543
6 Synthesis of Deoxyribonucleotides 543
7 Inhibitors of Nucleotide Biosynthesis 547
8 Nucleotide Salvage Pathway 550
9 Diseases Related to Nucleotide Biosynthetic Pathways 552
References 554

Part IX Response to Environmental Signalling

20 Two-Component Systems 557
Rani Gupta and Namita Gupta
1 Diversity of Two-Component Systems in Three Domains of Life 557
2 Types of Two-Component Systems 559
3 Sensor Kinases 561
4 Mechanism of Signal Sensing by Sensor Kinases 566
5 Response Regulators 569
References 572

21 Bacterial Response to Oxygen Availability 575
Rani Gupta and Namita Gupta
1 Introduction to Oxygen Sensors in *E. coli* 575
2 Rapid Response Sensors 576
3 Delayed Response Sensors 578
4 ArcAB- and FNR-Regulated Carbon Metabolism in *E. coli* in Response to Oxygen Availability 585
References 591

22 Regulation of Anaerobic Respiration 593
Rani Gupta and Namita Gupta
1 Anaerobic Respiration: An Introduction 593
2 Nitrate Respiration 593
3 Nitrate Reductases 594
4 Nitrite Reductases 596
5 Nitrate and Nitrite Transport in *E. Coli* 598
6 Two-Component System for NO_3^-/NO_2^- Sensing 599
7 Coregulation of Formate Dehydrogenase in Presence of Nitrate, Nitrite and Formate 601
8 Fumarate Respiration 601
9 DMSO and TMAO Respiration 602
References 608

23 Response to Phosphate Starvation and Osmotic Homeostasis 611
Rani Gupta and Namita Gupta
1 Response to Phosphate Starvation 611
2 Components of Pho Regulon 611
3 Two-Component PhoBR Regulates Pho Regulon in *E. Coli* 612
4 Cross-Regulation: Pi-Independent Regulation of Pho Regulon 614
5 Osmohomeostasis 615
6 Osmotic Stress and Turgor 615
7 Primary Response to Osmohomeostasis: ATP-Driven K^+ Uptake 616
8 Secondary Response: Osmoprotectant Accumulation 618
References 628

Part X Cell to Cell Signalling

24 Quorum Sensing, Bioluminescence and Chemotaxis 633
Rani Gupta and Namita Gupta
1 What is Quorum Sensing and Why do Bacteria Talk? 633
2 Discovery of Quorum Sensing 634
3 Signaling Molecules: Autoinducers 635
4 Quorum Sensing in Gram-Negative Bacteria: Bioluminescence 637
5 Quorum Quenching 643

6 Chemotaxis 644
7 Bacterial Movement 645
8 Proteins Required for Chemotaxis 647
9 Chemotactic Response 648
References 652

25 Competence and Sporulation in *Bacillus subtilis* 653
Rani Gupta and Namita Gupta
1 Competence: An Introduction 653
2 Competence Development in Different Bacteria 653
3 Genetic Basis of Competence in *B. Subtilis* 654
4 ComK: The Master Competence Regulator 655
5 Molecular Mechanism of Competence: Induction by Quorum Sensing 657
6 Sporulation in *Bacillus Subtilis* 658
References 668

Correction to: Fundamentals of Bacterial Physiology and Metabolism C1

About the Authors

Prof. Rani Gupta a Ph.D. in Botany from University of Delhi, is a Professor in the Department of Microbiology, University of Delhi. She has over 30 years of teaching experience in the subjects of Microbial Physiology and Metabolism, Prokaryotic and eukaryotic diversity, Bacteriology and Environmental Microbiology, besides mentoring Ph.D., M.Phil. and M.Sc. students. Her major areas of research interest include microbial enzymes of industrial applications and development of sustainable green solutions using enzymes to substitute chemical based processes. She has published over 130 highly cited research/review articles and is also a reviewer for several international journals of repute. She has had many national and international research collaborations over the years and has significant research achievements to her credit.

Dr. Namita Gupta is currently a Senior Manager at Foundation for Innovation and Technology Transfer (FITT), an academia-industry interface organization of the Indian Institute of Technology Delhi. She earned her Ph.D. in Microbiology at the University of Delhi and has over 15 years of professional experience in the areas of biotechnology-oriented research, patent research & IP solutions, technology valuation & transfers, techno-commercial evaluation of innovative technologies, technology financing and industrial collaborations.

Authors and Coauthors

Authors

The following have authored Chap. 5 of this book:

Yogender Pal Khasa, Ph.D.
Associate Professor, Department of Microbiology, University of Delhi South Campus, New Delhi, 110021

Shilpa Mohanty
Doctoral Student, Department of Microbiology, University of Delhi South Campus, New Delhi, 110021

Coauthors

The following have coauthored the chapters mentioned below:

Meenu Saini
Chapter 1
Doctoral Student, Department of Microbiology, University of Delhi South Campus, New Delhi, 110021

Amuliya Kashyap
Chapter 4
Doctoral Student, Department of Microbiology, University of Delhi South Campus, New Delhi, 110021

Shruti Bindal, Ph.D.
Chapter 3
Ex-Ph.D. Student, Department of Microbiology, University of Delhi South Campus, New Delhi, 110021

Richa Sharma, Ph.D.
Chapter 7
Assistant Professor, Department of Microbiology, Shaheed Rajguru College of Applied Sciences for Women, University of Delhi, Delhi, 110096

Sudha Choudhary, Ph.D.
Chapter 14
Associate Professor, Department of Microbiology, Ram Lal Anand College, University of Delhi, New Delhi, 110021

Sunita Aggarwal, Ph.D.
Chapter 15
Associate Professor, Department of Microbiology, Institute of Home Economics, University of Delhi, New Delhi, 110016

Part I
Prokaryotes: Bacterial and Archaeal Diversity and Cell Structure

Chapter 1
Diversity of Prokaryotes

Rani Gupta, Namita Gupta, and Meenu Saini

1 Three-Domain Classification

Robert Whittaker's much accepted five kingdom classifications of all living organisms (Whittaker, 1959) are now replaced by three-domain classification as proposed by Carl Woese using molecular chronometers, 16S/18S ribosomal DNA sequence homologies (Woese & Fox, 1977). The phylogenetic tree indicates that whole kingdom of life can be divided into bacteria, archaea and eukaryote. Interestingly, archaea are more closely related to eukaryotes than to bacteria (Fig. 1).

The two domains archaea and bacteria together are termed as prokaryotes and constitute a vast array of microorganisms occupying different niches. The diversity of these prokaryotic domains has been dealt with in this chapter. The first line of classification that separates archaea from bacteria is the differences in their 16 s rRNA gene, and the second is the ability of archaea to adapt in extreme environments (thermophilic, acidophilic, halophilic, etc.) in contrast to the mesophilic bacteria. Both archaea and bacteria are highly diverse in terms of their nutritional requirements and thus have extreme metabolic flexibility. This flexibility helps them to evolve independently in different habitats which increases their survival rate.

2 Classification and Discovery of Archaea

The concept of archaea as an independent domain originated for methanogens based on differences in 16s rRNA and tRNA gene sequences, two unusual coenzymes for methane formation and lack of peptidoglycan in their cell walls (Woese & Fox, 1977). The first complete genome structure of the archaeon *Methanococcus jannaschii* later confirmed that archaea are separate and distinct life forms as about two-third of their genes were different (Bult et al., 1996). Genes involved in energy production, cell division and metabolism were similar to those in bacteria, while genes for DNA replication, transcription and translation were similar to eukaryotes. Thus, it is believed

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_1

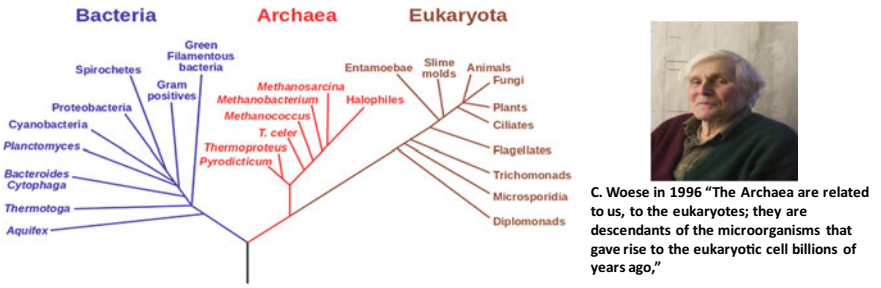


Fig. 1 Phylogenetic tree by Carl Woese based on three-domain classification system. *Source* Phylogenetic tree—NASA Astrobiology Institute; Scientist image—Institute for Genomic Biology, University of Illinois at Urbana-Champaign (Creative commons Attribution License)

that archaea came into existence 3–4 billion years ago and are the most ancient lineage that gave rise to both bacteria and archaea.

Archaea are metabolically highly diverse and can be chemoorganotrophs or chemolithotrophs and aerobic or anaerobic. Chemolithotrophy, wherein organisms use inorganic compounds as electron donors, is unique to prokaryotes. Archaea include methanogens (specifically found in this domain), sulfur and sulfate reducers, ammonia oxidizers and extremophiles existing in extremes of temperature, pH, salt and niches devoid of oxygen. However, some species are commonly found in much lesser extreme habitats such as soil sediments, oceans and even in human gut. In nature, they are significant contributors to the global carbon and nitrogen cycles and also have many biotechnological applications (Cavicchioli, 2007).

Archaea are classified into seven phyla which include two main ancient phyla and five other phyla, some of which have been reclassified and others are newly discovered (Fig. 2).

3 Phylum Euryarchaeota

The name Euryarchaeota is derived from the Greek word '*eurus*' meaning wide as it is a large and physiologically diverse phylum (Woese et al., 1990; Barns et al., 1996). It is divided into five major physiological and morphological groups as discussed below.

Methanogens: Anaerobic Chemolithotrophs

Methanogens are the largest group of cultured archaea. They produce methane as the end product during respiration, and the process is known as methanogenesis. They are stringent anaerobes and inhabit marshes, swamps, pond and lake muds, marine sands, intestinal tract of humans and animals, rumen of cattle and sewage treatment plants. They are obligate lithotrophs that obtain their energy by oxidizing compounds like H_2, formate, acetate, methanol and methylamines and utilizing the electrons generated to reduce CO_2 to form CH_4. Some genera can grow as autotrophs

Fig. 2 Classification of archaea into different phyla

utilizing hydrogen and CO_2 as sole sources of carbon and energy. Most methanogens are non-halophilic and mesophilic. The most notable exception is *Methanopyrus* that is a hyperthermophilic methanogen.

Box 1: Most Hyperthermophilic Archaeon: *Methanopyrus kandleri*

Methanopyrus kandleri is a hyperthermophile which was isolated from the black smoker fluid of the Kairei hydrothermal vents (fissure in earth's surface that releases geothermally heated water). It grows optimally at 100 °C and can withstand temperatures as high as 122 °C. It is an autotroph that produces methane from hydrogen and carbon dioxide only. The organism contains unsaturated phytanyl ether-linked lipids, not found in any other organism. These lipids help to stabilize its membrane at such unusually high growth temperatures (Takai et al., 2008).

Some unique features of this group include:

- Presence of several unusual cofactors such as tetrahydromethanopterin, methanofuran, coenzyme M, coenzyme F_{420} and coenzyme F_{430} that are involved in various reactions of methanogenesis.
- Diversity of cell wall: Some genera have cells wall containing pseudomurein (*Methanobacterium* and *Methanobrevibacterium*) which differs from eubacterial murein as N-acetyl muramic acid is replaced by *N*-acetyl talosaminuronic acid and the tetrapeptide is composed entirely of L-amino acids while others have cell walls composed of methanochondroitin (*Methanosarcina*). Some genera also have protein or glycoprotein walls (*Methanocaldococcus and Methanoplanus*), while *Methanospirillum* has S-layer cell walls.

Extreme Halophiles: Aerobic Chemoorganotrophs

The extreme halophiles belong to the order *Halobacteriales.* Haloarchaea are mostly obligate aerobes and require at least 8% NaCl for growth. Usually, the growth optimum is at 12–23% NaCl and most genera can grow at saturating conditions (36% NaCl) as well. They are chemoorganotrophs and use amino acids and organic acids as electron donors and require a number of growth factors such as vitamins for optimal growth. They stain gram negative, their cell shapes range from rods (*Halobacterium*) to cocci (*Halococcus*) to squares (*Haloquadratum*), and colonies are red to orange due to the presence of carotenoids that protect the cells against damaging effects of sunlight. They inhabit highly saline environments, e.g., salt lakes (Dead Sea and Great Salt Lake), heavily salted foods, e.g., certain fishes and meats.

Halophiles withstand high salt concentration by accumulating compatible solutes such as glycine, betaine, polyols and amino acids. Additionally, they build up K^+ concentration inside the cell much higher than Na^+ outside, thus maintaining positive water balance. In this case, the cellular proteins are rich in acidic residues that are shielded by cations that form a hydrated shell around the proteins. High salt concentration is necessary for their survival as when Na^+ concentrations fall, the acidic groups repel each other and the wall breaks apart causing cell lysis (Oren, 2008).

Box 2: Bacteriorhodopsin: Proton Pump for ATP Synthesis

Halobacterium salinarum is the best-studied member of the group Haloarchaea. It synthesizes a protein called bacteriorhodopsin which is similar to mammalian rhodopsin. Bacteriorhodopsin acts as a light-driven proton pump for ATP synthesis as it can capture light energy for movement of protons across the membrane and can support the growth of the organism under anoxic conditions. Bacteriorhodopsin is an integral membrane protein found embedded in purple membrane, a two-dimensional crystalline patch, and can occupy up to 50% of the total cell surface area of the archaea (Voet & Voet, 2004).

Source: Wikipedia. (Creative Commons Attributions License).

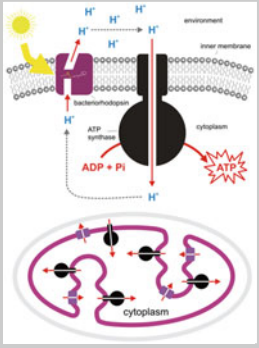


Table 1 Characteristic features of members of the order Thermoplasmatales

Organism	Optimum growth conditions (temperature and pH)	Nutritional requirement
Thermoplasma (cell-wall-less)	55 °C and pH 2	Chemoorganotroph
Ferroplasma (cell-wall-less)	35 °C and pH 1.2–1.7	Chemolithotroph/Chemoorganotroph
Picrophilus (S-layered)	47–65 °C and pH 0.7	Chemoorganotroph

Thermoplasms: High metabolic diversity

The order Thermoplasmatales includes thermophilic and acidophilic genera—*Thermoplasma, Picrophilus* and *Ferroplasma* . Of these, *Thermoplasma* and *Ferroplasma* are cell-wall-less while *Picrophilus* has an additional S-layer around its plasma membrane (Table 1).

Thermoplasma is a facultative aerobe that can grow chemoorganotrophically under aerobic or anaerobic conditions by sulfur respiration. Its species have been isolated from self-heating coal refuse piles and solfatara fields (hot sulfur-rich environments). It grows optimally at 55 °C and pH 2. To withstand extreme high temperature and low pH, its plasma membrane is strengthened by large amounts of caldarchaeol (diglycerol tetraethers), lipoglycan (lipid-containing polysaccharides) and glycoproteins. They lack a cell wall and were initially associated with the morphologically similar cell-wall-less bacteria Mycoplasmas due to their pleomorphic shape and formation of fried egg colonies (Darland et al., 1970).

Ferroplasma is a chemolithotroph related to *Thermoplasma*, and it oxidizes ferrous to ferric. Its species can also grow chemoorganotrophically on various carbon compounds as aerobe or facultative anaerobes. It is a strong acidophile with optimum growth pH of 1.2–1.7 but is not a thermophile as its optimum growth temperature is 35 °C. In mine tailings containing pyrite, it oxidizes ferrous to ferric as its energy source and generates acid causing acid mine drainage (Méndez-Garcia et al., 2015).

Picrophilus is an extreme acidophile that grows optimally at extreme pH 0.7. It is capable of growth at pH 0 as well and at temperatures between 47 and 65 °C. It is aerobic and grows chemoorganotrophically utilizing organic substrates. The membrane has an unusual arrangement of lipids mainly polar ether lipids that form a highly acid impermeable layer at very low pH. It is of interest as a model for extreme acid tolerance (Fütterer et al., 2004).

Extremely thermophilic S^0 reducers: Anaerobic chemoorganotrophs

This group includes the order *Thermococcales* and two main genera, *Thermococcus* and *Pyrococcus.* They are strictly anaerobic chemoorganotrophs that can metabolize proteins and other organic compounds with elemental sulfur as electron acceptor which is reduced to sulfide. The species of these genera are indigenous to anoxic thermal waters. Both the genera are hyperthermophiles. *Pyrococcus* grows optimally

between 70 and 106 °C, while *Thermococcus* grows between 55 and 95 °C. These two are morphologically similar having spherical cells with a tuft of polar flagella.

Box 3: *Pyrococcus furiosus*: Rushing Fireball

(*Pyrococcus* in Greek 'fireball' and *furiosus* in Latin 'rushing').

The scientific name of this organism is based on its round shape and its rapid doubling time at a temperature near 100 °C with very fast motility by the virtue of a tuft of lophotrichous flagella (Näther et al., 2006).

Source: Wikipedia (Creative Commons Attribution License).

Sulfate Reducers: Hyperthermphilic Anaerobic Chemolithotrophs/ Chemoorganotrophs

They belong to the order *Archaeoglobales* and include two main genera *Archaeoglobus* and *Ferroglobus*. They are named as sulfate reducers as they use sulfate instead of elemental sulfur as terminal electron acceptor.

Archaeoglobus grows anaerobically and can couple oxidation of H_2, lactate, glucose or other complex organic compounds to the reduction of various sulfur compounds. It is a hyperthermophile isolated form hot marine hydrothermal vents that grows optimally at 83 °C. In addition to being a sulfate reducer, *Archaeoglobus* also possesses the methanogen coenzyme F_{420} and methanopterin and can produce tiny amounts of methane.

Ferroglobus isolated from a shallow marine hydrothermal vent grows optimally at 85 °C. It has an S-layer covering outside the cell. It is not a sulfate reducer; instead, it is an anaerobic iron-oxidizing chemolithotroph that couples oxidation of ferrous to ferric to reduction of nitrate to nitrite. *F. placidus* was the first archaeon to be discovered that could oxidize iron coupled to the reduction of nitrate anaerobically (Hafenbradl et al., 1996).

4 Phylum Crenarchaeota: Hyperthermophiles

This phylum contains four orders, namely *Sulfolobales, Thermoproteales, Desulfurococcales* and *Caldisphaerales*. They are mostly hyperthermophiles (some species grow above 100 °C) and obligate anaerobes. Many are autotrophic chemolithotrophs

and chemoorganotrophs with diverse electron donors and acceptors. They are often found in geothermal heated water, deep-sea hydrothermal vents and terrestrial environments such as solfataras**.** All these habitats are hot and rich in sulfur compounds like H_2S or S^0.

Sulfolobales: Aerobic Chemolithotrophs

Order *Sulfolobales* contains thermoacidophilic cocci isolated from terrestrial volcanic habitats having temperatures as high as 100 °C. The key genera are *Sulfolobus* and *Acidianus.*

Sulfolobus inhabits sulfur-rich acidic thermal areas and grows optimally at 80 ºC and pH of 2–3. It is aerobic and grows chemolithotrophically by oxidizing H_2S or S^0 to H_2SO_4 using oxygen as terminal acceptor. It can also grow heterotrophically under oxic conditions and oxidize sugars via the non-phosphorylated Entner–Doudoroff pathway and a complete TCA cycle.

Box 4: *Sulfolobus solfataricus*: A Model Organism for Molecular Biology Studies of Archaea

The genome of *S. solfataricus* shows a high level of plasticity, and about 11% of the genome is made up of integrated insertion sequences. These mobile elements present in the genome indicate that they got inserted by horizontal gene transfer. Moreover, this organism shows strong eukaryotic features in terms of its mode of DNA replication, transcription and translation with many unique archaeal specific abilities like genes for cell wall synthesis. This organism is used as a model to study the methods of DNA replication, cell cycle, chromosomal integration, RNA processing and translation as these processes are similar to eukaryotic organisms in spite of the fact that *Sulfolobus* is a prokaryote (She et al., 2001).

Acidianus also lives in acidic solfataric springs and grows at high temperatures of 65–95 °C with an optimum at 90 °C. It is a facultative anaerobe as it can grow both aerobically and anaerobically using S^0. Under aerobic conditions, it oxidizes S^0 to H_2SO_4 using oxygen as terminal acceptor, while under anaerobic conditions it is a hydrogen chemolithotroph and uses S^0 as terminal acceptor and H_2 as donor, thereby forming H_2S.

Thermoproteales: Anaerobic chemolithotrophs

Order *Thermoproteales* includes three main genera *Thermoproteus*, *Thermophilum* and *Pyrobaculum.* Both *Thermoproteus* and *Thermophilum* are strict anaerobes that can carry out S^0-based anaerobic respiration. They are found in slightly acidic or neutral hot springs. Species of *Thermoproteus* can grow as chemolithotrophs on hydrogen as well as chemoorganotrophically on complex carbon substrates.

Pyrobaculum can grow optimally at 100 °C and has been isolated from terrestrial neutral or acidic hot springs and hydrothermal vents. It can respire aerobically and

also anaerobically using nitrate, ferric or elemental sulfur as electron acceptors and H_2 as an electron donor.

Desulfurococcales: Anaerobic Chemolitho- and Chemoorganotrophs

Order *Desulfurococcales* contains coccoid or disk-shaped hyperthermophiles isolated from submarine volcanic habitats. This order includes four main genera, namely *Desulfurococcus, Ignicoccus*, *Pyrodictium* and *Pyrolobus.*

Pyrodictium is a strict anaerobe, grows optimally at 105 °C and forms characteristic network of tubelike structures that connects the cells. It is a hydrogen chemolithotroph and uses sulfide as a terminal acceptor. It can also grow organotrophically on complex organic compounds.

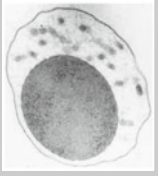
Pyrolobus fumarii grows optimally at 113 °C and is one of the most thermophilic organisms isolated till date that can survive autoclaving temperature (121 °C) for one hour. It is an obligate H_2 chemolithotroph that couples oxidation of H_2 to reduction of nitrate to ammonium, thiosulfate to H_2S or O_2 to H_2O. *Pyrodictium* and *Pyrolobus* are the examples of the most hyperthermophilic prokaryotes.

Desulfurococcus is a strictly anaerobic S^0 reducer that grows optimally at 85 °C.

Ignicoccus is also an anaerobe that grows optimally at 90 °C and uses H_2 as electron donor and S^0 as acceptor. Some species of *Ignicoccus* are hosts to a parasitic archaeon *Nanoarchaeum equitans. Ignicoccus hospitalis* is called as 'hospital fireball' (Huber et al., 2000).

Box 5: Ignicoccus: Modern Descendant of Eukaryotic Cell Origin

It is the only archaeon with a double membrane structure where the outer membrane is the site of energy generation while the inner membrane encloses cytoplasm and the enzymes responsible for biosynthesis. Outer and inner membranes are separated by a very large and voluminous periplasmic space containing membrane-bound vesicles which are expected to export substances outside the cell. This type of cell membrane assembly highly resembles eukaryotic cell structure. Thus, *Ignicoccus* is considered to be a 'modern descendant of ancestral cell type that gave rise to the origin of eukaryotic cells' (Rachel et al., 2002).



Source: Rachel et al. (2002). *(Creative Commons Attribution License).*

Caldisphaerales

Order *Caldisphaerales* contains a single genus *Caldisphaera* that are thermoacidophilic, aerobic and heterotrophic cocci.

5 Newly Discovered Archaea

Phylum Korarchaeota: Obligate Anaerobe, Chemoorganotroph

This phylum includes only a single species, namely *Candidatus*: *Korarchaeum cryptofilum* which means 'the hidden filament of youth.' Morphologically, the cells of *K. cryptofilum* are long and thin filaments of variable length. It was discovered in 1994 from Yellowstone National Park and is an obligate anaerobic chemoorganotrophic hyperthermophile growing at 85 °C (Barns et al., 1996).

Phylum Nanoarchaeota: A parasitic archaeon

Initially included in Euryarchaeota, this diverged as a separate phylum in 2002. It is represented by a single species *Nanoarchaeum equitans* which is one of the smallest cellular organisms (0.49 Mb). This organism can only grow parasitically, and it replicates when it gets attached to the surface of its host organism *Ignicoccus hospitalis* (Fig. 3). It cannot grow in pure cultures. The organism and its host were first isolated from a submarine hydrothermal vent and both grow at temperatures from 70 to 98 °C. The parasite depends on its host for many metabolic functions as genes required for biosynthesis of most amino acids, lipids, nucleotides and cofactors are missing in its genome. This organism has the smallest genome size (Huber et al., 2002).

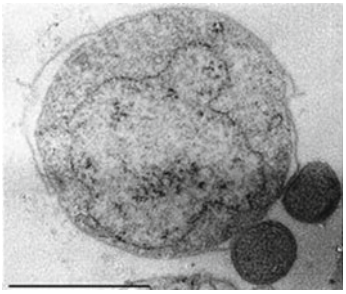


Fig. 3 Cells of *Ignicoccus hospitalis* 'the hospital fireball' (single large ball) and 300–500 nm tiny cells of *N. equitans* (two small balls) attached to its outer membrane. *Source* Wikipedia (Public Domain)

Phylum Thaumarchaeota: Chemolithotrophic Ammonia oxidizers

This phylum was proposed in 2008 based on phylogenetic data. They are believed to have diverged from the primary line of archaeal descent prior to divergence of Euryarchaeota and Crenarchaeota (Brochier-Armanet et al., 2008). Thaumarchaeota are ubiquitous in soils, marine waters, sediments and hot springs and are one of the most abundant and widespread phyla on earth. Organisms under this phylum are ammonia-oxidizing aerobic chemolithoautotrophs. The representative members of this phylum are *Crenarchaeum symbiosum*, *Nitrosopumilus maritimus*, *Nitrososphaera viennensis* and *Nitrososphaera gargensis*. They were initially called mesophilic crenarchaeotes for many years but genome mining of *C. symbiosum* showed that it lacks the typical crenarchaeal signatures and instead has several characteristic euryarchaeal signatures. Genome sequence analysis of *N. maritimus* confirmed that Thaumarchaeota constitute a unique phylum of archaea. *N. maritimus* can grow at very low concentrations of ammonia (typical of marine environments), chemolithotrophically oxidizing ammonia to nitrite (nitrification) (Walker et al., 2010).

Phylum Aigarchaeota: Lineage of Crenarchaeota and Thaumarchaeota

This phylum was proposed in 2011 and shows close relationship with mesophilic *Thaumarchaeota* and hyperthermophilic *Crenarchaeota* (Nunoura et al., 2011). The main representative member of this phylum is *Candidatus*: Caldiarchaeum subterraneum whose environmental genome was recovered from a mildly acidic, oxic subsurface filamentous 'streamer' community at 70 °C. *C. subterraneum* might grow chemolithotrophically utilizing hydrogen or carbon monoxide (CO) as electron donor in the presence of oxygen.

Phylum Lokiarchaeota: Archaeal host for emergence of eukaryotes

This is a novel candidate phylum proposed in 2015 after identification of a candidate genome (of a unicellular form named as *Lokiarchaeum*) in a metagenomic analysis of a mid-oceanic sediment sample taken near a hydrothermal vent in the Arctic Ocean (Spang et al., 2015). This discovery is a milestone in terms of its phylogenetic analysis which suggests a monophyletic grouping of Lokiarchaeota with eukaryotes where 3.3% of its genes correspond to only those found in eukaryotes such as genes coding for motor protein actin.

6 Diversity of Bacteria

With the advent of culture-dependent and culture-independent metagenomic approaches, more than 80 bacterial phyla are known today as compared to only 12 known phyla in 1987. However, less than 50% are culturable and characterized in laboratory conditions. Of these, over 90% species belong to four bacterial phyla, namely Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. All the phyla

within the bacterial domain have extreme metabolic diversity and have developed various modes of nutrient uptake and energy generation and even species belonging to the same phylum are metabolically diverse as they exist in different habitats. The bacteria are divided into five lineages majorly based on molecular differentiation and also on the basis of gram-staining.

(1) The deeply branching bacteria which are thermophilic and closely related to archaea
(2) Proteobacteria gram-negatives which show high morphological versatility
(3) Non-Proteobacteria gram-negatives
(4) The low G + C gram-positive bacteria
(5) The high G + C gram-positive bacteria.

7 The Deeply Branching Bacteria

They represent the most ancient bacterial lineage and are considered closest to the last universal common ancestor as they thrive in extreme environments that existed during the earliest life on earth and hence named deeply branching. These are represented by five bacterial phyla which include all thermophiles except *Deinococcus* which is a mesophilic extremophile able to withstand high amount of radiations.

Phylum **Aquificae** is the oldest branch of bacteria which includes obligate chemolithoautotrophic hyperthermophiles. The representative genus of this phylum is *Aquifex* whose Latin meaning is 'water-maker' as it produces water by carrying out oxidation of hydrogen (Huber et al., 1992). They are facultative aerobes or microaerophiles, have a temperature optimum of 85 °C, can grow up to 95 °C and are found near underwater volcanoes and hydrothermal vents.

Phylum **Thermotogae** represents the second oldest branch of hyperthermophilic bacteria with temperature optimum of 80 °C and can grow up to 90 °C. Genus *Thermotoga* includes species which are gram-negative and non-sporulating and forms a sheath-like envelope called a toga. They grow in active geothermal areas like hot springs and hydrothermal vents. It includes chemoheterotrophic fermentative anaerobes that can catabolize sugars and produce fermentation products like lactate, acetate, CO_2 and H_2. About 24% of the coding sequence of *Thermotoga* is similar to archaeal genes indicating horizontal gene transfer.

Thermodesulfobacterium is another known deeply branching thermophilic bacteria belonging to phylum **Thermodesulfobacteria**. It is a strict anaerobic sulfate reducer that couples oxidation of organic compounds such as lactate, pyruvate and ethanol to reduction of sulfate to H_2S.

The last phylum considered to be evolutionarily significant is **Deinococcus–Thermus** which includes genus *Deinococcus* and *Thermus*. Both are extremophiles having great biotechnological importance. *Thermus* is a thermophile which grows at an optimum temperature of 70ºC. It has been isolated from many geothermal systems and is the source of *Taq* DNA polymerase used worldwide in the PCR technique. The species *Deinococcus radiodurans* is extremely resistant to both radiation and

desiccation which is due to an interplay of its DNA repair system, arrangement of DNA in the cell and its ability to accumulate large amounts of manganese.

Box 6: *Deinococcus radiodurans*: World's Toughest Bacterium in Guinness Book of World Records

The name is derived from a mixture of Greek and Latin words meaning 'terrible berry that can survive exposure to radiation.' It is the most radiation-resistant organism known till date, and the key feature responsible for its resistance is its efficient DNA repair system and multiple copies of its genome. High doses of radiation lead to fragmentation of the organism's genomic DNA, but it can put the fragments back together with precision and absolute fidelity. The organism can also survive severe dehydration, cold and vacuum, and hence it has been nicknamed as **'Conan the Bacterium'** in reference to Conan the Barbarian, a fictional action hero (Gross, 2007).

Source: Gross (2007). *(Creative Commons Attribution License).*

8 The Proteobacteria

The phylum Proteobacteria was initially referred to as 'purple bacteria and relatives' by Carl Woese, but now it is a separate phylum in the bacterial domain. It is the largest group of gram-negative bacteria with more than 500 genera. The name Proteobacteria is derived from the ancient word 'Proteus' meaning Greek god of the sea capable of acquiring various shapes. Morphologically, they show high level of versatility including rods, cocci, spirilla, filamentous, budding and appendaged forms. They also show high metabolic diversity including photoautotrophs, chemolithotrophs and chemoheterotrophs.

Proteobacteria are divided into six classes, alpha, beta, gamma, delta, epsilon and zeta. Gammaproteobacteria is the largest class followed by Alphaproteobacteria and Betaproteobacteria.

Class Alphaproteobacteria

Alphaproteobacteria is the first class of Proteobacteria having a distinctive feature of being oligotrophic. Members of this class can thrive in very low nutrient conditions like deep oceanic sediments, glacial ice or deep undersurface soil. They are metabolically diverse including phototrophs, nitrogen fixers and even obligate intracellular parasites (Table 2).

A majority of the species belonging to Alphaproteobacteria are anoxic phototrophs which do not evolve oxygen during photosynthesis and are commonly known as purple non-sulfur photosynthetic bacteria mainly belonging to the genera *Rhodobacter* and *Rhodospirillum*. These were named 'purple non-sulfur' due to their orange-brown to purple-red appearance which is imparted to them by various carotenoid pigments and were originally known to be unable to use sulfide as an

Table 2 Some important metabolically diverse genera of class Alphaproteobacteria

Representative genera	Unique features
Rhizobium, *Bradyrhizobium* (symbiotic nitrogen fixers)	Chemoorganotrophs; fix nitrogen inside root nodules of leguminous plants
Azospirillum, *Rhodospirillum* (Free-living nitrogen fixers)	Chemoorganotrophs; fix atmospheric nitrogen and make it available to the plants
Nitrobacter, *Paracoccus* (denitrifiers)	Aerobic chemolithotrophs; carry out nitrite oxidation
Rhodospirillum, *Rhodobacter* (purple non-sulfur phototrophs)	Anoxygenic photosynthetic organisms
Hyphomicrobium, *Methylobacterium* (facultative methylotroph)	Chemoorganotrophs; grow on methanol, formate, formaldehyde Division by budding (*Hyphomicrobium*)
Acetobacter, *Gluconobacter* (acetic acid producers)	Chemoorganotrophs; produce acetic acid using ethanol as substrate
Agrobacterium tumefaciens, *Brucella, Bartonella*, *Rickettsia* (pathogens)	*Agrobacterium:* causes crown gall disease in plants *Brucella and Bartonella:* causes *B*rucellosis; Bartonellosis and trench fever in humans and animals *Rickettsia*: Obligate intracellular parasites; causes typhus fever, rocky mountain spotted fever and Q fever in humans
Sphingomonas (aromatic compound degrader)	Aerobic chemoorganotrophs; can degrade a variety of aromatic and organic compounds
Caulobacter (oligotroph)	Aerobic chemoorganotrophs; form stalks or prosthecae to attach to a solid substrate
Magnetococcus (Magnetic bacteria)	Obligate microaerophile; chemolithotrophs/chemoorganotroph Form spherical magnetosomes; align along the earth's geomagnetic field

electron donor for reduction of CO_2 to cell material. However, new studies show that they can use sulfide and not elemental sulfur. Some species can carry out fermentation in the absence of oxygen. Under aerobic growth, photosynthesis is genetically suppressed and the organism grows as chemoorganotrophs.

Box 7: Obligate Aerobic Anoxygenic Phototrophs

These differ from purple non-sulfur bacteria in being obligatory aerobic that grow as strict photoheterotrophs. They use bacteriochlorophyll *a* as the photosynthetic pigment along with various accessory carotenoid pigments that impart yellow, orange or pink color to them. The main genera included in this category are *Roseobacter* and *Erythrobacter* (Yurkov & Beatty, 1998).

Caulobacter is a typical oligotroph that produces stalks or prosthecae with a holdfast at one end to attach to a solid substrate. It is an aerobic chemoorganotroph and is widely distributed in freshwater lakes and streams. It serves as a model organism to study cell cycle regulation, asymmetric cell division and differentiation which is discussed in detail in Chap. 4: Cell Division. It produces two daughter cells, one of which is motile having a polar flagellum called as the swarmer cell required for swimming motility during chemotaxis and also for cell dispersal to new environments. After a short span, the swarmer cells differentiate into the other daughter cell called as the stalked cell which produces stalks or prosthecae with a holdfast at one end to attach to a solid substrate (Fig. 4).

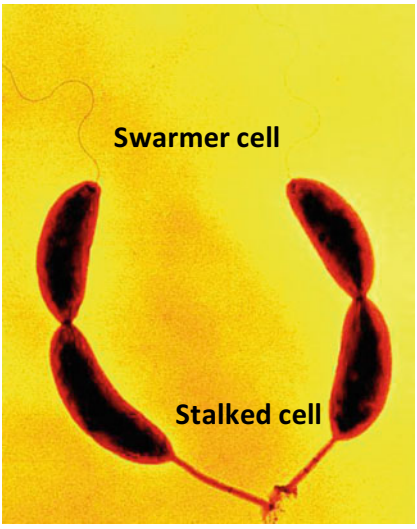


Fig. 4 Two types of daughter cells produced by *C. crescentus. Source* Wikipedia (Public Domain)

Box 8: Importance of Stalk in Caulobacter's Lifestyle

The stalk produced by the cells of *Caulobacter* may serve multiple purposes. Under phosphate starvation conditions, the length of the stalk increases and the elongated stalk with increased surface area and volume can extend away from the sessile cell to facilitate nutrient uptake in nutrient limiting environment and aid in cell growth and division. Another important function of the stalk is to provide buoyancy to the unattached cells of *Caulobacter* to facilitate its stay close to air/water interface as it is an aerobe.

Pelagibacter (*'pelagus'* meaning sea and *'bacter'* meaning rod-shaped bacterium), also known as 'bacterium of the sea,' is another example of an oligotroph which is a strict aerobic chemoorganotroph found in the photic zones of earth's oceans. *P. ubique* is considered to be the most abundant bacterial species inhabiting the earth and has the smallest genome among free-living organisms (Giovannoni et al., 2005).

Apart from phototrophs and oligotrophs, this class also comprises C1 compound-metabolizing bacteria also known by the name 'pink-pigmented facultative methylotrophs' as they produce pink colonies when grown on methanol, e.g., *Methylobacterium* species and the budding bacteria *Hyphomicrobium* (Fig. 5), nitrogen fixers, e.g., *Rhizobium*, *Bradyrhizobium*, *Azospirillum* etc., nitrifying bacteria like *Nitrobacter* and animal and human pathogens like *Brucella*, *Bartonella* and *Rickettsia* species which cause a variety of serious infections (Table 2). *Bartonella* and *Rickettsia* both use arthropods as their reservoir and transmission to humans and animals occur by arthropod's bite. Rickettsia are obligate intracellular parasites metabolically inactive outside the host cell.

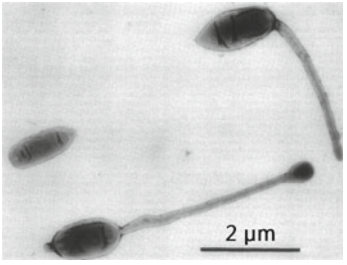


Fig. 5 Budding bacteria *Hyphomicrobium. Source* Moore and Hirsch (1973). (Creative Commons Attribution license)

Table 3 Few important human pathogens in class Betaproteobacteria

Causative agent	Infection
Neisseria gonorrhoeae	Gonorrhea (sexually transmitted disease)
Neisseria meningitidis	Meningitis (only bacteria to cause meningitis in epidemic form)
Burkholderia cepacia	Nosocomial infections (patients with cystic fibrosis and pneumonia)
Bordetella pertussis	Pertussis (whooping cough)

Class Betaproteobacteria

Class Betaproteobacteria comprises nearly 500 characterized species. In contrast to Alphaproteobacteria, organisms belonging to this class are copiotrophs with great nutritional requirements. Some members of this class are pathogenic, which are mostly aerobic chemoorganotrophs. Some of the human pathogens which cause life-threatening infections are listed in Table 3.

Another genus *Burkholderia* include aerobic chemoorganotrophic rods, and one of the important species *Burkholderia cepacia* is also known to be a plant pathogen and can cause soft rot disease in onions. However, it has some positive features also as it can promote plant growth in certain plant and can degrade various organic compounds and is thus an important organic matter recycler.

Betaproteobacteria also include phototrophic purple non-sulfur bacteria which have similar characteristics as mentioned in class Alphaproteobacteria but are copiotrophs, e.g., *Rhodocyclus* as well as obligate and facultative methylotrophs that can grow on C1 compounds (like methanol) but not on methane, e.g., *Methylophilus.* Some are known to produce polyhydroxybutyrates (PHBs), e.g., *Ralstonia.*

This class also includes other important species with various environmental implications such as:

1. **Ammonia Oxidizers**: These are obligate chemolithotrophs and oxidize ammonia into nitrite (nitrification) and play an important role in the nitrogen cycle, e.g., *Nitrosomonas* and *Nitrosospira.*
2. **The sewage mold**: *Sphaerotilus natans* is an aquatic periphyton. It is a filamentous sheathed bacterium that grows in contaminated streams and waters, in drainage, trickling filters, etc., and produces aggregates that plug pipes, irrigation systems and ditches. It is commonly known as sewage mold/sewage fungus.
3. **Flocculating bacteria in wastewater treatment**: *Zoogloea* is an aerobic chemoorganotroph, and its name is derived from a Greek word meaning 'living glue' as it produces a thick gelatinous capsule that binds cells together. This gelatinous mass can cause flocculation because of which it is important in wastewater treatment. The first species known under this genus was *Z. ramigera.*

4. **Generators of acid mine drainage**: *Thiobacillus thiooxidans* and *T. ferrooxidans* are found in sulfur- and iron-rich habitats such as sulfur springs and pyrite deposits. They are chemolithotrophs and carry out oxidation of iron and sulfur, and both are used in bioleaching and biomining for extraction of metal from their ores through oxidation. During the process, sulfuric acid is produced as the end product which is a major cause of acid mine drainage.

Class Gammaproteobacteria

Gammaproteobacteria are the largest and physiologically most diverse class of Proteobacteria with over 1500 characterized species. They include several medically, ecologically, and scientifically relevant groups.

1. **The phototrophic purple sulfur bacteria**

The representative genus of this group is *Chromatium.* They are strictly anaerobic photolithoautotrophs that utilize H_2S as an electron donor for CO_2 fixation. They can be isolated from anoxic zones of sulfur-rich aquatic habitats where sulfide is oxidized to elemental sulfur (S^0) that is stored intracellularly as refractile sulfur granules unlike green sulfur bacteria where sulfur is deposited outside the cells. The sulfur is further oxidized to sulfuric acid. They carry out anoxygenic photosynthesis via type II photosystem.

2. **The enteric bacteria**

It is the largest group of bacteria under the family Enterobacteriaceae within the class Gammaproteobacteria. They live in the intestines of animals and mammals and are thus named as enteric bacteria. They include non-sporing, rod-shaped facultative anaerobes that are either non-motile or have peritrichous flagella. Most also reduce nitrate to nitrite during anaerobic respiration. They are oxidase negative and catalase positive. They have simple nutritional requirements and can ferment sugars via EMP pathway to a variety of end products.

Based on their fermentation products, they are divided into two groups:

(a) Mixed-acid fermentation—Majority of enteric bacteria belong to this group such as *Escherichia, Proteus, Salmonella, Yersinia and Shigella.* The fermentation end products are lactate, acetate, succinate, formate (or H_2 and CO_2).
(b) Butanediol fermentation—This group includes *Enterobacter, Klebsiella, Serratia* and *Erwinia.* The major fermentation end products are butanediol, ethanol and CO_2.

Many enteric bacteria are pathogenic to humans, animals and plants, and important examples are listed in Table 4.

Table 4 Few important pathogenic bacteria belonging to Gammaproteobacteria

Organism	Infection (humans)
Escherichia coli	Gut or urinary tract infections
Salmonella typhi	Typhoid fever
Salmonella	Gastroenteritis
Shigella dysenteriae	Bacillary dysentery (a form of gastroenteritis)
Yersinia pestis	Bubonic and pneumonic plague
Yersinia enterocolitica	Diarrhea
Enterobacter aerogenes	Urinary tract infections
Klebsiella pneumonia	Pneumonia
Proteus	Urinary tract infection and sepsis
Serratia marcescens	Nosocomial infections
Erwinia	A plant pathogen; causes leaf spots and discoloration

3. The biofilm forming pseudomonads

Any gram-negative bacterium that is an aerobic rod with polar flagellation can be termed as pseudomonad and includes the genus *Pseudomonas.* They are aerobic chemoorganotrophs that are found in soil, aquatic systems and air. Many species are pathogenic. They are used as an experimental system for studying biofilm formation. *P. aeruginosa* is an opportunistic pathogen associated with respiratory and urinary infections. Important plant pathogens include *P. syringae* and *P. marginalis.*

They produce characteristic water-soluble pigments such as pyocyanin, phenazine and pyoverdins. *Pseudomonas* species are important biotechnologically for a variety of reasons. Many can degrade a wide variety of organic compounds including a wide spectrum of heterocyclic and aromatic organic compounds that are not attacked by other bacteria. *P. putida* is an excellent example known to degrade xenobiotics (Cao et al., 2009).

4. The comma-shaped vibrios

Members belonging to the genus *Vibrio* have unusual shape as they are slightly curved rods in the form of comma. Members of this order are flagellated straight or curved rods. They are facultative aerobes having a fermentative metabolism, and the majority are aquatic. Important pathogens within this group include *V. cholera* that causes cholera and *V. parahaemolyticus* that causes gastroenteritis. They are oxidase positive and thus differentiated from Enterobacteria. Most bioluminescent bacteria belong to this group and are typically found as symbionts of deep-sea animals such as *V. fischeri and V. harveyi.*

5. **The obligate methylotrophs**

They are obligately methanotrophic and aerobically oxidize methane/methanol to gain energy and build biomass. The representative genera are *Methylococcus* and *Methylomonas.*

6. **Microbial Nanowires: Shewanella**

Shewanella species are facultative anaerobes, non-spore formers, straight or curved rods with a single flagellum. They display a wide flexibility in terms of the terminal electron acceptors. They can use a large variety of metal electron acceptors including uranium, plutonium, chromium, selenite, etc., and carry out dissimilatory metal reduction. Thus, they are promising candidates for bioremediation of radionuclides. They are also of interest for microbial electrogenesis (generation of electricity by microbes) and microbial fuel cells as they are able to transport electrons from the terminal point in ETC to metal surfaces via nanowires which are electrically conductive. They use extension of the outer membrane as conductive appendages.

7. **Pathogens: Zoonotic and obligate intracellular parasites**

Class Gammaproteobacteria also consists of zoonotic pathogens which can infect humans through animals such as *Pasteurella multocida,* the causative agent of fowl cholera that causes Pasteurella infection in humans.

Other pathogenic species within this class are *Legionella* and *Coxiella.* Legionella is an intracellular parasite of protozoa, and *L. pneumophila* causes pneumonia-type illness called Legionnaires' disease and a mild flu-like illness called Pontiac fever.

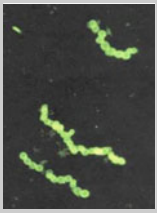
Coxiella burnetii is the only member of the genus *Coxiella.* It is an intracellular parasite of hosts such as insects, birds, rodents, sheep, goat and humans and survives within their phagolysosomes. It causes Q fever, a flu-like illness.

Box 9: *Buchnera aphidicola*: An Endosymbiont of Aphids

The characteristic feature of *B. aphidicola* is its very small genome size of less than 1 Mb. It is unculturable and is primarily an endosymbiont of pea aphid *Acyrthosiphon pisum* that feeds on plant sap. The genome size ranges from 600 to 650 kb that encodes about 500–560 proteins. It survives in host aphid within special cells termed bacteriocytes. Each bacteriocyte in turn contains numerous plasma membrane-derived vesicles termed symbiosomes harboring *Buchnera* cells. Due to the well-established symbiotic relationship with the aphid, *Buchnera* lost a number of genes by deletion during course of evolution resulting in smaller genomes and has a genome approximately one-seventh the size of the *E. coli* genome (Van Ham et al., 2003).

Apart from the above-mentioned groups, *Beggiatoa* is another species within the class which is filamentous and shows gliding motility. They are usually found in sulfur-rich environments and thrive as both heterotrophs and chemolithotrophs.

Box 10: *Thiomargarita namibiensis*: The Largest Bacterium Ever Discovered



It is the largest bacterium reported so far with a diameter of 100–300 μm which can grow as large as 750 μm in diameter. Cells of *Thiomargarita* contain microscopic granules of sulfur that give the cells a pearly luster when exposed to light, and thus the bacterium is named *Thiomargarita* which means 'sulfur pearl' based on the appearance of the cells. It was found in the ocean sediments of the continental shelf of Namibia, hence the name *namibiensis*.

Source: Wikipedia (Public Domain).

Class Deltaproteobacteria

Deltaproteobacteria are a small group of chemoorganotrophs that predominantly comprise aerobic bacteria along with some strict anaerobes that reduce sulfur compounds while oxidizing iron compounds.

The sulfate- or sulfur-reducing bacteria (SRB)

These are anaerobes that thrive in habitats such as marine sediments and nutrient-rich anoxic environments. They oxidize organic compounds such as lactate, formate, butyrate, propionate, pyruvate and aromatic compounds to acetate using sulfate as the terminal electron acceptor and converting it to H_2S. However, some species are incomplete oxidizers and acetate is not further oxidized, e.g., *Desulfovibrio*, whereas some species can completely oxidize acetate to CO_2, e.g., *Desulfobacter* and *Desulfurculus*.

Another organism *Syntrophobacter wolinii* is also a strict anaerobe, and it can reduce sulfate under certain conditions. In natural habitats, this bacterium interacts with H_2-consuming bacteria in a metabolic relationship called syntrophy where it oxidized propionate, thus producing acetate, CO_2 and H_2 in the presence of a H_2-consuming partner. In the absence of H_2-consuming partner, it can also grow by reducing sulfate by fermenting pyruvate or fumarate.

Bacterial Nanowires: *Geobacter*

Geobacter has gained a lot of interest in bioremediation and electricity generation similar to the genus *Shewanella* of Gammaproteobacteria. *Geobacter* was the first organism known for reducing iron and other metal compounds including radioactive metals and petroleum compounds into environmentally benign carbon dioxide during

oxidation of both metal and organic compounds. The electrons generated in the process can be passed via pili as nanowires grown between the species. Further, species of *Geobacter* (such as *G. metallireducens* and *G. sulfurreducens*) are able to cooperatively metabolize a mixture of chemicals, like ethanol and sodium fumarate, that neither could process alone.

The fruiting body formers: Myxobacteria

Myxobacteria are aerobic soil gliding bacteria that are rod-shaped and lack flagella. Most myxobacteria are predators of other microbes. They typically travel in swarms, exhibit A-C signaling and form fruit bodies.

The parasitic bacterium: *Bdellovibrio*

Bdellovibrio is an example of parasitic bacteria known to predate other bacteria (bacteriovorous) (Fig. 6). It is an aerobic bacterium with curved rod and polar flagellum. After attachment to its prey, it penetrates and accumulates intracellularly. The cell then rounds off to form a spheroplast like structure called the bdelloplast. *Bdellovibrio* continues to elongate and obtain nutrients from the host. It then undergoes multiple divisions to give rise to new cells that eventually lyse the host cell. The lysed bacteria appear as lytic zones similar to plaques of viruses.

Class Epsilonproteobacteria

Epsilonproteobacteria consist of a few known genera. Initially, this class included a single order Campylobacterales (*Campylobacter* and *Helicobacter*). In recent times, a new order *Nautiliales* (*Nautilia* and *Caminibacter*) has been proposed based on phylogenetic analysis.

Most of the known species are symbiont or pathogens and inhabit animal gut. Both *Campylobacter* and *Helicobacter* are microaerophilic, motile, helical or vibroid rods and are pathogenic to humans or animals. *C. jejuni* is a major cause of gastroenteritis, while *H. pylori* causes peptic ulcers. Another related genus is *Wolinella* which is

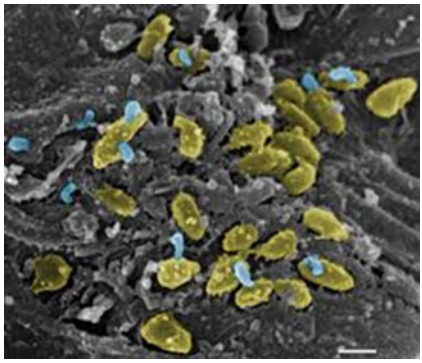


Fig. 6 *Bdellovibrio* (blue) seen parasiting *Pseudomonas tolaasii* (green) on mushroom surface. *Source* Saxon et al. (2014). (Creative Commons Attribution License)

anaerobic and serves as a symbiont in cattle rumen. These bacteria are both aerobic and anaerobic chemolithotrophs that utilize sulfur or hydrogen as electron donors.

Metagenomic studies have revealed occurrence of other *Epsilonproteobacteria* belonging to genus *Sulfurimonas* and *Nautilia* in hydrothermal vents and cold seep habitats.

Class Zetaproteobacteria

Zetaproteobacteria is represented by single species *Mariprofundus ferrooxydans* which grows as iron-oxidizing chemolithoautotroph and forms biofilms on the surface of rocks (Emerson et al., 2007; Singer et al., 2011). Metagenomic studies have revealed a large diversity of the unculturable Zetaproteobacteria.

9 The Non-proteobacteria Gram-Negatives

This group includes gram-negative bacteria which are not included in the phylum Proteobacteria and show high functional diversity. It includes phototrophs, chemolithotrophs, obligate intracellular pathogens, cell-wall-less bacteria with intracellular compartments, etc.

Phylum Cyanobacteria—Oxygenic photosynthetic bacteria

Cyanobacteria are widespread aerobic photosynthetic bacteria which can be isolated from soil, freshwater and marine habitats. They are essentially aerobic and carry out oxygenic photosynthesis. They have chlorophyll a and both type I and type II photosystems. Other pigments include water-insoluble carotenoids and phycobilins.

Box 11: Cyanobacteria: First Oxygen-Evolving Phototrophic Organisms on Earth

Earth's first phototrophs were anoxygenic that existed 3–4 billion years ago that used light energy to oxidize H_2S to elemental sulfur to synthesize complex organic carbon as food. However, molecular and chemical evidence indicates that around 3.5 billion years ago oxygenic photosynthesis first appeared on earth. Phototrophic organisms capable of using H_2O as electron donor to fix CO_2 as food evolved oxygen as a waste product and were called oxygen-evolving cyanobacteria. Accumulation of oxygen on earth led to gradual change in the atmosphere converting an anoxic environment to oxygen-rich environment and thus gave rise to evolution of many organisms capable of aerobic respiration (Whitton, 2012).

Prochlorophytes are another class of photosynthetic organisms which were initially classified in a separate phylum but have now been merged with cyanobacteria. Unlike cyanobacteria, they are unicellular and spherical and contain chlorophyll *b* in addition to chlorophyll *a* but lack phycobilins and appear greenish as grass in color.

Phylum Chlorobi—Anoxygenic Photosynthetic Green Sulfur Bacteria

The key genus is *Chlorobium* that is found in anoxic zones of sulfur-rich lakes. These are obligate anaerobic photolithoautotrophs which use H_2S, elemental sulfur and H_2 as the electron donors for CO_2 fixation that occurs by reverse citric acid cycle. They use type I photosystem to carry out photosynthesis and contain bacteriochlorophylls *c*, *d* and *e* and minor amounts of bacteriochlorophyll *a* that imparts a green color to the bacteria. When sulfide is oxidized, granules of sulfur are deposited outside the cells. Thus, they are named as green sulfur bacteria. Cells have diverse morphology ranging from rods to cocci to vibrios growing singly or in chains and clusters.

Phylum Chloroflexi—Anoxygenic Photosynthetic Green Non-sulfur Bacteria

This phylum includes both photosynthetic and non-photosynthetic bacteria. The phylum includes both photosynthetic green non-sulfur bacterial genus *Chloroflexus*, a thermophile, and non-photosynthetic genus *Herpetosiphon* that are aerobic organotrophs. *Chloroflexus* carries out anoxygenic photosynthesis and contains bacteriochlorophylls *a* and *c*. Unlike green sulfur bacteria, they use type II photosystem. They grow best as photoheterotrophs using simple carbon sources as source of electrons for photosynthesis. They can also grow in dark as aerobic chemoheterotrophs.

Phylum Acidobacteria: Newly discovered photoheterotroph

This is a newly devised phylum of bacteria and includes one key genus *Acidobacteria.* Its members are widespread in soils and may be important contributors to ecosystems. However, they are difficult to cultivate and only a few species have been cultured. Its members are physiologically and metabolically quite diverse. They include chemoorganotrophs as well as photoheterotrophs and can be aerobes or obligate anaerobes.

Box 12: Bacteroides-to-Firmicutes Ratio: Implication in Obese Patients

Firmicutes and Bacteroides make up the largest portion of the mouse and human gut microbiome. The relationship between gut microbiome and obese people has been known for a decade now. Members of the phylum Bacteroidetes and another phylum Firmicutes (gram positive) make up the largest portion of the human gut microbiome and have been considered an important factor involved in obesity. Studies have shown that in obese patients, composition of gut microbiota varies greatly as the number of bacteria belonging to phylum Firmicutes increases greatly than the number of Bacteroides. In fact, the number of these two groups of gut organisms are inversely proportional as in lean people the ratio of Bacteroides is higher than Firmicutes. Thus, study of this complex ecological relationship may provide effective treatments for obese patients (Ley et al., 2006).

Phylum Bacteroidetes: Part of gut microbiota/polysaccharide degraders

They are gram-negative bacteria that ferment complex polysaccharides and produce short-chain fatty acids. The most well-studied genus is *Bacteroides* that includes anaerobic, saccharolytic rods abundantly found in human and animal gut. *B. ruminis* is an important member of rumen flora. *B. fragilis*, the first isolated *Bacteroides* species, is a human pathogen associated with appendicitis (Rajilić-Stojanović & de Vos, 2014).

Another genus *Cytophaga* includes obligate aerobes that appear as individual rods or filaments or as trichomes having widespread occurrence in freshwater and soil. They exhibit gliding motility like myxobacteria but do not form fruiting bodies. They carry out degradation of complex polysaccharides/crystalline cellulose and are found in soils and freshwaters. Some species are fish pathogens such as *C. columnaris* and *C. psychrophila.*

Flavobacteriia and *Flexibacteria* are aerobic rods that are motile by gliding. They can decompose several polysaccharides and are abundant in soils and aquatic habitats.

Phylum Chlamydiae: Obligate intracellular parasites

They are non-motile cocci that are obligate intracellular parasites and rely on their hosts for most metabolites. They are called energy parasites as they are incapable of synthesizing ATP. They reproduce only within host cells forming two types of cells called elementary and reticulate bodies. Two important species known to cause humans diseases are *C. trachomatis*, causative agent of sexually transmitted infections and *C. psittaci* that causes psittacosis, a feverish pneumonia that occurs in birds and also transmitted to humans.

Life cycle of *Chlamydiae trachomatis*

The life cycle of Chlamydiae involves the role of two types of cells (Fig. 7). The small non-multiplying cell is called an elementary body which acts a means of transmission for the bacterium. Since the mode of transmission is air, the elementary bodies are resistant to drying. The other form is a vegetative form called the reticulate body which multiplies by binary fission inside the host cell to generate enough inoculum for transmission. The vegetative cells get converted to elementary bodies after a certain set of cell divisions, and then they lead to rupture of host cells to release elementary bodies in the air which can infect another nearby host and get converted into reticulate bodies. The approximate generation time measured for reticulate bodies is 2–3 h (Nunes & Gomes, 2014).

Phylum Dictyoglomi: Xylanase Production

The genus *Dictyoglomus* of this phylum is an extremely thermophilic anaerobic chemoorganotroph having a triple-layered wall. It shows relationship to phylum Thermotogae (Nishida et al., 2011). It is of biotechnological interest as it produces xylanase which is used in pretreatment of wood pulp.

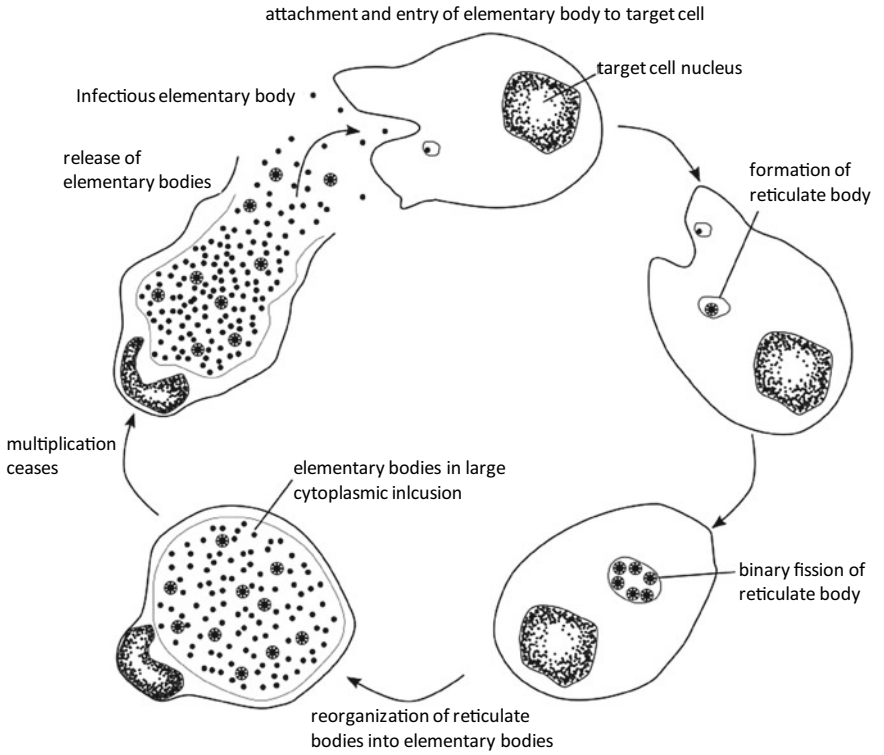


Fig. 7 Life cycle of *Chlamydiae*. *Source* Wikipedia (Public Domain)

Phylum Fibrobacteres: Cellulose degrader

The key genus *Fibrobacter* is strictly anaerobic with a fermentative metabolism. It can ferment various carbohydrates. It is found in rumen or gut of animals where it decomposes cellulose.

Phylum Fusobacteria: Pathogenic

Members are obligately anaerobic, non-spore-forming rods that dwell in sediments, gut and oral cavities. They ferment carbohydrates, peptides and amino acids. *Fusobacterium* species cause periodontal diseases, Lemierre's syndrome and topical skin ulcers in humans.

Phylum Gemmatimonadetes

Gemmatimonadetes are widespread in soil and are the most abundant phyla in soil and sewage as revealed by metagenomic studies. The first member of this phylum, *Gemmatimonas aurantiaca*, was isolated from activated sludge of a sewage treatment system in 2003. This bacterium exhibits both aerobic and anaerobic respiration.

Phylum Spirochaetes: Cork screw-shaped spirilla with flexible motility

Spirochaetes are chemoheterotrophic slender, long bacteria with a flexible, helical shape. The location of their flagella is unique and is called axial filaments which lies inside a flexible outer sheath and extends from both ends allowing twisting motion. The key genera included in this phylum are *Borrelia, Leptospira, Spirochaeta, Treponema* which can cause a variety of human diseases. *Leptospira* species cause leptospirosis, *Treponema pallidum* causes syphilis, and *Borrelia burgdorferi* causes Lyme disease.

Phylum Planctomycete: Intracellular compartmentalization/Cell-wall-less S-layered bacteria

Planctomycetes are a group of morphologically unique bacteria as their cell walls lack peptidoglycan and are of an S-layer type. They have membrane-bound internal compartments including anammoxosome, considered analogous to eukaryotic mitochondrion (Fig. 8). Anammoxosomes are involved in anammox reaction i.e. anaerobic oxidation of ammonia. Genus *Gemmata* has a nuclear envelop around the nucleoid, while some genera such as *Brocadiales* have the anammoxosome which is considered analogous to eukaryotic mitochondrion. Recently, an endocytosis like ability and a protein homologous to the eukaryotic protein clathrin which is important for clathrin-mediated endocytosis have been discovered in this phylum. All these

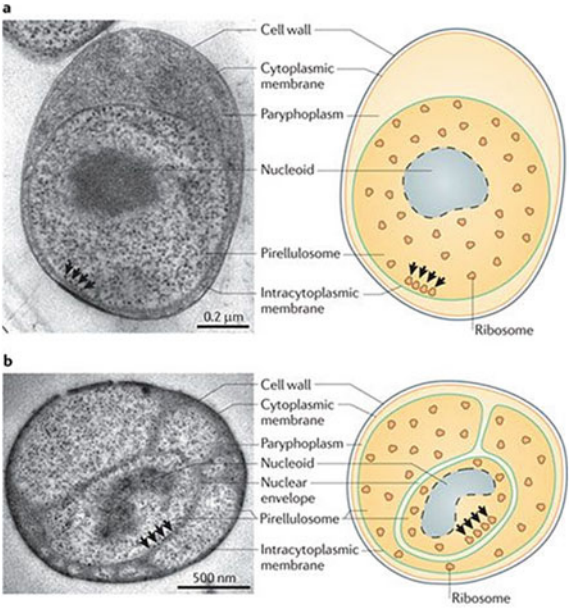


Fig. 8 Cell structure of Planctomycetes showing intracellular compartmentalization. *Source* Fuerst & Sagulenko (2011). With kind permission from Springer Nature

features make this phylum interesting to study origin and evolution of eukaryotic cells (Fuerst & Sagulenko, 2011).

Phylum Verrucomicrobia: Intracellular compartmentalization

Similar to the phylum Planctomycetes, phylum Verrucomicrobia also comprises intracellular compartments having membrane-bound nucleoid and ribosomes (Lee et al., 2009). They contain only a few described species; however, metagenomic studies have revealed their abundance in both soil and aquatic environments. They are aerobic or facultatively aerobic bacteria that can ferment sugars. They form cytoplasmic appendages called prosthecae. The key genera are *Verrucomicrobium* and *Prosthecobacter*.

Phyla Deferribacteres, Chrysiogenetes and Nitrospirae

Phyla Deferribacteres and Chrysiogenetes contain anaerobic chemoorganotrophs that can use various electron acceptors during anaerobic respiration. Most species can carry out nitrate respiration. The key genus of Deferribacteres is *Deferribacter* that is a thermophilic ferric ion reducer. It can also reduce nitrate and metal oxides. The key genus of phylum Chrysiogenetes is *Chrysiogenes*. *C. arsenates* can oxidize some organic compounds like acetate and use arsenate as electron acceptor. Some Chrysiogenetes can reduce nitrite, nitrate, elemental sulfur, thiosulfate and selenite during anaerobic respiration.

The key genus of phylum Nitrospirae is *Nitrospira* which is chemolithotrophic, oxidizes ammonia directly to nitrate and grows autotrophically. It is a complete ammonia oxidizer (comammox). Another member of this phylum is *Leptospirillum* which is an aerobic, acidophilic chemolithotrophic iron oxidizer found in acid mine drainage.

10 Firmicutes: The Low G + C Gram-Positive Bacteria

The phylum Firmicutes comprises the low G + C content gram-positive bacteria (having a 45–60% GC content). The name has been derived from Latin words '*firmus*' meaning strong and '*cutis*' meaning skin indicating their characteristic thick cell wall. All gram-positive bacteria were earlier included in the phylum Firmicutes. However, this phylum now comprises the low G + C bacteria (45–60% GC content) while the high G + C bacteria are placed in the phylum Actinobacteria (G + C content up to 70%).

Firmicutes have coccus or rod-like cells, and they mainly constitute the lactic acid bacteria, the spore formers, the non-spore formers, photosynthetic bacteria, Negativicutes and the cell-wall-less bacteria.

Lactic acid bacteria

These are non-sporulating, non-motile rods and cocci which produce lactic acid and are used extensively in food production and preservation. They are aerotolerant anaerobes and obtain energy only by substrate-level phosphorylation. They have limited biosynthetic capabilities and thus require many vitamins, amino acids, purines and pyrimidines for growth. There are two groups of lactic acid bacteria: homofermentative which produce a single fermentative product, lactic acid, and heterofermentative which produce ethanol and acetic acid along with lactic acid (Table 5).

The Spore formers

All endospore-forming bacteria are gram positive belonging to the genera *Bacillus*, *Clostridium* and *Sporosarcina.* The endospores contain the signature molecule called dipicolinic acid and high levels of calcium and can survive extreme conditions.

The genus *Bacillus* includes aerobic spore formers largely rods, while few are cocci with peritrichous flagella. They produce a number of extracellular enzymes, and many species produce antibiotics, e.g., polymyxin, gramicidin, etc. Most of

Table 5 Few important genera of lactic acid bacteria and their key features

Genus	Cell form and arrangement	Fermentation	Characteristics
Streptococcus	Cocci in chains	Homofermentative	Used in production of Swiss cheese; lyse erythrocytes when growing on blood agar; many species are pathogenic such as *S. pyogenes* (pus formation) and *S. mutans* (dental caries)
Pediococcus	Cocci in tetrads	Homofermentative	Used in making sauerkraut along with *Leuconostoc* and *Lactobacillus*
Lactobacillus	Rods	Mostly homofermentative	Common in dairy products, used for preparation of fermented milk products; involved in spoilage of beer, milk, wine and meat
Enterococcus	Cocci in chains	Homofermentative	*E. faecalis* is normal resident of intestinal tract in humans/animals and an opportunistic pathogen
Lactococcus	Cocci in chains	Homofermentative	Used in production of buttermilk and cheese
Leuconostoc	Cocci in chains	Heterofermentative	Find application in production of fermented milk products, fermentation of vegetables and wine; involved in food spoilage

these are non-pathogenic. Examples of *Bacillus* species and their unique features are represented in Table 6.

Clostridium is a genus comprising obligate anaerobes and spore formers and exhibits a fermentative type of metabolism. Clostridia lack a respiratory chain and can carry out only substrate-level phosphorylation. Most species of this genus are saprophytic organisms found in many places in the environment, most notably the soil, marine and freshwater anaerobic sediments and intestinal tract of humans and animals. However, the genus does contain some human pathogens. A few important species have been listed in Table 7.

Genus *Sporosarcina* is unique as they are the only cocci that produce endospores. It consists of strictly aerobic spherical cells that are arranged in tetrads or cubical packets of eight cells.

Desulfotomaculum is also a spore former anaerobe that reduces sulfate and sulfite to H_2S.

The non-spore formers

The most notable gram-positive bacteria that do not form endospores are *Staphylococcus, Listeria, Lactobacillus* and *Sarcina.*

Table 6 Biotechnological application of important *Bacillus* species

Bacillus sp.	Characteristic features
B. subtilis	Produces a consortium of enzymes, model organism for studying gene regulation, cell division, etc.
B. cereus	Causes food poisoning
B. stearothermophilus	Thermophilic, produces extremely heat-resistant endospores, associated with spoilage of canned foods
B. thuringiensis	Spores are commonly used as insecticide commercially called 'Bt toxin'
B. anthracis	Causes anthrax and is increasingly being exploited as a 'bioweapon'

Table 7 Important features of few *Clostridium* species

Clostridium sp.	Characteristic features
C. botulinum	Causes botulism
C. tetani	Causes tetanus and produces characteristic terminal spores
C. perfringens	Causes wound infections called gas gangrene
C. thermosaccharolyticum	Thermophilic, produces extremely heat-resistant endospores, associated with spoilage of canned foods
C. pasteurianum	Fixes atmospheric nitrogen
C. acetobutylicum	Used for industrial production of butanol
C. thermocellum *C. cellulolyticum* *C. cellulovorans*	Ferment cellulose with formation of acids and alcohol, decompose cellulose in anoxic habitats like rumen, possess a multi-enzyme complex called cellulosome

Staphylococcus is facultatively anaerobic, non-motile cocci that can undergo respiration as well as fermentation. Staphylococci are common commensals and parasites of humans and occasionally can cause infections. *S. epidermidis* is usually found on the skin or mucous membranes, while *S. aureus* is associated with pathological conditions such as boils, pimples, wound infections, pneumonia and toxic shock syndrome.

Listeria is a facultatively anaerobic non-spore-forming rod. *L. monocytogenes* causes listeriosis, a food-borne illness.

The genus *Lactobacillus* has already been described earlier in this chapter.

The genus *Sarcina* contains strict anaerobes, and they show unusual morphology as they divide into three perpendicular planes to give rise to eight or more cells. They are extremely acid-tolerant and can inhabit in human stomach. In fact, in case of certain gastrointestinal infections such as pyloric ulceration, there is faster growth of these bacteria.

The photosynthetic bacteria

Heliobacterium and *Heliophilum* are gram-positive anaerobic photosynthetic bacteria that have an unusual bacteriochlorophyll *g*. They have type I photosystem but are unable to grow autotrophically. They grow photoheterotrophically in light and fermentatively under dark conditions.

Negativicutes

These are gram-positive bacteria with an outer membrane. *Negativicoccus succinivorans* has unusual cell envelope as it has an inner and outer membrane like gram-negative bacteria but 16s rRNA gene sequence revealed its homology to gram-positive bacteria. It also ferments succinate (Marchandin et al., 2010).

Another member *Veillonella* is chemoheterotrophic occurring mostly as diplococci. It is an anaerobe and lives as a parasite in homeothermic animals.

The cell-wall-less bacteria (Tenericutes/Mollicutes)

These are the smallest independently replicating prokaryotes. They are distinguished by the lack of a cell wall (therefore called Mollicutes—soft skinned). They are commonly known as mycoplasmas because *Mycoplasma* is the best characterized genus of this group. As per some recent classification schemes, Tenericutes has been proposed as a separate phylum containing a single class Mollicutes (Ludwig et al., 2009).

Mycoplasmas do not stain gram-positively but are phylogenetically related to Firmicutes. They are sensitive to osmotic lysis but are resistant to a variety of cell wall targeting antibiotics such as β-lactams. They live in close association with animal and plant host cells, and some species have a special terminal structure that aids in attachment to the host cells. In animals and humans, they are known to cause diseases of respiratory and urinary tract. For example, *Mycoplasma pneumonia* causes a typical pneumonia in humans; *M. hominis* causes genital tract infections, and *Ureaplasma urealyticum* causes urinary tract infections (Metwally et al. 2014).

Another genus *Spiroplasma* consists of spiral-shaped cells and is an important pathogen of plants and arthropods that feed on them.

11 Actinobacteria: The High G + C Gram-Positive Bacteria

Actinobacteria have a G + C content of above 50%. They contain rod-shaped to filamentous aerobic bacteria that are commonly found in soil and plant materials. Most species are commensals though some are pathogenic such as *Mycobacterium.* Many Actinobacteria, especially *Streptomyces* sp., are well recognized as antibiotic producers. Some species also dwell on mucosal surfaces in humans and cause actinomycoses, ocular and periodontal infections. Some of the important genera under this phylum are discussed below.

The filamentous bacteria: Actinomycetes

These are known as actinomycetes meaning 'ray fungus' from the Greek words 'actis' meaning ray/beam and 'mykes' meaning fungus. They have a distinctive life cycle including the development of filamentous cells called hyphae that differentiate to form spores. Actinomycetes are primarily soil inhabitants and can degrade a wide variety of organic compounds.

Antibiotic Producers: Streptomyces Species

The genus *Streptomyces* is one of the largest groups comprising around 500 described species. They are soil-dwelling aerobes that grow as aerial filaments (sporophores) which divide to form non-motile conidial spores. Streptomyces produce over two-third of the clinically useful antibiotics such as streptomycin, neomycin, grisemycin and chloramphenicol.

Human Commensals

Actinobacteria also include human commensals which live in various parts of human body. Species belonging to the genus *Propionibacterium, Micrococcus, Bifidobacterium, Gardnerella* and *Nocardia* are examples of such organisms.

Propionibacterium are pleomorphic, non-sporing, non-motile rods that are facultative anaerobic or aerotolerant. They are common skin and intestinal inhabitants, and *P. acnes* is involved in causing skin acne. *Micrococcus* is an aerobe that also inhabits skin of humans.

Bifidobacterium is an anaerobic bacterium ubiquitously found in mouth, gastrointestinal tract and vagina of humans. Recently, it has gained importance to be used as gut-friendly probiotic species.

Another commensal organism is *Gardnerella* which is facultatively anaerobic, non-sporulating, non-motile coccobacilli. The only species of this genus is *G. vaginalis* which is part of vaginal microbiome of healthy women. However, there are

some pathogenic variants of *G. vaginalis*, the causative agent of bacterial vaginitis. *Nocardia* species are a part of oral microflora found in healthy gingiva and periodontal pockets.

Human Pathogens

Key organisms *Mycobacterium tuberculosis* that causes tuberculosis and *Mycobacterium leprae* that causes leprosy also belong to Actinobacteria. These two species are obligate parasites and do not occur as free-living forms. Mycobacteria are aerobic, non-motile straight or curved rods that may undergo branching or filamentous growth. They also have a high content of unique lipids called mycolic acids in their cell walls due to which they are acid-fast.

Another bacterium, *Corynebacterium diphtheria*, causes diphtheria. The cells of *Corynebacterium* are distinctly identified by their arrangement in a form of a 'V,' 'palisades' or 'Chinese letters.' This characteristic arrangement is because during division, only inner portion of the cell wall is synthesized allowing cells to bend (snapping binary fission).

Many species of the genus *Nocardia* are opportunistic pathogens which causes serious infections (commonly called nocardiosis) when inhaled or introduced traumatically in the body.

Industrially Useful strains

Phylum Actinobacteria also constitute some industrially important species.

Propionibacterium species ferment lactic acid, carbohydrates and alcohols and produce propionic acid, acetic acid and CO_2. *P. shermanii* is used in making Swiss cheese to create characteristic CO_2 bubbles and to get the unique flavor due to the production of propionic acid. In fact, *Propionibacterium* was first discovered from Swiss cheese. Another strain is *Corynebacterium glutamicum*, important for overproduction of the amino acid glutamate.

Symbiotic nitrogen fixers

Frankia are nitrogen-fixing bacteria that live in symbiosis with non-leguminous plants. They form filamentous hyphae that divide both transversely and longitudinally to form non-motile spores.

Actinobacteria also include unusual organisms like *Arthrobacter* species. *Arthrobacter* is derived from a Greek word meaning jointed small sticks (Fig. 9). They have an unusual rod–coccus growth cycle and are rods during exponential growth and cocci in their stationary phase. They undergo snapping binary fission in which the outer bacterial cell wall ruptures at a joint (Krulwich & Pate, 1971).

Some Actinobacteria are also called as maduromycetes based on the presence of the sugar residue madurose (3-*O*-methyl-D-galactose), e.g., *Actinomadura.* They are aerobic filamentous and form short chains of spores or sporangia having one or many spores.

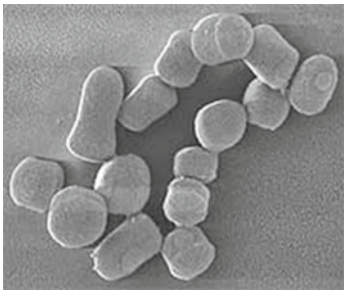


Fig. 9 Scanning electron micrograph of Arthrobacter cells. *Source* Wikipedia (Public Domain)

Thus, the diversity of prokaryotes including both archaea and bacteria clearly indicates that they are highly diverse metabolically as they occupy a different niche and inhabit a large portion of the earth and play many important roles within the environment.

Summary

- Woese & Fox (1977) presented a three-domain classification comprising archaea, bacteria and eukaryote.
- The concept of archaea as an independent domain originated for methanogens based on differences in 16s rRNA and tRNA gene sequences, two unusual coenzymes for methane formation and lack of peptidoglycan in their cell walls and later confirmed by complete genome sequencing of the archaeon *Methanococcus jannaschii.*
- Archaea are classified into seven phyla including two ancient phyla, Euryarchaeota and Crenarchaeota, and five other phyla, namely phylum Korarchaeota, phylum Nanoarchaeota, phylum Thaumarchaeota, phylum Aigarchaeota and phylum Lokiarchaeota.
- The phylum Euryarchaeota is a large and physiologically diverse phylum comprising methanogens, extreme halophiles, thermoplasms, extremely thermophilic S^0 reducers and sulfate reducers.
- Methanogens are stringent anaerobes, mostly non-halophilic, mesophilic and obligate chemolithotrophic methane producers. *Methanopyrus* a hyperthermophile is the first microbe to be discovered that can withstand temperatures as high as 122 °C.
- Haloarchaea are extreme halophiles that require at least 8% NaCl for growth and are mostly obligately aerobic chemoorganotrophs.
- Thermoplasms include the thermophilic and extremely acidophilic genera *Thermoplasma, Picrophilus* and *Ferroplasma.* Of these, *Thermoplasma* and *Ferroplasma* lack cell wall while *Picrophilus* has an S-layer outside its plasma membrane.

- Extremely thermophilic S^0 reducers are strictly anaerobic chemoorganotrophs that can metabolize proteins and other organic compounds.
- Phylum Crenarchaeota contains four orders, namely *Sulfolobales, Thermoproteales, Desulfurococcales* and *Caldisphaerales*, and members belonging to this phylum are mostly hyperthermophiles and obligate anaerobes. Many are autotrophic chemolithotrophs and chemoorganotrophs with diverse electron donors and acceptors.
- *Sulfolobus* and *Acidianus* are key genera belonging to the order *Sulfolobales.*
- Order Thermoproteales includes three main genera: *Thermoproteus, Thermophilum* and *Pyrobaculum.*
- Order *Desulfurococcales* contains coccoid or disk-shaped hyperthermophiles isolated from submarine volcanic habitats and includes *Pyrodictium* and *Pyrolobus*, the most hyperthermophilic prokaryotes.
- *Ignicoccus hospitalis* is called as 'hospital fireball' and a host to a parasitic archaeon *Nanoarchaeum equitans.* It is considered to be a modern descendant of ancestral cell type that gave rise to the origin of eukaryotic cells.
- Phylum Korarchaeota includes only a single species, namely *Candidatus*: Korarchaeum cryptofilum which is an obligate anaerobic chemoorganotrophic hyperthermophile growing at 85 °C.
- Phylum Nanoarchaeota is represented by a single species *Nanoarchaeum equitans* which is one of the smallest cellular organisms (0.49 Mb) and only grows parasitically on the surface of its host organism *Ignicoccus hospitalis.*
- Members of phylum Thaumarchaeota are aerobic chemolithoautotrophic ammonia oxidizers and may play an important role in biogeochemical cycles such as nitrogen and carbon cycle.
- Phylum Aigarchaeota has a close phylogenetic relationship with mesophilic Thaumarchaeota and hyperthermophilic Crenarchaeota. The main representative member of this phylum is *Candidatus*: Caldiarchaeum subterraneum.
- Phylum Lokiarchaeota is a novel candidate phylum proposed in 2015 after identification of a candidate genome of a unicellular form named as *Lokiarchaeum.* It is considered as an archaeal host for emergence of eukaryotes.
- The bacteria are divided into five lineages majorly based on molecular differentiation and also on the basis of gram-staining, namely the deeply branching bacteria which are thermophilic and closely related to archaea, Proteobacteria gram-negatives which show high morphological versatility, non-Proteobacteria gram-negatives, the low G + C gram-positive bacteria and the high G + C gram-positive bacteria.
- The deeply branching bacteria represent the most ancient bacterial lineage. Phylum Aquificae is the oldest branch of deeply branching bacteria which includes obligate chemolithoautotrophic hyperthermophiles followed by phylum Thermotogae, the second oldest branch of hyperthermophilic bacteria.
- The phylum Proteobacteria is the largest group of gram-negative bacteria with more than 500 genera, and the name is derived from the ancient word 'Proteus'

meaning Greek god of the sea capable of acquiring various shapes. Morphologically, they show high level of versatility including rods, cocci, spirilla, filamentous, budding and appendaged forms.

- Proteobacteria are divided into six classes, alpha, beta, gamma, delta, epsilon and zeta. Gammaproteobacteria is the largest class followed by Alphaproteobacteria and Betaproteobacteria.
- Alphaproteobacteria is the first class of Proteobacteria having a distinctive feature of being oligotrophic. Examples include *Caulobacter* and *Pelagibacter* (smallest genome among free-living organisms).
- Mycoplasma belonging to phylum Firmicutes is also one of the smallest genome organisms, but they live as obligate intracellular parasites.
- A majority of the species belonging to Alphaproteobacteria are anoxic phototrophs which are commonly known as purple non-sulfur photosynthetic bacteria mainly belonging to the genera *Rhodobacter* and *Rhodospirillum*, and this class also includes C1 compound-metabolizing bacteria like *Methylobacterium* species; nitrogen fixers like *Rhizobium* and *Bradyrhizobium*; nitrifying bacteria like *Nitrobacter*; and animal and human pathogens like *Brucella*, *Bartonella* and *Rickettsia* species which cause a variety of serious infections.
- Class Betaproteobacteria comprises copiotrophs, organisms with great nutritional requirements, and includes obligate chemolithotrophic ammonia oxidizers *Nitrosomonas* and *Nitrosospira*, phototrophic purple non-sulfur bacteria *Rhodocyclus*, pathogens causing life-threatening infections like *Neisseria*, facultative methylotrophs like *Methylophilus* and polyhydroxybutyrate (PHB) producers like *Ralstonia.*
- Gammaproteobacteria are the largest and physiologically most diverse class of Proteobacteria with over 1500 characterized species and include enteric bacteria, the pseudomonads, the comma-shaped vibrios, *Shewanella* species and pathogens like *Legionella* and *Coxiella.*
- *Buchnera aphidicola* is an endosymbiont of insects with a very small genome, while *Thiomargarita namibiensis,* largest bacterium reported so far, also belong to Gammaproteobacteria.
- Class Deltaproteobacteria includes microbial electrogenerator *Geobacter sulfurreducens,* the first organism which can oxidize organic compounds and metals including iron, radioactive metals and petroleum compounds into environmentally benign carbon dioxide, while the electrons generated in the process can be passed via pili as nanowires grown between the species. It also includes myxobacteria that exhibit gliding motility and form fruiting bodies during nutrient scarcity and the parasitic bacterium *Bdellovibrio* that attacks other bacteria.
- Class Epsilonproteobacteria include pathogenic *Campylobacter* and *Helicobacter* causing gastroenteritis and peptic ulcers, respectively, in humans**.**
- Zetaproteobacteria includes only *Mariprofundus ferrooxydans* which is an iron-oxidizing neutrophilic chemolithoautotroph.
- Phylum Cyanobacteria, belonging to non-proteobacterial gram-negatives, are essentially aerobic and carry out oxygenic photosynthesis. These were the first oxygen-evolving phototrophic organisms on earth.

- Phylum Chlorobi includes anoxygenic photosynthetic green sulfur bacteria, and the key genus is *Chlorobium.*
- Phylum Chloroflexi includes anoxygenic photosynthetic green non-sulfur bacteria, and the representative genus is *Chloroflexus* that can carry out anoxygenic photosynthesis
- Phylum Bacteroidetes makes up the largest portion of human gut microbiota and includes non-proteobacterial gram-negative bacteria that ferment complex polysaccharides and produce short-chain fatty acids.
- Phylum Chlamydiae includes obligate intracellular parasites that rely on their hosts for most metabolites. Also called energy parasites as they are incapable of synthesizing ATP.
- Members of the phylum Spirochaetes have cork screw-shaped morphology with flexible motility and include *Borrelia, Leptospira, Spirochaeta, Treponema* that cause a variety of human diseases. *Leptospira* species cause leptospirosis, *Treponema pallidum* causes syphilis, and *Borrelia burgdorferi* causes Lyme disease.
- Planctomycetes are a group of morphologically unique bacteria as their cell walls lack peptidoglycan and are of an S-layer type, and they have membrane-bound internal compartments including anammoxosome, analogous to eukaryotic mitochondrion, where anammox reaction that is anaerobic ammonia oxidation takes place.
- Phylum Nitrospirae includes the key genus *Nitrospira* which is a chemolithotroph which carries out commamox reaction, i.e., direct oxidation of ammonia to nitrate.
- The low G + C (45–60% GC content) gram-positive Firmicutes constitute the lactic acid bacteria, the spore formers like *Bacillus* species and clostridia, the non-spore formers like *Staphylococcus, Listeria, Lactobacillus* and *Sarcina*, photosynthetic bacteria *Heliobacterium* and *Heliophilum* that have an unusual pigment bacteriochlorophyll *g*, Negativicutes and the cell-wall-less bacteria.
- *Bacillus* species produce a number biotechnologically important enzymes and antibiotics such as polymyxin and gramicidin.
- Members of the genus *Clostridium* are human pathogens such as *C. botulinum and C. tetani* causing botulism and tetanus, respectively.
- Gram-positive bacteria with an outer membrane are called Negativicutes. An example is *Negativicoccus succinivorans.*
- The smallest independently replicating prokaryotes, distinguished by the lack of a cell wall, are called Tenericutes/Mollicutes (soft skinned). They are commonly known as mycoplasmas.
- Gram-positive bacteria with high G + C content above 50% are classified under the phylum Actinobacteria. This group includes commensals like *Propionibacterium, Micrococcus, Bifidobacterium, Gardnerella* and *Nocardia*; pathogens such as *Mycobacterium tuberculosis, Mycobacterium leprae* and *Corynebacterium diphtheria*; antibiotic producers *Streptomyces* species; and industrially important *Propionibacterium shermanii* and *Corynebacterium glutamicum.*

Questions

1 Give example/s for the following:

i. World's toughest bacterium
ii. Most abundant species on earth
iii. Organism with square-shaped cells
iv. An aerobic anoxic photoheterotroph
v. Pink-pigmented facultative methylotroph
vi. The first complete genome of which archaeon was reported?
vii. Archaeal species able to withstand temperatures as high as 122 °C
viii. The cell-wall-less archaea: modern descendants that gave rise to eukaryotes
ix. Organism known as rushing fireball
x. Organism known as hospital fireball
xi. The oldest deeply branching bacteria
xii. Example of an oligotroph
xiii. Polyhydroxybutyrates-producing bacteria
xiv. Bacteria which can carry out direct oxidation of ammonia to nitrate
xv. Example of microbial nanowire
xvi. The bacteria parasitic on other bacteria
xvii. Causative agent of Lyme disease
xviii. Bacteria with intracellular compartmentalization.

2. Why are Proteobacteria named so?
3. What are Negativicutes?
4. Explain life cycle of Chlamydiae.
5. What is bacteriorhodopsin?
6. What is the main difference between cyanobacteria and other photosynthetic bacteria?
7. Why planctomycete cell is considered to be close to eukaryotic cell?

References

Barns, S. M., Delwiche, C. F., Palmer, J. D., & Pace, N. R. (1996). Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proceedings of the National Academy of Sciences, 93*(17), 9188–9193.

Brochier-Armanet, C., Boussau, B., Gribaldo, S., & Forterre, P. (2008). Mesophilic Crenarchaeota: Proposal for a third archaeal phylum, the Thaumarchaeota. *Nature Reviews Microbiology, 6*(3), 245.

Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J.-F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., … Venter, J. C. (1996). Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii. Science, 273*(5278), 1058–1073.

Cao, B., Nagarajan, K., & Loh, K. C. (2009). Biodegradation of aromatic compounds: Current status and opportunities for biomolecular approaches. *Applied Microbiology and Biotechnology, 85*(2), 207–228.

Cavicchioli, R. (2007). Archaea. *Molecular and cellular biology*. ASM Press, Washington, DC.

Darland, G., Brock, T. D., Samsonoff, W., & Conti, S. F. (1970). A thermophilic, acidophilic mycoplasma isolated from a coal refuse pile. *Science, 170*(3965), 1416–1418.

Emerson, D., Rentz, J. A., Lilburn, T. G., Davis, R. E., Aldrich, H., Chan, C., & Moyer, C. L. (2007). A novel lineage of proteobacteria involved in formation of marine Fe-oxidizing microbial mat communities. *PLoS ONE, 2*(8), e667.

Fuerst, J. A., & Sagulenko, E. (2011). Beyond the bacterium: Planctomycetes challenge our concepts of microbial structure and function. *Nature Reviews Microbiology, 9*(6), 403.

Fütterer, O., Angelov, A., Liesegang, H., Gottschalk, G., Schleper, C., Schepers, B., Dock, C., Antranikian, G., Liebl, W. (2004). Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. *Proceedings of the National Academy of Sciences, 101*(24), 9091–9096.

Giovannoni, S. J., Tripp, H. J., Givan, S., Podar, M., Vergin, K. L., Baptista, D., Bibbs, L., Eads, J., Richardson, T. H., Noordewier, M., Rappé, M. S., Short, J. M., Carrington, J. C., Mathur, E. J. (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science, 309*(5738), 1242–1245.

Gross, L. (2007). Paradox resolved? The strange case of the radiation-resistant bacteria. *PLoS Biology, 5*(4), e108.

Hafenbradl, D., Keller, M., Dirmeier, R., Rachel, R., Roßnagel, P., Burggraf, S., Huber, H., & Stetter, K. O. (1996). Ferroglobus placidus gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe2+ at neutral pH under anoxic conditions. *Archives of Microbiology, 166*(5), 308–314.

Huber, R., Wilharm, T., Huber, D., Trincone, A., Burggraf, S., König, H., Reinhard, R., Rockinger, I., Fricke, H., Stetter, K. O. (1992). *Aquifex pyrophilus* gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. *Systematic and Applied Microbiology, 15*(3), 340–351.

Huber, H., Burggraf, S., Mayer, T., Wyschkony, I., Rachel, R., & Stetter, K. O. (2000). *Ignicoccus* gen. nov., a novel genus of hyperthermophilic, chemolithoautotrophic Archaea, represented by two new species, *Ignicoccus islandicus* sp nov and *Ignicoccus pacificus* sp nov. and *Ignicoccus pacificus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 50*(6), 2093–2100.

Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C., & Stetter, K. O. (2002). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature, 417*(6884), 63.

Krulwich, T. A., & Pate, J. L. (1971). Ultrastructural explanation for snapping postfission movements in *Arthrobacter crystallopoietes*. *Journal of Bacteriology, 105*(1), 408–412.

Lee, K. C., Webb, R. I., Janssen, P. H., Sangwan, P., Romeo, T., Staley, J. T., & Fuerst, J. A. (2009). Phylum Verrucomicrobia representatives share a compartmentalized cell plan with members of bacterial phylum Planctomycetes. *BMC Microbiology, 9*(1), 5.

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Microbial ecology: Human gut microbes associated with obesity. *Nature, 444*(7122), 1022.

Ludwig, W., Schleifer, K. H., & Whitman, W. B. (2009). Revised road map to the phylum Firmicutes. In *Bergey's Manual of Systematic Bacteriology* (pp. 1–13). Springer, New York, NY.

Marchandin, H., Teyssier, C., Campos, J., Jean-Pierre, H., Roger, F., Gay, B., Carlier, J. P., & Jumas-Bilak, E. (2010). *Negativicoccus succinicivorans* gen. nov., sp. nov., isolated from human clinical samples, emended description of the family Veillonellaceae and description of Negativicutes classis nov., Selenomonadales ord. nov. and Acidaminococcaceae fam. nov. in the bacterial phylum Firmicutes. *International journal of systematic and evolutionary microbiology*, *60*(6), 1271–1279.

Méndez-García, C., Peláez, A. I., Mesa, V., Sánchez, J., Golyshina, O. V., & Ferrer, M. (2015). Microbial diversity and metabolic networks in acid mine drainage habitats. *Frontiers in Microbiology, 6,* 475.

Metwally, M. A., Yassin, A. S., Essam, T. M., Hamouda, H. M., & Amin, M. A. (2014). Detection, characterization, and molecular typing of human Mycoplasma spp. from major hospitals in Cairo, Egypt. *The Scientific World Journal*, *2014*.

Moore, R. L., & Hirsch, P. (1973). First generation synchrony of isolated Hyphomicrobium swarmer populations. *Journal of Bacteriology, 116*(1), 418–423.

Näther, D. J., Rachel, R., Wanner, G., & Wirth, R. (2006). Flagella of *Pyrococcus furiosus*: Multifunctional organelles, made for swimming, adhesion to various surfaces, and cell-cell contacts. *Journal of Bacteriology, 188*(19), 6915–6923.

Nishida, H., Beppu, T., & Ueda, K. (2011). Whole-genome comparison clarifies close phylogenetic relationships between the phyla Dictyoglomi and Thermotogae. *Genomics, 98*(5), 370–375.

Nunes, A., & Gomes, J. P. (2014). Evolution, phylogeny, and molecular epidemiology of Chlamydia. *Infection, Genetics and Evolution, 23,* 49–64.

Nunoura, T., Takaki, Y., Kakuta, J., Nishi, S., Sugahara, J., Kazama, H., Chee, G.-J., Hattori, M., Kanai, A., Atomi, H., Takai, K., Takami, H. (2011). Insights into the evolution of Archaea and eukaryotic protein modifier systems revealed by the genome of a novel archaeal group. *Nucleic acids research, 39*(8), 3204–3223.

Oren, A. (2008). Microbial life at high salt concentrations: Phylogenetic and metabolic diversity. *Saline Systems, 4*(1), 2.

Rachel, R., Wyschkony, I., Riehl, S., & Huber, H. (2002). The ultrastructure of *Ignicoccus*: Evidence for a novel outer membrane and for intracellular vesicle budding in an archaeon. *Archaea, 1*(1), 9–18.

Rajilić-Stojanović, M., & de Vos, W. M. (2014). The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiology Reviews, 38*(5), 996–1047.

Saxon, E. B., Jackson, R. W., Bhumbra, S., Smith, T., & Sockett, R. E. (2014). Bdellovibrio bacteriovorus HD100 guards against *Pseudomonas tolaasii* brown-blotch lesions on the surface of post-harvest Agaricus bisporus supermarket mushrooms. *BMC Microbiology, 14*(1), 163.

She, Q., Singh, R.K., Confalonieri, F., Zivanovic, Y., Allard, G., Awayez, M.J., Christina, C. Y., Clausen, I. G., Curtis, B. A., De Moors, A., Erauso, G., Fletcher, C., Gordon, P. M. K., Heikamp-de Jong, I., Jeffries, A. C., Kozera, C. J., Medina, N., Peng, X., Thi-Ngoc, H. P., … Van der Oost, J. (2001). The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. *Proceedings of the National Academy of Sciences, 98*(14), 7835–7840.

Singer, E., Emerson, D., Webb, E. A., Barco, R. A., Kuenen, J. G., Nelson, W. C., Chan, C. S., Comolli, L. R., Ferriera, S., Johnson, J., Heidelberg, J. F., & Edwards, K. J. (2011). *Mariprofundus ferrooxydans* PV-1 the first genome of a marine Fe (II) oxidizing Zetaproteobacterium. *PloS One, 6*(9), e25386.

Spang, A., Saw, J. H., Jørgensen, S. L., Zaremba-Niedzwiedzka, K., Martijn, J., Lind, A. E., van Eijk, R., Schleper, C., Guy, L., & Ettema, T. J. (2015). Complex archaea that bridge the gap between prokaryotes and eukaryotes. *Nature, 521*(7551), 173.

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Hirayama, H., Nakagawa, S., Nunoura, T., & Horikoshi, K. (2008). Cell proliferation at 122 °C and isotopically heavy CH_4 production by a hyperthermophilic methanogen under high-pressure cultivation. In: *Proceedings of the National Academy of Sciences, 105*(31), 10949–10954.

Van Ham, R. C., Kamerbeek, J., Palacios, C., Rausell, C., Abascal, F., Bastolla, U., Fernández, J. M., Jiménez, L., Postigo, M., Silva, F. J., Tamames, J., Viguera, E., Latorre, A., Valencia, A., Morán, F., & Moya, A. (2003). Reductive genome evolution in *Buchnera aphidicola. Proceedings of the National Academy of Sciences, 100*(2), 581–586.

Voet, D., & Voet, J. G. (2004). Biochemistry. Hoboken. *John Wiley & Sons, 1*, 591.

Walker, C. B., De La Torre, J. R., Klotz, M. G., Urakawa, H., Pinel, N., Arp, D. J., Brochier-Armanet, C., Chain, P. S. G., Chan, P. P., Gollabgir, A., Hemp, J., Hügler, M., Karr, E. A., Könneke, M., Shin, M., Lawton, T. J., Lowe, T., Martens-Habbena, W., Sayavedra-Soto, L. A., … & Stahl, D. A. (2010). *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proceedings of the National Academy of Sciences, 107*(19), 8818–8823.

Whittaker, R. H. (1959). On the broad classification of organisms. *The Quarterly Review of Biology, 34*(3), 210–226.

Whitton, B. A. (Ed.). (2012). *Ecology of cyanobacteria II: Their diversity in space and time*. Springer Science & Business Media.

Woese, C. R., & Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Sciences, 74*(11), 5088–5090.

Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences, 87*(12), 4576–4579.

Yurkov, V. V., & Beatty, J. T. (1998). Aerobic anoxygenic phototrophic bacteria. *Microbiology and Molecular Biology Reviews, 62*(3), 695–724.

Chapter 2
Prokaryotic Cell Structure and Function

Rani Gupta and Namita Gupta

1 Morphology of Bacterial Cell

The average size of a bacterial cell ranges from as small as 0.3 μm in diameter for *Mycoplasma genitalium* to 100–300 μm in diameter for *Thiomargarita namibiensis* which can grow as large as 750 μm in diameter and visible to the naked eye. Another large bacterium *Epulopiscium fishelsoni* is about 600 μm in length and 80 μm in diameter. The average size of most bacteria is small; they increase their surface area-to-volume ratio which helps in quick absorption of nutrients from the environment and waste products are also excreted in a similar manner. The smallest archaea known till date is *Nanoarchaeum equitans* which is about 0.4 μm in diameter living parasitically on another archaeal host *Ignicoccus hospitalis*.

Three distinct types of bacterial shapes recognized are cocci, rods and spirals which can cluster themselves in different arrangements. Coccus (singular) or cocci are spherical cells whose average diameter is 0.5–2 μm. They can occur in several arrangements characteristic of a particular species, e.g., Diplococci which exist in pairs; *Micrococcus* forming tetrad arrangement of four cells; long cocci chains occur in case of *Streptococcus*, *Enterococcus* and *Lactococcus*; irregular patterns of grape-like bunches as observed in case of *Staphylococcus* and regular cuboidal arrangement of cells for *Sarcina*. In case of rods, which are cylindrical in shape, the average length is 1–10 μm and 0.25–1 μm diameter. Rods, also termed as bacillus (singular) or bacilli, often exist singly or in pairs (diplobacilli). In some cases, they occur in chains, e.g., *Bacillus subtilis*, *Bacillus megaterium* (long chains). Species like *Corynebacterium diptheriae* show palisade arrangement of cells in which long bacilli rods are aligned side by side like matchsticks. Further, some long rods twist to form rigid spiral shapes called Spirilla. Spirochetes are also spiral in shape, but they are flexible in movement. Some species show unusual morphology like comma-shaped vibrios, star-shaped genus *Stella* and square-shaped *Haloquardatum walsbyi*. Apart from this, some cells are pleomorphic that do not exhibit any characteristic shape and show variable shapes when grown in pure cultures, e.g., *Mycoplasma* and *Corynebacterium* (Fig. 1).

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_2

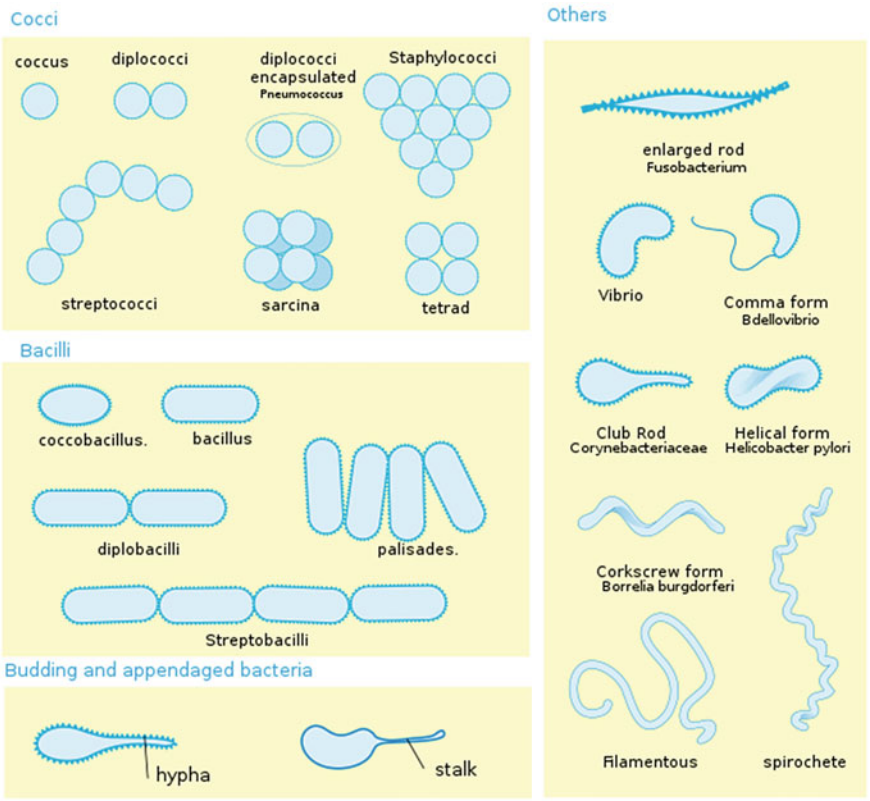


Fig. 1 Different types of bacterial cell shapes and arrangements. *Source* Wikipedia. (Public Domain)

2 Cell Organization

A typical bacterial cell is divided into three main parts: the cell envelope, the cytoplasm and the extracellular appendages (Fig. 2). The cell envelope maintains the integrity of the cell and mainly comprises innermost plasma membrane, cell wall, periplasmic space between plasma membrane and cell wall and the outer most layers surrounding the cell wall. The cytoplasm contains nucleoid as genetic material, ribosomes as protein synthesis machinery and inclusion bodies dispersed all over the cytoplasmic space. The extracellular appendages like fimbriae, pili and flagella serve multiple functions and help cells in conjugation, attachment and locomotion. In addition to the main components, bacterial cells also form endospores which are dormant structures that help bacteria to survive in adverse environmental conditions.

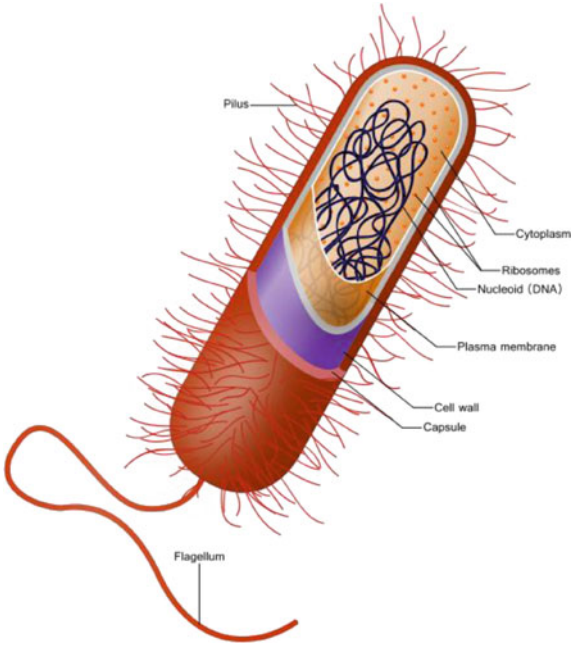


Fig. 2 Structure of a typical bacterial cell. *Source* Wikimedia Commons (Creative Commons Attribution license)

3 Bacterial Membranes

Plasma membrane or cytoplasmic membrane is the most important layer of the cell envelope as it surrounds the cytoplasm and defines the cell shape. No cell can survive without a plasma membrane as it is the essence of living state. It is very thin with a thickness of around 8–10 nm and appears as two dark lines separated by a light line when seen under electron microscope (Willey et al., 2008).

Functions of plasma membrane

(1) Membrane maintains the integrity of the cell due to its selective permeability which acts as barrier allowing selective diffusion or transport of solutes and nutrients across the cell and also waste excretion outside the cell.

(2) The second major function is that the membrane serves as a site for energy conservation and generates proton motive force to carry out various metabolic processes pivotal to cell like ATP biosynthesis, motility and transport of solutes and ions in and out of the cell (Depelteau et al., 2019).

(3) Photosynthesis, lipid biosynthesis and synthesis of cell wall constituents also take place in the plasma membrane. For photosynthesis, certain pigments and enzymes are present in infoldings of the membrane extending into the cytoplasm, and these extensions are known as chromatophores. Other important

cellular processes like DNA replication, cell division, protein trafficking and secretion are also carried out by several membrane-associated proteins.

Structure of plasma membrane: Singer and Nicolson's Fluid Mosaic Model

The structure of plasma membrane is well explained as the Fluid Mosaic Model proposed by Singer and Nicolson. It is based on the fact that bilayered phospholipids provide fluidity to the membrane and proteins embedded in the membrane (Singer & Nicolson, 1972). The phospholipids are amphipathic in nature with a variable polar head, glycerol-3-phosphate moiety and two non-polar tails made up of fatty acids (usually C14-C20) linked together by an ester bond. The glycerol moiety with one saturated fatty acid chain at C-1 position, one monounsaturated fatty acid chain at C-2 position and a phosphate group attached to it at C-3 position is constitutively called as phosphatidic acid. Different bacteria have variable polar chemical head groups attached to phosphate of phosphatidic acid via phosphodiester bond to form different phospholipid molecules present in the membrane as depicted in Fig. 3. Out of all, three major kinds of phospholipids found in *E. coli* are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin having ethanolamine, glycerol and phosphatidylglycerol as polar head groups (Raetz & Dowhan, 1990; Dowhan, 1997).

The polar end of the membrane bilayer faces the cytoplasm and the external environment and being hydrophilic interacts with water. The non-polar tails being hydrophobic face each other in the bilayer and make the hydrophobic core (Fig. 4). The protein content of the membrane is quite high and two types of proteins can be found which interact with each other during crucial cellular processes: **Integral membrane proteins** which are firmly embedded in the phospholipid bilayer and **peripheral membrane proteins** which are associated with the membrane surface from outside or inside through interaction with integral proteins (Fig. 5).

Membrane fluidity

The fluidity of the membrane is essential for lateral movement of proteins and is mainly maintained by the number of saturated and unsaturated fatty acids present in

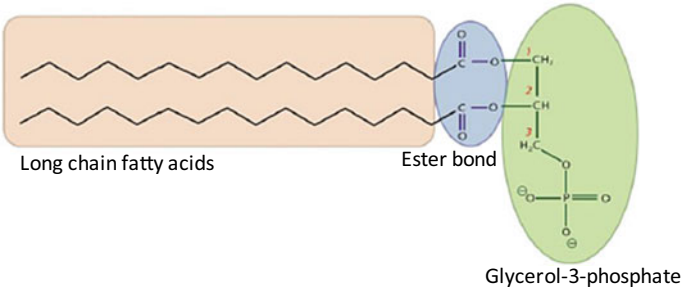


Fig. 3 Structure of phosphatidic acid. *Source* Caforio and Driessen (2017). With kind permission from Elsevier

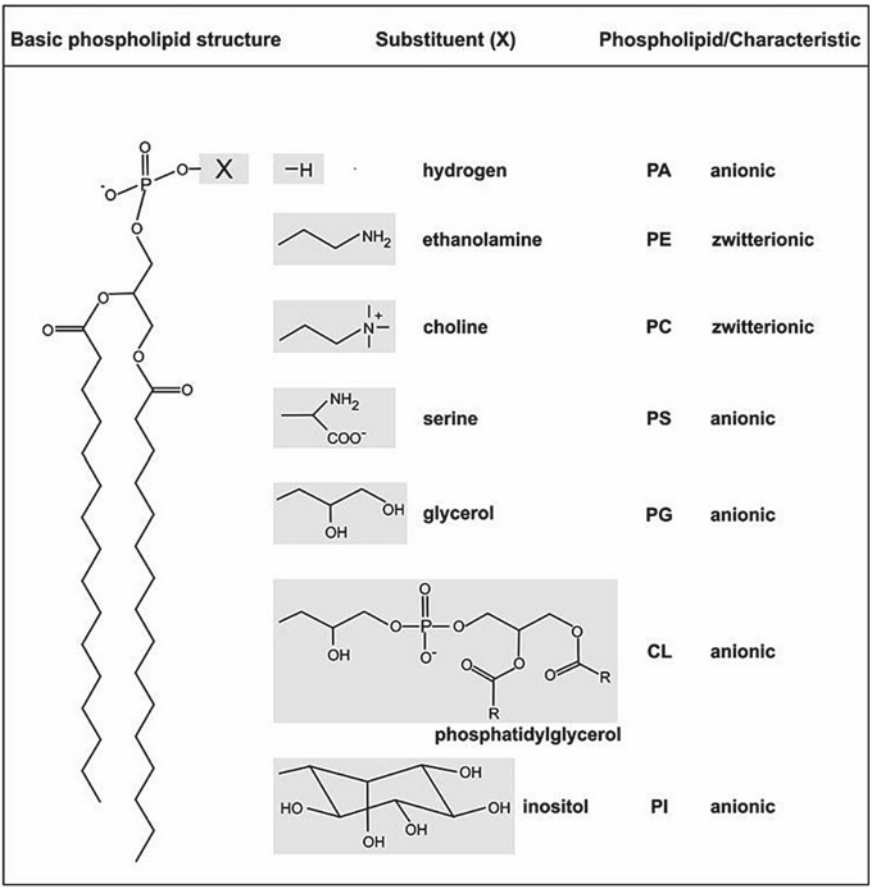


Fig. 4 Common head groups found in phospholipids of bacterial membranes. PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol. *Source* Aktas et al. (2014). (Creative Commons Attribution license)

the bilayer which is largely modulated by environmental conditions specially temperatures. Thermophiles which grow at high temperatures have higher ratio of saturated fatty acids to unsaturated fatty acid content while reverse is true for psychrophiles. In psychrophiles, besides unsaturation, other low-temperature-induced changes in bacterial membranes are the presence of short-chain and branched fatty acids instead of long- and straight-chain fatty acids. Some cold-adapted microbes also contain polyunsaturated fatty acids that remain flexible even at very cold temperatures (Sohlenkamp & Geiger, 2016).

In addition, many bacterial membranes are further stabilized due to the presence of sterol-like molecules called hopanoids which have pentacyclic structure (Fig. 6). The hopanoids are similar to eukaryotic sterols such as cholesterol and modify plasma

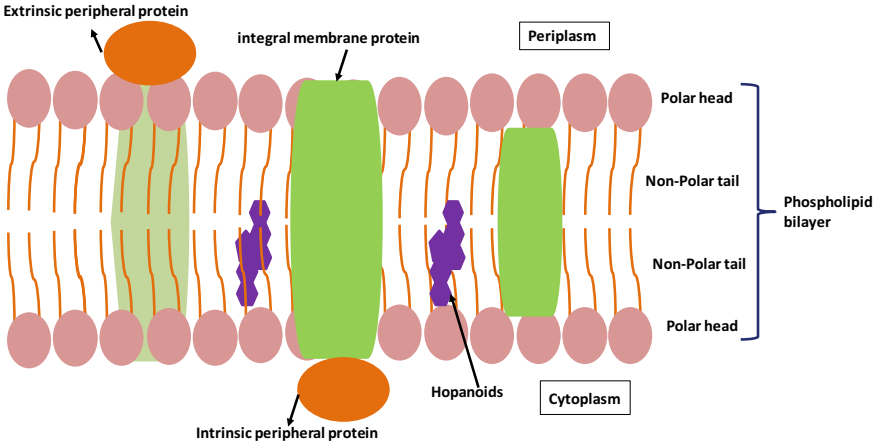


Fig. 5 Structure of bacterial plasma membrane

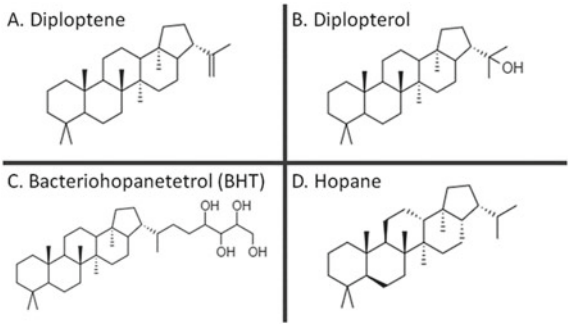


Fig. 6 The basic structure of hopanoid present in bacterial cell membranes. *Source*Wikipedia (Creative Commons Attribution License)

membrane properties influencing membrane permeability for their survival during environmental stress. In *Streptomyces*, hopanoids are formed in the aerial hyphae to minimize loss of water across the membrane (Poralla et al., 2000). In *Frankia*, due to the presence oxygen-sensitive nitrogenase enzyme, hopanoids make the lipid bilayer tightly packed to restrict the entry of oxygen (Berry et al., 1993).

Archaeal membranes

Archaeal cells are also surrounded by plasma membrane; however, the composition of the lipids varies greatly from that of bacteria. They lack fatty acids and instead contain multiple copies of a 5-carbon branched hydrocarbon molecule called isoprene unit which is highly methylated, cyclic and unsaturated and form isoprenoid chains of variable lengths (Fig. 7). The methyl groups, saturation/desaturation ratio and cyclic structures are responsible for maintaining the fluidity and permeability of the archaeal

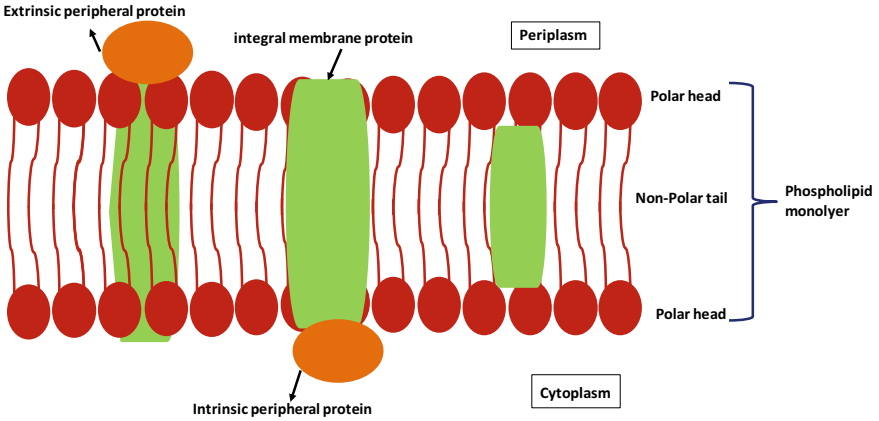


Fig. 7 Structure of archaeal tetraether phospholipid monolayer

membranes. These are linked to glycerol through glycerol-1-phosphate moieties via ether linkages rather than ester linkages (Fig. 8) as ether linkages are more resistant to chemical and heat treatment and can withstand extremes of conditions in which archaea survive. The glycerol molecules may be linked to a phosphate group similar to bacteria or to a carbohydrate group (Shimada et al., 2008).

The arrangement of lipids is very diverse in archaea and can be classified into two major types:

1. **Glycerol diether lipids**: These have a phytanyl group consisting of 5 isoprene units (20-C side chain) attached to glycerol molecules through ether linkage and form a bilayered structure. The 20-C diether is commonly termed as archaeol. Various other modifications in the basic diether structure are also found in various archaeal species as shown as in Fig. 9a. For example, the halophilic and some thermophilic archaea have macrocyclic glycerol diethers or tetritol

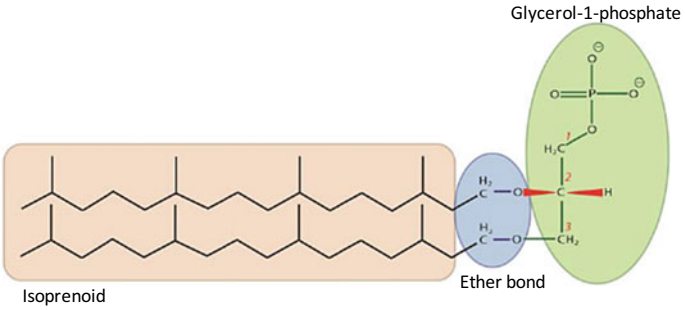


Fig. 8 Phospholipid moiety of archaeal membrane. *Source* Caforio and Driessen (2017). With kind permission from Elsevier

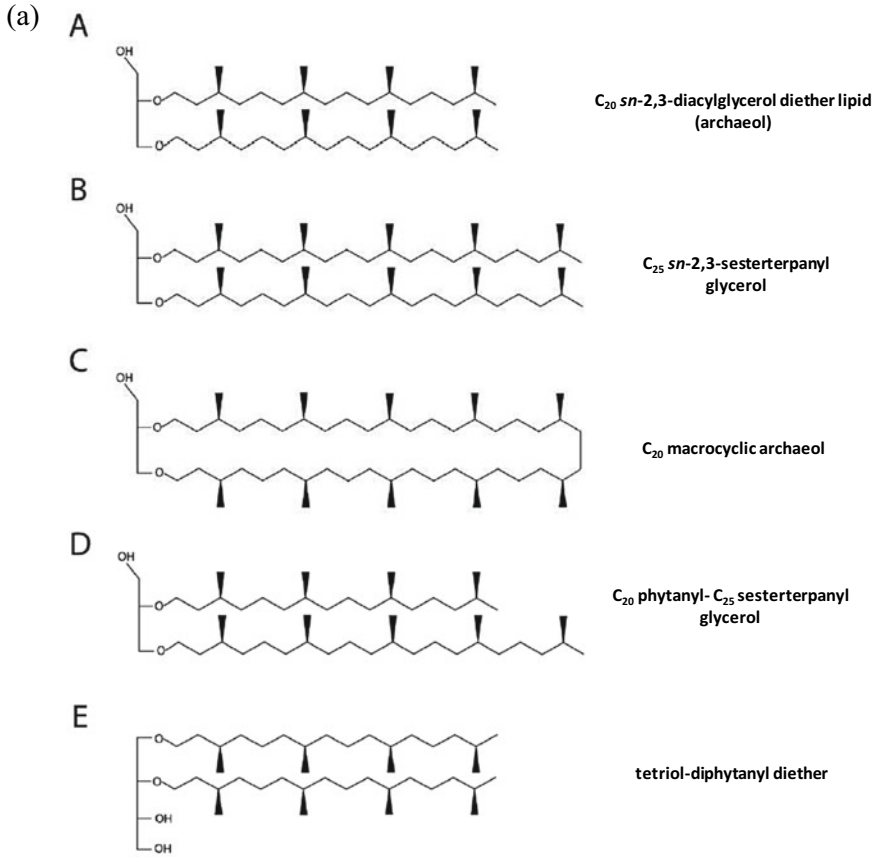


Fig. 9 Modifications of archaeal **a** diether and tetraether **b** lipids and their structures. *Source* Caforio and Driessen (2017). With kind permission from Elsevier

diethers in their lipids to cope up with extreme salinity and temperatures (Comita et al., 1984; De Rosa et al., 1986).

2. **Diglycerol tetraether lipids**: These have two glycerol residues linked by two phytanyl groups (40-C side chain). The 40-C diglycerol tetraether is commonly termed as caldarchaeol as it was the first tetraether lipid to be characterized (Langworthy, 1977). The ends of the phytanyl side chain pointing inward from each glycerol molecule are covalently linked thus making this a monolayer structure and termed as the H-shaped caldarchaeol (Koga & Morii, 2005). In *Sulfolobus*, these tetraethers cyclize in the form of pentacyclic rings which may be as many as eight in numbers. These ringed lipids are called crenarchaeol. It is more rigid than the lipid bilayer. Sulfolobales also contain nonitol tetraether and calditol tetraethers. (Fig. 9b; Untersteller et al., 1999).

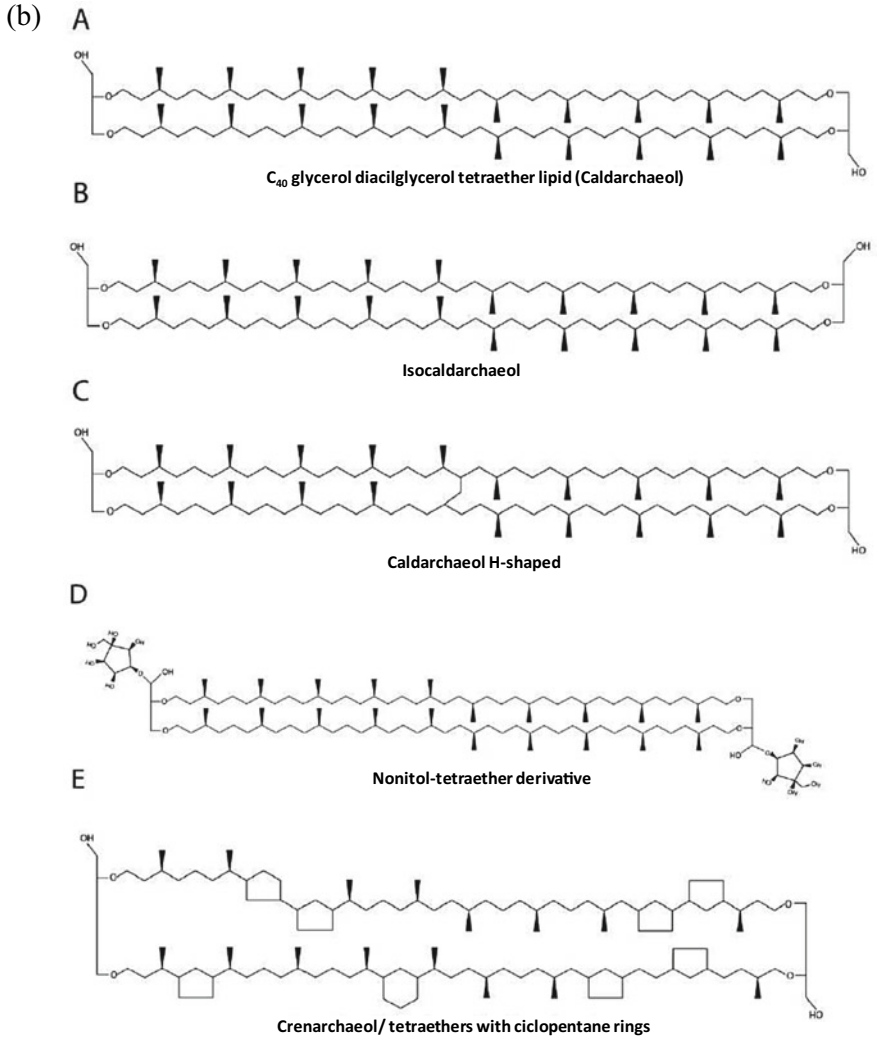


Fig. 9 (continued)

Diversity of membrane rigidity requirements is related to the diverse habitat that archaea live in and changes are necessary to maintain fluidity and stability. Lipid monolayers are widely distributed among hyperthermophilic archaea as they are more rigid and resistant to heat than the lipid bilayers. Typical example of archaea having tetraether lipid monolayers and cyclopentane rings is *Thermoplasma,* an acido-thermophile without a cell wall. Presence of such lipids leads to tight membrane packing and reduces the permeability of membrane toward protons at elevated temperatures (Shimada et al., 2008). The major differences between bacterial and archaeal membranes have been listed in Table 1.

Table 1 Differences between bacterial and archaeal membranes

Bacterial membrane	Archaeal membrane
Membrane thickness is 8–10 nm	Membrane thickness is 4–5 nm
Contains 14-C to 20-C linear fatty acid chains	Contains 20C/40C branched isoprenoid hydrocarbon chains
Glycerol linked to phosphates at C3 position	Glycerol linked to phosphates or sugars or sulfates at C1 position
C1 and C2 position occupied by ester linked fatty acids	C2 and C3 position occupied by ether linked fatty acids
Form only bilayered membrane structures	Form both bilayered and monolayered membrane structures
No cyclic structures	Form Pentacyclic isoprene structures to maintain hydrocarbon chain length
Hopanoids present to stabilize the plasma membrane	Hopanoids absent

Box 1: Archaeosomes Mediated Drug Encapsulation and Delivery

Archaeosomes constitute a novel family of liposomes wherein ether lipids such as archaeol (diether) and caldarchaeol (tetraether), the characteristic feature of archaea, are used for membrane preparation. These are desirable since they can withstand harsh conditions such as high temperature and low pH and can easily be autoclaved. However, they find limited application at present as they mediate drug delivery at a much slower rate than the conventional liposomes. But they are considered perspective candidates for developing drug delivery systems (Kaur et al., 2016).

4 Cell Wall

The bacterial cells are surrounded by a complex cell wall made up of sugars and amino acid polymers known as **peptidoglycan or murein**. Cell wall is a rigid structure which prevents bursting of the cell due to osmotic pressure. Bacterial cell wall also plays various other vital functions such as providing attachment sites for bacteriophages, consists of antigenic determinants, plays important role in cell septation during division and provides a rigid platform for extracellular appendages like flagella, fimbriae and pili.

Based on differences in thickness and composition of their cell wall, bacteria can be distinguished into two broad categories as **gram-positive and gram-negative bacteria**. This grouping is based on differential staining technique (**Gram's staining)** developed by **Hans Christian Gram**. In this technique, basic dye crystal violet along

with iodine as mordant is retained only in gram-positive bacteria due to thick cell wall while in gram-negatives, it is lost upon solvent washing as the cell wall in this group is not only thinner but also rich in lipids. Upon counter staining with safranin, gram positives appear blue, while gram-negatives stain pink. This classification was confirmed by sophisticated methods of electron microscopy where iodine was replaced by an electron-opaque marker, potassium trichloro(Q2-ethylene)-platinum (II), in order to visualize and follow cellular perturbations throughout the staining process (Beveridge & Davies, 1983).

Composition of Gram-positive and Gram-negative cell wall

The peptidoglycan layer of gram-positive bacteria is relatively thick (around 20–80 nm) and continuous; and accounts for 50–90% of the dry weight of the cell wall. In addition to peptidoglycan, gram-positive cell wall is rich in characteristic phosphorylated polyols termed as teichoic acids which are linked to both peptidoglycan and to cell membrane. Teichoic acid which is linked to cell wall via sugar moieties is termed as 'wall teichoic acid' and to cell membrane via lipids is termed as 'lipoteichoic acid'.

On the other hand, peptidoglycan layer of gram-negative bacteria is thinner with very few layers of around 5–10 nm and comprises only 10% of the cell wall. Major part of a gram-negative cell wall (or cell envelope) is contributed by an additional plasma membrane which is located outside the peptidoglycan layer and is termed outer membrane. The structural organization of the outer membrane is similar to that of the cytoplasmic membrane (about 7.5 nm thick) and may provide the additional support required to withstand the turgor pressure from within the cell. At certain regions, it may adhere to inner membrane called **Bayer patches** which in turn breaks the continuity of the peptidoglycan layer (discontinuous). Further, in gram-negative bacteria, lipoproteins (also known as Braun's lipoproteins) are covalently and non-covalently attached to the peptidoglycan layer and outer membrane, respectively. Moreover, lipopolysaccharides (LPS) are also present in gram-negative cell envelope as the outer most layer. LPS is a complex lipopolysaccharide having three distinct components, inner lipid A, core polysaccharide and outermost O-antigen (Table 2 and Fig. 10).

Protoplast, Sphaeroplast and L forms

Wall-less bacterial cells generated on treatment with either cell wall hydrolyzing enzyme lysozyme or in presence of cell wall synthesis inhibitor is called protoplast in gram-positive bacteria and sphaeroplast in case of gram-negatives since it has outer membrane also. These wall-less forms generally lyse due to osmotic stress; however, few can still survive and regenerate and these are specifically termed as 'L forms.'

Periplasm

The periplasmic space is the region between the outer and inner membranes of gram-negative bacteria. Periplasm is the site for protein folding, oxidation and transport. It also houses several hydrolytic enzymes, proteins that specifically bind

Table 2 Differences between gram-positive and gram-negative cell wall

Gram-positive cell wall	Gram-negative cell wall
Peptidoglycan layer is thicker (20–80 nm)	Peptidoglycan layer is thin (5–10 nm)
Peptidoglycan constitutes 50–90% of the dry weight of the cell wall	Peptidoglycan constitutes only 10% of the dry weight of the cell wall
Do not have outer membrane	Outer membrane constitutes the major part
No periplasmic space	Periplasmic space present
Other macromolecules present are teichoic acid; wall techoic acid and lipoteichoic acid	Other macromolecules present are lipoproteins, lipopolysaccharides and porins
Lipid content is comparatively less	High lipid content due to outer membrane
Gram-positive bacteria are called '**mono-derm**' as contain cytoplasmic membrane (exception, Dienococcus and Negitivicutes: gram-positive but also have an outer membrane lacking LPS; gram positive diderm)	Gram-negative bacteria are called '**di-derm**' as they have two membranes, cytoplasmic and an outer membrane

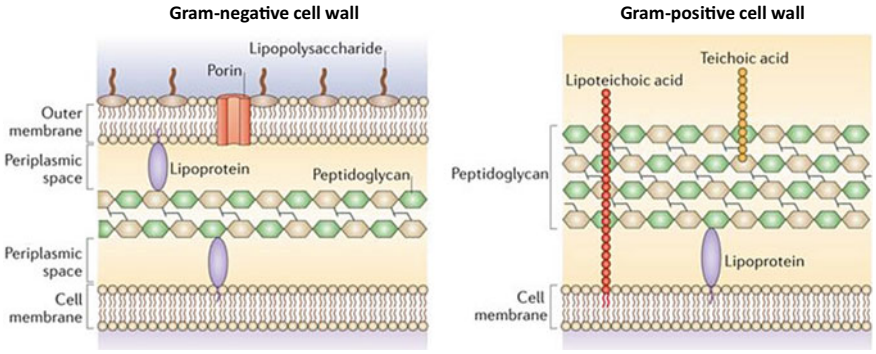


Fig. 10 Structural differences in gram-negative and gram-positive cell walls. *Source* Brown et al. (2015). With kind permission from Springer Nature

sugars, amino acids and inorganic ions. Moreover, β-lactamases and aminoglycoside-modifying enzymes (phosphorylation or adenylation) which are important for antibiotic resistance are present in periplasm (Miller & Salama, 2018).

Archaeal cell wall

The chemical composition of archaeal cell wall is very different from bacteria. They do not possess any outer membrane except in *Ignicoccus*, the only archaeon to possess a double membrane structure. The main polysaccharide present in archaeal cell wall is pseudomurein which differs from polysaccharide murein present in bacterial peptidoglycan in three ways:

1. Archaeal cell wall contains *N*-acetyltalosaminuronic acid in place of *N*-acetylmuramic acid.
2. The disaccharide is formed by β-1,3-glycosidic bonds instead of β1,4-glycosidic bonds as seen in bacteria.
3. The peptidoglycan backbone is cross-linked by *L*-amino acids rather than D-amino acids as in bacteria.

Other polysaccharides such as polymers of glucose, glucuronic acid, galactosamine uronic acid, etc. also form thick cell wall in some archaea such as *Methanosarcina*. Most archaea are also surrounded by a layer of proteins and glycoproteins in place of a cell wall which is called as S-layer. S-layer is also present in bacteria as an external layer to cell wall; however, in archaea S-layer is considered to be cell wall as it surrounds the plasma membrane. On the basis of presence of S-layer and other polysaccharides, the cell wall of archaea can be divided into different types as shown in Table 3 and Fig. 11.

The function of archaeal cell wall is similar to bacteria, and it protects cell from osmotic lysis and also maintains the shape of the cell. Since archaea have pseudopeptidoglycan layer in cell wall containing pseudomurein, they cannot be attacked by

Table 3 Variety of archaeal cell walls

Cell wall type	Characteristic features	Examples
Only S-layer	Made up of proteins and glycoproteins which arrange themselves in various symmetries like hexagon, tetragon and trimers	Methanogens-*Methanolobus* and *Methanococcus* Halophiles-*Halobacterium* Extreme thermophiles-*Sulfolobus*, *Thermoproteus*, *Pyrodictium*, *Picrophilus* etc.
S-layer plus an external protein sheath	A protein sheath is present as outer most covering external to S-layer	*Methanospirillum*
S-layer plus methanochondroitin	Thick layer of methanochondriotin, a polysaccharide present external to S-layer	*Methanosarcina*
Pseudomurein between S-layer and plasma membrane	The outer most S-layer and inner most plasma membrane separated by pseudomurein	*Methanothermus* and *Methanopyrus*
Pseudomurein	No S-layer, only pseudomurein as the outer most covering similar to peptidoglycan layer of gram-positive bacteria	*Halococcus*, *Natrococcus*, *Methanobacterium*, *Methanosphaera* *Methanobrevibacter* etc.
Outer membrane with pore-forming proteins	Outer membrane is present resembling outer membrane of gram-negative bacteria	*Ignicoccus hospitalis*
Cell wall less archaea	No external layer to plasma membrane is present	*Thermoplasma* and *Ferroplasma*

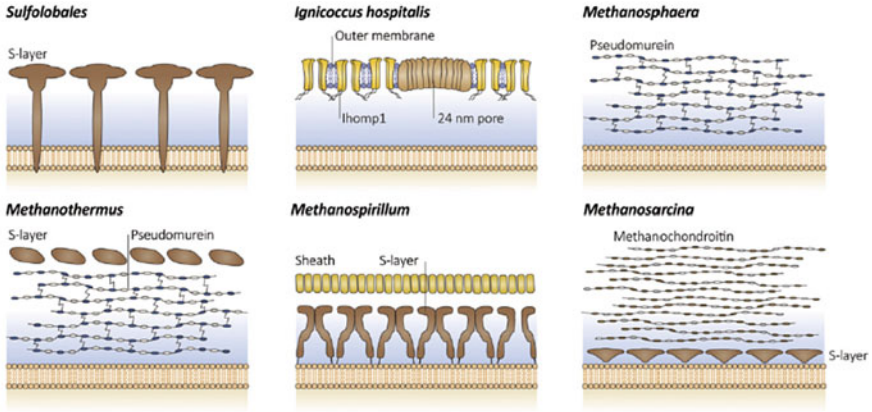


Fig. 11 Schematic representation of different cell walls found among archaeal domain. *Source* Albers and Meyer (2011). With kind permission from Springer Nature

lysozyme and penicillin antibiotic. Some archaea like *Thermoplasma* and *Ferroplasma* are called as 'cell wall-less' archaea as they lack cell wall and are only surrounded by plasma membrane as the outermost covering.

5 Bacterial Capsule

Bacterial capsule, if present, forms the outermost layer of the cell and the bacterial colonies appear mucoid. Capsules are characteristics of most of the gram-negative bacteria; however, some gram-positive bacteria like *Bacillus megaterium*, *Streptococcus pyogenes*, *S. pneumoniae*, *S. agalactiae*, *Staphylococcus epidermidis,* etc. also have capsular envelope. It is a very large structure mostly consisting of well-organized polysaccharide layer which is not easily washed off. Although most bacteria consist of polysaccharide capsules, in some bacteria it is proteinaceous such as poly D-glutamic acid capsule of *Bacillus anthracis.* Capsules are highly antigenic called 'K-antigen' like LPS O-antigens and fimbriae and flagella F-antigen. The composition of capsule is highly variable and thus has been utilized for serotyping the strains. The Quellung reaction, also called as the Neufeld reaction, has been used to serotype *Streptococcus pneumoniae* in diagnostic settings. In this reaction, antibody binds to the antigenic capsule making it opaque and large allowing the capsular bacteria to be visualized under light microscope.

Functions—Capsules confer many advantages to bacteria in their natural environment:

1. **Acts as a virulence factor**: Capsulated bacteria evade phagocytosis, complement mediated lysis and cell-mediated immune mechanism which makes them

more virulent than their non-capsulated counterparts such as capsulated strains of *Streptococcus pneumoniae*.
2. **Prevents desiccation**: Capsules are highly hydrated which protects the bacteria against desiccation.
3. **Adhesion and biofilm formation**: Capsules are highly mucoid and help in attachment to host cell, substratum and to each other.
4. **Acts as carbohydrate reserve**: Dextrans present in capsule act as sugar reserves and are utilized during starvation.
5. **Vaccination**: Capsular polysaccharides conjugated with protein carriers, such as the tetanus toxoid or diptheria toxoid, stimulates a robust immune response.
6. They exclude bacterial viruses and most hydrophobic toxic materials such as detergents.

Slime layer and Glycocalyx

Capsule is an organized structure surrounding the bacterial cell; however, in some bacteria extracellular polysaccharide layer is unorganized and is easily removable. This unorganized layer is referred to as **slime layer**. It is mostly composed of exopolysaccharides, glycoproteins and glycolipids. Like capsule, slime layer confers protection to bacterial cells from desiccation and may help to survive against many chemical sterilants and antibodies, and also allows bacterial adhesion to smooth surfaces such as body implants and catheters. Slime is often produced by gliding bacteria to facilitate motility, e.g., myxobacteria.

Glycocalyx (literally meaning 'sugar coat') broadly refers to any polysaccharide layer surrounding the bacterial cell. It is a term that encompasses both capsule and slime layer; some microbiologists refer to all capsules as glycocalyx and do not differentiate microcapsules.

S-layer

This proteinaceous outer layer is interconnected to peptidoglycan in gram-positive bacteria, LPS in gram-negative bacteria and pseudomurein in archaea. Planctomycetes contain only S-layer as outer covering since they lack cell wall.

The term 'S-layer' was first accepted for general use in 'First International Workshop on Crystalline Bacterial Cell Surface Layers, Vienna (Austria)' in 1984. It was defined as 'Two-dimensional arrays of proteinaceous subunits forming surface layers on prokaryotic cells' in 1987.

The major function of S-layer is protection as it is the outer most layer of the cellular envelope while it serves as adhesion site and virulence factor in pathogens.

Function

Since S-layer-carrying organisms are ubiquitous in the biosphere and because S-layers represent one of the most abundant cellular proteins, it is now obvious that these metabolically expensive products must provide the organisms with an advantage of selection in very different habitats.

1. **Cell shape determination**: S-layers have a shape determining and maintaining function.
2. **Adhesion sites**: S-layers from Bacillaceae were found to function as adhesion sites for cell-associated exoenzymes. The high-molecular-weight exo-amylase from two *B. stearothermophilus* wild-type strains were bound to the S-layer surface in a density that did not disturb diffusion of nutrients or metabolites through the S-layer lattice. S-layer-associated exoenzymes were also described for *T. thermohydrosulfurigenes.*
3. **Protection**: S-layers from gram-negative bacteria such as *Aeromonas salmonicida*, *Campylobacter fetus*, *Aquaspirillum serpens* and *Caulobacter crescentus* were found to protect the cells from attack by bacterial parasites such as *Bdellovibrio bacteriovorus*, but they could not shield them from other predators like protozoa.
4. **Virulence**: S-layers can contribute to virulence when they are present as a structural component of the cell envelope of pathogens.
5. **Molecular sieves**: Due to presence of pores identical in size and morphology (2–8 nm), they work as precise molecular sieves, providing sharp cutoff levels for the bacterial cells.
6. **pH and osmotic pressure**: S-layers may protect bacteria from changes in pH and osmotic pressure.

Application Potential

1. **Ultrafiltration**: S-layers from various Bacillaceae were shown to be suitable for the production of isoporous ultrafiltration membranes with well-defined molecular weight cutoffs.
2. **Bioanalytical sensors/immunoassays/affinity microparticles/affinity membranes**: S-layers are used as immobilization matrices for binding of monolayers of functional molecules (e.g., enzymes, antibodies and immunogens) in a geometrically well-defined way and exploited for the production of bioanalytical sensors, immunoassays, affinity microparticles and affinity membranes.
3. **Vaccines**: Whole-cell preparations or partially purified cell products are currently used as attenuated vaccines against fish pathogens.
4. **Nanostructure technologies**: S-layers are self-assembly systems that exploit the molecular scale manufacturing precision of biological systems and hence are prime candidates for production of defined nanostructures.

6 Bacterial Cytoplasm

The cytoplasm of bacteria is a colorless gel-like matrix enclosed by plasma membrane together called as protoplast. The liquid component of cytoplasm is called cytosol in which structures such as ribosomes, inclusion bodies and plasmids are scattered throughout. Cytoplasm is the site for performing many cellular functions such as

DNA replication, cell division, growth and metabolism. The cytoplasmic composition of archaea is more or less similar to bacteria performing similar kind of functions. Prokaryotic cytoplasm does not contain any membrane bound organelles as opposed to eukaryotes and can be divided into five main constituents serving different purposes.

Cytoskeleton

Cytoskeleton consists of structural components which are mainly involved in cell division events and also in determining shape of the cells. They show both structural and functional similarity toward eukaryotic counterparts; however, they are less complex. Three cytoskeletal proteins of organisms *E. coli*, *B. subtilis* and *Caulobacter crescentus* have been studied in great details, viz. FstZ, MreB/Mbl and CreS.

FstZ is the first cytoskeletal protein to be recognized in *E. coli* and is homologous to eukaryotic tubulin protein. It is present in most bacteria and is involved in cell division as it forms a ring at the centre of the dividing cell. This triggers recruitment of other proteins required for formation of new cell wall between dividing cells to separate the daughter cells (Anderson et al., 2004). The role of FstZ protein in cell division has been discussed in detail in Chap. 4: Cell Division. This protein is also involved in cell division in archaea. However, another tubulin homologue CetZ is present in certain Euryarchaeotes which is only involved in conferring rod shape to the cells and not involved in cytokinesis.

MreB protein found in many bacteria is mainly involved in determining cell shape and is homologous to eukaryotic actin protein. Its role in maintaining rod shape of *E. coli* cells has been studied and *E. coli* cells deficient or mutant for MreB protein are spherical in shape. This protein can polymerize to form filaments similar to actin microfilaments. It is supposed to interact with proteins involved in cell length growth such as PBP2 (penicillin binding protein 2) and is involved in directing the machinery needed for peptidoglycan synthesis to allow peptidoglycan synthesis along the length of the cell (Varma & Young, 2009). Besides its role in providing shape to the cell, MreB is also suggested to help in chromosome segregation (Kruse et al., 2003), localization of proteins to poles (Gitai et al., 2004) and resistance to external mechanical stress (Jones et al., 2001). A similar protein Mbl with similar function is found in *B. subtilis* (Daniel & Errington, 2003). In archaea, MreB and another actin homologue crenactin found specifically in crenarachaeotes is responsible for rod shape of the cells.

CreS or crescentin protein is particularly present in *C. crescentus* and is responsible for providing the characteristic curved shape to the bacterium. It is homologous to eukaryotic intermediate filaments keratin and lamin, and it can assemble as filaments without requirement of any cofactor or ATP. CreS deficient or mutant *Caulobacter* cells become straight rods (Ausmees et al., 2003).

Ribosomes

These are the protein synthesis machinery which are loosely scattered throughout the cytoplasm of the bacterial cell. Ribosomes are made up of proteins and ribonucleic acid molecules and are made up of two subunits, one large subunit called as 50S

Table 4 Differences among bacterial, archaeal and eukaryotic ribosomes

Ribosomal characteristics	Bacteria	Archaea	Eukaryotes
Ribosome type	70S	70S	80S
Subunits	50S (large subunit) 30S (small subunit)	50S (large subunit) 30S (small subunit)	60S (large subunit) 40S (small subunit)
rRNA genes	50S-23S and 5S rRNA 30S-16S rRNA	50S-23S and 5S rRNA 30S-16S rRNA	60S-28S, 5.8S and 5S rRNA 40S-18S rRNA
Ribosomal proteins	55	68	82

and a small subunit called as 30S which together form a ribosome molecule called as 70S. Comparison of eukaryotic ribosomal composition to bacterial and archaeal ribosomes (Table 4) points out the main differences among all the three domains of life as ribosomes are conserved in all domains and serve the same purpose of messenger RNA (mRNA) translation and protein synthesis from mRNA.

Nucleoid

The bacterial genome is not enclosed in any membrane and is present as discrete irregularly shaped region known as nucleoid. Nucleoid majorly contains the double stranded DNA and some molecules of RNA and proteins. The nucleoid can be seen clearly using an electron microscope or under a light microscope after staining the DNA with Feulgen stain. The length of bacterial genomic DNA is much larger than the size of the cell, still DNA is packed compactly as a single chromosomal unit. This condense packing of DNA is facilitated by supercoiling, a mechanism which results in DNA looping, by different topoisomerase enzymes which participate in underwinding and overwinding of DNA (Champoux, 2001). Supercoiling is further assisted by certain nucleoid-associated proteins (NAPs) which are similar to histones in eukaryotes and help in bending and folding of the chromosome into a compact structure. These NAPs are also involved in regulation of bacterial gene expression (Dillon & Dorman, 2010). Some bacteria have more than one chromosome such as *Vibrio cholerae* and some show polyploidy such as *Epulopiscium.*

Archaea also have the double-stranded DNA as the genetic material which is packed compactly as nucleoid. Members of the phylum euryarchaeota are suggested to have histones associated with their DNA to form nucleosomes similar to eukaryotes. However, archaea possess only four histone proteins as compared to eukaryotes which involve eight histones in chromosomal packing. The histone molecules are hypothesized to prevent denaturation of DNA in extreme temperatures in which thermophilic archaea survive. Crenarchaeotes, on the other hand, pack their DNA into chromosome similar to bacteria employing supercoiling and NAPs.

Plasmids

These are extrachromosomal DNA molecules other than genomic DNA present in most bacteria. They are also double-stranded but exist as autonomous replicating bodies independent of chromosomal DNA. Some plasmids can also exist episomally which can integrate into the bacterial chromosome and also as independent replicons. Plasmids are mostly circular but can be linear also. They code for a few genes only in contrast to chromosomal DNA which codes for lots of proteins. The genes coded by plasmids are not essential for bacterial growth but can provide selective advantage to the cell such as antibiotic resistance. Some species possess numerous plasmids, for example, *Borellia burgdorferi* has 12 linear and nine circular plasmids. The plasmids can also be transferred to other bacterial cells through formation of a tube-like structure between two cells called as sex pilus via a process called as conjugation and the plasmids are conjugative (Smillie et al., 2010). Other plasmids which cannot be transferred to other cells are non-conjugative plasmids. In addition, plasmids can be majorly classified on the basis of selective advantage they provide to the bacteria:

1. **R plasmids**: These carry resistance genes against antibiotics and other growth inhibitors and thus allow bacteria, which are otherwise sensitive to these antibiotics, to grow in their presence. They are conjugative and can confer antibiotic resistance in other bacteria through transfer. Example is RP4 plasmid in *Pseudomonas.*
2. **Col plasmids**: These produce bacteriocins secreted by certain bacteria which are toxic to other closely related species and kill them. Example is ColE1 plasmid in *E. coli* which codes for colicin E1.
3. **Virulence plasmids**: These carry virulence genes and make the bacteria carrying such plasmids pathogenic. Example is Ti plasmid of *Agrobacterium tumefaciens* which is transferred to plants and is responsible for crown gall disease in plants.
4. **Metabolic/degradative plasmids**: These carry genes for enzymes responsible for degrading unusual substances such as toluene. Example is CAM plasmid for degrading camphor in *Pseudomonas.*

Inclusions

These are cytoplasmic aggregates which store organic or inorganic substances in insoluble form as inclusion bodies. These inclusions can be granular and crystalline while some are amorphous. These have single layers of membrane made up of phospholipids and proteins around them and are scattered throughout the cytoplasm. They can store organic carbon compounds as energy reservoirs and also maintain osmotic pressure by releasing the osmotic stress in the cytoplasm due to insoluble substances present as inclusions and not dissolved in cytoplasm. Different types of storage inclusions are found among bacteria which are described briefly here.

Carbon storage inclusions: Carbon storage granules found in bacteria are poly-β-hydroxyalkonates (PHA) of varying length of which poly-β-hydroxybutyrate (PHB) is the most commonly occurring. PHBs are lipid granules formed from the polymerization of β-hydroxybutyrate through ester linkage. These are synthesized by cells

when the carbon source is in abundance inside the cell and can be used as carbon and energy source later on. Archaea also store carbon in the form of PHAs. Another type of carbon storage granules prevalently found in bacteria are glycogen inclusions which is a polymer of glucose. These also serve as carbon and energy source in nutrient-depleting conditions.

Inorganic storage inclusions: These include polyphosphate and sulfur granules found in many bacteria. Polyphosphate granules are highly advantageous for cells as they serve as storage reservoir of phosphates which are hydrolyzed to release phosphate when needed. The free phosphate thus available can be used for synthesis of nucleic acids, phospholipids and in some organisms for ATP synthesis in phosphate limiting conditions. Sulfur granules are found in some photosynthetic bacteria (green and purple sulfur bacteria as described in Chap. 1: Diversity of Prokaryotes) which use hydrogen sulfide as electron donor during metabolism. Hydrogen sulfide gets converted to elemental sulfur during the photosynthetic reaction and the sulfur can get deposited either inside or outside the cells as sulfur granules.

Apart from the usual storage inclusions, bacteria also possess some unique inclusion bodies which serve specific functions of a particular kind. These include carboxysomes, magnetosomes and gas vesicles.

Carboxysomes have large polyhedral coating made up of proteins inside which an enzyme called carbonic anhydrase is enclosed which can convert carbonic acid into CO_2. The CO_2 produced and entrapped in the carboxysome is converted to sugars by another enzyme RubisCO present inside. Thus, these carboxysomes act as the site of CO_2 fixation and are important for cells. These can be found in cyanobacteria and other CO_2-fixing bacteria (Fig. 12).

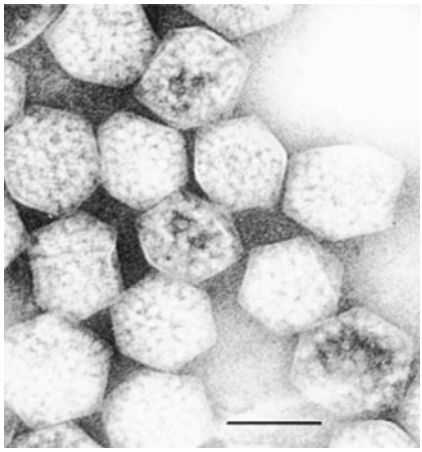


Fig. 12 Negatively stained image of carboxysomes (*Halothiobacillus neapolitanus*) with interior filled with RuBisCO molecules. *Source* Tsai et al. (2007). (Creative Commons Attribution license)

Magnetosomes are formed from iron oxide mineral magnetite (Fe_3O_4) or sulfur-containing greigite (Fe_3S_4) which arrange themselves as intracellular chains enclosed within the invagination of the plasma membrane. Magnetosomes are mostly found in aquatic organisms (magnetotactic bacteria) which allow these bacteria to exhibit magnetotaxis in which the bacterial cells orient along the Earth's magnetic field. Another suggested function of magnetosomes for aquatic organisms is that they guide the cells toward the bottom of the oceans where they grow best at lower concentrations of oxygen. Examples of magnetotactic bacteria are *Magnetococcus*, *Magnetospirillum* (Fig. 13).

Gas vesicles are present in certain planktonic bacteria such as cyanobacteria and also in archaea. They are small, hollow and cylindrical structures made up of proteins which aggregate together to form gas vacuoles which in turn provide buoyancy to the cells to float at the surface or at that depth of the water body where light, oxygen and nutrients are present in sufficient amounts. Gas vesicles are impermeable to water and solutes but permeable to atmospheric gases. Collapsing of vesicles results in the loss of the ability to float and bacteria sink downward.

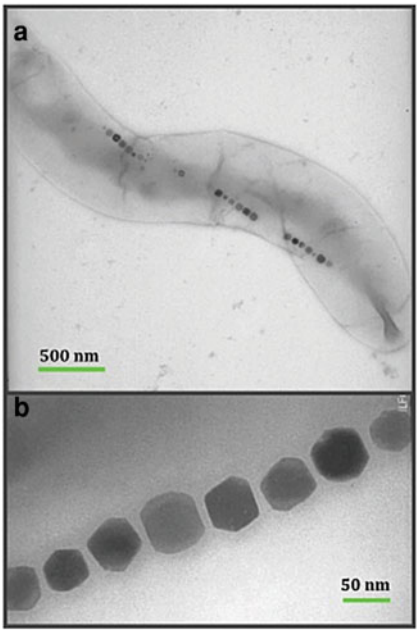


Fig. 13 Magnetosomes in *Magnetospirillum magneticum* as visible by TEM analysis (a) at 500nm; (b) at 50nm. *Source* Ginet et al. (2011). (Creative Commons Attribution License)

7 Extracellular Appendages

Bacteria possess extracellular structures like fimbriae, pili and flagella as a means to thrive in different environmental conditions they are exposed to. These structures are present external to cell envelope and help bacteria in performing various functions such as attachment to some solid surface, horizontal gene transfer and also in motility.

Fimbriae and pili: External appendages for adhesion

Fimbriae: They are short hair-like filamentous structures extending outward from the surface of the cell with up to 1000 fimbriae per cell. These are made up of proteins arranged helically as long tubes (in micrometers) with a diameter of about 3–10 nm. The main function of fimbriae is in the cell's attachment to some solid surface such as rocks to form biofilms and also on a liquid surface to form pellicles. Some pathogenic bacteria also use fimbriae to attach to host tissues for disease progression as in case of *Salmonella* which causes salmonellosis, *Neisseria gonorrhea* which causes gonorrhea and *Bordetella pertussis* which causes whooping cough.

Pili: These are also protein filaments and are similar to fimbriae except that they are longer in length, and only one or a few pili are present external to cell envelope. These two terms, pili and fimbriae, can be used interchangeably sometimes as they serve similar function; however, some pili like sex pili are specifically called pili and are present in cells having conjugative plasmids. The synthesis of pilus occurs by the addition of the subunits of the oligomeric protein pilin to the base of the pilus. Pili can be divided into two major types based on their structure and function:

a. **Adhesion pili/Type IV pili**: These are synonymous to fimbriae and help in adhesion of bacteria to specific surface such as human tissues. These are commonly found in gram-negative pathogens such as *Neisseria*, *E. coli*, *Vibrio cholerae* and certain gram-positive pathogens like *Streptococcus pyogenes* and *Clostridium.* They are also called as type IV pili, and they enhance the virulence of the pathogens as they aid in invasion of the host tissue through formation of microcolonies, biofilms. Pili also act as antigens present at the surface of pathogenic bacteria recognized by the host immune system and some bacteria can produce pili of different composition with altered antigenicity to evade off specific host immune responses. These pili are also responsible for certain forms of unusual motility exhibited by bacteria such as twitching motility on solid surfaces and also act as receptor for attachment of certain viruses, bacteriophages and bacteria and get coated by them as seen under electron microscope.

In *Vibrio cholera*, the disease is caused due to a toxin secreted by a lysogenic bacteriophage. Type IV pili serve as the binding site for the toxin secreting bacteriophage. The expression of the toxin gene incorporated in the bacterial genome is coregulated to the expression of pilus on the surface of the bacterium and hence the pilus is named as toxin coregulated pilus (TCP). TCPs aggregate to bring bacterial cells closer to each other and form microcolonies that protect them to evade the host defense mechanism (Li et al., 2008).

Some bacteria having type IV pili exhibit jerky movements on a solid surface called as twitching motility which is a type of gliding motility. In such bacteria, type IV pili are located towards the ends of the rod-shaped cells where they first extend while attached to the solid surface and then retract to drag the cell forward with force which is an energy driven process occurring as a result of ATP hydrolysis. Examples of bacteria showing twitching motility include *Pseudomonas*, *Moraxella* and *Myxococcus*.

Box 2: *Myxococcus xanthus*: Twitching and Gliding Motility on Solid Surfaces

This bacterium exhibits two unique forms of motility, viz. twitching motility, which introduces jerky movements, also called as social (S) motility as it occurs in a coordinated manner involving large number of cells and gliding motility which is a smooth movement of cells, also called as adventurous (A) motility as it is observed in single cells moving independently. Twitching motility is due to the presence of type IV pili and gliding motility occurs due to the presence of some proteins similar to flagellar movement proteins. Both the processes are energy consuming and require ATP hydrolysis (Willey et al., 2008).

In some bacteria, type IV pili are also involved in genetic transfer via the process of transformation, a process of DNA uptake by a recipient bacterial cell from the neighboring donor cells and its integration into the genome of recipient cell by homologous recombination. *Neisseria meningitidis*, a gram-negative pathogen causing meningitis, expresses type IV pili for recognition and binding of short DNA uptake sequences (DUSs) present in the donor DNA. Basic structure of type IV pili has been presented in Fig. 14.

Many archaea such as *Methanococcus maripaludis*, *Sulfolobus acidocaldarius* also possess type IV pili as adhesives thus enabling archaeal cells to adhere to different substrates. The structure of the archaeal pilus is similar to its bacterial homologue; however, some differences are also there one being the presence of a central hollow lumen (Chaudhury et al., 2018).

b. **Conjugative/sex pili**: The sex pilus facilitates horizontal gene transfer between two bacteria through a process called conjugation, a process of transfer of genetic material between two mating pairs. Sex pili are different from adhesion pili in that they are larger with a diameter of around 9–10 nm. They are formed as a tube-like structure which extends from a donor bacterium and reaches the recipient cell to form a ladder like structure known as mating bridge which allows the transfer of DNA of conjugative plasmid to the recipient cell. Many bacteria possess sex pilus, and some can have up to 10 sex pili per cell. The most common and well-studied example of sex pilus is of *E. coli* encoded by F factor plasmid.

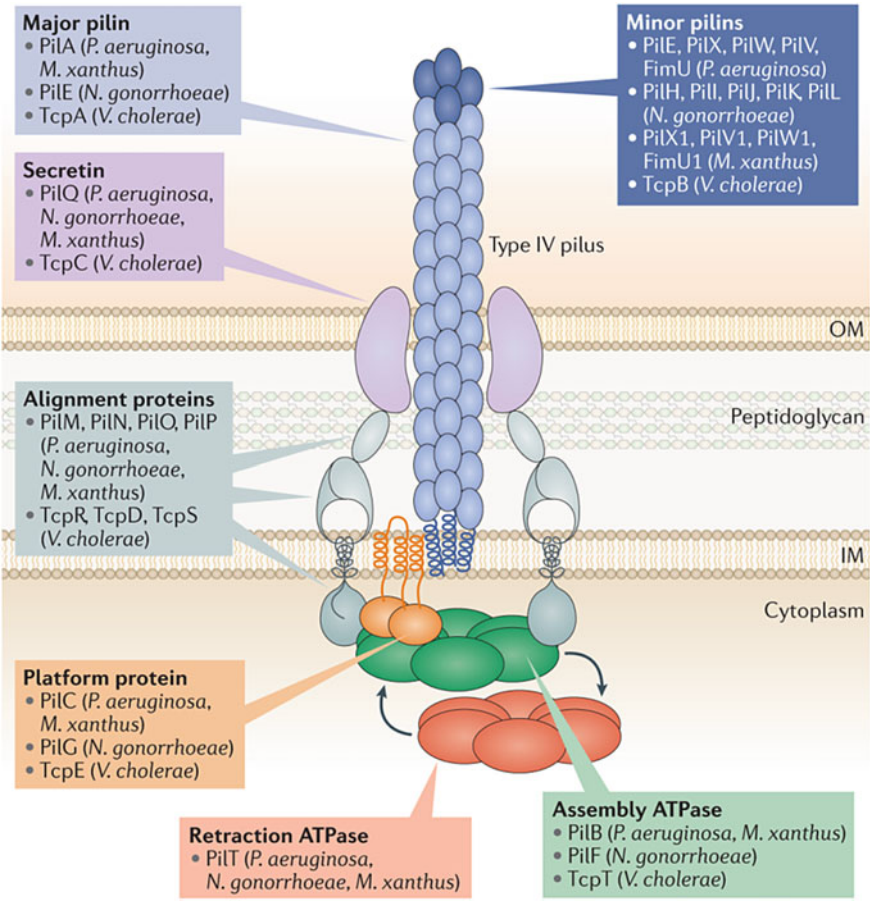


Fig. 14 Basic structure of type IV pili. *Source* Craig et al. (2019). With kind permission from Springer Nature

Flagella: Extracellular appendage for motility

Bacterial flagellum (plural flagella) is a hollow tube (20 nm wide and 20 μm long)-like structure that protrudes from the cell envelope toward outside. The main function of flagella is to provide motility to the cells in liquid medium. In some organisms, they act as virulence factors and in some they are important for attachment to a surface.

Arrangement of Flagella

The distribution and arrangement of flagella is different for different organisms. Based on the position where flagella are attached to the cell surface, the flagellar arrangement can be of four major types (Fig. 15).

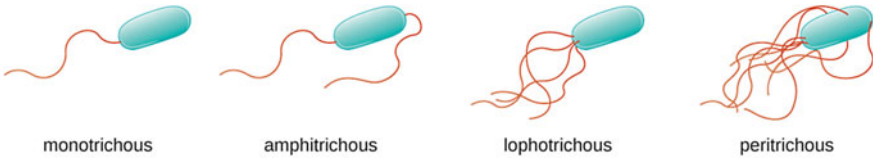


Fig. 15 Different types of flagellar arrangement. *Source* Keenleyside (2019). (Creative Commons Attribution License)

1. **Monotrichous flagellation**: The bacteria have only one flagellum attached to the surface. They are named so as mono means 'one' and trichous means 'hair.' This type of flagellation is also called as polar flagellation if the flagellum is present at one of the two ends as seen in *Vibrio cholerae*.
2. **Amphitrichous flagellation**: The bacteria have one flagellum attached at both the ends and hence the name 'amphi' which means on both sides. Only one flagellum is active at one point of time and bacteria can switch directions based on which side to move using either of the two flagella.
3. **Peritrichous flagellation**: In this type of flagellation, flagella are spread evenly over the entire surface of the bacterium and thus the name 'peri' meaning around. *E. coli* has peritrichous flagella.
4. **Lophotrichous flagellation**: This is in contrast to polar flagellum as it consists of a tuft of polar flagella present at one of the two poles of the cell. Lopho means 'tuft or cluster' and hence the name.

Basic structure of flagella

Flagella are very thin helical structures not visible under light microscope. However, using transmission electron microscopy, the basic structure of a flagellum has been studied. A flagellum is composed of three components, viz. the filament, the hook and the basal body or motor of flagella.

The filament is the longest part of the flagellum which is exposed outside the cell. It is a polymer of the protein flagellin which associates itself in protofilaments which come together to form a rigid cylinder helical in shape. In gram-negative organisms such as *E. coli*, *Salmonella typhimurium*, *Caulobacter crescentus* and *Vibrio alginolyticus*, 11 protofilaments cluster together parallelly along the filament axis to form a filament. The molecular mass of the protein flagellin ranges from 30 to 60 KDa, and its amino acid sequence is highly conserved among bacterial species. The hook is also a filamentous structure which is broader than the filament and connects filament to the basal body making the connection flexible. The basal body is a rod-like structure which is slightly complex as it has rings which remain anchored in the cell envelope and varies in gram-negative and gram-positive bacteria. Basal body of gram-negative organisms such as *E. coli* consists of four rings, viz. L, P MS and C. The L ring is the outermost ring which is embedded in the lipopolysaccharide of the outer membrane, P ring is next which is embedded in the peptidoglycan layer of the cell wall, the third ring MS is linked to the plasma membrane, and the fourth

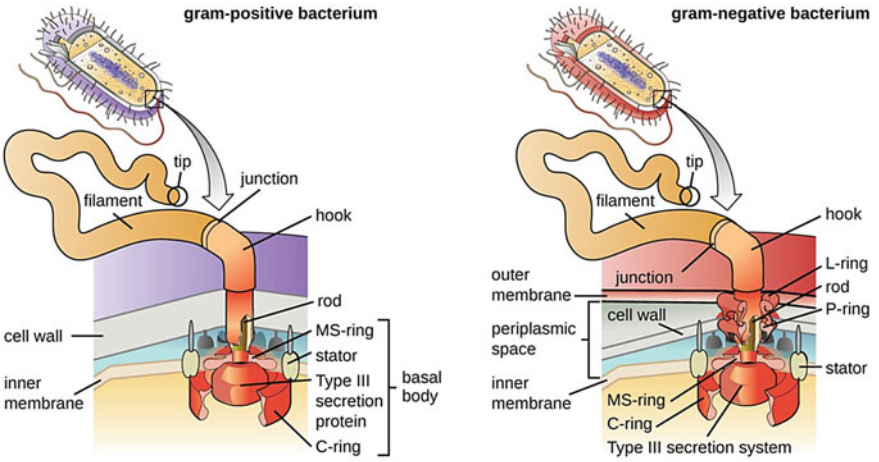


Fig. 16 Differential flagellar arrangement in gram-positive and gram-negative bacteria showing basic parts of flagella, viz. basal body, hook and filament. *Source* Keenleyside (2019). (Creative Commons Attribution License)

ring C is the innermost linked to the cytoplasm. In gram-positive bacteria, there is no outer membrane structure and only two types of rings are present, the outer ring which is anchored in the peptidoglycan cell wall and the inner ring anchored in the inner ring anchored in the plasma membrane (Fig. 16).

Flagellar movement

The flagella of bacteria act as a motor which results in the rotation of the helical filament like a boat propeller which leads to movement of bacteria called as swimming. The swimming motility can be of two types: run which is smooth and fast and tumble which changes the orientation of the cell and moves the cell from one point to another. The helical filaments can rotate both clockwise and counter-clockwise depending on the type of movement. Usually, if rotation is anti-clockwise, cells move forward and run and if rotation is clockwise, they tumble (Fig. 17).

The driving force for rotation of filaments is imparted to the cell by motor. The motor is located at the base of the flagellum and is composed of two main components: the rotor and the stator. The rotor part is formed by basal body of the flagellum along with *Fli* proteins and the stator is composed of *Mot* proteins, MotA and MotB surrounding the basal body. Together rotor and stator generate torque that is transmitted to the hook and filament and results in rotation. The torque is generated due to a proton motive force (pmf) across the membrane which is generated by movement of protons from outside to inside via membrane channels formed by MotA and MotB proteins. For a single rotation, around 1000 protons are translocated across the membrane and the rotations are counted as revolutions per second. For example, *E. coli* can rotate at a rate of 270 revolutions per second and is able to move both anti-clockwise (run) and clockwise (tumble). The switching of directions occurs in

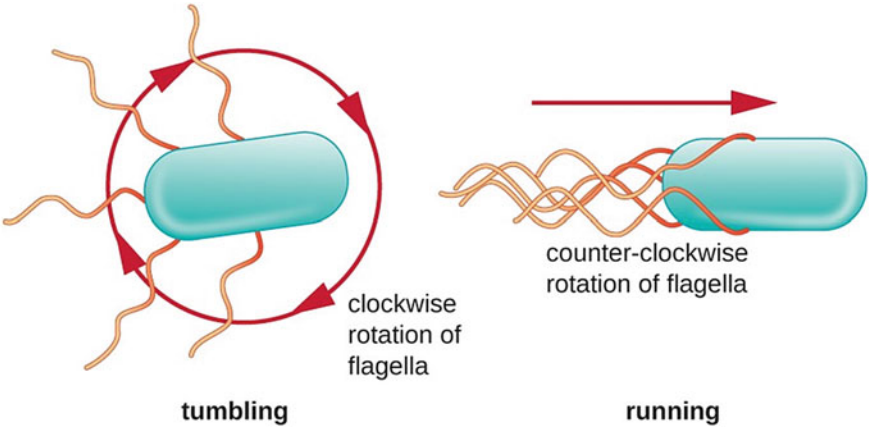


Fig. 17 Clockwise and anti-clockwise rotation of flagella resulting in directional movement of bacteria. *Source* Keenleyside (2019). (Creative Commons Attribution License)

response to external stimulus due to a motor switch regulated by Fli proteins specially FliG protein which interacts with stator.

Archaellum: Archaeal Flagellum for locomotion

There is a widespread distribution of archaella in the archaeal domain. Archaellum plays similar role in making the cells motile like bacterial flagella; however, there are major differences in the structure and functioning of the two as listed in Table 5. Due to the differences, the archaeal flagellum has been renamed as archaellum as it is specific in structure and function.

The archaeon *Halobacterium salinarum* is the first and best studied archaea for flagellar motility. The cells of *H. salinarum* swim at a speed about one-tenth of the speed of the bacterium *E. coli*. The reason for this huge difference in motility rate is due to the difference in the torque generated for rotation of the archaella. In archaea, rotation of the archaella is driven by ATP hydrolysis rather than by pmf in case of *E. coli*.

Extracellular structures specific to archaea

Cannulae

Cannulae are hollow tubular structures uniquely found on the surface of the thermophilic archaeon *Pyrodictium.* They have an outside diameter of 25 nm and are made up of three homologous glycoproteins. The cells grow in a network of cannulae which are proposed to anchor cells to each other. During the process of cell division, the newly generated daughter cells remain connected to each other via cannulae ultimately leading to the formation of a dense network of cells and cannulae. Cannulae are extremely resistant to heat and denaturing agents. The function of cannulae is not

Table 5 Major structural and functional differences between archaeal and bacterial flagella

Archaellum	Flagellum
Thinner in diameter around 10–14 nm in dimeter	Thick structure of around 20 nm diameter
Structural similarity to bacterial type IV pili	No structural similarity to type IV pili
Made up of 7–15 proteins, no homology to flagellar proteins	Made up of over 30 different proteins
Flagellin protein modified at N-terminal via N-linked oligosaccharides	No modification of flagellin protein
Archaellum is not hollow	Flagellum is a hollow structure
Flagellin subunits added to the base of the filament for its synthesis and growth	Flagellins subunits added at the tip of the filament for its synthesis and growth
No basal body rings and hooks may or may not be present	Basal body consisting of two/four rings and hook connects basal body to the filament
Flagellar rotation is slower as energy is provided by ATP hydrolysis	Flagellar rotation is faster as energy is provided by proton motive force
Counterclockwise rotation of archaellum results in reverse motility	Counterclockwise rotation of flagellum results in forward running motility
Clockwise rotation of archaellum leads to forward movement of the cell	Clockwise rotation of flagellum leads to tumble movement for reorientation of the cell

known properly; however, in addition to anchoring cells, it might help in cell-to-cell communication for nutrient exchange as well as exchange of genetic material.

Some bacteria also possess unusual surface structures called spinae which are similar to cannulae in being tubular in shape. Example of a bacterium producing spinae is *Roseobacter* which forms similar network of cells and spinae thus connecting cells over long distances.

Hami

Hami (singular: hamus) are another type of archaeal cell surface appendages which are of particular interest due to their unique shape. They are filamentous structures having a helical base, and a tripartite barbed grappling hook is present at the opposite end. The presence of tiny grappling hooks suggests their role in attachments of cells to different surfaces. Archaeal species bearing hami are found in biofilm communities in association with a bacterium. This association appears like a string-of-pearl-like arrangement as observed in case of filamentous sulfur-oxidizing bacterium *Thiothrix* which, growing in cold and sulfuric springs, forms the outer whitish part of the pearl and the connecting string or thread and the archaea filled in the interior of the pearl.

Bacterial Endospores: Structure and location

Endospores are dormant structures formed during a process called as sporulation in which the vegetative cells undergo a programmed event of differentiation and get converted into a spore. Endospores are not produced by all bacterial species but certain genera of the phylum firmicutes like *Bacillus*, *Clostridium*, *Sporosarcina* with

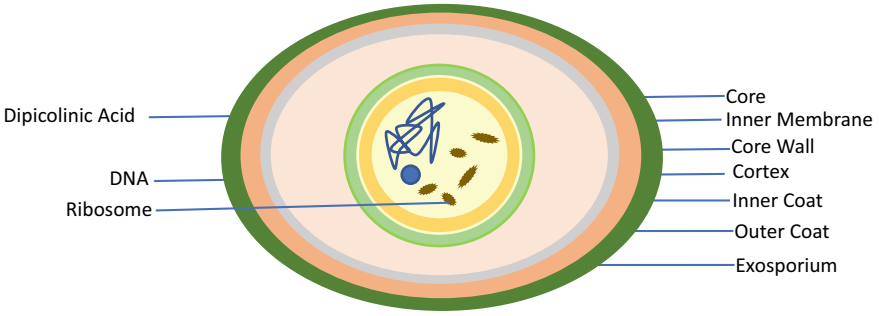


Fig. 18 Structure of a bacterial endospore

species of *Bacillus* being the best-studied organisms. The location of the spore within the cell can also vary and depends on the species in which it is being formed. Three main types of endospore positioning inside the cell are central (middle of the cell), terminal (toward the end) and sub-terminal (between middle and end). The diameter and shape of the spore can also vary as they can be larger than the vegetative cells and can be spherical or elliptical respectively.

Endospore is a metabolically inactive resting structure resistant to a variety of harsh external conditions such as extreme heat, chemicals, desiccation and ultraviolet and gamma radiations. The structure of endospore is quite different from that of vegetative cell and is mainly made up of three layers, viz. cortex, coat and exosporium. The innermost portion of the spore is the core which is similar to vegetative cell cytoplasm consisting of organelles such as nucleoid and ribosomes and is surrounded by an inner membrane and a thin core wall called as germ cell wall. The germ cell wall contains peptidoglycan units required by the spore during its reactivation to form a vegetative cell to form the cell wall. The core wall is surrounded by a thick layer of loosely cross-linked peptidoglycan called as cortex specific to endospore. Outside the cortex is the spore coat which is made up of several layers of spore-specific proteins present in a highly cross-linked manner. After coat layers, the outermost layer exosporium is a very thin and delicate covering which is composed of glycoprotein units (Fig. 18). Thus, the endospore is surrounded by a variety of layers which provide it the ability to withstand adverse environmental conditions.

Reactivation of spore to vegetative cell

The formation of spore is a complex process, and it requires around 8 h which is described in detail in Chap. 25: Competence and Sporulation in *Bacillus subtilis*. However, when favorable conditions are perceived, the spore transforms back into active vegetative form within a few minutes. This reactivation occurs in three distinct stages, viz. **activation, germination and outgrowth**. Activation occurs when spores are subjected to heat for a period of time and it prepares them for germination. The next step germination is the key step which breaks the dormancy of the spore when nutrients are supplied in the medium. During germination, the spore loses most of its properties such as resistance to desiccation, heat and loss of refractility which leads

the spore for outgrowth resulting in water uptake and activation of cellular processes such as DNA, RNA and protein synthesis. Due to water intake, the spore swells and bursts to release the vegetative cell which continues to grow until unfavorable conditions are sensed again.

Summary

- The average size of most bacteria is small; they increase their surface area-to-volume ratio which helps in quick absorption of nutrients from the environment, and waste products are also excreted in a similar manner.
- Three distinct types of bacterial shapes recognized are cocci, rods and spirals which can cluster themselves in different arrangements.
- A typical bacterial cell is divided into three main parts: the cell envelope, the cytoplasm and the extracellular appendages.
- The cell envelope maintains the integrity of the cell and comprises innermost plasma membrane, cell wall, periplasmic space between plasma membrane and cell wall and the outermost layers surrounding the cell wall.
- The cytoplasm contains nucleoid as genetic material, ribosomes as protein synthesis machinery and inclusion bodies dispersed all over the cytoplasmic space.
- The extracellular appendages like fimbriae, pili and flagella serve multiple functions and help cells in conjugation, attachment and locomotion.
- In addition to the main components, bacterial cells also form endospores which are dormant structures that help bacteria to survive in adverse environmental conditions.
- Plasma membrane or cytoplasmic membrane is the most important layer of the cell envelope as it surrounds the cytoplasm and defines the cell shape.
- The major functions of plasma membrane are: maintaining the integrity of the cell due to its selective permeability; serving as a site for energy conservation and generating proton motive force; and as site for photosynthesis, lipid biosynthesis and synthesis of cell wall constituents.
- The structure of plasma membrane is explained as the Fluid Mosaic Model proposed by Singer and Nicolson. It is a phospholipid bilayer: The phospholipids are amphipathic in nature with a variable polar head, glycerol-3-phosphate moiety and two non-polar tails made up of fatty acids linked together by an ester bond.
- The three major kinds of phospholipids found in *E. coli* are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin having ethanolamine, glycerol and phosphatidylglycerol as polar head groups.
- The polar end of the membrane bilayer faces the cytoplasm and the external environment and being hydrophilic interacts with water. The non-polar tails being hydrophobic face each other in the bilayer and make the hydrophobic core.
- Two types of proteins are present in the plasma membrane: Integral membrane proteins which are firmly embedded in the phospholipid bilayer and peripheral membrane proteins which are associated with the membrane surface from outside or inside through interaction with integral proteins.

- The fluidity of the membrane is essential for lateral movement of proteins and is mainly maintained by the number of saturated and unsaturated fatty acids present in the bilayer.
- Many bacterial membranes are further stabilized due to the presence of sterol-like molecules called hopanoids which have pentacyclic structure.
- The composition of archaeal plasma membrane varies greatly from that of bacteria. They lack fatty acids and instead contain multiple copies of 5-carbon isoprene units which are highly methylated, cyclic and unsaturated and form isoprenoid chains of variable lengths. These are linked to glycerol through glycerol-1-phosphate moieties via ether linkages rather than ester linkages.
- The arrangement of lipids in archaeal membranes can be classified into two major types: (1) glycerol diether lipids that have a phytanyl group consisting of 5 isoprene units (20-C side chain) attached to glycerol molecules through ether linkage and form a bilayered structure. The 20-C diether is commonly termed as archaeol; (2) diglycerol tetraether lipids have two glycerol residues linked by two phytanyl groups (40-C side chain). The 40-C diglycerol tetraether is commonly termed as caldarchaeol.
- The bacterial cells are surrounded by a complex cell wall made up of sugars and amino acid polymers known as peptidoglycan or murein.
- Based on differences in thickness and composition of their cell wall, bacteria can be distinguished into two broad categories as gram-positive and gram-negative bacteria.
- In case of gram-positive bacteria, the peptidoglycan layer is relatively thick (around 20–80 nm) and continuous; and accounts for 50–90% of the dry weight of the cell wall. In addition to peptidoglycan, the cell wall is rich in Teichoic acids which are linked to both peptidoglycan and to cell membrane.
- In case of gram-negative bacteria, the peptidoglycan layer is thinner with very few layers of around 5–10 nm and comprises only 10% of the cell wall. Major part of the cell wall is contributed by an additional plasma membrane which is located outside the peptidoglycan layer and is termed outer membrane. Further, lipoproteins (also known as Braun's lipoproteins) are covalently and non-covalently attached to the peptidoglycan layer and outer membrane, respectively. Moreover, lipopolysaccharides (LPS) are also present in gram-negative cell envelope as the outer most layer. LPS is a complex lipopolysaccharide having three distinct components, inner lipid A, core polysaccharide and outermost O-antigen.
- The periplasmic space is the region between the outer and inner membranes of gram-negative bacteria. Periplasm is the site for protein folding, oxidation, transport and houses several hydrolytic enzymes, proteins that specifically bind sugars, amino acids and inorganic ions. β-lactamases and aminoglycoside-modifying enzymes which are important for antibiotic resistance are also present in periplasm.
- The main polysaccharide present in archaeal cell wall is pseudomurein. It differs from bacterial murein in three ways: (1) contains N-acetyltalosaminuronic acid in place of N-acetylmuramic acid; (2) the disaccharide is formed by β-1,3-glycosidic

bonds instead of β1,4-glycosidic bonds; and (3) the peptidoglycan backbone is cross-linked by L-amino acids rather than D-amino acids.

- Most archaea are also surrounded by a layer of proteins and glycoproteins in place of a cell wall which is called as S-layer.
- Some archaea like *Thermoplasma* and *Ferroplasma* are called as 'cell wall-less' archaea as they lack cell wall and are only surrounded by plasma membrane as the outer most covering.
- Bacterial capsule, if present, forms the outermost layer of the cell, and the bacterial colonies appear mucoid. Capsules are characteristics of most gram-negative bacteria; however, some gram-positive bacteria like *Bacillus megaterium*, *Streptococcus pyogenes*, *S. pneumoniae*, *S. agalactiae*, *Staphylococcus epidermidis,* etc. also have capsular envelope.
- Capsules confer many advantages to bacteria in their natural environment such as they act as a virulence factor, protect the bacteria against desiccation, help in adhesion and biofilm formation, act as carbohydrate reserve, help in vaccination as they stimulate a robust immune response and exclude bacterial viruses and most hydrophobic toxic materials such as detergents.
- In some bacteria, an unorganized and easily removable extracellular polysaccharide layer referred to as slime layer is present. It is mostly composed of exopolysaccharides, glycoproteins and glycolipids. Slime is often produced by gliding bacteria to facilitate motility, e.g., myxobacteria.
- Surface layer (S-layer) is a self-assembled proteinaceous outer layer which may or may not be glycosylated. It occurs in some bacteria but is a characteristic feature of most archaea.
- The cytoplasm of bacteria is a colorless gel-like matrix enclosed by plasma membrane together called as protoplast. The liquid component of cytoplasm is called cytosol in which structures such as ribosomes, inclusion bodies and plasmids are scattered throughout. Cytoplasm is the site for performing many cellular functions such as DNA replication, cell division, growth and metabolism.
- Cytoskeleton consists of structural components which are mainly involved in cell division events and also in determining shape of the cells. Three cytoskeletal proteins of organisms *E. coli*, *B. subtilis* and *Caulobacter crescentus* have been studied in great details, viz. FstZ, MreB/Mbl and CreS.
- FstZ, homologous to eukaryotic tubulin protein, is present in most bacteria and is involved in cell division as it forms a ring at the centre of the dividing cell. MreB protein found in many bacteria is mainly involved in determining cell shape and is homologous to eukaryotic actin protein. CreS or crescentin protein is homologous to eukaryotic intermediate filaments keratin and lamin. It is particularly present in *C. crescentus* and is responsible for providing the characteristic curved shape to the bacterium.
- Ribosomes are the protein synthesis machinery which are loosely scattered throughout the cytoplasm of the bacterial cell. They are made up of proteins and ribonucleic acid molecules and have two subunits, one large subunit called as 50S and a small subunit called as 30S which together form a ribosome molecule called as 70S.

- Bacterial genome is not enclosed in any membrane and is present as discrete irregularly shaped region known as nucleoid. It majorly contains the double stranded DNA and some molecules of RNA and proteins.
- Plasmids are extrachromosomal DNA molecules other than genomic DNA present in most bacteria. They are also double stranded but exist as autonomous replicating bodies independent of chromosomal DNA. Some plasmids can also exist episomally which can integrate into the bacterial chromosome and also as independent replicons.
- The genes coded by plasmids are not essential for bacterial growth but can provide selective advantage to the cell such as antibiotic resistance.
- Inclusion bodies are cytoplasmic aggregates which store organic or inorganic substances in insoluble form. Different types of storage inclusions found among bacteria include: (1) carbon storage inclusions—these are poly-β-hydroxyalkonates (PHA) of varying length of which poly-β-hydroxybutyrate (PHB) is the most commonly occurring; (2) inorganic storage inclusions: These include polyphosphate and sulfur granules found in many bacteria.
- Some bacteria also possess some unique inclusion bodies which serve specific functions of a particular kind. These include carboxysomes, magnetosomes and gas vesicles.
- Bacteria possess extracellular structures like fimbriae, pili and flagella as a means to thrive in different environmental conditions they are exposed to.
- Fimbriae are short hair-like filamentous structures extending outward from the surface of the cell with up to 1000 fimbriae per cell. These are made up of proteins arranged helically as long tubes. The main function is in the cell's attachment to some solid surface such as rocks to form biofilms and also on a liquid surface to form pellicles. Some pathogenic bacteria also use fimbriae to attach to host tissues for disease progression.
- Pili are also protein filaments and are similar to fimbriae except that they are longer in length, and only one or a few pili are present external to cell envelope.
- Pili can be divided into two major types based on their structure and function: Adhesion pili/Type IV pili and Conjugative/sex pili.
- Adhesion pili/Type IV pili are synonymous to fimbriae and help in adhesion of bacteria to specific surface such as human tissues. These are commonly found in gram-negative pathogens such as *Neisseria*, *E. coli*, *Vibrio cholerae* and certain gram-positive pathogens like *Streptococcus pyogenes* and *Clostridium.*
- Conjugative/sex pili facilitate horizontal gene transfer between two bacteria through a process called conjugation, a process of transfer of genetic material between two mating pairs. They are formed as a tube-like structure which extends from a donor bacterium and reaches the recipient cell to form a ladder like structure known as mating bridge which allows the transfer of DNA of conjugative plasmid to the recipient cell.
- The most common and well-studied example of sex pilus is of *E. coli* encoded by F factor plasmid.
- Bacterial flagella are hollow tube (20 nm wide and 20 μm long)-like structures that protrude from the cell envelope toward outside. The main function of flagella

is to provide motility to the cells in liquid medium. In some organisms, they act as virulence factors and in some they are important for attachment to a surface.

- Based on the position where flagella are attached to the cell surface, the flagellar arrangement can be of four major types: monotrichous, amphitrichous, peritrichous and lophotrichous.
- A flagellum is composed of three components, viz. the filament, the hook and the basal body or motor of flagella.
- The filament is the longest part of the flagellum which is exposed outside the cell. It is a polymer of the protein flagellin which associates itself in protofilaments which come together to form a rigid cylinder helical in shape.
- The hook is also a filamentous structure which is broader than the filament and connects filament to the basal body making the connection flexible.
- The basal body is a rod-like structure which has rings which remain anchored in the cell envelope and varies in gram-negative and gram-positive bacteria.
- Basal body of gram-negative organisms such as *E. coli* consists of four rings. L ring is the outermost which is embedded in the lipopolysaccharide of the outer membrane, P ring is next which is embedded in the peptidoglycan layer of the cell wall, the third ring MS is linked to the plasma membrane, and the fourth ring C is the innermost linked to the cytoplasm.
- In gram-positive bacteria, there is no outer membrane structure, and only two types of rings are present, the outer ring which is anchored in the peptidoglycan cell wall and the inner ring anchored in the inner ring anchored in the plasma membrane.
- The flagella of bacteria act as a motor which results in the rotation of the helical filament like a boat propeller which leads to movement of bacteria called as swimming. The swimming motility can be of two types: run which is smooth and fast and tumble which changes the orientation of the cell and moves the cell from one point to another.
- Archaellum: Archaeal Flagellum for locomotion
- The archaeal flagellum known as archaellum plays similar role in making the cells motile like bacterial flagella; however, there are major differences in the structure and functioning of the two.
- Endospores are dormant structures formed during a process called as sporulation in which the vegetative cells undergo a programmed event of differentiation and get converted into a spore. Endospores are not produced by all bacterial species but certain genera of the phylum firmicutes like *Bacillus*, *Clostridium*, *Sporosarcina.*
- Endospore is a metabolically inactive resting structure resistant to a variety of harsh external conditions such as extreme heat, chemicals, desiccation and ultraviolet and gamma radiations.

Questions

1. Give examples of the following arrangements found in cocci
    a. Arrangement in pair

   b. Arrangement in tetrad
   c. Long cocci chain
   d. Grape-like bunches
   e. Regular cuboid arrangement

2. Give examples for the following cell shapes of bacteria

   a. Star shaped
   b. Spiral
   c. Square shape
   d. Pleomorphic

3. Differentiate between composition of bacterial and archaeal cell membrane.
4. What are archaeosomes?
5. Differentiate between composition of bacterial and archaeal cell wall.
6. What are L-forms? Why are they so called?
7. Differentiate between slime layer, glycocalyx and S-layer.
8. Differentiate between ribosomes of bacteria, archaea and eukaryotes.
9. What are carboxysomes and magnetosomes?
10. Give one example each for following flagellar attachment

    a. Monotrichous
    b. Amphitrichous
    c. Peritrichous
    d. Lophotrichous

11. Differentiate between archaellum and bacterial flagellum.
12. What are extracellular appendages in archaea?
13. Draw and label structure of a bacterial endospore.

References

Aktas, M., Danne, L., Möller, P., & Narberhaus, F. (2014). Membrane lipids in *Agrobacterium tumefaciens*: Biosynthetic pathways and importance for pathogenesis. *Frontiers in Plant Science, 5,* 109.

Albers, S. V., & Meyer, B. H. (2011). The archaeal cell envelope. *Nature Reviews Microbiology, 9*(6), 414–426.

Anderson, D. E., Gueiros-Filho, F. J., & Erickson, H. P. (2004). Assembly dynamics of FtsZ rings in *Bacillus subtilis* and *Escherichia coli* and effects of FtsZ-regulating proteins. *Journal of Bacteriology, 186*(17), 5775–5781.

Ausmees, N., Kuhn, J. R., & Jacobs-Wagner, C. (2003). The bacterial cytoskeleton: An intermediate filament-like function in cell shape. *Cell, 115*(6), 705–713.

Berry, A. M., Harriott, O. T., Moreau, R. A., Osman, S. F., Benson, D. R., & Jones, A. D. (1993). Hopanoid lipids compose the Frankia vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. *Proceedings of the National Academy of Sciences, 90*(13), 6091–6094.

Beveridge, T. J., & Davies, J. A. (1983). Cellular responses of *Bacillus subtilis* and *Escherichia coli* to the gram stain. *Journal of Bacteriology, 156*(2), 846–858.

Brown, L., Wolf, J. M., Prados-Rosales, R., & Casadevall, A. (2015). Through the wall: Extracellular vesicles in gram-positive bacteria, mycobacteria and fungi. *Nature Reviews Microbiology, 13*(10), 620–630.

Caforio, A., & Driessen, A. J. (2017). Archaeal phospholipids: Structural properties and biosynthesis. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1862*(11), 1325–1339.

Champoux, J. J. (2001). DNA topoisomerases: Structure, function, and mechanism. *Annual Review of Biochemistry, 70*(1), 369–413.

Chaudhury, P., Quax, T. E., & Albers, S. V. (2018). Versatile cell surface structures of archaea. *Molecular Microbiology, 107*(3), 298–311.

Comita, P. B., Gagosian, R. B., Pang, H., & Costello, C. E. (1984). Structural elucidation of a unique macrocyclic membrane lipid from a new, extremely thermophilic, deep-sea hydrothermal vent archaebacterium, *Methanococcus jannaschii*. *Journal of Biological Chemistry, 259*(24), 15234–15241.

Craig, L., Forest, K. T., & Maier, B. (2019). Type IV pili: Dynamics, biophysics and functional consequences. *Nature Reviews Microbiology, 17*(7), 429–440.

Daniel, R. A., & Errington, J. (2003). Control of cell morphogenesis in bacteria: Two distinct ways to make a rod-shaped cell. *Cell, 113*(6), 767–776.

De Rosa, M., Gambacorta, A., & Gliozzi, A. (1986). Structure, biosynthesis, and physicochemical properties of archaebacterial lipids. *Microbiological Reviews, 50*(1), 70.

Depelteau, J. S., Brenzinger, S., & Briegel, A. (2019). *Bacterial and archaeal cell structure*. Elsevier.

Dillon, S. C., & Dorman, C. J. (2010). Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. *Nature Reviews Microbiology, 8*(3), 185–195.

Dowhan, W. (1997). Molecular basis for membrane phospholipid diversity: Why are there so many lipids? *Annual Review of Biochemistry, 66*(1), 199–232.

Ginet, N., Pardoux, R., Adryanczyk, G., Garcia, D., Brutesco, C., & Pignol, D. (2011). Single-step production of a recyclable nanobiocatalyst for organophosphate pesticides biodegradation using functionalized bacterial magnetosomes. *PLoS One, 6*(6).

Gitai, Z., Dye, N., & Shapiro, L. (2004). An actin-like gene can determine cell polarity in bacteria. *Proceedings of the National Academy of Sciences, 101*(23), 8643–8648.

Jones, L. J., Carballido-López, R., & Errington, J. (2001). Control of cell shape in bacteria: Helical, actin-like filaments in *Bacillus subtilis*. *Cell, 104*(6), 913–922.

Kaur, G., Garg, T., Rath, G., & Goyal, A. K. (2016). Archaeosomes: An excellent carrier for drug and cell delivery. *Drug Delivery, 23*(7), 2497–2512.

Keenleyside, W. (2019). *Microbiology: Canadian edition.*

Koga, Y., & Morii, H. (2005). Recent advances in structural research on ether lipids from archaea including comparative and physiological aspects. *Bioscience, Biotechnology, and Biochemistry, 69*(11), 2019–2034.

Kruse, T., Møller-Jensen, J., Løbner-Olesen, A., & Gerdes, K. (2003). Dysfunctional MreB inhibits chromosome segregation in *Escherichia coli*. *The EMBO Journal, 22*(19), 5283–5292.

Langworthy, T. A. (1977). Long-chain diglycerol tetraethers from *Thermoplasma acidophilum*. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 487*(1), 37–50.

Li, J., Lim, M. S., Li, S., Brock, M., Pique, M. E., Woods, V. L., Jr., & Craig, L. (2008). Vibrio cholerae toxin-coregulated pilus structure analyzed by hydrogen/deuterium exchange mass spectrometry. *Structure, 16*(1), 137–148.

Miller, S. I., & Salama, N. R. (2018). The gram-negative bacterial periplasm: Size matters. *PLoS biology, 16*(1).

Poralla, K., Muth, G., & Härtner, T. (2000). Hopanoids are formed during transition from substrate to aerial hyphae in *Streptomyces coelicolor* A3 (2). *FEMS Microbiology Letters, 189*(1), 93–95.

Raetz, C. R., & Dowhan, W. (1990). Biosynthesis and function of phospholipids in *Escherichia coli*. *Journal of Biological Chemistry, 265*(3), 1235–1238.

Shimada, H., Nemoto, N., Shida, Y., Oshima, T., & Yamagishi, A. (2008). Effects of pH and temperature on the composition of polar lipids in *Thermoplasma acidophilum* HO-62. *Journal of Bacteriology, 190*(15), 5404–5411.

Singer, S. J., & Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science, 175*(4023), 720–731.

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P., & de la Cruz, F. (2010). Mobility of plasmids. *Microbiology and Molecular Biology Reviews, 74*(3), 434–452.

Sohlenkamp, C., & Geiger, O. (2016). Bacterial membrane lipids: Diversity in structures and pathways. *FEMS Microbiology Reviews, 40*(1), 133–159.

Tsai, Y., Sawaya, M. R., Cannon, G. C., Cai, F., Williams, E. B., Heinhorst, S., Kerfeld, C. A., & Yeates, T. O. (2007). Structural analysis of CsoS1A and the protein shell of the *Halothiobacillus neapolitanus* carboxysome. *PLoS biology, 5*(6).

Untersteller, É., Fritz, B., Bliériot, Y., & Sinaÿ, P. (1999). The structure of calditol isolated from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius. Comptes Rendus De l'Académie Des Sciences-Series IIC-Chemistry, 2*(7–8), 429–433.

Varma, A., & Young, K. D. (2009). In *Escherichia coli*, MreB and FtsZ direct the synthesis of lateral cell wall via independent pathways that require PBP 2. *Journal of Bacteriology, 191*(11), 3526–3533.

Willey, J. M., Sherwood, L., & Woolverton, C. J. (2008). *Prescott, Harley, and Klein's microbiology* (7th ed.). New York: McGraw-Hill.

Chapter 3
Bacterial Cell Wall Biosynthesis and Inhibitors

Rani Gupta, Namita Gupta, and Shruti Bindal

The bacterial cell wall forms a sacculus around the cell membrane and provides resistance to osmotic stress and imparts mechanical strength to the cell. Peptidoglycan layer forms a mesh with the help of glycan chains that are cross-linked by peptide bridges. Formation and degradation of this sacculus are a dynamic process wherein various synthases and hydrolases take part in its synthesis and cleavage, respectively. Consistent growth and/or shedding and integrity of cell wall are of utmost importance for the survival of bacteria. A bacterial cell without a cell wall would not be able to survive in open environment. Therefore, cell wall and enzymes involved in its synthesis are regarded as potential targets for inhibiting the growth or killing of harmful bacteria.

1 Components of Bacterial Cell Wall

Peptidoglycan

Peptidoglycan is responsible for imparting rigidity and strength to the bacterial cell wall. As mentioned earlier, peptidoglycan is composed of polymers of sugar and amino acids which are cross-linked with each other and thus results in a rigid but flexible structure, and it depends on the extent of cross-linking. Peptidoglycan consists of repeating groups of β1,4-linked *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) disaccharides. *N*-acetyl muramic acid residue also has four to five amino acid peptide chain. Of these, tetrapeptide is composed of alternating L and D amino acids, viz. L-alanine (R1), D-glutamate (R2), L-lysine or diaminopimelic acid (R3; varies among bacteria) and D-alanine (R4) with an additional D-alanine at R5 position which is cleaved during transpeptidation or cross-linking. Peptidoglycan helical strands are held together by cross-linking wherein R4 amino acid of one chain is cross-linked to R3 amino acid of neighboring chain, which is either L-lysine or diaminopimelic acid (m-Dpm) the latter being uniformly present in gram-negative peptidoglycans (Vollmer et al., 2008). The cross-linking may be direct or through a

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_3

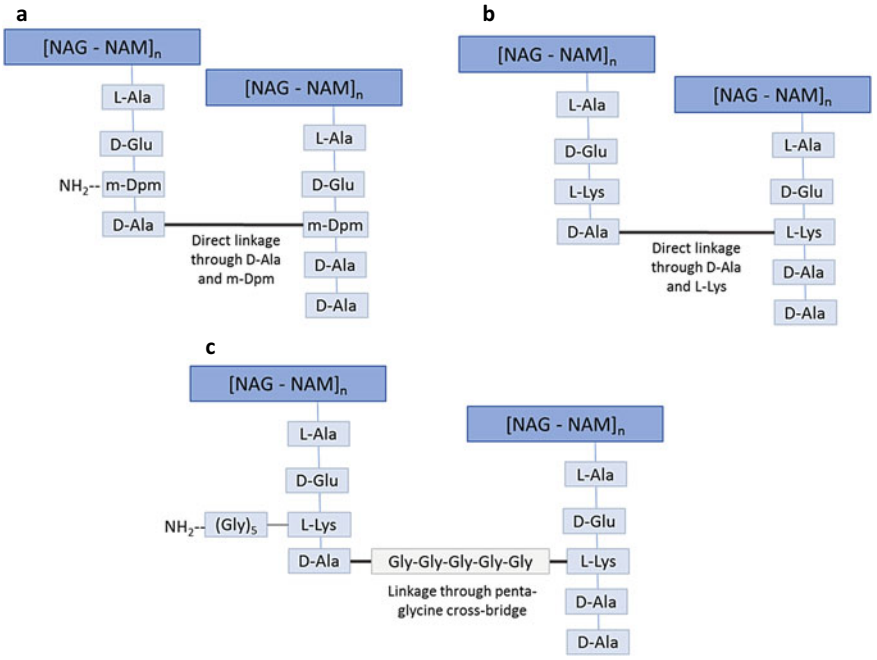


Fig. 1 Cross-linking of peptidoglycan units through wall peptides. **a** Direct linkage through D-Ala and m-Dpm; **b** direct linkage through D-Ala and L-Lys; and **c** linkage through penta-glycine cross-bridge

peptide bridge as in *Staphylococcus aureus* where a glycine pentapeptide is present (Fig. 1).

Peptidoglycan cross-linking extent varies from species to species, and it provides rigidity to the structure. It may be 40–60% as in *E. coli* and other gram-negative bacteria and as high as 90% as in *Staphylococcus*. The glycan chains arrange themselves in helically twisted conformation such that the peptide bridges radiate in all directions from the axis of the backbone and hence allow interpeptide cross-linking with neighboring chains. The peptidoglycan forms a three-dimensional network surrounding the cell membrane, which is known as **murein sacculus**.

Orientation of peptidoglycan layer with respect to the plasma membrane has been proposed to have two models: layered model (parallel orientation) and scaffold model (perpendicular orientation). Glycan chains run perpendicular to the plasma membrane in scaffold model while they are parallel in layered model (Fig. 2). The spaces between these matrices are supposed to harbor respective macromolecules of gram-positive and gram-negative cell walls. However, scientists believe that parallel model which is a layered model is more consistent with experimental data than the scaffold model. Although the orientation of peptidoglycan is controversial, its basic structure remains the same (Dmitriev et al., 2003).

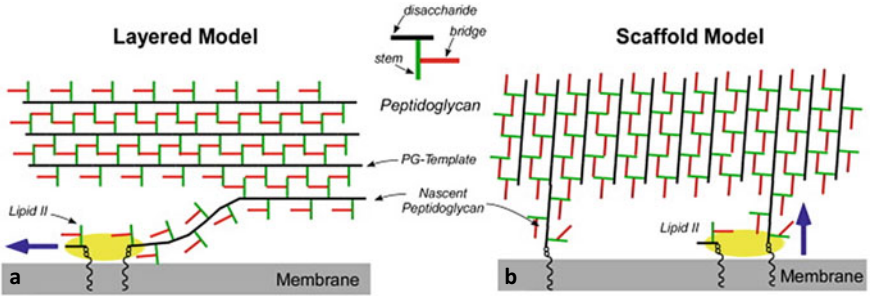


Fig. 2 **a** Layered model (parallel orientation) verus **b** scaffold model (perpendicular orientation). The blue arrows show the direction of PG chain elongation. *Source* Kim et al. (2015). With kind permission from Elsevier

Teichoic Acids (major accessory components of gram-positive cell wall)

Teichoic acids are phosphodiester-linked polyol repeats found in bacterial cell walls of certain gram-positive bacteria. Teichoic acid was discovered by Armstrong et al. in 1958 (Brown et al., 2013). These polyol phosphate polymers are either ribitol or glycerol linked by phosphodiester bonds. They also have ester-linked D-alanine, *N*-acetylglucosamine, *N*-acetylgalactosamine and glucose as substituent groups. These groups vary with bacterial species and can act as antigenic determinants.

Teichoic acids can either be covalently linked to peptidoglycan called **wall teichoic acids or WTA** or connected to the cell membrane via a lipid anchor, called as **lipoteichoic acid** (Fig. 3). Since these polymers are highly negatively charged, they serve as cation-sequestering molecules (Neuhaus & Baddiley, 2003). The wall teichoic acid is linked to peptidoglycan by disaccharide formed by *N*-actyl glucosamine and *N*-acetyl muramic acid polymer which in turn has one or two polyol phosphate units attached to C4 of *N*-acetylmuramic acid (Fig. 4) (Brown et al., 2013).

WTAs play various roles in bacterial cell wall and help the organism in many ways. Many biosynthetic enzymes of WTAs and peptidoglycan are reported to interact with each other, and rod-shaped bacterial cells lacking WTA synthesis genes lose their shape and become spherical. This implies that WTAs have a major role in cell division and growth. WTAs bear negative charge, and they interact with metal cations as well as protons in the medium. This helps in maintaining integrity of the cell wall as cation binding reduces the repulsion between phosphate groups. Moreover, proton binding influences the local pH of the cell wall and thus modulates the function of some enzymes localized at that area. Ion binding also reported to prevent fluctuation in osmotic pressure in and out of the cell. Hydrophobic nature of WTAs affects binding of various extracellular molecules to cell surface, for example, β-lactam antibiotics. WTAs are also known to play an important role in biofilm formation (Brown et al., 2013).

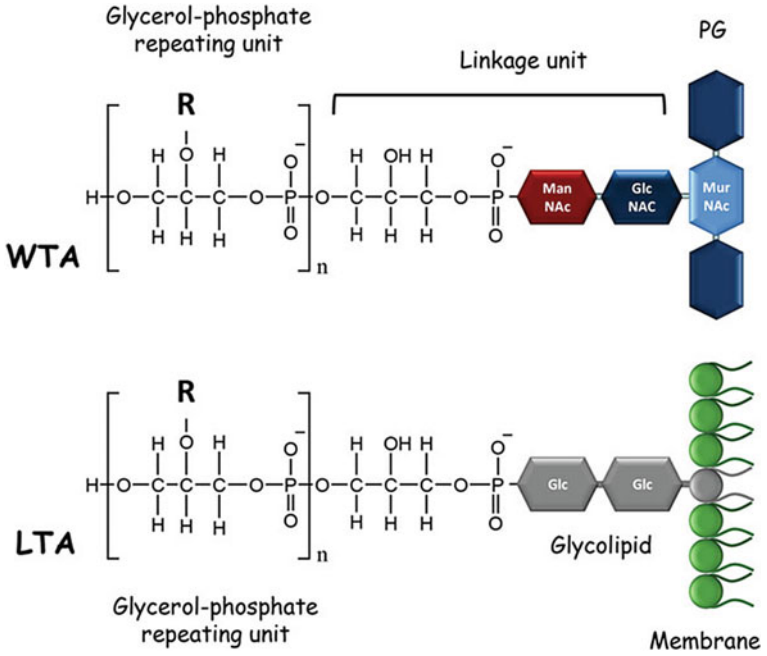


Fig. 3 Schematic representation of wall teichoic acid (WTA) and lipoteichoic acid (LTA) structure. Wall teichoic acid showing peptidoglycan attached to disaccharide which in turn is attached to phosphodiester polyol. *Source* Chapot-Chartier and Kulakauskas (2014). (Creative Commons Attribution License)

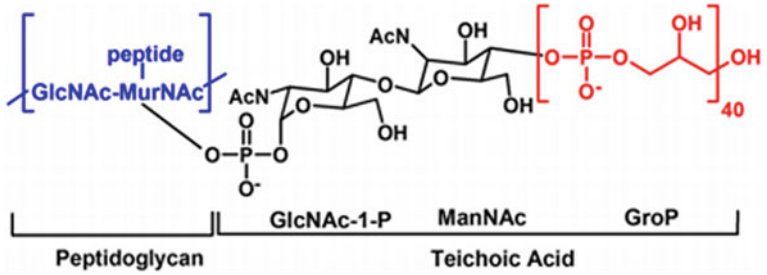


Fig. 4 Structural representation of the wall teichoic acid polymer from *B. subtilis* 168 where it is attached to peptidoglycan through the 6-hydroxyl of *N*-acetylmuramic acid. *Source* D'Elia et al. (2009). With kind permission from Elsevier

Lipopolysaccharides (major accessory components of gram-negative cell wall)

The gram-negative cell envelope is majorly composed of outer membrane, while peptidoglycan shares a small part of it. Though outer membrane is mostly like the plasma membrane, it also contains other macromolecules besides phospholipids. One

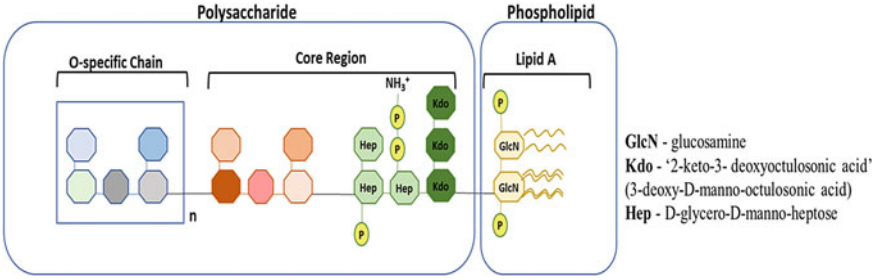


Fig. 5 General structure of LPS from gram-negative enterobacteria

of the main components is lipopolysaccharide (LPS). This is divided into two regions: phospholipid lipid A, which is phosphoglycolipid, and polysaccharide composed of core polysaccharide and O-specific chain (Fig. 5).

Fatty acid moieties of lipid A are characteristic of bacterial species, and it is attached to a disaccharide of glucosamine phosphate through amino groups of the latter. Fatty acids such as stearic acid (C18), palmitic acid (C16), myristic acid (C14), lauric acid (C12) and caproic acid (C6) are commonly found in lipid A region of LPS (Fig. 6) (Erridge et al., 2002).

Lipid A region of LPS is responsible for anchoring LPS to the outer membrane. It is also accountable for toxicity during infections by various gram-negative pathogens such as *E. coli*, *Salmonella* and *Shigella*, and thus known as **endotoxin**. It is responsible for eliciting the innate immune response and usually causes symptoms like vomiting, diarrhea and gas when humans are infected by entero-pathogens. Lipid A can also elicit endotoxic shock when secreted in large amounts into bloodstream.

Core polysaccharide: This region of LPS is more or less conserved among bacterial species. The core polysaccharide has two parts, the outer core and the inner core. The outer core (or the hexose region) is variable and generally consists of common hexose sugars such as glucose, galactose, *N*-acetyl galactosamine and *N*-acetyl glucosamine. On the other hand, the more conserved inner core is formed of unusual sugars like 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-mannoheptose (Hep) and is present in LPS of almost all organisms. The only exceptions till date are LPS of *Acinetobacter* and *Burkholderia cepacia* which contain 2-keto-D-glycero-D-talo-octonic acid (Ko) in place of Kdo. The bond between lipid A and Kdo residue can be hydrolyzed below pH 4.4 with release of O-antigen into the medium. Both inner and outer core sugar residues can be substituted with charged groups like phosphate, pyrophosphate, 2-aminoethylphosphate and 2-aminoethylpyrophosphate. In the inner core, it maintains a close association with the Ca^{2+} and Mg^{2+} ions that are required for membrane structure and function (Erridge et al., 2002).

O-specific polysaccharide: It is the farthest end of LPS and is made up of different sugar molecules. Sugars, such as glucose, galactose, rhamnose, mannose, including dideoxyhexoses, such as abequose and colitose, form a four- or five-membered branched sequence and behave as a single unit. O-specific polysaccharide consists of

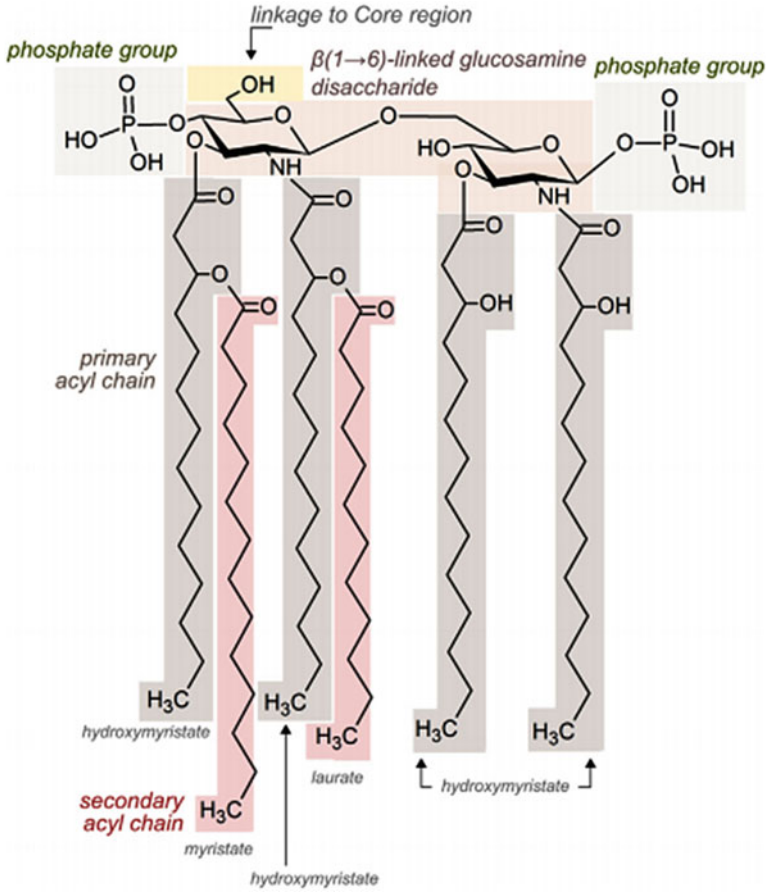


Fig. 6 Detailed structure of *E. coli* lipid A; *E. coli* lipid A contains a (1 → 6)-linked glucosamine disaccharide backbone (light brown); primary acyl chains (light gray) directly linked to the sugar moieties; secondary acyl chains (light red) esterified to hydroxyl groups of primary acyl chains. *Source* Steimle et al. (2016). With kind permission from Elsevier

many repeats (up to 50 repeats) of such oligosaccharide units and forms a long chain. Arrangement of these oligosaccharide units is highly species- and strain-specific. LPS containing O-specific polysaccharide region is called smooth LPS, while LPS lacking this region is known as rough LPS.

O-specific polysaccharide is also called O-antigen because during infection O-specific polysaccharide comes in contact with the host cell at first and behaves as an antigen (Alexander & Rietschel, 2001).

2 Proteins Present in Outer Membrane

Apart from phospholipids and LPS, outer membrane also contains various proteinaceous macromolecules like lipoproteins and porins. **Lipoproteins** are amino peptide chains linked with lipid moiety at their N-terminal. This class of lipoproteins covalently attached to peptidoglycan is called murein lipoproteins, and they help in the anchoring of outer membrane to peptidoglycan layer. In *E. coli*, about one-third of the murein lipoprotein is bound to the peptidoglycan. Some lipoproteins are also present on the outer leaflet of the outer membrane, but their functions are yet to be elucidated.

Porins are another major class of membrane-bound protein present exclusively in gram-negative bacteria. These are water-filled pores embedded in the outer membrane and provide size-specific internalization of nutrients while providing outer membrane its selective permeability. They are the most abundant outer membrane proteins that impart a sieving capability to membrane and offer protection against the toxic extracellular components while sufficiently allowing nutrient uptake (details are provided in Chap. 6: Solute Transport).

3 Biosynthesis of Bacterial Cell Wall

The peptidoglycan layer is composed of polysaccharides with alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) saccharide groups which are cross-linked in between by a peptide bridge. The formation of this structure requires various enzymatic reactions acting on different substrates in different compartments of the cell.

The cell wall synthesis pathway can be divided into the following four steps:

(i) Synthesis of precursor molecules
(ii) Formation of bactoprenol-disaccharide pentapeptide
(iii) Translocation of bactoprenol-disaccharide pentapeptide to periplasmic side; and
(iv) Polymerization and elongation.

In this section, each step of cell wall synthesis is discussed in detail along with the antibiotics that are used to inhibit cell wall synthesis.

Synthesis of precursor molecules

The formation of precursor molecules, viz. UDP-NAG and UDP-NAM-pentapeptide, can be divided into three major reaction steps that take place in cytoplasm:

i. Synthesis of UDP-NAG
ii. Conversion of UDP-NAG to UDP-NAM
iii. Formation of UDP-NAM-pentapeptide.

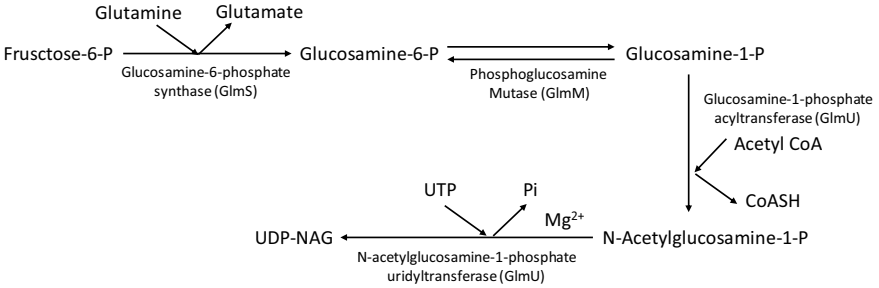


Fig. 7 Conversion of fructose-6-phosphate to UDP-NAG

Synthesis of UDP-NAG: It initiates from fructose-6-phosphate through four sequential reactions catalyzed by three enzymes, viz. GlmS (glucosamine-6-phosphate synthase), GlmM (phosphoglucosamine mutase) and GlmU (glucosamine-1-phosphate acetyltransferase and *N*-acetylglucosamine-1-phosphate uridyltransferase) (Fig. 7).

GlmS is a dimeric enzyme which acts as an amidotransferase which transfers amino group from glutamine to D-fructose-6-phosphate and converts it to D-glucosamine-6-phosphate, glucosamine-6-phosphate isomerases to glucosamine-1-phosphate by GlmM, an isomerase with mutase activity. Finally, UDP-NAG is formed by first transfer of acetyl group, and then, *N*-acetyl glucosamine is transferred to UDP molecule to form UDP-NAG in the presence of Mg + 2 and UTP with release of inorganic phosphate molecule. These reactions are catalyzed by a bifunctional enzyme GlmU (Barreteau et al., 2008).

Conversion of UDP-NAG to UDP-NAM—Some of UDP-NAG is subsequently converted to UDP-NAM in two steps, catalyzed by enolpyruvate transferase (MurA) and UDP-NAG enolpyruvate reductase (MurB) enzymes (Fig. 8). At this stage, antibiotic 'fosfomycin' can inhibit the conversion of UDP-NAG to UDP-NAM by inhibiting MurA enzyme. Fosfomycin is a structural analog of phosphoenolpyruvate (PEP) and forms a suicidal intermediate with MurA enzyme and inhibits the catalysis.

Formation of UDP-NAM-pentapeptide—UDP-NAM is further modified by incorporating alternating L- and D-amino acids in a form of a chain, albeit sequentially. Each amino acid is incorporated by specific ligase enzyme: L-alanine by MurC; D-glutamic acid by MurD; meso-diaminopimelic acid by MurE; and D-alanyl-D-alanine

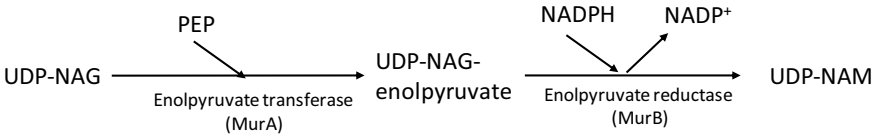


Fig. 8 Conversion of UDP-NAG to UDP-NAM

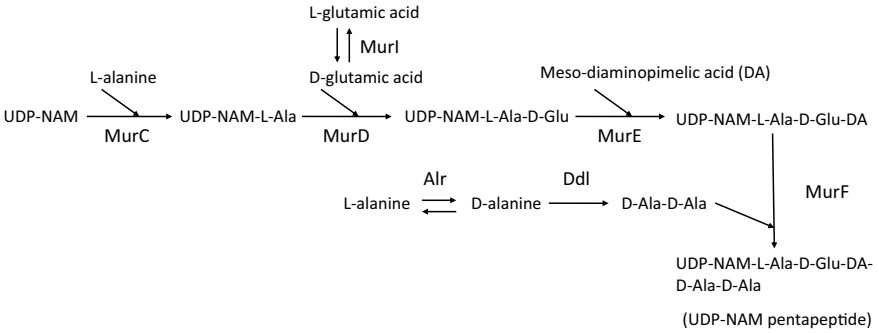


Fig. 9 Attachment of peptide to UDP-NAM showing addition of amino acids by respective ligases

by MurF. Moreover, racemases (MurI and Alr) are involved in the conversion of L-amino acids, viz. L-glutamic acid and L-alanine to their D-isomers, viz. D-glutamic acid and D-alanine, respectively. Terminal two D-alanine molecules are firstly joined together to form a dipeptide by a ligase enzyme, DdlA, and then is joined to the peptide chain linked to UDP-NAM moiety. Antibiotic 'D-cycloserine' inhibits both D-alanine racemase (Alr) and D-alanine ligase (DdlA) enzymes and thus inhibits this step of precursor generation (Fig. 9) (Barreteau et al., 2008).

Formation of bactoprenol-disaccharide-pentapeptide

The membrane-embedded bactoprenol is synthesized by undecaprenyl pyrophosphate synthase (UppS) enzyme on the cytoplasmic side of the membrane. UppS consecutively condenses eight isopentenyl pyrophosphates (IPP) to a farnesyl pyrophosphate (FPP) molecule with the formation of new cis-bonds and results in the formation of undecaprenyl pyrophosphate (C55-PP) which is dephosphorylated by undecaprenyl pyrophosphate phosphatase (UppP) to produce undecaprenyl phosphate (C55-P).

Bactoprenol-disaccharide-pentapeptide is then formed by subsequent action of two integral membrane enzyme, viz. phospho-NAM-pentapeptide translocase (MraY) and glycosyltransferase MurG. Firstly, MraY catalyzes the transfer of phospho-NAM-pentapeptide moiety from UDP-NAMpp to bactoprenol and yields UMP and bactoprenol-NAM-pentapeptide or **lipid I**. Next, MurG transfers NAG moiety from UDP-NAG to lipid I to produce bactoprenol-NAM-(pentapeptide)—NAG or **lipid II** (Fig. 10). This bactoprenol—disaccharide-pentapeptide or lipid II is then transferred to the outer side of the membrane from inner side by an enzyme known as flippase (Liu & Breukink, 2016).

Formation of lipid I can be inhibited by 'tunicamycin' antibiotic as it inhibits the catalytic action of MraY glycosyltransferase enzyme. Lipid II (bactoprenol-disaccharide-pentapeptide) is only allowed to translocate outside.

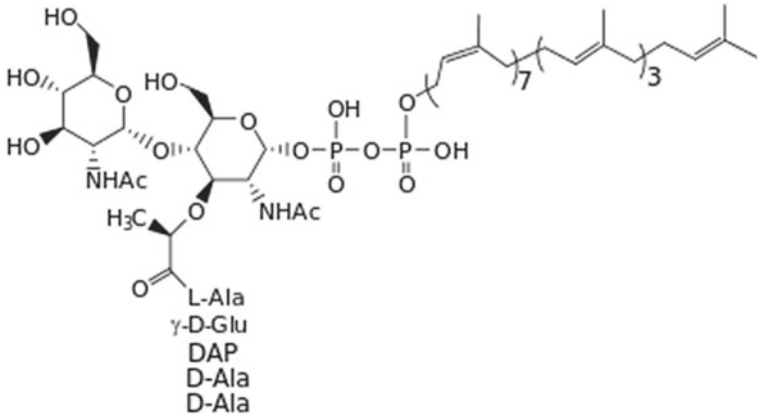


Fig. 10 Lipid II. *Source* Borbás and Herczegh (2011). With kind permission from Elsevier

Translocation of bactoprenol-disaccharide pentapeptide to periplasmic side by enzyme flippases

At this step, special enzymes known as flippases come into action and help in translocation of bactoprenol-disaccharide pentapeptide from the inner surface of the cytoplasmic membrane to the outer surface. Polytopic membrane proteins like FtsW, RodA, SpoVE and MurJ are suggested to catalyze this step and function as lipid II flippases. Complete mechanism by which these flippases assist the translocation of bactoprenol-disaccharide pentapeptide and which enzyme acts as a major flippase during cell wall synthesis is not known as yet. However, FtsW, RodA and SpoVE belong to the shape, elongation, division and sporulation (SEDS) family of enzymes and are suggested to play role as a flippase during division, elongation and sporulation, respectively. On the other hand, MurJ belongs to the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily of proteins and is reported to be important during cell wall synthesis in *E. coli* (Fig. 11).

Polymerization and elongation

As soon as bactoprenol-disaccharide pentapeptide moiety faces the periplasmic side of cytoplasmic membrane, periplasmic located glycosyltransferases and DD-transpeptidases catalyze the transfer of disaccharide-pentapeptide to the growing peptidoglycan chain and cross-link peptidoglycan chains via peptide bond formation, respectively (Fig. 12). Here, newly synthesized bactoprenol-disaccharide-pentapeptide is transferred to growing end of peptidoglycan with release of bactoprenol pyrophosphate. For the next cycle, bactoprenol is regenerated by its dephosphorylation.

Antibiotic ‘bacitracin’ inhibits its dephosphorylation by forming a metal-dependent complex with the lipid carrier bactoprenol pyrophosphate and thus interrupts the recycling of bactoprenol.

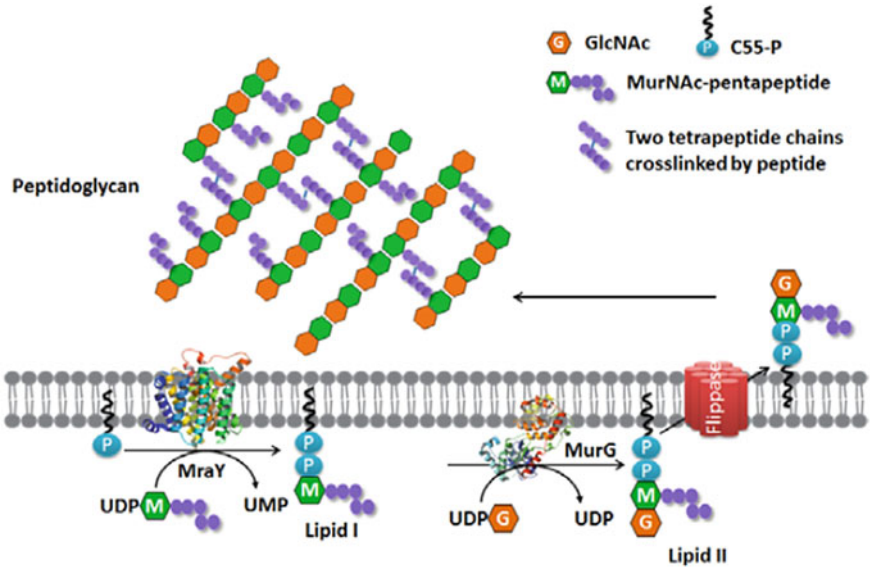


Fig. 11 Translocation of bactoprenol-disaccharide pentapeptide to periplasmic side. *Source* Liu and Breukink (2016). (Creative Commons Attribution License)

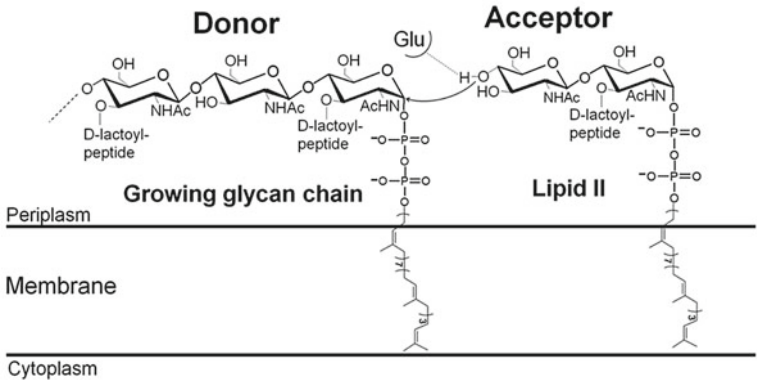


Fig. 12 Polymerization of peptidoglycan chain by glycosyltransferase. *Source* Derouaux et al. (2013). (Creative Commons Attribution License)

Cross-linking of two peptidoglycan chains takes place by transpeptidation in two steps. The first is release of terminal D-alanine residue of one of the pentapeptides. This cleavage step provides energy for the formation of a new peptide bond between D-alanine of this pentapeptide stem and non-alpha amino group of the dibasic amino acid in a second pentapeptide stem or the last amino acid of the peptide cross-bridge if it exists.

Penicillin-binding proteins

Penicillin-binding proteins (PBPs) are of two types: high-molecular-weight (HMW) PBPs and low-molecular-weight (LMW) PBPs. They are enzymes which exhibit glycosyltransferase and/or DD-transpeptidase activities belonging to the family of acyl serine transferases.

HMW PBPs are anchored to the cytoplasmic membrane by an N-terminal, signal peptide. The C-terminal domain is the penicillin-binding domain, which exhibits the transpeptidation activity and cross-links the peptidoglycan chains. Both the domains are located outside the cytoplasmic membrane. Major function of HMW PBPs is transpeptidation and transglycosylation reactions during peptidoglycan synthesis. LMW PBPs have DD-carboxypeptidases activity (although some exhibit transpeptidase or endopeptidase activity). Summary of cell wall biosynthesis pathway along with inhibitors is presented in Fig. 13 and Table 1.

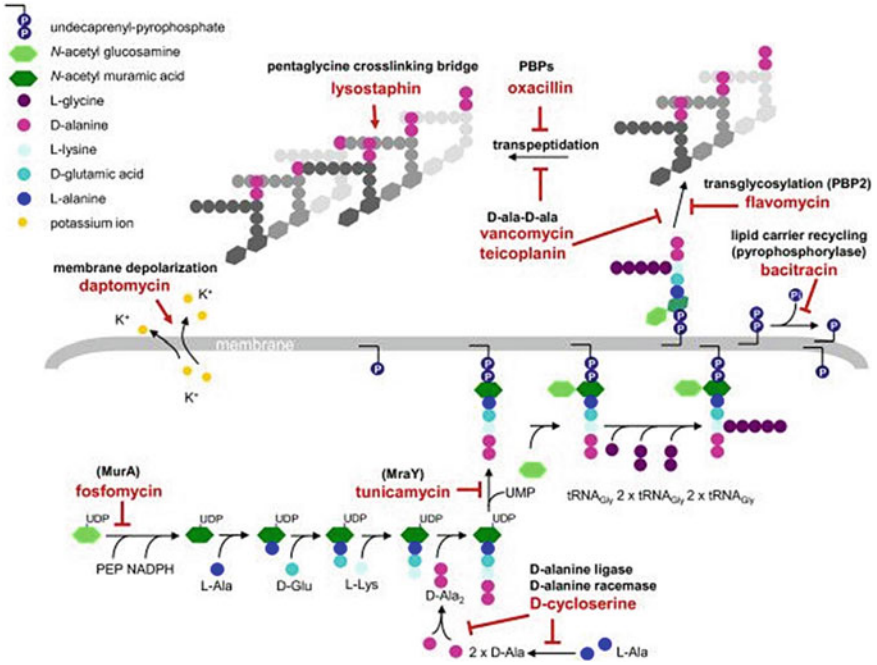


Fig. 13 Complete pathway of peptidoglycan synthesis along with the antibiotics (in red) inhibiting at various steps. *Source* Dengler et al. (2011). (Creative Commons Attribution License)

Table 1 Action of different enzymes involved in cell wall synthesis and antibiotics/inhibitors against some of these enzymes

Enzymes	Reaction catalyzed	Inhibitor (s)
Enolpyruvate transferase (MurA)	PEP + UDP-NAG → UDP-NAG-enolpyruvate + Pi	Fosfomysin
UDP-NAG enolpyruvate reductase (MurB)	UDP-NAG-enolpyruvate → lactyl ether of UDP-NAM	
Ligase enzyme MurC	Ligate L-alanine to UDP-NAM	
Ligase enzyme MurD	Ligate D-glutamic acid to UDP-NAM-L-ala	
Ligase enzyme MurE	Ligate meso-diaminopimelic acid (DA) to UDP-NAM-L-ala-D-glu	
Ligase enzyme MurF	Ligate D-alanyl-D-alanine to UDP-NAM-L-ala-D-glu-DA	
Racemase MurI	Convert L-glutamic acid to D-glutamic acid	
Racemase Alr	Convert L-alanine to D-alanine	D-cycloserine
Ligase enzyme DdlA	Ligate 2 D-alanine together	D-cycloserine
Undecaprenyl pyrophosphate synthase (UppS)	Synthesize C55-PP	
Undecaprenyl pyrophosphate phosphatase (UppP)	Dephosphorylate C55-PP to C55-P or bactoprenol	
Phospho-NAM-pentapeptide translocase (MraY)	UDP-NAMpp + Bactoprenol → UMP + bactoprenol-NAM-pentapeptide (lipid I)	Tunicamycin
Glycosyltransferase MurG	UDP-NAG + lipid I → bactoprenol-NAM-pentapeptide-NAG (lipid II)	
Phosphorylase	Lipid carrier recycling	Bacitracin
PBP2	Transglycosylation	Flavomycin; vancomycin; teicoplanin
PBPs	Transpeptidation	Oxacillin

4 Lipopolysaccharide Synthesis

Lipid A biosynthesis in *E. coli* is also known as Raetz pathway and is composed of nine enzyme-catalyzed reactions. Like peptidoglycan synthesis, lipid A biosynthesis also occurs in different compartments of the bacterial cell. The first three steps are catalyzed by cytoplasmic enzymes; fourth and fifth reactions are catalyzed by peripheral membrane enzymes, while remaining four steps are catalyzed by integral membrane enzymes.

The first reaction of lipid A biosynthesis is the acylation of the sugar nucleotide UDP-NAG. The UDP-NAG acyltransferase (LpxA) enzyme, that catalyzes the first reaction, is quite selective in nature and its active site functions as an accurate hydrocarbon ruler. For example, in *E. coli*, it is highly selective for β-hydroxymyristate,

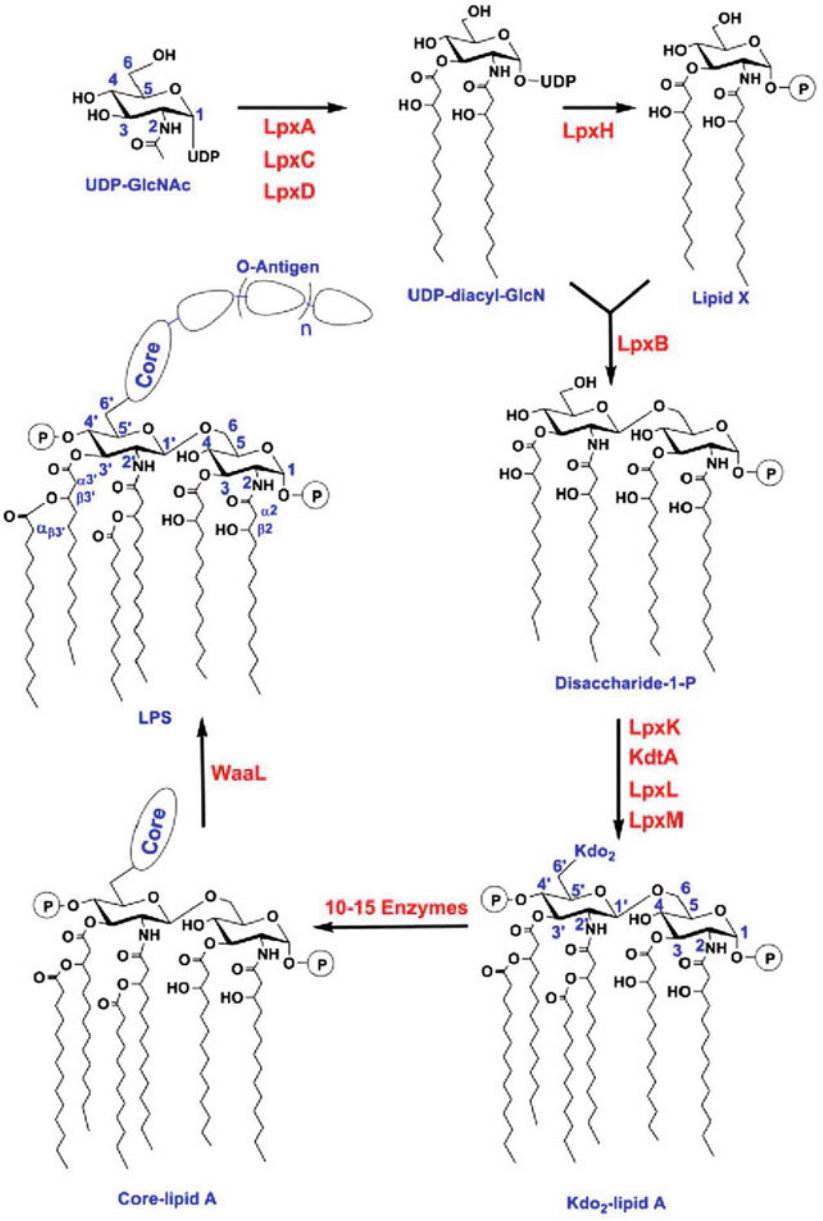


Fig. 14 Biosynthetic pathway of lipopolysaccharide in bacteria. *Source* Wang and Quinn (2010). With kind permission from Elsevier

that is, a 14-carbon acyl chain, and shows two times faster incorporation with a 14-carbon molecule than 12- or 16-carbon acyl chains.

Under physiological conditions, acylation of UDP-NAG by LpxA is unfavorable due to very low equilibrium constant, of approximately 0.01, for this reaction. Therefore, a quick deacetylation of UDP-3-O-(acyl)-NAG, formed in the previous reaction, is done by a zinc metalloenzyme LpxC, so that the lipid A biosynthesis pathway can proceed further. Thus, reaction catalyzed by LpxC is the committed step of Raetz pathway and is also an excellent target for drug development against gram-negative bacteria.

After deacetylation, a second β-hydroxymyristate moiety is added to the more stable deacetylated product by LpxD using ACP thioesters as substrate similar to LpxA, thus generating UDP-2,3-diacylglucosamine.

Lipid A biosynthesis pathway is now carried forward by peripheral membrane proteins LpxH (a pyrophosphatase) and LpxB (a disaccharide synthase). Pyrophosphatase LpxH cleaves the pyrophosphate bond of UDP-2,3-diacylglucosamine to form 2,3-diacylglucosamine-1-phosphate, also known as lipid X. Following this cleavage, the disaccharide synthase LpxB catalyzes the condensation of another molecule of UDP-2,3-diacylglucosaminem with lipid X in order to generate a β, 1′-6 linked disaccharide (Wang & Quinn, 2010).

Now, this disaccharide is handed over to integral membrane enzymes that catalyze the remaining four steps of the pathway. Foremost, the disaccharide is phosphorylated at its 4′ position by a specific kinase, LpxK. This phosphorylation step results in a product, named as lipid IV_A, that possesses some properties of endotoxins (Raetz & Whitfield, 2002).

Next to lipid IV_A formation, two Kdo residues are transferred by a bifunctional WaaA enzyme (also known as KdtA) and finally in order to generate a six acyl chain product, the late acyltransferases, LpxL and LpxM add lauroyl and myristoyl chains to the KDO_2-lipid IV_A, thus giving the final product, KDO_2-lipid A (Fig. 14).

KDO_2-lipid A is now joined to core oligosaccharide and then flipped from inner leaflet to the outer leaflet of the inner membrane by MsbA, an ABC transporter. Finally, O-antigen is added by several enzymatic reactions to form LPS, which is then transported to the outer leaflet of the outer membrane (Emiola et al., 2015).

Summary

- Bacterial cell wall is composed of peptidoglycan formed by alternating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) saccharide groups which are cross-linked in between by a peptide bridge.
- The cell wall synthesis pathway can be divided into the following four steps, viz. synthesis of precursor molecules; formation of bactoprenol-disaccharide pentapeptide; translocation of bactoprenol-disaccharide pentapeptide to periplasmic side; and polymerization and elongation.
- Synthesis of precursor molecules takes place in the cytoplasm.

- UDP-NAG is synthesized from fructose-6-phosphate followed by the formation of UDP-NAM from UDP-NAG. Peptide chain is linked to form UDP-NAM-pentapeptide.
- UDP-NAG is converted to UDP-NAM in two steps catalyzed by enolpyruvate transferase (MurA) and UDP-NAG enolpyruvate reductase (MurB) enzymes.
- Undecaprenyl phosphate (C55-P) or usually known as bactoprenol is hydrophobic C_{55} isoprenoid molecule which acts as a membrane carrier for the translocation of monomer unit of peptidoglycan, i.e., β (1–4) linked NAM-pentapeptide-NAG, to the periplasmic surface of the inner membrane.
- MraY catalyzes the transfer of phospho-NAM-pentapeptide moiety from UDP-NAMpp to bactoprenol and yields UMP and bactoprenol-NAM-pentapeptide or **Lipid I**.
- MurG catalzses formation of bactoprenol-NAM-(pentapeptide)—NAG or **Lipid II**.
- Bactoprenol-disaccharide-pentapeptide or lipid II is flipped to the outer side of the membrane the enzyme flippase.
- Formation of lipid I can be inhibited by 'tunicamycin' antibiotic as it inhibits the catalytic action of MraY glycosyltransferase enzyme.
- Periplasmic located glycosyltransferases and DD-transpeptidases catalyze the transfer of disaccharide-pentapeptide to the growing peptidoglycan chain and cross-link peptidoglycan chains via peptide bond formation, respectively.
- Antibiotics like oxacillin, vancomycin and flavomycin act upon these glycosyltransferases and DD-transpeptidases and inhibit their functioning.
- Flavomycin, vancomycin and teicoplanin also inhibit cell wall synthesis.
- Penicillin-binding proteins (PBPs) are the enzymes which exhibit glycosyltransferase and/or DD-transpeptidase activities and are classified as high-molecular-weight (HMW) PBPs and low-molecular-weight (LMW) PBPs. Beta-lactam antibiotics, such as oxacillin, bind the transpeptidase active domain.
- Lipid A biosynthesis in *E. coli* is also known as Raetz pathway and is composed of nine enzyme-catalyzed reactions.

Questions

1. Enumerate major steps in the synthesis of bacterial cell wall
2. What is the precursor molecule for the synthesis of bacterial cell wall? Write various biochemical reactions required for its synthesis
3. What is the mode of action of fosfomycin antibiotic?
4. What is the site of action of D-cycloserine?
5. What is the role of enzyme flippases?
6. Which step of cell wall synthesis will be inhibited in the presence of tunicamycin?
7. What is the other name of Raetz pathway?
8. Differentiate between lipid I and lipid II
9. What is teichoic acid? List differences between WTA and LTA
10. Explain the role of teichoic acid

11. What is antigenic in outer membrane of gram-negative bacteria?
12. Answer the following in one word.

    i. Antibiotic which inhibits alanine dipeptide addition to NAM
    ii. Lipid carrier of cell wall peptidoglycan
    iii. Other name of peptidoglycan
    iv. Amino acid present in the interbridge between two peptidoglycan chain in *Staphylococcus aureus*
    v. A unique amino acid present in all gram-negative bacteria
    vi. Acidic polysaccharide found in the cell wall of gram-positive bacteria
    vii. Enzyme which hydrolyzes peptidoglycan
    viii. Sugar present in cell wall of archaea
    ix. Unique lipid found in cell membrane of mycoplasma
    x. Part of lipopolysaccharide that is associated with toxicity
    xi. Enzyme which creates openings in the peptidoglycan during division
    xii. Specific biochemical reaction inhibited by penicillin during cell wall synthesis
    xiii. Antibiotic which inhibits NAG to NAM conversion
    xiv. Inhibitor for synthesis of lipid I
    xv. Antibiotic which inhibits phosphatase action on bactoprenol-disaccharide pyrophosphate
    xvi. Lipoprotein that holds the outer membrane of gram-negative bacteria with peptidoglycan
    xvii. Other name of lipid A synthesis pathway.

References

Alexander, C., & Rietschel, E. T. (2001). Invited review: Bacterial lipopolysaccharides and innate immunity. *Journal of Endotoxin Research, 7*(3), 167–202.

Barreteau, H., Kovač, A., Boniface, A., Sova, M., Gobec, S., & Blanot, D. (2008). Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiology Reviews, 32*(2), 168–207.

Borbás, A., & Herczegh, P. (2011). Synthesis of lipid II phosphonate analogues. *Carbohydrate Research, 346*(12), 1628–1632.

Brown, S., Santa Maria, Jr., J. P., & Walker, S. (2013). Wall teichoic acids of gram-positive bacteria. *Annual review of microbiology, 67*, 313–336.

Chapot-Chartier, M. P., & Kulakauskas, S. (2014). Cell wall structure and function in lactic acid bacteria. *Microbial Cell Factories, 13*(S1), S9.

D'Elia, M. A., Millar, K. E., Bhavsar, A. P., Tomljenovic, A. M., Hutter, B., Schaab, C., Moreno-Hagelsieb, G., & Brown, E. D. (2009). Probing teichoic acid genetics with bioactive molecules reveals new interactions among diverse processes in bacterial cell wall biogenesis. *Chemistry & Biology, 16*(5), 548–556

Dengler, V., Meier, P. S., Heusser, R., Berger-Bächi, B., & McCallum, N. (2011). Induction kinetics of the *Staphylococcus aureus* cell wall stress stimulon in response to different cell wall active antibiotics. *BMC Microbiology, 11*(1), 16.

Derouaux, A., Sauvage, E., & Terrak, M. (2013). Peptidoglycan glycosyltransferase substrate mimics as templates for the design of new antibacterial drugs. *Frontiers in Immunology, 4,* 78.

Dmitriev, B. A., Toukach, F. V., Schaper, K. J., Holst, O., Rietschel, E. T., & Ehlers, S. (2003). Tertiary structure of bacterial murein: The scaffold model. *Journal of Bacteriology, 185*(11), 3458–3468.

Emiola, A., George, J., & Andrews, S. S. (2015). A complete pathway model for lipid A biosynthesis in *Escherichia coli*. *PLoS ONE, 10*(4), e0121216.

Erridge, C., Bennett-Guerrero, E., & Poxton, I. R. (2002). Structure and function of lipopolysaccharides. *Microbes and Infection, 4*(8), 837–851.

Kim, S. J., Chang, J., & Singh, M. (2015). Peptidoglycan architecture of Gram-positive bacteria by solid-state NMR. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1848*(1), 350–362.

Liu, Y., & Breukink, E. (2016). The membrane steps of bacterial cell wall synthesis as antibiotic targets. *Antibiotics, 5*(3), 28.

Neuhaus, F. C., & Baddiley, J. (2003). A continuum of anionic charge: Structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiology and Molecular Biology Reviews, 67*(4), 686–723.

Raetz, C. R., & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual Review of Biochemistry, 71*(1), 635–700.

Steimle, A., Autenrieth, I. B., & Frick, J. S. (2016). Structure and function: Lipid A modifications in commensals and pathogens. *International Journal of Medical Microbiology, 306*(5), 290–301.

Vollmer, W., Blanot, D., & De Pedro, M. A. (2008). Peptidoglycan structure and architecture. *FEMS microbiology reviews*, *32*(2), 149–167.

Wang, X., & Quinn, P. J. (2010). Lipopolysaccharide: Biosynthetic pathway and structure modification. *Progress in Lipid Research, 49*(2), 97–107.

Part II
Bacterial Cell division and Growth

Chapter 4
Cell Division

Rani Gupta, Namita Gupta, and Amuliya Kashyap

Bacteria are prokaryotic cells that lack a nucleus and organelles with one or two chromosomes constituting their genetic material. Bacteria divide asexually by binary fission, a process in which cell increases in size to prepare for division following which a cleavage furrow develops in the cell membrane, and subsequently, the two daughter cells separate from each other. By this process, a bacterium creates an exact copy of itself. All the organisms within a colony being genetic equals, this process is also referred to as cellular cloning. Binary fission is different from mitosis employed by eukaryotic cells for division and growth in terms that binary fission lacks formation of spindle fibers for segregation of daughter chromosomes. However, both the processes are similar in that the organism first duplicates its DNA and subsequently divides into two daughter cells. Binary fission is also a simpler process in comparison with mitosis, making it a faster process, while mitosis includes checkpoints that regulate cell cycle progression from one stage to the other reflecting the complex nature of eukaryotic cells.

A newly formed bacterial cell must complete a plethora of tasks before it attains a stage of division and complete a cell cycle. A bacterial cell cycle is constituted by distinct-independent processes that operate simultaneously in a highly regulated, coupled and a coordinated manner. These processes include: DNA replication, segregation of sister chromosomes, precise positioning of division machinery and ultimately equal cytokinesis. Therefore, not only a bacterium has to replicate its DNA and double in size but also precisely position its division machinery to produce daughter cells with equal cell mass and chromosome. It is vital for a bacterial cell to regulate and coordinate all these processes both spatially and temporally to achieve equal division. These processes are 99.7% accurate as a result of which under normal conditions, DNA less cells are rarely formed.

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_4

1 Bacterial Cell Cycle

In eukaryotes, there are cell cycle checkpoints that ensure that the initiation of one step is dependent on the prior step. But, in bacteria, the cell cycle consists of overlapping set of parallel processes, and individual steps involved in different processes seem to be only loosely linked to one another. The bacterial cell cycle consists of three well-defined periods, viz B-period or the pre-replicative period, C-period or the replication period and D-period also called the division period. B-period is similar to the G1 phase of the eukaryotic cells and is the time taken by bacterial cell to prepare for the cascade of events expected ahead during cell cycle. During this period, the cell mass increases. This period spans between birth of a newly formed daughter cell and just before initiation of DNA replication.

C-period is the DNA replication period and is divided into three stages: initiation, elongation and termination. It functionally corresponds to S phase of eukaryotic cell cycle and is considered as the key phase. During this period, separation of sister chromosomes occurs concurrently with DNA replication. In eukaryotes, chromosomal segregation occurs in the M-phase which is separated from the S phase by intermittent G2 phase. In this G2 phase, replication is completed and some time passes before chromosomes are segregated. However, in bacteria, replication and segregation occur concomitantly in the C-phase, and hence, a separate G2 phase is absent. In fast-growing cells, C-period can be sub-divided into C1 and C2 period, where C1 is the period for replication of pre-initiated inherited chromosome, while C2 is the time taken for initiation of another round of replication (Wang & Levin, 2009). Following C-period is the D-period where the cell ultimately divides into two daughter cells. This division is accomplished by assembly of multiple proteins into a complex termed as divisome and is described in later sections of the chapter.

Box 1: Central Dogma

The 'Central Dogma of Bacterial Cell Division Cycle' also known as BCD dogma suggests that before the cell divides into two daughter cells, the process of chromosome replication and doubling of cell mass should complete in order to ensure viability of daughter cells. As per this theory, the cell mass at the time of replication initiation, termed Mi, is roughly constant per *oriC* (Zaritsky & Woldringh, 2015). According to the theory, a cell cycle completes (C + D) minutes later, when the cell mass reaches Mi X $2^{(C+D)/t}$. The parameters considered in this theory however differ between species and vary depending on environmental conditions, mutations and presence of inhibitory drugs.

The concept of multi-fork replication in fast-growing *E. coli* was further explained by this model. To maintain the balance, multi-fork replication is initiated with constant progression rates by the faster growing and dividing cells. The model states that when the sum of C and D periods is less than the doubling time of bacteria,

then a B-period or gap between division and replication initiation will be observed. However, when the sum total of C and D periods is greater than the doubling time of bacteria, an overlapping cell cycle will be observed (Cooper & Helmstetter, 1968). The latter is exactly what is observed in fast-growing cells which then adopt to multi-fork replication to ensure genome integrity in the daughter cells. However, the model has certain limitations. The model considers the process of replication as the key point in deciding the fate of cell cycle. It overlooks the effect of nutrient-dependent and metabolic changes on events occurring after replication initiation. The model also fails in explaining the reliability of single round of replication initiation per cell cycle.

Cooper–Helmstetter Model

Stephen Cooper and Charles E. Helmstetter proposed a model that specified the relationship between chromosome replication and division of *E. coli* B/r strain. Nutrient availability is one of the strongest determinants of cell size and of the time taken to complete one cell cycle in both *E. coli* and *B. subtilis.* When the cells are grown in nutrient-rich media, the generation times are shorter and the size can be almost double the size of their slow-growing counterparts. Nutrient-dependent increase in cell size is basically a means to accommodate additional amount of DNA generated at faster growth rates. The generation time of *E. coli* is 20 min, while the time taken for replication of the complete chromosome is 40 min. This situation requires cells to initiate a second round of replication before termination of the previous round. This results in multi-fork replication allowing shorter generation time to maintain faster growth rates (Fig. 1).

2 Chromosomal Replication and Segregation

The process of replication is initiated by highly conserved AAA^+ ATPase, DnaA. Replication initiation depends on the concentration of DnaA and on the methylation status of the ori region (Mott & Berger, 2007). DnaA binds unique and specific DNA locus adjacent to the origin *oriC* and causes DNA melting (Fig. 2). However, for DnaA to bind at its specific site on the chromosome, it must reach certain threshold concentration in the cell which is attained at a specific cell size. This cell size at which the DnaA has reached a critical concentration is termed as initiation mass required to trigger replication. Strand separation upon DnaA binding allows access to DNA polymerase III along with its accessory proteins. The replication fork thus generated proceeds bi-directionally around the circular chromosome till it reaches the terminus site, *terC*. DNA methylation also plays an important role in governing initiation of DNA replication. There are multiple GATC sites present in or near *oriC* and in the *dnaA* promoter region. These GATC sites in the origin region must be fully methylated for replication to initiate. This is due to the fact that methylation of DNA enhances the extent of its bending which in turn is important for replication initiation. In addition to these two factors, it has also been demonstrated in *E. coli* that in response to certain stress conditions such as amino acid or carbon starvation,

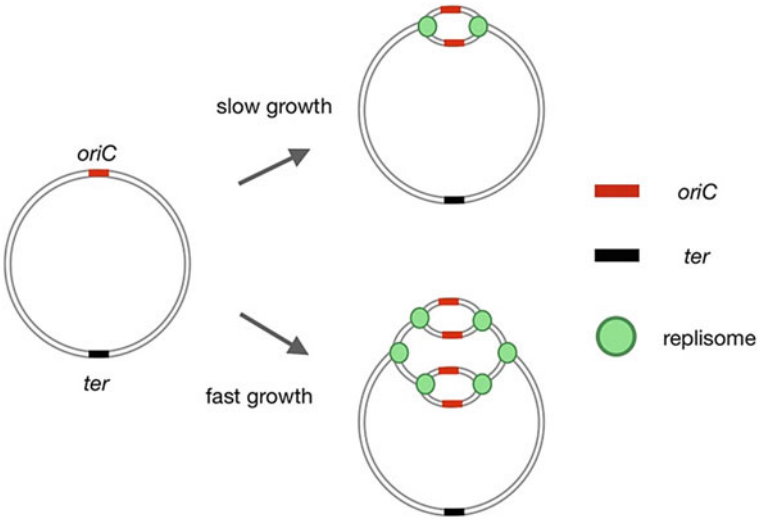


Fig. 1 In rapidly growing cells, a new round of replication begins even before the preceding round has completed. This leads to multiple forks of replication which makes sure that at least one round of replication finishes completely before cytokinesis. This in turn ensures that each daughter cell receives a complete set of chromosome after division. *Source* Trojanowski et al., 2018. (Creative Commons Attribution License)

small nucleotides of guanosine tetraphosphate (ppGpp) also regulate transcription, and their concentration is found to be inversely proportional to bacterial growth rate. Therefore, the presence of these inhibitors will affect transcription and hence DnaA synthesis in the cell. This generates a nutrient-dependent control of DnaA expression and hence replication initiation.

After initiation, the replication continues from origin to the terminus accompanied by movement of replicating point, called the replication fork. The replication in *E. coli* is bi-directional where two replication forks form and move in opposite directions. A number of proteins interact at the replication fork forming a complex termed as the 'replisome assembly' and further elongate the DNA. The core of the replisome consists of helicase protein DnaB which causes further melting of template DNA (Fig. 2). It forms a hexamer of identical subunits and requires ATP and helicase loader protein DnaC for its activity. DnaB helicase moves along DNA duplex causing separation of two single strands in an ATP-dependent manner. The separated strands are inhibited from subsequent reannealing due to the presence of single-strand-binding protein, SSB, which immediately binds the separated single strands, thereby preventing duplex formation. Under stress conditions, replication fork can often break down leading to 'replisome collapse,' and such collapsed forks are reinitiated by proteins PriA, PriB, PriC and DnaT. These proteins are altogether termed as 'replication restart primosome.' PriA and PriC recognize collapsed forks followed by sequential recruitment of PriB and DnaT. After the DNA has unwound, RNA polymerase, called the primase (DnaG), synthesizes a short RNA sequence

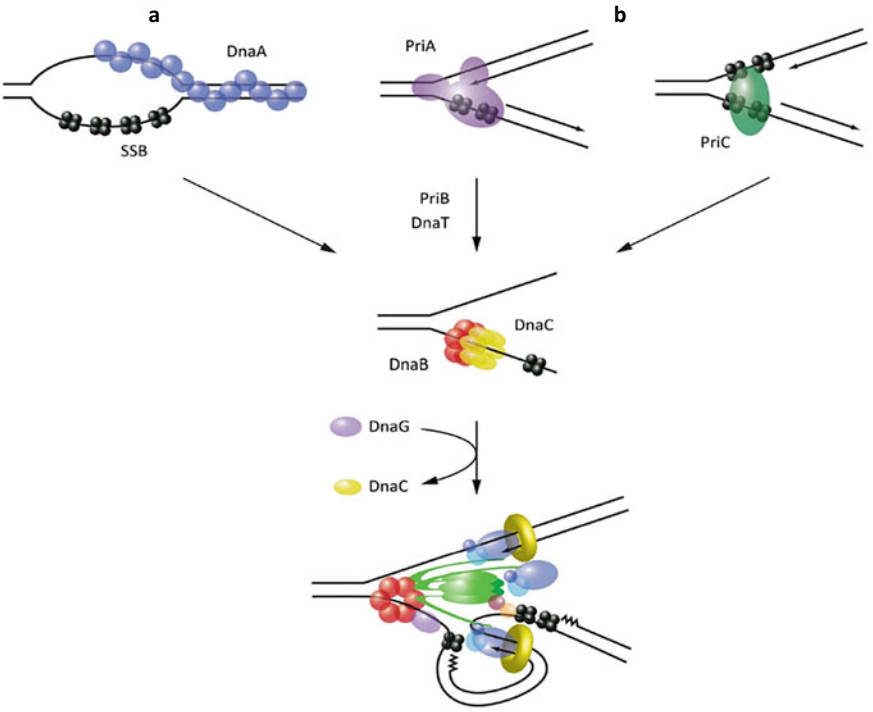


Fig. 2 *E. coli* DNA replication is initiated after binding of DnaA at *oriC* forming the initial complex. DnaA binding leads to melting or strand separation followed by SSB binding. Abandoned replication forks are recognized by PriA and PriC. All the three pathways lead to DnaB (helicase) binding along with the loader protein DnaC. DnaB unwinds DNA followed by polymerization by DNA Pol III. *Source* Beattie and Reyes-Lamothe (2015). (Creative Commons Attribution License)

complementary to template DNA strand. Primase disassociates from the DNA duplex after synthesizing a short primer, and this is further elongated by DNA polymerase III. During elongation, the strand that runs 5′-3′ in the replication fork is termed as the leading strand with continuous DNA synthesis, while the other is termed as the lagging strand formed as short pieces termed Okazaki fragments with each fragment requiring new primer. The Okazaki fragments are further joined as a single strand by DNA ligase, and the replication process is therefore termed 'semi-discontinuous.' The process of replication occurs till the terminator site *ter* (or *terC*) is reached following which topoisomerase IV and DNA gyrase lead to decatenation and separation of newly replicated DNA. After replication, parent and the daughter chromosome are resolved with the help of recombinases and DNA translocases at the terminus region. These recombinases and translocases are specific to bacteria such as XerCD recombinase and FtsK translocase in *E. coli* while RipX and CodV recombinases and SpoIIIE DNA translocase in *B. subtilis* (Beattie & Reyes-Lamothe, 2015).

Origin segregation—Following replication, the parent and the newly replicated daughter chromosome need to segregate. In both *E. coli* and *B. subtilis*, duplicated

chromosomes begin to separate from one another even before the completion of DNA replication. Unlike eukaryotes where there is formation of microtubule spindle fibers dedicated to pull daughter chromosomes away from each cell, prokaryotes utilize mechanisms that topologically and temporally regulate the segregation of chromosome and cytokinesis. In the bacterial cell, the replication machinery is tethered to the cell center and the newly replicated sister duplexes are pushed in opposite direction before cytokinesis. The chromosome remains highly organized throughout the replication process, and in a newborn cell, the origin is centered at the cell mid-point. After replication of the origin, the two copies of *oriC* migrate toward opposite poles in the cell. Subsequently, as the remaining regions of the chromosome are replicated, they separate from one another and trail their respective *oriC* toward opposite poles. Therefore, the first region in the chromosome to be segregated is origin-proximal region, and in most bacteria, origin is segregated actively by the ParAB*parS* partitioning system, with nearly 65% bacteria harboring this system (Badrinarayanan et al., 2015). Although the parABS system is very widespread, it is absent in *E. coli*. It was initially discovered in *Caulobacter crescentus* and deletion of this system often results in significant increase in number of anucleate cells indicating its importance in chromosome segregation. The system consists of three functional components:

1. *parS* sites that are *cis* acting DNA sequences located near the origin
2. ParB protein that specifically binds *parS* sites and
3. ParA which is a P-loop ATPase that binds *parS*-ParB nucleoprotein complex.

After the origins replicate, ParB spreads across the regions near the *parS* sites bridging distant portions of DNA by forming a nucleoprotein complex. ParA then forms dynamic filaments in the cell that segregate the *parS*-ParB sites by either pushing or pulling the nucleoprotein complex toward opposite poles. One of the origins after replication remains anchored at one pole by binding PopZ protein (pole organizing protein). It is a polarly localized protein that binds ParB*parS* complex anchoring it to one pole, while the complex at the other origin comes in contact with ATP-bound ParA. ParA-ATP can bind DNA non-specifically, but ParA-ADP shows weak DNA binding. Further, ParA has intrinsic weak ATPase activity which is stimulated by ParB. Therefore, ParA in ATP-bound state interacts with the ParB*parS* complex (Fig. 3), upon which its intrinsic ATPase activity is stimulated by ParB. Now, in the ADP bound state, ParA leaves the ParB-*parS* sites, and these alternate cycles of binding and leaving DNA generate either a pushing or a pulling force to move ParB-*parS* toward opposite poles causing segregation of *ori* (Surtees & Funnell, 2003).

In *B. subtilis*, similar mechanism operates for chromosomal segregation but involves proteins Soj and SpoJ, homologues of ParA and ParB, respectively, for chromosome segregation during vegetative phase (Badrinarayanan et al., 2015) while DivIVA, RacA and SpoIIIE exhibit crucial role in chromosome segregation during sporulation. DivIVA does not seem to get directly involved in the segregation process but assists movement of duplicated origin toward the poles. RacA is a DNA-binding protein that binds GC-rich inverted repeats near the origin region forming a highly stable nucleoprotein complex for facilitating the chromosome movement toward the

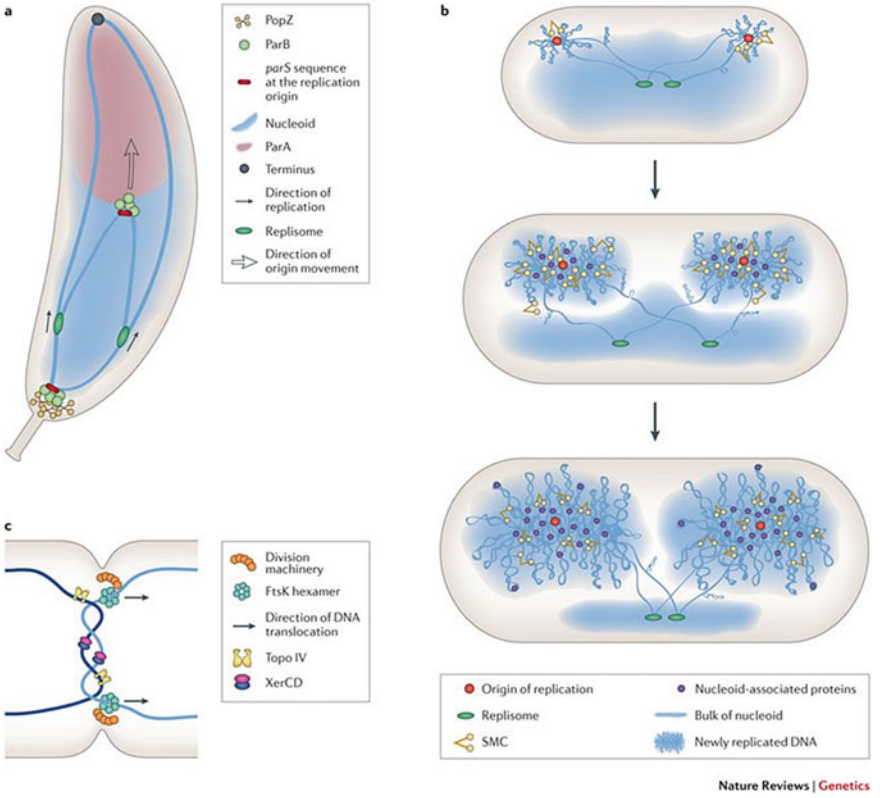


Fig. 3 **a** Segregation of origin in *C.crescentus* by ParAB*parS* partitioning system: Protein ParB binds *parS* sites present around replication origin. ParB*parS* present at one origin remains polarly localized by binding PopZ protein at one pole while complex at the other origin interacts with ATP-bound ParA. ATPase activity in ParA is then stimulated by ParB and ParA leaves the DNA complex as inactive ParA. This results in retraction of ParB*parS* complex toward the opposite pole and hence segregation of origins. **b** After newly replicated *oriC* have separated and moved to opposite poles, bulk chromosome segregation is facilitated by SMC complex and other proteins. **c** Segregation of *ter* region: Newly replicated sister chromosomes are separated by DNA translocase FtsK that pumps them toward opposite poles. Topoisomerase IV de-entangles sister chromosomes by eliminating topological constraints while XerCD recombinase them from the dimeric forms. *Source* Wang et al. (2013). With kind permission from Springer Nature

opposite poles of the cell. SpoIIIE is a DNA translocase which leads the completion of segregation process. After the origins are segregated by partitioning system, they are anchored at the opposite poles by certain proteins that bind specific DNA regions. In *B. subtilis*, RacA protein accumulates at the pole and binds a RacA-binding motif proximal to *oriC*, hence anchoring it to the cell pole. No such polar anchoring complex has been identified in *E. coli.*

Bulk chromosome segregation—Once the chromosomal origins separate after replication, remaining bulk chromosome trails their respective origins. Soon after the

origins separate, the remaining chromosome is condensed lengthwise by concerted action of supercoiling and structural maintenance of chromosome (SMC) complex proteins (Wang et al., 2013). These proteins drive lengthwise compaction of chromosomes driving them away from each other, and they ultimately separated by Topoisomerase IV that eliminates pre-catenated entanglements (Fig. 3). The functional analog of SMC complex in *E. coli* is the MukBEF complex which is utilized for chromosome segregation (Murray & Sourjik, 2018). This complex clusters around the origin region and contributes to chromosome segregation; however, the exact mechanism by which MukBEF complex functions to segregate chromosome is still unclear. Here, it is important to note that the generation time of *E. coli* is 20 min, while the time taken for replication of the complete chromosome is 40 min. This is because the organism has the capacity to initiate a second round of replication before termination of the previous round, and hence, multi-fork replication allows for such shorter generation times.

Terminus Separation—After sequential segregation of *oriC* and bulk chromosome, it is the terminus that has to separate for achieving ultimate chromosome segregation. Segregation of *ter* regions requires dedicated machinery as replication of circular chromosomes can result in catenated forms. As mentioned earlier, topoisomerase IV plays a very important role here. It de-entangles and relaxes the sister chromosomes, eliminating any topological constraint which allows movement of sister chromosomes to opposite poles. Another major contributor to the *ter* segregation machinery is the divisome protein FtsK, a DNA translocase that pumps newly replicated sister chromosomes to opposite poles of the cell. FtsK also directly activates recombinase XerCD that carries out site-directed recombination between sister chromosomes in order to resolve them from the dimeric forms (Fig. 3).

Spatial Regulation of DNA replication in dimorphic bacterium *Caulobacter cresentus*

Besides *E. coli* and *B. subtilis*, another organism *C. cresentus* has long been a significant system for uncovering unfamiliar mechanisms that operate during bacterial cell cycle. *C. cresentus* belongs to the class of α-proteobacteria and is an aquatic gram-negative oligotrophic bacterium inhabiting nutrient-poor environment. It is an important model to study cell cycle regulation, asymmetric cell division and cellular differentiation. The bacterium attains its distinct crescent shape due to the presence of crescentin, a bacterial cytoskeletal protein which is homologous to eukaryotic intermediate filament proteins. It exhibits an elaborate life cycle featuring regulated changes in the cell shape during asymmetric cell division. It also strikingly displays dimorphic cell cycle with two types of cells, stalked and swarmer cells. A newborn swarmer cell is equipped with pili and a single flagellum at one of its pole. It is incapable of DNA replication and dedicates its energy toward motility and dispersal. With time, the swarmer undergoes differentiation at its flagellar pole. It begins to secrete a polysaccharide adhesive called the holdfast which mediates permanent attachment to surfaces. The swarmer cell soon loses its flagella and pili and develops a thin extension of its cell envelope termed as the stalk. This newly developed stalked cell is reproductively mature and divides to produce daughter swarmer cells, which

then grow further in similar dimorphic manner. Now, does a cell decides that DNA replication will occur only in the stalked cells but not in the swarmer cells? This differentiation is achieved by virtue of a protein called CtrA that belongs to the response regulator family of transcription factors. It prevents DNA replication by binding to the chromosomal origin of replication but undergoes temporal degradation during the swarmer-stalked transition. CtrA regulation also leads to developmental asymmetry between the two cell types.

Soon after a stalked cell initiates DNA replication, CtrA is synthesized and activated. As the stalked cell further progresses in the cell cycle toward division, two important regulatory histidine kinases DivJ and PleC migrate toward opposite poles in the cell (Fig. 4). DivJ appears first, migrates to the stalked pole, and its signaling leads to CtrA localization, degradation and inactivation (Hughes et al., 2012). This results in one round of DNA replication after which PleC migrates to the opposite pole. When both DivJ and PleC are present in the stalked cell, the effect of PleC predominates meaning CtrA will be active, and its signaling will promote dispersal and activity of CtrA. This differential signaling allows only one round of DNA replication per division cycle or one can say that multi-fork replication does not occur in this organism. When the cell begins to constrict during the pre-divisional stage, the two cellular daughter compartments differentially inherit either DivJ or PleC. DivJ takes the stalked cell compartment, thereby abolishing CtrA activity in the stalked daughter cell and allowing immediate initiation of new round of replication. On the other pole, PleC maintains CtrA activity in the swarmer compartment, thereby

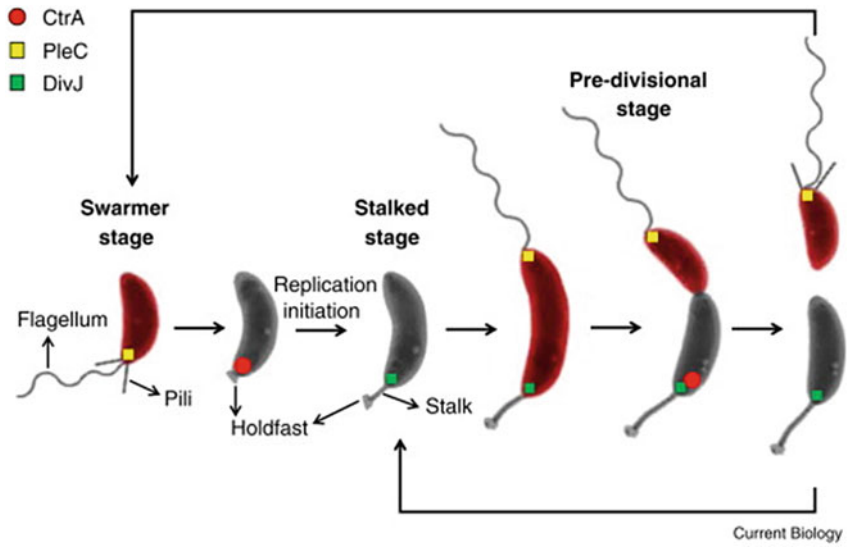


Fig. 4 Assymetric cell division in *C. cresentus*. Spatiotemporal pattern of three proteins, CtrA (red), PleC (yellow) and DivJ (green) regulates and drives differential asymmetry during progression of dimorphic cell cycle. *Source* Hughes et al., (2012) With kind permission from Elsevier

inhibiting DNA replication in the daughter swarmer cell until its timed degradation. Therefore, this differential localization of two different regulatory proteins at opposite poles drives developmental asymmetry between the daughter cells in *C. crescentus.*

As found in other bacteria, chromosomal segregation in *C. cresentus* is accomplished by ParABS partitioning system. Also as mentioned earlier, during replication of DNA, the polymeric protein PopZ mediates firm attachment of origin of replication to the cell pole both in the beginning and toward the end of cell cycle. As the replication machinery moves from one pole to the other, the newly duplicated chromosome along with the origin is translocated from one cell arm to the other or toward opposite poles in the cell. Similar movement of newly duplicated DNA toward opposite poles is also seen in *Myxococcus xanthus.*

Box 2: Cell Division in Archaea: Reminiscent of Eukaryotic Mitosis

Understanding about archaeal cell division still lags behind what is known about bacterial cell division. In fact, rather than simulating bacterial cell division, archaeal cell cycle resembles the features observed during eukaryotic cell cycle. Studies done on cell cycle of archaeal model organism *Sulfolobus* have yielded intriguing results. It is the best characterized archaeal genus, and understanding in terms of molecular and mechanical functioning of chromosomal replication has improved a lot. *Sulfolobus* spp. are extremophiles belonging to the phylum *Crenarcheaota* that thrive best in hot acid environments of 80 °C and pH3. Cell division in this archaea resembles eukaryotic mitotic cell cycle. The very first similarity lies in the fact that the daughter chromosomes in members of this genus are not segregated immediately after replication contrary to bacterial cell cycle where replication and segregation are concomitant. This feature clearly resembles eukaryotic cell cycle where intermittent G2 phase separates the replicative S phase and segregation M phase. In fact, in *Sulfolobus* spp., G2 phase consumes more than 50% of the cell cycle (Bernander, 2007), while the G1 phase is constituted by a very short interval (<5% of the doubling time) and S phase lasts for about one-third of the cell cycle. Alternating phases of replication and mitosis constitute the core of the *Sulfolobus* cell cycle, and inhibition of chromosome segregation results in cell division block. This specifies that checkpoint-like mechanisms function in *Sulfolubus* to stringently regulate replication, segregation and division.

Further, the genus *Sulfolobus* interestingly has three origins of replication just as multiple origins found in eukaryotes (Lundgren et al., 2004). The chromosome replication proteins are also homologous to those of eukaryotes. Bi-directional replication is initiated from each of the three origins giving rise to six replication forks, each polymerizing with the same rate. The rate of DNA polymerization is tenfold lower than that seen in *E. coli*, again sharing similarity with eukaryotes in terms of their

slower rates. As seen in eukaryotic replication, the Okazaki fragments synthesized during lagging strand replication in *Sulfolobus* are also smaller in length.

In archaea, the daughter chromosomes are segregated by two proteins SegA and SegB, similar to bacterial ParABS partitioning system. SegA is similar to bacterial ParA, but no counterparts of SegB are present, although it is thought to be functionally equivalent to ParB. In fact, the role of SegB is not clear, but it binds DNA at specific sequences. SegA, like ParA, polymerizes and exerts a pulling or pushing force to segregate chromosomes. Although the segregation process is similar to that seen in bacteria, cytokinesis in *Sulfolobus* spp. is similar to eukaryotic process. Cytokinesis is done using proteins similar to ESCRT proteins found in eukaryotes (Makarova et al., 2010). ESCRT proteins in eukaryotes are involved in endocytosis and named after the function performed by them: endosomal sorting complex required for transport. These ESCRT like division proteins found in archaea form a ring at the cell center that constricts to divide cell into equal halves.

The features mentioned above have been demonstrated in members of *Crenarcheaota,* and it should be kept in mind that not all archaeal groups exhibit these characteristics. Some archaeal groups lack a G2 phase and segregate the daughter chromosomes immediately after replication or parallelly at the same time. The chromosomal segregation system SegAB is also restricted to only few archaeal groups. In some groups, MinD homologue is used to segregate the sister chromosomes. Also, as seen in bacteria, many archaeal organisms belonging to Euarchaeota phylum utilize FtsZ protein that forms a septum at cell mid-point during division (Lindas et al., 2008).

3 Cytokinesis/Cell Division

Chromosomal replication and segregation is followed by cytokinesis where the parent cytoplasm divides equally into the progenies by a tightly spatially and temporally regulated system such that the cell divides exactly at the cell mid-point and only after chromosome has replicated completely. In *E. coli*, cell division is conducted by a dynamic protein complex of at least 10 essential proteins and is termed as 'divisome' or 'septasome.' In addition to 10 essential proteins, approximately, 15 non-essential proteins are involved as well. The genes coding for proteins involved in forming the divisome hyperstructure were named as filamentous temperature sensitive (Fts) as mutations in these genes led to formation of filamentous phenotype at non-permissive temperatures. The divisome ensures proper constriction, septal peptidoglycan synthesis and ultimately cell separation.

Essential Cell Division proteins/Divisome Machinery

FtsZ is the most essential structural protein which forms the Z-ring or the division septum at the cell mid-point. It also functions as a scaffold for the recruitment of other downstream proteins involved in formation of divisome assembly. FtsZ is therefore responsible for guiding the assembly, location and shape of division septum. It is a

highly conserved protein across major bacterial groups and in the archaeal branch of euryarchaea but is absent in the crenarchaea and few other bacterial groups. Chloroplasts in most photosynthetic eukaryotes also utilize nuclear-encoded FtsZ for division. This is a result of their endosymbiotic origin, and for the same reason, Ftsz has been found to be involved in mitochondrial division in several primitive eukaryotes; however, it is lost in higher eukaryotes including fungi, animals and plants.

FtsZ is a soluble protein structurally similar to tubulin and consists of three distinct domains: an N- terminal domain, a tubulin-like core domain and a C-terminal peptide (Fig. 5). N-terminal domain is variable while the C-terminal peptide is conserved and is essential for interaction with other cell division proteins (FtsA and ZipA) in order to anchor Z-ring to the cell membrane. The core domain binds and hydrolyzes GTP; binding of GTP induces its self-assembly into protofilaments. The core domain is highly conserved and contains sequences homologous to highly conserved tubulin motif. A single amino acid mutation in this region leads to conversion of GTPase activity of FtsZ to ATPase. The monomers of FtsZ are found throughout the cell in about 3000–20,000 copies. These monomers form protofilaments and a large number of short overlapping protofilaments assemble into bundles to form the Z-ring. FtsZ monomers polymerize from 'head to tail' and disassemble upon GTP hydrolysis. Both assembly and disassembly of FtsZ protofilaments depend on the concentration of FtsZ.

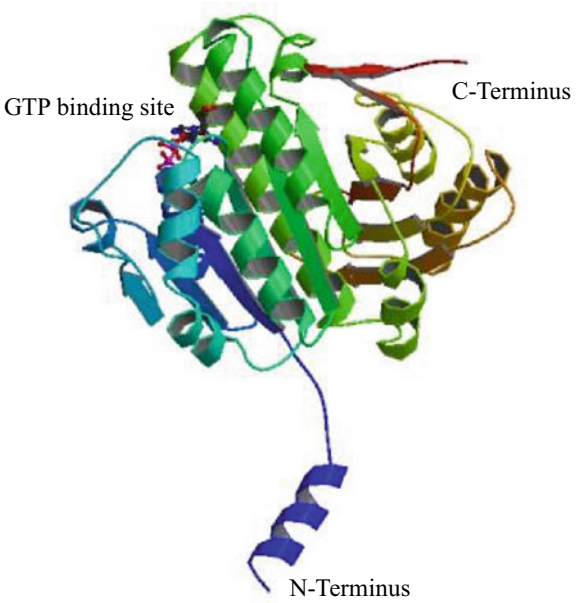


Fig. 5 Crystal structure of FtsZ from *Methanocaldococcus jannaschii* (PDB: 1FSZ) consists of a GTP-binding sequence and a C-domain that interacts with FtsA and ZipA

During cell-division, association of FtsZ to the cell membrane is fundamentally important. FtsZ seems to have no affinity for the cell membrane, but its attachment to the cell membrane is essential for maintaining the structural integrity of Z-ring during septation and most likely for transmitting a constrictive force on the membrane. In *E. coli*, FtsZ anchors to the inner membrane by two other essential proteins, FtsA and ZipA.

ZipA (FtsZ interacting protein A) is a biotopic membrane protein containing four domains: a transmembrane anchor domain at the N-terminus, a charged domain, a proline and glutamine enriched domain followed by a globular domain at the C-terminus also named as the FtsZ-binding domain (FZB) (Fig. 6). A membrane topology of ZipA with N-terminus located in periplasm and cytoplasmic C-terminus is rare in bacteria. ZipA is dispersed throughout the cell and is recruited during division by FtsZ in order to form Z-ring. The C-terminal peptide domain of FtsZ interacts with the C-terminal globular domain of ZipA and anchors itself to the inner membrane.

FtsA is an actin-like protein which along with ZipA functions as an anchor protein, aiding in attachment of FtsZ to the cell membrane. However, in *B. subtilis*, it has been seen that FtsA deletion mutants divide poorly but can still survive. FtsA protein consists of two domains with a central core forming an interdomain nucleotide-binding site. Each domain is further divided into two subdomains (Fig. 7). The

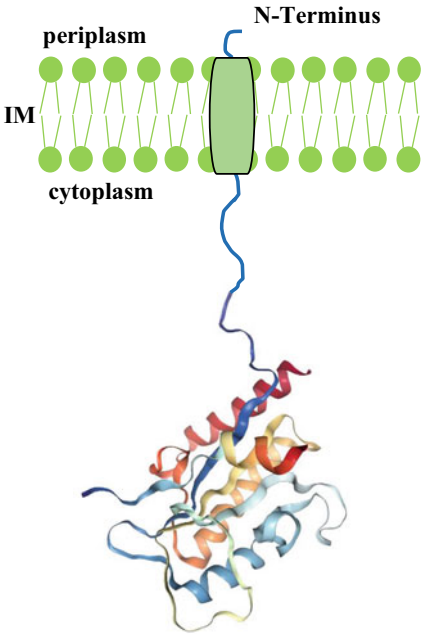


Fig. 6 ZipA is a single-pass transmembrane protein with N-terminal transmembrane anchor domain and a C-terminus FtsZ-binding domain. Crystal structure is only available for FtsZ-binding domain. These two domains are linked by a proline and glutamine-enriched domain (PDB 1F47)

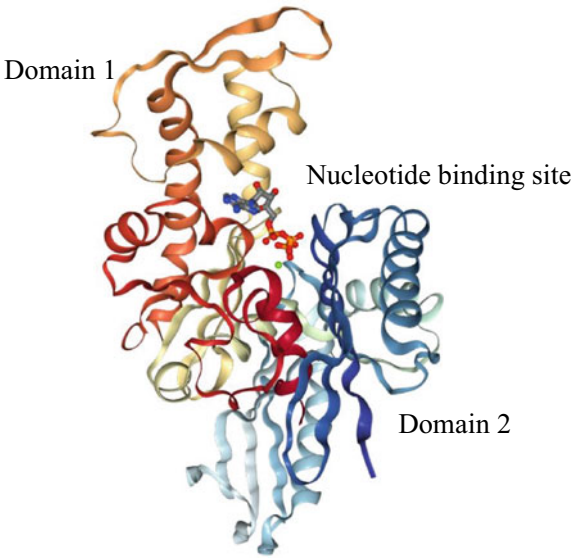


Fig. 7 Crystal structure of FtsA from *T. maritima* (PDB: 1E4G). FtsA consists of two domains with a central core forming an interdomain nucleotide-binding site

presence of ATP-binding site has also been shown in the nucleotide-binding site indicating that FtsA has the ability to bind ATP. The extended C-terminus of FtsA contains 15 amino acids that are highly conserved and form an amphipathic helix. This helix is the membrane targeting sequence of FtsA, and removal of this sequence results in formation of cytoplasmic rods rather than a functional protein that interacts with the membrane. FtsA also harbors a region that contains several charged residues, and this region is proposed to be involved in FtsZ binding. Studies have shown that FtsA self-integrates to form dimers, and furthermore, dimerization is important for Z-ring integrity. Substitution mutations destabilizing the FtsA dimer lead to destabilization of the Z-ring. In *B. subtilis*, FtsA is a dimer, while in *Streptococcus pneumonia*, FtsA polymerizes into higher order corkscrew-like structural helices formed by pairs of paired filaments. These polymers form in a nucleotide-dependent manner and are more stable when formed in the presence of ATP than ADP.

FtsZ is most likely the first protein to localize at the cell mid-point upon initiation of cell division. Assembly of FtsZ along with ZipA and FtsA is called 'proto-ring' due to their involvement in the initial stages of cytokinesis. Once these proteins are assembled and tethered to the cell membrane at cellular mid-point, remaining essential proteins are recruited. These proteins (FtsK, FtsQ, FtsL, FtsB, FtsW, PBP3 or FtsI, and FtsN) are either single-pass or multi-pass inner membrane proteins and are described below:

FtsK is important for the newly replicated daughter chromosomes to segregate completely before cell begins to divide at the cell center. This requires complete clearance of DNA from the cell mid-point before whole septation process has occurred. This clearance is facilitated by multifunctional protein FtsK, also called as DNA translocase. It is septum-located translocase containing three domains: an N-terminal domain which is an integral inner membrane domain with four transmembrane helices, a linker domain rich in proline and glutamine, and a C-terminal domain (Fig. 8). The C-terminal is the DNA translocase domain which forms hexameric ring structures through which DNA duplexes can pass. FtsK is also required for recruitment of preformed FtsBQL complex.

FtsK assembles at the cell mid-point during late stages of cell division, and by this time, it is expected that the newly replicated sister chromosomes have undergone decatenation followed by segregation. However, if this process is delayed, then DNA will still remain at the cell center and undergo breakage due to septal formation. Therefore, in such conditions, FtsK plays a vital role to speed up the segregation process of two daughter chromosomes before cell plate formation.

FtsBLQ complex (composed of FtsQ, FtsL and FtsB) is a molecular connector coordinating a multitude of transient reactions during cell division (den Blaauwen & Luirink, 2019). They form a ternary complex in 1:1:1 stoichiometry. These proteins

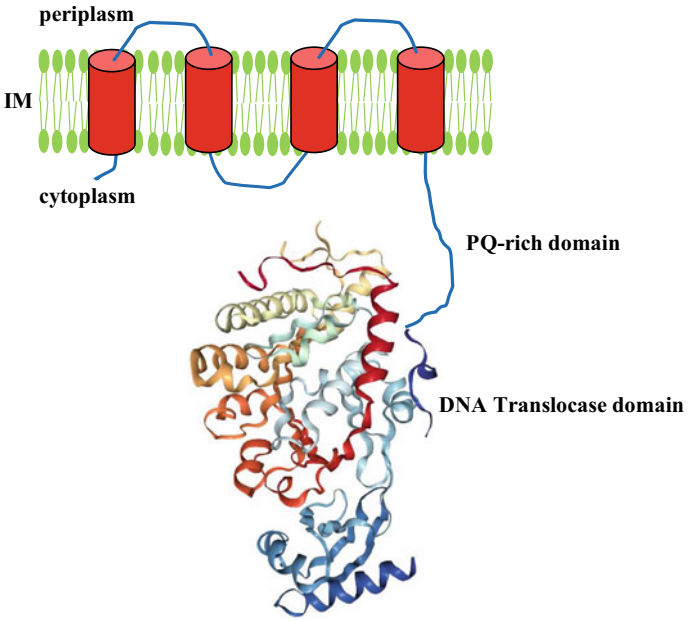


Fig. 8 FtsK consists of an integral inner membrane domain at its N-terminus arranged as four transmembrane helices, a linker domain rich in proline and glutamine (PQ-rich domain) located in cytoplasm, and cytoplasmic C-domain. The C-terminal is the DNA translocase domain which has been shown to form hexameric ring structures through which DNA duplexes can pass (PDB: 2IUS)

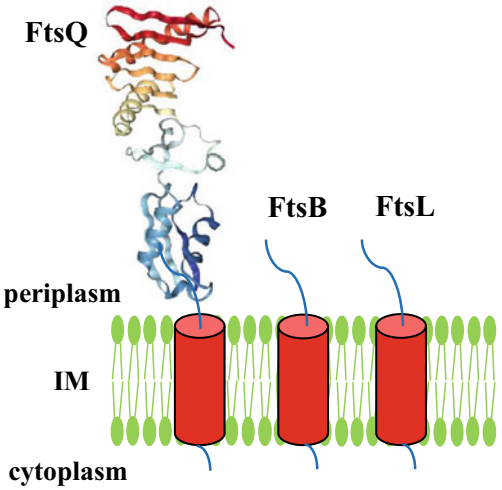


Fig. 9 FtsBLQ forms a complex, and each protein consists of a small cytoplasmic N-domain, transmembrane helix and a long periplasmic domain. The crystal structure has only been solved for the periplasmic domain of FtsQ (PDB: 2VH1)

are bitopic membrane proteins forming a divisome sub-complex that appears to bridge cytoplasmic cell division proteins to the ones located in the periplasm. Majority of bacterial genomes examined show conservation of homologues of FtsQ, FtsL and FtsB. Of these proteins, FtsQ seems to play central role in formation of divisome. It consists of a tail region located in the cytoplasm, a periplasmic and a transmembrane domain (Fig. 9). Although it evidently holds a pivotal role in network of cell division proteins, the exact role is still not known. Bacterial two-hybrid studies showed that FtsQ interacts with FtsA, FtsK, FtsL, FtsB, FtsW, PBP3 and FtsN. Co-immunoprecipitation further confirmed these interactions.

FtsL also consists of three domains: cytoplasmic N-domain, transmembrane helix (Fig. 9) and C-terminal periplasmic domain which is important for interaction with FtsQ but no other cell division proteins. The N-terminal cytoplasmic domain recruits cell division protein FtsW, involved in later stages of division. Interaction between FtsL and FtsB takes place via their transmembrane helical domains and a part of the periplasmic domain containing coiled coil. FtsB is composed of a small cytoplasmic N-domain, a transmembrane domain and a huge periplasmic domain. The periplasmic domain at its C-terminus interacts with FtsQ, while the cytoplasmic domain along with the transmembrane helix and membrane proximal part of periplasmic domain interact with FtsL, PBP3 and presumably FtsW. The functions of FtsB and FtsL are not clear, and not much is known beyond the fact that they interact with the divisome.

FtsW is an essential cell division protein involved in bridging cell division and septal peptidoglycan synthesis. It is present virtually in all bacteria that have peptidoglycan. It is a transmembrane protein with ten transmembrane domains formed by ten helices (Fig. 10). It recruits PBP3 to the mid-cell. PBP3 is a penicillin-binding protein

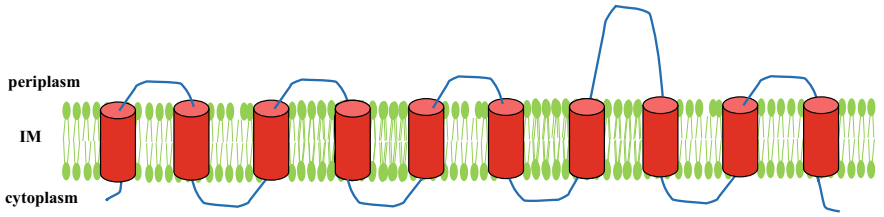


Fig. 10 FtsW that synchronizes septal peptidoglycan synthesis with cell division consists of 10 transmembrane helices and a large loop between helix 7 and 8

that catalyzes cross-linking of peptidoglycan for generating new poles. FtsW belongs to shape, elongation, division and sporulation (SEDS) protein family that function in concert with a penicillin-binding protein catalyzing the synthesis of peptidoglycan; however, their exact mode of action remains unknown. As no function other than recruitment of PBP3 has been linked to FtsW, it is speculated to integrate signals between proteins residing in cytoplasm and those in the periplasmic sites.

Septal penicillin-binding proteins (PBP)—PBP2 and PBP3 have been identified important in maintaining cell shape and cell division. Mutation in *mrdA*, gene encoding for PBP2, resulted in formation of round-shape phenotype indicating that this protein is involved in peptidoglycan synthesis with the cylindrical part of the cell. On the other hand, *ftsI* mutant, gene encoding PBP3, resulted in filamentous phenotype indicating role of PBP3 in synthesis of septal peptidoglycan. Both PBP2 and PBP3 belong to class B of penicillin-binding proteins and possess peptidoglycan transpeptidase activity allowing them to cross-link the peptide moieties within the peptidoglycan layer. It has been suggested that these two proteins differ in their substrate specificity with PBP3 exhibiting preference for the peptidoglycan precursors with a tripeptide side chain while PBP2 for ones with a pentapeptide side chain.

PBP3 has a short intracellular N-terminal region followed by single-spanning transmembrane domain and a periplasmic domain. The catalytic residues reside in the C-domain, also referred to as penicillin-binding domain. N-terminal domain, including the transmembrane domain, is responsible for interaction with FtsW and is important for localization of PBP3 to the division site. Genetic and biochemical studies have shown PBP3 interaction with FtsA, FtsN, FtsQ and FtsW. PBP3 and FtsW have also been shown to form a sub-complex.

PBP1B, a class A penicillin-binding protein encoded by *mrcB*, also localizes at division site during cell division. It has the ability to both polymerize the glycan strands of peptidoglycan by transglycosylation activity and to cross-link the peptide moieties by transpeptidation. Both PBP3 and PBP1B interact directly and possibly enlarge the peptidoglycan during cell division. PBP1B also interacts with FtsN which in turn can interact with PBP3. Recently, another protein PBP5 with role in maintaining cell morphology by regulating the number of available pentapetides for transpeptidation was found to localize at the cell septum.

FtsN is the last of the known essential cell division proteins and is also a single-spanning transmembrane protein. It is only found among γ-proteobacteria and is

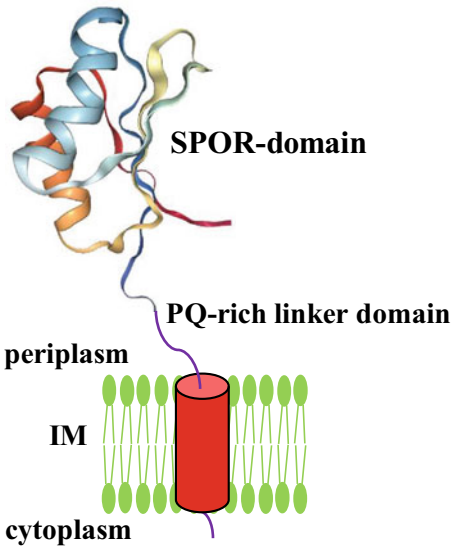


Fig. 11 FtsN is a single-spanning transmembrane protein consisting of a cytoplasmic N-domain, followed by a transmembrane helix. Long proline and glutamine-rich linker is present in the periplasm which is followed by a domain called as SPOR, with peptidoglycan-binding activity (PDB: 1UTA)

formed by a cytoplasmic N-domain, a large periplasmic C-domain and interconnecting transmembrane domain (Fig. 11). The short cytoplasmic domain interacts with FtsA. The periplasmic portion of the protein consists of a long proline and glutamine-rich linker followed by a domain called as SPOR, with peptidoglycan-binding activity. FtsN is suggested to have a 'triggering role' in constriction of Z-ring. The SPOR domain has been suggested to specifically bind septal peptidoglycan which is transiently available during the constriction process. Depletion of FtsN in the cell has also been shown to cause ordered disassembly of the divisome complex and hence is suggested to play a role in maintaining its stability. FtsN also directly interacts with FtsQ and PBP3. FtsN fails to be recruited to the divisome in the absence of FtsA or FtsQ.

Assembly of the divisome

The overall process of divisome formation is well explained by recruitment of proteins as sub-complexes (Fig. 12). The assembly of various sub-complexes depends upon the proteins already recruited and not on proteins downstream of them. Cell division basically happens in two steps that occur in a time-dependent manner: The first is the formation of FtsZ proto-ring which localizes at the cell center and the second is the subsequent recruitment of protein sub-complex that catalyze and regulate septal peptidoglycan synthesis. Upon initiation of cell division, FtsZ is the first protein to localize at the cell mid-point following which FtsA and ZipA assemble into a ring and bind directly to FtsZ forming a proto-ring tethering FtsZ to the cell

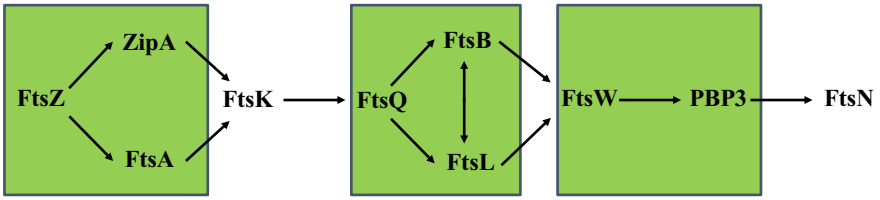


Fig. 12 Sequential recruitment of proteins constituting the divisome complex. Arrows indicate the order of recruitment, and proteins forming sub-complex are illustrated in green boxes

membrane. This assembly is completely dependent on localization of FtsZ and not any of the downstream proteins. These three proteins are solely responsible for the recruitment of other proteins involved in forming the divisome. FtsK, a DNA translocase, is then recruited at the site where FtsZ, FtsA and ZipA have pre-localized. Its recruitment also depends on the assembly of FtsZ, FtsA and ZipA rather than on downstream proteins FtsQ and PBP3. FtsK is followed by FtsQ which acts as an interaction hub within the divisome and is essential for recruiting FtsL and FtsB, the two of which also require each other for their localization. FtsL and FtsB are followed by FtsW which requires both of these preceding proteins for its localization. FtsW is followed by PBP3 which requires FtsW to localize, and they form the third sub-complex. Ultimately, FtsN is the last essential protein to be recruited at the divisome and its localization depends on PBP3.

Recent studies provide insights into the roles of FtsBLQ and FtsN that, respectively, inhibit and stimulate temporal regulation of septal peptidoglycan synthesis. FtsN also directly interacts with PBP1b leading to stimulate its glycosyltransferase activity. On the other hand, FtsBLQ has been shown to inhibit this activity in PBP1b. This suggested that FtsBLQ keeps a check on septal peptidoglycan synthesis until the cell is ready for it. Therefore, the initiation of peptidoglycan synthesis at the septum is partly regulated by activity of FtsBLQ and FtsN that function antagonistically on PBP1b.

Box 3: Quick guide to different divisome complex proteins

FtsZ: Tubulin like

ZipA: FtsZ interacting protein

FtsA: Actin like

FtsBLQ: Molecular interconnector

FtsW: Recruits penicillin-binding proteins to the divisome

FtsK: DNA translocase

FtsN: Required for mid-cell localization of amidases; plays triggering role in constriction of Z-ring.

4 Membrane Constriction and Septum Progression

During cell division, a cell has to elongate and pinch-off at the center by septal constriction. During this process, new peptidoglycan synthesis at the constricted site has to occur followed by regeneration of poles in the two daughter cells. Entire bacterial cell envelope maintains the cell shape and osmotic balance by virtue of stress-bearing peptidoglycan cell wall (PG). In gram-positive bacteria, the entire cell envelope is composed of sacculus (cell wall) that encases the inner membrane (IM), while an additional outer membrane (OM) enclosing the cell wall is present in gram-negative bacteria forming a tripartite envelope (IM, PG, OM). Electron microscopic studies have shown that the distance between outer membrane, cell wall and inner membrane strikingly remains the same throughout the process of cell division. Hence, it is obvious that cell must have mechanisms that physically and functionally coordinate the whole process of constriction in order to maintain cell envelope integrity during division. Gram-negative bacteria employ a constrictive mode of cell division in which synthesis of new septum and its splitting occurs concurrently with outer membrane invagination. In gram-positive bacteria, however, septal cross-wall is first completely synthesized followed by its splitting for separating the daughter cells (Fig. 13). Thus, the overall division process involves constriction of the entire sacculus, splitting of septum for separation into two daughter cells and fresh peptidoglycan synthesis to fill in the gaps.

As mentioned earlier, during division, all layers of the cell envelope constrict together, and there are mechanisms that physically and functionally coordinate the whole process. To achieve this synchrony across cell envelope layers, bacteria utilize IM-associated multicomponent machinery that span the entire envelope. This machinery can be divided into two distinct types: 1. elongasome, the cell elongation machinery and 2. divisome, the cell division machinery. The elongasome is organized by actin homolog—MreB that mediates lateral PG synthesis along the length of the cell causing cell elongation, whereas the divisome is organized by FtsZ, a tubulin homolog, which mediates the new pole synthesis at the dividing septum.

The polymerization of PG starts with lipid II precursor, synthesized on the inner face of the cytoplasmic membrane, and is subsequently translocated through this membrane by MurJ flippase. Bacterial cell wall synthesis is orchestrated by a repertoire of peptidoglycan synthases (PG synthases) that include (i) glycosyltransferases (GTases) which polymerize glycan strands from the precursor saccharide moiety lipidII, (ii) transpeptidases (TPases) that cross-link peptides between adjacent glycan strands and (iii) bifunctional PG synthases that carry out both the activities. In *E.* coli, the transpeptidases PBP2 and PBP3 are essential subunits of elongasome and divisome, respectively. Similarly, PBP1A and PBP1B are two major bifunctional PG synthases participating in elongation and division, respectively (Egan & Vollmer, 2013).

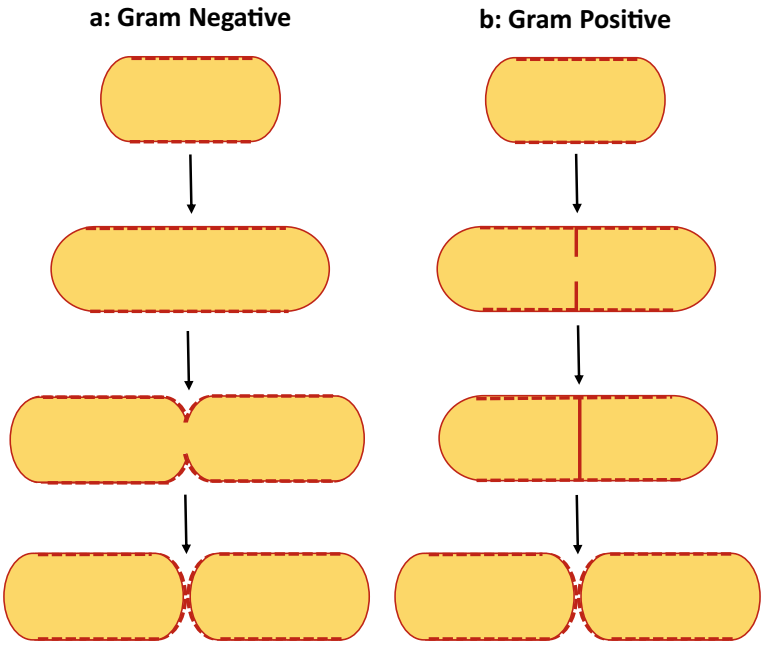


Fig. 13 During division, rod-shaped bacteria elongate by adding new peptidoglycan (red dashes) into lateral cell wall. Gram-negative bacteria employ constrictive mode of cell division where new septum synthesis, its splitting and invagination of the outer membrane occur simultaneously. In gram-positive bacteria, septal cross-wall is first completely synthesized before splitting it for cell separation

Generation of constriction force

After all the proteins which are involved in formation of a divisome have assembled at the cell mid-point, the constriction process starts by pulling the cell envelope inward. However, no contractile element is found outside the cell membrane, and consequently, constriction force for pulling the cell envelope inward seems to be exerted from the cytoplasm. There are evidences that FtsZ, which assembles in bundles to form the Z-ring, acts as the main motor in generating the driving force required for constriction (Rico et al. 2013). There are two mutually non-exclusive mechanisms that have been proposed to describe how FtsZ may exert a pulling force: bending of FtsZ and FtsZ condensation.

In the bending model, the motor force is generated exclusively by FtsZ, in a cycle of polymerization, membrane attachment, conformational change, depolymerization and nucleotide exchange, all driven by GTP hydrolysis. During this process, the curvature of FtsZ polymers is modified upon GTP hydrolysis, and mechanical force is transferred to the membrane. According to the condensation model, the constriction force is generated by lateral attraction between different FtsZ filaments that interact to form a compact structure. Increase in the lateral attractions causes compression of the

protofilaments and hence a shift from a loose spiral extending along the cell length to a compact ring at the mid-cell. The FtsZ ring undergoes further condensation along the cell diameter forming a thick annular structure, its diameter decreasing with progression of constriction. The belt-like annular ring constricts to a 'pinch-point' until the two halves of the cell are separated. Two FtsZ associated proteins, ZapA or ZapB, have been suggested to assist in this mechanism. After completion of constriction, the ring disassembles and the septum closes. It is however not known if upon disassembly, the component proteins of divisome complex are degraded or recycled.

Splitting of septum and PG synthesis

As mentioned earlier, it is important to note that in gram-negative bacteria IM constriction, PG synthesis and PG hydrolysis occur simultaneously and are coordinated by divisome. In the divisome, localization of FtsZ at the cell mid-point recruits other downstream divisome proteins including PG synthases and amidases required, respectively, in PG synthesis and splitting of septum. While FtsZ generates the driving force for constriction, septal PG synthesis is principally catalyzed by PBP3 (transpeptidase) and PBP1B (bifunctional PG synthase) at the leading edge of the inward-moving septum (Fig. 14).

The septal cleavage is catalyzed by tightly regulated periplasmic amidases and occurs closely after PG synthesis and adjacent to the invaginating outer membrane. *E. coli* has four amidases that hydrolyze the amide bond between N-acetyl muramic acid and L-alanine releasing respective peptides from glycan strands. The four amidases are the soluble periplasmic AmiA, AmiB and AmiC, and the outer membrane lipoprotein AmiD. Of these, AmiB and AmiC localize to the division site, while AmiA is more diffused within the periplasm. These proteins play major roles in cleavage of the septum during cell division to allow separation of the two daughter cells. Mutants lacking two or more amidases form chains of non-separated cells that have increased outer membrane permeability. About one-third of the newly synthesized septal peptidoglycan is removed immediately during septum formation by these septum-splitting hydrolases. The amidases need to be highly regulated as abrupt activities will pose

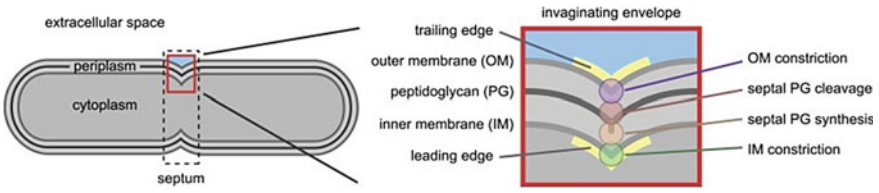


Fig. 14 Constriction of tripartite cell envelope in gram-negative bacteria; IM constriction, PG synthesis and PG hydrolysis occur simultaneously and are coordinated by divisome. FtsZ generates the driving force for constriction, while septal PG synthesis is principally catalyzed by PBP3 and PBP1B at the leading edge of the inward-moving septum. Septal cleavage is catalyzed by periplasmic amidases closely after PG synthesis and adjacent to the invaginating outer membrane. *Source* Gray et al. (2015). (Creative Commons Attribution License)

potential danger to peptidoglycan in a normal cell. It has been suggested that these amidases require specific activator proteins EnvC or NlpD which are also recruited at the divisome site and are activated by FtsN. EnvC is a periplasmic protein which activates AmiA and AmiB, while NlpD is an outer membrane lipoprotein that activates AmiC. FtsN is required for mid-cell localization of AmiB, AmiC and its activator NlpD but not for activator EnvC.

Outer membrane constriction during cell division

In gram-negative bacteria, besides cell membrane and sacculus, an additional outer membrane constitutes the cell envelope. It protects the bacteria against many antibiotics and antibacterial enzymes by preventing their access to intra cellular targets. Abundant OM proteins like Lpp, Pal, OmpA interact by covalent and non-covalent interactions with PG, tethering the OM tightly to peptidoglycan layer. The deletion of *pal* or *lpp* causes reduced outer membrane integrity and increased release of outer membrane vesicles into the culture media. The constriction of OM is promoted by the energy-transducing Tol system that also localizes to the cell mid-point during later stages of cell division, again in a divisome-dependent manner. Tol system is conserved in gram-negative bacteria and comprises of the IM components: (i) TolQ, TolR and TolA that form into a complex; (ii) TolB in the periplasm; and (iii) OM lipoprotein Pal (Fig. 15) (Gray et al., 2015). These proteins localize to the mid-cell during division depending on FtsN. All these proteins are encoded in two adjacent operons, and loss of any of these component causes delayed OM constriction and defects in OM integrity leading to blebbing and leakage. Functionally, TolQR, a homolog of flagellar motor MotAB, harnesses proton motive force and energizes TolA. Transfer of this force causes TolA to adopt an extended conformation allowing it to interact with respective periplasmic and OM components, TolB and Pal. It is

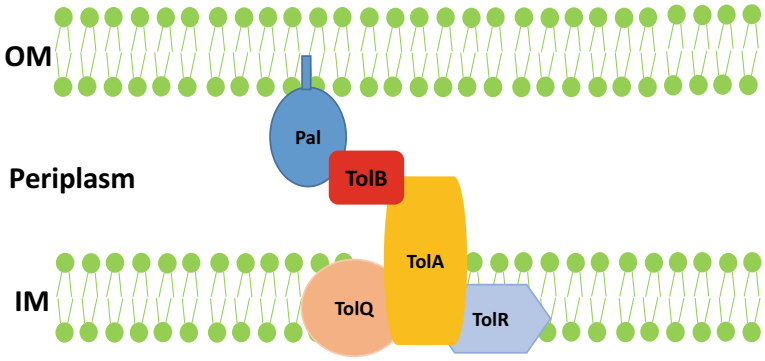


Fig. 15 Tol-Pal system involved in outer membrane constriction in gram-negative bacteria: TolQ, TolR and TolA are present in the inner membrane (IM) and form a complex; TolB is the periplasmic counterpart, while Pal is the outer membrane (OM) lipoprotein. TolQR harnesses proton motive force and energizes TolA which adopts an extended conformation to interact with respective periplasmic and OM components, TolB and Pal. These cycles of Tol-Pal interaction and release promote OM invagination

believed that these cycles of Tol-Pal interaction and release promote OM invagination. However, the exact mechanism by which Tol system promotes OM constriction remains to be completely elucidated along with how Tol-facilitated OM constriction is coordinated with septal PG synthesis.

5 Regulation of Septum/Z-Ring Formation

The most important aspect of Z-ring formation is that it must be formed at the cell mid-point and that the septum commences to constrict only after complete chromosome has replicated This ensures that cell division leads to daughter cells of similar size and with equal amount of nucleoid. Therefore, it is essential to control Z-ring formation both spatially and temporally, and this is achieved by two regulatory systems, viz. Min system and nucleoid occlusion. The Min system ensures that the septum is not formed at the poles where there is no DNA, while nucleoid occlusion system restricts Z-ring assembly over the nucleoid. The combinational effect of both the systems precisely positions FtsZ ring at the cell center only after complete chromosome has replicated.

A. The Min System

The Min system was identified about thirty years ago when Adler and colleagues discovered an *E. coli* mutant that often proliferated to produce cells which were smaller in size. These cells lacked nucleoid but were metabolically active for hours and were termed as minicells. In these cells, cytokinesis could not occur properly in the cell center and instead occurred aberrantly at cell pole leading to pinching of DNA-free minicells (Rowlett & Margolin, 2015). These minicells were identified to be a defective mutant of certain proteins later recognized as part of a system named Min system due to small sized minicells.

Generally, rod-shaped bacteria like *E. coli* select the cell mid-point for proper cytokinesis by virtue of certain proteins that negatively regulate the formation of central Z-ring. The Min system maintains a concentration of such negatively acting proteins toward the poles leaving only the cell center for formation of Z-plate leading to mid-point cytokinesis. The *E.coli* mutant discovered by Adler and colleagues was accordingly identified to be a defective mutant of these proteins allowing Z-ring formation to occur anywhere across the cell, hence causing improper cytokinesis leading to formation of minicells (Rowlett & Margolin, 2013).

Components of the Min System

The Min system comprises the three proteins, viz MinC, MinD and MinE, that function systematically to spatially restrict the formation of Z-ring precisely at the cell center. This regulation is achieved by discouraging the haphazard assembly of FtsZ protofilaments anywhere in the cell. Of the three Min proteins, only MinC directly interacts with FtsZ to inhibit its assembly into the Z-ring. The monomers of FtsZ bind to each other in a ‘head to tail’ manner forming longer protofilaments

that arrange themselves into bundles by lateral interactions (Fig. 16). The MinC protein comprises two distinct domains: amino-terminus and the carboxy-terminus domain. Two MinC monomers bind via the C-terminus domain to form active dimers. The N-terminus domain strongly inhibits the longitudinal head to tail interaction of FtsZ monomers, while the carboxy-terminus antagonizes the lateral interaction between the protofilaments. Active MinC protein thus causes shortening of the FtsZ protofilaments while inhibiting their arrangement into bundles, thereby preventing Z-ring formation. This also implies that if the MinC protein is present throughout the cell, formation of Z-ring and hence cytokinesis would never occur. In order to prevent this, the other two Min proteins, MinD and MinE, function to restrict the localization of MinC toward the poles at all times in a normal cell. MinD-MinE protein complex

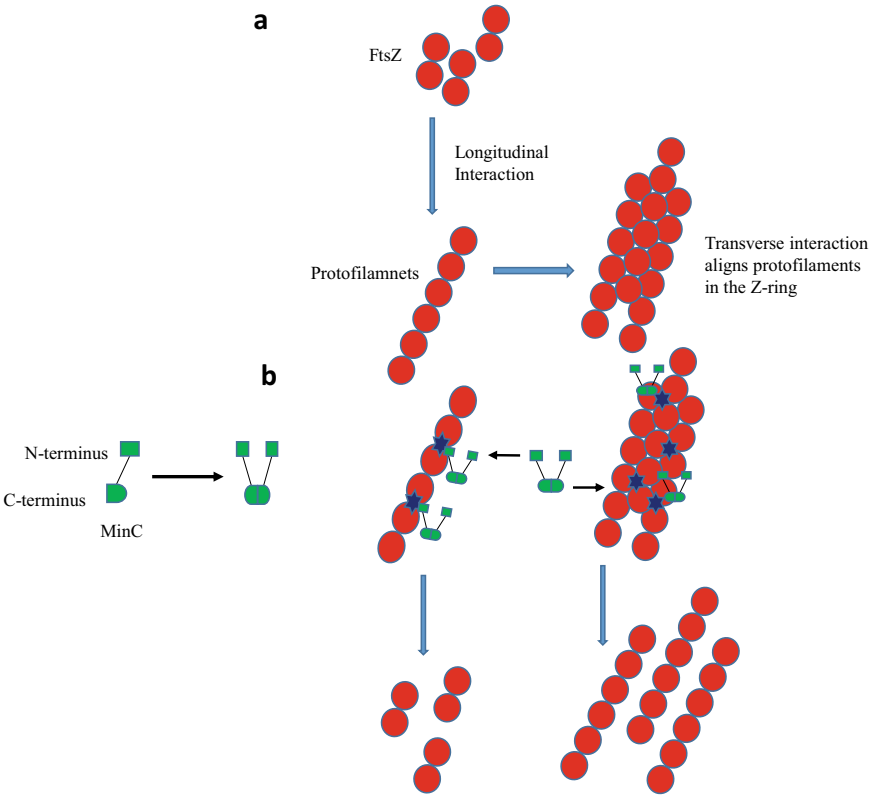


Fig. 16 **a** FtsZ units interact longitudinally to form the longer protofilaments that later arrange into bundles by lateral interaction. **b** The MinC protein consists of two distinct C and N-terminus domain and form dimers by C-domain interaction. The N-domain inhibits the longitudinal interaction between FtsZ causing shortening of the existing protofilaments. C-domain antagonizes the lateral interaction between the protofilaments releasing them from the bundles; this overall prevents Z-ring formation

functions as a ship that oscillates between the two cellular poles in order to spatially restrict the inhibitor protein MinC, away from the cellular mid-point.

The Min oscillator system

In *E. coli*, MinC concentration is maintained as a gradient toward the poles which is not stagnant but oscillates between the two poles via well-coordinated roles of Min system proteins. The MinD and MinE protein together create an oscillating system which acts to ship MinC protein toward the cell poles (Fig. 17). MinD protein directly binds MinC and the cell membrane while the MinE protein imparts the sense to MinCD complex to assume its role as a spatial regulator.

The C-terminus of MinC which is important for its dimerization also interacts with MinD. MinD is a ParA family ATPase which forms dimers upon ATP binding. These dimers of MinC and MinD copolymerize as alternating units to form the

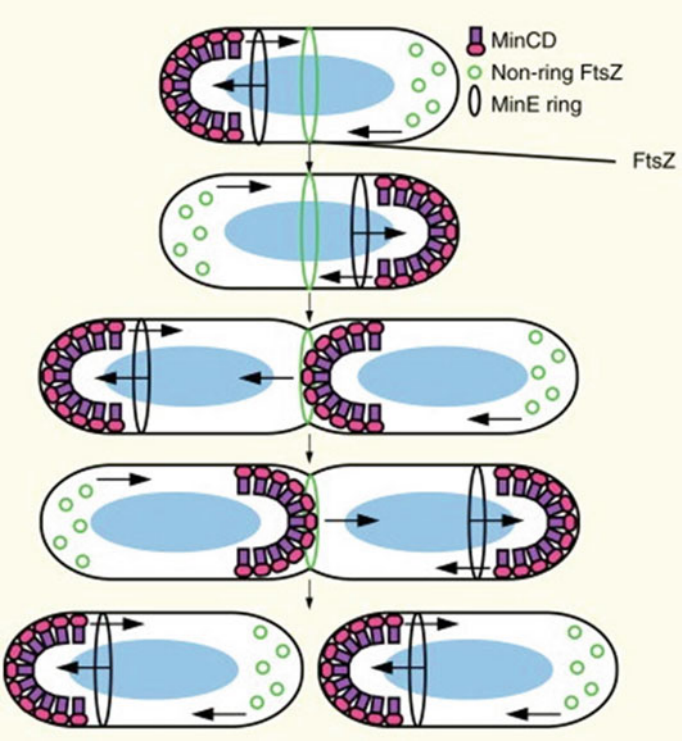


Fig. 17 In *E. coli*, MinE forms an inhibitor ring to spatially restrict the MinCD complex at one cell pole, while FtsZ resides far at the other. MinE stimulates ATPase activity in MinD causing conformational change, monomerization, and releasing free MinD and MinC into the cytosol. They diffuse and cycle toward the other pole causing FtsZ to oscillate to the opposite pole. Due to lack of remaining substrate at the existing pole, MinE ring now moves to the opposite pole to dislodge the reassembled MinCD. This forms a MinC free central zone where Z-ring forms freely, allowing equal mid-point division. *Source* Rowlett and Margolin (2013). With kind permission from Elsevier

MinCD complex. The C-terminal membrane targeting sequence of MinD allows it to bind the cell membrane in dimeric-ATP-bound state along with MinC, hence indirectly associating it to the membrane. This complex however does not form multiple discrete foci but only assemble at the cellular pole membrane. Here, MinE comes into play.

The MinE protein restricts the MinCD complex to the poles and causes dislodging of the complex. MinE protein binds ATP-bound-MinD dimers and stimulates the ATPase activity causing conformational change, monomerization, displacement of MinC and hence dislodging of MinD. This leads to release of free MinD and MinC into the cytosol which moves toward the other pole where MinE is still absent. In this series of movement, MinE always lags behind MinD. While MinE continues to dislodge the membrane-bound MinCD complex at one pole, the free MinC and MinD diffuse and assemble far away from the original site, the opposite pole. After MinE finishes disassembly of MinCD complex, no substrate remains at the original site, and it moves to the opposite edge where MinCD complex has now reassembled. This back-and-forth movement continues in the sequence of: MinD, MinC followed by MinE. Along with dislodging MinCD complex, MinE simultaneously prevents its accessibility to the membrane at the cell center. The FtsZ protofilaments form a Z-ring at the cellular mid-point, and therefore, it is essential that the Z-ring inhibitor MinC is absent at this region. This is accomplished as MinE dimers form an inhibitory ring structure toward the cell pole allowing MinCD assembly only onto the membrane at the poles and toward the cell center just a little away from the mid-point. The ring is formed near mid-cell and follows the edge of MinD concentration by shrinking toward the pole.

The overall MinE activity spatially restricts the MinCD complex only and only at the poles. The functions of various Min system proteins can be summarized as follows :

(1) MinC: FtsZ and hence Z-ring inhibitor
(2) MinD: Binds MinC and cell membrane
(3) MinE: Spatial regulator of MinCD; forms the inhibitor ring and dislodges the MinCD complex.

Hence, the dynamics between MinD and MinE keep inhibitor protein MinC away from the cell mid-point at all times while oscillating it between the poles.

An *E. coli* mutant lacking MinE will result into formation of lethal filaments due to defect in ability to form the Z-ring. Lack of MinE will lead to MinCD assembly throughout the cell membrane inhibiting the formation of Z-ring and henceforth impede cytokinesis resulting in filamentous cells. On the other hand, excess MinE in a mutant cell will relieve MinCD from the center and also from the poles. This will allow development of Z-ring at the poles as well, resulting in formation of minicells. Similar phenotype will be obtained in cell mutant in MinC or MinD proteins.

B. Nucleoid Occlusion

The bacterial cell division involves formation of a mid-point septum that divides a cell into two identical daughter cells with same cytoplasmic mass and nuclear material. This process however requires a high degree of coordination. The Min system prevents formation of Z-plate aberrantly anywhere in the cell allowing only mid-cell cytokinesis. However, at the same time, it is important to temporally synchronize the formation of the Z-ring and nucleoid replication. Both Z-ring and nucleoid replication occur at the cellular mid-point, so it is quite possible that developing Z-ring can come down onto the chromosome causing its bisection and break. Therefore, both these events must be synchronized in such a way that Z-ring formation commences just about when DNA is completely replicated. This temporal regulation of septum formation such that Z-ring does not form over chromosomal DNA is achieved by phenomena called nuclear occlusion. This second system inhibits cell division in the vicinity of nucleoid ensuring that chromosome is not bisected by the division septum.

It was originally postulated that active transcription and translation occurring around the nucleoid produces a short-range inhibitor that prevents septum formation. This effect called the nucleoid occlusion was important to maintain cell integrity and in selecting mid-cell site for cell division. The molecular basis of this process was deciphered after discovery of the nucleoid occlusion factors; Noc (nucleoid occlusion) in *B. subtilis* and SlmA (synthetic lethal with a defective Min system) in *E. coli*. Deletion mutants of these proteins produced division septum over unsegregated nucleoid and bisection of the chromosome whereas over-production of these proteins led to longer cells (Wu & Errington, 2012).

Molecular mechanism of Nucleoid Occlusion

The two nucleoid occlusion proteins, Noc and SlmA, act at the level of FtsZ polymers which are required in forming Z-ring at the mid-cell point. Two mechanisms are known by which they prevent FtsZ polymerization. One mechanism postulates that SlmA is stimulated upon DNA binding which then interacts with FtsZ-GTP altering its polymerization. It consumes the GTPase activity of FtsZ required to form polymers and thus inhibit it from forming protofilaments. The other mechanism states that rather than inhibiting the assembly of FtsZ polymers, SlmA protein prevents their assembly into higher order bundles required for forming the Z-ring. The SlmA protein consists of two helices in the amino-terminus domain that form a helix-turn-helix motif presumably involved in DNA binding, while there are several hydrophobic residues present in its carboxy-terminus. These residues interact with several glutamate residues present in the carboxy domain of FtsZ independent of its GTP-binding domain. The SlmA bound FtsZ can therefore polymerize into protofilaments, but two protofilaments sandwiching the same SlmA will be restricted from assembling into higher order bundles. By either of the mechanism, the formation of Z-ring is inhibited and the concentration of Noc or the SlmA protein has to be controlled temporally such that septum formation occurs only after complete chromosome segregation (Fig. 18).

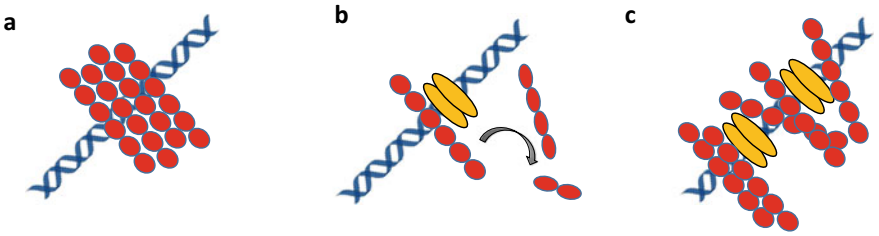


Fig. 18 Molecular mechanism of nucleoid occlusion; **a**: in a SlmA free zone, FtsZ polymerizes into protofilaments (red) that assemble as bundles to form the Z-ring; **b**: according to one hypothesis, dimers of SlmA (yellow) bind to SBS and interact with FtsZ-GTP leading to disruption of protofilaments; **c**: according to other mechanism, SlmA dimers bound to SBS restrict the assembly of protofilaments into higher order bundles that are required for forming a Z-ring

Binding sites of Noc and SlmA and prevention of cell division

The Noc and SlmA proteins are localized to nucleoid by binding chromosome at the Noc-binding site (NBS) and SlmA-binding site (SBS), respectively. These sites are highly specific for Noc and SlmA binding and are scattered across the chromosome but are absent in the *Ter* region. NBS is a 14 bp palindromic sequence and is distributed across *B. subtilis* chromosome except for a large gap at the *Ter* region. Noc probably forms dimers and binds at NBS spreading across 1-2 kb along the chromosome from NBS. Like the NBS, SBS is also a palindrome with 20–50 copies present in the *E. coli* genome. Noc and SlmA however show no homology and belong to different classes of DNA-binding proteins (Tonthat et al., 2011). Noc is a ParB family member and SlmA contains a putative helix-turn-helix N-terminal motif and a coiled coil C-terminus.

Bacterial chromosome replicates bi-directionally from a single origin, *oriC*, and the two replication forks meet about half way around the circle at terminus region, *ter.* After replication at the origin, the parent and the newly formed *oriC* move apart to quarter positions of the cell or to opposite poles. This is followed by progressive segregation of the remaining part, and division into two daughter cells occurs by formation of a septum between the segregated sister chromosomes. The terminus *Ter* region is segregated in the end and is last to be removed from the cellular mid-point. As the NBS or SBS are absent in this region, the concentration of Noc or SlmA will fall only during the later stages of chromosome segregation. Location of these sites on chromosome therefore optimally positions Noc or SlmA to act as negative regulator of cell division across the replication of proximal chromosome except during replication of the terminus. The absence of these proteins will allow FtsZ to polymerize and assemble into bundles in the Z-ring or septum which forms concomitantly with replication of the *Ter* region. Hence, this mechanism acts as a temporal regulator and fine-tunes the coordination between chromosome segregation and cell division in such way that division machinery is allowed to assemble only when Noc or SlmA free terminus region begins to replicate and Noc or SlmA bound *oriC* has moved away from the cell mid-point (Fig. 19).

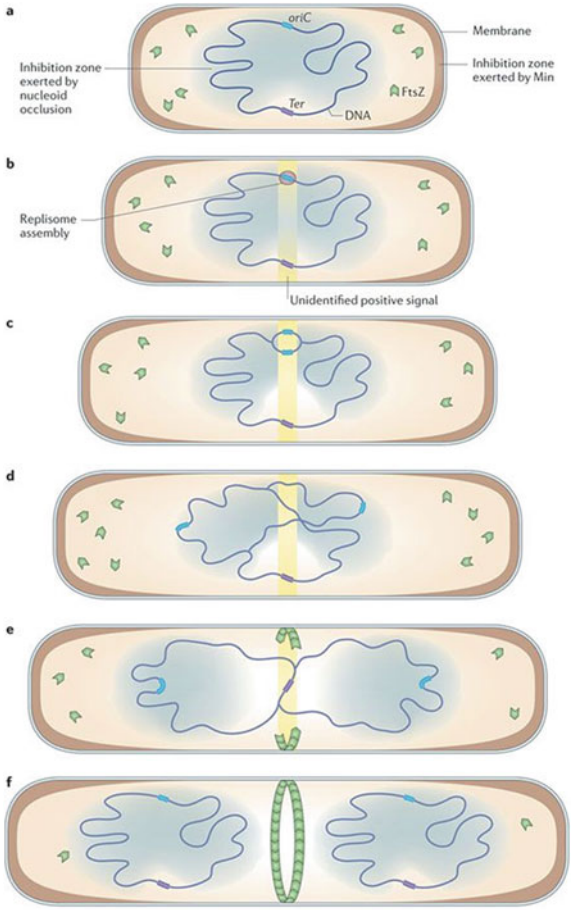


Fig. 19 In a bacterial cell, that has not yet reached the later stages of chromosome replication (**a**–**d**), nucleoid occlusion exerted by the nucleoid and the Min system together inhibit FtsZ and formation of Z-ring anywhere in the cell. In later stages of cell division, (**e**) the nucleoid occlusion protein bound *oriC* moves to the quarter or terminal positions of the cell. As the replication fork reaches the *Ter* region, nucleoid occlusion and a Min free zone allow FtsZ to polymerize and assemble into Z-ring at the cell mid-point. Once the replication and segregation of the *Ter* region are completed (**f**), the sister chromosomes move to the quarter positions and the reminder of the cell machinery is assembled to divide the cell into two equal daughter cells with equal amount of chromosome. *Source* Wu and Errington (2012). With kind permission from Springer Nature

In rod-shaped bacteria such as *B. subtilis* and *E. coli*, inhibitory effect of the Min system is sufficient for preventing cell division before segregation of the sister chromosomes. However, in round-shaped bacteria, Min system is absent, and therefore, nucleoid occlusion system is expected to play a significant role in allowing only mid-point cell division. Indeed Noc deletion mutant of *S. aureus* produced multiple Z-rings and DNA breaks confirming the importance of nucleoid occlusion

in cocci. Though NBS sequences have not yet been identified in *S. aureus* chromosome, sequences similar to *B. subtilis* NBS are found in the proximal half of its chromosome near the *oriC* and are absent in the *Ter* region.

There have been evidences that systems other than nucleoid occlusion may play a part in allowing correct cell division. A *noc min* double mutant of *B. subtilis* or a *slmA min* double mutant of *E. coli* produced normal cells by dividing at the cell mid-point. This points toward the existence of unidentified nuclear occlusion systems in these bacteria that are yet to be discovered.

6 Divisome Proteins as Antimicrobial Targets

Ever since the discovery of antibiotic compounds, bacteria have co-evolved by adapting counter mechanisms rendering drug ineffective. Some of these well-known mechanisms are mutations, efflux pumps, enzymatic clearance of drug and altering the drug target. With evolvement of superbugs which are practically resistant to all currently available drugs, there is an alarming need of novel targets to counter attack the adaptive genetic machinery of bacteria. The cell division proteins, due to understanding of their essentiality, their mechanism and their protein– protein interaction, can be explored as novel targets for the discovery of new-generation antibacterial agents.

The major divisome protein FtsZ is an essential cell division protein conserved in virtually all eubacteria and archaea. It is the cytoskeleton protein involved in formation of extremely dynamic Z-ring or the septum at the mid-cell point for equal cell division. Owing to its essentiality during bacterial cell division, it has been validated as a highly promising drug target for antimicrobial intervention. FtsZ is regarded as the homologue of eukaryotic cytoskeleton protein tubulin due to similarity in their structural and functional features. This brings up the question of whether the drug designed against FtsZ would also inhibit tubulin, if administered in eukaryotes. It is required for a drug to be highly specific in its mode of action, effecting only the molecule it was designed against. The two proteins however have limited sequence similarity of about 10–18%, with large difference in the C-terminus amino acid sequence and demonstrate significant differences in their nucleotide binding (Li & Ma, 2015). Moreover, tubulin is a heterodimer of α and β subunits and hence exhibits polarity, while FtsZ is a homodimer. Therefore, specific inhibitors designed against FtsZ, targeting regions variable than tubulin, will develop an antimicrobial drug with limited cytotoxicity to eukaryotic cells. Table 1 summarizes few of the natural inhibitors of FtsZ. Though a number of natural compounds are known to inhibit FtsZ in one way or the other, they have poor bioavailability and mostly tend to have other accompanying effects as well. However, understanding of their mode of action with advancements in bioinformatics tools can lay a foundation for exploring and rationally designing drugs capable of specifically targeting FtsZ.

The divisome complex FtsBLQ is also considered as a novel target for rational drug designing. FtsBLQ is initially required in divisome assembly and acts as a molecular

Table 1 Few known natural inhibitors of FtsZ

Compound	Natural source	Effect on FtsZ
Cinnamaldehyde	*Cinnamomum cassia*	Broad-spectrum activity against *E. coli* and *B. subtilis* In the presence of the compound, FtsZ protofilaments became thinner and shorter
Viriditoxin	*Aspergillus viridinutans*	Inhibits FtsZ polymerization and GTP hydrolysis
Totarol	*Podocarpus totara*	Perturbs FtsZ assembly by causing binding induced conformational changes in FtsZ, inhibiting its polymerization
Dichamanetin	*Uvaria chamae*	Inhibits GTPase activity in FtsZ
Berberine	*Berberis aquifolium*	Inhibits FtsZ assembly, thus affecting Z-ring morphology by disturbing its typical mid-cell localization and reducing the frequency of Z-ring per unit cell length by almost 50% Inhibits gram-positives, gram-negatives and most importantly MDR *M. tuberculosis* and methicillin-resistant *Staphylococcus aureus*

connector coordinating a multitude of transient reactions in the divisome. Interference with FtsBLQ complex will either result in premature division or inhibit divisome assembly in the first place, potentially leading to cell lysis. Inhibition of protein interactions is not considered as a choice for designing antimicrobial compounds as resistance development to such compounds is relatively easier. However, the multitude of interactions that FtsBLQ complex coordinates in the divisome would require multiple mutations to overcome the effect of drug. Among other considerations for FtsBLQ as a drug target are its low abundance, relatively accessible location, and the absence of human homologues. Crystal structures of most of these divisome proteins are available, and this knowledge further complements rational design of modulators capable of interfering with multitude of protein interactions in the divisome.

Summary

- Bacteria divide asexually by binary fission which is different from mitosis of eukaryotic cells as it lacks formation of spindle fibers for segregation of daughter chromosomes.
- A bacterial cell cycle includes: DNA replication, segregation of sister chromosomes, precise positioning of division machinery and ultimately equal cytokinesis; all divided into three well-defined periods, viz B-period, C-period or the replication period and D-period.
- B-period is similar to the G1 phase of the eukaryotic cell cycle, while C-period is the DNA replication period further divided into three stages: initiation, elongation and termination. It functionally corresponds to S phase of eukaryotic cell cycle.
- In fast-growing cells, multi-fork replication is observed and C-period is subdivided into C1 and C2 periods; C1 is the period for replication of pre-initiated

inherited chromosome, while C2 is the time taken for initiation of another round of replication.

- 'Central Dogma of Bacterial Cell Division Cycle' also known as BCD dogma suggests that before the cell divides into two daughter cells, the process of chromosome replication and doubling of cell mass should complete in order to ensure viability of daughter cells.
- Replication initiation depends on the concentration of DnaA and on the methylation status of the *ori* region. The replication in *E.coli* is bi-directional where two replication forks form and move in opposite directions.
- Newly replicated *oriC* is segregated by ParAB*parS* partitioning system in most bacteria, while *E. coli* utilizes MukBEF complex. Origin separation is followed by segregation of bulk chromosome and terminus region.
- Assymetric cell division in dimorphic bacterium *Caulobacter crescentus* is controlled by regulated system involving CtrA protein that inhibits DNA replication.
- During bacterial division, a number of proteins termed as filamentous temperature sensitive (Fts) assemble at the cell mid-point in order to drive equal cell division. About 10 such proteins assemble into sub-complexes to form 'divisome' or 'septosome.'
- FtsZ is a tubulin homolog and is the most essential structural protein which forms the Z-ring or the division septum at the cell mid-point. It is most likely the first protein to localize at the cell mid-point upon initiation of cell division and functions as a scaffold for the recruitment of other downstream proteins.
- Biotopic membrane protein ZipA and actin-like FtsA function as anchor proteins aiding in attachment of FtsZ to the cell membrane. Assembly of FtsZ along with ZipA and FtsA is called 'proto-ring' due to their involvement in the initial stages of cytokinesis.
- The clearance of newly replicated daughter chromosomes is facilitated by multifunctional protein FtsK, also called as DNA translocase.
- In the divisome assembly, FtsBLQ complex has been considered as a molecular connector coordinating a multitude of transient reactions in the divisome.
- FtsW is an essential cell division protein involved in bridging cell division and septal peptidoglycan synthesis; also recruits PBP3 at the cell center.
- FtsN has a 'triggering role' in the constriction of Z-ring. Depletion of FtsN in the cell has been shown to cause ordered disassembly of the divisome complex and hence is suggested to play a role in maintaining its stability.
- There are evidences that FtsZ acts as the main motor in generating the driving force required for constriction. Two mutually non-exclusive mechanisms that have been proposed for this: bending of FtsZ and FtsZ condensation.
- During constriction, septal cleavage is catalyzed by tightly regulated periplasmic amidases and occurs closely after PG synthesis and adjacent to the invaginating outer membrane.
- In gram-negative bacteria, constriction of OM is promoted by the energy-transducing Tol-Pal system that also localizes at the cell mid-point during later stages of cell division, again in a divisome-dependent manner.

- It is essential to control Z-ring formation both spatially and temporally, and to achieve this, two regulatory systems, viz. Min system and nucleoid occlusion system, operate in the cell.
- In *E. coli*, MinE forms an inhibitor ring to spatially restrict the MinCD complex at one cell pole, while FtsZ resides far at the other. The functions of Min system proteins can be summarized as: MinC: FtsZ and hence Z-ring inhibitor; MinD: binds MinC and cell membrane; and MinE: spatial regulator of MinCD and forms the inhibitor ring to dislodge the MinCD complex.
- In *E. coli*, MinC concentration is maintained as a gradient toward the poles which is not stagnant but oscillates between the two poles via well-coordinated roles of Min system proteins. The MinD and MinE protein together create an oscillating system which acts to ship MinC protein toward the cell poles.
- Nucleoid occlusion temporally regulates septum formation such that Z-ring does not form over chromosomal DNA by inhibiting cell division in the vicinity of nucleoid. Two nucleoid occlusion proteins, Noc and SlmA- act at the level of FtsZ polymers, and in a SlmA-free zone, FtsZ can polymerize to form the Z-ring.

Questions

1. How binary fission in bacteria differs from eukaryotic mitosis?
2. Schematically represent three phases of cell cycle?
3. What is central dogma of bacterial cell cycle?
4. The generation time of *E. coli* is 20 min. However, it takes 40 min. for its complete chromosome replication. Explain.
5. Define the term 'Initiation mass' with reference to cell division?
6. Which is the key enzyme for initiation of replication?
7. What are the important factors for initiation of replication?
8. Why DNA replication process is called semi-discontinuous?
9. Explain replisome assembly in *E. coli*?
10. What are the functions of different components of ParABparS system? How does the chromosome segregate after replication in most of the bacteria other than E. coli?
11. Explain the role of two regulatory histidine kinases, DivJ and PleC, in asymmetric cell division seen in *Caulobacter crescentus*?
12. Which proteins of divisome are considered as perspective drug targets for designing antimicrobials?
13. What is nucleoid occlusion?
14. Why is FtsZ named so?
15. Explain polymerization and depolymerization of FtsZ?
16. What is the function of FtsK?
17. Schematically represent sequential recruitment of sub-complexes involved in divisome assembly?
18. Explain how FtsZ helps in constriction of cell envelope at the septum?
19. Explain how Tol-system facilitates outer membrane constriction in gram-negative bacteria?

20. In the light of emergence of MDR pathogens, why do you think divisome proteins can be ideal drug targets for designing antimicrobials?
21. What are the differences in cell wall constriction seen in gram-positive and gram-negative bacteria?

References

Badrinarayanan, A., Le, T. B., & Laub, M. T. (2015). Bacterial chromosome organization and segregation. *Annual Review of Cell and Developmental Biology, 31,* 171–199.

Beattie, T. R., & Reyes-Lamothe, R. (2015). A replisome's journey through the bacterial chromosome. *Frontiers in Microbiology, 6,* 562.

Bernander, R. (2007). The cell cycle of Sulfolobus. *Molecular Microbiology, 66*(3), 557–562.

Cooper, S., & Helmstetter, C. E. (1968). Chromosome replication and the division cycle of Escherichia coli Br. *Journal of Molecular Biology, 31*(3), 519–540.

Den Blaauwen, T., & Luirink, J. (2019). Checks and balances in bacterial cell division. *MBio, 10*(1), e00149-e219.

Egan, A. J., & Vollmer, W. (2013). The physiology of bacterial cell division. *Annals of the New York Academy of Sciences, 1277*(1), 8–28.

Gray, A. N., Egan, A. J., van't Veer, I. L., Verheul, J., Colavin, A., Koumoutsi, A., ... Simorre, J. P. (2015). Coordination of peptidoglycan synthesis and outer membrane constriction during Escherichia coli cell division. *elife 4*, e07118.

Hughes, V., Jiang, C., & Brun, Y. (2012). Caulobacter Crescentus. *Current Biology: CB, 22*(13), R507.

Lindås, A. C., Karlsson, E. A., Lindgren, M. T., Ettema, T. J., & Bernander, R. (2008). A unique cell division machinery in the Archaea. *Proceedings of the National Academy of Sciences, 105*(48), 18942–18946.

Li, X., & Ma, S. (2015). Advances in the discovery of novel antimicrobials targeting the assembly of bacterial cell division protein FtsZ. *European Journal of Medicinal Chemistry, 95,* 1–15.

Lundgren, M., Andersson, A., Chen, L., Nilsson, P., & Bernander, R. (2004). Three replication origins in Sulfolobus species: Synchronous initiation of chromosome replication and asynchronous termination. *Proceedings of the National Academy of Sciences, 101*(18), 7046–7051.

Makarova, K. S., Yutin, N., Bell, S. D., & Koonin, E. V. (2010). Evolution of diverse cell division and vesicle formation systems in Archaea. *Nature Reviews Microbiology, 8*(10), 731–741.

Mott, M. L., & Berger, J. M. (2007). DNA replication initiation: Mechanisms and regulation in bacteria. *Nature Reviews Microbiology, 5*(5), 343–354.

Murray, S. M., & Sourjik, V. (2018). Self-organised segregation of bacterial chromosomal origins. *bioRxiv*, 304600.

Rico, A. I., Krupka, M., & Vicente, M. (2013). In the beginning, Escherichia coli assembled the proto-ring: An initial phase of division. *Journal of Biological Chemistry, 288*(29), 20830–20836.

Rowlett, V. W., & Margolin, W. (2013). The bacterial min system. *Current Biology, 23*(13), R553–R556.

Rowlett, V. W., & Margolin, W. (2015). The min system and other nucleoid-independent regulators of Z ring positioning. *Frontiers in Microbiology, 6,* 478.

Surtees, J. A., & Funnell, B. E. (2003). Plasmid and chromosome traffic control: How ParA and ParB drive partition. *Current Topics in Developmental Biology, 56,* 145–180.

Tonthat, N. K., Arold, S. T., Pickering, B. F., Van Dyke, M. W., Liang, S., Lu, Y., ... Schumacher, M. A. (2011). Molecular mechanism by which the nucleoid occlusion factor, SlmA, keeps cytokinesis in check. *The EMBO journal*, *30*(1), 154–164.

Trojanowski, D., Hołówka, J., & Zakrzewska-Czerwińska, J. (2018). Where and when bacterial chromosome replication starts: A single cell perspective. *Frontiers in Microbiology, 9,* 2819.
Wang, J. D., & Levin, P. A. (2009). Metabolism, cell growth and the bacterial cell cycle. *Nature Reviews Microbiology, 7*(11), 822–827.
Wang, X., Llopis, P. M., & Rudner, D. Z. (2013). Organization and segregation of bacterial chromosomes. *Nature Reviews Genetics, 14*(3), 191–203.
Wu, L. J., & Errington, J. (2012). Nucleoid occlusion and bacterial cell division. *Nature Reviews Microbiology, 10*(1), 8–12.
Zaritsky, A., & Woldringh, C. L. (2015). Chromosome replication, cell growth, division and shape: A personal perspective. *Frontiers in Microbiology, 6,* 756.

Chapter 5
Growth Physiology and Kinetics

Yogender Pal Khasa and Shilpa Mohanty

1 Growth Curve in Bacteria

The term 'growth' is defined as an increase in the cellular constituents leading to a surge in the cell number or cell size. The phenomenon of binary fission or budding increases the cell number, whereas repeated cycles of nuclear division which are not followed by cell division result in increased cellular size. The microbial growth is measured by understanding the underlying mechanism of different cellular processes taking place within a cell, contributing to a rise in cell size or cell number. However, it is difficult to study the growth pattern of individual cells. Hence, estimation of the total cell number is adopted as a feasible approach to measure growth pattern of microorganisms.

Generally, prokaryotic cells utilize the binary mode of replication, which involves the splitting up of a cell to daughter cells by the phenomenon of cell elongation, chromosome replication, DNA segregation and finally septum formation. However, the process of cell division is regulated in such a way that the complete copy of the genome is received by both the daughter cells. The other modes of bacterial replication include budding in yeast, fragmentation in *Spirogyra*, baeocyte (small cells) production in cyanobacterium *Stanieria* and transverse septa formation in segmented filamentous bacteria (Waterbury & Stanier, 1978; Angert, 2005; Hedblom et al., 2018).

Upon inoculation of a bacterial culture into a liquid medium, the cells start utilizing the nutrients present in the growth medium, which leads to an increase in the cellular biomass. In a closed system, where no further media is added or removed, the bacterial population undergoes a series of growth phases. The microbial growth pattern is generally divided into four stages: lag, logarithmic or exponential, stationary and death phases (Fig. 1).

Lag phase

The inoculation of the bacterial culture, i.e., primary inoculum into a fresh cultivation medium, results in a period where no apparent increase in the number of cells occurs.

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_5

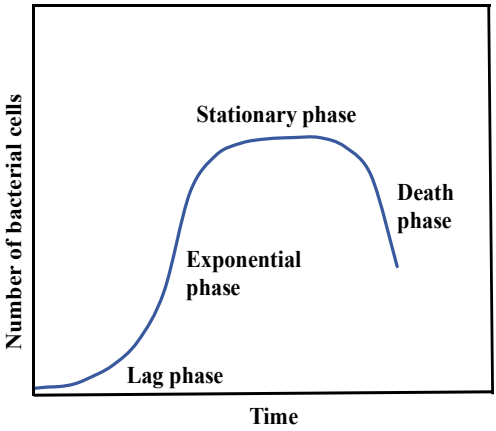


Fig. 1 Bacterial growth curve representing four phases of growth

This stage of cell growth is known as the lag phase. However, despite poor bacterial growth, the cells are viable as they are synthesizing new cellular components to adapt to newer conditions and prepare themselves for subsequent growth. To achieve optimal growth, the cell must have a sufficient amount of enzymes, coenzymes, ATP, cofactors and ribosomes to efficiently start replication, transcription and translation.

Exponential Phase

The exponential or the logarithmic phase represents the stage at which the microbial cells are actively dividing with their maximum growth rate. Microbial proliferation rate is governed by cultivation conditions and their genetic capability. During log phase, the bacterial cells exhibit balanced growth, where the cells divide at a regular time interval that remains constant over several generations (Hagen, 2010). In this phase, the cellular constituents are synthesized at relatively constant rates. However, changes in growth conditions may lead to an unbalanced growth where the cellular components such as proteins and nucleic acids are produced at different rates. Microorganisms synthesize several products during the exponential phase that are required by them for their optimal growth. These primary metabolites include amino acids, vitamins, enzymes, ethanol and certain organic acids. Many of these compounds are beneficial for human use and have high commercial potential (Singh et al., 2017).

Stationary Phase

The log phase is followed by a stationary phase in which the cell turnover number remains constant. In this phase, the growth rate matches the death rate resulting in a condition of no net growth rate. Several factors like nutrient deprivation, inaccessibility of oxygen in case of aerobic culture and toxic waste accumulation steer a microbial cell toward the stationary phase. During this phase, there is a drop in the number of viable cells due to cell lysis. This may be followed by cryptic growth

of some cells that utilize the cellular constituents released after lysis of other cells that are genetically programmed to commit suicide. These suicidal cells exhibit a phenomenon of altruism, in which they aid in the survival of other cells in the population while sacrificing themselves by programmed cell death (PCD). However, an alternate scenario exists when new cell formation ceases, but the population remains metabolically active. In that case, secondary metabolites such as antibiotics and hormones are synthesized due to metabolite deregulation.

Box 1: Programmed cell death (PCD)

Programmed cell death (PCD) is a genetically induced phenomenon that is activated in a certain proportion of bacterial cells as a part of response system toward stress conditions such as nutrient starvation, infection by phage, exposure to radiation and stress due to the oxidative environment. The most common way by which PCD is induced is by the toxin–antitoxin (TA) mechanism. The TA system is useful in several cellular processes such as synthesis of peptidoglycan, conferring resistance to bacteriophages and development of biofilms, among others. The TA loci are present in bacterial and archaeal populations in the chromosomal and extra-chromosomal DNA. They are organized into an operon containing two genes constitutively expressing a stable toxin that is responsible for the disruption of a major cellular process and an unstable antitoxin that acts in an antagonistic manner to the toxin. When a cell is growing under normal conditions, the toxin and antitoxin bind to each other, thereby making the toxin ineffective. However, under stressed conditions like nutrient deprivation, the antitoxins are degraded, making the toxin free and active, which disrupt cellular processes significantly. There are three different types of TA systems (type I, type II and type III) which are classified based on the function of antitoxin and the components of the TA system (Allocati et al., 2015).

Death Phase

The stationary phase is succeeded by the death phase, which is marked by an exponential decrease in the cell number. The death phase commences due to deprivation of nutrients and energy, accumulation of toxic wastes and activation of autolytic enzymes. The period for which a bacterial population may survive such adverse conditions varies as there are some bacteria which form spores and cysts that are resting, but viable and have the potential to germinate under favorable growth conditions.

2 Effect of Environmental Factors on Microbial Growth

Microorganisms can withstand and survive in extreme environmental conditions and are thus present in habitats which are otherwise inhospitable to other organisms. *Picrophilus torridus*, for example, is considered by far to be the most acidophilic organism, which can endure a pH of as low as 0.7. *Halobacterium* can thrive in high salt concentrations and requires high concentrations of NaCl to grow. *Saccharomyces rouxii*, an osmo-tolerant yeast, can survive in low water activity conditions of as low as 0.6 and thus can grow in high sugar concentrations. The archaeon, *Pyrolobus fumarii* can grow at a temperature of as high as 113 °C (Fütterer et al., 2004). There are several other such microbes which can thrive and flourish in extreme conditions and are therefore named as extremophiles. Several environmental factors, such as solute concentration, temperature, pH, oxygen and radiation, have a profound impact on microbial growth and physiology. Microorganisms have evolved different mechanisms to combat the stress conditions to survive environmental fluctuations. The effect of the environmental factors on microbial growth and strategies that aids them in adapting and surviving the harsh environment has been discussed below.

Effect of solutes and water activity on microbial growth

The concentration of solutes present outside a microbial cell plays a critical role in its survival in the natural environment. A typical microbial cell retains certain chemicals in its cytoplasm that aids in generating an osmotic or turgor pressure that prevents the cell from shrinking by adjusting to the external environment (Pilizota and Shaevitz, 2013). The free water molecules present in the external environment are measured in terms of water activity (a_w), which is crucial for the maintenance of cellular physiology of a microbial cell. The a_w of pure water is taken to be 1, and the value for a NaCl and $MgCl_2$ saturated solution is 0.75 and 0.3, respectively. It has been observed that the lowest value of a_w where life can sustain is 0.6 (Bolhuis et al., 2006). As cells encounter an environment which has a low water activity, they tend to lose water in order to maintain an osmotic equilibrium with the outside environment. A temporary loss in the turgor pressure will be counteracted by an increase in the intracellular concentration of solutes, which will subsequently lead to the restoration of osmotic balance. This phenomenon is termed as osmoregulation (Beales 2004).

Many microorganisms belonging to bacteria, archaea and eukaryotes can flourish in high salt concentrations. Halophiles can survive high salt concentrations ranging from 0.6 M commonly found in sea to greater than 5 M, which is the saturation limit. They are found in varied types of habitats including natural brines, artificial salterns and deep salt mines. *Halobacterium* spp., *Salinibacter ruber, Dunaliella salina* and *Staphylococcus aureus* are some of the commonly observed halophiles. To survive in high salt concentrations, archaea employ a 'salt-in' strategy and gather intracellular KCl, which equals to the concentration of NaCl present in the external environment. Moreover, their enzyme functionalities are adapted to high salt requirements (~4–5 M). The intracellular accumulation of potassium ions (K^+) plays a major role in adjusting the bacterial cell to high osmotic stress conditions. One of the mechanisms

by which K^+ ions help enzymes to achieve optimal activity is by stabilizing the active site as observed in *Haloferax volcanii* DNA ligase. Plasma membrane and cell wall of *Halobacterium* spp., found commonly in the Dead Sea and the Great Salt Lake in Utah, require higher sodium ions for their stability. However, certain halophiles (bacterial as well as eukaryotic) use a 'salt-out' strategy, thereby eliminating salt and amassing compatible solutes (DasSarma & DasSarma, 2015). Although the prime molecules required for maintaining an osmotic balance are inorganic ions such as K^+ and Na^+, organic solutes known as osmolytes or compatible solutes play an essential role in responding to osmotic stress. These organic solutes are usually a product of metabolism of nutrients such as carbohydrates and proteins and belong to the class of sugars, amino acids and polyols along with their derivatives as well as ectoines and betaines. They are either synthesized by the cell directly (glutamate, ectoine, proline, trehalose, sucrose) or imported from the external medium (glycine betaine). However, they are selectively transported across the cell membrane, which is governed by osmotic pressure present in the external environment. The most common exogenous compatible solute is glycine betaine in bacteria and higher plants along with the amino acid proline. *Listeria monocytogenes* has been demonstrated to adapt to high osmotic pressure by the accumulation of several compounds such as proline-rich peptides (Sleator et al., 2003; Beales, 2004; Roberts, 2005).

Effect of temperature on microbial growth

Temperature is one of the most crucial factors which affects microbial growth. In a microbial cell, the rate of enzyme-catalyzed reactions increases with temperature. It is a general phenomenon that as the growth temperature steadily moves toward the optimal value, every 10 °C rise in the temperature is accompanied with the doubling of the growth rate. With increasing temperature, enzymes catalyze reactions faster, and hence a higher microbial growth is observed. However, beyond a certain value, high temperatures prove to be fatal as it tends to denature enzymes and proteins of the cell, melts the lipid bilayers and disintegrates microbial membranes. Every organism is suited to grow above a minimum temperature and grows best at an optimum temperature, and there exists a maximum temperature above which it cannot grow.

On the basis of the optimal temperature range for growth, there are four classes of microorganisms: psychrophiles, mesophiles, thermophiles and hyperthermophiles. Psychrophiles are organisms which can grow at a temperature as low as 0 °C. Their optimal growth occurs at a temperature of 15 °C or below and maximum at 20 °C. Organisms which can grow at 0 °C but have an optimum of 20–40 °C are known as psychrotolerant as they can survive and grow at low temperature. These psychrotolerant organisms are commonly isolated from Antarctic and Arctic regions, and examples include the green alga *Chlamydomonas nivalis* the causative agent of red snow, *Polaromonas* found in sea ice and *Psychromonas*, one of whose species has been reported to grow at a temperature as low as −12 °C. Other genera containing psychrophilic species include *Pseudomonas, Alcaligenes, Photobacterium* and archaeon *Methanogenium*. The proteins and enzymes of such cold-adapted organisms are modified to survive in such conditions. One such modification includes higher levels of α-helix as compared to β-sheets as the latter makes protein

structure more rigid. In cold conditions, higher content of α-helix enhances the flexibility of protein structure. Their membrane contains polyunsaturated fatty acids (PUFAs), which are generally not observed in prokaryotic organisms and aids in maintaining the flexibility of the membrane. Another adaptation which helps these psychrophiles to thrive in a cold environment is the synthesis of cold-shock proteins and cryoprotectants like glycine betaine.

Mesophiles are organisms which possess an optimal temperature range of 20–50 °C. Majority of the groups of microorganisms lie within this range. Thermophiles are organisms which grow at temperatures above 55 °C. They show a slow growth at 45 °C and have optima ranging between 55 and 65 °C. The most common habitats of these microbes include hot springs and water lines, compost, self-heating haystacks and artificial hot water environments such as water heaters. In general, prokaryotes tolerate higher temperature as compared to eukaryotes, and most of the thermophilic prokaryotes belong to the group of archaea. Another class of thermophiles, known as the hyperthermophiles, survives at temperatures greater than 80 °C. Their optimum temperature ranges between 80 and 113 °C, and they cannot grow well even below 55 °C. They are majorly found in hot springs and hot regions of the sea floor. Examples of such organisms include *Pyrococcus abyssi* and *Pyrodictium occultum* which are found in marine waters. *Methanopyrus*, a methanogenic archaeon, can grow at 122 °C. Thermophiles and hyperthermophiles have highly thermostable enzymes and proteins which can function at such high temperatures only. Solutes such as di-inositol phosphate, mannosylglycerate and diglycerol phosphate are synthesized at increased levels in some hyperthermophiles and prevent proteins against thermal degradation. The presence of high content of lipids with saturated fatty acids helps to maintain the stability and integrity of the bacterial membrane. On the other hand, archaea which constitute the majority of the hyperthermophiles do not possess fatty acids in their membrane. Their membrane adaptation involves the presence of C40 hydrocarbons along with repeating units of isoprene linked to glycerol phosphate via ether bonds. Another unique adaptation in archaea is the arrangement of the membrane into a lipid monolayer rather than a lipid bilayer that protects it from melting at high temperatures. The enzymes from thermophiles and hyperthermophiles like Taq DNA polymerase of *Thermus aquaticus* and *Pfu* polymerase from the hyperthermophile *Pyrococcus furiosus* are commonly used for DNA amplification in polymerase chain reaction (PCR) technique (Ishino and Ishino, 2014; Kengen, 2017).

Effect of pH on microbial growth

Microorganisms can grow in a wide pH range with an optimum lying between 4 and 9. However, there are certain extremophiles which can grow at pH values less than 3 and more than 9. Generally, fungi favor a much more acidic environment as compared to bacteria and protists which thrive well around neutral pH values. Usually, the optimal pH lies between 3 and 8 for bacterial species. In the case of yeasts, the range is slightly on the acidic side (3 to 6) while it is 3 to 7 for most of the molds.

A change in the hydrogen ion concentration of the surrounding environment causes disruption of the microbial membrane, inhibition of cellular enzymes and other

transport proteins and hence affects the microbial growth. pH variations also affect the degree of ionization of nutrients, thereby leading to the development of nutrient-deficient conditions. As a result, organisms have evolved different mechanisms by which they combat such pH variations.

Organisms which can grow within a neutral pH range of 5.5–7.9 are termed as neutrophiles. Organisms which flourish in acidic environments are termed as acidophiles. They are usually found in habitats such as thermal soils associated with volcanic activity and acidic hot springs having pH values below 5.5. Many archaeal species can grow at these acidic conditions. *Picrophilus oshimae, Picrophilus torridus, Ferroplasma acidarmanus* and *Sulfolobus acidocaldarius* can grow at extremely low pH values. *Picrophilus torridus* is a thermoacidophilic archaeon that grows in a habitat having a pH value of 0.7 and a temperature of 60 °C. The *Picrophilus* membrane exhibits high stability toward acidic conditions and low proton permeability and is known to lose its function when exposed to neutral pH conditions (Fütterer et al., 2004). In order to maintain the intracellular pH near neutrality and maintain a proper proton motive force (pmf) across the membrane, the bacterial cell modifies their membrane potential to prevent the entry of protons. Membrane of obligate acidophiles such as *Alicyclobacillus acidocaldarius*, *Alicyclobacillus acidiphilus* and *Acidiphilium* sp. is extremely impervious to protons (Siliakus et al., 2017). Another mechanism by which a bacterial cell responds to acidic conditions is via the proton-translocating ATPase that extrudes protons out of the cell.

Alkaliphiles are the group of organisms that can survive at high alkaline conditions where the pH is usually 9 or above and are commonly found in soda lakes, alkaline hydrothermal vents and alkaline serpentinizing regions. They generally have pH optima of 9, but extremophiles are able to grow at extremely high pH values of around 12. For example, *Bacillus* species such as *B. firmus* and *B. alcalophilus* can withstand an extremely high external pH of ~11. In one of the strategies used to combat such extreme pH, the cell wall acts as an important defense barrier as it possesses a high fraction of negatively charged carboxylic groups that repel the hydroxyl ions and attract the H^+ ions (Morozkina et al., 2010; Preiss et al., 2015).

Effect of pressure on microbial growth

Oceans having an average depth of 3,800 m impart a pressure of 38 megapascals (MPa) on marine organisms which is 380 times more as compared to atmospheric pressure. High hydrostatic pressure inhibits several cellular processes that include motility, cell division, replication, transcription and translation, disruption of nucleic acid and protein structure, and membrane function. In the case of mesophiles, a pressure of about 50 MPa leads to inhibition of cell growth. High pressure tends to modify the cellular shape of bacterial cells such as in case of *E. coli* which turns filamentous with multiple nuclei when subjected to a pressure of 40–50 MPa. Microbes surviving in hydrothermal vents show a great degree of osmotic adaptation as in case of *Photobacterium profundum*, where both monomers and oligomers of β-hydroxybutyrate accumulate in response to increasing hydrostatic pressure. Piezophiles or barophiles can grow at a depth of greater than 10 km. They need a minimum of 70–80 MPa

of pressure to survive, and a pressure drop below 50 MPa ceases their growth and survival (Abe, 2007; Morozkina et al., 2010; Jebbar et al., 2015).

Effect of oxygen on microbial growth

Bacterial species can grow in a diverse range of conditions, where they may adopt different strategies for respiration under aerobic and anaerobic environment. A typical bacterial respiratory chain consists of dehydrogenases specific for a particular substrate that transfers reducing equivalents from donor to quinone acceptors, where the electrons are subsequently accepted by terminal electron acceptors (Borisov & Verkhovsky, 2015). The energy production via oxidative phosphorylation and synthesis of products such as tyrosine and cholesterol require the presence of oxygen. Facultative anaerobes can grow under anaerobic conditions but show better growth under aerobic conditions. Aerotolerant organisms are not affected by the presence or absence of oxygen. Obligate anaerobes, on the other hand, are extremely sensitive to oxygen that causes their death if exposed. Microaerophiles are a group of microbes that require oxygen for growth, but at a lower level as compared to the atmospheric levels. The high concentrations of oxygen prove to be fatal for cell survival, as a result of which several defensive mechanisms have been established to tolerate toxic levels of oxygen. Reactive oxygen species (ROS) tend to accumulate in a microbial cell as a by-product of aerobic respiration. Superoxide (O_2^-) anion and hydrogen peroxide (H_2O_2) are extremely reactive and damage the cell components. Enzymes such as superoxide dismutase and catalase play a vital role in protecting against ROS. Superoxide dismutase helps in converting the strong oxidizing agent superoxide anion (O_2^-) to hydrogen peroxide, where the reaction is as follows:

$$2O_2{}^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} O_2 + H_2O_2$$

The catalase enzyme prevents the accumulation of hydrogen peroxide by the following reaction:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Certain transcription factors such as Yap1 and Skn7 are the part of yeast oxidative stress response system. These transcription factors are activated at high oxygen levels induced by hydrogen peroxide. In such conditions, they are responsible for the activation of thioredoxin and thioredoxin reductase (Lee et al., 1999).

3 Diauxic Growth

Microbial population tends to show a biphasic logarithmic growth when supplied with two different carbon sources in their growth medium. The two exponential phases are separated by a lag phase, in which microbial growth is limited. This concept was

first introduced by the French scientist Jacques Monod while growing *E. coli* cells on two different carbon sources, i.e., glucose and lactose, which showed a biphasic growth where glucose was preferentially consumed. After glucose depletion from the culture medium, a lag phase was observed, followed by lactose utilization (Fig. 2).

Molecular basis of diauxic growth was explained by Jacques Monod in the year 1947. On the basis of regulation of *lac* operon (Fig. 3), he described the phenomenon of catabolite repression, which aids bacterial cells in preferential uptake of a single carbon source, while inhibiting the uptake of other less preferred nutrient sources.

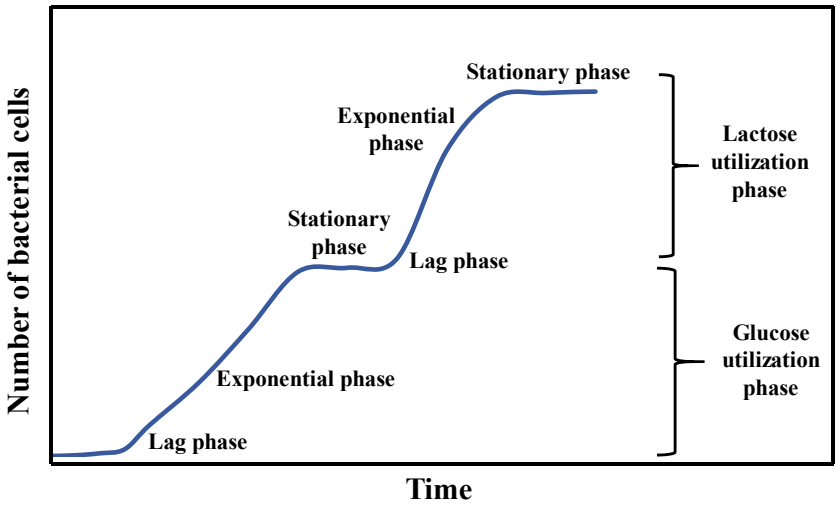


Fig. 2 Bacterial diauxic growth behavior in the presence of glucose and lactose

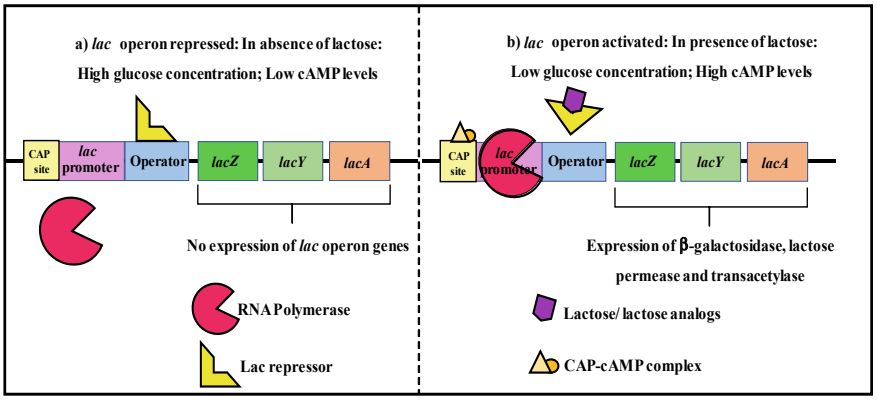


Fig. 3 Regulation of *lac* operon of *E. coli*: **a** cAMP-dependent catabolite repression by glucose and **b** derepression by lactose

Concentration of glucose in the growth medium is a major regulator of *lac* operon. Since glucose is a preferred carbon source as compared to lactose, the cellular machinery has certain adaptations that do not allow the expression of genes for lactose metabolism when glucose is present. This helps the cell to conserve its energy, as it readily utilizes glucose before synthesizing any new enzymes required for lactose breakdown. This phenomenon of catabolite repression involves one of the cell's essential secondary messengers, cyclic adenosine monophosphate (cAMP). The concentration of cAMP is inversely proportional to the concentration of glucose in the surroundings. The decrease in glucose concentration is accompanied by an increase in the intracellular cAMP levels, which in turn acts as an allosteric effector molecule for another DNA-binding protein catabolite activator protein (CAP) involved in lactose metabolism. Encoded by the *crp* gene, CAP itself is not capable of binding to the CAP site located in the *lac* operon and hence requires its effector molecule, cAMP, to bind to its site. Once the CAP protein binds to the DNA with the aid of cAMP, it enables the DNA to physically interact with RNA polymerase that increases its affinity for the *lac* operator region and allows for the transcription of the *lac* operon genes. Hence, an enhanced concentration of glucose causes a decline in cAMP concentration, thereby preventing the binding of CAP and subsequent activation of *lac* operon genes (Griffiths et al., 1999) (Fig. 3).

4 Effect of Recombinant Protein Production on Bacterial Growth

The expression of recombinant proteins often confers metabolic stress on the cells. The specific growth rate of induced recombinant cells expressing heterologous proteins is lower as compared to uninduced or plasmid-free cells. The reduction in the growth rate is attributed due to the diversion of cellular resources toward recombinant protein expression. This 'metabolic burden' on cells may result in the activation of genes involved in stress responses. Thus, the simultaneous synthesis of stress response proteins, as well as foreign proteins, is associated with an elevated demand for maintenance energy and higher respiration rates. Moreover, in the event of high-level protein expression, the accumulation of toxic wastes stimulates the production of undesired metabolites such as acetic acid that further hampers the cell growth. The by-product build-up further creates nutrient and oxygen-limiting conditions that induce starvation and anaerobiosis, respectively, thereby contributing to a significant decline in cell growth. The following are a few examples of the stress response systems that are activated during recombinant protein production (Hoffmann and Rinas, 2004; Chou, 2007).

(a) **Heat-shock response**

Expression of recombinant proteins by bacterial cells may result in the activation of a heat-shock-like response that arises due to the accumulation of proteins that have failed to fold correctly. Such misfolded proteins get deposited to form insoluble

aggregates known as inclusion bodies leading to expression of heat-shock proteins. These proteins are a part of a normal stress response toward elevated temperatures, and their expression is controlled by the sigma factor σ^{32}. The DnaK protein encoded by the *dnaK* gene is a chaperone that binds to the aggregating proteins and aids in quality control. Heat-shock proteins like Lon protease, encoded by the *lon* gene, catalyze the degradation of abnormally folded proteins synthesized during stress conditions. In many cases, expression of the heterologous protein is also associated with a high transcription and expression rates of heat-shock proteins such as DnaK and GroEL (Hoffmann and Rinas, 2004).

(b) **Stringent response**

During nutrient deprivation conditions and different stress signals, the stringent response system gets activated in bacterial cells. The most important feature of such a response is the production of certain small secondary messenger molecules such as guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp). The ppGpp molecule helps in the activation of the stress signal cascade by binding to the RNA polymerase in gram-negative bacteria and regulating the proportion of initiating nucleoside triphosphates (iNTPS) in gram-positive bacteria such as *Bacillus subtilis*. Besides, it is responsible for the transcriptional down-regulation of genes involved in translation and also activates transcription of stress response genes. Stringent response is normally activated when there is a limitation of aminoacylated tRNA, which leads to a roadblock in the synthesis of stable RNA molecules. A stringent-like response can also get activated during the expression of heterologous proteins when the amino acid composition of the desired protein is different from native proteins of the host cell. The presence of rare amino acids in the foreign protein results in their depletion in the host cell and leads to stringent response. Moreover, over-production of recombinant proteins also results in a decline in the amino acid pool, thereby causing an arrest in the protein synthesis. One of the ways by which this hurdle in recombinant protein expression can be combated is by supplementing the growth media with amino acids or with complex nutrients such as casamino acids and peptone (Hoffmann & Rinas, 2004; Boutte & Crosson, 2013).

5 Measurement of Microbial Growth

The measurement of microbial growth rate and cell density is essential to study cellular physiology and metabolism. Although several methods are available for measuring microbial growth, there are challenges that one may face during growth measurement. Many organisms tend to form clumps or adopt filamentous structures when they are grown under slightly unfavorable conditions, making the measurement of microbial biomass difficult. In addition, unfavorable growth conditions also lead to the development of resistant stages (e.g., spores), which results in erroneous measurement. The inoculum's growth conditions also play an important role in determining

the growth kinetics as cells take time to adjust to the new environmental conditions. The term 'growth' can be defined in terms of an increase in cell number or biomass, and several methods are available by which the generation time or microbial growth rate can be calculated. Various growth measurement techniques along with their advantages and disadvantages are listed below.

Measurement of microbial cell number

1. **Direct counting method**

(a) **Microscopic cell counts**

The use of a counting chamber for the enumeration of microorganisms is one of the most common techniques, which is considered to be easy, quick and reasonable. Another advantage of this method is that it can provide extra information regarding the morphology and size of the microbial cells. The different counting chambers that are used include the Petroff–Hausser chamber which is generally used for the counting of prokaryotic cells and the hemocytometer that aids in both prokaryotic and eukaryotic cell counting. These counting chambers are slides that are constructed in such a way that they hold chambers with a grid engraved on them. The depth of these cavities is predetermined. The microbial cell count depends on the volume of the chamber and dilutions of the microbial culture sample. Staining of prokaryotic cells with dyes is a common technique that enables cell counting with more ease. Other strategies for visualization of these cells include the usage of a phase-contrast or fluorescence microscope.

A hemocytometer is a specialized microscopic slide that consists of squares having a defined area in which a particular volume of cells is loaded. By counting the number of cells in the defined area and applying a suitable formula, the cell number per milliliter (ml) of suspension can be calculated. A hemocytometer consists of four corner big squares which are further divided into sixteen smaller squares (Fig. 4).

A drop of the cell suspension is placed onto the hemocytometer, which is then observed under a light microscope. The cells present in the four corner squares along with those lying at the bottom and the left-hand boundaries are calculated. Each of the 16 small squares has a side of 1 mm and a depth of 0.1 mm. Therefore, the volume of the small square in a hemocytometer is calculated using the formula, width × height × depth, i.e., $1 \times 1 \times 0.1 = 0.1\ \text{mm}^3 or 10^{-4}$ ml. The number of cells present in 1 ml of a sample can then be calculated as follows:

$$\frac{\text{Average number of cells}}{0.1\ \text{mm}^3 \text{volume}} = \frac{\text{Number of cells counted in four corner squares}}{4}$$

Therefore,

$$\frac{\text{The total number of cells}}{\text{ml}} = \frac{\left(\frac{\text{Average number of cells}}{0.1\ \text{mm}^3\ \text{volume}}\right) \times \text{Dilution Factor}}{10^{-4}}$$

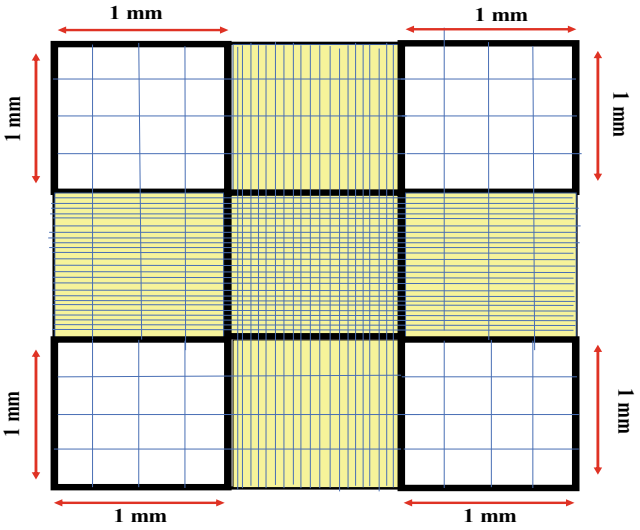


Fig. 4 A pictorial representation of a hemocytometer

However, a hemocytometer is plagued by several disadvantages. Direct counting method tends to be tedious and error-prone due to the adsorption of the microbial cells onto the surfaces of pipettes and other glassware. Further, this method of documenting the total count of microbial cells does not differentiate between live and dead cells and cannot be used for samples with a low microbial load. In order to differentiate between the live and dead cells, a cell viability test can be performed using the trypan blue exclusion method. This method works on the principle that living cells possess intact cell membranes and thus do not allow the entry of dyes. However, dead cells get stained with the dye easily. Therefore, whenever a cell suspension is mixed with the dye, the viable cells will exclude the dye and will remain unstained whereas the non-viable cells will take up the dye and will appear blue in color which will help in differentiating between the live and dead cells (Strober, 1997; Sanders, 2012).

The counting of cell number using a hemocytometer is a low-cost and versatile method used in conjunction with different stains for enumeration of different cell types irrespective of their shape and size. However, this process is time-consuming and laborious and may prove to be cumbersome while dealing with large amounts of samples. These disadvantages give rise to a need for automated methods that will reduce the working time as well as errors associated with manual counting. The principle behind automated cell count machines is the presence of a digital camera that takes images for cell count, which are further analyzed using specialized software. Although automated techniques aid in saving time and reduce workload, they still have certain limitations. They are compatible only with a few stains and may face difficulties while analyzing

different cell types. These disadvantages are attributed mostly to limitations in the hardware and the associated software (Cadena-Herrera et al., 2015).

(b) **Coulter counter method**

This is an electronic counting technique which is regularly used in the field of clinical hematology. Coulter counters are especially beneficial for enumeration of larger microorganisms including non-filamentous yeasts as well as protists. However, this method is not suitable for cell counting of filamentous organisms. In addition, small size and elongated shape of bacterial cells and their tendency to form filaments make this counting strategy unfit for them. In this technique, a fixed volume of appropriately diluted microbial cell suspension is allowed to pass through a small orifice which lies between two saline-filled compartments containing the electrodes. The electrodes are responsible for measuring the electric resistance as electric current passes through the orifice. Each time a microbial cell flows through the orifice, there is an increase in the electrical resistance and a drop in the conductivity, thereby generating a voltage pulse. The drop in electrical conductivity is attributed to the high electrical conductance of saline solution as compared to bacterial cell. The method also provides an opportunity to study the cell size, as the voltage pulse generated is proportional to the size of the microbial cells. However, one of the major reasons behind the failure of this technique is the clumping or aggregation of the cells. The problem of clumping can be circumvented by choosing an organism that does not have an inherent property of clump formation or by using specific physical methods of cell separation like blending or treatment with mild ultrasonic waves. An additional drawback of electronic counters is frequent blocking of the orifice. However, ultrafiltration of all the reagents and selecting solvents that do not lead to the generation of particulate matter are few methods that can be employed to prevent the blocking of the orifice.

(c) **Flow cytometry**

Microbes belonging to the group of protists and yeasts can be enumerated using flow cytometry, which is a rapid technique for cell counting. Various biological facets of eukaryotic cells can be studied using this technology. This strategy has also been adopted to study the prokaryotes. In this technique, a microbial cell suspension is allowed to flow in a stream of fluid, which is then analyzed using laser light of varying degree of frequencies and angles. Biomass of the microbial population is examined based on light-scattering phenomenon along with fluorescent dyes that are used to stain chemical components of the cell such as DNA. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin–chlorophyll–protein (PerCP), Alexa Fluor 405 and Alexa Fluor 488 are some of the commonly used fluorescent dyes used in flow cytometry.

2. **Colony counting method**

The invention of agar plates for culturing microorganisms not only aided in the isolation of pure strains of a particular microbial culture but also helped in counting colonies arising from a single viable cell. Several plate-based microbial

colonies' counting approaches are used for enumeration of microbial cells. In this method, only those cells that are viable and reproduce to form colonies are counted in terms of colony-forming units (CFUs). The plating methods commonly used for colony counting are described below.

(a) **Pour-plate technique**
In the pour-plate technique, a diluted microbial sample is mixed with molten agar medium, which is then poured in a sterile petri-plate and allowed to solidify. The technique is suitable for enumeration of microorganisms present in a mixed culture sample. However, care should be taken while employing this method as the agar must be cooled down to 45 °C before pouring into the petri-plates as a higher temperature may kill the microbial cells. Since the colonies grow in and on the surface of the agar, this technique also allows for isolation and identification of microorganisms having different oxygen requirements. Microaerophiles or those microorganisms that have low oxygen requirements as compared to aerobic organisms usually grow within the agar plates.

(b) **Spread-plate technique**
The spread-plate technique is routinely used for counting of microbial cells present in a small volume of sample and finds applications in experiments concerning isolation, screening and enrichment of microorganisms. An appropriately diluted microbial culture is spread onto the surface of agar plates with the help of a glass, wire or Teflon-based spreader or a turntable. These agar plates upon incubation yield uniformly dispersed microbial colonies for counting. The number of colonies obtained on the culture plates is multiplied with the dilution factor to obtain the total number of microbial cells present in the culture sample. The spread-plate technique enables the growth of the microbial colonies over the surface of the agar plate only.

(c) **Thin-layer plate technique**
In this method, a sample of the microbial culture is mixed with a small volume of molten soft agar of concentration 0.6–0.75%, followed by its pouring onto agar plates. After layering the soft molten agar, the plates are allowed to cool and solidify. The soft-agar overlay technique is often used in plaque assays to enumerate the infectious bacteriophages or viruses (of size 100–200 nm) that infect bacterial cells. Since viruses are obligate parasites, they cannot exist and propagate outside their host cells. To determine the number of infectious particles present in a sample, the host bacterial cells are mixed with the phage particles to encourage the infection process. In this strategy, the mixing of an appropriately diluted phage sample with a bacterial cell suspension is done, followed by pouring onto the surface of agar plates by mixing it with soft molten agar. The soft agar is gently shaken to uniformly distribute it over the surface of the solidified agar medium and subsequently incubated in an incubator. Following incubation, the bacterial cells (in range of approximately 10^8 cells) grow over the surface of the plate forming a lawn. However, in the presence of infectious phage particles which are replicating within the bacterial cells, a zone of lysis starts to develop on the plate that gives rise to cleared regions

within the lawn known as a plaque. Since a plaque arises from a single phage that subsequently replicates and releases progeny particles, calculation of the plaque-forming units (pfu) is carried out to determine the phage titers (Sanders, 2012).

(d) **Layered-plate technique**

The principle of the layered-plate technique is similar to the thin-layered strategy, except for the fact that an extra layer of agar is poured onto the surface of the set soft-agar medium to ensure the growth of all microorganisms present in the sample at the subsurface region. The advantage of such an approach is that since all the colonies are formed at the subsurface section of the plate, they are much smaller and more compact. The strategy thus enables screening of a higher number of colonies as compared to other methods. Additionally, this method provides flexibility while supplying nutrients for the growth of the microbial cells. The basal layer of the petri-plates is prepared using minimal media for long-term storage. The stocks of molten soft agar can be prepared and mixed with high concentrations of the additional nutrients whenever required by the microbial population.

(e) **Membrane filter technique**

Bacterial cells in water samples are often enumerated using the membrane filter method. In this strategy, bacterial cells are filtered by passing the water samples through a membrane filter. There are two ways by which the bacterial cell count can be performed. The first is the direct count method, wherein the membrane-trapped bacterial cells are stained via fluorescent dyes like acridine orange or staining DNA using 4′,6-diamidino-2-phenylindole (DAPI), which are then counted with the help of a microscope. The staining enables easy visualization of the bacterial cells against the black background. The second is a plate-based method, wherein the membrane filter containing the trapped bacterial cells is placed over an agar plate or on a liquid media-soaked pad. This is subsequently incubated to obtain individual colonies. In order to examine the purity of water samples, this method is frequently used in conjunction with a selective media that specifically selects for particular microorganisms.

The success of a colony counting method depends on the uniform distribution of the colonies on the plate. Although the viable cell count method helps in the detection of live microbial cells, erroneous results arise if the cells are clumped and have not been dispersed evenly. A plate used for colony counting should preferably contain 30–300 colonies to maintain the accuracy of the procedure. Moreover, there are several strains of bacterial species that grow inefficiently when plated on a solid agar media. For example, bacterial species belonging to the genus *Legionella*, *Brucella,* and *Leptospira* are slow-growing and fastidious and therefore require sophisticated culturing techniques (Doern, 2000).

(f) **Most Probable Number (MPN)**

The most probable number (MPN) method is a technique that is employed to estimate the number of viable organisms in a sample that otherwise cannot be enumerated using plating methods. It is generally used to determine water quality where an indicator is used to detect the presence of contaminants. MPN

method is based on replicate inoculation of tubes containing liquid broth with the bacterial sample and assessing the number of tubes that are showing growth. The bacterial culture samples are diluted using tenfold serial dilution method, and each of the dilutions is used to inoculate three replica tubes containing nutrient broth. The test samples are appropriately diluted to compare all the three replica tubes. As the dilution fold of a test sample increases, the probability that one of the three tubes will fail to receive inoculum will also increase, thereby showing no growth. After the tubes have shown the growth/no-growth pattern, the results are deciphered using the MPN table provided by the Bacterial Analytical Manual (BAM) of the US Food and Drug Administration (FDA). The table compares the replicas of three dilutions each separated by a $\log_{10}$ unit, that is, 0.1, 0.01 and 0.001. However, according to the user's choice, different dilution sets can be used, and the result may be interpreted by taking the dilution factor given in the table and used in the experimental study into account. According to the result pattern given in the MPN table, if all the three tubes of each of the dilution show growth and turbidity, then the result will be recorded as 333 which will correspond to a 95% confidence interval and MPN value of > 1100 (number of colonies in 100 ml of sample). The MPN method is suitable for samples where the growth rate and kinetics of the microbial population residing within the test sample are highly variable (Sutton, 2010).

3. **Estimation of cellular biomass**

The growth of microbial cells is also accompanied by an increase in cell biomass. There are several ways by which the microbial biomass can be measured.

(a) **Wet cell weight (WCW) and Dry cell weight (DCW) estimation**

The wet weight of microbial cells is calculated by separating the liquid media from the solid content, followed by measuring the weight of the cellular mass. Since the biomass is not dried in the oven, the measured value also includes the weight of the constituents of the liquid media enclosed within the interstitial spaces of the cells leading to an erroneous calculation.

The measurement of cellular biomass using the dry cell weight method involves growing the cells in a liquid media that are separated from the media constituents by centrifugation followed by drying of cell pellet in an oven at a high temperature of about 60 °C till constant weight. This technique is especially useful for the estimation of filamentous fungal biomass. However, there are several problems associated with this strategy as it has low sensitivity and is time-consuming. In addition, drying in the oven may lead to the degradation or loss of some of the volatile components at a high temperature. Moreover, if the relative humidity of the room in which weighing is carried out is high, moisture absorbancy by the dried biomass may produce inaccurate results.

(b) **Measurement of turbidity**

Turbidity-based methods are commonly used for measuring bacterial growth as they are simple to perform and do not cause damage to the bacterial cells. A beam of a light ray is made to pass through the bacterial cell suspension, and

the amount of light that passes through and reaches the detector is estimated. The principle behind this technique is that when a light ray passes through a sample containing cells, it gets scattered. The amount of light that gets scattered is directly proportional to the cellular biomass, thereby setting a linear relationship within a certain limit. However, when the bacterial cell concentration is very high, then the linearity tends to deviate. As a light beam passes through a cell suspension, the intensity of light decreases due to absorption by the cells. The relation between cell density and the amount of transmitted light can be best explained with the help of Beer–Lambert law. Mathematically, Beer–Lambert law is as follows:

$$\frac{I}{I_0} = 10^{-x.L} \tag{1}$$

where, I_O = intensity of incident light, I = intensity of the light that has been transmitted, x = density of cell suspension, L = length of the light path.
The law states that the intensity of light that is transmitted (I) through the cells of density x reduces as the logarithm of x to the base 10. As the density of the cell population increases, the amount of light that is transmitted also reduces exponentially. Therefore, the above equation can be represented as follows by taking $\log_{10}$ on both sides:

$$\log_{10}\frac{I}{I_0} = -x.L \tag{2}$$

However, in a spectrophotometer, instead of measuring the amount of light that is transmitted, the amount of light that is absorbed by the bacterial cells is measured, thereby establishing a direct relationship between cell density and the amount of light absorbed. Since the amount of light that is absorbed is directly proportional to the cell density, Eq. (2) can be represented as follows:

$$\log_{10}\frac{I_0}{I} = x.L \tag{3}$$

The logarithmic fraction of I_0/I is referred to as absorbancy (A) or turbidity or optical density (OD), which is measured at a wavelength of 600 nm. Therefore, the OD_{600} of a sample tends to increase with an increase in the cell density.

$$\log_{10}\frac{I_0}{I} = A = OD_{600} = x.L \tag{4}$$

The optical density of a sample is measured with the help of a spectrophotometer or colorimeter. If the absorbancy of an unknown sample is to be determined, then a standard curve consisting of known sample concentrations versus their optical density has to be plotted (Behera et al., 2012).
The Beer–Lambert law is a combination of two laws, where the Lambert law states that the amount of light that is absorbed by a particular medium does not depend on incident light's intensity. Therefore, the amount of light that is transmitted is expressed as a ratio of the intensity of the light that is transmitted versus the intensity of the incident light.

$$\frac{I}{I_0} = T \tag{5}$$

where T = transmittance; I = intensity of transmitted light and I_O = intensity of the incident light.
Beer's law states that the absorbance of light by a particular medium depends on its concentration and length of the light path. In the spectrophotometric analysis, the length of the light path is the width of the cuvette referred to as pathlength.

$$A \propto c.L \tag{6}$$

where A = absorbance of the solution; c = concentration of a solution and L = pathlength. In the case of a spectrophotometric analysis based on normal cuvettes, the pathlength is specified to be 1 cm (10 mm).
Therefore, the absorbance of a given solution is given by:

$$A = \varepsilon.c.L$$

In the above equation, ε is the molar extinction coefficient of the solution. It is a measure of how strongly a solution absorbs light at a particular wavelength. To determine the concentrations of DNA and proteins, the spectrophotometer is set at a wavelength of 260 and 280 nm, respectively. In the case of turbidity-based measurements of growth, the maximum absorbance of the cells is observed at a wavelength of 600 nm, which lies within the visible range of light. The optical density is generally measured at a wavelength of 600 nm, i.e., OD_{600} for the estimation of bacterial growth.

4. **Other methods of measuring microbial growth**
The extent of microbial growth can be correlated with the amount of its cellular constituents. Several parameters determine the use of a particular cell component as an indirect tool for measuring microbial growth. The concentration of

that compound must be constant and present in all the organisms. In addition, a reliable and sensitive extraction method needs to be developed for the quantitative measurement of that particular compound. The chemical components like adenosine triphosphate (ATP), D-muramic acid, D-alanine and fatty acids of bacterial origin can be assessed. Adenosine triphosphate (ATP) is the energy currency of the cell that is available in all the organisms. A sensitive technique available for its quantitative estimation is the luciferin–luciferase system. In the enzymatic reaction, ATP reacts with the luciferin–luciferase complex resulting in a light emission that is detected. The method is easy, rapid as well as robust and even aids in the detection of unculturable organisms. However, there are problems while quantitating ATP, especially from the soil biomass. The process of extraction of ATP from the cells remains incomplete, and after the extraction, it is prone to chemical or enzymatic degradation. The algal community present in the benthic regions of water systems are the primary producers of freshwater community. The quantitative determination of algal biomass is carried out by measuring the amount of chlorophyll present in the algal cells. The most conventional method used for the estimation of chlorophyll a involves its extraction using acetone, followed by its spectrophotometric analysis. Phospholipids are another class of chemical compounds that are found in the bacterial cell membranes and can be quantitatively measured. The estimation of phospholipids is rapid and represents an accurate way of calculating microbial biomass. The quantitative determination of microbial biomass is also done by the estimation of other cellular constituents like ergosterol, chitin, carbon and amino acids (Martens, 1995; Frostegard and Baath, 1996; Kahlert and McKie, 2014).

6 Growth Kinetics of Batch Fermentation

Microbial growth can be viewed as a response to changes in the natural environment where a suitable cultivation media helps in the synthesis of compounds required for several biological processes. Microbial cells require nutrients for energy generation as well as for the production of biological macromolecules that increase cellular biomass.

Binary fission is the most common route of bacterial reproduction, wherein a cell divides into two individual cells. These individual daughter cells further divide to form a total of four individual cells. Mathematically this increase in the cell number is expressed in terms of geometric progression as follows:

$2^0 \ldots 0.2^1 \ldots 0.2^2 \ldots 0.2^3 \ldots 0.2^{\,m}$ or $1 \ldots 2 \ldots 0.4 \ldots 0.8 \ldots 0.16 \ldots 0.2^{\,m}$ where m is the number of generations.

If cell death is not taken into consideration, then with each generation time, bacterial cells double their population. The total bacterial population can be calculated based on the initial bacterial number that is used as the inoculum (M_0), the final bacterial population (M) after a certain period and the number of bacterial generations (m). Mathematically, these variables can be elucidated using the following

equation:

$$M = M_0 \times 2^m$$

By taking logarithmic values ($\log_{10}$) on both sides, we get:

$$\log_{10} M = \log_{10} M_0 + m\log_{10} 2$$

$$\log_{10} M - \log_{10} M_0 = m\log_{10} 2$$

$$\log_{10} \frac{M}{M_0} = \log_{10} 2^m$$

$$m = \frac{\log_{10} M - \log_{10} M_0}{\log_{10} 2}$$

The value of $\log_{10} 2$ is 0.301 which can be substituted in the above equation, to give us the following final equation:

$$m = \frac{\log_{10} M - \log_{10} M_0}{0.301} \text{or } m = 3.3\left(\log_{10} M - \log_{10} M_0\right)$$

If the number of the initial and final bacterial population is known, then using the above formula, the number of bacterial generations can be calculated.

The generation time (t_d) of a rapidly dividing bacterial population is defined as the time duration required by the bacterial cells to double its population. Using the knowledge of the number of generations (m) in the time period (t), the generation or the doubling time of the actively growing bacterial cells in their exponential phase can be determined as follows:

$$t_d = {}^{t}/_{m}$$

where m is equal to: $3.3(\log_{10} M - \log_{10} M_0)$.

The generation time is an inherent property of a microorganism. Different bacterial species possess varied generation times that further depends on the nutrients and the fluctuations in the surroundings.

In bacterial growth kinetics, the number of bacterial generations per unit time is expressed in terms of growth rate (μ) which can be described in the following terms:

$$\mu = \frac{m}{t} = \frac{\text{Number of generations}}{\text{Total time period}}$$

where m is the number of bacterial generations and t is the time. Since t/m represents the generation time (t_d), μ is represented as the reciprocal of generation time (t_d):

$$\mu = 1/t_d$$

Experimentally, the growth rate of a microorganism can be calculated by inoculating it in a suitable growth medium and measuring its turbidity with respect to time. Knowledge of the growth rate of the microorganism is used to grow and cultivate it in suitable media. Batch, fed-batch and continuous cultivation strategies are generally employed to grow microbial cells. In batch culture, microorganisms are grown in a closed environment with specific amounts of nutrients. Since no new media is supplemented to sustain the growth of these microorganisms after nutrient depletion, this type of culture exhibits the four phases of growth: lag, log or exponential, stationary and death phases. The slow growth of the cells marks the lag phase as the microorganisms adapt to the new environment. The period of adaptation is followed by the log phase, where the well-adapted cells grow at an exponential rate, thereby achieving a constant maximum growth rate. The cells growing in the logarithmic phase show an upsurge in cell number as well as dry weight.

In the exponential phase, the rate of change of biomass in batch mode is proportional to the biomass of cells present, which is depicted as follows:

$$\begin{aligned} \frac{dx}{dt} &\propto x \\ \frac{dx}{dt} &= \mu x \\ \mu &= \frac{1}{x} \cdot \frac{dx}{dt} \end{aligned} \tag{7}$$

where $\frac{dx}{dt}$ is the rate of change of cellular biomass; x is the concentration of biomass (g/l); t is the time (hours); and μ is the specific growth rate (hours^{-1}) which is defined as the rate of change of biomass per unit biomass.

The net specific growth rate (μ_{net}) of a microbial population can be obtained after subtracting the death rate of the cells (k_d) from the total specific growth rate (μ_g).

$$\mu_{net} = \mu_g - K_d$$

However, by neglecting the death rate K_d (hours^{-1}), during the exponential phase, the net specific growth rate of the cells is equal to the gross specific growth rate of the system.

$$\mu_{net} = \mu_g$$

By Eq. (7), we get that the rate of increase in the cellular biomass growing in the exponential phase is directly proportional to the initial concentration of the cells. Now integrating both the sides of Eq. (7), we get:

$$\int \frac{dx}{dt} = \int \mu x$$

$$\int \frac{dx}{x} = \mu \int dt$$

Now taking the limits, i.e., time from $0 \rightarrow t$ and biomass from $x_0 \rightarrow x_t$, we get:

$$\ln\ x_t - \ln\ x_0 = \mu t \tag{8}$$

$$\ln\ x_t = \ln\ x_0 + \mu t \tag{9}$$

Or

$$x_t = x_0 e^{\mu t}$$

where x_0 is the initial concentration of cells at $t = 0$; x_t is the concentration of microbial biomass at time t; and e is the base of the natural logarithm.

From Eq. (9), it is deduced that when a graph of the natural logarithm of cell concentration is plotted against time, it will yield a straight line in the logarithmic phase. The slope of the line will give specific growth rate (μ) of the microbial population. Since the microorganisms will be reproducing at their maximum growth rate in the exponential phase, the specific growth rate (μ) will be represented as the $\mu_{\max}$.

By rearrangement of Eq. (9), we get:

$$\ln \frac{x_t}{x_0} = \mu t \tag{10}$$

The doubling time (t_d) of the microbial population is the time taken by the cells to double their population. This indicates that if the initial microbial biomass is represented by x_0, then after t_d, the final biomass will be twice of x_0, i.e., 2 x_0. Therefore, by putting the respective values in Eq. (10) we get:

$$\ln \frac{2x_0}{x_0} = \mu t_d$$

$$\ln 2 = \mu t_d$$

The value of the natural logarithm of 2 is 0.693; therefore, the above equation becomes:

$$t_d = \frac{0.693}{\mu}$$

Hence, if the doubling time of the microbial cells is known, then the specific growth rate can be easily calculated.

The microbial cells growing in a batch culture rapidly utilize the nutrients present in the medium and grow at an exponential rate. At this stage, they also synthesize several growth-associated products known as primary metabolites. However, after a certain period of growth, microbes face the challenges of nutrient deprivation, toxic waste accumulation and by-product formation. These fluctuations in a closed system induce changes in the cellular metabolism that results in unbalanced growth. This type of growth is exhibited by cells in response to stress conditions that may lead to variations in the intracellular composition. In such a scenario, the change in cell number may be constant, but variations in cell mass may be observed. The exponential phase is marked by an enhanced level of reproduction rates, whereas the deceleration phase is highlighted by the strategies employed by a cell to adapt to the unfavorable conditions.

The termination of the deceleration phase initiates the stationary phase where the value of μ_{net} equals zero. In this phase, the growth rate and the death rate are equal, and the net specific growth rate becomes zero. However, even though the net specific growth rate approaches zero, the cells are not unviable; they are metabolically active and continue to synthesize non-growth-associated products known as secondary metabolites. The production of certain secondary metabolites such as antibiotics and hormones is more pronounced during this stage.

The bacterial growth cycle culminates with the death phase. The deficiency of key nutrients in the medium and accumulation of toxic wastes adversely affects cellular health leading to a decline in the growth rate of the cells and the microbial population. Similar to the growth rate, the death rate of the microbial cells also follows an exponential pattern.

$$\frac{dx}{dt} = -k_d x \tag{11}$$

where k_d is the death rate constant; $\frac{dx}{dt}$ is the rate of change of biomass; and x is the microbial biomass.

By rearranging Eq. (11) and integrating it on both sides, we get:

$$\int \frac{dx}{x} = \int -k_d .dt$$

Taking limits $x_s \rightarrow x$ and $0 \rightarrow t$, we get:

(x_s is the cell biomass after the stationary phase, and x is the cell biomass in death phase at time t)

$$\ln\ x - \ln\ x_s = -k_d.t$$

$$\ln\frac{x}{x_s} = -k_d.t$$

Taking ln on both sides:

$$\frac{x}{x_s} = e^{-k_d t}$$

$$\Rightarrow \ln\frac{x}{x_s} = -k_d\ t$$

Monod Model of Batch kinetics

Jacques Monod developed a mathematical model based on the effect of nutrient concentrations on bacterial growth in batch fermentation. The studies were carried out with *Escherichia coli* as the model microorganism, and observations were made based on its growth in different glucose concentrations. Monod kinetics for batch culture is similar to the Michaelis–Menten enzyme kinetics, which states that the growth rate of the bacterial population is dependent on only one growth-limiting substrate. Analogous to Michaelis–Menten equation, the V, $V_{\max}$ and K_m are replaced by μ, $\mu_{\max}$ and K_s, respectively. Therefore, the equation for Monod kinetics can be written as follows:

$$\mu = \frac{\mu_{\max}.S}{K_s + S} \tag{12}$$

where μ is the specific growth rate; $\mu_{\max}$ is the maximum specific growth rate; S is the concentration of growth-limiting substrate; and K_s is the saturation constant.

The value of K_s is defined as the substrate concentration at which the specific growth rate achieves the value of half of the maximum growth rate ($\mu_{\max}$). The saturation constant highlights the affinity of an organism toward a specific substrate. If the value of K_s is high for a particular substrate, then the affinity of the bacterial population toward that substrate is low, and if the K_s is low, then it denotes a high affinity for the substrate. The smaller value of K_s indicates that the bacterial population is growing at a specific growth rate which is close to the maximum specific growth rate ($\mu_{\max}$) of the population. The maximum specific growth rate, i.e., $\mu_{\max}$, depends on the organism whose growth is being monitored, whereas the K_s value depends on the available nutrient (Fig. 5).

Taking the inverse of Eq. (12) results,

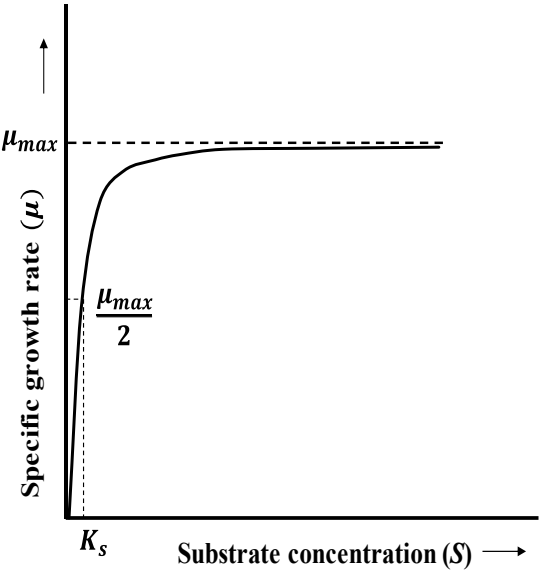


Fig. 5 Effect of a growth-limiting substrate concentration on the specific growth rate of a microorganism

$$\frac{1}{\mu} = \frac{K_s + S}{\mu_{\max}.S}$$

$$\frac{1}{\mu} = \frac{K_s}{\mu_{\max}.S} + \frac{S}{\mu_{\max}.S}$$

$$\frac{1}{\mu} = \frac{K_s}{\mu_{\max}.S} + \frac{1}{\mu_{\max}}$$

Since the above equation follows the equation of a straight line, i.e., $y = mx + c$, graphically it is represented in Fig. 6.

Growth Yield Coefficients

The microbial growth yield coefficient (Y) is the measure of the conversion efficiency of the substrate to product and biomass. Nutrients available in the growth media are used for the generation of microbial biomass. Mathematically, the substrate utilized for biomass production is represented in the form of true or theoretical yield coefficient as follows:

$$Y^t_{X/S} = \frac{\Delta x}{\Delta S}$$

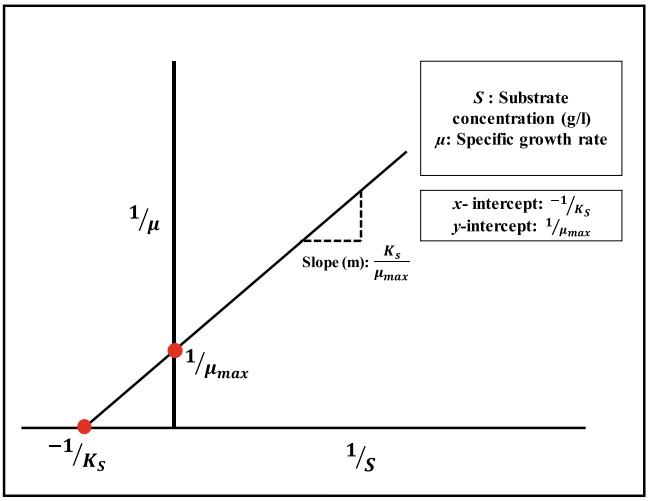


Fig. 6 A Lineweaver–Burk plot of $\frac{1}{\mu}$ plotted against $\frac{1}{s}$. The x-axis and y-axis intercepts are given by $-\frac{1}{K_s}$ and $\frac{1}{\mu_{\max}}$, respectively, where K_s denotes the affinity of an organism toward a substrate. The slope (m) of the graph is given by $\frac{K_s}{\mu_{\max}}$

where $Y^{t}{}_{x/S}$ is the moles or mass of biomass produced per mole or mass of substrate consumed.

Δx is the net moles or mass of biomass produced, and ΔS is the net moles or mass of substrate consumed.

Similarly,

$$Yx_{/ATP} = \frac{\Delta x}{\Delta ATP} = \frac{\text{biomass produced}}{ATP \text{ consumed}}$$

$Yx_{/ATP}$ is the units of biomass generated per mole of ATP consumed

$$Yx_{/O_2} = \frac{\Delta x}{\Delta O_2} = \frac{\text{biomass produced}}{\text{oxygen consumed}}$$

$Yx_{/O_2}$ is the units of biomass produced per unit of oxygen consumed

$$Y_{P/S} = \frac{\Delta P}{\Delta S} = \frac{\text{product produced}}{\text{substrate consumed}}$$

$Y_{P/S}$ is the amount of product synthesized in moles or mass per unit of substrate consumed

$$Y_{CO_2/S} = \frac{\Delta CO_2}{\Delta S} = \frac{\text{Carbon dioxide produced}}{\text{substrate consumed}}$$

$Y_{CO_2/S}$ is the units of carbon dioxide evolved per unit of substrate consumed.

Where Δx is the net biomass in moles or mass; ΔATP is the net ATP in moles; ΔO_2 is the net oxygen in moles or mass; ΔP is the net product in moles or mass; ΔS is the net substrate in moles or mass; and ΔCO_2 is the net moles or mass of carbon dioxide.

However, the substrate provided to the microbial population is utilized not only for growth and reproduction, but some of it is diverged and assimilated for cell maintenance as well as for energy and metabolite production.

Therefore, the observed yield $\left(Y^o_{x/S}\right)$ is represented as:

$$Y^o_{x/S} = \frac{\Delta x}{\Delta S'}$$

where

$$\Delta S' = \Delta S_{\text{biomass}} + \Delta S_{\text{maintenance}} + \Delta S_{\text{product}} + \Delta S_{\text{growth}}$$

Respiratory quotient

The ratio of carbon dioxide produced to oxygen consumed in living cells is known as respiratory quotient (RQ). It is represented as:

$$RQ = \frac{\text{moles of } CO_2 \text{formed}}{\text{moles of } O_2 \text{utilized}}$$

The rate of change of oxygen concentration during growth is directly proportional to the cellular biomass.

$$-\frac{dO_2}{dt} \propto x$$

(The negative sign denotes the reducing concentration of oxygen as it is being utilized)

where $\frac{dO_2}{dt}$ is the rate of change of oxygen concentration and x is the cell biomass

$$-\frac{dO_2}{dt} = q_{O_2} x$$

$$q_{O_2} = -\frac{1}{x}.\frac{dO_2}{dt} \tag{13}$$

where q_{O_2} is the specific oxygen consumption rate.

Now,

$$Y_{x/O_2} = \frac{\Delta x}{\Delta O_2}$$

Multiplying and dividing the right-hand side by Δt

$$Y_{x/O_2} = \frac{\Delta x}{\Delta O_2} \cdot \frac{\Delta t}{\Delta t}$$

$$Y_{x/O_2} \cdot \frac{\Delta O_2}{\Delta t} = \frac{\Delta x}{\Delta t} \tag{14}$$

$$\text{It is known that: } \frac{dx}{dt} = \mu x \tag{15}$$

Now putting Eq. (14) in Eq. (15), we get:

$$Y_{x/O_2} \cdot \frac{\Delta O_2}{\Delta t} = \mu x$$

$$Y_{x/O_2} \cdot \frac{\Delta O_2}{\Delta t} \cdot \frac{1}{x} = \mu, \text{ where } \frac{1}{x} \cdot \frac{\Delta O_2}{\Delta t} = q_{O_2}$$

Hence,

$$Y_{x/O_2} \cdot q_{O_2} = \mu$$

$$q_{O_2} = \frac{\mu}{Y_{x/O_2}}$$

From the above equation, it is evident that the oxygen consumption rate of aerobic culture depends upon the specific growth rate of microbial culture.

Maintenance Energy

Microbial cells perform several cellular functions such as cell repair, transport of materials across the cell membrane, uptake of nutrients from the external environment, osmoregulation and motility. The energy required to carry out these housekeeping functions is termed as maintenance energy.

The rate at which the substrate will be utilized for the maintenance of cellular function is represented via the maintenance coefficient (m) as follows:

$$-\frac{dS}{dt} \propto x$$

(the negative sign denotes the reducing concentrations of the substrate as it is being utilized)

$$-\frac{dS}{dt} = mx$$

where m denotes the maintenance coefficient

$$m = -\frac{1}{x}.\frac{dS}{dt}$$

Growth and Non-growth-Associated Products

Microorganisms can synthesize several products and metabolites during their course of growth and reproduction. However, not all the products are produced at the same time simultaneously. Certain substances such as amino acids, enzymes and hormones are the primary metabolites synthesized during the exponential phase and hence are referred to as growth-associated products. During growth-associated product formation, the concentration of the product (P) synthesized is directly proportional to the microbial biomass (x).

$$P \propto x \Rightarrow \frac{dP}{dt} \propto x$$

$$\frac{dP}{dt} = q_p.x$$

$$q_p = \frac{1}{x}.\frac{dP}{dt} \tag{16}$$

where $\frac{dP}{dt}$ is the rate of change of product concentration; x is the microbial biomass; and q_p is the specific product formation rate.

In growth-associated product formation, there is a direct correlation between specific product formation rate (q_p) and growth rate of the microorganism (μ) which is derived as follows:

$$Y_{P/x} = \frac{\Delta P}{\Delta x}$$

Multiplying and dividing the right-hand side with Δt, we get:

$$Y_{P/x} = \frac{\Delta P}{\Delta x}.\frac{\Delta t}{\Delta t}$$

$$Y_{P/x} = \frac{\Delta P}{\Delta t}.\frac{\Delta t}{\Delta x} \tag{17}$$

$$\frac{\Delta P}{\Delta t} = Y_{P/x} . \frac{\Delta x}{\Delta t} \text{ Where } \quad \mu = \frac{1}{x} . \frac{dx}{dt} \tag{18}$$

Therefore, substituting (16) and (18) in Eq. (17) we get:

$$q_p . x = Y_{P/x} . \mu x$$

$$q_p = Y_{P/x} . \mu$$

or

$$q_p \propto \mu$$

where $Y_{P/x}$ is constant under ideal growth conditions using any specific substrate. Hence, in this case, the specific product formation rate, denoted by q_p, is directly proportional to the specific growth rate of cells, that is, μ.

Substances such as antibiotics or other secondary metabolites are produced during the stationary phase of the microbial growth and are referred to as non-growth-associated products. During their synthesis, the specific growth rate is zero and the specific product formation rate (q_p) is constant and also independent of μ.

$$q_p = \beta; \text{ Where } \quad \beta \text{ is a constant}$$

Certain secondary metabolites, lactic acid and xanthan gum are mixed-growth-associated products. These types of products are synthesized in both the stationary and growth phases. The equation representing mixed-growth-associated product formation is:

$$q_p = \alpha\mu + \beta$$

Such that: If α equals zero, then only non-growth-associated product formation takes place; if β equals zero, then only growth-associated product formation occurs, and then α will equal $Y_{P/x}$ (Fig. 7).

7 Continuous Stirred Tank Reactor (CSTR)

Batch cultures show four different stages of growth that occur due to the changing concentration of nutrient and accumulation of by-products in the cultivation medium. In contrast, continuous cultures do not pass through these changes due to the addition of fresh nutrients along with continual removal of spent media. Continuous

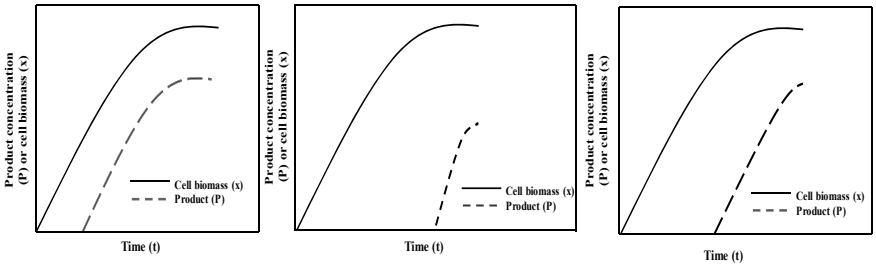


Fig. 7 Growth-associated, non-growth-associated and mixed-growth-associated product formation

culture strategy provides microorganisms with steady optimal environmental conditions and enables maintenance of microbial growth and synthesis of uniform quality products for a longer duration as compared to batch culture. In continuous cultures, a steady-state phenomenon is obtained where the concentration of substrate, product and biomass remains constant. Hence, CSTR is an ideal strategy to study the way microorganisms respond to the changing environmental conditions during growth. In CSTR, the oxygen requirements of the culture are met by the impeller and sparger. The impeller agitates and provides aeration through the formation of air bubbles. It also plays an important role in the uniform mixing of fermentation media. The high oxygen demand of aerobic microorganisms is fulfilled by the air sparger located at the bottom of a culture vessel/fermenter. Probes are used to aid in real-time determination of pH, temperature and dissolved oxygen (DO) levels in the culture vessel. The presence of a cooling jacket around the fermenter vessel or cooling coil inside it helps in the maintenance of the desired temperature.

Specific growth rate in a continuous culture

The rate of the flow of the medium in and out of the fermenter vessel and the culture volume are denoted by F and V, respectively. The ratio of F to V, that is, F/V gives the dilution rate (D), which is defined as the rate of flow of nutrient medium per unit volume (Fig. 8).

In a CSTR, the net increase in the cellular biomass will be given by subtracting the output of cells from the growth in the fermenter vessel. This is represented as follows:

$$\text{Net biomass increase in CSTR} = \text{Growth in CSTR} - \text{Output of cells from CSTR}$$

Now, for a bioreactor of working volume V and time interval dt, the equation can be written as:

$$V.dx = V.\mu x.dt - Fx.dt \tag{19}$$

By dividing Eq. (19) by $V.dt$, we get:

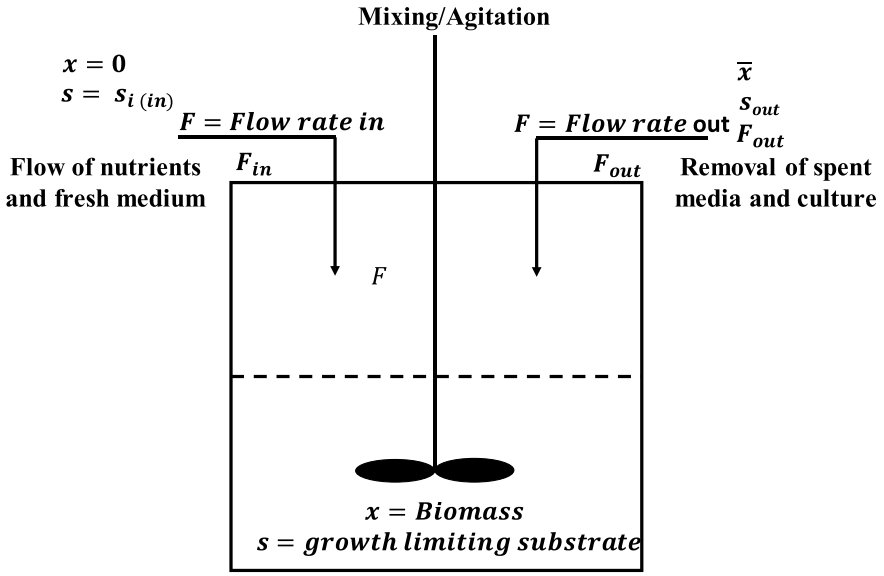


Fig. 8 A diagrammatic representation of a continuous stirred tank reactor (CSTR)

$$\frac{dx}{dt} = \mu x - Dx \tag{20}$$

At steady state, $\frac{dx}{dt} = 0$; hence,

$$\mu = D$$

When $\mu = D$, Eq. (12) can be written as:

$$D = \frac{\mu_{\max}.\overline{S}}{K_s + \overline{S}} \tag{21}$$

where $\overline{S}$ is the steady-state substrate concentration.

At steady state, the dilution rate (D) is used to control the desired specific growth rate. By arranging Eq. (21), the $\overline{S}$ can be calculated as:

$$\overline{S} = \frac{K_s.D}{\mu_{max} - D}$$

In the case of steady-state conditions, where the rate of change of biomass is zero, the growth rate (μ) is equal to the dilution rate (D). In such a scenario, the substrate and cell concentration remain constant; i.e., there is no net accumulation of biomass or substrate. However, if $\mu > D$, then biomass accumulation will occur

in the bioreactor and a substrate-limiting condition will develop. On the other hand, if $\mu < D$, then washout of the cells will occur with substrate accumulation. So, it is always beneficial to run a CSTR close to μ_{max} to avoid washout and loss in productivity of the bioreactor.

8 Fed-Batch Culture

The late twentieth century witnessed major development in the field of bioprocess engineering with high-level production of industrial enzymes and therapeutic proteins. A significant rise in the demand for proteins such as antibodies and industrial enzymes laid stress on the need to explore modifications of the existing production systems and innovative strategies for yield improvement. The product concentration achieved at the end of a fermentation run depends on the productivity of the recombinant strain, the maximum cell biomass attained and the cell survival in a high-cell-density fermentation. In batch fermentation strategy, it is very difficult to achieve maximum protein production, as a limited amount of nutrients is supplemented at a concentration that is non-toxic to the cells. Since it is a closed system, nutrient deprivation in the later stages of fermentation does not allow for high-cell-density cultivation (HCDC). This limitation in carrying out high-cell-density fermentation can be resolved by employing the strategy of fed-batch cultivation. Fed-batch cultures involve continuous or semi-continuous supplementation of nutrients into the fermentation medium without the removal of spent media. This strategy is especially useful when the substrate is inhibitory in nature. The intermittent supply of substrate thus aids in the prevention of substrate accumulation and catabolite repression.

In fed-batch culture, the growth-limiting nutrient is supplemented in a concentrated form, which helps in enhancing the duration of logarithmic phase and maximizes product concentration by increasing viable cellular biomass at high-cell-density concentrations. The mechanism of operating a fed-batch culture is similar to a batch culture. The inoculum is added at a low concentration to a fermenter medium whose composition is similar to that of the batch. The cells grow at an exponential rate utilizing nutrients supplemented in the media. Following nutrient depletion, as the cells start approaching the stationary phase, a fraction of the cells and product are recovered and supplemented with fresh nutrients. This process is known as intermittent-harvest fed-batch strategy, which can be repeated several times to achieve high cell density and enhanced product yields. This approach is mostly used in viral vaccine production.

In contrast, a traditional fed-batch strategy is commonly employed for recombinant proteins and antibody production. In this case, the cells growing at an exponential rate are supplemented with 10–15 times concentrated basal or complex feed medium either continuously or intermittently. The fresh nutrients are added proportionately in accordance with cellular concentration. Such a strategy enables lengthening the production phase of cells and allows for higher production of desirable products. Here, the culture broth is not removed. To make up for the additional nutrients, the

initial volume of the fed-batch culture is kept low, i.e., 40–50% less as compared to its maximum volume. Although it is important to keep the initial volume low enough to adjust for the additional medium constituents, it should have a minimum level such that the fermenter impeller remains submerged, a factor critical for proper mixing and aeration. In order to run a successful fed-batch culture, the medium and the feeding strategy need to be designed appropriately. The feed has to be added at a particular concentration in suitable stoichiometric ratios. The feeding rate of concentrated nutrients should match with the consumption rate of the cells, such that the substrate concentration is maintained below the inhibitory level. In addition, the feed has to be supplied in a concentrated form to avoid its accumulation in the bioreactor, which would subsequently increase the culture volume. The feeding strategy further depends on the control mechanism and mode of feeding. The control mechanism is designed such that the concentration of one or two nutrients is kept flexible while the rest of the feed is provided according to stoichiometric ratios that match with the rate of consumption by the cells. The mode of feeding, on the other hand, can be intermittent or continuous. In most of the fermenter runs, continuous mode of feeding is preferred. The feed can be supplied at a constant or variable interval, with either a fixed or variable feed volume using a peristaltic pump (Costa et al., 2014).

Feeding strategies used in fed-batch culture

The nutrient feeding strategy can be classified into three different types depending on the mode of feeding. These include constant feeding, stepwise increased feeding and exponential or continuous feeding (Fig. 9).

In the case of constant feeding strategy, concentrated feed medium is supplied at a constant rate, such that over a period of time, both fermenter volume and the cell concentration increase. The stepwise increased feeding strategy involves supplementation of nutrients at a rate that increases in a stepwise or linear manner. The exponential feeding strategy is the most suitable method to achieve a high cell density in a fermenter. In this approach, the nutrients are supplied at an exponential rate such that the cell growth also occurs in a logarithmic manner. The rate of nutrient feeding is increased in proportion to the cell growth which aids in the maintenance of a

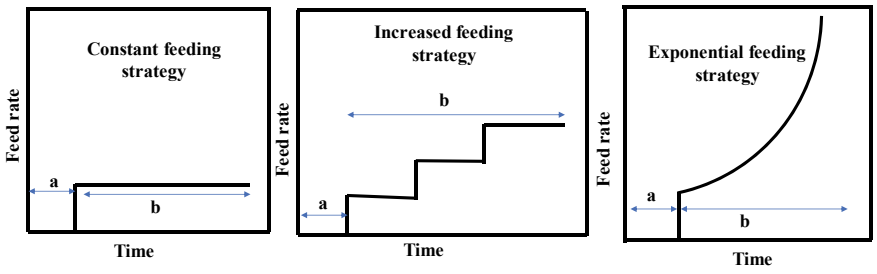


Fig. 9 Different nutrient feeding strategies to achieve high cell density in fed-batch cultivation; (i) constant feeding strategy; (ii) stepwise increased feeding strategy; and (iii) exponential feeding strategy, where a is batch phase and b denotes the fed-batch phase

constant specific growth rate, i.e., where:

$$\mu = \frac{1}{x} \cdot \frac{dx}{dt}$$

The fed-batch feeding profile is regulated using the following equation:

$$F = F_0 . e^{\mu t}$$

where F_0 is the initial feed rate, F is the final feed rate, μ is the specific growth rate, t is the time and e is the base of the natural logarithm.

There are several ways to devise the most appropriate feeding strategy. One way of regulating the rate of feeding involves determining the concentration of essential nutrients in the culture broth. This is the most convenient method to understand how much amount of feed should be applied and at what rate and time intervals. If the concentration of nutrients is known, then at low nutrient concentrations, the feed can be supplemented at correct time intervals. For example, in case of mammalian cells such as myeloid-derived cells, glucose and glutamine play a significant role in maintaining the viability of the cells. Therefore, they are commonly used as a control for measuring the nutrient concentrations. There are online as well as offline systems available by which nutrient concentration can be measured.

In another mechanism, the nutrient feeding can be designed so as to control the culture pH at a set point to avoid acid or base accumulation. The principle behind this strategy is that when glucose is consumed, acid production takes place that tends to lower the pH of the medium. In the case of mammalian cells, the amount of lactic acid produced is measured to determine the nutrient consumption rate and subsequent feeding profile. Depending on the amount of lactic acid production during fermentation, the rate at which base is to be added can be determined.

An alternate method by which feeding can be controlled is by measuring the culture turbidity. Controlling the nutrient feeding at a rate proportional to turbidity is the most straightforward strategy that is useful when the cells are growing in the exponential phase to maintain a constant specific growth rate. However, when the logarithmic phase ceases, the feed rate has to be modified or adjusted according to the growth requirement to avoid overfeeding and nutrient accumulation.

Nutrient feeding strategy can also be designed on the basis of microbial oxygen uptake rate (OUR). This strategy is very accurate, sensitive and dependable, and effectively determines the feed rate profile. The oxygen consumption rate, unlike pH, is not dependent on external factors such as presence of buffers or production of other acidic metabolites by the cells. A slight change in the OUR can be precisely measured, which will reflect the changes in the metabolic rates of the cells. There are online measurement methods to assess the relationship between OUR and nutrient consumption rate of the cells (Wlaschin and Hu, 2006).

A comparative analysis of a few basic features of batch, fed-batch and CSTR has been given in Table 1.

Table 1 Features of batch, fed-batch and continuous cultures

S. No.	Batch culture	Fed-batch culture	Continuous culture
1	It is a closed system, where the batch components are not removed or added. The nutrients are supplemented at the start of the fermentation run, and then the microorganism is allowed to grow without further nutrient supplementation	It is initially operated in the batch mode. However, after a particular time interval, the fresh nutrients are added at a continuous or semi-continuous rate	It is an open system, where fresh media components, nutrients and other additives are added and spent media containing cells, product and other by-products is removed continuously
2	Due to its closed nature, the four phases of growth—lag, logarithmic, stationary and death phases—are observed. The system is transient where the growth rate tends to zero toward the end of the fermentation	The cells are maintained in the logarithmic phase by supplementation of fresh nutrients and media components. However, after a certain period, the growth declines due to the accumulation of toxic wastes and other metabolic by-products	The system is maintained in the logarithmic state. The growth and the product formation rates are maintained for a long duration, and once steady state is achieved, the cell biomass and nutrient concentration remain constant inside the fermenter
3	The batch mode of fermentation is popular as it is relatively simpler, mechanically reliable and more flexible	The fed-batch mode of fermentation has advantages over the batch mode of culturing as it gives higher cell density and enhanced volumetric productivity. A better control over nutrient feeding can be achieved without toxic waste or by-product accumulation	The continuous culture aids in achieving high yields and product titers as the growth can be maintained for a prolonged duration, thereby helping in attaining high product titers. However, the continuous operational conditions in a chemostat (a system in which the chemical composition is kept at a controlled level by continuous addition of fresh medium and removal of culture liquid containing leftover nutrients, metabolic end products and microorganisms) may put pressure on the cells

(continued)

Table 1 (continued)

S. No.	Batch culture	Fed-batch culture	Continuous culture
4	The nutrients are supplied initially in the fermentation media at a particular quantity, which gradually declines, leading to the cessation of growth	The nutrients are supplied at a point where nutrient deprivation occurs. However, care has to be taken to avoid nutrient accumulation that may lead to growth inhibition	The nutrients are supplied continuously. The spent media is withdrawn, and simultaneously, fresh media is supplemented to maintain a desired growth rate of the cells
5	There is no change in the volume of the fermenter as no media components are added or removed	The volume of the fermenter increases as fresh nutrients are added at periodic intervals to the fermenter, but the spent media is not removed	There is no change in the volume of the fermenter as the amount of spent media that is removed from the fermenter is replaced by the fresh nutrients
6	Represented mathematically as: $\mu = \frac{\mu_{max}.S}{K_s+S}$	Represented mathematically as: $x = x_0 e^{\mu t}$ $F = F_0.e^{\mu t}$	Represented mathematically as: $D = \frac{F}{V}$ At steady state, $\mu = D$

9 Applications of High-Cell-Density Cultivation Strategies

The strategy of high-cell-density cultivation (HCDC) is used to enhance the microbial biomass and increase the volumetric product concentration. The primary goal of the fermentation industry is to obtain a maximized volumetric productivity to have cost-effective production of the desired product. The term 'volumetric productivity' denotes the amount of product synthesized in a fixed volume within a specified period. Initially, the concept of HCDC was introduced for the production of single-cell protein (SCP), ethanol and microbial biomass from yeast cultures. Later, it was broadened to include the cultivation of other mesophilic organisms and derive commercially and industrially important products. With the advent of recombinant DNA technology (RDT), *E. coli* is the most preferable host for heterologous protein production at large scale.

Although HCDC has been successfully used for the over-production of industrially relevant proteins, several problems are associated with this approach. There are certain microorganisms that are fastidious, and their specific requirements may prove to be a challenge for bioengineers while designing an efficient production system. A high-scale fermenter has several associated problems such as solubility of gases and other substrates in media, substrate-limiting conditions and growth inhibition due to substrate accumulation. Stability of media components and products, toxic effects of products, by-product accumulation, product degradation, enhanced demands for oxygen, high heat, foaming and CO_2 generation are some of the other major roadblocks that one may encounter while running a high-scale fermenter. The commonly

observed metabolic by-products which accumulate during microbial growth in the presence of high concentrations of carbon source include acetate, propionate, lactate and ethanol in the case of *E. coli, Bacillus subtilis, L. lactis* and *S. cerevisiae*, respectively. By limiting the amount of carbon source in the medium or by using metabolic engineering approach, one can aim to reduce the accumulation of these undesirable by-products during the fermentation process (Riesenberg & Guthke, 1999).

A large number of biomolecules and proteins, including both primary and secondary metabolites, have been produced using large-scale fermentation processes. Several strains of *Corynebacterium*, *Brevibacterium* and *Serratia* are commonly employed for amino acid production. Several modified strains of *C. glutamicum* have been developed using genetic engineering strategies, for the over-production of L-glutamate and aromatic amino acids like tryptophan. Vitamins are another class of primary metabolites that have been produced using microbial fermentation. Several organic acids such as acetate, pyruvate, succinate and lactate have been produced using metabolically engineered strains of *E. coli* and *C. glutamicum.* The treatment of bacterial infections has been made possible by the advent of β-lactam antibiotics such as penicillin G, cephalosporin G, cephamycin G and other derivatives. Genetic engineering of the parent strain, *Penicillium chrysogenum*, has been carried out resulting in high-level production of β-lactam antibiotics. Several other biopharmaceutical compounds such as rapamycin from *Streptomyces hygroscopicus* and artemisinin from engineered *E. coli* and *S. cerevisiae* strains have been synthesized using microbial fermentation (Adrio & Demain, 2010).

Summary

- Growth is defined as an increase in the cellular constituents, which leads to a surge in the cell number or cell size. The study of microbial growth is beneficial as it helps in understanding the cellular physiology when grown under different cultivation conditions.
- Microorganisms can be cultured using batch, fed-batch and continuous approach. Batch culture is a closed system where once the inoculum is added to the growth medium, no further nutrient supplementation is done.
- During batch fermentation, a bacterial population undergoes four phases of the growth cycle: the lag phase, in which the cells are metabolically active and prepare themselves for division by synthesizing new cellular components such as ATP, enzymes and other cofactors; the logarithmic phase, in which the cells grow exponentially at their maximum specific growth rate (μ_{max}); the stationary phase that represents a stage at which the microbial growth rate is equal to the death rate. Upon nutrient starvation and by-product accumulation, the cell growth ceases, where the specific growth rate becomes zero; and the death phase, where nutrient deprivation and toxic by-product accumulation lead to an exponential decline in the growth.
- The phenomenon of diauxic growth is observed when bacterial culture while growing in a medium supplemented with two utilizable sugars such as glucose and lactose shows a biphasic growth behavior. The bacterial cells preferentially

consume the most readily utilizable carbon source, i.e., glucose, which is accompanied by a lag and an exponential phase. During glucose utilization, the *lacZ* and *lacY* genes of the *lac* operon required for the lactose breakdown and uptake are not transcribed since the *lacI* repressor molecule binds to the operator region of the operon and inhibits the transcription by RNA polymerase. In the presence of high glucose, intracellular cAMP levels are low that further prevents the CAP protein binding to the CAP site. However, with glucose utilization, the intracellular cAMP level increases, enabling the CAP protein to bind to the CAP site. Further, the lactose molecule acts as an inducer that interacts with the *lac* repressor at its effector site and changes its conformation such that it no longer binds to the operator. The conformational change further facilitates RNA polymerase binding to the promoter and initiates the *lac* operon gene transcription.

- Microorganisms adapt to fluctuating and extreme environmental conditions via modification of their membrane, expression of different transporters, synthesis of compatible solutes and also by the presence of efficient DNA repair mechanisms, proteins and enzymes.
- There are several microscopic and plate-based methods that can be used to measure microbial growth. Hemocytometer and Coulter counter are examples of counting chambers that aid in microbial number determination. Pour plate and spread-plate techniques are used for the calculation of colony-forming units (CFUs). Spectrophotometric measurements, determination of wet cell weight (WCW) and dry cell weight (DCW) and quantitative estimation of ATP, chitin and ergosterol are some other methods frequently used for the assessment of microbial biomass.
- In batch kinetics, the specific growth rate is defined as the rate of change of biomass per unit biomass. It is represented as $= \frac{1}{x}.\frac{dx}{dt}$, where x is the cellular biomass and $\frac{dx}{dt}$ is the rate of change of biomass.
- The doubling time of a bacterial population is defined as the time taken by the cells to double their population. It is represented as $t_d = \frac{0.693}{\mu}$, where t_d is the doubling time and μ is the specific growth rate. Using Monod model for batch kinetics, the specific growth rate (μ) can be calculated as: $\mu = \frac{\mu_{max}.S}{K_s+S}$, where S is the substrate concentration and K_s is the substrate concentration at which the specific growth rate achieves half the value of maximum growth rate ($\mu_{\max}$).
- The fed-batch mode of cultivation is a strategy in which fresh nutrients are added upon depletion. It is a frequently used method for bacterial cultivation, where the large-scale production of recombinant proteins in high-cell-density bacterial cultures is achieved.
- The continuous stirred tank reactor (CSTR) works on the principle of steady state, where fresh nutrients are supplied to the bacterial culture, and simultaneously media containing the cells and product are regularly removed. Therefore, there is no net change of biomass, a condition known as steady state, i.e., $\frac{dx}{dt} = 0$. The CSTR cultivation strategy is commonly used for the estimation of kinetic parameters of growth.

Questions

1. Define microbial growth and also discuss four phases of bacterial growth in a batch culture.
2. What are compatible solutes? Explain with few examples.
3. How does *Deinococcus radiodurans* tolerate the high-intensity ionizing radiations?
4. Name the enzymes involved in protecting cells against reactive oxygen species (ROS).
5. Define diauxic growth. What are the three structural genes involved in *lac* operon?
6. What is the specific growth rate of a microorganism? How is it represented mathematically?
7. Differentiate between growth-associated, non-growth-associated and mixed-growth-associated product formation.
8. What is the specific product formation rate of a microorganism? Does it have any relation with the specific growth rate in case of growth-associated product formation? If yes, then demonstrate it mathematically.
9. What is maintenance energy? Discuss its significance.
10. What is programmed cell death and deliberate on its induction mechanism?
11. Name three fluorescent dyes used in the flow cytometry technique.
12. Define K_s. How does it play a role in the selection of an ideal substrate for microbial growth?
13. What is the volumetric productivity of a bioreactor?
14. How is aeration provided in a fermenter?
15. What are the different nutrient feeding strategies adopted to achieve high cell density in a fed-batch operation?
16. Derive the relation between specific growth rate (μ) and dilution rate (D) in a CSTR maintained at steady state.
17. Calculate the specific growth rate of a microorganism, if its doubling time is 30 min.
18. Explain the effect of following conditions on the cell biomass and substrate concentration in a CSTR (a) $\mu > D$, (b) $\mu = D$ and (c) $\mu < D$.

References

Abe, F. (2007). Exploration of the effects of high hydrostatic pressure on microbial growth, physiology and survival: Perspectives from piezophysiology. *Bioscience, Biotechnology, and Biochemistry, 71*(10), 2347–2357.

Adrio, J. L., & Demain, A. L. (2010). Recombinant organisms for production of industrial products. *Bioengineered Bugs, 1*(2), 116–131.

Allocati, N., Masulli, M., Di Ilio, C., & De Laurenzi, V. (2015). Die for the community: An overview of programmed cell death in bacteria. *Cell Death and Disease, 6*(1), e1609.

Angert, E. R. (2005). Alternatives to binary fission in bacteria. *Nature Reviews Microbiology, 3*(3), 214.

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: A review. *Comprehensive Reviews in Food Science and Food Safety, 3*(1), 1–20.

Behera, S., Ghanty, S., Ahmad, F., Santra, S., & Banerjee, S. (2012). UV-visible spectrophotometric method development and validation of assay of paracetamol tablet formulation. *Journal Analysis Bioanalytical Techniques, 3*(6), 151–157.

Bolhuis, H., Palm, P., Wende, A., Falb, M., Rampp, M., Rodriguez-Valera, F., et al. (2006). The genome of the square archaeon Haloquadratum walsbyi: Life at the limits of water activity. *BMC Genomics, 7*(1), 169.

Borisov, V. B., & Verkhovsky, M. I. (2015). Oxygen as Acceptor. *EcoSal Plus*, *6*(2).

Boutte, C. C., & Crosson, S. (2013). Bacterial lifestyle shapes stringent response activation. *Trends in Microbiology, 21*(4), 174–180.

Cadena-Herrera, D., Esparza-De Lara, J. E., Ramírez-Ibañez, N. D., López-Morales, C. A., Pérez, N. O., Flores-Ortiz, L. F., & Medina-Rivero, E. (2015). Validation of three viable-cell counting methods: Manual, semi-automated, and automated. *Biotechnology Reports, 7,* 9–16.

Chou, C. P. (2007). Engineering cell physiology to enhance recombinant protein production in *E. coli*. *Applied Microbiology and Biotechnology, 76*(3), 521–532.

Costa, A. R., Rodrigues, M. E., Henriques, M., Oliveira, R., & Azeredo, J. (2014). Feed optimization in fed-batch culture. In *Animal cell biotechnology* (pp. 105–116). Humana Press.

DasSarma, S., & DasSarma, P. (2015). Halophiles and their enzymes: Negativity put to good use. *Current Opinion in Microbiology, 25,* 120–126.

Doern, G. V. (2000). Detection of selected fastsidious bacteria. *Clinical Infectious Diseases, 30*(1), 166–173.

Frostegård, Å., & Bååth, E. (1996). The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biology and Fertility of Soils, 22*(1–2), 59–65.

Fütterer, O., Angelov, A., Liesegang, H., Gottschalk, G., Schleper, C., Schepers, B., et al. (2004). Genome sequence of Picrophilus torridus and its implications for life around pH 0. *Proceedings of the National Academy of Sciences, 101*(24), 9091–9096.

Griffiths, A. J., Gelbart, W. M., Miller, J. H., & Lewontin, R. C. (1999). Regulation of the lactose system. In *Modern genetic analysis*. WH Freeman.

Hagen, S. J. (2010). Exponential growth of bacteria: Constant multiplication through division. *American Journal of Physics, 78*(12), 1290–1296.

Hedblom, G., Reiland, H., Sylte, M. J., Johnson, T. J., & Baumler, D. J. (2018). Segmented filamentous bacteria—metabolism meets immunity. *Frontiers in Microbiology, 9,* 1991.

Hoffmann, F., & Rinas, U. (2004). Stress induced by recombinant protein production in Escherichia coli. In *Physiological stress responses in bioprocesses* (pp. 73–92). Springer.

Ishino, S., & Ishino, Y. (2014). DNA polymerases as useful reagents for biotechnology—The history of developmental research in the field. *Frontiers in Microbiology, 5,* 465.

Jebbar, M., Franzetti, B., Girard, E., & Oger, P. (2015). Microbial diversity and adaptation to high hydrostatic pressure in deep-sea hydrothermal vents prokaryotes. *Extremophiles, 19*(4), 721–740.

Kahlert, M., & McKie, B. G. (2014). Comparing new and conventional methods to estimate benthic algal biomass and composition in freshwaters. *Environmental Science: Processes and Impacts, 16*(11), 2627–2634.

Kengen, S. W. (2017). Pyrococcus furiosus, 30 years on. *Microbial Biotechnology, 10*(6), 1441–1444.

Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., & Toledano, M. B. (1999). Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *Journal of Biological Chemistry, 274*(23), 16040–16046.

Martens, R. (1995). Current methods for measuring microbial biomass C in soil: Potentials and limitations. *Biology and Fertility of Soils, 19*(2–3), 87–99.

Morozkina, E. V., Slutskaia, E. S., Fedorova, T. V., Tugaĭ, T. I., Golubeva, L. I., & Koroleva, O. V. (2010). Extremophilic microorganisms: Biochemical adaptation and biotechnological application (review). *Prikladnaia Biokhimiia I Mikrobiologiia, 46*(1), 5–20.

Pilizota, T., & Shaevitz, J. W. (2013). Plasmolysis and cell shape depend on solute outer-membrane permeability during hyperosmotic shock in *E. coli. Biophysical Journal, 104*(12), 2733–2742.

Preiss, L., Hicks, D. B., Suzuki, S., Meier, T., & Krulwich, T. A. (2015). Alkaliphilic bacteria with impact on industrial applications, concepts of early life forms, and bioenergetics of ATP synthesis. *Frontiers in Bioengineering and Biotechnology, 3,* 75.

Riesenberg, D., & Guthke, R. (1999). High-cell-density cultivation of microorganisms. *Applied Microbiology and Biotechnology, 51*(4), 422–430.

Roberts, M. F. (2005). Organic compatible solutes of halotolerant and halophilic microorganisms. *Saline Systems, 1*(1), 5.

Sanders, E. R. (2012). Aseptic laboratory techniques: Plating methods. *JoVE (Journal of Visualized Experiments), 63,* e3064.

Siliakus, M. F., van der Oost, J., & Kengen, S. W. (2017). Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. *Extremophiles, 21*(4), 651–670.

Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2017). Microbial metabolites in nutrition, healthcare and agriculture. *3 Biotech, 7*(1), 15.

Sleator, R. D., Gahan, C. G., & Hill, C. (2003). A postgenomic appraisal of osmotolerance in Listeria monocytogenes. *Applied and Environment Microbiology, 69*(1), 1–9.

Strober, W. (1997). Monitoring cell growth. *Current Protocols in Immunology*, *21*(1), A–3A.

Sutton, S. (2010). The most probable number method and its uses in enumeration, qualification, and validation. *Journal of Validation Technology, 16*(3), 35–38.

Waterbury, J. B., & Stanier, R. Y. (1978). Patterns of growth and development in pleurocapsalean cyanobacteria. *Microbiological Reviews, 42*(1), 2.

Wlaschin, K. F., & Hu, W. S. (2006). Fedbatch culture and dynamic nutrient feeding. In *Cell culture engineering* (pp. 43–74). Springer.

Part III
Transport Across Cell Membrane

Chapter 6
Solute Transport

Rani Gupta and Namita Gupta

1 Biological Membranes as Transport Barrier: A General Account

Biological membranes are composed of lipid bilayers that are selectively permeable barriers surrounding distinct cellular compartments. Plasma membrane or cell membrane separates the cellular contents from the extracellular environment. Biological membranes are composed of mainly phospholipid molecules that are arranged as a bilayer with their hydrophobic tails pointing inward and their hydrophilic heads outward, exposed to water. Due to their hydrophobic core, the membranes are impermeable to polar, hydrophilic and large biological molecules and ions, and these can be transported only via specific transporter proteins. The bulk lipids provide fluidity to the membrane. Biological membranes are quite flexible and can adapt to changes in the shape and size of cell and intracellular organelles associated with cell growth and movement. In spite of their flexibility, they are very durable and resistant to damage. They can seal over temporary breaks in their continuity allowing two membranes to fuse (like in exocytosis) or compartments to undergo fission (like in cell division). Membranes also have a large array of embedded, integral and peripheral proteins. The diversity of these proteins varies in different cells and may change in response to varying environmental conditions. The bulk lipid in membrane provides a fluid matrix for proteins to rotate and laterally diffuse for physiological functioning.

2 Transport Across Membranes: Passive and Active Transport

There are two types of transport systems operating to move substances across membranes—passive transport and active transport (Fig. 1 and Table 1).

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_6

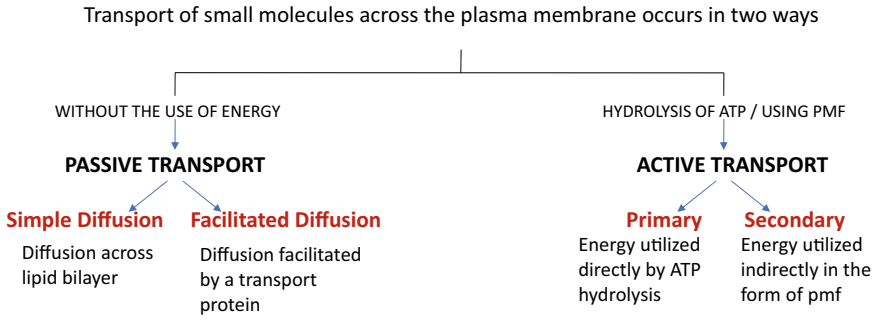


Fig. 1 Types of transport system across plasma membrane

Table 1 Differences between passive and active transport

Passive transport	Active transport
Does not require energy	Energy in the form of ATP or proton motive force (PMF) is required
Occurs down the concentration gradient	Occurs against concentration gradient
Rate of transport is slower	Rate of transport is faster

Passive transport

Passive transport does not require energy and is dependent upon concentration gradient, size and charge of the solute and hydrophobicity. In passive transport, the solute particles diffuse across the membrane from an area of high to low concentration till equilibrium is reached. The rate of diffusion is dependent upon size and degree of hydrophobicity of the substance. Diffusion can be of two types:

- Simple diffusion
- Facilitated diffusion

Simple diffusion: In simple diffusion, small non-charged molecules or lipid soluble molecules move from areas of high concentration to areas of low concentration. The molecule simply dissolves in the phospholipid bilayer, diffuses across it and then dissolves at the other side of membrane. This process is independent of membrane proteins and energy. It is a non-selective process. The rate of uptake decreases as more and more molecules are acquired till the concentration of the molecules is equal at both sides of the membrane. Oxygen, carbon dioxide and most lipids move by simple diffusion.

Factors affecting simple diffusion:

- Size of concentration gradient (chemical + electrical) between inside and outside of membrane—larger the gradient, higher is the rate of diffusion.
- Permeability of cell membrane—if membrane is non-permeable then rate of uptake decreases.

- Lipid solubility of solute—oil/water partition coefficient is a measure of lipid solubility of solute. Higher the ratio, higher is the rate.
- Size of solute—smaller solutes will diffuse faster.
- Charge of solute—charged solutes tend to attract a shell of water molecules around themselves, thus weakly or uncharged molecules diffuse faster.

Osmosis: It is a type of simple diffusion in which water molecules diffuse through a selectively permeable membrane from areas of higher water concentration to areas of lower water concentration.

Facilitated diffusion: During facilitated diffusion, substances move into or out of cells down their concentration gradient through transport proteins embedded in the cell membrane. Facilitated diffusion allows the passage of charged or polar molecules that cannot fit between the phospholipids. There are specialized membrane proteins (facilitators) that bind to specific substrates through many weak, non-covalent interactions leading to a conformational change in the protein. There is a drastic difference in the rates of transport achieved between simple and facilitated diffusion with a much higher rate of transport in the latter process. Facilitated diffusion systems are not very common in bacteria. The most well-known example is the glycerol facilitator in *E. coli*, *Salmonella typhimurium*, *Pseudomonas* and *Bacillus* and glucose facilitator in *Zymomonas mobilis* and *Saccharomyces cerevisiae.*

Mechanisms of Facilitated Diffusion

It is carrier-facilitated diffusion wherein a carrier protein picks up one molecule of the solute, undergoes a conformational change to release the molecule on the other side and then repeats the process until equilibrium is reached. Examples include:

- Glycerol uniporter in *E. coli*: A glycerol carrier protein is present in cell membrane through which glycerol enters the cell. However, the carrier is not specific for glycerol, and many more straight-chain polyols, such as urea, glyceraldehydes and glycine, can pass through these channels. In *E. coli,* there is no other example of such a non-specific pore in inner cytoplasmic membrane. The uniqueness of this facilitator is that glycerol is taken up at a faster rate, but it does not allow accumulation inside the cell; i.e., it never reaches saturation.

Kinetics of simple and facilitated diffusion

The facilitating protein may be specific for a molecule, or it may be non-specific. Such type of diffusion is easily distinguished by their saturation kinetics; i.e., at a certain concentration of solute, the uptake becomes constant. This is in contrast to simple diffusion which is linear along the difference in substrate concentration across membrane. Hence, in diffusion, the initial rate of uptake is linear with difference in substrate concentration. However, in facilitated diffusion, it is a rectangular hyperbola approaching maxima (Fig. 2).

For simple diffusion, the solute must be somewhat soluble in the lipidic phase of bilayer while it is not required when a facilitator is involved. Water is an exception which moves across cell membrane by simple diffusion/osmosis.

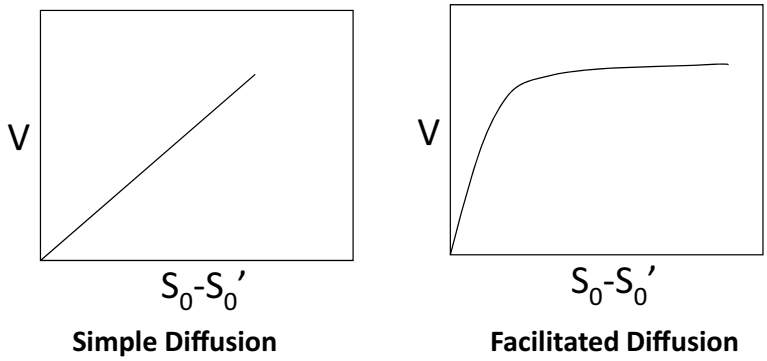


Fig. 2 Diffusion curves as a function of substrate concentration

Simple diffusion has to be considered as a simple passing in and out of a solute depending upon concentration across the membrane. Therefore, supposing that a solute is present in a concentration S_0, its initial velocity of inward movement will be directly proportional to concentration outside.

$$V \ \alpha \ S_0$$

V' is the outward movement considering S_0' is the concentration inside the cell at any time. Hence, V'α S_0'. The movement will be given by subtracting the two, i.e.,

$$V - V' \ \alpha \ S_0 - S_0'$$

$$V \ \alpha \ S_0 - S_0'$$

At equilibrium, V will become zero since solute on both sides will be equal. Now, considering a cell, there will be certain width of the membrane (l), so the overall rate will become

$$V\alpha \frac{S_0 - S_0'}{1}$$

In addition to these, several other parameters like solubility of solute, viscosity, temperature, etc., will also affect overall diffusion rates. For all these parameters, a diffusion constant D can be considered as constant for a particular membrane and at a set physical condition. The equation can thus become:

$$V = \frac{D\left(S_0 - S_0'\right)}{1}$$

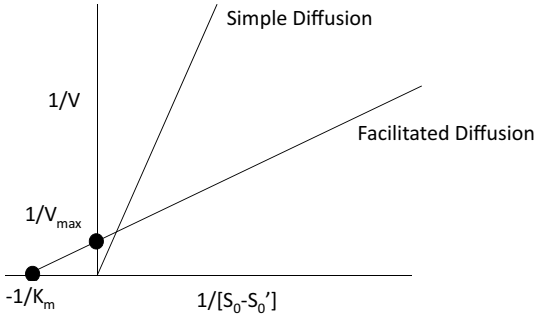


Fig. 3 Double reciprocal curves for simple and facilitated diffusion

This expression would be linear with intercept at zero, and D/l becomes the slope. Thus, a linear plot between V and $S_0 - S_0'$ indicates that the solute is taken up by simple diffusion.

Now considering the case of **facilitated uptake** where backward movement is nil since it is mediated by a facilitator protein. Here, uptake reaches maxima at a certain substrate concentration indicating saturation just like in an enzymatic reaction. Therefore, equation of facilitated diffusion is same as Michaelis–Menten equation:

$$\begin{aligned} V &= V_{\max}[S_0] \\ &= K_m + \left[S_0'\right] \end{aligned}$$

Thus, by studying rate of transport as a function of substrate concentration, it is easy to distinguish simple diffusion from facilitator carrier mediated one. If double reciprocal curves are plotted, then in simple diffusion, there will be no intercept and axis will pass through zero as V_{max} is infinite and $1/V_{max}$ would be zero, while for facilitated diffusion, intercept on Y-axis would be $1/V_{max}$ and on X-axis would be $-1/K_m$. If the double reciprocal curve is nonlinear, then it might be possible that more than one transport system is involved (Fig. 3).

Another distinction is that in facilitated diffusion stereo-selectivity is also observed which again suggests that it is the conformation of carrier protein which decides the substrate. Both simple and facilitated diffusion ultimately produce the same result, i.e., equilibrium of solute concentration. Facilitated diffusion shows saturated kinetics, while simple diffusion does not.

Active transport

Active transport is the movement of solute against a concentration gradient through a transport protein, and thus, it requires energy either in the form of ATP or proton motive force (PMF). Microbial cells need active transport for the following two reasons:

- Microbes live in low nutrient concentration habitat
- Active transport is required for transporting and concentrating solutes intracellularly.

Active transport is classified as either primary active transport or secondary active transport.

Primary active transport: It uses ATP as an energy source during transport of molecules against their concentration gradient across the cell membrane. ATP hydrolysis by ATPases causes a conformational change in the transport protein allowing particles to influx or efflux.

Secondary active transport: Secondary active transport utilizes PMF as an energy source for facilitated uptake of solute. This system functions independent of direct ATP coupling. This type of transport is well exemplified by mitochondria, bacteria and lower eukaryotes.

Energetics of transport

Microorganisms generally grow in diluted solutions and need to scavenge essential nutrients from their environment. Many solutes or ions can be taken up by simple or facilitated diffusion along their concentration gradient, and such processes also result in release of energy (exergonic). Some solutes or ions, on the other hand, are accumulated against their concentration gradient inside the cell with the help of active transport at an expense of some energy (endergonic).

Movement of a solute along or against its concentration gradient can be explained by Gibbs free energy equation

$$\Delta G = \Delta H - T\Delta S$$

where ΔS represents the change in entropy of the system. If a solute moves from high to low concentration, then it results in an increase in the entropy of the system and a negative free energy ($-\Delta G$) which implies release of energy. While in reversed situation, where the solute moves from low to high concentration, entropy of the system decreases which results in a positive free energy ($+\Delta$G) indicating energy consumption during the process.

In case of the transportation of solutes or ions across the cell membrane, free energy of the system depends on two factors.

- Concentration gradient
- Electrical gradient.

which is collectively known as **electrochemical gradient**.

$$\mathbf{\Delta}\boldsymbol{G} = \boldsymbol{RT}\ \mathbf{ln}\,([X]_{\mathbf{in}}/[X]_{\mathbf{out}}) + ZF\mathbf{\Delta}V\ \mathbf{or}\ \mathbf{2.3RTlog}\,([X]_{\mathbf{in}}/[X]_{\mathbf{out}}) + ZF\mathbf{\Delta}V$$

(in case of uncharged molecules like sugars, $\boldsymbol{ZF\Delta V}$ will be **zero**).

$\boldsymbol{R}$	**gas constant = 2 Cal/mol/K**
$\boldsymbol{T}$	**temperature in Kelvin**
$\boldsymbol{[X]_{in}}$	**Concentration inside the cell in mM**
$\boldsymbol{[X]_{out}}$	**Concentration outside the cell in mM**
$\boldsymbol{Z}$	**charge on the solute or ion**
$\boldsymbol{F}$	**Faraday's constant = 23,062 cal released as one mole of charge moves down a voltage gradient of 1 V (1000 mV)**
$\boldsymbol{\Delta V}$	**membrane potential**.

For uncharged solutes, only concentration gradient ($\boldsymbol{\Delta G = RT \ln ([X]_{in}/[X]_{out})}$ is considered.

Transportation of a solute against its concentration gradient is coupled with the transportation/translocation of another solute/ion along its concentration gradient to make the process feasible.

For example, uptake of an amino acid like glutamic acid into the cell, where its concentration is 0.5 mM from external environment/medium where its concentration is 0.005 mM, i.e., from low concentration to high concentration against a membrane potential of -60 mV 37 °C, would require.

$$\Delta G = RT \ln ([X]_{in}/[X]_{out}) + ZF\Delta V$$
$$\Delta G = (2)(310) \ln (0.5/0.005) + (-1)(23,062)(-0.060)$$
$$\Delta G = 2855.20 + 1383.72 = 4238.9 \text{ Cal/mol} = +4.2 \text{ kcal/mol}$$

Now the energy required to move 1 mol of glutamic acid into the cell can be provided by the exergonic reaction of translocating sodium ions inside the cell along its concentration gradient.

Let us suppose the concentration of Na^+ ions inside and outside the cell is 10 mM and 100 mM, respectively; then, the energy released during the uptake of 1 mol of Na^+ ions would be:

$$\Delta G = RT \ln ([X]_{in}/[X]_{out}) + ZF\Delta V$$
$$\Delta G = (2)(310) \ln (10/100) + (+1)(23,062)(-0.060)$$
$$\Delta G = -1427.60 + 1383.72 = -2811.32 = -2.8 \text{ kcal/mol}$$

But, we need a total of 4.2 kcal/mol of energy to transport 1 mol of glutamic acid. Therefore, in order to move 1 mol of glutamic acid, 2 mol of Na^+ ions should be translocated along its concentration gradient to provide enough energy for the endergonic reaction.

Box 1: Experimental identification of the source of energy for transport as ATP OR PMF

- Decouple ATP hydrolysis and Δp and perturb (increase or decrease) them independently
  - Mutants defective in ATP synthase (*unc mutants*)
  - Add inhibitors of ATP synthase (N, N'-dicyclohexylcarbodiimide DCCD)
- Perturbing intracellular levels of ATP—Once ATP synthase is inactivated, bacteria rely on substrate-level phosphorylation.
  - Substrate-level phosphorylation can be lowered by:
    - Starvation such that reserved energy is utilized
    - Using inhibitor of substrate-level phosphorylation (e.g., arsenate)
  - Substrate-level phosphorylation can be increased by:
    - Feeding cells energy source like glucose; however, this can also be a source of pmf by respiration. Therefore, respiration can be prevented by adding an inhibitor like cyanide or growing cells anaerobically
- Perturbing Δp
  - Ionophores that collapse proton potential like valinomycin
  - Addition of substrates that feed directly into electron transport chain (ETC) like succinate or lactate.

3 Membrane Transport Proteins

Cell membranes allow water and non-polar molecules to permeate by simple diffusion. The passage of various polar molecules, such as ions, sugars, amino acids, nucleotides and many cell metabolites, is aided by special membrane transport proteins. These proteins occur in many forms and in all types of biological membranes. Each protein transports a particular class of molecule (such as ions, sugars or amino acids) and even within the class, only certain molecular species are transported. Transport of molecules such as water and urea that can diffuse across the phospholipid bilayers is often accelerated by transport proteins. Cells use membrane transport proteins to maintain intracellular concentration that varies significantly from the extracellular environment.

The polypeptide chains of membrane transport proteins traverse the lipid bilayer multiple times (multipass transmembrane proteins). By forming a continuous protein pathway across the membrane, these proteins allow specific hydrophilic solutes to cross the membrane without coming into direct contact with the hydrophobic interior

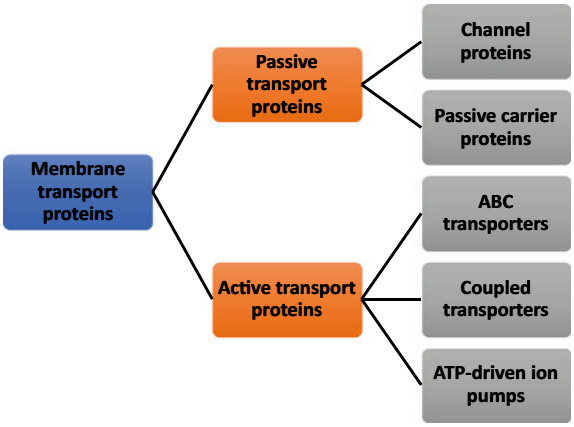


Fig. 4 Different classes of transport proteins

of the lipid bilayer. Different types of transport proteins are classified into channel proteins, carrier proteins, specific transporters and ATP-driven ion pumps (Fig. 4).

Channels: Channel proteins are composed of one or more α subunits and contain a pore region through which the solutes pass at high flux rates. Most of the channel proteins transport ions. They open to make a hole in the membrane through which ions can diffuse down their gradients. All channel proteins share the following two features:

- they facilitate a thermodynamically favorable movement of molecules
- they have an affinity and specificity for the molecule that they transport.

Most channel proteins are highly selective for a particular solute type such as Na^+, Ca^{2+} and K^+. Others are less selective such as general cation or anion channels. They are composed of multiple protein subunits or domains that traverse the lipid bilayer multiple times. Most ion channels are formed from 4 to 6 protein subunits, each comprising membrane-spanning alpha helices. The subunits associate in a circular formation, resulting in a central hydrophilic pore perpendicular to the membrane. Channel proteins have a selectivity filter that allows them to discriminate among different solutes. The selectivity filter is a collection of amino acid residues concentrated in the interior of the channel protein. As particles pass into the channel protein, an electrostatic interaction occurs between the amino acid residues and the ion which allows the channel protein to identify the ion.

Channel proteins transport the solutes down their concentration or electrochemical gradients, an energetically favorable reaction. They form a protein-lined passageway across the membrane through which multiple water molecules or ions move simultaneously. Consequently, transport through channel proteins occurs at a rapid rate of up to 10^8 molecules per second. Channel proteins carry out passive transport since no energy source, other than pre-existing electrochemical gradient, is involved.

To control diverse cellular functions, channel proteins are regulated by gating. As a response to a specific trigger, gating allows the channel protein to undergo a conformational change that causes the channel to open or close, allowing or disallowing its specific particle to pass. When open, the channel provides a continuous pathway through the bilayer. Channel proteins can be rapidly activated making them ideal for fast processing of signals. A number of different mechanisms can physically or chemically modulate the channel proteins.

Voltage gating: A change in the electrical potential of the cell membrane causes a conformational change in the channel protein, allowing it to open. This allows an influx or efflux of ions which in turn depolarizes the membrane. Such proteins are well characterized in excitable neuronal and muscle tissues. Here, passage of an impulse down a neuron causes reduction in the voltage which leads to opening of sodium channels in the adjacent portion of the membrane. This allows influx of Na^+ ions into the neuron, and the nerve impulse is thus continued.

Ligand gating: Binding of a ligand at an allosteric binding site of the protein causes a conformational change of the channel protein, leading to an influx or efflux of ions. The protein is returned to its original shape on release of the ligand. The nicotinic acetylcholine receptor located on the postsynaptic side of the neuromuscular junction is an example.

Other gating: Others methods by which channel proteins may be gated include light activation, mechanical activation, temperature activation or secondary messenger activation. Light-activated protein channels have a photo switch through which a photon causes a conformational change causing them to open or close. The channel rhodopsins that serve as sensory photoreceptors in unicellular green algae, controlling phototaxis, are the only natural occurring example of such channels. In case of mechanically activated protein channels, a mechanical stimulus causes their opening or closing. Such channels are involved in touching, hearing and balancing sensations in humans. Ligand-gated protein channels are typically linked to second messenger gating.

Examples of channel proteins

Porins

Gram-negative bacteria have a two-layered cellular envelope: the cytoplasmic membrane and the outer membrane (OM). The outer membrane is an asymmetrical bilayer that effectively acts as a permeability barrier providing protection from external environment without compromising the nutritional requirements. This dual capacity is achieved via well-fabricated macromolecules comprising highly hydrophobic lipid bilayer within which specific water-filled pores are embedded. These hydrophilic pores termed 'Porins' provide size-specific internalization of nutrients while providing OM its selective permeability. Porins were discovered by Hiroshi Nikaido in 1977 (Nikaido et al., 1977) and are the most abundant outer membrane proteins that impart a sieving capability to the membrane. They also offer protection against the toxic extracellular components while sufficiently allowing

nutrient uptake. Although widespread in the OM of gram-negative bacteria, porins are also found in the cell wall of some gram-positive *Mycobacteria.*

Porins allow transmembrane passage of hydrophilic compounds up to a certain size exclusion limit. Small molecules of <600 Da such as nutrients or waste products can diffuse freely across the membrane through porin pathways. Such porins are termed as 'general diffusion' porins bringing about non-specific transport and are present in high copy number. In *E. coli,* the porins OmpF, OmpC and PhoE act as non-specific channels with no substrate-binding sites. Substrate-specific porins are expressed under certain growth limitation conditions at low copy number. Examples include LamB and Tsx that allow passage of solutes maltodextrins and nucleosides, respectively (Benz, 1988). Bacterial porins have also been shown to interact with a variety of host tissues aiding in adhesion and eliciting innate and adaptive immune responses. Porins of *S. typhimurium* have shown to inhibit phagocytic activity and induce complement activation. Therefore, besides acting as channels, the role of porins is quite diverse. They can act as receptors of bacteriocins, bacteriophages, immune moieties like antibodies, interferons or epithelial cells (Achouak et al., 2001).

The first crystal structure of porin was published in 1990–91 from outer membrane protein of *Rhodobacter capsulatus*. It was a trimeric porin with a β-barrel structural motif of approximately 30A° in diameter. Later, several porin structures were developed revealing high architectural conversation (Zeth & Thein, 2010). Although some porins like OmpA in *E. coli* are monomeric, others like OmpF are organized as trimers. Each monomeric barrel in the trimer is formed by 16 antiparallel β-strands stabilized by extensive hydrogen bonding. These strands are connected by short turns at the periplasmic side, and longer and irregular cell surface exposed hairpin loops that account for the hydrophilicity. One of these external loops, L3, folds toward the central axis constricting the external channel entrance and lumen of the pore. It folds enough to sufficiently constrict the barrel determining the size exclusion limit. The flexibility of this loop is important in solute passage.

Porin-mediated antibiotic resistance

The synthesis of porins is controlled at genetic level and is also influenced by environmental conditions. The number of porins in bacteria can be 10^6 copies per cell, but cells can, to some level, control the permeability in response to environmental stimuli by regulating the expression of porins. Expression of porin OmpC and OmpF is regulated by a two-component system including sensor kinase EnvZ and response regulator OmpR. Porins generally have size exclusion limits approaching the size of many antibiotics, limiting their diffusion across the OM and therefore contributing to the intrinsic resistance. Therefore, mutations affecting the expression or functioning of porins can lead to increased antibiotic resistance. Such mutation can broadly have three effects: loss of porin (A); or reduced porin expression (B); or reduced conductance or size of porin channel (C) (Pagès et al., 2008) (Fig. 5).

Porin loss and reduced porin expression of OmpF in *E. coli* leads to resistance development to beta-lactam class of antibiotics. Similarly, 20-fold reduction in expression level of OmpF porin was observed in a mutant with point mutations in the promoter region in an *Enterobacter cloacae* isolate which led to ertapenem

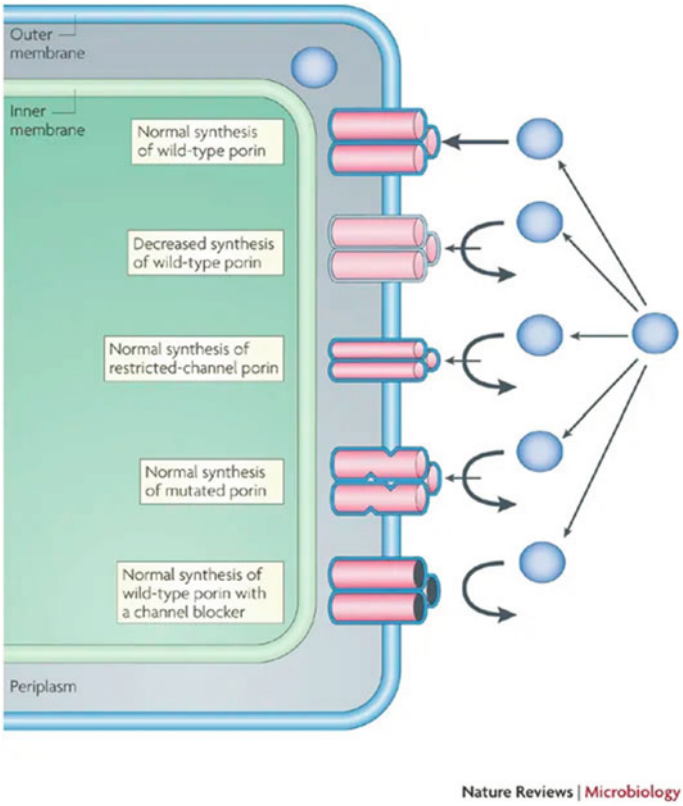


Fig. 5 Diagrammatic representation of altered antibiotic transport as a result of mutations effecting expression and function of porin. *Source Pagès et al.* (2008). *With kind permission from Springer Nature*

resistance (Fernández & Hancock, 2012). However, in clinical isolates of *P. aeruginosa*, resistance to carbapenems was due to point mutation in the OprD gene which resulted in early termination of translation, a deletion or insertion element within the porin-encoding gene (Richardot et al., 2015).

Antibiotic resistance can also occur due to mutations leading to alteration in porin function. A G-to-D mutation in loop 3 of OmpF/OmpC results in reduction of conductance and causes resistance to beta-lactams. Mutations in loop 3 of the porin PIB of *Neisseria gonorrhoea* confer resistance to penicillins and tetracycline (Fernández & Hancock, 2012). Similarly, mutations in the constriction zone can also restrict the path of antibiotics conferring resistance. Therefore, molecular insights in the structure, function and regulation of porins are important in understanding mechanism of antibiotic resistance, further enabling identification of novel strategies in drug development.

General non-specific porins (OmpF, OmpC, PhoE)

OmpF, OmpC and PhoE are classified as general diffusion porins and are conserved throughout the phylum of γ-proteobacteria. These are homotrimers of 16-stranded β–barrels forming a size-selective defined channel. Predominantly, such general porins show no particular substrate specificity and allow movement of hydrophilic-charged molecules >600 Da including antibiotics. However, OmpF and OmpC of *E. coli* prefer cations while PhoE selects phosphates and anions. Unlike other OMPs, the pore in the general diffusion porins is constricted by the inwardly folded extracellular loop, the third loop 'L3', giving it an hourglass-like shape. This loop, together with the opposite barrel wall, forms the constriction zone that contributes significantly to the size exclusion limit, ion selectivity and permeability properties of the pore. The constriction loop has a motif, PEFGG, conserved among enterobacterial porins (Koebnik et al., 2000).

OmpA

The OmpA protein is one of the most abundant OMPs of *E. coli* that occur at $>10^5$ copies per cell, like murein lipoprotein (Lpp) and other general diffusion porins. OmpA consists of two domains: 170 residue long membrane bound N-domain and 155 amino acid long periplasmic C-domain that interacts with the peptidoglycan layer. This porin spans the cell envelope and allows interaction between outer membrane and peptidoglycan, thus maintaining structural integrity. Further, the absence of OmpA and Lpp compromises the cell shape. They also serve as receptors for various bacteriophages (K3, M1, Ox2) and colicins (Colicin K, Colicin L) (Koebnik et al., 2000). OmpA also serves as a model protein in elucidating structure and function of small monomeric proteins (Burgess et al., 2008).

OmpX

OmpX is a structural homolog of OmpA and was first characterized in *Enterobacter cloacae*. It belongs to a family of highly conserved proteins, with functional significance in virulence by neutralizing host defense mechanisms. For instance, OmpX protein (Ail) in *Yersinia enterocolitica* contributes to its virulence, as OmpX is essential for the organism's adhesion to and internalization into mammalian cells (Kolodziejek et al., 2012). OmpX protein is composed of the basic β-barrel architecture, as in OmpA (Koebnik et al., 2000).

Substrate-specific porins (LamB)

LamB is an *E. coli* porin which exhibits substrate specificity for maltose, and likewise, the sucrose-specific porin ScrY exists in *S. typhimurium*. Both these porins are homotrimers consisting of 18-stranded antiparallel β-barrels. The *lamB* gene in *E. coli* is induced as a part of *mal* regulon in the presence of maltose or maltodextrins (Koebnik et al., 2000).

Other porins

Apart from general porins listed above, all gram-negative bacteria have several high-molecular-weight OMPs such as TonB-dependent receptors (e.g., FhuA and FepA), responsible for transport of large substrates such as iron-siderophore complexes or vitamin B12, respectively (Noinaj et al., 2010). Phospholipase A (OMPLA), another OMP, is involved in colicin release from *E. coli* and is implicated in the virulence of *Campylobacter* and *Helicobacter* strains (Dekker, 2000). OMPLA represents the only outer membrane enzyme whose three-dimensional structure is available. Although OMPLA is known to hydrolyze phospholipids, it is physiological relevance in the outer membrane is still not completely understood.

4 Specialized Channel Proteins

Aquaporins: one of the most important examples of general diffusion by porins

Aqua porins are so named as they are responsible for bulk water transport and are thus abundant in cells involved in water transport such as the mammalian kidney cells. They are integral membrane proteins and conserved through all domains of life. However, in case they are missing, especially in certain microbes, water transport occurs solely by general diffusion across membrane.

Aquaporins were discovered by in 2003 by Peter Agre and Roderick MacKinnon who shared the Nobel Prize in Chemistry for this discovery. Peter Agre, a hematologists, discovered a polypeptide in red cells that were identical to a polypeptide in the kidney. He injected the complementary mRNA of the protein into *Xenopus laevis* oocytes. When these oocytes were dropped into distilled water—they burst, which was contradictory of their nature as membrane of these oocytes are generally impermeable. Agre and his group then realized that the product of mRNA injected caused the membrane to become highly permeable to water. They named it CHIP28 (channel-forming integral membrane protein of 28 kDa) which was later known as aquaporin 1 (Brown, 2017).

Structurally, aquaporins are membrane integral proteins with their N- and C-termini facing toward cytoplasmic side linked to six transmembrane right-handed α-helices resulting in five inter-helical loop regions A-E (Fig. 6a). Different aquaporins contain different peptide sequences due to which size of the pore is different between aquaporins. Pore size directly affects what molecules are able to pass through, and likewise, small pore sizes only allow small molecules like water. Among the five inter-helical loops, a 3D 'hourglass' structure is formed by loops B and E which are hydrophobic loops and have a conserved motif, asn-pro-ala (NPA). These hydrophobic residues lining the channel wall generate a local positive electrical field allowing water to enter in a single file. Also, positive charge provided by arginine-195 (Arg) and histidine-180 (His) residues results in further repulsion of protons facilitating water to move forward (Fig. 6b) (Agre, 2006).

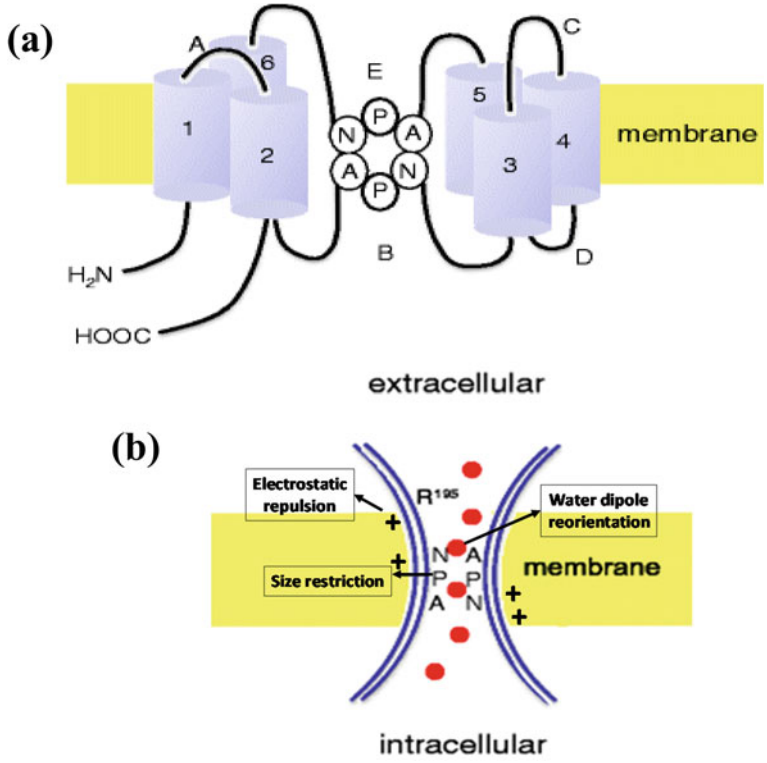


Fig. 6 Schematic representation of aquaporin **a** transmembrane view; **b** water in flow. *Source Adapted from Herrera and Garvin* (2011). *With kind permission from Springer Nature*

Mechanosensitive channels

All organisms, from single-celled bacteria to multicellular plants and animals, experience one or the other kind of mechanical forces from outside (shear force, gravity, touch) or inside (osmotic pressure and membrane deformation) the cell and also respond to these forces to grow and develop. One of the mechanisms of response is through mechanosensitive channels which are located in the cell membrane and respond to the changes in tensions in lipid bilayer of cytoplasmic membrane. Their major physiological role is to protect cell integrity during the shifts from high to low osmolarity condition. Under hypertonic medium, gram-negative bacteria maintain the cell integrity by accumulating potassium and glutamate inside the cell which counter the water loss from the cell. However, when these cells are suddenly shifted to hypo-osmotic environment, water starts entering the cell and builds up a turgor pressure on the cell wall. Increase in this turgor pressure beyond a certain limit could result in cell bursting and death. Under such situations, bacterial cell maintains its integrity by gating mechanosensitive channels to release excess ions outside the cell (Fig. 7). In open state, mechanosensitive channels can create a transient pore of the

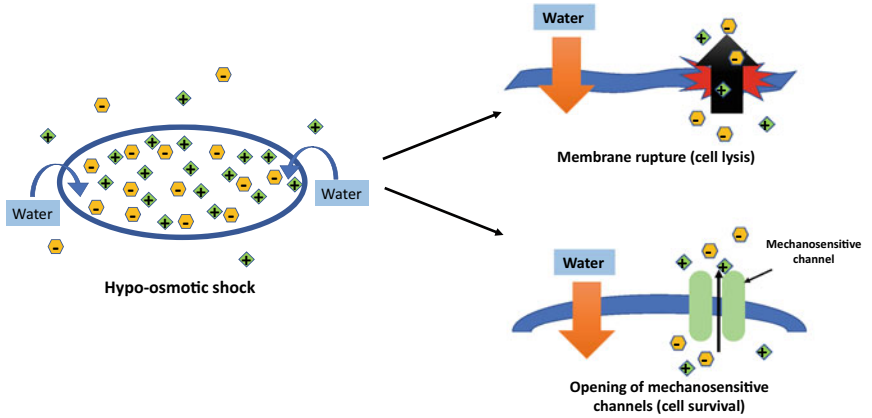


Fig. 7 Diagrammatic representation showing functioning of mechanosensitive channels in hypo-osmotic condition

size ranging from 6 Å to 30 Å. Mechanosensitive channels in bacteria are non-specific in nature toward the ions or molecules that pass through them, while eukaryotic channels are specific in nature. Mechanosensitive channels are well established in both prokaryotes and eukaryotes and are most extensively studied in *E. coli*. There are three major classes of mechanosensitive channels in *E. coli,* viz. MscL (large), MscS/MscK (small/kalium, i.e., potassium) and MscM (mini). This classification is based on their single-channel conductance and pore size. Gating of mechanosensitive channels is triggered by tension in lipid bilayer which is also known as stretch model of gating mechanism. Besides this model, gating can also be triggered by spring-like tether model where tethers are attached to the channels which act as spring mechanism of a shutter (Kung et al., 2010; Haswell et al., 2011).

5 Membrane Transport Proteins: Carriers

Carrier proteins bind the solutes on one side of the membrane, undergo a conformational change and release the solutes on the other side of the membrane. Compared with channel proteins, carrier proteins have a much slower rate of transport (~1000 molecules per second). Carrier proteins are involved in both active and passive transports. During active transport, they couple the energy stored in ATP, electrochemical gradient or other energy sources with uphill transport of solutes against their concentration gradient. Carrier proteins are of two types: transporters and ATP-powered pumps.

Transporter proteins couple the energy stored in ATP of electrochemical membrane gradients to facilitate movement of solutes across the cell membranes.

They are involved in the movement of a wide variety of ions and molecules. Transporters are of the following three types:

- **Uniporters**: 'Uni' means 'one' and 'porter' meaning transport; these transporters are associated with the transport of one molecule at a time. They transport the molecules down a concentration gradient and are generally associated with passive transport (or facilitated diffusion). However, they require electrochemical gradient and membrane potential as a driving force for transport, for example glucose transporter of *Zymomonas mobilis*, glycerol and lysine transporters of *E. cosli*.
- **Symporters**: 'Sym' means 'together'; these transporters move one molecule downhill to facilitate uphill movement of another molecule against the concentration gradient, transporting both molecules in the same direction. Examples include lactose and H^+ transport by lactose permease, $Na^{+/}$glucose system.
- **Antiporters**: 'Anti' means 'against'; these transport two or more different molecules across the cellular membrane in opposite directions. During this transport, one molecule is moved along the concentration gradient (downhill), while the other is moved against the gradient (uphill). They show ping-pong kinetics; i.e., one molecule is transported across the membrane, while the other molecule is carried back but in the opposite direction. Examples are Na^+/Ca^{2+}exchanger and Na^+/H^+ antiports.

Symporters and antiporters catalyze 'uphill' movement of certain molecules, and they carry out active transport, but unlike pumps, they do not hydrolyze ATP (or any other molecule) during transport. Because they rely on the gradients generated by ion pumps, they are also known as secondary active transporters. They are also referred to as cotransporters or coupled transporters because of their ability to transport two different solutes simultaneously.

Box 2: Lactose transport—an example of symport

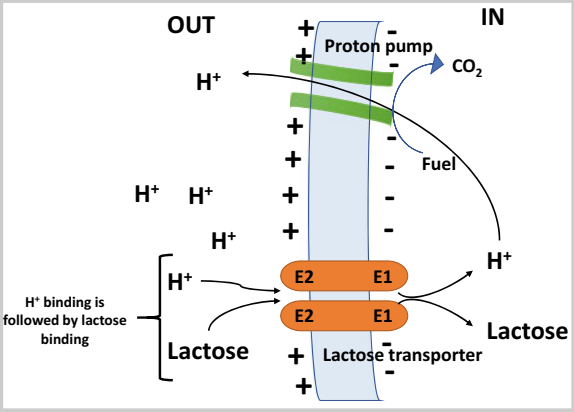
Lactose permease contains two binding sites for lactose with varying affinities. Site E-1 faces the cytoplasm and has low affinity for lactose binding, while site E-2 is present on the exterior surface and has high affinity for lactose. E-1 and E-2 are inter-convertible conformations of lactose permease which depends on the binding and release of both H^+ and lactose. The involvement of a specific permease was confirmed by using N-ethylmaleimide-β-galactoside, an inhibitor of lac permease which specifically inhibits lactose transport.

Movement of lactose from outside to inside

H^+ ions and lactose bind to E-2 site of lactose permease sequentially (H^+ followed by lactose); this is followed by conformation change in lactose permease, and E-2 changes to E-1 and results in release of both H^+ and lactose in the cytoplasm. Releasing of H^+ and lactose initiates the conversion of E-1 to E-2.

The driving force of lactose import

1. 2, 4-dinitrophenol which uncouples ATP and proton gradient stops lactose transport proving that lactose transport is pmf mediated.
2. Energy-depleted cells will demonstrate uptake of lactose analog if pmf of outside environment is lowered.
3. Lactose transport in synthesized vesicle can be demonstrated in the presence of lactic acid if ADP is not available, showing that electron supplied by lactate oxidation can generate pmf and thus facilitate lactose transport.
4. The lactose/H + symport is further supported by acidification of cytoplasm of energy-depleted cells if incubated with lactose and thus subsequently can produce ΔP.



Lactose permease transports lactose along with protons as a symporter

ATP-powered pumps use the energy of ATP hydrolysis by ATPases to move molecules/ions against a concentration gradient across the cell membrane. The best-understood example is the Na^+/ K^+-ATPase or Na^+/ K^+ pump in animal cells.

They are classified into four groups: P-class pumps, F-class pumps, V-class pumps and ABC superfamily. Of these, P-, F- and V-classes transport only ions, while ABC superfamily is the one which transports small molecules. The characteristic features of these groups are shown below (Table 2).

6 Special/Unique Transport Proteins: Ionophores

Ionophores are a special class of compounds or proteins which facilitate the transport of ions across the lipid bilayer even in the absence of the protein pore for that particular ion and disrupt the membrane potential which leads to cytotoxicity. They

Table 2 Different classes of ATP-powered pumps and their mechanism of action

S.No	Type	Mechanism	Example
1	P-class pumps	• Phosphorylation of the alpha subunit of the protein by ATP induces a conformational change that allows transport	• Na^+/ K^+-ATPase in animal cell membranes
2	F-class pumps	• Pumps only protons • No phosphorylation-dephosphorylation of the proteins involved	• ATP synthase in bacterial plasma membranes, eukaryotic mitochondria and chloroplasts • Linked to electron transport chain
3	V-class pumps	• Pumps only protons • Activity does not involve a phosphoprotein intermediate	• H^+ pump in animal lysosomal/endosomal membranes and plant vacuole membranes • Responsible for maintaining lower pH inside the organelles as compared to surrounding cytosol • Generate a proton gradient at the expense of ATP leading to pH values as low as 1
4	ABC superfamily	• Typical **A**TP-**b**inding **c**assette (ABC) • Composed of four core domains: • Two transmembrane domains that recognize various substrates and undergo conformational changes (powered by ATP hydrolysis) to transport the substrate across the membrane • Two cytoplasmic nucleotide-binding domains (ATP-binding cassette) that have a highly conserved structure • Bacterial ABC transporters have a high affinity ligand/solute binding protein to capture the substrate. These are periplasmic in gram-negative bacteria and anchored to the outer face of cell membrane in gram-positive bacteria	• >100 different transport proteins found in various organisms • Bacterial importers of biomolecules like ions, amino acids, sugars • Bacterial exporters (efflux) of toxins, drugs, etc. • Eukaryotic efflux pumps

Outer membranes of gram-negative bacteria have specialized large pores called porins that have diameters of ~10 Å and transport molecules which are <600 Da

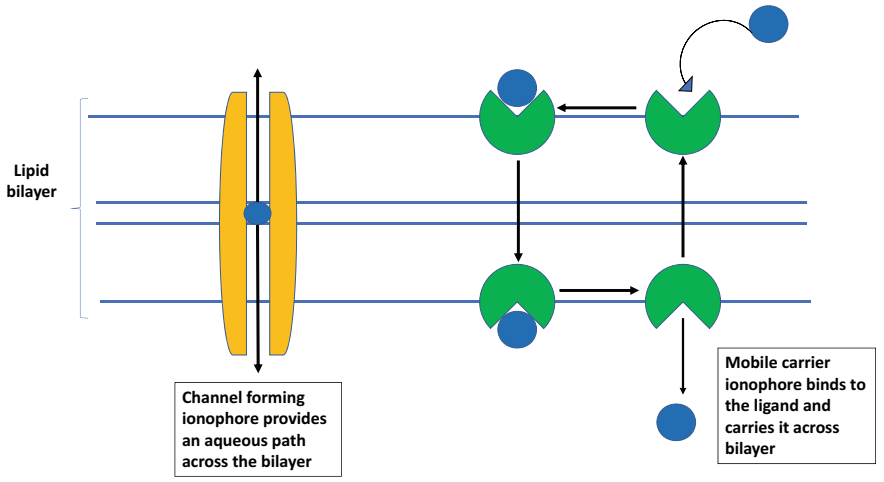


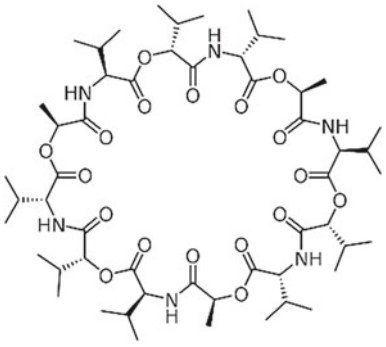
Fig. 8 Channel-forming and carrier-type ionophores. *Source* Adapted from Stillwell (2016). With kind permission from Elsevier

are generally produced by many microorganisms as a part of the defense mechanism against other microbes. Many synthetic membrane-spanning ionophores are also available and investigated and are used as antibiotics.

Ionophores contain a hydrophilic pocket for ion binding while the exterior surfaces of the ionophores are hydrophobic in nature which allows the movement of the ionophore-ion complex across hydrophobic lipid bilayer (Stillwell, 2016).

There are two types of ionophores (Fig. 8):

1. **Carrier ionophore** is a circular molecule that binds reversibly to a single ion at a time and shields its charge from the surrounding environment. The masking of the charge facilitates the diffusion of the ion across lipid bilayer. Most popular example of carrier ionophore is **valinomycin** from *Streptomyces*, a depsipeptide. It is a circular molecule, made up of three repeats of D-α-hydroxyisovaleryl-D-valyl-L-lactoyl-L-valyl. It transports specifically K^+ ions across cell membrane.



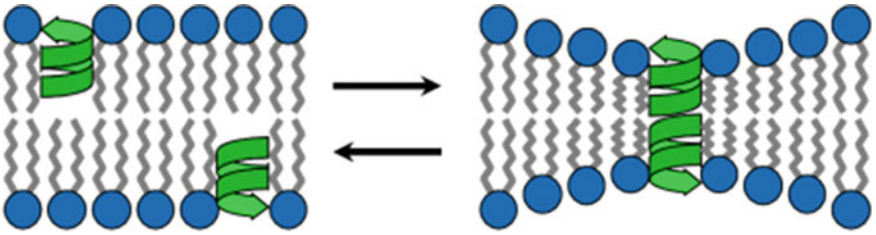


Fig. 9 Dimerization of two monomers of gramicidin A forms the functional channel. *Source David and Rajasekaran* (2015). *(Creative Commons Attribution License)*

Valinomycin ring closely surrounds the K^+ ion, which interacts with six oxygen atoms of valinomycin. K^+ is thus hidden in the middle of the molecule, and the charged ion is shielded from the non-polar membrane.

Channel formers bore into the lipid bilayer with the help of its hydrophobic exterior surface and provide a hydrophilic pore for ions to pass through it without any repulsion from hydrophobic core of lipid bilayer. One of the examples of such ionophore is gramicidin A.

Gramicidins are linear pentadecapeptide antibiotics that are produced by soil bacterium *Bacillus brevis*. Gramicidin A is a helical tube-like structure that spans the cell membrane as a dimer formed by end-to-end joining of two monomer molecules. Gating (opening and closing) of a gramicidin channel involves reversible dimerization. Dimerization by joining of two monomers opens the gate, while the gate is closed in the monomer form, (Fig. 9).

7 Iron Transport in Bacteria: Concerted Action of Primary and Secondary Active Transport

Iron is an essential nutrient for bacterial growth. However, due to its insolubility, it is limited in the environment. Thus, bacteria employ specific proteins known as siderophores or iron-binding proteins for iron chelation.

In gram-negative bacteria, after its uptake, iron has to pass through two membranes (outer and inner membranes) before reaching cytosol. The following steps are involved in this:

1. Ferric ions present in the environment are bound by siderophores such as enterochelin in *E. coli* (also known as enterobactin)
2. Siderophore binding proteins (IroN) present in outer membrane of gram-negative bacteria facilitate movement of iron bound siderophores to the periplasm. This is an energy requiring process which involves a complex of three inner membrane proteins **TonB, ExbB and ExbD**. After reaching the periplasm, the iron bound siderophore is transported inside the cell by an ABC transporter

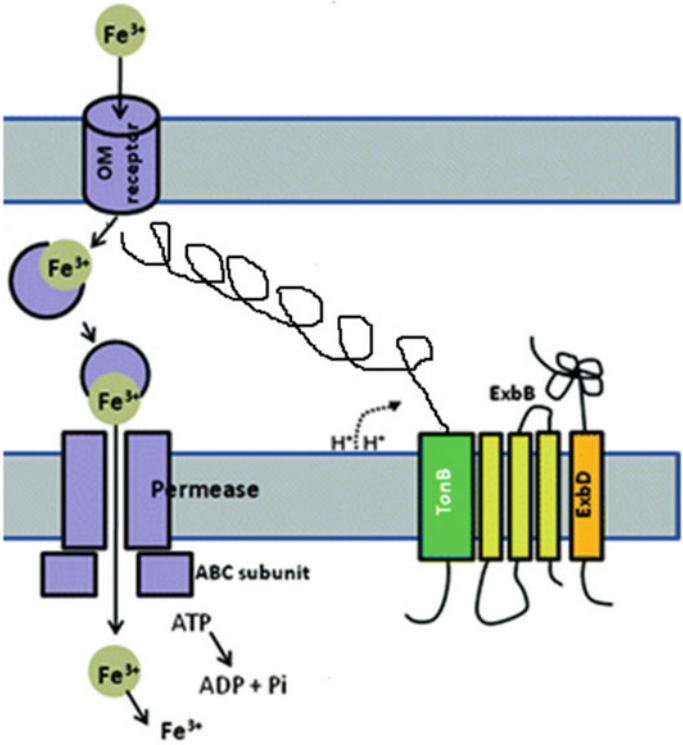


Fig. 10 Iron transport in bacteria. *Source* Adapted from Krewulak and Vogel (2008). *With kind permission from Elsevier*

system involving a periplasmic binding protein, transmembrane protein and NBD (IroE, FepD, FepG. FepC).

3. In the cytoplasm, an esterase such as ferric enterochelin esterase (Fes) of *E. coli* cleaves the enterochelin allowing the release and reduction of iron.

The above mechanism involves both primary and secondary active transport components of the cell. The inner membrane transporter proteins **TonB, ExbB and ExbD** (Fig. 10) generate a proton motive force which assists the internalization of iron bound siderophore into the periplasm (secondary active transport) while translocation of the iron bound siderophore into the cytoplasm of the cell is assisted by ABC transporter (primary active transport) (Krewulak & Vogel, 2008).

Gram-positive bacteria, in contrast, do not have an outer membrane, and thus, the iron bound siderophore can be directly internalized through ABC transporters.

Box 3: How to study transport?

1. Ortho-nitrophenyl-β-galactoside (ONPG) assay is the easiest to study lactose transport. The problem is that galactose is further metabolized and this can affect uptake rate.
2. Radiolabeling and pulse chase experiments are the easiest and reproducible. However, they require sophisticated instrumentation and radioactivity safety issues.
3. Mutants which are defective in metabolism but not in transport can be employed.
4. Glucose analogs such as methyl α-glucoside and 2-deoxyglucose, both of which cannot be metabolized beyond hexose phosphate.
5. Liposome technology—Isolate cytoplasmic membrane vesicles. Membrane vesicles in bacteria were pioneered by H.R. Kobach in 1995. These vesicles can be made such that:
   - they lack enzymes
   - they can be manipulated by introducing energy sources to understand their role.

Summary

- Biological membranes are composed of lipid bilayers that are selectively permeable barriers surrounding distinct cellular compartments.
- Due to their hydrophobic core, the membranes are impermeable to polar, hydrophilic and large biological molecules and ions, and these can be transported only via specific transporter proteins.
- There are two types of transport systems operating to move substances across membranes—passive transport and active transport.
- Passive transport does not require energy and involves movement of molecules down the concentration gradient, while active transport requires energy in the form of ATP or PMF and takes place against the concentration gradient.
- Passive transport employs the power of diffusion which can be of two types, simple diffusion and facilitated diffusion, wherein diffusion is facilitated by a transport protein.
- Active transport mechanisms employ specific protein molecules variously called as carriers, permeases, porters, translocases, translocators and transporters.
- Microbial cells need active transport because they usually live in low nutrient concentration habitat and for transporting and concentrating solutes intracellularly.
- Primary active transport uses the energy found in ATP, while secondary active transport is a type of energy-mediated transport where proton motive force facilitates the uptake of solute.

- Channel proteins, most of which transport ions, open to make a hole in the membrane, through which ions can diffuse down their gradients.
- Besides acting as channels, the role of porins is quite diverse; they can act as receptors of bacteriocins, bacteriophages, immune moieties like antibodies, interferons or epithelial cells.
- Mutations affecting the expression or functioning of porins can lead to increased antibiotic resistance. Such mutations can broadly have three effects: porin loss; reduced conductance or size of porin channel; or lower level of expression of porins.
- Aquaporins (AQP) are integral membrane proteins that serve as channels in the transfer of water, and in some cases, small solutes across the membrane.
- Major physiological role of mechanosensitive channel is to protect cell integrity during the shifts from high to low osmolarity condition.
- Carriers bind solutes on one side of the membrane undergo a conformational change and release the solutes on the other side of the membrane.
- Carrier proteins are of two types: transporters and ATP-powered pumps.
- Transporters couple the energy stored in electrochemical membrane gradients to facilitate movement of solutes across the cell membranes.
- There are three types of transporters: uniporters, antiporters and symporters.
- ATP-powered pumps are ATPases that use energy of ATP hydrolysis to move ions or small molecules across a membrane against a chemical concentration gradient or electric potential.
- Ionophores are a special class of membrane-spanning proteins that induce the transport of ions and are generally produced by certain bacteria as defense mechanism. They are either carrier ionophores or channel formers.
- Carrier ionophores work by binding to an ion and shielding its charge. Masking of the charge allows the diffusion of ions across the lipid bilayer. Example includes K^+ ionophore Valinomycin.
- Channel formers bore into the lipid bilayer by virtue of their hydrophobic exterior surface and form a hydrophilic pore through which ions easily pass without any repulsion from lipid hydrophobic core. Example includes antibiotic Gramicidin produced by *Bacillus brevis.*
- Iron transport is an example of transport that involves both primary and secondary active transport components of the cell.

Questions

1. Differentiate between passive and active transports.
2. Device an experiment to show that glycerol transport in *E. coli* is not simple diffusion but is an example of facilitated diffusion.
3. How many Na^+ ions need to be transported outside to import 1 mol of glutamic acid across cell membrane? Provided concentration of glutamic acid inside and outside of the cell is 0.5 M and 0.005 M, respectively; concentration of Na^+ inside and outside of the cell is 10 mM and 100 mM, respectively; and membrane potential is 60 mM at 37 °C.

4. How will you demonstrate whether a transport mechanism is a primary active transport or secondary active transport?
5. How were aquaporins discovered?
6. What are mechanosensitive channels? How do they function to protect cell against bursting in hypo-osmotic conditions?
7. Explain lactose transport across cell membrane of *E. coli*.
8. How does valinomycin kill bacteria?
9. Lactose transport is an example of which type of transport?
10. Which channels protect against lysis in hypo-osmotic conditions?
11. Name an inhibitor of ATPase.
12. What is an inhibitor of substrate-level phosphorylation?
13. Give an example of carrier ionophore.
14. Give an example of channel ionophore.
15. What is the other name of outer membrane proteins in *E. coli*?
16. What is the contribution of Peter Agre for which he was conferred Nobel prize?
17. Which conserved moiety of aquaporins is responsible for water transport?

References

Achouak, W., Heulin, T., & Pagès, J. M. (2001). Multiple facets of bacterial porins. *FEMS Microbiology Letters, 199*(1), 1–7.

Agre, P. (2006). The aquaporin water channels. *Proceedings of the American Thoracic Society, 3*(1), 5–13.

Benz, R. (1988). Structure and function of porins from gram-negative bacteria. *Annual Reviews in Microbiology, 42*(1), 359–393.

Brown, D. (2017). The discovery of water channels (Aquaporins). *Annals of Nutrition and Metabolism, 70*(Suppl. 1), 37–42.

Burgess, N. K., Dao, T. P., Stanley, A. M., & Fleming, K. G. (2008). β-barrel proteins that reside in the *E. coli* outer membrane in vivo demonstrate varied folding behavior in vitro. *Journal of Biological Chemistry, 283*(39), 26748–26758.

David, J. M., & Rajasekaran, A. K. (2015). Gramicidin A: A new mission for an old antibiotic. *Journal of Kidney Cancer and VHL, 2*(1), 15.

Dekker, N. (2000). Outer-membrane phospholipase A: Known structure, unknown biological function: MicroReview. *Molecular Microbiology, 35*(4), 711–717.

Fernández, L., & Hancock, R. E. (2012). Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. *Clinical Microbiology Reviews, 25*(4), 661–681.

Haswell, E. S., Phillips, R., & Rees, D. C. (2011). Mechanosensitive channels: What can they do and how do they do it? *Structure, 19*(10), 1356–1369.

Herrera, M., & Garvin, J. L. (2011). Aquaporins as gas channels. *Pflügers Archiv-European Journal of Physiology, 462*(4), 623.

Koebnik, R., Locher, K. P., & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: Barrels in a nutshell. *Molecular Microbiology, 37*(2), 239–253.

Kolodziejek, A. M., Hovde, C. J., & Minnich, S. A. (2012). Yersinia pestis Ail: Multiple roles of a single protein. *Frontiers in Cellular and Infection Microbiology, 2,* 103.

Krewulak, K. D., & Vogel, H. J. (2008). Structural biology of bacterial iron uptake. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1778*(9), 1781–1804.

Kung, C., Martinac, B., & Sukharev, S. (2010). Mechanosensitive channels in microbes. *Annual Review of Microbiology, 64,* 313–329.

Nikaido, H., Song, S. A., Shaltiel, L., & Nurminen, M. (1977). Outer membrane of Salmonella XIV. Reduced transmembrane diffusion rates in porin-deficient mutants. *Biochemical and Biophysical Research Communications*, *76*(2), 324–330.

Noinaj, N., Guillier, M., Barnard, T. J., & Buchanan, S. K. (2010). TonB-dependent transporters: Regulation, structure, and function. *Annual Review of Microbiology, 64,* 43–60.

Pagès, J. M., James, C. E., & Winterhalter, M. (2008). The porin and the permeating antibiotic: A selective diffusion barrier in gram-negative bacteria. *Nature Reviews Microbiology, 6*(12), 893.

Richardot, C., Plésiat, P., Fournier, D., Monlezun, L., Broutin, I., & Llanes, C. (2015). Carbapenem resistance in cystic fibrosis strains of Pseudomonas aeruginosa as a result of amino acid substitutions in porin OprD. *International Journal of Antimicrobial Agents, 45*(5), 529–532.

Stillwell, W. (2016). Membrane transport—Chap. 19. In W. Stillwell (Ed.), *An introduction to biological membranes: Composition, structure and function* (2nd ed., pp. 423–451). London: Elsevier.

Zeth, K., & Thein, M. (2010). Porins in prokaryotes and eukaryotes: Common themes and variations. *Biochemical Journal, 431*(1), 13–22.

Chapter 7
ABC Transporters and Group Translocation

Rani Gupta, Namita Gupta, and Richa Sharma

1 Historical Background of ABC Transporters

ATP-**b**inding **c**assette (ABC) transporters are ubiquitous in distribution among both prokaryotes and eukaryotes. They mediate active transport by ATP hydrolysis and thus form the primary transport system. In microorganisms, they are associated with uptake of variety of ions and molecules and are also important for resistance to antimicrobials. They have gained much importance due to the fact that they are linked to several diseases and genetic disorders in humans.

The physiology of uptake systems is well studied in *E. coli* and *Salmonella typhimurium* where there are three main categories of transport systems: PEP-PTS system, shock-sensitive system mediated by ATP and shock-insensitive system mediated by proton motive force (PMF). ABC transporters fall in the category of shock-sensitive transporters. These systems comprise substrate binding periplasmic proteins for uptake of solute. The proteins are lost due to osmotic shock making the system shock-sensitive. For the same reason, these transport systems are absent from isolated membrane vesicles. On the other hand, shock-insensitive transport systems do not involve periplasmic proteins and are fully retained in isolated vesicles. However, there is no demarcation of transport systems with respect to uptake of various biomolecules, viz. amino acids, inorganic ions, sugars, etc., and in fact, transport of single molecule may occur by multiple transport systems. Hyde et al. (1990) coined the term ABC transporter (for **A**TP-**b**inding **c**assette) for all those systems which had a periplasmic substrate-binding protein and transmembrane ATP-binding protein. The first periplasmic protein sequence was reported for histidine transporter (His J) of *Salmonella typhimurium* in 1982. It was followed by report of maltose transporter (Mal K) from *E. coli* which was observed to share high sequence similarity with histidine permease from *S. typhimurium* (Gilson et al., 1982). Later, in 1985, Higgins and group reported another oligopeptide transporter system dependent on periplasmic substrate-binding protein Opp A along with three membrane-bound components Opp B, Opp C and Opp D. They also reported three additional membrane-bound proteins His G, His M and His P. They, for the first time, identified a nucleotide-binding

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_7

sequence on Opp D which shared high homology at exactly same position with similar proteins from earlier reported histidine and maltose transport systems, i.e., His P and Mal K proteins, respectively. Functionality of this domain as nucleotide-binding site was determined by affinity assay by binding to Cibacron Blue affinity column and also by using ATP affinity analogue 5′ FSBA which competes with ATP. In 1986, the first eukaryotic ABC transporter was reported for multidrug-resistant protein (Higgins, 2001).

2 ABC Transporters: Substrate Specificity, Domain Organization and Mechanism of Transport

ABC transporters are highly diverse in function as they are not only involved in uptake but at the same time are exporters too. A variety of molecules from charged inorganic ions to biomolecules, amino acids, sugars, proteins and polysaccharides are transported by these systems. Although they exhibit substrate specificity, they may also allow multi-substrate as in the case of oligopeptide transporters and multidrug transporters.

ABC transporters are essential for export of cell wall polysaccharides during its genesis, antibiotic production and interestingly also offer antifungal resistance in fungi. Apart from transport function, ABC transporters have been characterized to serve as channels and also play a regulatory role as exemplified by regulation of potassium channel in *S. typhimurium* and *E. coli* by Sap/Trk systems. Hence, ABC transporters can be categorized as follows:

1. Importers in prokaryotes
2. Exporters in both prokaryotes and eukaryotes
3. Functions other than transport again in both prokaryotes and eukaryotes.

Domain organization

ABC transporter basically has four domains, two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). These domains can be formed independently by four different polypeptides as in the case of oligopeptide transporter or can be fused in a variety of manners as presented in Fig. 1. All the four domains can even fuse together to form a single domain as in P-glycoproteins. However, certain transporters lack some domains such as histidine and maltose transporters and the earliest known ones have only single gene for ATP-binding domain. Proline and betaine transporters have one transmembrane and one ATP-binding domain. In such cases, polypeptides interact to form homodimers to provide four basic domains found in other systems.

The transmembrane domains are highly hydrophobic integral membrane proteins and span the membrane by α-helices. They are diverse in number and can vary from 6 to 10 per subunit. The nucleotide-binding domains are in cytoplasm face of the membrane.

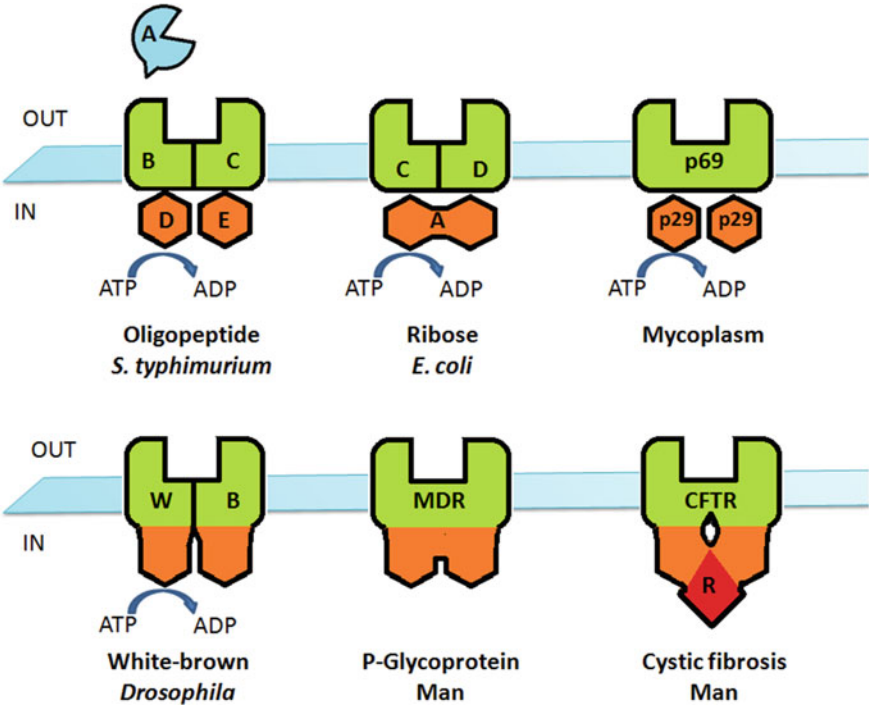


Fig. 1 Domain organization of ABC transporters

In addition to transmembrane domain and nucleotide-binding domains, each of the transport systems for import has periplasmic substrate-binding proteins (SBPs). SBPs bind substrate externally and transport it across the membrane-bound transport complex. In gram-positive bacteria, such proteins are anchored outside the cell via lipid groups. Among all the three complexes, the most conserved is NBD, while SBPs are highly diverse. This is true with respect to their function as NBDs perform ATPase activity throughout, while SBPs bind to different substrates and need to be diverse. The SBPs have high affinity and specificity for substrates, and they also facilitate movement of solute from outside to inside and hence provide directionality. SBPs are absent in export systems (Fig. 2).

Nucleotide-binding domain: Nucleotide-binding domains are highly conserved. They have two sub-domains: one resembles recA protein and the other is a helical sub-domain. There are several conserved motifs, of which the most important are the P-loops present in recA like domain and the LSGGQ motifs in the helix like domain. The two NBDs assemble in head to tail manner so that P-loop of one faces LSGGQ motif of other. The domain interphase forms the ATP-binding site, and hence, in the absence of ATP, there is a gap in the interphase which closes when ATP is bound (Fig. 3). In each transport cycle, two molecules of ATP are hydrolyzed.

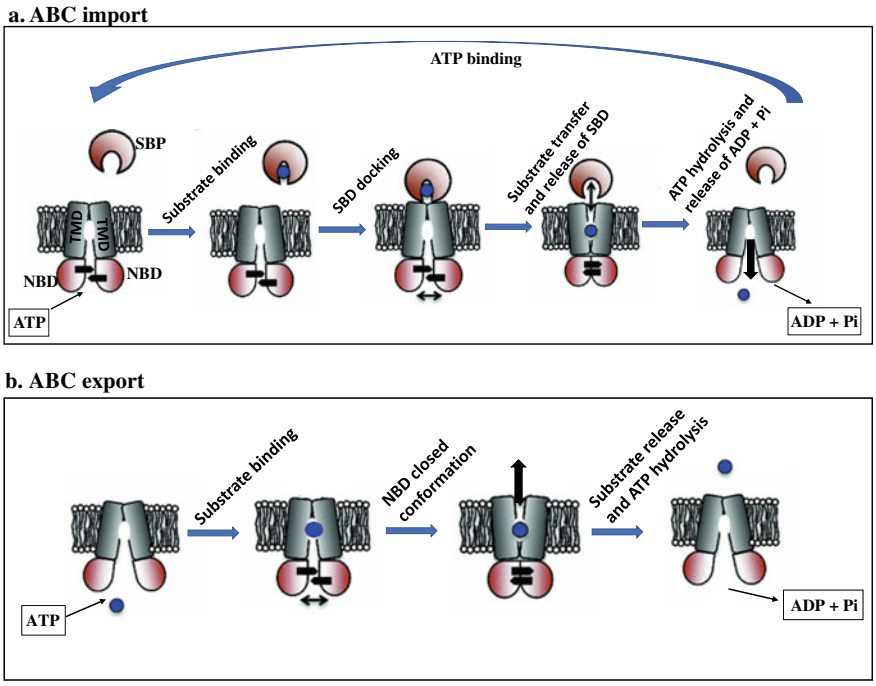


Fig. 2 Schematic representation of a. ABC import system and b. ABC export system. Adapted from: Swier et al. (2016). With kind permission from Springer Nature

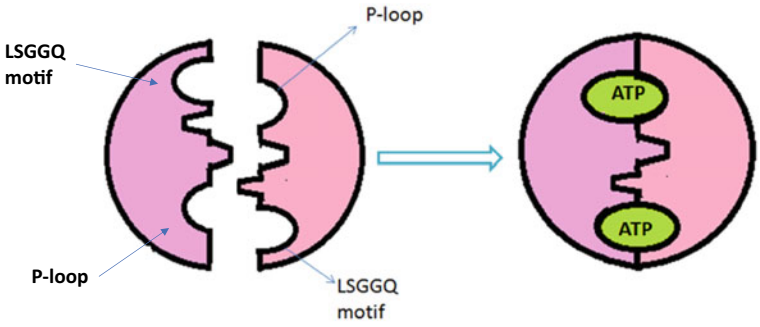


Fig. 3 Nucleotide-binding domains (NBD) in open and closed conformations

Coupling ATP hydrolysis and transport

The elucidation of the mechanism of coupling ATP hydrolysis and transport has been made possible through crystal structures where it is proposed that there has to be effective coordinated molecular motion between TMD and NBD. Architecturally,

TMDs are presented as α helices and interact with grooves formed by NBD's sub-domains. ATP binding and its hydrolysis change the conformation of NBDs which in turn affects movement of TMDs. These conformation changes were clearly observed in crystal structure of Sar1866, a multidrug ABC transporter where both nucleotide-bound and free-state conformations have been obtained. In a simplified version, it can be put forth in the following stages:

1. Periplasmic SBP binds the substrate and gets associated with TMD on periplasmic side.
2. The conformational change is passed down to NBD that facilitates ATP binding to NBD and the gap between sub-domains closes.
3. This results in movement of α-helices of TMDs which results in bringing them closer, and TMDs flip from facing inward to facing outward, facilitating import of solute inside.
4. The flipping of TMD from facing inward to outward is simultaneously coupled to the release of substrate from periplasmic protein-binding site into the translocating tunnel.
5. The substrate-binding to transient-binding site in translocation path facilitates inward movement of substrate coupled to ATP hydrolysis which helps in flipping TMD conformation from outward facing to inward. Inward facing conformation finally occludes the substrate in the cytoplasm.

The coupling mechanism broadly seems to be uniform, but TMDs are variable with respect to their topology (Linton, 2007; Locher, 2008).

Types of ABC transporters

ABC transporters can be divided into two main types, i.e., importers and exporters. Structure of ABC importers and exporters is presented in Fig. 4. Importers can be further divided on the basis of the structure of TMD and their substrates (Swier et al., 2016) into the following two groups:

1. Type I or small ABC importers
2. Type II or large ABC importers.

Type I or small ABC importers—The TMD of Type I importers have 12 helices, i.e., only six helices per subunit. The substrates such as ions, sugars and amino acids bind to periplasmic proteins and are further transported by TMD. The extensively studied Type I importers are HisPQM for histidine, molybdate–tungstate transporter (ModBC) and MalFGK for maltose. The detailed mechanism of transport has been discussed above.

Type II or large ABC importers—Type II ABC importers have 20 transmembrane segments with 10 helices in each TMD. These are used for uptake of larger substrates such as metal ions and vitamins, e.g., Haem (HIF) or vitamin B12 (BtuCD) (Hvorup et al., 2007).

ABC exporters—ABC exporters are widely distributed among both prokaryotes and eukaryotes including humans. They have wide substrate specificity with a common

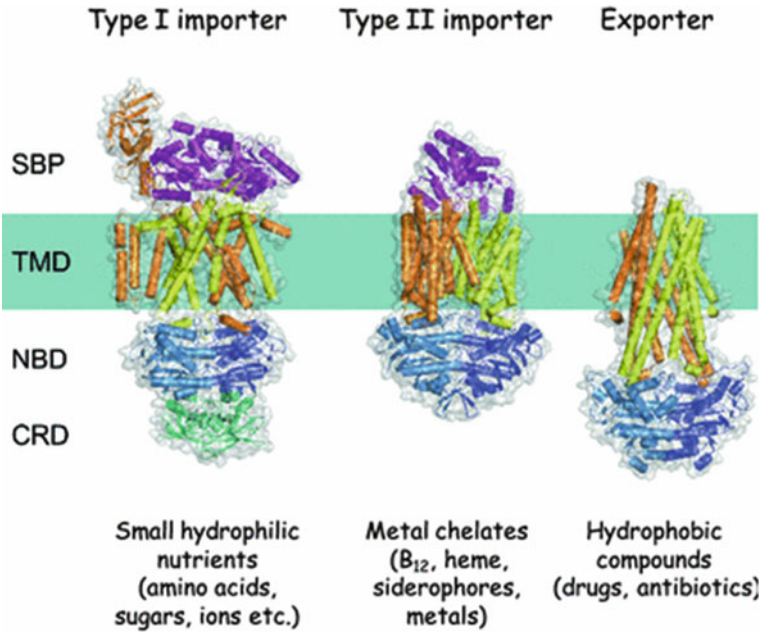


Fig. 4 Structures of ABC exporters and importers. *Source* Swier et al. (2016). With kind permission from Springer Nature

topology having 12 TMD like Type I importers but lack the periplasmic-binding proteins. The major distinction is that these helices extrude out of the cytoplasmic membrane, and the TMD helices which embrace each other are tightly bound to NBD.

ATP binding converts the inward-facing pocket of substrate-bound TMD to outward-facing conformation. The export of the substrate is accompanied by ATP hydrolysis with reversion of the conformation to again inward facing. The ABC exporter which has been most studied is of the Sav1866 protein from *Staphylococcus aureus*.

ABC transporters play an important role in various physiological processes such as nutrient uptake, antibiotic resistance, protein secretion, growth and uptake of metal ions. As a consequence, many ABC transporters play a crucial role in virulence of pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus pneumonia* and *Yersinia* spp. ABC transporters are also important prospective candidates for designing antimicrobials and vaccines. They are ideal drug targets because they are crucial for survival as well as virulence of the bacteria in the host. At the same time, importer type ABC transporters are not found in mammalian hosts, and thus, specific drugs can be designed to block the functionality of prokaryotic ABC transporters (Garmory & Titball, 2004; Tanaka et al., 2018).

3 Bacterial Multidrug Efflux Pumps

Efflux pumps constitute the most prominent resistance determinant, which is ubiquitous in bacteria to mammals, signifying their importance in both clinical and environmental settings. Most of the pathogens harbor inherently chromosomally encoded efflux pumps; however, they also acquire the genes from the environment via conjugative plasmids and horizontal gene transfers. Therefore, antibiotic resistance as a result of efflux pumps maybe: (a) intrinsic, where constitutively basal level present in a pathogen renders resistance toward an antibiotic; (b) acquired, where increased expression levels of chromosomally encoded efflux pumps are as a result of mutation/horizontal gene transfer/conjugative plasmid; and (c) phenotypic, transient, where an efflux pump is expressed at increased levels in the presence of an effector (Hernando-Amado et al., 2016) (Fig. 5).

In comparison with the conventional knowledge about resistance genes harbored by pathogens, MDR efflux pumps confer certain added features, which render them as favorite candidates for antibiotic resistance among ESKAPE group of pathogens (made up of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), such as:

(i) MDR efflux pumps are ubiquitous in nature, present in both prokaryotes and eukaryotes, irrespective of their distribution and pathogenic index. Several reports on diverse pathogens have emphasized on the contribution of MDR efflux pumps to the acquisition of multidrug resistance.

(ii) Such efflux systems are not substrate-specific and are able to extrude antibiotics belonging to different classes and other structurally dissimilar compounds, solvents, etc. Therefore, efflux pumps significantly differ from other modes of drug resistance (e.g., β-lactamases) that acts on a specific group of antibiotics. Any single efflux pump can extrude a broad range of structurally diverse substrates; hence, its inhibition will increase the bacterial susceptibility to several antimicrobials.

(iii) Distribution of efflux pumps are complicated in nature: A single organism can possess 10 different MDR efflux pumps belonging to different families; or a single family of efflux pump is conserved throughout different species of specific bacterial genera. Although expression of a single efflux pump can confer MDR phenotype, simultaneous over-expression of more than one MDR efflux system in a pathogen could be alarming.

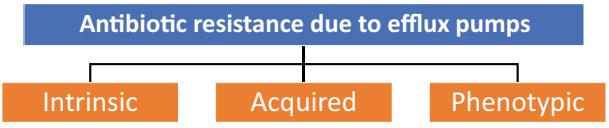


Fig. 5 Types of antibiotic resistance shown by efflux pumps

Box 1: Alternative Roles of Multidrug Efflux Pumps Beyond Antibiotic Resistance!

MDR efflux pumps correspond to 10% of the transporter genes present in bacterial species and are very aptly referred to as detoxification element (Piddock, 2006). Undoubtedly, these ancient molecules were evolutionarily preserved due to their physiologically relevant roles. MDR efflux pumps have critical role in bacterial physiology and ecological behavior besides drug resistance and hence are equally abundant in both clinical as well as non-clinical environments (Alvarez-Ortega et al., 2013). Listed below are few of the alternative roles MDR efflux pumps play, which rightly suggest these transporters to be master regulators in cellular metabolism and physiology.

- Bacterial pathogenicity
- Cell-to-cell communication
- Biofilm formation
- Efflux of bile salts
- Efflux of plant antimicrobials
- In vitro colonization and persistence
- Biocide resistance
- Plant–bacteria or bacteria–bacteria interactions
- Heavy metal tolerance.

Discovery of bacterial efflux pumps

Antibiotic efflux leading to bacterial resistance was first reported in *E. coli*, expelling tetracycline by plasmid-encoded proteins and conferring resistance to the same (McMurry et al., 1980). Though this resistance mechanism was novel, it was believed to occur via acquisition of resistance genes, contributing resistance to structurally similar group of antibiotics. Later on, discovery of chromosomally encoded efflux pump conferring resistance to multiple structurally diverse drugs challenged this paradigm. However, Mallea et al. (1998) elucidated the first efflux mechanism involved in extrusion of multiple antibiotics in *Enterobacter* spp., and AcrAB-TolC type RND efflux pumps were found to be predominant in most of the multidrug-resistant isolates of *Enterobacter aerogenes* (Blanco et al., 2016).

Classification of bacterial efflux pump families and their distribution

Bacterial efflux pumps responsible for multidrug resistance (MDR) can be categorized broadly into three evolutionary distinct and diverse efflux superfamilies based on their bioenergetics, structure and transport mechanisms:

(1) ATP-binding cassette-ABC type (driven by ATP hydrolysis);
(2) Major facilitator superfamily-MFS type (drug/proton or cation antiporters); and
(3) Resistance-nodulation-division-RND type tripartite efflux system which are also drug/proton antiporters (Yamaguchi et al., 2015).

MDR efflux transporters are broadly divided into two groups: transporters, which require ATP as energy source (primary active transporters, such as ABC efflux pumps), and transporters, which utilize PMF as energy source (secondary active transporters). Both types are ubiquitous in bacteria. Though single-component efflux transporters are prevalent in both gram-positive and gram-negative bacteria, multi-component transenvelope efflux transporters are exclusively present in gram-negative bacteria owing to their membrane architecture. It is noteworthy that a single organism may have multiple efflux pumps and even variants of a single efflux pump. Also, a given efflux pump may extrude different antibiotics belonging to same/different classes of antibiotics. The major efflux pumps are described below:

- **SMR and PACE efflux pumps**—The **s**econdary **m**ultidrug **t**ransporters (SMR) are the smallest known efflux pumps, belonging to the DMT superfamily. They are 100–120 amino acids long and contain four transmembrane (TM) helices. Present in both gram-positive and gram-negative bacteria, these efflux pumps utilize PMF to extrude multiple antibiotics and noxious compounds. Well-studied model is EmrE in *Escherichia coli* (consisting of 110 residues), functioning as an antiparallel homodimer, extrudes erythromycin, tetracycline, ethidium and proflavine out of the cell. Other representatives of SMR families are QacC and SepA in *Staphylococcus aureus* and KpnEF in *K. pneumoniae* (Srinivasan & Rajamohan, 2013). The proteobacterial antimicrobial compound efflux (PACE) transporters exemplified by *acinetobacter* chlorhexidine efflux (AceI) in *A. baumannii* are similar to SMR family, as they are 150-amino acid long with two tandem TM domains (Splenger et al., 2017).
- **MFS efflux pumps**—**M**ajor **f**acilitator **s**uperfamily (MFS) is the largest superfamily of secondary transporters, and its members are distributed in bacteria, archaea and eukaryotes. MFS pumps consist of 12–14 transmembrane domains and are of 400–600 amino acids in length. These transporters utilize PMF and function as symports, uniports and antiports with various substrates such as ions, sugars, oligosaccharides and antibiotics such as fluoroquinolones and tetracycline (Splenger et al., 2017). NorA (chromosomally encoded), QacA and QacB (plasmid acquired) in *S. aureus* and PmrA in *Streptococcus pneumoniae* are the extensively studied MFS efflux pumps in gram-positive bacteria. However, in gram-negative bacteria, these pumps are prevalent as tripartite structure with an adaptor and outer membrane protein. Examples include EmrAB-TolC in *E. coli* that effluxes cotrimoxazole and other hydrophobic uncouplers and FarAB-MtrE in *Neisseria gonorrhoeae* that effluxes fatty acids. Nevertheless, there are other well-studied MFS transporters which are single component in nature, e.g., MdtM, QepA and MdfA in *E. coli* that extrude bile salts, fluoroquinolones and chloramphenicol, tetracyclines, trimethoprim and fluoroquinolones, respectively (Hernando-Amado et al., 2016).
- **MATE efflux pumps**—Efflux transporters belonging to multidrug and toxic compound extrusion (MATE) families have been described for both gram-positive bacteria and gram-negative bacteria such as *Vibrio parahaemolyticus* (NorM), *Haemophilus influenzae* (HmrM), *Clostridium difficile* (CdeA), *Pseudomonas aeruginosa* (PmpM), *Enterobacter cloacae* (EmmDr) and *S. aureus* (MepA). MATE secondary efflux transporters consist of 12 alpha-helical TM regions

and range from 400–700 amino acids in length. They are capable of extruding structurally diverse substrates, viz. antibiotics such as fluoroquinolones, chloramphenicol, ampicillin and other compounds such as ethidium, triethylammonium, metformin and cimetidine. (Splenger et al., 2017). MATE efflux pumps can utilize two energy sources: PMF and sodium ion gradient. Though MATE transporters extrude similar substrates as RND pumps, they are single component tranporters unlike tripartite RND efflux systems.

- **RND efflux pumps**—**R**esistance-**n**odulation-**d**ivision superfamily of efflux pumps functions as drug/proton antiporter and unlike other efflux transporters are believed to be exclusively present in gram-negative pathogens. However, recent studies have revealed RND type monomers in gram-positive bacteria as well such as in *S. aureus*, *B. subtilis* and *Clostridium difficile* (Venter et al., 2015). RND superfamily of transporters consists of seven families including the hydrophobic/amphiphilic efflux (HAE) family, the SecDF protein-secretion accessory protein (SecDF) family and the heavy metal efflux (HME) family. Transporters of the HAE subfamily in *E. coli* include five efflux transporters, AcrAB, AcrAD, AcrEF, MdtAB and MdtEF. AcrAB-TolC efflux pumps in *E. coli* and MexAB-OprM from *P. aeruginosa* are the two extensively studied RND efflux systems. Over-expression of RND efflux pumps has been associated with MDR phenotype in many clinically significant opportunistic pathogens. AcrAB-TolC efflux pumps are also known to have functional significance in both clinical and non-clinical setups, conferring intrinsic and/or acquired resistance to multiple antibiotics, bile salts, toxins, detergents, dyes, free fatty acids, solvents, etc. (Anes et al., 2015).

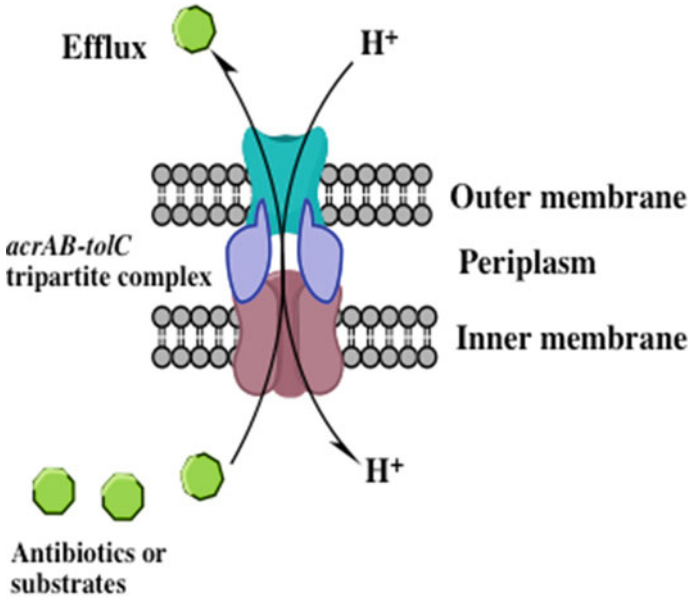


Fig. 6 Simplistic model describing the function of tripartite AcrAB-TolC type of RND efflux pump system, which utilizes proton motive force for transport of antibiotics/substrates out of the cell

AcrAB-TolC tripartite efflux pumps belong to the HAE subfamily of the RND superfamily. This efflux pump is organized as a tripartite complex consisting of three major components: a transmembrane RND transporter (AcrB) in the inner membrane involved in substrate recognition and transfer, an outer membrane protein (TolC) providing an exit channel for substrate out of cell, and a periplasmic membrane fusion adaptor protein (AcrA) that acts as a connecting link between both AcrB and TolC, and three of these proteins together form an effective efflux unit (Fig. 6).

Such pumps are highly efficient in extrusion of substrates, both from cytoplasmic and periplasmic space. They utilize PMF gradient as energy source and are drug/proton antiporters. The proton dislocation in these proteins helps in substrate transport across the membranes. This PMF is generated by hydrolysis of ATP, catalyzed by membrane-bound ATPases and by oxidative metabolism, and hence forms the driving force for the activity of RND efflux pump. AcrAB-TolC efflux pumps are primarily associated with multiple antibiotic resistance in many clinically significant gram-negative pathogens including *Enterobacter* spp., *E. coli*, *P. aeruginosa*, *Salmonella* spp. and *Klebsiella* spp. (Anes et al., 2015).

A comparative account of major efflux pumps with respect to distribution, energy requirement, and substrate is presented in Table 1.

Box 2: How to Measure Efflux Activity by Multidrug Efflux Pumps? Blair and Piddock (2016)

- Most common approach used for determining efflux activity is by drug susceptibility measurements (for instance, minimum inhibitory concentration—MIC). An isolate with greater expression of an efflux pump is supposed to be less susceptible to various antimicrobials as compared to the isolates with lower efflux pump expression. Additionally, the presence of an inhibitor like carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or phenylalanine-arginine beta-naphthylamide (PAβN) in MIC assays along with controls without inhibitor can significantly tell the difference in fold change in MIC values. Thus, isolates with increased drug susceptibility in the presence of an efflux inhibitor are presumed to over-express one or more efflux pumps. However, this method lacks sensitivity and is not a direct measurement of efflux activity.
- Direct measurement of efflux activity involves various fluorescent substrates of these efflux pumps like ethidium bromide, Nile red, Hoechst H33342, etc. There are two important ways of determining efflux activity: either by measuring how much substrates are expelled directly or how much substrates are accumulated in the bacterial cell, which indirectly refers to the efflux activity of the bacterium. One greater advantage of this method is that it helps in measuring efflux kinetics and is more sensitive.

Table 1 Comparative characterization of multidrug efflux pumps of bacteria

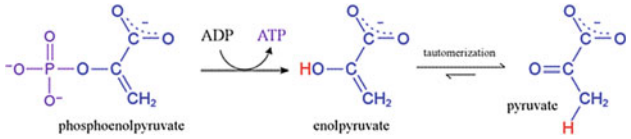
	The ATP-binding cassette (ABC) superfamily	The major facilitator superfamily (MFS)	The small multidrug resistance (SMR) family	The resistance-nodulation-division (RND) superfamily	MATE (Like RND)	PACE (like SMR)
Energy source	Drug/ATP antiporter driven by ATP hydrolysis	Proton/monovalent ions- based drug symporter/antiporter/uniporter	Drug/proton antiporter uses PMF	Drug/proton antiporter uses PMF	PMF and sodium ion gradient	
Components composition	Multicomponent, multidomain system; total size C1000 residues, 6 transmembrane spanners, approximately 500 sequenced members	Size C400-600 residues; 12 or 14 transmembrane spanners, approximately 500 sequenced members	Homodimers, subunit size: C100 residues with 4 transmembrane helices, 10 sequenced members	Multicomponent, subunit size: C1000 residues, 12 transmembrane spanners, 16 sequenced members	Single component, 12 alpha-helical TM region and range from 400–700 a.a	100 a.a. and two tandem TM domains
Distribution	Bacteria, archaea and eukaryotes	Bacteria, archaea and eukaryotes	Only in prokaryotes	Prokaryotes (mainly gram-negative bacteria)	Gram-positive and gram-negative	
Substrates exported	Antibiotics, sugars, amino acids, ions, vitamins, iron complexes, peptides, proteins, complex carbohydrates	Antibiotics, anions, sugars, metabolites and other substrates	Drugs and other substrates	Multiple antibiotics, toxic dyes, detergents, bile salts, etc	Diverse substrate antibiotics and their compounds	
Subfamily	28 families out of which only 3–4 are drug efflux pumps	17 families wherein MDR efflux pumps are found in three families	Two subfamilies; one for drugs and other for other substrates	Seven families including three specific for drugs, metal ions and lipopolysaccharides, respectively	MOP superfamily	
Example	LmrA, and MacB	NorA, PmrA, MdtM	Qac and EmrE	AcrB and MexB	NorM, CedA, PmpM	AceI

Prepared from: Saier et al. (1998)

4 Group Translocation: PEP-PTS System

The phosphoenolpyruvate (PEP): carbohydrate transferase system (PTS) is different from other primary and secondary transport systems which are directly driven by ATP or PMF. The system is referred to as group translocation system. Group translocations are a specialized form of active transport wherein molecules are modified as they are transported across the cell membrane or into the cell. The molecule is chemically altered during its transport across a membrane so that once inside the cytoplasmic membrane, it will not leak out of the cell and can be accumulated.

PEP-PTS system was first discovered by Roseman (1969). This system catalyzes the translocation of certain carbohydrates called PTS sugars. Sugars such as rhamnose, arabinose and xylose are non-PTS sugars. Carbohydrates are transported as phosphorylated derivatives at the expense of PEP, a high energy compound generated during glycolysis. PEP is a more energetic phosphoryl donor than even ATP. The phosphate group of pyruvic acid is released on hydrolysis, and the enol form is tautomerized into the more stable keto form. The keto form has 76 kJ/mol more bond energy than the enol form, and this enol-keto tautomerization leads to release of high energy.



PEP-PTS system facilitates transport of sugar as phosphorylated sugar wherein the phosphoryl donor is phosphoenolpyruvate (PEP). Sugars are taken up in their phosphorylated forms instead of as free sugars leading to intracellular accumulation of the sugar phosphate esters. PEP also stores similar energy as ATP, and thus, an ATP molecule is generated by pyruvate kinase reaction (Fig. 7). It is also well

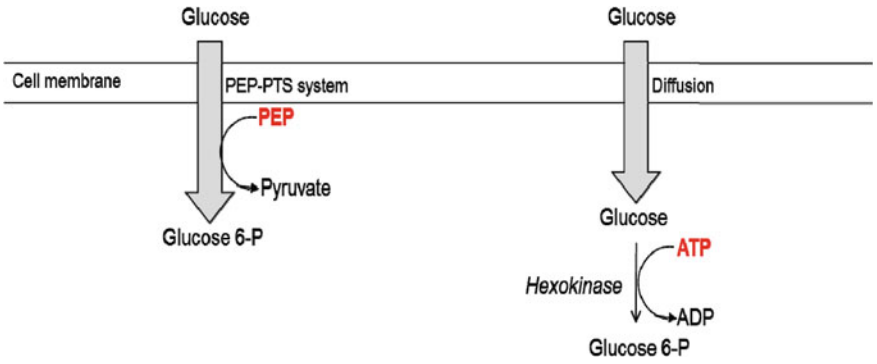


Fig. 7 Glucose phosphorylation during transport through PEP-PTS system versus diffusion

known that non-PTS carbohydrates require more ATP for both their uptake and phosphorylation before their breakdown. Thus, PEP-PTS transport system conserves ATP and hence is found widely among obligate and facultative anaerobes which generate energy via substrate-level phosphorylation. It is completely absent from eukaryotes and many aerobic eubacteria; however, it has been reported in archaea. Apart from translocation of carbohydrates, the PTS proteins may phosphorylate other proteins related to chemostasis and metabolic regulations.

Advantages of PEP-PTS system

- Obligate and facultative anaerobes generate ATP only via substrate-level phosphorylation and thus have a low ATP pool
- Instead of using ATP to phosphorylate glucose via the action of hexokinase, these organisms use another high energy compound, PEP.

Generalized scheme for carbohydrate translocation by PEP-PTS system

PEP-PTS is a multicomponent system that involves enzymes of the plasma membrane and those in the cytoplasm. PTS proteins are well characterized in enteric bacteria, *E. coli* and *Salmonella typhimurium.* A large number of PTS translocations and phosphorylations from a variety of bacteria have been described. The general scheme for carbohydrate uptake is presented in Fig. 8.

This is not a single-step reaction and involves multiple proteins, and the phosphate transfer takes place across three proteins before it is donated to carbohydrate, viz. EI, HPr (Histidine protein) and EII. EI and HPr are cytosolic proteins, and phosphate from PEP is first transferred to EI and then to HPr:

$$\text{EI} + \text{PEP} \rightarrow \text{P} + \text{Pyruvate}$$

$$\text{EI - P} + \text{HPr} \rightarrow \text{HPr - P} + \text{EI}$$

EI and HPr are called PTS proteins and are present in all PTS systems. However, EII is carbohydrate specific permease complex and may have four domains *A*, *B*, *C* and *D* for specific functions like binding to the carbohydrate, for phosphorylation, to catalyze the transport of carbohydrate into the cell, etc. The domain organization is again carbohydrate-specific. It may be a single membrane-bound polypeptide having three domains such as in mannitol EII^{Mtl} or it may be made up of two or more proteins (wherein one protein is cytoplasmic while the other protein is membrane-bound). Domain C is membrane-bound and helps in transport of the carbohydrate. In glucose EII^{Glc} of *E. coli*, EIIA is soluble and EIIBC is membrane-bound, while in mannose, EIIAB is soluble and EIIC is membrane-bound. The phosphoryl group from HPr travels through EIIA to EIIB and finally to membrane-bound integral protein EIIC (which makes the translocation channel as it also has the substrate-specific binding site).

$$\text{HPr - P} + \text{EIIA} \rightarrow \text{HPr} + \text{EIIA - P}$$

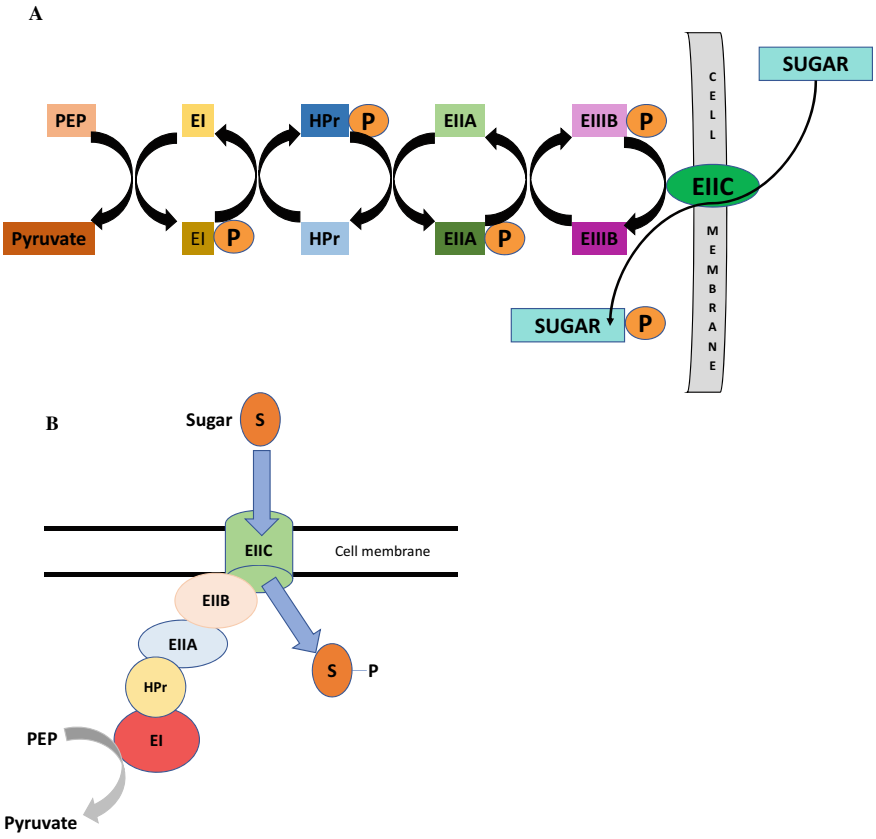


Fig. 8 Mechanism of glucose transport by PEP-PTS system: schematic depiction of sequential phosphorous transfer from PEP to EIIC (which forms homodimer in the membrane) yielding sugar phosphate (S-P)

$$\text{EIIA - P} + \text{EIIB} \rightarrow \text{EIIB - P} + \text{EIIA}$$

$$\text{EIIB - P} + \text{Carbohydrate(out) - EIIC} \rightarrow \text{EIIB} + \text{Carbohydrate - P(in)} + \text{EIIC}$$

Glucose, mannose and mannitol PTS systems of *E. coli* are the most common and well-studied. Domain and polypeptide organization of the integral proteins can vary with respect to different sugars. Of the three transport proteins, EIIC is always membrane-bound. In some cases, like fructose and mannose, there are two membrane proteins EIIC and EIID instead of only one in other systems. EIIA and EIIB can be cytoplasmic or they can be membrane-bound in cases where they are fused to EIIC. For example, in glucose PTS, all the three proteins are fused together and are thus membrane-bound, while in cellobiose, PTS, EIIA and EIIB are cytoplasmic while

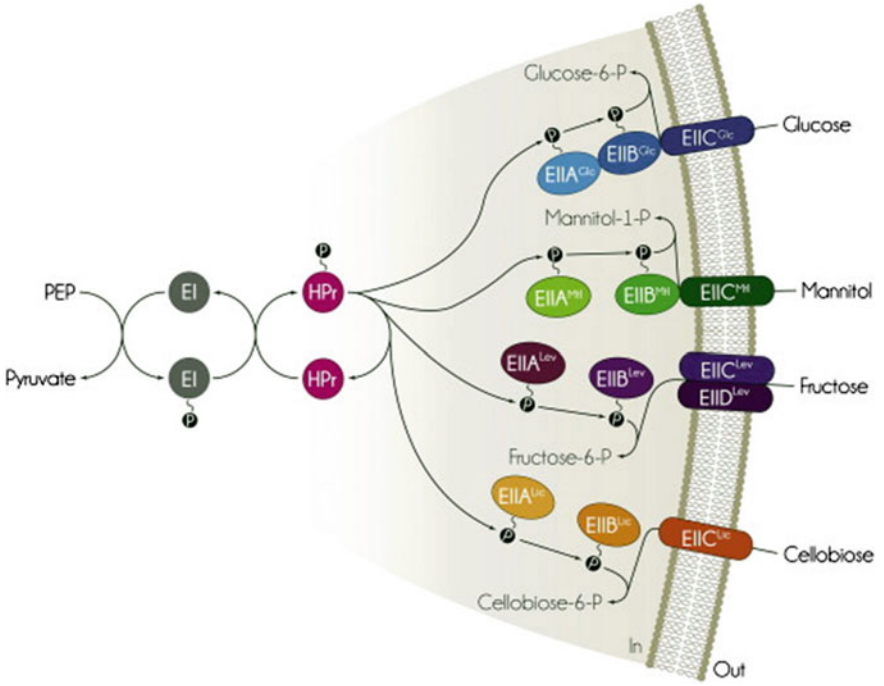


Fig. 9 PEP-PTS system for transfer of glucose, mannitol, fructose and cellobiose in *E. coli. Source* Galinier and Deutscher (2017). With kind permission from Elsevier

EIIC is membrane-bound. In mannitol PTS, only EIIA is cytoplasmic while EIIB is fused with EIIC (Fig. 9).

Characterization of PTS proteins EI and HPr: Protein characterization, Domain organization and phosphorylation (Postma et al., 1993; Siebold et al., 2001)

EI Protein: EI protein of most gram-positive and gram-negative bacteria has been reported to be a monomer of 64 kDa encoded by a gene named *pstI*. Studies have shown that autophosphorylation of EI protein using PEP requires dimerization of the protein and divalent cations such as Mg^{2+} and Mn^{2+}. Dimerization is facilitated by a sulfhydryl group, while phosphorylation occurs at N-3 position of histidyl residue of each monomer (e.g., His-189 of *E. coli* EI protein). EI protein contains two domains, one at N-terminal and other at C-terminal. Carboxy terminal is necessary for autophosphorylation by PEP and plays a key role in dimerization as well, while N-terminal is necessary for reversible phosphorylation of HPr protein as it was observed in an in vitro phosphorylation experiment that this domain can only be reversibly phosphorylated by HPr-P and not by either PEP or EI-P. The genes of several EI proteins from various sources have been sequenced and show high conservation around the site of the phosphorylating histidine residue. Interestingly,

the gene sequence also showed similarities with pyruvate phosphate dikinase and PEP synthase, which also phosphorylate using either PEP or ATP as phosphate donor during generation of PEP from pyruvate.

HPr Protein: HPr proteins are small proteins with molecular weight of only 9–10 kDa encoded by *pstH* gene. HPr proteins have conserved sequences in the region of phosphorylation among many gram-positive and gram-negative bacteria. The HPr protein of *E. coli* and *S. typhimurium* was found to be identical, and only one conservative exchange was observed in HPr from *Klebsiella pneumonia.* The detailed structural studies have confirmed the role of His-15, the phosphorylating residue of HPr. The role of active residue was also confirmed by site-directed mutagenesis where mutating His-15 to alanine lost phosphorylating potential with EI or PEP. Protonated N-3 atom of His-15 residue at physiological pH makes the N-1 atom a better nucleophile for EI-P. On phosphorylation, hydrogen bond involving N-3 atom breaks following a conformational change. The phosphoryl transfer potential for hydrolysis of HPr-P from *S. typhimurium* was found to be −13 kcal/mol, close to that of original PEP. This suggests that HPr-P retains most of the phosphoryl transfer potential of PEP which enables it to transfer phosphate further to other proteins involved in chemotaxis and metabolic regulation. In *E. coli*, Arg-17 residue has also been reported to be important for proper functioning of the HPr protein beside His-15 residue as mutation of Arg-17 to Gly/Lys in *E. coli* resulted in a protein which was inactive in mannose transfer. Further, molecular modeling studies of HPr and $EIIA^{Glc}$ from *B. subtilis* showed that both the proteins had complementary surface topographies which can facilitate protein–protein interaction during phosphate transfer.

Characterization of EII domain

EII protein may be a membrane-bound monomer or a multimer with at least one of the subunits being membrane-bound. EII protein always has three domains, IIA, IIB and IIC, except in the mannose system where IID is additionally present.

The sequence comparisons of different EII proteins suggest that there are at least four classes. The sequences of each class are heterogenous and share only 25% identities while the members of different classes show only conserved motifs. Most EIIs share several conserved features:

i. Three distinct domains representing IIA, IIB and IIC which may be all free or fused. If fused, they have a flexible linker region.
ii. A hydrophilic domain IIA with a conserved histidine residue which interacts with HPr-P (ca. 100 residues).
iii. A hydrophilic domain IIB having an active cysteine that interacts with IIA (ca. 100 residues) on any side
iv. A IIC membrane-bound domain of nearly 250 residues which includes 6–8 transmembrane helices and at least one large hydrophilic loop that includes histidine and a GXXE motif.
v. In most cases, three domains are connected by linkers which may be a general linker having several charged amino acids or a specific one such as PA linker

with Pro-Ala repeats or Q linker having hydrophilic residues (Gln, Asn and His) and Ser, Thr and Ala residues.

Translocation catalyzed by EIIs: Through a large number of biochemical, mutational and complementation studies, a consensus model for translocation of carbohydrates has been proposed.

i. The periplasmic PTS substrate binds to the substrate-binding site of EIIC. If EII is not phosphorylated or $EIICB^{Glc}$ is not complexed with EIIA, then in such situations, there is only a slow facilitated diffusion of sugar, if at all.
ii. Phosphorylation of IIB site at cysteine allows rapid translocation of PTS sugar by conformational change.
iii. After phosphorylation of the bound sugar by IIB-P domain, dissociation of the phospho-carbohydrate into the cytoplasm.

Thus, translocation is normally coupled to phosphorylation.

Catabolite regulation by PTS system

Inhibition of utilization of other sugars until glucose is present is called glucose repression or catabolite repression. This occurs by different mechanisms:

- **Inducer Exclusion** is inhibition of uptake and metabolism of other sugars in the presence of glucose. This regulates the uptake of non-PTS sugars such as lactose, maltose, rhamnose, arabinose and xylose. This is possible by inhibition of transport and other catabolic enzymes by building up of dephosphorylated forms of EIIA as a result of glucose phosphorylation via phosphorylation of $EIICB^{Glc}$. In the presence of glucose, dephosphorylated forms of EIIA build up and subsequently block permeases of other sugars (such as lactose permease and maltose permease) and inhibit their entry into the cell (Fig. 10) (Brückner & Titgemeyer, 2002; Perez-Alfaro, 2014).

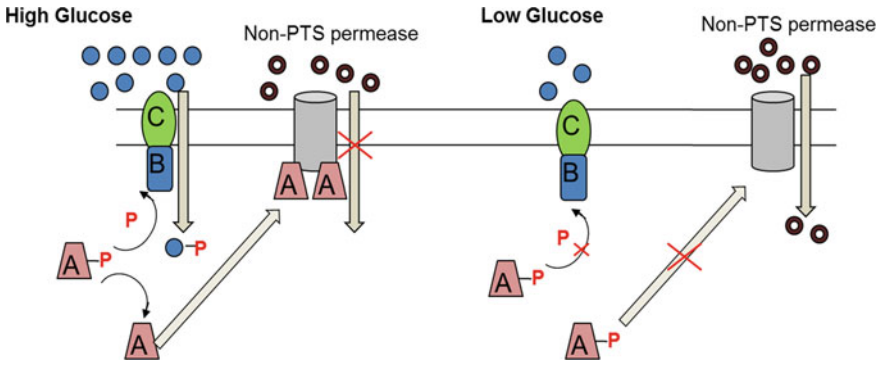


Fig. 10 Diagrammatic representation of inducer exclusion phenomenon of inhibition of uptake of non-PTS sugars in the presence of glucose (A, B and C represents EIIA, EIIB and EIIC, respectively)

However, in gram-positive bacteria, this is mediated by HPr protein, wherein non-PTS sugars are not utilized in the presence of glucose, even if they are phosphorylated and taken up by the cell. In this case, an increase in fructose 1,6-diphosphate (FDP) concentration inside the cell during glucose metabolism results in a relay of phosphorylation reactions as FDP allosterically activates an ATP-dependent protein kinase which phosphorylates HPr protein of PTS at a serine residue. This Ser-phosphorylated HPr protein then in turn activates a sugar phosphate phosphatase that dephosphorylates the phosphorylated non-PTS sugars present in the cytoplasm. The dephosphorylation of these non-PTS sugars then facilitates their expulsion from the cell (Ye & Saier, 1996) (Fig. 11).

- **cAMP-mediated catabolite repression in enteric bacteria**—If phosphorylated IIA-P fails to get dephosphorylated in the presence of low glucose, it activates adenylate cyclase by phosphorylating it. Adenylate cyclase forms cAMP which along with CRP (or CAP) activates transcription of catabolic operons for breakdown of sugars other than glucose. Likewise, when glucose is present, IIA-P is dephosphorylated by IICB and thus is not able to activate adenylate cyclase, and transcription for degradation of other sugars is inhibited (Fig. 12) (Gottschalk, 1979).

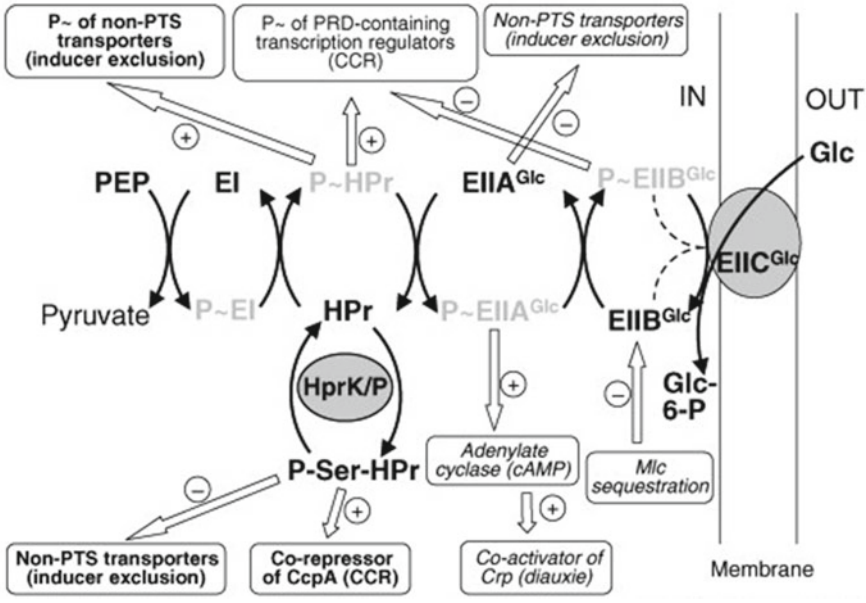


Fig. 11 EIIA- and HPr-mediated catabolite repression and inducer exclusion of non-PTS sugars in the presence of glucose (EIIA-mediated regulation is observed in enteric bacteria while HPr-mediated regulation is observed in gram-positive bacteria). *Source* Adapted from Deutscher (2008). With kind permission from Elsevier

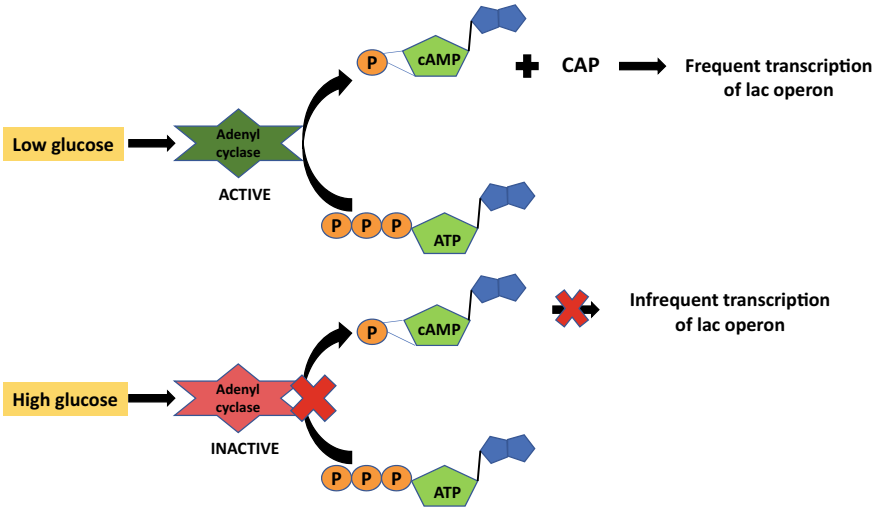


Fig. 12 cAMP-mediated catabolic repression by glucose in *E. coli*

Involvement of PEP-PTS system in catabolism of sugars which are repressed in presence of glucose has been proved in mutants lacking HPr or EI proteins. The reason is that in the absence of these, EIIA cannot be phosphorylated which is needed for activating adenylate cyclase. *E. coli* has a well-documented requirement of PTS proteins for growth on non-PTS sugar. This was further substantiated by the observation that on supplementing cAMP, HPr or EI mutants recovered and could grow on non-PTS sugars.

HPr negative mutants failed to uptake certain non-PTS sugars. This further suggests that HPr mutants would lack phosphorylation of $EIIA^{Glc}$, and the non-phosphorylated form would accumulate which is capable of binding the transporters of non-PTS sugars like lactose, xylose, melibiose, etc. (Deutscher et al., 2006).

cAMP-independent carbon catabolite repression in low GC gram-positive bacteria—Carbon catabolite repression (CCR) in low GC content gram-positive bacteria like *B. subtilis* is independent of cAMP-based repression (Saier et al., 1996). Instead, CCR in gram-positive bacteria mainly involves negative regulation by a global transcriptional regulator catabolite control protein A (CcpA), which is expressed constitutively and belongs to LacI/GalR repressor family, along with its two phosphorylated cofactors HPr(Ser46-P) and Crh(Ser46-P) (Hueck and Hillen, 1995; Lorca et al., 2005). It also involves PEP-PTS system for uptake of carbon sugars; however, unlike enteric bacteria, the phosphorylated sugar metabolites are sensed by a bifunctional kinase HprK/P (Dahl, 2002). Hpr kinase is responsible for the phosphorylation of the cofactors Hpr and Crh at position 46 occupied by a serine residue and is metabolite-controlled as its kinase activity is triggered in the presence

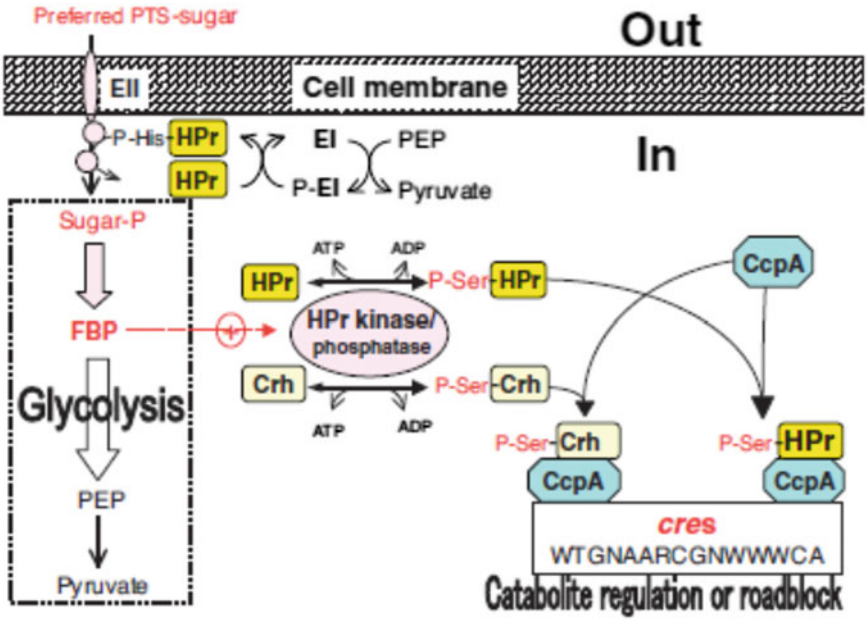


Fig. 13 Mechanism of CcpA/Hpr/Crh-mediated CCR in *Bacillus subtilis*. The term roadblock is used for complete blocking of transcription of the gene when *cre* site is present within the gene itself. *cres* denotes *cre* site whose base composition and nucleotide length might vary depending on the bacteria. *Source* Fujita et al. (2007). With kind permission from Taylor & Francis

of high concentration of fructose-1,6-bisphosphate and ATP, while it gets inhibited in the presence of high concentration of Pi (Jault et al., 2000). The transport of the preferred carbon sugar inside the cell results in phosphorylation of the sugar which in turn is sensed by HprK leading to the activation of its kinase activity. The activated HprK then phosphorylates Hpr/Crh protein at serine 46 position. The transcriptional regulator CcpA forms a complex with the phosphorylated form of Hpr/Crh and binds effectively to cis-acting *cre* (catabolite responsive element) site present either in the promoter region of the gene or within gene itself. The *cre* site is 14–16 bp long having a typical palindromic nucleotide motif with the consensus sequence 'TGWAANCGNTNWCA' where W is A or T, N is any base. CcpA in complex with HPr(Ser46-P) is mainly responsible for strong catabolite repression in comparison with CcpA/Crh(Ser46-P) complex. In addition, fructose-1,6-bisphosphate also interacts with CcpA to form a ternary complex (CcpA/Hpr(Ser-P)/FBP) which has been reported to show highest DNA-binding affinity (Schumacher et al., 2007). CcpA complex binding at the *cre* site represses the transcription of genes involved in non-preferred metabolism (Fujita, 2009; Cai et al., 2012). In several *Bacillus* and *Lactobacillus* species, CcpA-mediated catabolite repression has been reported to be involved in the regulation of metabolite production, carbon catabolism (Chen et al., 2018; Antunes et al. 2011), aerobic and anaerobic metabolism (Zotta et al., 2012),

stress tolerance (Li et al., 2016) and metabolite production as inactivation of *ccpA* gene resulted in partial or complete relief from CCR for these regulatory processes (Zeng et al., 2017) (Fig. 13).

Summary

- There are three main categories of transport system well characterized in bacteria. These are shock-sensitive system mediated by ATP and shock-insensitive system mediated by proton motive force (PMF) and group translocation- PEP-PTS system.
- **A**TP-**b**inding **c**assette (ABC) transporters are ubiquitous in distribution among both prokaryotes and eukaryotes. They mediate active transport by ATP hydrolysis and thus form the primary transport system.
- ABC transporters can be categorized as: importers in prokaryotes; exporters in both prokaryotes and eukaryotes; and functions other than transport again in both prokaryotes and eukaryotes.
- ABC transporter basically has four domains, two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). ATP binding and its hydrolysis change the conformation of NBDs which in turn affects movement of TMDs.
- The flipping of TMD from facing inward to outward is simultaneously coupled to the release of substrate from periplasmic protein-binding site into the translocating tunnel.
- Primary active transporters, e.g., ABC efflux pumps, utilize ATP as energy source and secondary active transporters utilize proton motive force.
- Bacterial efflux pumps are responsible for multidrug resistance and can be categorized broadly into three evolutionary distinct and diverse efflux super-families based on their bioenergetics, structure and transport mechanisms: ATP-binding cassette-ABC type; major facilitator superfamily-MFS type and resistance-nodulation-division-RND type.
- Group translocations are a specialized form of active transport wherein molecules are modified as they are transported across the cell membrane or into the cell.
- PEP-PTS system was first discovered by Saul Roseman in 1964. This system catalyzes the translocation of certain carbohydrates called PTS sugars.
- PEP-PTS system facilitates transport of sugar as phosphorylated sugar wherein the phosphoryl donor is phosphoenolpyruvate (PEP), and it involves multiple proteins, EI, histidine protein (HPr) and EII.
- EI and HPr are called PTS proteins and are present in all PTS systems. However, EII is carbohydrate-specific permease complex and may have four domains A, B, C and D for specific functions.
- The phosphoryl group from HPr travels through EIIA to EIIB and finally to membrane-bound integral protein EIIC which makes the translocation channel as it also has the substrate-specific binding site.
- Inhibition of utilization of other sugars till glucose is present is called glucose repression or catabolite repression.

- In the presence of glucose, dephosphorylated forms of EIIA build up and subsequently block permeases of other sugars (such as lactose permease and maltose permease) and inhibit their entry into the cell called inducer exclusion.
- Inducer expulsion is another mode by which non-PTS sugars are not utilized in the presence of glucose. Rather, they are expelled even if they are phosphorylated by up-regulation of sugar phosphatases which dephosphorylate non-PTS sugars, thereby facilitating their expulsion out of the cell.

Questions

1. What are shock-sensitive and shock-insensitive transportation?
2. What are the different domains of ABC transporter?
3. Elucidate mechanism of coupling of ATP hydrolysis and transport in an ABC transporter.
4. What are different possibilities of antibiotic resistance conferred by efflux pumps?
5. Compare and contrast three the major families of efflux pumps.
6. What is group translocation? What is the advantage of PEP-PTS system?
7. Present the generalized scheme of glucose uptake by PEP-PTS system.
8. What is inducer exclusion and inducer expulsion phenomenon?
9. Mutants defective in HPr are generally defective in uptake of both PTS and non-PTS sugars. Explain?

References

Alvarez-Ortega, C., Olivares, J., & Martínez, J. L. (2013). RND multidrug efflux pumps: What are they good for? *Frontiers in Microbiology, 4,* 7.

Anes, J., McCusker, M. P., Fanning, S., & Martins, M. (2015). The ins and outs of RND efflux pumps in *Escherichia coli*. *Frontiers in Microbiology, 6,* 587.

Antunes, A., Martin-Verstraete, I., & Dupuy, B. (2011). CcpA-mediated repression of Clostridium difficile toxin gene expression. *Molecular Microbiology, 79*(4), 882–899.

Blair, J. M., & Piddock, L. J. (2016). How to measure export via bacterial multidrug resistance efflux pumps. *MBio, 7*(4), e00840-e916.

Blanco, P., Hernando-Amado, S., Reales-Calderon, J., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Blanca Sanchez, M., & Martinez, J. (2016). Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. *Microorganisms, 4*(1), 14.

Brückner, R., & Titgemeyer, F. (2002). Carbon catabolite repression in bacteria: Choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS Microbiology Letters, 209*(2), 141–148.

Cai, J., Tong, H., Qi, F., & Dong, X. (2012). CcpA-dependent carbohydrate catabolite repression regulates galactose metabolism in Streptococcus oligofermentans. *Journal of Bacteriology, 194*(15), 3824–3832.

Chen, C., Lu, Y., Wang, L., Yu, H., & Tian, H. (2018). CcpA-dependent carbon catabolite repression regulates fructooligosaccharides metabolism in Lactobacillus plantarum. *Frontiers in Microbiology, 9,* 1114.

Dahl, M. K. (2002). CcpA-independent carbon catabolite repression in Bacillus subtilis. *Journal of Molecular Microbiology and Biotechnology, 4*(3), 315–322.

Deutscher, J. (2008). The mechanisms of carbon catabolite repression in bacteria. *Current Opinion in Microbiology, 11*(2), 87–93.

Deutscher, J., Francke, C., & Postma, P. W. (2006). How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiology and Molecular Biology Reviews, 70*(4), 939–1031.

Fujita, Y. (2009). Carbon catabolite control of the metabolic network in Bacillus subtilis. *Bioscience, Biotechnology, and Biochemistry, 73*(2), 245–259.

Fujita, Y., Miwa, Y., Tojo, S., & Hirooka, K. (2007). *Carbon catabolite control and metabolic networks mediated by the CcpA protein in Bacillus subtilis* (pp. 91–110). Trivandrum, India: Global regulatory networks in Bacillus subtilis. Transworld Research Network.

Galinier, A., & Deutscher, J. (2017). Sophisticated regulation of transcriptional factors by the bacterial phosphoenolpyruvate: Sugar phosphotransferase system. *Journal of Molecular Biology, 429*(6), 773–789.

Garmory, H. S., & Titball, R. W. (2004). ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. *Infection and Immunity, 72*(12), 6757–6763.

Gilson, E., Higgins, C. F., Hofnung, M., Ames, G. F. L., & Nikaido, H. (1982). Extensive homology between membrane-associated components of histidine and maltose transport systems of Salmonella typhimurium and Escherichia coli. *Journal of Biological Chemistry, 257*(17), 9915–9918.

Gottschalk, G. (1979). Regulation of bacterial metabolism. In G. Gottschalk (Ed.), *Bacterial metabolism* (pp. 142–166). New York: Springer.

Hernando-Amado, S., Blanco, P., Alcalde-Rico, M., Corona, F., Reales-Calderon, J. A., Sanchez, M. B., & Martinez, J. L. (2016). Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials. *Drug Resistance Updates, 28,* 13–27.

Higgins, C. F. (2001). ABC transporters: Physiology, structure and mechanism–an overview. *Research in Microbiology, 152*(3–4), 205–210.

Hueck, C. J., & Hillen, W. (1995). Catabolite repression in Bacillus subtilis: A global regulatory mechanism for the Gram-positive bacteria? *Molecular Microbiology, 15*(3), 395–401.

Hvorup, R. N., Goetz, B. A., Niederer, M., Hollenstein, K., Perozo, E., & Locher, K. P. (2007). Asymmetry in the structure of the ABC transporter-binding protein complex BtuCD-BtuF. *Science, 317*(5843), 1387–1390.

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., & Higgins, C. F. (1990). Structural model of ATP-binding proteing associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature, 346*(6282), 362.

Jault, J. M., Fieulaine, S., Nessler, S., Gonzalo, P., Di Pietro, A., Deutscher, J., & Galinier, A. (2000). The HPr kinase from Bacillus subtilis is a homo-oligomeric enzyme which exhibits strong positive cooperativity for nucleotide and fructose 1, 6-bisphosphate binding. *Journal of Biological Chemistry, 275*(3), 1773–1780.

Li, C., Sun, J. W., Zhang, G. F., & Liu, L. B. (2016). Effect of the absence of the CcpA gene on growth, metabolic production, and stress tolerance in Lactobacillus delbrueckii ssp. bulgaricus. *Journal of Dairy Science, 99*(1), 104–111.

Linton, K. J. (2007). Structure and function of ABC transporters. *Physiology, 22*(2), 122–130.

Locher, K. P. (2008). Structure and mechanism of ATP-binding cassette transporters. *Philosophical Transactions of the Royal Society B: Biological Sciences, 364*(1514), 239–245.

Lorca, G. L., Chung, Y. J., Barabote, R. D., Weyler, W., Schilling, C. H., & Saier, M. H. (2005). Catabolite repression and activation in Bacillus subtilis: Dependency on CcpA, HPr, and HprK. *Journal of Bacteriology, 187*(22), 7826–7839.

Mallea, M., Chevalier, J., Bornet, C., Eyraud, A., Davin-Regli, A., Bollet, C., & Pages, J. M. (1998). Porin alteration and active efflux: Two in vivo drug resistance strategies used by *Enterobacter aerogenes. Microbiology, 144*(11), 3003–3009.

McMurry, L., Petrucci, R. E., & Levy, S. B. (1980). Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli. *Proceedings of the National Academy of Sciences, 77*(7), 3974–3977.

Pérez-Alfaro, R. S. (2014). Regulatory switches for hierarchical use of carbon sources in E. coli. *Network Biology*, *4*(3), 95.

Piddock, L. J. (2006). Multidrug-resistance efflux pumps? not just for resistance. *Nature Reviews Microbiology, 4*(8), 629.

Postma, P. W., Lengeler, J. W., & Jacobson, G. R. (1993). Phosphoenolpyruvate: Carbohydrate phosphotransferase systems of bacteria. *Microbiology and Molecular Biology Reviews, 57*(3), 543–594.

Roseman, S. (1969). The transport of carbohydrates by a bacterial phosphotransferase system. *The Journal of General Physiology, 54*(1), 138–184.

Saier, JR, M. H., Paulsen, I. T., Sliwinski, M. K., Pao, S. S., Skurray, R. A., & Nikaido, H. (1998). Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. *The FASEB Journal, 12*(3), 265–274.

Saier, M. H., Chauvaux, S., Jr., Cook, G. M., Deutscher, J., Paulsen, I. T., Reizer, J., & Ye, J. J. (1996). Catabolite repression and inducer control in Gram-positive bacteria. *Microbiology, 142*(2), 217–230.

Schumacher, M. A., Seidel, G., Hillen, W., & Brennan, R. G. (2007). Structural mechanism for the fine-tuning of CcpA function by the small molecule effectors glucose 6-phosphate and fructose 1, 6-bisphosphate. *Journal of Molecular Biology, 368*(4), 1042–1050.

Siebold, C., Flükiger, K., Beutler, R., & Erni, B. (2001). Carbohydrate transporters of the bacterial phosphoenolpyruvate: Sugar phosphotransferase system (PTS). *FEBS Letters, 504*(3), 104–111.

Spengler, G., Kincses, A., Gajdács, M., & Amaral, L. (2017). New roads leading to old destinations: Efflux pumps as targets to reverse multidrug resistance in bacteria. *Molecules, 22*(3), 468.

Srinivasan, V. B., & Rajamohan, G. (2013). KpnEF, a new member of the Klebsiella pneumoniae cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance. *Antimicrobial Agents and Chemotherapy, 57*(9), 4449–4462.

Swier, L. J., Slotboom, D. J., & Poolman, B. (2016). ABC importers. In A. George (Ed.), *ABC Transporters-40 Years on* (pp. 3–36). Amsterdam, The Netherlands: Springer International Publishing.

Tanaka, K. J., Song, S., Mason, K., & Pinkett, H. W. (2018). Selective substrate uptake: the role of ATP-binding cassette (ABC) importers in pathogenesis. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1860*(4), 868–877.

Venter, H., Mowla, R., Ohene-Agyei, T., & Ma, S. (2015). RND-type drug efflux pumps from Gram-negative bacteria: Molecular mechanism and inhibition. *Frontiers in Microbiology, 6,* 377.

Yamaguchi, A., Nakashima, R., & Sakurai, K. (2015). Structural basis of RND-type multidrug exporters. *Frontiers in Microbiology, 6,* 327.

Ye, J. J., & Saier, M. H. (1996). Regulation of sugar uptake via the phosphoenolpyruvate-dependent phosphotransferase systems in *Bacillus subtilis* and *Lactococcus lactis* is mediated by ATP-dependent phosphorylation of seryl residue 46 in HPr. *Journal of Bacteriology, 178*(12), 3557–3563.

Zeng, L., Chakraborty, B., Farivar, T., & Burne, R. A. (2017). Coordinated regulation of the EIIMan and fruRKI operons of Streptococcus mutans by global and fructose-specific pathways. *Applied and Environmental Microbiology*, *83*(21).

Zotta, T., Ricciardi, A., Guidone, A., Sacco, M., Muscariello, L., Mazzeo, M. F., Cacace, G., & Parente, E. (2012). Inactivation of ccpA and aeration affect growth, metabolite production and stress tolerance in Lactobacillus plantarum WCFS1. *International Journal of Food Microbiology, 155*(1–2), 51–59.

Chapter 8
Protein Secretion

Rani Gupta and Namita Gupta

All microorganisms synthesize proteins in cytoplasm and need to export them to outer environment to serve variety of functions including cell development via. biogenesis of extracellular appendages, nutrition acquisition, antibiotic resistance and also virulence. Therefore, there exist several secretory mechanisms for export of proteins without compromising their structural and functional integrity. By and large bacterial cell (gram positive and negative) utilizes the generalized secretory pathway such as Sec pathway and Tat pathway ubiquitous to all domains of life. Major differences in these pathways are that Sec system transports unfolded proteins, while Tat system transports folded proteins (Natale et al., 2008). Although both the systems involve similar components, their mechanism of transport is entirely different. Bacterial secretion systems have been well reviewed by Green and Mecsas (2016).

1 Sec System for Transport of Unfolded Proteins

This is the most prevalent system in all life forms, bacteria, archaea, thylakoid membrane of plants and endoplasmic reticulum of eukaryotes. The main components of this system are: (i) a target protein, (ii) a transmembrane channel and (iii) a motor protein having ATPase function. The basic mechanism involves recruitment of a target protein to Sec translocase, the membrane integrated channel, from where, with the help of a peripheral molecular motor-associated ATPase, the translocation of protein is facilitated across the membrane (Natale et al., 2008).

Mechanism of recruitment of protein to Sec translocase: There are basically two methods by which the target protein reaches the protein channel: cotranslational targeting and post-translational targeting (Fig. 1) (Freudl, 2018).

- Cotranslational targeting: In this mechanism, signal recognition particle (SRP) binds to the nascent protein as it emerges out of ribosome, and this ternary complex is targeted to the Sec translocase. The Sec translocase has SRP receptors (SR or

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_8

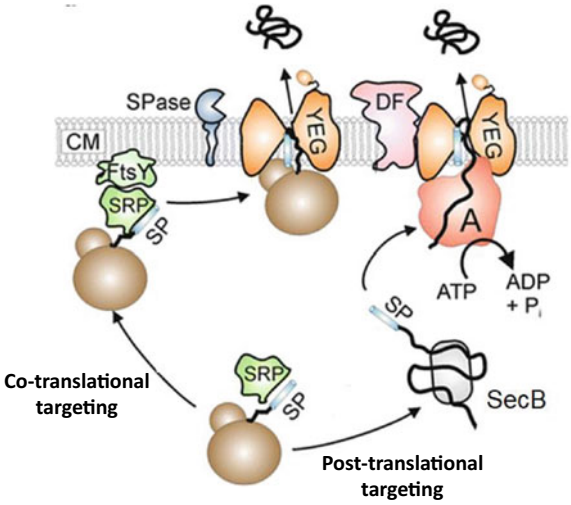


Fig. 1 Sec transport system showing cotranslational and post-translational targeting of unfolded protein; Spase is the signal peptidase, SP is the signal peptide, FtsY is SRP receptor, SRP is the signal recognition particle, YEG is Sec translocator, A is the SecA peripheral ATPase, SecB is the secretion specific chaperone. *Source* Freudl (2018). (Creative Commons Attribution License)

FtsY), and through these receptors, the target protein is transported from SRP to Sec translocase. Majority of membrane-bound proteins are secreted by this pathway, and only, a few proteins are exported to the extracellular environment using this method. However, in endoplasmic reticulum of eukaryotes, all secretory proteins are targeted by this mechanism.

- Post-translational targeting: This involves a secretion-specific chaperone SecB which binds to protein that is released from the ribosome. SecB maintains unfolded state of the protein which can be translocated through Sec translocase.

Sec signal peptide: The structures and functions of signal sequences are conserved and are around 20 amino acids in length divided into n-region, h-region and c-region. N-region is generally composed of positively charged amino acids followed by h-region which is formed of hydrophobic amino acids and a polar carboxy c-region which has a cleavage site for Type I peptidase which cleaves the protein as it is transported across the membrane or just after translocation. The hydrophobic core forms the alpha helix, while the charged n-region maintains electrostatic interaction with phospholipids. Both n and h regions are recognized by SRP elements.

Sec translocase: The transmembrane channel, also known as protein conducting channel (PCC), consists of three integral membrane proteins, SecY, SecE and SecG, and one peripherally associated protein, SecA that functions as ATPase. Sec translocase functions similar to an ABC transporter.

Mechanism of translocation: Peripherally associated SecA accepts protein either from SecB or ribosome SRP complex and pushes unfolded protein through the Sec translocase channel, and it is finally exported as a result of the energy released from ATP hydrolysis and proton motive force (pmf).

2 Tat System for Transport of Folded Proteins

Tat system translocate proteins which have already attained structure after translation. This is important for protein which requires cofactors assembly prior to translocation. However, it is not the only reason for Tat-dependent transport as there are proteins which acquire cofactor after translocation, for example, copper–sulfur cluster-containing nitrous oxide reductase. The Tat translocase is also consisted of two or more membrane-integrated proteins TatA and TatC or TatA, TatB and TatC. Energy for translocation is obtained from proton motive force. There are a large set of bacterial proteins which are cofactor independent but are transported by Tat system. Moreover, many hetero-dimeric proteins require Tat system to get translocated outside the cell membrane because only one of the subunits contains the signal peptide and other subunit(s) must be carried together. Thus, here, folding of the protein is essential so that the protein subunits can associate with each other and translocated simultaneously (Freudl, 2018; Natale et al., 2008).

Tat translocase: Translocation of unfolded proteins by Sec translocase system requires an approximately 12 Å wide channel which can be provided by an aqueous channel. By contrast, translocation of folded proteins requires a channel of diameter ranging from 20 to 70 Å. Thus, Tat system is highly distinct and unique from other protein transporters. In *E. coli* and plant chloroplast, three-component TatABC system exists wherein Tat B and C form an integral membrane complex consisting of multiple copies of each protein. TatB is present as a tetrameric unit with one transmembrane spanning helix, while TatC is a dimer with six transmembrane helices. TatA, present as dispersed protomers when not involved in translocation, contains one transmembrane helix (TMH) followed by a basic amphipathic helix (APH) and a highly charged unstructured C-terminal tail. TMH and APH together form an L-shaped arrangement because of the presence of a glycine residue at their junction which functions as a helix breaker (Green & Mecsas, 2016).

Tat signal peptide: Tat signal has similar topology as Sec signal; however, it is generally composed of 40 amino acids with larger n-region. Also, it has two characteristic arginine residues at n- and h-region interface composing a motif defined as Z-R-R-x-ȼ-ȼ where Z is polar and ȼ is hydrophobic residue. H-region is generally 13–20 uncharged residues and is less hydrophilic than Sec signal. C-region functions as Sec avoidance signal and has positively charged amino acid. Due to the presence of twin arginine residues, it is also called ‘twin arginine translocation system’ (Palmer & Berks, 2012). The differences between Sec and Tat secretion systems is presented in Table 1.

Table 1 Comparison of Sec and Tat secretion systems

Sec system	Tat system
Translocation of unfolded protein	Translocation of folded protein
Signal is smaller of around 20 a.a	Signal is around 40 a.a. long with twin arginine at n- and h-region interface
Channel for Sec system is narrow	Size of channel varies depending upon the dimension of folded polypeptide
Translocation is a multistep event	It is a single-step event

Folding of Tat substrate: Tat substrates must fold before they are targeted to translocase, and the system must ensure that there is no mis-targeting to Sec translocase or pre-mature targeting to Tat translocase. Generally, common chaperones like DnaK and SlyD interact with wide range of Tat signals, and they associate with the polypeptide immediately upon its translation. Also, in the absence of these chaperones, many more similar chaperones can function. However, for cofactor containing proteins, specific chaperones are required. Association of chaperones protects the polypeptide in several ways, (i) protect against pre-mature translocation, (ii) protect degradation of Tat signal before recognition by Tat system, (iii) stabilizes apo-protein when cofactor is limiting, (iv) prevent translocation before insertion of cofactor and folding, leading to oligomerization of substrate protein, also known as 'proof-reading.'

Although general chaperones are sufficient for protein folding, however, there may be involvement of additional quality control steps to ensure that only folded protein are translocated.

Mechanism of translocation: TatBC complex binds to the substrate protein in an energy-independent manner with twin arginine interacting with TatC and passenger domain forming close association with TatB. Further, with the help of proton motive force (pmf), substrate gets more tightly associated with TatBC complex and results in recruitment and polymerization of TatA protomers. Now, this TatABC substrate complex flips toward the periplasmic side with N-terminal of signal peptide facing the cytoplasm and its C-terminal facing the periplasm for the action of signal peptidases. After cleavage, the signal peptide is possibly released to the periplasm (Fig. 2).

Box 1: Signal peptidases

Independent of the type of translocation machinery, signal peptides of the translocated proteins are cleaved by two types of signal peptidases present at the external side of the cell membrane. **Type I signal peptidases** recognize the signature sequence $A_{-3}X_{-2}A_{-1}$ where alanine residue at -1 position is highly crucial for its recognition while **Type II signal peptidases**, called as lipoprotein signal peptidases, recognize and cleave the consensus sequence $L_{-3}(A/S/T)_{-2}(G/A)_{-1}C_{+1}$ where cysteine residue at $+1$ position is highly conserved and is lipidated prior to cleavage (Paetzel et al., 2002).

3 Protein Secretion in Gram-Negative Bacteria

The transport of proteins requires passage through cytoplasmic membrane in all bacteria, but in gram-negative bacteria, proteins need to additionally cross the periplasmic space before they cross the outer membrane. Sec or Tat translocation systems assist the substrate protein to cross the inner membrane (or cytoplasmic membrane) from cytoplasm. Thus, in gram-positive bacteria, protein is directly exported outside, while gram-negative bacteria require one more step (or molecular machinery) to translocate periplasmic located proteins to the extracellular medium.

Gram-negative bacteria possess different types of molecular machineries to facilitate translocation of proteins or effector molecules from periplasm to outside of the cell. These systems are known as Sec-dependent secretion systems and are named as Type II, V, VIII and IX secretion systems. Moreover, gram-negative bacteria are also able to translocate proteins or effector molecules directly from the cytoplasm of the cell to the extracellular medium in a single step without the need of Sec or Tat pathway. These systems are known as Sec-independent secretion systems and are named as Type I, III, IV and VI secretion systems (Fig. 3) (Bocian-Ostrzycka et al., 2017).

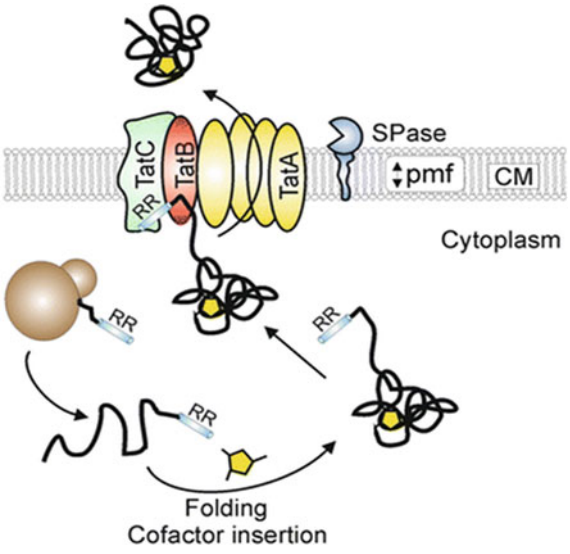


Fig. 2 Tat transport system for the secretion of folded proteins (RR refers to twin arginine). *Source* Freudl (2018). (Creative Commons Attribution License)

4 Sec-dependent Secretion Systems of Gram-Negative Bacteria

Type II secretion system (T2SS)—It is a conserved secretion system in gram-negative bacteria wherein proteins are transported from the periplasmic space to the extracellular environment. The proteins secreted through this system have varied biological functions but are generally nutritionally important hydrolytic enzymes such as lipases and proteases. These exoproteins may be mono- or oligomeric. Bacterial pathogens also utilize the T2SS for transport of virulence factors such as cholera toxin of *V. cholerae*.

This system is coupled with the Sec or Tat secretion pathways which deliver the proteins to the periplasm. Naturally, a Sec- or Tat-cleavable signal at the N-terminus becomes a prerequisite for protein transport through this system. Another precondition for transport through T2SS is protein folding. Since this system can transport only folded proteins, unfolded proteins crossing the cytoplasmic membrane by Sec pathway must be folded in the periplasm in order to be secreted.

T2SS is composed of 12–15 proteins present as four different sub-assemblies both in inner and outer membranes. These are outer membrane complex, inner membrane platform, secretion ATPase and pseudopilus (von Tils et al., 2012).

Outer membrane complex: This is a homo-multimeric protein channel of 12–15 subunits composed of a protein called secretin Gsp-D. These subunits are assembled to form an empty-ring type β-barrel structure having a central cavity of 50–80 Å spanning the outer membrane (OM). The cavity consists of a C-terminus facing the outside environment, a transmembrane domain embedded in the OM and an N-terminus

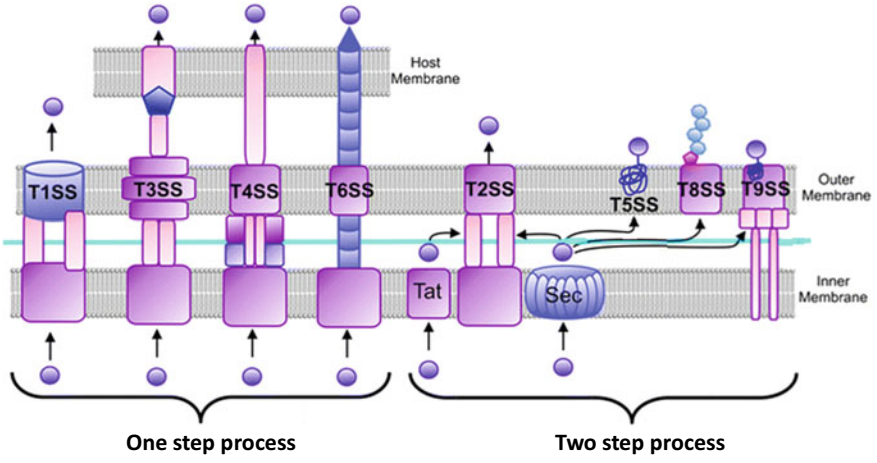


Fig. 3 Sec-dependent and Sec-independent secretion systems in gram-negative bacteria. *Source* Bocian-Ostrzycka et al. (2017). (Creative Commons Attribution License)

toward the periplasm. The N-terminus interacts with Gsp-C of the inner membrane platform to make a connecting channel for secretion of proteins extracellularly.

Inner membrane platform: It consists of Gsp proteins that span the inner membrane. Four proteins make up this platform: Gsp-C, Gsp-F, Gsp-L and Gsp-M.

- Gsp-C: This is a self-assembling homodimeric bitopic (single-pass) inner membrane protein. It interacts at its C-terminus with the N-terminus of Gsp-D to form a connecting channel through which the secretory proteins pass out of the cell. The contact of Gsp-C and Gsp-D causes the secretion gate to open and pump the secretory proteins out of the cell. This interaction also determines the specificity of secretion.
- Gsp-F: This is a polytopic (multipass) integral membrane protein having three transmembrane regions, two large cytoplasmic domains and a small periplasmic loop. The N-terminus of Gsp-F interacts with Gsp-L and may be involved in binding the secretion ATPase and providing stability to the secretion complex.
- Gsp-L: This is a bitopic inner membrane protein having an N-terminal cytoplasmic domain, a transmembrane domain, and a C-terminal periplasmic domain. Gsp-L provides stability to the secretion complex and also interacts with Gsp-E, the ATPase of the system.
- Gsp-M: This is a dimeric bitopic protein having a short N-terminal cytoplasmic domain, a transmembrane domain in the cytoplasmic membrane and a C-terminal periplasmic domain. When associated together, Gsp-C, Gsp-L and Gsp-M stabilize each other and protect each other from proteolysis.

Secretion ATPase: This is the Gsp-E ATPase which is closely associated with the inner membrane platform on the cytoplasmic side of the plasma membrane. It belongs to the traffic ATPase superfamily that functions as distinct dynamic hexamers that have two nucleotide-binding motifs. Additionally, it has three conserved regions: the Asp Box, the His Box and the tetracysteine motif. Gsp-E lacks any hydrophobic or transmembrane domains. Interaction with Gsp-L associates Gsp-E with the cytoplasmic membrane. The interaction between Gsp-L and Gsp-E stimulates oligomerization of Gsp-E which then curtails ATPase activity.

Pseudopilus: It is a pilus-like structure composed of Gsp-G, Gsp-H, Gsp-I, Gsp-J and Gsp-K which are its constitutive elements and Gsp-O which is a prepilin peptidase involved in their maturation. The mature pseudopilins are bitopic periplasmic proteins that have a conserved N-terminal hydrophobic domain and a periplasmic C-terminal globular domain.

Gsp-G is the most abundant and is therefore called the major pseudopilin. It self-assembles and protrudes out of the cell as a long fibrillar structure called a hyper-pseudopilus (HPP). Gsp-H, Gsp-I, Gsp-J and Gsp-K are minor pseudopilins. Gsp-I initiates fiber formation, Gsp-K helps in pseudopilus elongation and interaction with Gsp-G, and Gsp-H provides the hinge between the tip and the core of the pseudopilus.

The pseudopilus is formed upon polymerization of individual mature pseudopilins into a right-handed helix consistent with the Type IV pilus structure.

The secretion ATPase Gsp-E assembles and disassembles these pseudopilus subunits. Retraction of the pseudopilus allows secretory proteins to enter the system while its extension forces them out through the Gsp-D channel.

Mechanism of transport by T2SS: T2SS substrate proteins are transported to periplasm via the Sec or Tat system. Proteins transported by the Sec pathway are then folded in the periplasm and remain there until secretion by T2SS. Pre-pseudopilins are translocated to the periplasm via the Sec pathway. Gsp-O cleaves and converts the pre-pseudopilins into mature pseudopilins. The secretion ATPase Gsp-E provides energy for the formation of the pseudopilus. Gsp-C and Gsp-D interact to form a connecting channel through the inner and outer membranes. Retraction of the pseudopilus lets secretory proteins enter the system followed by extension which pushes the secretory proteins out through secretin Gsp-D into the extracellular environment like a piston (von Tils et al., 2012) (Fig. 4).

Type V secretion system (T5SS)—It is another Sec-dependant system wherein either membrane-integrated proteins are transported to the bacterial outer membrane or secreted proteins are transported to the extracellular milieu. All members of this group contain a signal peptide which is present at the N-terminus and mediates the export of the protein across the cytoplasm via the Sec pathway. This transport is energy-independent and uses free energy of protein folding as the sole energy source. Owing to the fact that extrinsic factors are not required for transport, T5SS presents itself as a potential tool for secretion of biotechnologically important proteins or enzymes. T5SS can be sub-divided into five types (5a-e) based on organization of different domains, size of translocator protein, direction of transport and requirement

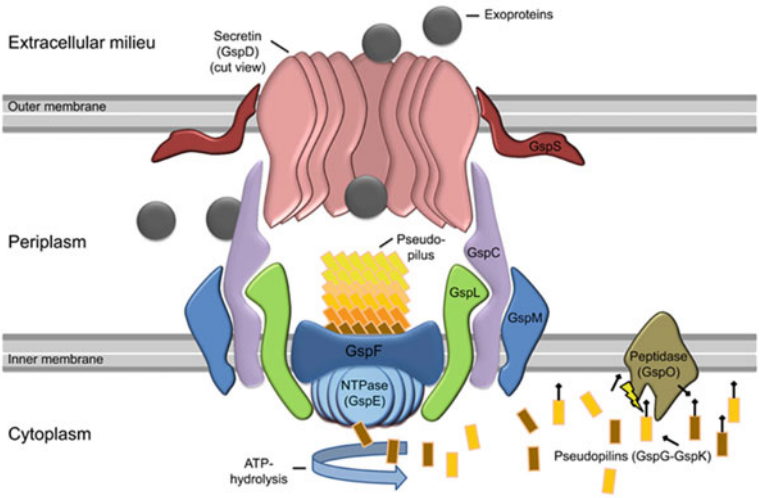


Fig. 4 Type II secretion system. *Source* von Tils et al. (2012). (Creative Commons Attribution License)

Table 2 Distinguishing features of various Type V secretion systems

Feature	T5aSS	T5bSS	T5cSS	T5dSS	T5eSS
Location of three domains or secretion peptide	Contains the signal peptide, translocator and passenger all in a single peptide	The translocator and passenger domains are separate peptides, each with its own signal	The three domains are on a single peptide, but three different strands coil together to make up the passenger and translocator domain	Contains the signal peptide, translocator and passenger all in a single peptide	Contains the signal peptide, translocator and passenger all in a single peptide
Translocator protein	Translocator is made of 14 antiparallel β-strands	The translocator is made up of 16 antiparallel β-strands	The translocator is made up of 12 antiparallel β-strands, four each coming from the three peptide chains	The translocator is made up of 16 antiparallel β-strands	Translocator is made of 14 antiparallel β-strands
Direction of transport	C- to N-terminus	C- to N-terminus	C- to N-terminus	C- to N-terminus	N-to C-terminus
Translocator folding domain	Can require BamA for initiation of translocator folding	Contains an intrinsic POTRA domain in place of BamA	Requires BamA for initiation of translocator folding	Contains an intrinsic POTRA domain in place of BamA	Can require BamA for initiation of translocator folding

of extrinsic factors as they all have translocator folding domains (Table 2 and Fig. 5) (Leo et al. 2012).

Type 5a secretion system (T5aSS) is also known as the autotransporter system. The amino acid sequence contains all the information needed for transport, and they are therefore independently transferred across the outer membrane. These are present in a wide range of gram-negative bacteria where they play essential roles such as host colonization factors, invasion proteins, toxins and adherence proteins, e.g. pertactin from *Bordetella pertussis*, Hap protein from *Haemophilus influenzae* and antigen 43 from *Escherichia coli.* There are three structural motifs in an autotransporter protein: a translocator domain, a signal peptide and a passenger domain.

Type 5b secretion system (T5bSS) is also known as two-partner system (TPS). Secretion by this system is similar to the autotransporter system except that the passenger domain (TpsA) and the translocator domains (TpsB) are located on separate polypeptide chains encoded by a single operon. Like the autotransporters, these are also found in gram-negative bacteria and have a role in cell adhesion and pathogenesis such as

hemagglutinin (FHA) of *Bordetella pertussis* and adhesins (HMW1 and HMW2) from *Hemophilus influenza.*

Type 5c secretion system (T5cSS) is also known as the oligomeric coiled-coil (Oca) system or the trimeric autotransporter adhesins (TAAs) and consists of trimeric autotransporters. These proteins are usually adhesins (cell surface anchorage proteins) that are not cleaved after the transport, e.g. YadA of *Yersinia enterocolitica* and SadA of *Salmonella.*

Type 5d secretion system (T5dSS) links Type Va and Vb secretion systems. It consists of a single peptide the passenger and β-barrel translocator domain connected with a POTRA domain. This system has only been discovered recently, and the phospholipase PlpD of *Pseudomonas aeruginosa* is the only identified member of this group till date.

Type 5e secretion system (T5eSS) differs from T5aSS only by the direction of transport. While the classical autotransporters transport the passenger from C- to N-terminus, T5eSS transports passenger in the opposite direction that is from N- to C-terminus, e.g. intimin of *E. coli* and invasin of *Yersinia* spp.

Type VIII secretion system (T8SS)—It is a Sec-dependent secretion system that specifically transports short bristle-like fibers to the surface of bacteria. In pathogenic bacteria, the tips of these fibers contain adhesin that helps attach the bacterial cell to their host. These differ from both pili and flagella in structure and function, and a bacterial cell may have a 1000 of them on their surface. These unique classes of extracellular fibers are called 'curli' in *E. coli* and aggregative fimbriae in *Salmonella.* These are often confused with amyloid fibers which are disease-associated prion

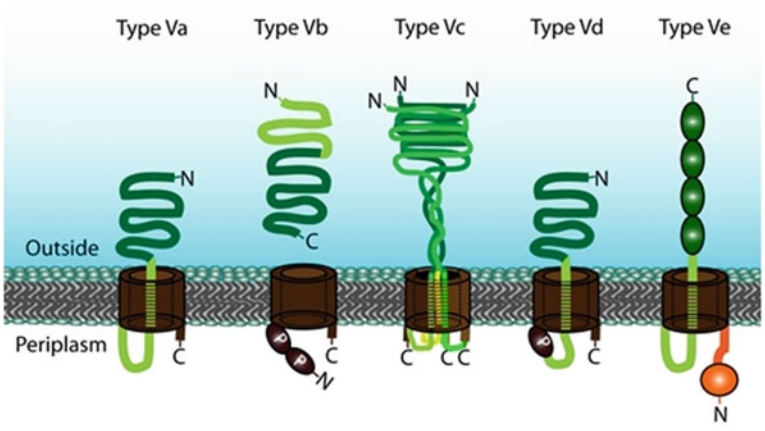


Fig. 5 Topology models of the different Type V secretion systems. The translocation domain is displayed in brown, linker/Tps regions in light green, passenger domains in dark green and periplasmic domains in orange. POTRA domains are labeled (P). For clarification of the topology, N- and C-termini are indicated. *Source* Leo et al. (2012). (Creative Commons Attribution License)

proteins. However, unlike amyloid fibers which are formed due to protein misfolding, these curli fibers are presented by the dedicated Type VIII secretory pathway. In *E.coli*, the curli phenotype is important for biofilm formation and host cell adhesion (Evans & Chapman, 2014).

Type VIII secretion system in *E. coli* is coded by two operons: one of them (csgBAC) codes for structural genes and the other (csgDEFG) codes for transport proteins (Fig. 6).

Structural proteins—csgBAC operon codes for three structural genes.

- CsgA: This is the major structural protein that forms the amyloidogenic core of the protein. It contains a 22-amino acid long N-terminal signal sequence which is recognized by GscG for transport.
- CsgB: This is the minor fiber protein which nucleates polymerization of CsgA.
- There is no reported role for the third gene *csgC* which is present on the csgBAC operon. It is not clear whether it actually codes for a protein or is simply redundant.

Transport proteins

- CsgD: This is a transcriptional factor which regulates the synthesis and secretion of fibers by T8SS.
- CsgG: This forms the barrel-shaped translocator pore in the OM.
- CsgE: This is a periplasmic protein which can be thought of as a modulator protein that directly binds CsgA and prevents its pre-polymerization and also controls the specificity and pore-like properties of CsgG.
- CsgF: This is a surface-exposed protein which is required for CsgB-mediated nucleation of CsgA.

Mechanism of transport and fiber formation—In response to specific signals, the transcription factor CsgD up-regulates the synthesis of structural and transport-associated T8SS proteins. The Sec pathway then delivers these proteins to the periplasm. CsgG assembles into a pore in the outer membrane, and CsgE binds both CsgA and CsgG. CsgA and CsgB are secreted as soluble monomeric proteins in assembly ready conformation. Upon reaching the extracellular milieu, with the help of CsgF, the CsgB protein forms a nucleation core and CsgA subunits assemble on it forming the curly phenotype.

Regulation of T8SS—It is seen that the exhibition of curli phenotype is higher at 30 °C, low salt concentrations, nutrient limitation and microaerophilic conditions. CsgD is the central protein for regulation of curli formation. It has an N-terminal receiver domain and a C-terminal DNA-binding domain. The expression of csgD is regulated in multifarious ways.

The stationary phase sigma factor RpoS interacts with Crl or MlrA, and the positive cooperation of the two regulates transcription of csgD. Crl is maximally stable below 30 °C, and therefore, the curli phenotype is seen at lower temperatures.

Apart from RpoS, there are three two-component systems that regulate csgD expression:

- The OmpR/EnvZ system that responds to changes in osmolarity
- The CpxA/R system that responds to stresses at the cell envelope including misfolding of periplasmic proteins
- The Rcs pathway that responds to membrane stress.

The Rcs pathway is also involved in capsule formation and biofilm generation. The curli phenotype is thereby coupled to formation of biofilms.

Type IX secretion system (T9SS)—This is involved in the secretion of some adhesins, some hydrolytic enzymes and apparatus responsible for gliding motility in the Chlorobi– Fibrobacteres–Bacteroidetes group (CFB group). The proteins secreted by this system usually remain anchored to the cell surface. It is notable that the gene sequences of the essential components of T9SS are unique to the CFB group. T9SS substrates contain an N-terminal Sec signal and an 80 amino-acid long C-terminal signal for transport through the OM. These substrates are usually modified by attachment to membrane glycans or glycolipids such as the LPS, e.g. porK-N (*Porphyromonas gingivalis*) and gldK-N (*Flavobacterium johnsoniae*) (Glew et al., 2017).

Discovery of Type IX Secretion System—This was discovered in *Porphyromonas gingivalis*, a gram-negative, non-motile, anaerobic bacterium which is the causative agent of periodontitis. It produces a potent proteolytic enzyme called gingipain, an essential virulence factor that counters host defense mechanism. Gingipains

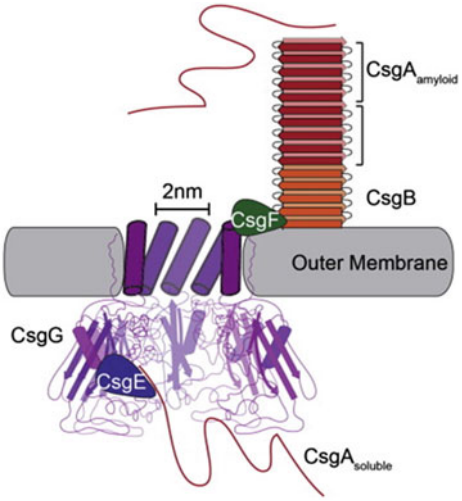


Fig. 6 Type VIII secretion system (the curli biogenesis system): CsgA is the major structural protein that forms the amyloidogenic core of the protein; CsgB is the minor fiber protein that nucleates polymerization of CsgA; CsgF allows CsgB-mediated nucleation of CsgA; CsgG forms barrel-shaped translocator pore in the OM; CsgE is a modulator protein that directly binds CsgA and prevents its pre-polymerization. *Source* Evans and Chapman (2014). With kind permission from Elsevier

are secreted abundantly and are mainly attached onto the outer membrane surface but are also released into the extracellular milieu. Gingipain is known to bind heme/hemoglobin, thereby allowing its acquisition. As a result, *P. gingivalis* forms black-pigmented colonies on blood agar plate. However, white/beige mutants could occasionally be observed. Such spontaneous mutant phenotype was associated with defective surface expression of gingipain. High-throughput transposon mutagenesis led to the identification of *porT*, the first gene implicated in encoding a protein involved in gingipain secretion. Gingipains were accumulated as inactive proenzymes in the periplasm due to defective secretion system in this mutant. Since this system was discovered in a porphyrin-negative or colorless mutant of *P. gingivalis*, the proteins of the system have been given the prefix 'por' indicating porphyrin (Lasica et al., 2017). Moreover, genome analysis revealed that all the genes of the secretory system of *P. gingivalis* were new. These genes could only be found in *Bacteroidetes* phylum such as *P. gingivalis* itself, *Cytophaga hutchinsonii* and *Prevotella intermedia.*

Structural and functional components

- Cytoplasmic and IM components: The proteins PorX and PorY are involved in a two-component system that regulates the expression of T9SS genes. The inner membrane proteins PorL and PorM form an energy transducer complex that uses proton motif force to power the assembly of T9SS and export of substrate proteins.
- Periplasmic components: Three lipoproteins, PorK, PorW and PG1058, along with the protein PorN form the periplasmic structural components of T9SS. PorN-PorK form a ring-shaped structure that is anchored into the OM. PorN also stabilizes the PorL-PorM complex.
- OM and surface components: Majority of T9SS components are located at the OM. These include PorP, PorQ, PorT, PorU, PorV, ProZ, PG0534, PG0189 and Sov. PorQ, PorU, PorV and PorZ which form an attachment complex on the cell surface. PorV is used as a shuttle that delivers the substrates to the attachment complex. PorU is a sortase that cleaves the C-terminal signal of the substrate and also facilitates its attachment to the membrane via its transpeptidase activity (Fig. 7).

Mechanism of secretion—T9SS is regulated at the transcriptional level by PorX-PorY two-component system. PorY is the sensor kinase that gets activated by unidentified signals and autophosphorylates at His193. The phosphate moiety is then transferred to Asp58 in the receiver domain of the response regulator, PorX. PorX interacts with the sigma factor SigP to regulate the transcription of T9SS genes and with the cytoplasmic domain of PorL to modulate the overall structural architecture of T9SS.

Substrates are delivered to the periplasm by the Sec pathway in an unfolded state. After cleavage of the N-terminal signal peptide, PorV brings the substrate to the attachment complex, and PorU attaches the substrate to membrane glycans or glycolipids while cleaving the C-terminal signal. Hence, the substrate remains tethered to the outer surface of the cell. The energy of transport comes from proton motif force, and the IM components PorL and PorM are involved in energy transduction.

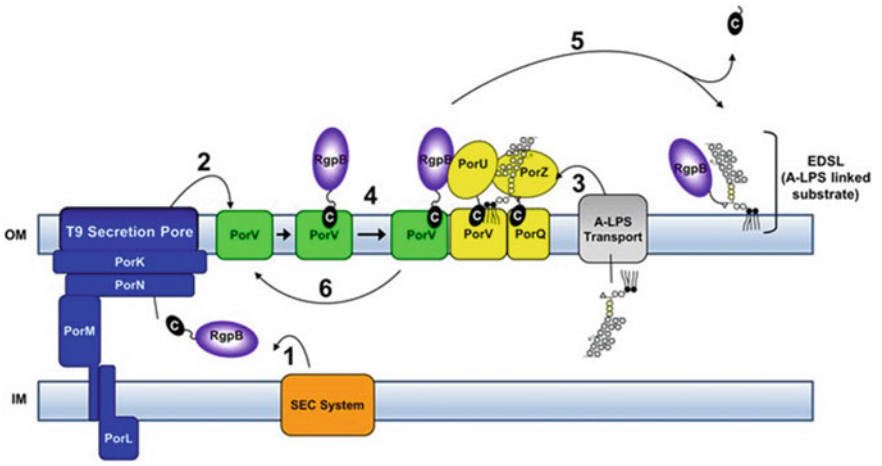


Fig. 7 Model of Type IX Secretion: T9SS substrates have a conserved C-terminal domain (C) that directs their secretion across the outer membrane (OM) via periplasm. *Source* Glew et al. (2017). (Creative Commons Attribution License)

5 Sec-independent Secretion Systems of Gram-Negative Bacteria

Type I secretion system (T1SS)—It is the simplest system which functions like ABC transporter and is present in gram-negative bacteria. It allows the transport of unfolded proteins/substrate directly from cytoplasm to extracellular medium in a single step. T1SS substrates are Sec-independent and mostly contain a signal peptide at C-terminal which can be recognized by T1SS and is not cleaved during secretion (Delepelaire, 2004).

Machinery and mechanism of T1SS—T1SS is composed of three proteins:

- ATP-binding cassette protein (ABC) comprising of a nucleotide-binding domain (NBD) and a transmembrane domain (TMD). It is localized in the cytoplasmic membrane and is responsible for specific recognition of signal peptide of its substrate and catalyzes ATP to provide energy to transport the substrate.
- Membrane fusion protein (MFP) provides a link between inner and outer membranes of the cell as it spans through the periplasmic space and interacts with ABC protein in the cytoplasmic membrane and OMP in the outer membrane during protein secretion. Its N-terminus, located in the cytoplasmic membrane, is also believed to play some role in substrate selection.
- Outer membrane protein (OMP) is a TolC family protein which forms a water-filled channel throughout the outer membrane. This channel is broad toward outside and constricted at its periplasmic side. The pore generated by OMP helps in translocation of unfolded protein to the extracellular medium.

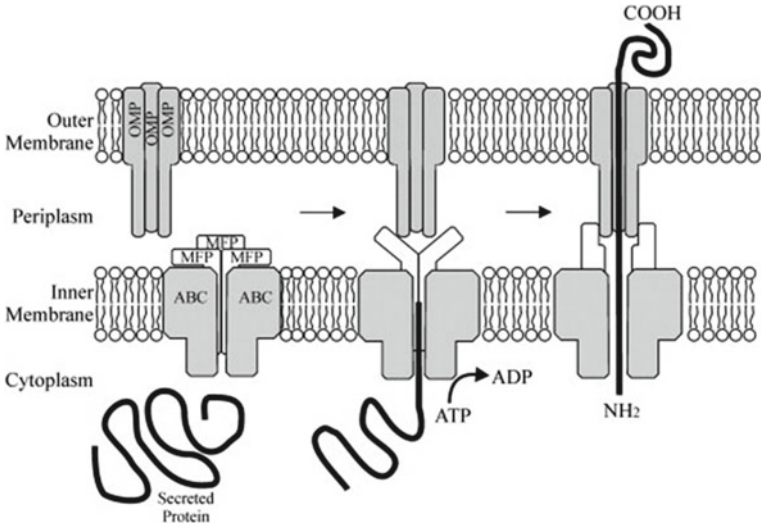


Fig. 8 Type I secretion system. ABC: transmembrane ATP-binding protein; MFP: membrane fusion protein; and OMP: outer membrane protein. *Source* Wells & Henderson (2013). With kind permission from Elsevier

Interaction of substrate signal sequence with ABC protein initiates a series of interaction between various components of T1SS system which leads to protein secretion directly to the extracellular medium in unfolded state (Fig. 8) (Lenders et al., 2013; Wells & Henderson, 2013).

Protein features—Proteins that are secreted through T1SS system have some peculiar features such as (i) they contain signal peptide sequence at their C-terminus except in bacteriocins where it is at N-terminus; (ii) have distinctive glycine rich repeats that specifically bind calcium ions, as calcium-binding RTX motifs prevent pre-mature protein folding inside the cell due to low calcium concentration; however, some substrates also contain different kinds of repeats having homology with adhesion molecules; (iii) contain very few or no cysteine residue; and (iv) are very acidic in nature with *pI* close to 4. The protein substrates secreted via the T1SS can be as small as <10KDa such as bacteriocins to gigantic ones such as adhesins (Linhartová et al., 2010; Guo et al., 2017). Some of the proteins which are secreted through T1SS system are alpha-hemolysine of *E. coli* (HylA), metalloproteases of *Erwinia chrysanthemi*, lipases and HasA hemophores of *Serratia marcescens.*

Alpha hemolysin (HylA) protein secretion is the first and best described example of T1SS system. *E. coli* hemolysis toxin HylA is encoded by hylCABD operon. Three components HylB, HylD and TolC form the T1SS machinery. Of these, HylB and HylD are specific for this protein, while TolC is a multifunctional outer membrane protein-coded separately.

HylC is an acyltransferase that acylates lysine residues that results in hemolysin activation. This acylation occurs post-translationally and does not affect hemolysin

secretion. HylB belongs to ABC transporter family and couples ATP hydrolysis with export of toxin. HylD is a membrane fusion protein anchored in the cytoplasmic membrane. TolC is a trimeric outer membrane protein that forms a transperiplasmic channel with tunnel domain projecting toward periplasmic space. The hemolysin protein HylA carries a carboxy terminal signal sequence, and thus, the secretion commences from C-terminus to N-terminus (Fig. 8).

Mechanism of export of hemolysin A:

1. HylA interacts with HylB-HylD complex on the cytoplasmic side through its C-terminal signal sequence.
2. After this interaction, HylD induces interaction with TolC.
3. As masses of HylD trimer are similar to that of TolC, this interaction results in the formation of a cylindrical structure of identical diameter.
4. Hemolysin A is then moved from HylD to TolC through a continuous tunnel.
5. Transport of hemolysin A is then accomplished as a result of energy released during ATP hydrolysis.

Box 2: Live attenuated bacterial vaccines

Attenuated live vaccines can be prepared by fusing heterologous antigens to the C-terminal of alpha hemolysin (HylA), and these can be secreted via Type I systems. These antigens can be used to generate polyclonal or monoclonal antibodies. Apart from the advantage of direct extracellular secretion of heterologous antigens, there is no size limitation for the antigen. Thus, even larger heterologous antigens can be produced using this system (Gentschev et al., 2002).

Type III secretion system (T3SS)—It is used for direct delivery of effector molecules into eukaryotic host cells including those of plants, animals and protists. This secretion system is present both in symbiotic and pathogenic bacteria wherein the effector molecules are delivered into the target and alter a wide range of metabolic pathways and cellular functions. The system is well recognized as nano-machinery or nano-syringes called 'injectisomes' due to their close functional similarity to a needle and syringe. These syringes form channels through the cell membranes of bacteria and host such that the effector proteins can be directly injected into the host cytoplasm.

Components of the T3SS may be present on virulence plasmids or in the genome. In both cases, they are a part of an operon along with effector protein molecules, their chaperones and transcription factors. Though the secretory proteins are species specific, the secretory system is well conserved among species. T3SS is evolutionarily related to flagella and has more than 24 types of proteins. The major components of the T3SS comprise a syringe-like array of proteins which span through double

membrane along with a needle-like structure protruding outside, which establishes contact with the host (Deng et al., 2017).

Structural composition of the T3SS—T3SS comprises numerous substructures, including a basal body, an inner membrane export apparatus, a cytoplasmic ring (C-ring), a cytosolic ATPase complex and a translocation pore.

Substrate recognition—All T3SS secretory proteins have a secretion signal of around 20 amino acid residues at their N-terminus. These signals are not well conserved but are typically enriched for proline, threonine, serine and isoleucine. Recently, it has been shown that secondary structures in the mRNAs of certain *Yersinia* spp. effector proteins can drive cotranslation and secretion through the T3SS. It has also been shown that some effector proteins in *Salmonella* spp. employ RNA complexed with the RNA-binding chaperone Hfq as a secretion signal. Thus, the T3SS may employ a divergent peptide signal, an RNA, an RNA-chaperone complex or two or more of these combined for substrate recognition.

Host cell recognition and delivery of effecter molecules—Transcription of T3SS is regulated by many environmental and host-derived signals. These include pH, temperature, availability of oxygen and chemical signals from the host.

The limited pore size of the needle makes protein unfolding prior to secretion indispensable. Multi-cargo chaperones bind to the substrates in the cytoplasm, and these complexes are targeted to ATPase. The ATPase recognizes signal in the substrate as well as the chaperone. Substrate unfolding and release of chaperone occur in a single step using energy from ATP hydrolysis. Further, efficient secretion also requires proton motive force for propelling the needle filament proteins for delivery of the substrate directly into the host cytoplasm (Fig. 9).

Type IV Secretion System (T4SS)—It is a diverse group involved in transporting a variety of substrates peculiarly DNA as nucleoprotein complex and in secretion of protein effector molecules. They are present in both gram-negative and gram-positive bacteria and in some archaeal groups. These systems are involved in genetic transfer via conjugation allowing dissemination of genetic elements such as antibiotic resistance cassette among bacterial populations. T4SSs evolved for conjugative transport of genetic material and later adapted other functions such as transport of effector proteins such as toxins required in establishing bacterial pathogenicity.

These systems are Sec-independent and, like other transport systems, rely on ATPases like VirB4 that energize conformational changes in the transport machinery. Besides VirB4, these systems comprise two other major proteins—coupling protein T4CP and relaxases. T4CP such as VirD4 of the VirB/D4 system of *Agrobacterium tumefaciens* is responsible for recruiting substrates to the transport machinery while relaxases form a stable nucleoprotein complex with the ssDNA allowing subsequent delivery by the coupling protein.

These systems can be functionally characterized into three major groups: conjugative, effector translocators and DNA release/uptake systems.

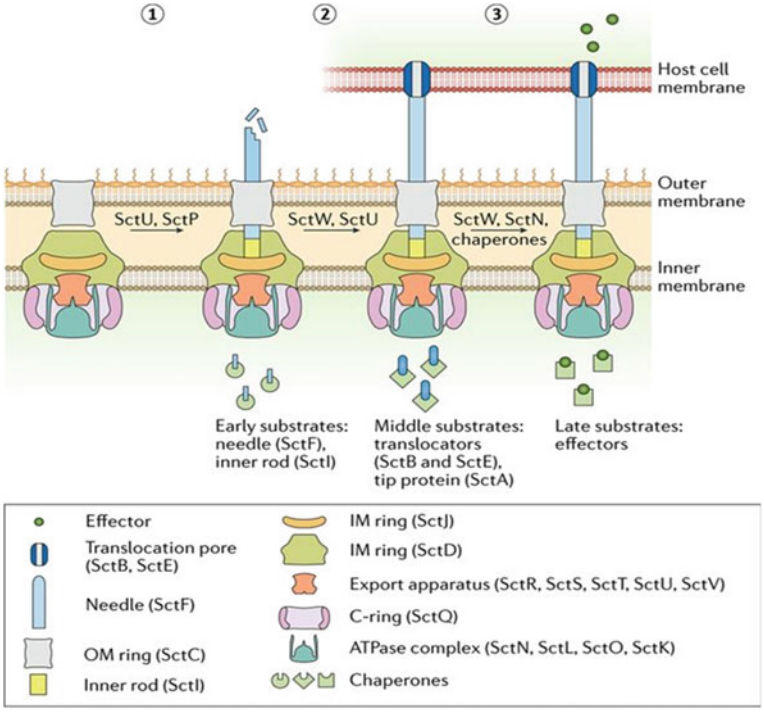


Fig. 9 Type III secretion system assembly and mechanism of transport. *Source* Deng et al. (2017). With kind permission from Springer Nature

- Conjugation machinery: These are coded on self-transmissible plasmids that also harbor other genes for selective advantage such as antibiotic resistance cassettes, metabolic or virulence gene or by transposons integrated in the chromosome. These elements code for T4SSs and its machinery including excisionase and integrase. It begins with DNA substrate processing when proteins assemble at the origin-of-transfer (oriT). This complex, termed relaxosome, is processed further by nicking and unwinding of dsDNA. The relaxase binds at 5‘ end of OriT, and this complex is targeted to the transport machinery by VirD4 coupling protein homologs. The ssDNA is then secreted into the recipient cell. In case of integrative transposons, there are recombinase–excisionase system that excises transposable elements into circular intermediates which are later secreted like the self-transmissible plasmids. Inside the recipient cell, homologous recombination causes integration of these elements into the chromosome.
- DNA release/uptake systems: These systems lack coupling protein, relaxosome and some other components of the T4SSs. They can be exemplified by the ComB system of *Helicobacter pylori* which lacks a relaxase and evolved to import DNA

from the extracellular milieu. On the other hand, *Neisseria gonorrhoeae* gonococcal genetic island (GGI) utilizes T4SS containing a relaxase to allow secretion of DNA directly into the extracellular environment.

- Effector Translocators: These systems are majorly characterized in gram-negative pathogens and are crucial in bacterial pathogenesis. They transfer the effector molecules such as virulence factors into the host cell. Examples include transport of pertussis toxin in *Bordetella pertussis*, CagA oncoprotein in *H. pylori* or VirE2 in *A. tumefaciens.* CagA protein has been visualized at the tip of pilus in *H. pylori* and is possibly injected directly into the host cell. The effector proteins are accompanied by chaperones and are also recruited to the transport machinery by a coupling protein. A notable exception is the transport of pertussis toxin as it lacks a perceptible coupling protein, and the bacteria directly secrete its substrate into the extracellular environment due to the absence of pilus structure (Fig. 10).

Type VI Secretion System (T6SS)—It is characterized as a contractile puncturing nanomachine that intriguingly works like a speargun to release effector proteins directly into a foreign cell. This machinery facilitates transfer of proteins to adjacent cells in a contact-dependent manner. T6SS provides a competitive edge to bacterial cells by enabling their interaction with one another to distinguish between self and foreign strains occupying the same niche. As a consequence, bacteria can selectively form communities by antagonizing non-self species. Another advantage that T6SS impart to virulent bacterial cells is their interaction to eukaryotic cells during pathogenesis.

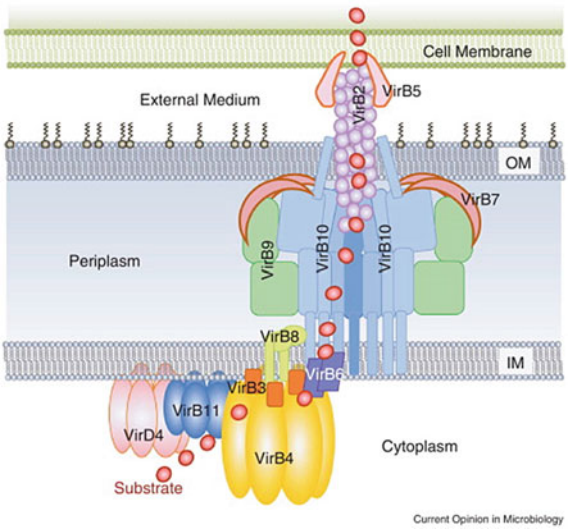


Fig. 10 Type IV secretion system. *Source* Waksman and Orlova (2014). With kind permission from Elsevier

The involvement of T6SS genes in protein secretion was first noticed in a fish pathogen *Edwardsiella tarda* in 2004 while performing proteome analysis of the secreted proteins. However, T6SS was actually discovered in 2006 in *Vibrio cholerae* and named Type VI secretion system (Pukatzki et al., 2006). The genes encoding T6SS have now been identified in almost one-third of gram-negative bacterial genomes sequenced till date. They have widespread occurrence, especially in organisms belonging to Proteobacteria phylum including pathogenic strains such as *E. coli*, *V. cholerae*, *Pseudomonas aeruginosa and Francisella tularensis* and also in symbiotic organisms like *Rhizobium leguminosarum.*

Architecture of T6SS—The structural assembly of T6SS is similar to an inverted bacteriophage tail extending outward through the bacterial cell surface. T6SS apparatus is composed of 13 core proteins that form the basic structure required for its function. The final assembly of T6SS includes three main complexes (Fig. 11) (Cianfanelli et al. 2016).

- Inverted phage tail-like complex: This complex is the main effector protein delivery module and has a dynamic tubular structure. The first component of this complex is a hollow tube formed by assembly of a protein hemolysin-coregulated protein (Hcp) into hexameric stacked rings. The second component is the puncturing device formed at the tip of the hollow tube. It is made up of a spike protein valine-glycine repeat protein G (VgrG) which is capped by a proline alanine alanine arginine (PAAR) domain-containing protein. VgrG protein is generally present as a trimeric structure analogous to the T4 phage tail spike. The third component consists of a contractile sheath that encases the tail tube. It is formed by repeating units of two proteins VipA and VipB (TssB and C). The sheath structure can disassemble coupled with Clp-V-mediated hydrolysis that takes place with the help of cytoplasmic Clp-V ATPase. This enables sheath to undergo multiple rounds of extension and contraction which is contradictory to phage tail which can contract only once.
- Base-plate complex: It is composed of four proteins, viz: TssE, TssF, TssG and TssK. These proteins interact with the tail-like complex in the cytoplasm, and both are recruited to the cell envelope together.
- Envelope spanning complex: This complex has three components: the inner membrane spanning proteins TssL and TssM, and the outer membrane spanning protein TssJ. These proteins span the cell periplasm from inner to outer membrane, thus anchoring the tail complex to the cell membrane.

Mechanism of T6SS—T6SS functions in a contact-dependent manner, and upon sensing a condition that mimics antagonism, the attacker cell secretes certain effector molecules into the periplasmic space of the target cell. The tail tube of the T6SS is a 'tail within a tail structure.' The outer ring or the sheath acts a spring which contracts upon stimulation, propelling the inner tail tube into the periplasm of the recipient foreign cell like a rocket. The inner tube along with puncturing tip only punctures the outer membrane for delivery of effector protein and not the cytoplasmic membrane which resembles the mechanism exhibited by T4 bacteriophage for DNA transfer.

After protein secretion, the contracted sheath is disassembled by the action of ClP-V ATPase, and core proteins are recycled for another round of contraction.

Effector Molecules—Effector molecules released by the attacker cell into the periplasm of the foreign bacteria range from lethal toxins to peptidoglycan-degrading enzymes, lipases that degrade the cell membrane and nucleases among few others. Many effector molecules have been identified and biochemically characterized in different bacterial pathogens. These include:

- VgrG in *V. cholerae:* Although VgrG is present at the tip of the tail forming a structural component, it also has effector function by virtue of its muraminidase or actin-crosslinking activity present in its carboxy terminus domain.
- Tse1, Tse2 and Tse3 in *P. aeruginosa*: Tse1 and Tse3 exhibit lytic activity targeting peptide and sugar portions of the peptidoglycan layer while Tse2 is a toxin.

P. aeruginosa and *V. cholerae* also produce lipases and phospholipases as effector molecules. Since lipids are ubiquitous, lipase effectors can target both eukaryotic and bacterial cells. DNase effectors in *Agrobacterium tumefaciens* exhibit an antibacterial DNase activity.

The attacking bacteria that produce the effector molecule remain unsusceptible to it by synthesizing an immunity protein coded by genes for the T6SS machinery. Immunity gene is present right adjacent to the effector protein coding gene. Therefore, the attacking cell will be able to kill $T6SS^-$ foreign cell only but not the $T6SS^+$ cell as the later will harbor the immunity protein to prevent self-intoxication, thus rendering effector molecule and killing ineffective. Tsi2 is one such cognate immunity protein against Tse2.

T6SSs allow competitive colonization among bacterial population as they provide a mechanism to prevent colonization by another strain or species. In gut commensal *Bacteroides fragilis*, T6SS is utilized by non-toxic strain to competitively eradicate enterotoxigenic *B. fragilis* and establish a stable niche. Another example is the T6SS-mediated intraspecies killing seen in oppositely swarming *Proteus mirabilis* populations where characteristic no-growth Dienes boundaries are formed. A typical example of interbacterial competitive antagonism employing an active T6SS apparatus is the survival of *P. aeruginosa* isolated from cystic fibrosis patients. Similar competition is seen in *Serratia marcescens, Burkholderia thailandensis, V. cholerae and Acinetobacter baumannii.* T6SSs are also up-regulated in monospecies biofilm formation, producing isogenic populations within a mixed community. Intracellular pathogen *Francisella tularensis* employs T6SS to produce certain effector molecules that allow its escape from phagosomes and thus its survival intracellularly in macrophages. However, the mechanism by which these effectors function is still unclear.

Regulation of T6SS—T6SS imparts an advantage to the bacterial survival in its environmental conditions. The expression of T6SS machinery is however energetically very costly and therefore is tightly regulated. A number of conditions can trigger up-regulation of T6SS operon. These include environmental conditions like

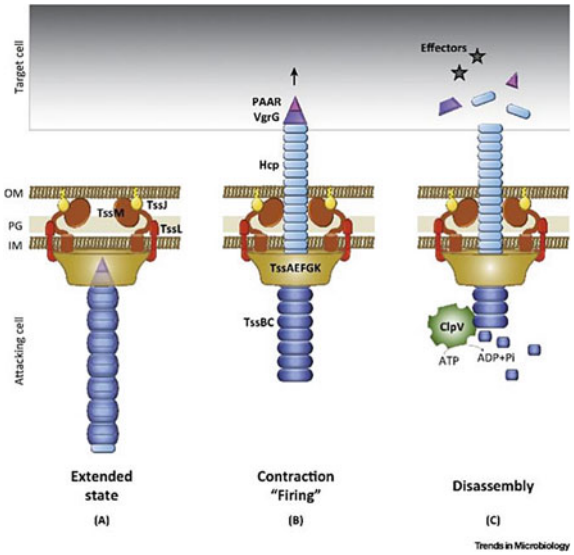


Fig. 11 Type VI secretion system. *Source* Cianfanelli et al. (2016). With kind permission from Elsevier

temperature, pH, salinity, iron concentration, sub-inhibitory antibiotic concentration, density-dependent regulatory mechanisms termed quorum sensing and physical alterations like membrane perturbation or surface sensing.

6 Protein Secretion in Gram-Positive Bacteria

Gram-positive bacteria contain a single phospholipid membrane covered with a thick layer of peptidoglycan from outside. Although Sec and Tat secretion pathways exist and are fully functional in gram-positive bacteria as well, but these pathways are sometimes not sufficient for translocating particular proteins to extracellular medium. Thus, gram-positive bacteria also possess some specialized machineries, exclusively present in them (Anné et al., 2016).

Sec A2—This system, which was first reported from *Mycobacterium tuberculosis*, is now known in other gram-positive bacteria such as *Bacillus subtilis, Clostridium difficile* and *Corynebacterium glutamicum*. In these bacteria, besides the conventional SecA protein of the Sec pathway, a second transport-associated ATPase is presently termed as SecA2. For the convenience of terminology, the conventional SecA protein is also named SecA1; both SecA1 and SecA2 are two non-interchangeable SecA protein homologs (Feltcher & Braunstein, 2012). Of these, SecA1 is essential, indispensable and is responsible for export via the canonical Sec pathway discussed earlier

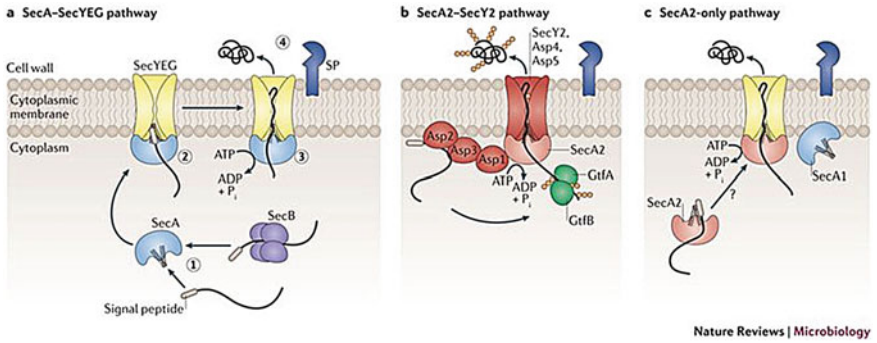


Fig. 12 SecA2 secretion system as compared to SecA system. *Source* Feltcher and Braunstein (2012). With kind permission from Springer Nature

in the chapter. SecA2 is dispensable and is involved in exporting a few proteins such as the virulent proteins in pathogens like *M. tuberculosis*, *S. aureus* or *Listeria monocytogenes*. Substrates targeted to SecA2 have a canonical signal peptide required for export; however, protein targeting via SecA2 depends on certain structural features present in the mature protein (Braunstein et al. 2019). SecA2 is a multi-substrate system that can be sub-categorized into two kinds based on the presence of accessory membrane proteins (Fig. 12). In some bacteria, the SecA2 protein is accompanied by another accessory membrane SecY2 protein forming a SecA2-SecY2 system. This system transports highly glycosylated proteins and the proteins that are incompatible with SecA1-SecYEG pathway. SecA2 systems without accessory membrane proteins are present in some bacteria which function in part with Sec pathway utilizing the SecYEG channel. It exports more diverse proteins than the SecA2-SecY2 system.

Sortases—Sortases are the enzymes that, unlike other secretion systems, do not secrete out protein to the extracellular space but attach them to cell surface through covalent linkage to the cell wall. They also play a crucial role in polymerization of pilin proteins to form 'pili' on the cell surface and in the attachment of adhesion-mediating large surface glycoproteins. Sortases are absent in bacteria lacking cell wall such as the mollicutes.

Cell surface proteins help microorganisms in adhesion, receiving environmental stimuli and signals and many-a-time surface proteins function as virulent factors during infections. Therefore, being a carrier of surface proteins, sortases can be a potential drug target to make an otherwise infectious microbe ineffective.

Sortases are cysteine transpeptidases. They catalyze transfer of their substrate (or protein) to an amino acid on the cell surface. They recognize their substrate by a special signature sequence or motif (generally LPXTG) at the C-terminal. The tail of C-terminal has an array of positively charged residues which retard the movement of protein passing through the external membrane, thus assisting membrane-bound sortases to process their substrate. The substrate proteins for this process are exported

Table 3 Classes of sortases with their recognition motif and substrate proteins

Class of sortases	Recognition motif	Substrate proteins
A (Housekeeping)	LPXTG	Most surface proteins
B	NP(Q/K)(T/S)(N/G/S)(D/A)	Iron or heme acquisition proteins
C	(I/L)(P/A)XTG	Pilin subunits
D	LPNTA	Endospore envelope proteins
E	LAXTG	Pili
F	–	–

through Sec secretion system, and their leader sequences at the N-terminal are already cleaved off by signal peptidases (Spirig et al., 2011).

Types of sortases—These cysteine transpeptidases are of four major types: A, B, C and D, based on phylogenetic relationship. However, recently, two more classes (E and F) found in Actinobacteria have been added in this classification (Table 3). Functions of these new classes are still not clear (Spirig et al., 2011).

- SortasesA are present in Firmicutes and are most extensively studied. They are the housekeeping sortases that recognize LPXTG motif at the C-terminal and target various functionally distinct proteins to cell envelope.
- SortasesB help in anchoring iron acquisition proteins to the cell envelope. They recognize different sorting motif, NP(Q/K)(T/S)(N/G/S)(D/A), in place of LPXTG. They also have some polymerization ability for pili secretion on cell surface.
- SortasesC are majorly involved in pilus assembly by catalyzing the polymerization of pilin subunits.
- Sortases D are still not well characterized but reports suggest their role in sporulation-related proteins.

Box 3: Injectosomes: Machinery for effector transport into host cell

Like T3SS (injectisomes) and T4SS systems in gram-negative bacteria, gram-positive bacteria also contain a functionally similar system for the translocation of effector molecules directly from the bacterial cytoplasm to the cytoplasm of the host cell. The system was first observed in *Streptococcus pyogenes* and is involved in the injection of virulence factor NAD glycohydrolase into keratinocytes (Madden et al., 2001).

Type VII secretion system (T7SS)—It is a Sec-independent secretion system. This is the premier secretion system associated with the virulence of *Mycobacterium tuberculosis* and has been extensively studied in pathogenic mycobacteria.

Mycobacteria possess a high lipid content cell wall called the mycomembrane. This dense hydrophobic, waxy layer serves as an effective barrier against environmental stresses, antibiotic therapy and protein transport. Thus, T7SS is essential for transporting virulence-associated proteins across the cell membrane and subsequently the mycomembrane (Ates et al. 2016; Clemmensen et al. 2017).

T7SS substrates—Two types of substrates are transported through this system: antigenic proteins of the ESX family and PE and PPE proteins which are largely mycobacterium specific. Most of these substrates contain a 'YxxxD/E' secretion signal. Till date, there are five T7SS gene clusters recognized in *Mycobacterium* sp. Three out of these, namely ESX1, ESX3 and ESX5, are known for their role in virulence.

All Type VII substrates are transported as heterodimers. The most worked-out cluster ESX1 is involved in secretion of EsxA and EsxB antigens along with other secretion-associated proteins. The EsxA and EsxB form a heterodimer, and their transport is codependent. The codependence is however not a property of all T7SS protein substrates.

T7SS machinery—The T7SS components include pore-forming membrane proteins, an ATPase, a subtilisin-like protease and intracellular conserved chaperones that recognize the Type VII substrates. In the ESX1 secretory apparatus, the antigens EsxA and EsxB, the ATPases EccA1 and EccCb1 hydrolyze ATP for energy generation and target the antigens to inner membrane channel composed of EccD, AccB, EccE, EspB and EspF. The secretion signal is recognized and cleaved by protease MycP1 in the periplasm (Fig. 13).

Mechanism of transport—Many studies have been conducted on various components of ESX1 transport system. Nearly, 20 virulence-associated proteins transported by ESX1 have been identified. The translocation and pore-forming mechanism is however still not clear. It is supposed that the secretion occurs in two steps. First, the translocation to the cytoplasm is initiated by respective ATPases via C-terminal secretion recognition motifs. Subsequently, the substrates are targeted to inner membrane channel. Along with it, several other substrates are also cotransported. The signal is cleaved by a protease in the periplasm and proteins are secreted out.

T7SS of other bacteria—Homologs of T7SS are also found in other gram-positive bacteria such as members of *Nocardiaceae, Bifidobacteriales, Propionibacteriales* and *Frankiales.* However, several of the T7SS components essential in mycobacteria apart from ATPase and protease are not found in Firmicutes. The T7SS of mycobacteria and Firmicutes is therefore distinct and are termed T7SSa and T7SSb, respectively.

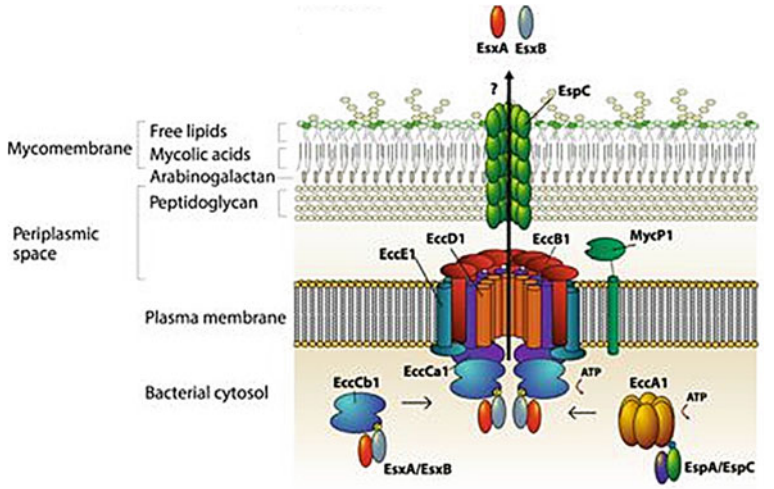


Fig. 13 Type VII secretion system. *Source* Clemmensen et al. (2017). (Creative Commons Attribution License)

Box 4: Discovery of T7SS

While studying differences between virulent *Mycobacterium tuberculosis* and avirulent *M. bovis* BCG, scientists observed that *M. tuberculosis* culture filtrate had an additional Early Secreted Antigenic Target (ESAT-6 or EsxA). Later, a specific 9.5 kb deletion in the genome of the avirulent strain which they designated as 'RD1' was recognized. When the 9 gene long RD1 region encoding ESX was introduced into *M. microti*, an avirulent mycobacterial species used as live attenuated vaccine, virulence was recovered. This conclusively proved the role of ESX secretion in virulence conferred by *M. tuberculosis.* ESX had no predictable homologs with other known secretion systems. Hence, T7SS was discovered (Clemmensen et a., 2017).

Summary

- Bacterial cells (gram positive and negative) utilize the generalized secretory pathways such as Sec and Tat pathways that are ubiquitous to all domains of life.
- Sec system transports unfolded proteins while Tat system transports folded proteins.
- Sec is the most prevalent system found in all life forms and has three main components: (i) a target protein, (ii) a transmembrane channel and (iii) a motor protein having ATPase function.

- The basic mechanism involves recruitment of target protein to Sec translocase. Target protein reaches the protein channel either through cotranslational targeting or post-translational targeting.
- Tat system translocates proteins which have already attained structure after translation.
- In *E. coli*, three-component TatABC system exists wherein Tat B and C form an integral membrane complex with multiple copies of each protein.
- Sec or Tat translocation in gram-positive bacteria results in the export of the substrate protein to extracellular environment; however, in case of gram-negative bacteria, this step results in the translocation of substrate protein to the periplasmic space.
- Gram-negative bacteria possess nine different types of molecular machineries (Type I to Type IX secretion systems except T7SS) to facilitate translocation of proteins or effector molecules from periplasm to outside of the cell.
- Type II, V, VIII and IX secretion systems are Sec-dependent, while Type I, III, IV and VI secretion systems are Sec-independent as they can translocate substrate protein directly from cytoplasm to extracellular medium.
- The proteins secreted through T2SS have a broad range of biological functions, and it can transport only folded proteins.
- T2SS is composed of 12–15 proteins present as four different sub-assemblies (outer membrane complex, inner membrane platform, secretion ATPase and pseudopilus) both in inner and outer membranes.
- T5SS is a Sec-dependant system. This transport does not require ATP or proton gradient and uses free energy of protein folding as the sole energy source and hence termed autotransporter and translocates proteins to cell surface or into extra-cellullar milieu.
- T8SS is a Sec-dependent secretion system that specifically transports short bristle-like fibers to the surface of bacteria responsible for 'curli' phenotype of *E. coli.*
- T9SS is required for the secretion of some adhesins, some hydrolytic enzymes and apparatus responsible for gliding motility in the Chlorobi–Fibrobacteres–Bacteroidetes group (CFB group).
- T9SS was discovered in a porphyrin-negative (colorless mutants) *Porphyromonas gingivalis*, the proteins of the system have been given the prefix 'por' indicating porphyrin.
- T1SS is the simplest and the first discovered system which is similar to ABC transporter.
- T3SS is well recognized as nano-machinery or nano-syringes called 'injectisomes' due to their close functional similarity to a needle and syringe. This system also plays role in flagella development.
- T4SS is a diverse group involved in transporting a variety of substrates peculiarly DNA as nucleoprotein complex and in secretion of protein effector molecules in both gram-negative and gram-positive bacteria and in some archeal groups.
- T6SS is characterized as a contractile puncturing nanomissile that works like a speargun to release effector proteins across the cell envelope into a foreign cell.

- Under stress conditions, bacteria are reported to require SecA2 enzyme for export of various stress-related proteins and for the maintenance of cell's integrity.
- Sortases catalyze the transfer of their substrate (or protein) to an amino acid on the cell surface by transpeptidation attachment.
- T7SS is associated with the virulence of *Mycobacterium tuberculosis* and has been extensively studied in pathogenic mycobacteria.

Questions

1. Differentiate between Sec and Tat secretory system.
2. What are Type I and Type II signal peptides?
3. Explain mechanism of export through Type II secretory system.
4. Explain why T5SS is supposed to be desired transport for export of biotechnological important proteins?
5. Which secretion system results in 'curli' phenotype of *E. coli*? Explain mechanism of transport and fiber formation.
6. Why proteins of Type IX secretion system are prefixed 'por'? How is T9SS regulated?
7. Explain Type I secretion system considering hemolysin transport across *E. coli* cell membrane.
8. Write a note on injectisome and differentiate it with injectosome.
9. Type VI secretion system organization represents inverted bacteriophage and function in a contact-dependent manner. Explain.
10. Which secretory system is able to transport DNA? Explain its functioning.
11. What are sortases?
12. Explain how proteins are transported through cell membrane of gram-positive bacteria?
13. How T7SS was discovered?

References

Anné, J., Economou, A., & Bernaerts, K. (2016). Protein secretion in gram-positive bacteria: From multiple pathways to biotechnology. In F. Bagnoli. & R. Rappuoli. (Eds.), *Protein and sugar export and assembly in gram-positive Bacteria. Current topics in microbiology and immunology* (Vol. 404), (pp. 267–308). Springer, Cham.

Ates, L. S., Houben, E. N., & Bitter, W. (2016). Type VII secretion: A highly versatile secretion system. *Microbiology spectrum*, *4*(1).

Bocian-Ostrzycka, K. M., Grzeszczuk, M. J., Banaś, A. M., & Jagusztyn-Krynicka, E. K. (2017). Bacterial thiol oxidoreductases—from basic research to new antibacterial strategies. *Applied Microbiology and Biotechnology, 101*(10), 3977–3989.

Braunstein, M., Bensing, B. A., & Sullam, P. M. (2019). The two distinct types of SecA2-Dependent export systems. *Microbiology spectrum*, *7*(3).

Cianfanelli, F. R., Diniz, J. A., Guo, M., De Cesare, V., Trost, M., & Coulthurst, S. J. (2016). VgrG and PAAR proteins define distinct versions of a functional type VI secretion system. *PLoS Pathogens, 12*(6), e1005735.

Clemmensen, H. S., Knudsen, N. P. H., Rasmussen, E. M., Winkler, J., Rosenkrands, I., Ahmad, A., ... & Aagaard, C. (2017). An attenuated Mycobacterium tuberculosis clinical strain with a defect in ESX-1 secretion induces minimal host immune responses and pathology. *Scientific reports*, *7*, 46666.

Delepelaire, P. (2004). Type I secretion in gram-negative bacteria. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1694*(1–3), 149–161.

Deng, W., Marshall, N. C., Rowland, J. L., McCoy, J. M., Worrall, L. J., Santos, A. S., ... & Finlay, B. B. (2017). Assembly, structure, function and regulation of type III secretion systems. *Nature Reviews Microbiology*, *15*(6), 323.

Evans, M. L., & Chapman, M. R. (2014). Curli biogenesis: Order out of disorder. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1843*(8), 1551–1558.

Feltcher, M. E., & Braunstein, M. (2012). Emerging themes in SecA2-mediated protein export. *Nature Reviews Microbiology, 10*(11), 779.

Freudl, R. (2018). Signal peptides for recombinant protein secretion in bacterial expression systems. *Microbial Cell Factories, 17*(1), 52.

Gentschev, I., Dietrich, G., & Goebel, W. (2002). The E. coli α-hemolysin secretion system and its use in vaccine development. *Trends in microbiology*, *10*(1), 39–45.

Glew, M. D., Veith, P. D., Chen, D., Gorasia, D. G., Peng, B., & Reynolds, E. C. (2017). PorV is an outer membrane shuttle protein for the type IX secretion system. *Scientific Reports, 7*(1), 1–14.

Green, E. R., & Mecsas, J. (2016). Bacterial secretion systems: An overview. *Microbiology Spectrum, 4*. https://doi.org/10.1128/microbiolspec. VMBF-0012-2015.

Guo, S., Stevens, C. A., Vance, T. D., Olijve, L. L., Graham, L. A., Campbell, R. L., ... & Braslavsky, I. (2017). Structure of a 1.5-MDa adhesin that binds its Antarctic bacterium to diatoms and ice. *Science Advances*, *3*(8), e1701440.

Lasica, A. M., Ksiazek, M., Madej, M., & Potempa, J. (2017). The type IX secretion system (T9SS): Highlights and recent insights into its structure and function. *Frontiers in Cellular and Infection Microbiology, 7,* 215.

Lenders, M. H., Reimann, S., Smits, S. H., & Schmitt, L. (2013). Molecular insights into type I secretion systems. *Biological Chemistry, 394*(11), 1371–1384.

Leo, J. C., Grin, I., & Linke, D. (2012). Type V secretion: Mechanism (s) of autotransport through the bacterial outer membrane. *Philosophical Transactions of the Royal Society B: Biological Sciences, 367*(1592), 1088–1101.

Linhartová, I., Bumba, L., Mašín, J., Basler, M., Osička, R., Kamanová, J., ... & Morová, J. (2010). RTX proteins: A highly diverse family secreted by a common mechanism. *FEMS microbiology reviews*, *34*(6), 1076–1112.

Madden, J. C., Ruiz, N., & Caparon, M. (2001). Cytolysin-mediated translocation (CMT): A functional equivalent of type III secretion in gram-positive bacteria. *Cell, 104*(1), 143–152.

Natale, P., Brüser, T., & Driessen, A. J. (2008). Sec-and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—distinct translocases and mechanisms. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1778*(9), 1735–1756.

Paetzel, M., Karla, A., Strynadka, N. C., & Dalbey, R. E. (2002). Signal peptidases. *Chemical Reviews, 102*(12), 4549–4580.

Palmer, T., & Berks, B. C. (2012). The twin-arginine translocation (Tat) protein export pathway. *Nature Reviews Microbiology, 10*(7), 483–496.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., ... & Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences*, *103*(5), 1528–1533.

Spirig, T., Weiner, E. M., & Clubb, R. T. (2011). Sortase enzymes in Gram-positive bacteria. *Molecular Microbiology, 82*(5), 1044–1059.

von Tils, D., Blädel, I., Schmidt, M. A., & Heusipp, G. (2012). Type II secretion in Yersinia—a secretion system for pathogenicity and environmental fitness. *Frontiers in Cellular and Infection Microbiology, 2,* 160.

Waksman, G., & Orlova, E. V. (2014). Structural organisation of the type IV secretion systems. *Current Opinion in Microbiology, 17,* 24–31.
Wells, T. J., & Henderson, I. R. (2013). Type 1 and 5 secretion systems and associated toxins—*Chapter 16.* In M.S. Donnenberg (Ed.), *Escherichia coli* (pp. 499–532). Academic Press.

Part IV
Central Metabolic Pathways

Chapter 9
Glycolysis and Gluconeogenesis

Rani Gupta and Namita Gupta

Carbon metabolic pathways are called central metabolic pathways as these are the key pathways to generate energy and intermediates for the synthesis of biomolecules viz. proteins, lipids, etc. Another major function of carbon metabolism is precursor generation for several anapleurotic reactions, which produce intermediates for other metabolic pathways. The major carbon metabolic pathways are glycolysis/glycolytic pathway/Embden–Meyerhoff–Parnas pathway (EMP), pentose phosphate pathway (PPP) and Entner–Doudoroff (ED) pathway.

Glycolysis and pentose phosphate pathway are ubiquitous, present in both prokaryotes and eukaryotes, and occur in cytosol. Apart from these, bacteria utilize alternate pathway for glucose breakdown during fermentative mode of growth. The major fermentation pathway is Entner–Doudodorff (ED) pathway which is mainly present in prokaryotes with few exceptions among eukaryotes such as *Penicillium notatum*, *Aspergillus niger* and *Entamoeba histolytica.*

Further, during growth on non-carbohydrate sources such as pyruvate, lactate, acetate, fatty acids and amino acids, glucose can be synthesized via the gluconeogenic pathway to supply glycolytic precursors required for various biosynthetic reactions.

1 Glycolysis: Embden–Meyerhoff–Parnas Pathway

Glycolytic pathway is an anaerobic pathway and does not require oxygen essentially for conversion of glucose to pyruvate. However, glycolysis is categorized as aerobic or anaerobic depending upon the oxidation of NADH generated during glycolysis. If NAD^+ is generated through oxidative phosphorylation, it is aerobic glycolysis, and if it is generated through fermentation during production of lactic acid/alcohol, it is called anaerobic glycolysis (Fig. 1).

Before proceeding to the mode of glucose oxidation to pyruvate, let us first recall glucose uptake in *E. coli.* Glucose is phosphorylated by the PEP-PTS system or by

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_9

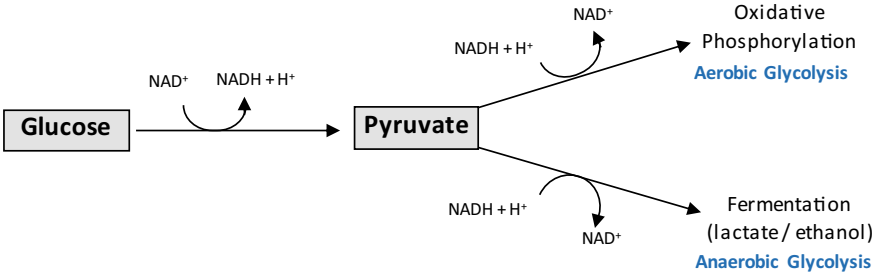


Fig. 1 Glycolytic pathway under aerobic and anaerobic condition

the action of hexokinase. We will now quickly recapitulate different steps of the glycolytic pathway. The glycolytic pathway can be divided into two stages (Fig. 2):

The first stage is the *energy utilizing stage* wherein glucose is converted to glucose-6-phosphate to fructose-6-phosphate and then to fructose-1,6-bisphosphate utilizing two moles of ATP per mole of glucose.

The second stage is *the energy generation stage* where pyruvate is generated with net gain of two ATP molecules per mole of glucose along with generation of two molecules of NADH + H^+.

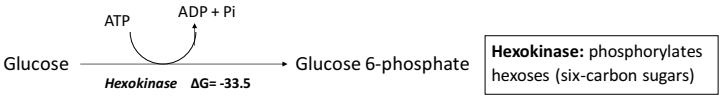
Gibbs free energy

Before going further to the glycolysis pathway and its reactions, let us understand the concept of free energy in context of biochemical reactions that take place during various cellular metabolic processes.

- *ΔG—free energy change at reaction conditions; ΔG^{O}'—free energy change at standard conditions*
- *ΔG values close to zero indicate the reactions are close to equilibrium and reversible.*
- *ΔG values far from zero indicate an irreversible reaction that favors one direction.*

Stage 1: Energy utilizing steps/preparatory phase

Step 1: Phosphorylation of glucose: Glucose is either phosphorylated during its uptake by PEP-PTS system or is phosphorylated by hexokinase which converts glucose to glucose-6-phosphate. The negative charge of the phosphate prevents passage of the sugar phosphate through the cell membrane thereby ensuring entrapment of glucose in the cell.



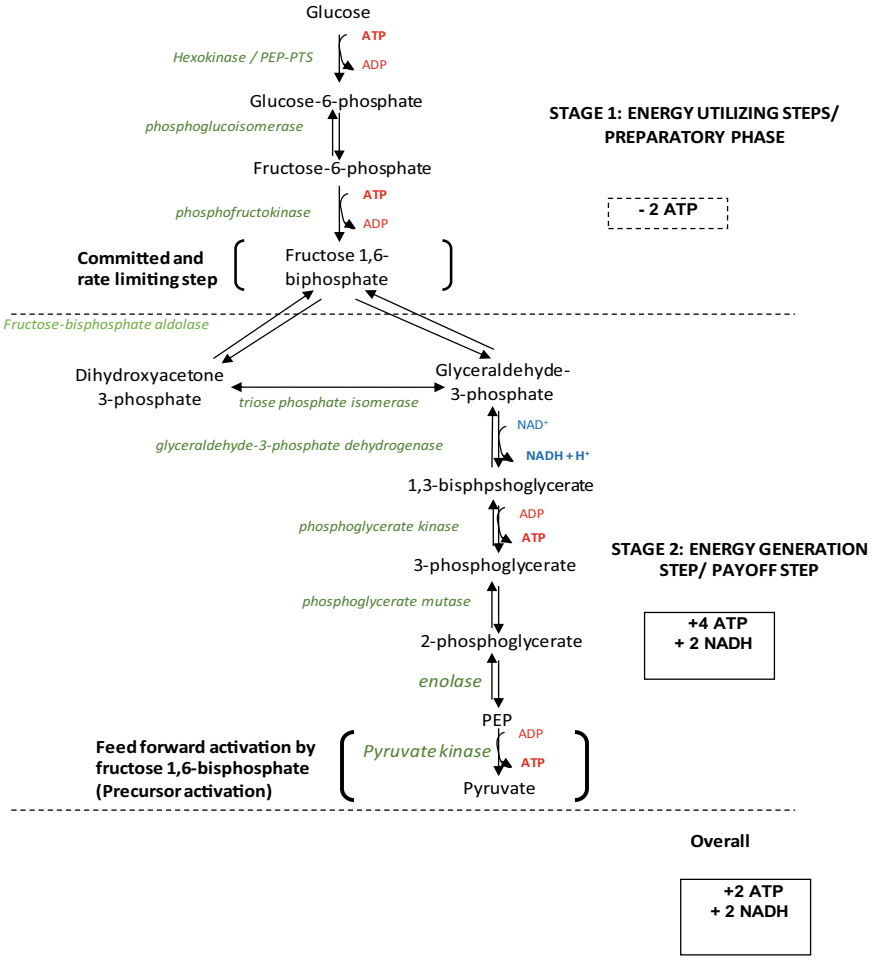
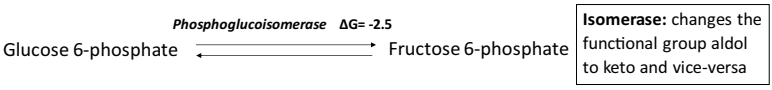


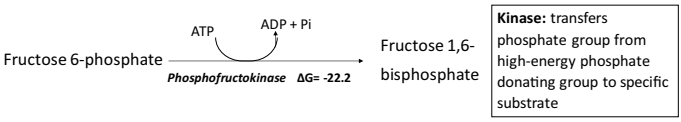
Fig. 2 Glycolytic pathway depicting regulatory steps and net energy and reductant changes

Step 2: Isomerization to fructose-6-phosphate: Glucose-6-phosphate is converted to fructose-6-phosphate by isomerisation from aldol form to keto form. The reaction is catalyzed by phosphoglucoisomerase and is a reversible, non-rate limiting reaction.



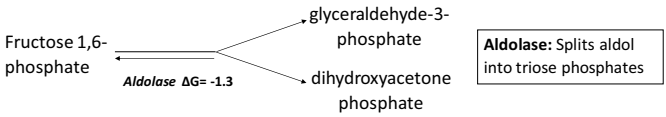
Step 3: Phosphorylation of fructose-6-phosphate: This reaction is the most important, irreversible reaction of glycolysis. The reaction is catalyzed by phosphofructokinase (PFK). This is a committed step and also a rate limiting step as it is controlled

by concentrations of its substrates, ATP and fructose-6-phosphate. ADP/AMP acts as positive modulators for this enzyme, while ATP and citrate are the negative modulators. In eukaryotes only, fructose 2,6-bisphosphate is an activator of this enzyme.

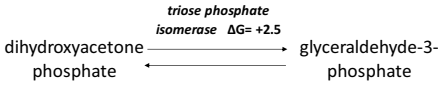


Stage 2: Energy generation step/payoff step

Step 4: Cleavage of Fructose-1,6 bisphosphate: Aldolase enzyme cleaves fructose-1,6 bisphosphate into two 3C compounds, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. This is a reversible reaction. Only glyceraldehyde-3-phosphate can proceed through the further steps.

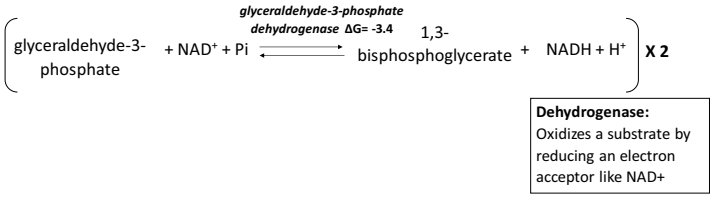


Step 5: Isomerization of triosephosphate: The next step is rapid isomerisation of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate by triose phosphate isomerase.



Finally, two molecules of 3-phosphoglyceraldehyde are produced. All the subsequent intermediates/products of glycolytic pathway are derivatives of trioses.

Step 6: Oxidation of Glyceraldehyde-3-phosphate: The oxidation of glyceraldehyde-3-phosphate to its corresponding acid is the first redox reaction of glycolytic pathway involving NAD^+, and it also requires inorganic phosphate. Thus, NADH and a new high energy anhydride linkage to phosphate are formed.

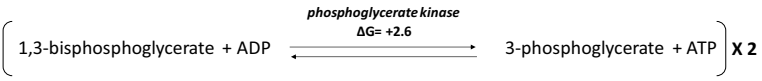


- *The cellular pool of NAD^+ is limited. For sustained glucose oxidation, NADH + H^+ must be immediately re-oxidized which is done either by electron transport chain or by fermentation.*
- *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a sulfydryl protein and can be inhibited by oxidizing agents. Thus, glycolysis is inhibited by iodoacetate as it oxidizes active SH group of the enzyme cysteine residue by acetylating it and releasing HI.*

$$\text{SH - Enz} + CH_3COOI \rightarrow \text{Enz - SOOCCH}_3 + HI$$

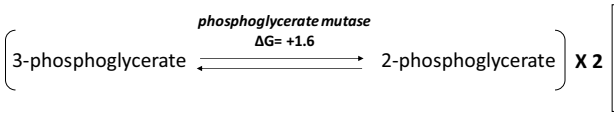
- *This is the reaction where the first high energy compound is generated. The oxidation of aldehyde to a carboxyl group along with high energy phosphate is conserved at C1 of 1,3 bisphosphoglycerate which conserves much of the free energy of oxidation of glyceraldehyde-3-phosphate.*
- *This is also an example of substrate-level phosphorylation in which the production of high energy phosphate is coupled with oxidation of substrate.*

Step 7: Formation of ATP from 1,3-bisphosphoglycerate and ADP: The high energy phosphate of 1,3-bisphosphoglycerate results in ATP synthesis and is a reversible reaction catalyzed by phosphoglycerate kinase. Thus, 1 mol of glucose is converted to two molecules of phosphoglycerate, thereby forming two molecules of ATP. These ATP molecules balance the two ATP molecules used initially in the energy utilizing steps of glycolysis.



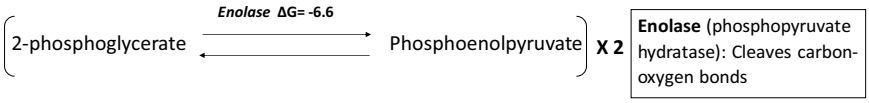
Step 8: Conversion of 3-phosphoglycerate to pyruvate: This is done via three steps:

i. Shift of phosphate from third carbon to second carbon by phosphoglycerate mutase

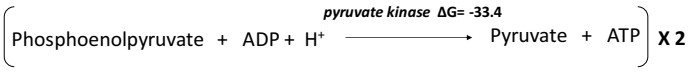


Mutase: Catalyses transfer of a functional group from one position to another in a same molecule

ii. Dehydration of 2-phosphoglycerate to form phosphoenolpyruvate by enolase. Dehydration of 2-phosphoglycerate results in energy distribution changes that creates a high energy enol phosphate linkage. This is also a reversible reaction.



iii. Conversion of PEP to pyruvate catalyzed by pyruvate kinase. This reaction is the third irreversible reaction resulting in generation of ATP and large change in free energy.



Stoichiometry of Glycolysis

Starting from glucose, the overall stoichiometry of glycolysis is as follows:

$$\text{Glucose} + 2\text{ADP} + 2\text{Pi} + 2\text{NAD}^{+} \rightarrow 2\text{Pyruvate} + 2\text{NADH} + 2\text{H}^{+} + 2\text{H}_2\text{O}$$

Further, during the electron transport cycle, 2.5 ATPs are produced per NADH. Thus, one round of glycolysis yields seven ATP under respiratory conditions (Table 1).

Glycolysis as a source of anapleurotic reactions for the generation of biomolecules

Glycolytic reactions also provide precursors for many pathways as illustrated in Table 2.

Table 1 Stoichiometry of Glycolysis

Reaction	No. of energy charge formed/used	No. of reducing equivalents formed
Glucose → Glucose 6-phosphate	(−1) ATP	
Fructose 6-phosphate → Fructose 1,6-bisphosphate	(−1) ATP	
2 glyceraldehyde-3-phosphate → 1,3-bisphosphoglycerate		2 NADH
2 1,3-bisphosphoglycerate → 2 3-phosphoglycerate fumarate	2 ATP	
2 phosphoenolpyruvate → 2 pyruvate	2 ATP	
Total	2 ATP	2 NADH
No. of ATP formed per glycolytic cycle under respiratory conditions	7 ATP	

Table 2 Anapleurotic role of glycolytic pathway

Precursors/glycolytic intermediates	Biomolecules
Glucose-6-phosphate	Polysaccharides, pentose phosphates and aromatic amino acids
Fructose-6-phosphate	Amino sugars such as muramic acid
Dihydroxyacetone phosphate	Phospholipids
3-phosphoglycerate	Glycine, serine, cysteine
Phosphoenolpyruvate	Aromatic amino acids, lactyl portion of muramic acid

2 Regulation of Glycolysis

Glycolysis is primarily regulated at the steps catalyzed by the enzymes phosphofructokinase and pyruvate kinase, both of which are irreversible reactions.

1. **Regulation of phosphofructokinase**—Phosphofructokinase 1 (PFK-1) catalyzes the irreversible phosphorylation of fructose 6-phosphate. This is the most important control point of glycolysis and is regulated by several factors:

    i. PFK-1 is allosterically inhibited by higher concentrations of ATP as it signifies abundance of high energy compounds in the cell. Conversely, it is activated by high concentrations of ADP/AMP as it signals low energy charge of cell. For the same reasons, it is also inhibited by high citrate levels that indicate low metabolic rates of Krebs cycle meaning low energy needs or high available energy in the cell.

Thus, PFK can accept ATP both as substrate and as modulator.This is is facilitated by the varying binding affinities of the substrate-binding sites and the regulator sites. PFK-1 has low K_m for ATP as substrate and high K_m for ATP as inhibitor. Thus, when ATP accumulates, it acts as an inhibitor as there is no need for catabolism.

ii. PFK-1 is feedback inhibited by phosphoenolpyruvate.

iii. In eukaryotes only, fructose 2,6-bisphosphate is a potent activator of PFK-1 enzyme. Fructose 2,6-bisphosphate is formed by the action of a second phosphofructokinase PFK-2. While in bacteria, PFK-2, present as a minor isozyme of PFK-1, shows hyperbolic kinetics with fructose-6-phosphate.

2. **Regulation of pyruvate kinase**—Pyruvate kinase shows precursor activation or feed forward activation by fructose-1,6 bisphosphate. This activation links the two kinase activities, *i.e.*, if PFK activity is high, fructose-1,6 bisphosphate will be more that in turn will activate the forward reaction by the pyruvate kinase, thus forming the final product, pyruvate.

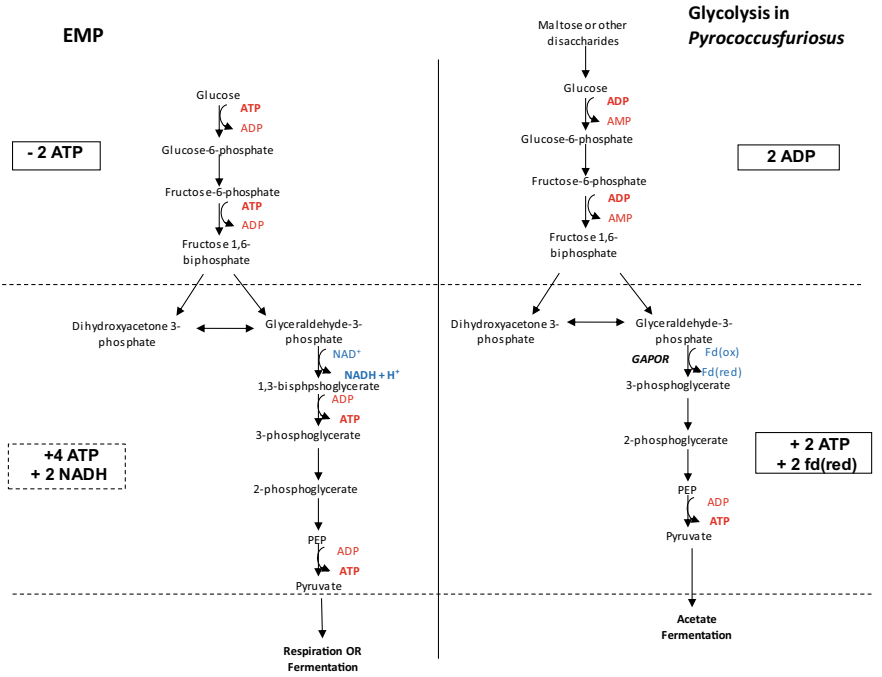


Fig. 3 Comparison between conventional EMP and glycolytic pathway of *Pyrococcus furiosus*

3 Modification of Glycolysis in *Pyrococcus Furiosus*

Pyrococcus furiosus has a modified glycolytic pathway (Verhees et al., 2003; Sato & Atomi, 2011) (Fig. 3). The key differences are as follows:

1. It does not take up glucose, instead maltose or other disaccharides are the substrates which are converted to monosaccharides by specific enzymes.
2. Instead of ATP, ADP is the high energy phosphate utilized.
3. Glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase activities are absent. Instead, it has glyceraldehyde-3-phosphate ferrodoxin oxidoreductase (GAPOR) which is a tungsten (metallo) enzyme.
4. Pyruvate is oxidized to acetyl CoA and CO_2 via the action of pyruvate: ferrodoxin oxidoreductase. Finally, acetyl CoA is converted to acetate by the action of ADP-dependent acetyl CoA synthetase.

4 Atypical Glycolysis in *Clostridium Thermocellum*

Clostridium thermocellum has an atypical glycolytic pathway. This was studied with cells growing on cellobiose (Zhou et. al., 2013). The key differences are as follows:

1. Intracellular glucose is phosphorylated by glucokinase using GTP instead of ATP.
2. Two genes have been annotated for phosphofructokinase which have different cofactor specificities: One is ATP linked, while the other is pyrophosphate (PPi) linked. Higher expression has been reported for PPi-linked PFK.
3. Pyruvate kinase is absent. Further, the annotated pyruvate-phosphate dikinase is not important for conversion of PEP to pyruvate. Instead the gluconeogenic enzymes come into play. PEP is converted to pyruvate via the malate shunt involving the following three enzyme activities (Fig. 4):

    a. GDP-linked PEP carboxykinase
    b. NADH-linked malate dehydrogenase
    c. NADH-linked malic enzyme.

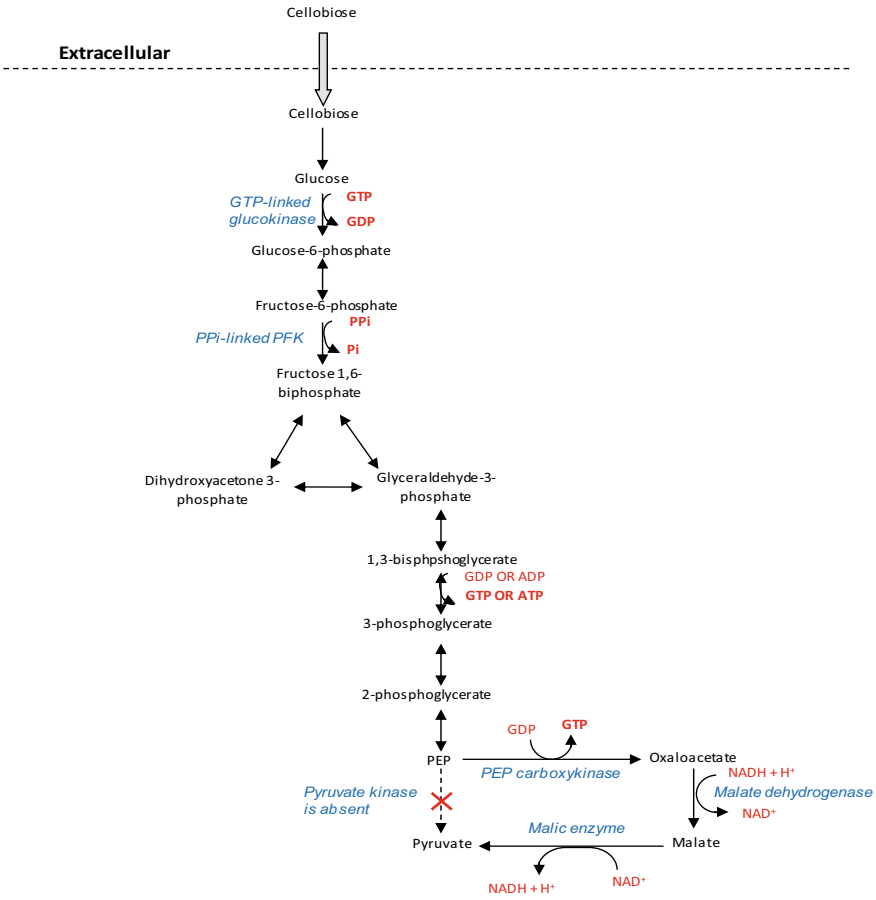


Fig. 4 Glycolytic pathway in *Clostridium thermocellum*

4. Both GTP and ATP are produced during the glycolytic pathway; however, only GTP is used.
5. Pyrophosphate provided by biosynthetic reactions is insufficient for sugar catabolism. Thus, alternative routes for PPi generation must exist. Various alternate ways of generating PPi in a variety of organisms have been reviewed by Zhou et al. (2013):

    a. ATP pyrophosphatase activity as is present in *Spirochaeta thermophila*
    b. PPi-dependent acetate kinase as present in *Enatamoeba histolytica* or PPi-linked PEP carboxykinase as present in *Propionibacterium shermanii*. However, in *C. thermocellum*, acetate kinase has been reported to be ATP dependent, while PEP carboxykinase is GDP dependent. Thus, these are unlikely to be the routes for PPi generation.
    c. Membrane-bound ion pumping inorganic phosphatase as present in *Rhodospirillum rubrum*. A gene encoding a proton-pumping pyrophosphatase has been found in *C. thermocellum* genome. However, its expression has been obtained only at the transcription level and not at the translation level.
    d. ADP-glucose synthase activity (ATP + glucose 1-P ↔ ADP-glucose + PPi) involved in formation and degradation of glycogen has been observed in various cellulolytic bacteria such as *Clostridium cellulolyticum*. ADP-glucose synthase has been found in *C. thermocellum* growing on cellobiose. Also, the genome contains other genes required for glycogen cycling. Thus, this pathway may be main source of pyrophosphate required for glycolysis. This needs to be further validated.
    e. It should be noted that PPi-dependent glycolysis has been reported from non-cellulolytic *Actinomyces naeslundii* also. PPi-linked PFK activity is also present in *Entamoeba histolytica*. Thus, it is likely that many more organisms have this atypical pathway.

5 Methylglyoxal Pathway: An Off-Shoot of Glycolysis

Methylglyoxal, also known as 2-oxopropanal or pyruvaldehyde, is a dicarbonyl compound that is formed as a by-product of glycolysis in several organisms. In *E. coli*, the increased intracellular uptake of glucose 6-phosphate or other carbon substrates such as xylose, glycerol, gluconate, lactose activates the methyl glyoxal pathway. Methylglyoxal is produced from dihydroxyacetone phosphate (DHAP) by the enzyme methylglyoxal synthase (Fig. 5).

High DHAP concentrations induce while high phosphate concentrations inhibit methylglyoxal synthesis. Triose phosphate isomerase plays an important role in determining the levels of DHAP as it converts glyceraldehyde 3-phosphate into DHAP. Under low levels of phosphate, glyceraldehyde 3-phosphate dehydrogenase is inhibited, and triosephosphate isomerase converts glyceraldehyde 3-phosphate to DHAP.

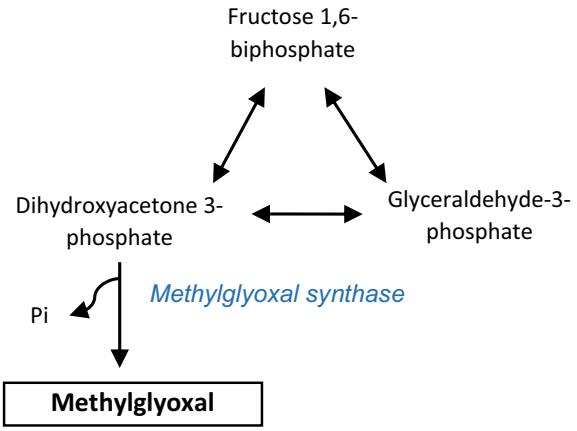


Fig. 5 Conversion of dihyroxyacetone phosphate to methylglyoxal under phosphate starvation conditions

Elevated levels of DHAP activate methylglyoxal synthase leading to production of methylglyoxal.

The reaction releases a phosphate group, and no ATP is produced. It is believed that the main purpose of this pathway is to relieve the cells from stress caused by elevated levels of sugar phosphates.

Methylglyoxal is highly reactive and is very toxic to cells as it can interact with DNA, RNA and proteins. Growth of *E. coli* ceases at millimolar concentrations. Due to the highly cytotoxic nature of methyglyoxal, its production is tightly controlled. It was observed that even after a 900-fold over-expression of methylglyoxal synthase in *E. coli*, methylglyoxal accumulation was very low (Weber et al., 2005). This is achieved by up-regulation of enzymes which degrade methyl glyoxal such as methylglyoxal reductases, methylglyoxal dehydrogenases, aldolase reductases and glutathione-dependent glyoxalase system. In *E. coli*, the glutathione-dependent glyoxalase system is the most common pathway that converts methylglyoxal to a less toxic product. This will be dealt in Chap. 17: Glutathione and Polyamines in bacteria.

6 Gluconeogenesis

Gluconeogenesis is synthesis of glucose from non-carbohydrate sources such as pyruvate, lactate, acetate, fatty acids and amino acids. The need of synthesizing glucose when an organism is grown on non-carbohydrate source is due to the requirement of precursors which are generated during glucose oxidation via. glycolysis and pentose phosphate pathway. All these precursors are required for synthesis of a variety of biomolecules specially reductant and ribose sugars for nucleotide synthesis.

Gluconeogenesis is not a simple reversion of glycolysis as reversing all the reactions of glycolysis would be impossible due to the reactions that require a large change in free energy viz. conversion of glucose to glucose-6-phosphate, fructose-6-phosphate to fructose-1, 6-bisphosphate and PEP to pyruvate. Therefore, out of the ten reactions involved in glycolysis, only seven are reversible for the synthesis of glucose from pyruvate.

Thus, alternative reactions are required for the following three steps:

I. Conversion of pyruvate to PEP
II. Conversion of fructose 1,6-bisphosphate to fructose 6-phosphate
III. Conversion of glucose 6-phosphate to glucose.

Conversion of pyruvate to PEP

First alternative reaction is required for the irreversible reaction of pyruvate kinase which converts PEP to pyruvate. In gluconeogenesis, pyruvate is converted to PEP by two modes (Fig. 6):

Pyruvate is carboxylated first to oxaloacetate by the action of pyruvate carboxylase which is then converted to PEP by PEP carboxykinase: This is the most common mode for conversion of pyruvate to PEP.

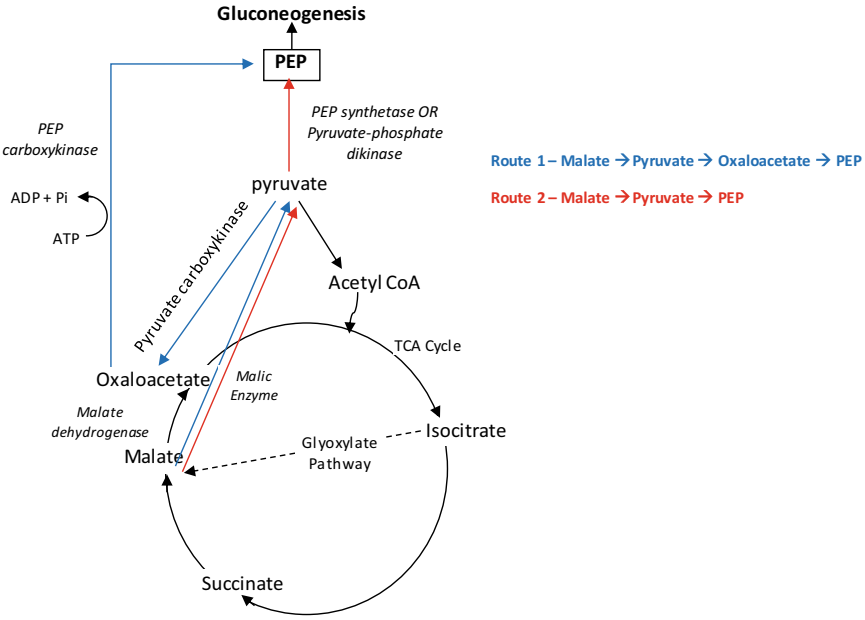
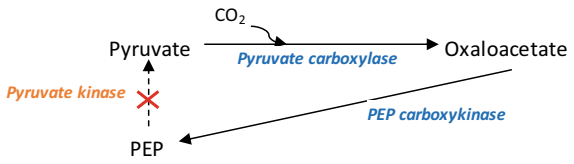
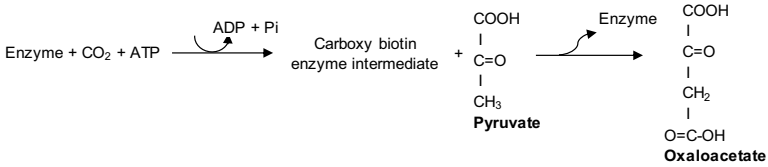


Fig. 6 Alternative routes for PEP generation during gluconeogenesis



Glycolytic enzymes are marked in orange while gluconeogenic enzymes are marked in blue.

Pyruvate carboxylase: Pyruvate carboxylase is a biotin-dependent enzyme like any other carboxylase. Here, biotin is covalently linked to ε-amino group of lysine. At the expense of ATP, the biotin linked pyruvate carboxylase is activated, and pyruvate gets carboxylated.



Acetate activation of pyruvate carboxylase: Pyruvate carboxylase is allosterically activated by acetyl CoA. Elevated acetyl CoA may indicate high requirement of oxaloacetate which may happen under any of the two conditions:

- When oxaloacetate is depleted due to its removal for anapleurotic reactions and its synthesis is required to continue TCA. Conversely, at low level of acetyl CoA, pyruvate carboxylase is inactive, and pyruvate is oxidized by TCA cycle. This is the non-gluconeogenic action of pyruvate caboxylase.
- When organism is starved or growing on non-carbohydrate sources indicating requirement for glucose. Here, oxaloacetate formed by pyruvate carboxylase will be used for synthesis of glucose by gluconeogenesis. This is the gluconeogenic action of pyruvate caboxylase.

In conditions when an organism is growing on non-carbohydrate sources, pyruvate can be generated during utilization of proteins/amino acids/organic acids. For this, an important enzyme is the **malic enzyme** which can convert organic acids such as succinate and malate to pyruvate. It is a ubiquitous enzyme that catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2, with the concomitant reduction of the cofactor $NAD(P)^+$ to NAD(P)H. In eukaryotes, malic enzyme has been found in the cytosol and/or in mitochondria and in the chloroplasts in plants.

PEP carboxykinase: Another enzyme which is required for generation of PEP from pyruvate is PEP carboxykinase which converts oxaloacetate to PEP. It catalyzes the decarboxylation and mononucleotide-dependent phosphorylation of oxaloacetate to form phosphoenolpyruvate. It requires ATP (or GTP) as a cofactor. This conversion is the first committed step of gluconeogenesis.

PEP generation in organisms where pyruvate carboxylase is absent

In organisms where pyruvate carboxylase is absent, alternative enzymes come into play for PEP synthesis. Various scenarios are possible as summarized below:

(a) **In organisms where pyruvate carboxylase is absent and PEP carboxykinase is active in the direction of oxaloacetate → PEP**

In such situations, malate can be converted to oxaloacetate (by the action of malate dehydrogenase). Oxaloacetate can then be converted to PEP by the action of PEP carboxykinase.

(b) **In organisms such as *E.*coli where pyruvate carboxylase is absent and PEP carboxykinase is active only in the direction of PEP → oxaloacetate and not in the direction of oxaloacetate → PEP**

Here, PEP needs to be synthesized directly from pyruvate without intervening oxaloacetate formation (as PEP carboxykinase cannot convert oxaloacetate to pyruvate). This is accomplished by the action of the enzymes PEP synthetase or pyruvate-phosphate dikinase which phosphorylate pyruvate by ATP via consumption of energy of two phosphate bonds.

PEP synthetase: PEP synthetase of *E. coli* transfers pyrophosphate group from ATP to an immidazole group of histidine in the enzyme. A phospho group is hydrolyzed to ensure sufficient monophosphorylated intermediates. This reacts with pyruvate to form PEP.

$$\text{Pyruvate} + H_2O + \text{ATP} \rightarrow \text{PEP} + \text{AMP} + \text{Pi}$$

Pyruvate-phosphate dikinase: It involves similar reaction as PEP synthetase but requires inorganic phosphate, and the reaction is in two steps (Fig. 7).

Step i. Inorganic phosphate serves as nucleophile in place of water as in PEP synthetase.

$$\text{Pyruvate} + \text{ATP} + \text{Pi} \rightarrow \text{PEP} + \text{AMP} + \text{PPi}$$

Step ii. The pyrophosphate (PPi) formed in this reaction is hydrolyzed by pyrophosphatase.

$$\text{PPi} + H_2O \rightarrow 2H_3PO_4$$

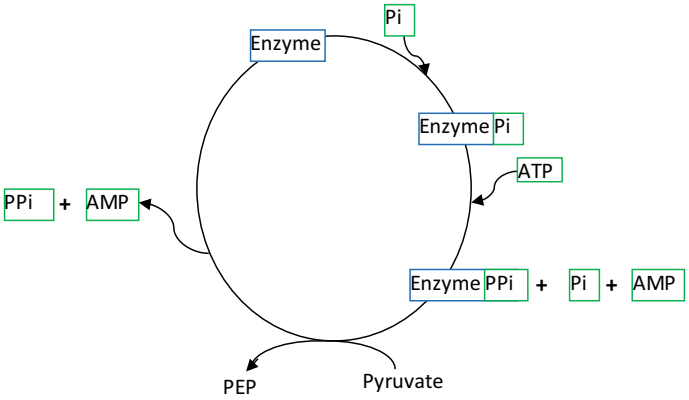


Fig. 7 Mechanism of action of pyruvate-phosphate dikinase reaction

It has been postulated that during the reaction of pyruvate-phosphate dikinase, ATP binds to the enzyme resulting in diphosphorylated enzyme which reacts with inorganic phosphate to release a molecule of pyrophosphate and monophosphorylated enzyme. The monophosphorylated enzyme transfers phosphate to pyruvate to form PEP and also generates its dephosphorylated form. Thus, pyruvate-phosphate dikinase exists in three different stages of phosphorylation: dephosphorylated to diphosphorylated to monophosphorylated forms and back to dephosphorylated form with the conversion of pyruvate to PEP.

Kinetic and positional analysis studies have shown that Pi binds first and then ATP is bound to the enzyme and AMP is only released when inorganic phosphate has reacted to form PPi.

Conversion of Fructose 1,6-bisphosphate to Fructose 6-phosphate

This occurs by the action of the enzyme fructose 1,6-bisphosphatase (FBPase). In eukaryotes, FBPase is regulated by both AMP and fructose-2,6-bisphosphate, while in bacteria, little is known about FBPase regulation. In *E. coli*, it is inhibited by AMP, but fructose-2,6-bisphosphate is absent in bacteria. Regulation of FBPase by AMP alone is unlikely, because concentrations of AMP in cells are relatively constant.

Conversion of Glucose 6-phosphate to Glucose

This occurs by the action of the enzyme glucose 6-phosphatase.

Substrates for gluconeogenesis

The precursors for gluconeogenesis are all those compounds which result in net generation of glucose. They include intermediates of Kreb's cycle, lactate, glycerol and amino acids which generate α-ketoacids after deamination. Some example of gluconeogenic substrates and the possible pathways for their conversion to glucose are depicted in Table 3.

Table 3 Gluconeogenic substrates and the pathways for their conversion to glucose

Gluconeogenic substrate	Pathway
Glycerol	Glycerol → glycerol phosphate → dihydroxyacetonephosphate → phosphoglyceraldehyde → Reverse glycolysis → glucose
Amino acids	α-keto acid → TCA intermediate → Reverse glycolysis → glucose
Aspartic acid	aspartic acid → oxaloacetate → Reverse glycolysis → glucose
Glutamate	glutamate → α-ketoglutarate → Reverse glycolysis → glucose
Alanine	alanine → pyruvate → Reverse glycolysis → glucose
Lactate	lactate → pyruvate → Reverse glycolysis → glucose

Gluconeogenesis and glycolysis are coordinated in a way that at a given time only one of these pathways is active while the other is inactive. This is advantageous as if both the pathways are active at the same time the net result would be hydrolysis of four nucleotide triphosphates (2 ATP + 2 GTP) per reaction cycle. This coordination is accomplished by controlling the activities of the distinctive enzymes of each pathway. Further, glucose determines the rate of glycolysis, while lactate and other precursors of glucose determine the rate of gluconeogenesis.

The most important control point is interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate. AMP stimulates phosphofructokinase, whereas ATP and citrate inhibit it. This is because high level of AMP indicates low energy charge and the need for ATP generation while high level of ATP indicates high energy charge of the cell. For the same reasons, fructose 1,6-bisphosphatase is inhibited by AMP and activated by PEP. In *E.* coli, fructose 1,6-bisphosphatase is a homotetramer that has an active and an inactive conformation. Binding of AMP to the enzyme leads to its inactive conformation, while binding of PEP leads to its active conformation (Fig. 8).

ATP is a direct allosteric modulator for phosphofructokinase as it binds to the enzyme and inhibits it. Similarly, AMP and PEP are the allosteric modulators of fructose 1,6-bisphosphatase. Here, AMP is a negative modulator, while PEP is a positive modulator.

Other than these, many other metabolites act as indirect modulators for these two enzyme as they determine the energy charge of the cell, i.e., ATP/AMP + ADP ratio. If energy charge of the cell is high, phosphofructokinase is inhibited, thereby inhibiting glycolysis. At the same time, this turns on gluconeogenesis by stimulating fructose 1,6-bisphosphatase activity. Conversely, low energy charge turns on glycolysis as fructose 1,6-bisphosphatase is inhibited. Examples of a few metabolites that modulate energy charge of the cell are provided in Table 4.

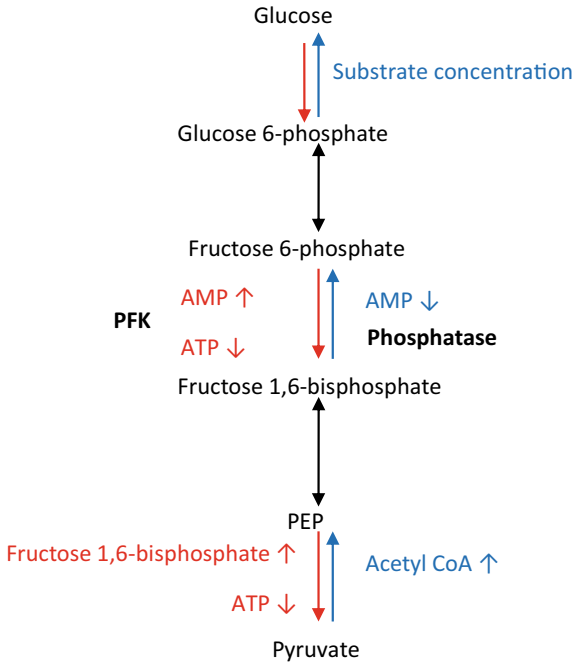


Fig. 8 Major control points and modulators of glycolysis (red) vs gluconeogenesis (blue)

Table 4 Metabolites that modulate energy charge of the cell

Compound	Indicates high/low energy
Citrate	High energy
Acetyl CoA	High energy
Alanine	High energy (inhibits pyruvate kinase)
Fructose 1,6-bisphosphate	High energy (inhibits PFK)

7 Key Scientists and Their Contribution to EMP Pathway

'Glycolysis' is a Greek word for 'splitting of sugar'. The elucidation of glycolytic pathway was a result of simultaneous work on muscle physiology and alcoholic fermentation by yeasts. The major contribution toward the microbial pathway was from French wine industry in late nineteenth century. Some of the key contributors are as follows:

Louis Pasteur	Demonstrated that alcoholic fermentation occurs in presence of live yeast cells. The view-point was formalized as VITALIST theory, i.e., living cells have a vital force
Buchner brothers (Hans Ernst August Buchner and Eduard Buchner)	First time demonstrated fermentation by cell-free yeast extract and refuted Pasteurs view of live cells Buchner brothers were preparing yeast paste by grinding it with sand. They filtered the extract through cheese cloth and wanted to preserve the juice. As the juice was supposed to be used as nutritional source, they did not preserve by conventional antiseptic, phenol but added sugar since it is a kitchen preservative. Suddenly, they noticed bubbles and realized that they have demonstrated cell-free alcoholic fermentation. However, this discovery was not paid attention for more than 10 years
Arthur Harden and William John Young	For the first time, measured CO_2 generated after incubating fresh yeast juice with glucose Harden and Young observed that CO_2 evolution decayed after sometime and it could be restored by addition of inorganic P. They observed that inorganic P disappeared during the course of incubation and thus suggested the formation of organic P which later isolated. We know this today as fructose 1,6-bisphosphate (FBP). Further, when FBP was added to fresh yeast juice, ethanol and CO_2 are formed Glucose?+?Pi???FBP???ethanol?+?CO_2 Discovered that low molecular weight cofactors are required for glucose fermentation Harden and Young demonstrated that fresh yeast juice can ferment glucose but dialyzed juice fails to do so and even did not make sugar phosphates. While searching for the dialyzable factor, they noticed that dialyzed juice regained activity by mixing the dialysate (liquid outside sac) suggesting that there are some low molecular weight activators. Next they showed that this low molecular weight activator is thermostable. Thus, they concluded that even phosphorylation of sugar requires heat stable low molecular weight cofactors which today we know as NAD^+/ADP/ATP/Mg^{2+}/TPP/K, etc. Arthur Harden along with Hans Karl August Simon von Euler-Chelpin was awarded the Nobel Prize in Chemistry in 1929 for the discovery of NAD^+
Carl Neuberg	Showed that reduction of acetaldehyde to ethanol was the last reaction of the fermentation process During various investigations, it was found that acetaldehyde could be reduced to ethanol by yeast. To confirm whether this was the last step in alcoholic fermentation, Neuberg added sodium bisulfite, a carbonyl trap. He found that there was no alcohol, instead there were equal amounts of glycerol, CO_2 and bisulfate adduct of acetaldehyde. Appearance of CO_2 at this stage suggested that precursor of acetaldehyde would be pyruvate as it was already known that yeast has pyruvate decarboxylase which can decarboxylate pyruvate to acetaldehyde and CO_2. This was later demonstrated by Embden

(continued)

(continued)

Gustav Embden	Demonstration of three carbon intermediates By using iodoacetate, Embden demonstrated accumulation of three carbon compounds, dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (PGA) from yeast extract fermenting either glucose or FBP. Therefore, Embden for the first time suggested aldolase reaction, though the enzyme was isolated later by Meyerhof Glucose/FBP???DHAP?+?PGA This was further confirmed by him by adding fluoride to actively fermenting yeast where he found new three carbon compounds, 2- and 3- isomers of phosphoglycerate. Thus, it was presumed that DHAP was reduced to PGA which in turn was oxidized to 3-phosphoglycerate and 2-phosphoglycerate Later, the requirement of reducing equivalents was established for this oxidation reduction reaction
Otto Fritz Meyerhof	Discovered hexokinase He demonstrated that fresh rabbit muscle extract converted glucose to lactate; however, lactate formation decreases with old muscle extract. Further, lactate formation is restored on adding dialyzed yeast extract to aged muscle extract. Following this experiment, Meyehof isolated hexokinase which loses its activity on standing *Hexokinase* Glucose?+?ATP???G6P???F6P?+?ATP???FBP???ethanol?+?CO_2
Jakub Karol Parnas	Otto Meyerhoff and Jakub Parnas continued the work on glycolysis after the sudden death of Embden in 1933. Finally, all the steps involved in glycolysis and the complete EMP pathway were proposed

Summary

- The major carbon metabolic pathways are glycolysis/glycolytic pathway/Embden–Meyerhoff–Parnas pathway (EMP), pentose phosphate pathway (PPP) and Entner–Doudoroff (ED) pathway.
- Glycolysis is a ubiquitous pathway present in all domains of life.
- Glycolysis is divided into two stages: The first stage is the energy utilizing stage, while the second stage is the energy generation stage.
- In the first stage, glucose is converted to glucose-6-phosphate to fructose-6-phosphate and then to fructose-1,6-bisphosphate utilizing two moles of ATP per mole of glucose.
- In the second stage, pyruvate is generated with net gain of two molecules each of ATP and NADH + H^+ per mole of glucose.
- Following energy generation via the electron transport system, seven molecules of ATP are generated from one round of glycolysis.
- Further aerobic oxidation of NADH is categorized as aerobic glycolysis, while anaerobic oxidation through fermentation is called anaerobic glycolysis.

- Glycolysis is a source of anapleurotic reactions for the generation of important biomolecules such as dihydroxyacetone phosphate, which is used as a precursor for phospholipid synthesis.
- Phosphofructokinase is allosterically inhibited by ATP, whereas pyruvate kinase shows precursor activation by fructose-1,6 bisphosphate.
- A modified glycolytic pathway exists in *Pyrococcus furiosus* wherein it does not take up glucose, instead use maltose or other disaccharides as the substrates. Instead of ATP, ADP is the high energy phosphate generated.
- *Clostridium thermocellum* has an atypical glycolytic pathway wherein the enzyme pyruvate kinase is absent. Instead, PEP is converted to pyruvate via the malate shunt. Also, there are two types of phosphofructokinase enzymes: One is ATP linked, while the other is pyrophosphate linked.
- During growth on non-carbohydrate sources such as pyruvate, lactate, acetate, fatty acids and amino acids, glucose can be synthesized via the gluconeogenic pathway to supply glycolytic precursors required for various biosynthesic reactions.
- Gluconeogenesis is not a simple reversion of glycolysis as three out of the ten reactions of glycolysis are not reversible due to requirement of large change in free energy.
- Alternative reactions are required for the steps involving conversion of pyruvate to PEP, Fructose 1,6-bisphosphate to fructose 6-phosphate and glucose 6-phosphate to glucose.
- The most common mode for conversion of pyruvate to PEP is via the action of enzymes pyruvate carboxylase and PEP carboxykinase.
- Pyruvate carboxylase carboxylates pyruvate to oxaloacetate. It is a biotin-dependent enzyme and is allosterically activated by acetyl CoA.
- PEP carboxykinase converts oxaloacetate to PEP. This conversion is the first committed step of gluconeogenesis.
- In organisms where pyruvate carboxylase is absent, PEP synthesis is via the action of other enzymes such as malate dehydrogenase, PEP synthetase and pyruvate-phosphate dikinase.
- During gluconeogenesis, fructose 1,6-bisphosphate is converted to fructose 6-phosphate by the enzyme fructose 1,6-bisphosphatase.
- During gluconeogenesis, glucose 6-phosphate is converted to glucose by the enzyme glucose 6-phosphatase.
- Gluconeogenesis and glycolysis are coordinated in a way that at a given time only one of these pathways is active while the other is inactive**.**
- Glucose determines the rate of glycolysis, while lactate and other precursors of glucose determine the rate of gluconeogenesis.
- The most important control point is the interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate.

Questions

1. What is the product of glycolysis under aerobic conditions?
2. Expand GAPDH.
3. Which reaction in EMP pathway requires inorganic phosphate?
4. Which reaction of glycolysis is an example of precursor activation?
5. Which enzyme is unique to glycolytic pathway of *Pyrococcus furiosus*?
6. What is gluconeogenesis?
7. How is PEP synthesized in *E. coli*?
8. Give details about a gluconeogenic enzyme.
9. Give details about a biotin-dependent enzyme.
10. Write the irreversible steps of EMP pathway.
11. How is PEP generated during gluconeogenesis in *E. coli* and other bacteria?
12. What is atypical about glycolysis in *Clostridium thermocellum*?
13. What is the key determinant for operation of gluconeogenesis?
14. What is aerobic and anaerobic glycolysis?
15. Describe regulation of glycolysis.
16. Explain how iodoacetate inhibits glycolysis.
17. PFK is an allosteric enzyme. Explain how ATP can function both as a substrate and as a regulator for its activity.
18. Explain the importance of the following enzymes in gluconeogenesis:
    i. pyruvate cdarboxylase
    ii. PEP carboxykinase
    iii. PEP synthetase and pyruvate-phosphate dikinase
    iv. Malic enzyme
19. What is methyloxal and under which conditions is it formed?

References

Sato, T., & Atomi, H. (2011). Novel metabolic pathways in Archaea. *Current Opinions in Microbiology, 14*(3), 307–314.

Verhees, C. H., Kengen, S. W. M., Tuininga, J. E., Schut, G. J., Adams, M. W. W., De Vos, W. M., & Der Oost, J. V. (2003). The unique features of glycolytic pathways in Archaea. *Biochemical Journal, 375*(Pt 2), 231–246.

Weber, J., Kayser, A., & Rinas, U. (2005). Metabolic flux analysis of *Escherichia coli* in glucose-limited continuous culture. II. Dynamic response to famine and feast, activation of the methylglyoxal pathway and oscillatory behavior. *Microbiology, 151,* 707–716.

Zhou, J., Olson, D. G., Argyros, D. A., Deng, Y., van Gulik, W. M., van Dijken, J. P., & Lynd, L. R. (2013). Atypical glycolysis in *Clostridium thermocellum*. *Applied and Environmental Microbiology, 79*(9), 3000–3008.

Chapter 10
Pentose Phosphate Pathway

Rani Gupta and Namita Gupta

1 Reactions of Pentose Phosphate Pathway

Pentose phosphate pathway (PPP) is one of three key central metabolic pathways. It is ubiquitous, present in both prokaryotes and eukaryotes, and occurs in cytosol. It is a major source of the reductant, NADPH and pentose sugars for the biosynthesis of nucleotides. Also, PPP is the main pathway for pentose utilization. It is also known as hexose monophosphate shunt or the Warburg–Lipmann–Dickens–Horecker pathway. The main functions of PPP pathway are:

- It is a major source of the reductant NADPH which is required in synthesis of fatty acids, triacylglycerides and steroids.
- It provides pentose sugars for anabolism of amino acids, vitamins, nucleotides and cell wall constituents.
- PPP is the main pathway for breakdown of pentose sugars.
- Its flux is regulated to prevent oxidative stress and redox homeostasis.

However, no ATP is directly produced in this pathway. The pathway consists of two irreversible oxidations and a series of reversible interconversions of sugar phosphates. Pentose pathway occurs in cytosol. Unlike glycolysis and citric acid cycle where reactions are directional, here interconversion of sugar phosphates can take place in a variety of ways. Pentose phosphate pathway is composed of two phases, oxidative or irreversible phase and non-oxidative or reversible phase (Fig. 1). Pentose phosphate pathway also contributes to serve as a source of biosynthetic intermediates for the synthesis of nucleotides, amino acids, vitamins, lipopolysaccharide and also fatty acids. It also plays an important role in counteracting oxidative stress by maintaining metabolic and redox homeostasis (Stincone et al. 2015).

Oxidative Phase:
There are two oxidative irreversible reactions which finally result in breakdown of glucose-6-phosphate to ribulose-5-phosphate and CO_2 along with generation of reductant, $NADPH+H^+$.

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_10

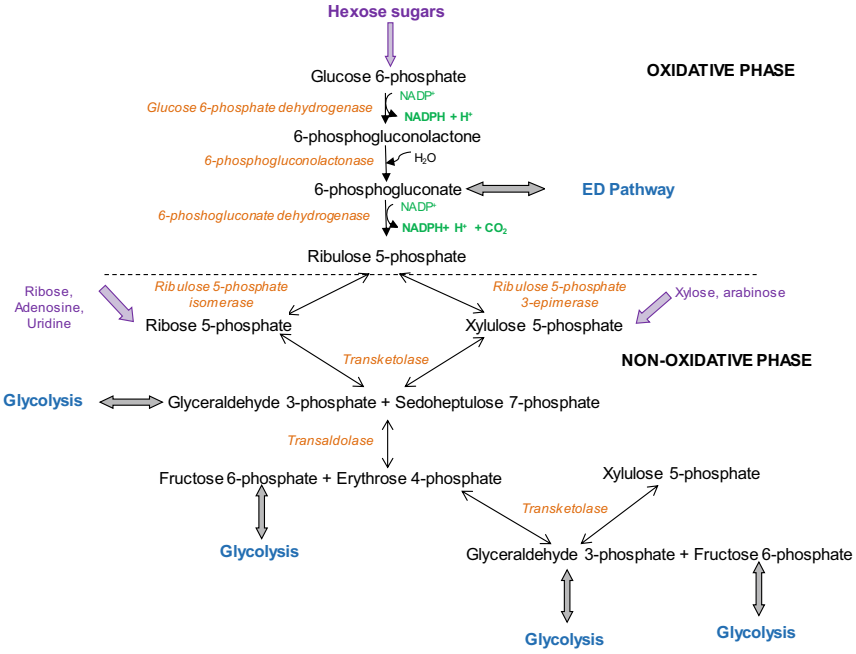
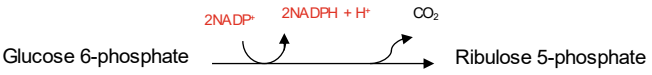
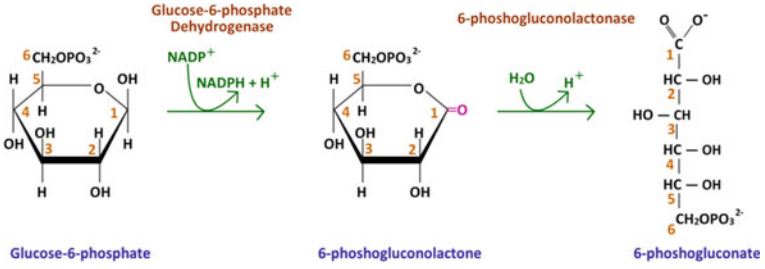


Fig. 1 Pentose phosphate pathway and the enzymes involved



1. **Dehydrogenation of glucose-6-phosphate**:
This reaction is catalyzed by the enzyme glucose-6-phosphate dehydrogenase commonly called G6PDH. This is specific for $NADP^+$ as coenzyme. This is an irreversible reaction, and PPP is regulated at this step primarily by the availability of reductant. The enzyme is inhibited by high $NADPH/NADP^+$ ratio.



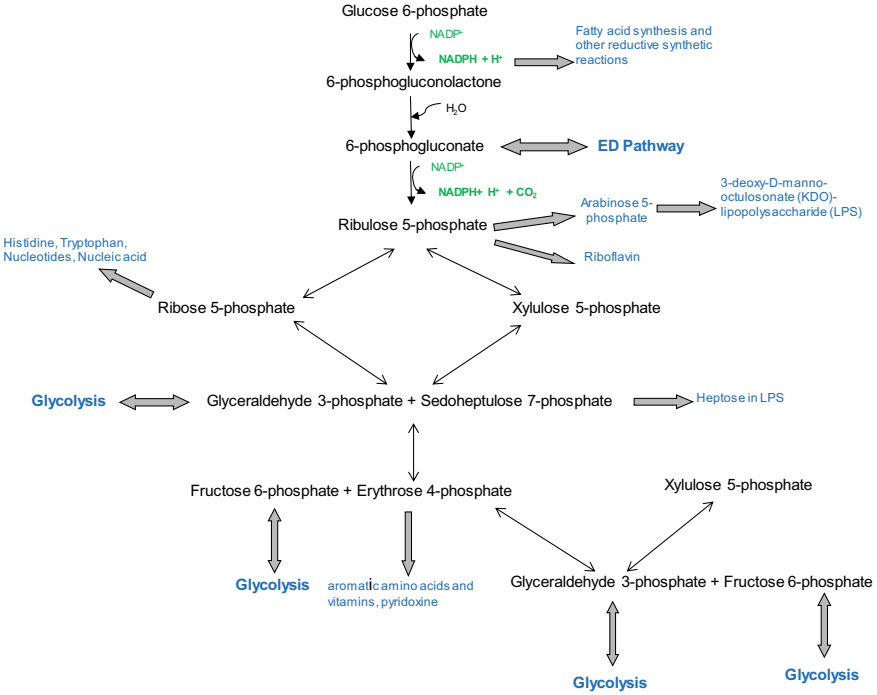
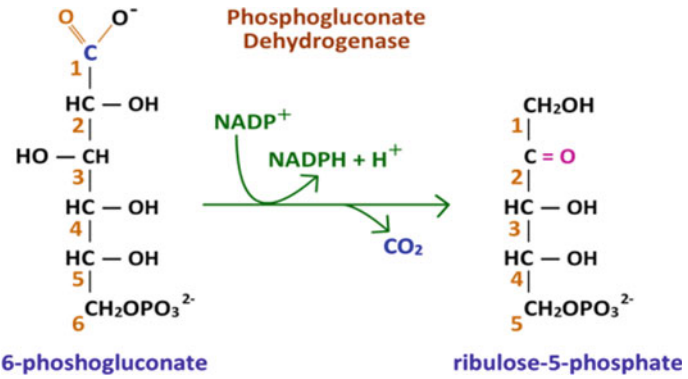


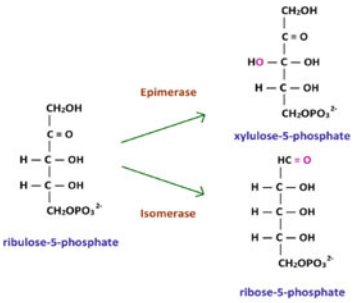
Fig. 2 Pentose phosphate pathway as a source of biosynthetic intermediates

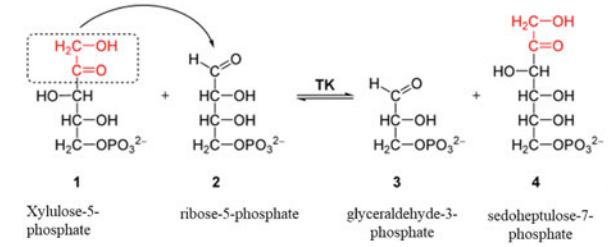
2. **Oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate** Oxidative decarboxylation of 6-phosphogluconate is catalyzed by 6-phosphogluconate dehydrogenase which is an irreversible reaction and uses $NADP^+$ as coenzyme. The reaction results in conversion of 6-carbon sugar to 5-carbon sugar, ribulose-5-phosphate, with concomitant evolution of CO_2.



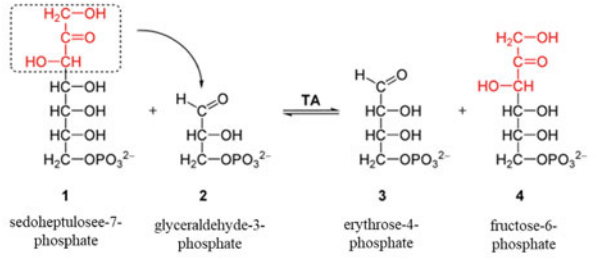
Non-oxidative reversible reactions—Sugar re-arrangements:

There are several reactions starting with ribulose-5-phosphate, where 5-carbon, 7-carbon, 6-carbon and 3-carbon compounds are generated. Thus, ribulose-5-phosphate is converted to ribose-5-phosphate required for nucleotide biosynthesis and also phosphoglyceraldehyde and fructose-6-phosphate where it links with glycolysis for complete oxidation of sugar.





Transketolase Reaction



Transaldolase Reaction

Thumb-rule of Transketolase and Transaldolase Reactions

- Both catalyze transfer of carbon moiety from a ketose donor to an aldose acceptor
- The α carbon of the ketose donor has the -OH moiety on the left side (i.e. the donor is a D-sugar)
- Transketolase catalyzes transfer of 2-carbon molecule while transaldolase catalyzes transfer of 3-carbon molecule

Pentose phosphate pathway as a source of biosynthetic intermediates

Pentose phosphate pathway generates several biomolecules which serve as intermediates in different metabolic pathways as depicted in Fig. 2.

Linking pentose phosphate pathway to glycolysis

As can be seen, pentose phosphate pathway acts as a source of precursors for various pathways. Both PPP and glycolysis feed into each other at various points. This interlinking is important in various scenarios, some of which are highlighted below:

- When the requirement of reductant (NADPH) is more than requirement of pentoses (such as ribose-5-phosphate) for biosynthetic pathways (such as nucleotide synthesis)—In such conditions, the pentoses instead of being fed into the biosynthetic pathway are converted to glyceraldehyde-3-phosphate and fructose-6-phosphate by the action of transketolase and transaldolase. Both these intermediates are then fed into glycolysis.
- In bacteria lacking stage 1 of EMP pathway such as phosphofructokinase activity and also enzymes of Entner–Doudoroff pathway (*i.e.*, 2-keto-3-deoxyphosphogluconate dehydratase—KDPG dehydratase), e.g., in species of *Thiobacillus*—In such conditions, PPP is the major catabolic pathway. Here, glucose is oxidized to glucose-6-phosphogluconate and then to phosphoglyceraldyde via PPP. Finally, phosphoglyceraldehyde is oxidized to pyruvate via stage 2 of EMP pathway. Thus, glucose can be completely oxidized in bacteria with a defective or mutated enzyme of stage I EMP via the pentose phosphate pathway.
- When requirement of pentoses for incorporation into nucleotides is high—In such conditions, the reversible non-oxidative sugar arrangements can synthesize ribose sugars starting from fructose-6-phosphate. Thus, 5-carbon ribose sugar can be synthesized from 6-carbon sugar.

2 Pentose Phosphate Pathway During Oxidative Stress Response

Pentose phosphate pathway plays an important role in counteracting oxidative stress (Stincone et al. 2015) by the following means:

- Maintaining metabolic and redox homeostasis via $NADP^+$ to NADPH reduction
- Synthesizing ribose 5-phosphate required for increased DNA synthesis due to DNA damage under stress
- Activating stress-responsive gene expression.

On exposure to an oxidizing agent such as H_2O_2, activity of pentose phosphate pathway increases rapidly in cells. This transition is brought about by metabolic and gene regulatory mechanism as outlined below:

- Within a few seconds of exposure, an oxidative inhibition of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) occurs which blocks glycolysis and increases the flux toward pentose phosphate pathway. Additionally, pyruvate kinase (PYK) is also inhibited due to the reactive oxygen species

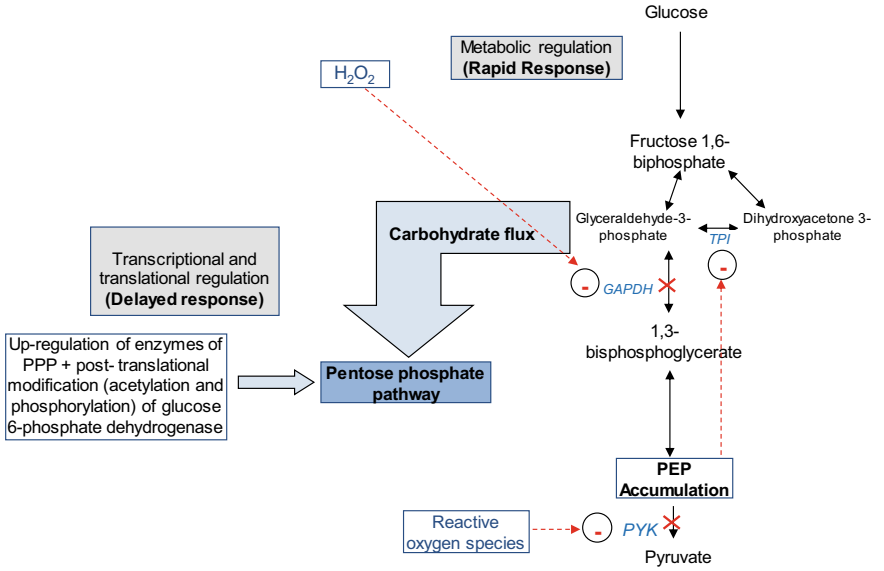


Fig. 3 Regulation of pentose phosphate pathway during oxidative stress

that leads to accumulation of its substrate PEP. This in turn causes feedback inhibition of several glycolytic enzymes including triose phosphate isomerase (TPI), which is also regulated by cellular redox. This leads to an increase in the flux toward PPP (Fig. 3).

- This is followed by a transcriptional up-regulation of PPP enzymes and post-translational modifications that increase the activity of glucose 6-phosphate dehydrogenase (G6PDH). This tight regulation prevents over-production of NADPH and PPP intermediates during normal growth while facilitating a rapid response under stress conditions.

Organization of genes of pentose phosphate pathway (Sprenger, 1995)

The genes corresponding to the main enzyme of pentose phosphate pathway are depicted in Table 1. All genes of the PPP are monocistronic except *rpe* and *tktB*. All the genes are constitutive in expression except *rpiB* which is regulated by RpiR.

gnd -

- Monocistronic located close to *his* and *rfb* gene clusters.
- It is regulated by growth rate and high titers are observed in glucose grown cells than in acetate grown cells.
- It is highly polymorphic in populations of *E. coli* and other enteric bacteria.

rpe -

- It is a multicistronic gene

Table 1 Genes corresponding to main enzymes of PPP

Enzyme	Gene
Glucose 6-phosphate dehydrogenase	*zwf*
6-phosphogluconolactonase	*pgl*
NADP-dependent 6-phoshogluconate dehydrogenase	*gnd*
Ribulose 5-phosphate isomerase	*rpi*
Ribulose 5-phosphate 3-epimerase	*rpe*
Transaldolase	*talB*
Transketolase	*tktA*

- *rpe* is clustered with other genes required in the biosynthesis of aromatic amino acids (*aroK*, *aroB*) and tryptophan (*trpS*).
- It serves as a first example of coregulation with other biosynthetic enzymes.

rpi -

- There are two isozymes of this enzyme encoded by *rpiA* and *rpiB*. Of these, *rpiB* is mostly restricted to bacteria and is absent in mammals.
- *E. coli* has both *rpiA* and *rpiB*.
- *rpiA* is a homodimer and is constitutive in nature. It is the main enzyme in *E. coli*.
- *rpiB* is inducible in presence of ribose or ribose 5-phosphate producing compounds; however, its expression is low even under induced conditions. Its induction is done by RpiR-mediated de-repression in the presence of ribose or ribose 5-phosphate producing compounds.
- Many pathogenic organisms have only *rpiB* which is essential for synthesis of ribose sugar, a precursor for nucleotide synthesis. Further, *rpiB* is absent in mammals, and thus, it has been recognized as a potential drug target.

tkt -

- Transketolases are linked to several pathways for catabolism of pentoses and production of ribose 5-phosphate.
- There are two genes, *tktA* and *tktB* encoding transketolase activity that have 74% identity.

tal -

- There are two genes, *talA* and *talB* encoding transaldolase activity; however, only *talB* function has been established.

Box 1: RpiB: Potential Drug Target (Louriero et al. 2015; Kaur et al. 2012)

Ribose 5-phosphate isomerase B (RpiB) can be a potential drug target for targeting several pathogenic diseases. RpiA is present in most eukaryotic organisms; however, RpiB exists mostly in prokaryotes with few exceptions in protozoal parasites and fungi. RpiA and RpiB share very low sequence identity and are structurally very different; thus molecules can be designed to selectively target RpiB.

It has already been looked upon as promising therapeutic target for protozoan diseases such as African sleeping sickness caused by *Trypanosoma brucei*, Giardiasis by *Giardia lamblia* and Leishmaniasis by *Leishmania donovani*. Recent reports of RpiB knockdown from *Trypanosoma brucei* show lower bloodstream infectivity proving its potential as drug target. RpiB has also been characterized from a pathogenic fungus, *Coccidioides immitis*, causing coccidioidomycosis commonly known as Valley fever. Anti-tuberculosis drugs can also be designed as *Mycobacterium tuberculosis* also has RpiB. Thus, designing inhibitors specific for RpiB can be a potential approach to target various diseases.

Box 2: Human Metabolic Diseases Due to Deficiency/Mutations in PPP Enzymes (Wamelink et al. 2008)

- **G6PDH (Glucose-6-phosphate dehydrogenase) deficiency**—It is the most common heritable enzyme defect in humans that occurs most often in males. It leads to neonatal hyperbilirubinemia and chronic hemolytic anemia.
- **RPI (Ribulose 5-phosphate isomerase) deficiency**—It is the rarest human disorder with only one patient diagnosed of this disease (in 1984) in medical history. As a consequence of the RPI disorder, the patient developed a brain disease called leukoencelopathy and peripheral neuropathy. The diagnosed case of RPI deficiency was reported in 1999. It took 15 years for the experts to establish a relation between the cause and effect. No new case of this disease has been reported thereafter.
- **TAL (Transaldolase) deficiency**—It is a rare disorder with about 30 patients worldwide. The leading symptoms of TAL deficiency are coagulopathy, thrombocytopenia, hepatosplenomegaly, hepatic fibrosis and dysmorphic features.
- **H6PDH (hexose-6-phosphate dehydrogenase) deficiency**—This is associated with hirsutism, oligomenorrhea, obesity, acne and infertility. H6PDH catalyzes the first two reactions of endoluminal PPP thus producing NADPH

within the endoplasmic reticulum. It is distinct from the cytosolic glucose-6-phosphate dehydrogenase (G6PDH). It uses a separate pool of $NADP^+$ and is capable of oxidizing several phosphorylated hexoses.

3 Pentose Phosphate Pathway in Heterofermentative Lactate Fermentation

Heterofermentative lactate fermentation leads to lactate production via pentose phosphate pathway using an interesting phosphoketolase reaction wherein xylulose-5-phosphate is cleaved to form phosphoglyceraldehyde and acetyl phosphate using inorganic phosphate (Fig. 4). This enzyme requires thiamine pyrophosphate (TPP) as

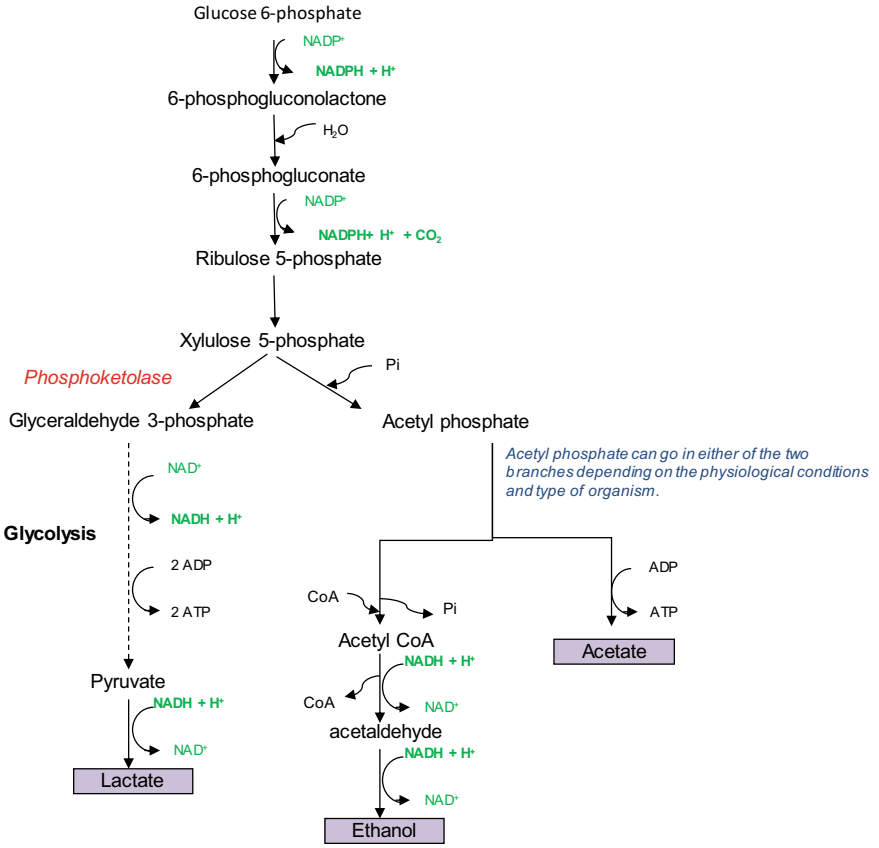


Fig. 4 Phosphoketolase pathway in heterofermentative lactate fermentation

a cofactor. This reaction is catalyzed by phosphoketolase; hence it is called the phosphoketolase pathway. Heterofermentative lactic acid bacteria include leuconostocs, some lactobacilli, *Oenococci* and *Weissella* species.

The phosphoglyceraldehyde is oxidized to pyruvate using one NADH + H^+. Pyruvate is then converted to lactate regenerating one NAD^+. This is the same pathway that is followed in homofermentative bacteria.

The acetyl phosphate has two possible fates, depending on the environmental conditions:

1. Acetyl phosphate can be reduced into acetaldehyde and then to ethanol using 2 mol of reductants produced during oxidation of glucose. Thus, all the three moles of reductants are regenerated. In this case, the final end products are lactate and ethanol. *Leuconostoc* preferentially produces lactate and ethanol under low aeration conditions as this helps in re-generation of reductants.
2. Acetyl phosphate can be converted to acetate by the action of acetate kinase. This reaction yields one molecule of ATP. In this case, the final end products are lactate and acetate. *Leuconostoc* preferentially produces lactate and acetate in an aerated environment that helps in generation of energy in the form of ATP.

4 Pentose Degradation in Archaea

Archaea do not use pentose phosphate pathway for the degradation of pentoses. Instead, degradation of pentoses involves firstly their oxidation by glucose dehydrogenase, which in addition to glucose has extended catalytic specificity toward C5 sugars such as arabinose and xylose. Subsequently, these oxidized pentoses are broken down by ED pathway. This pathway has been discussed in greater detail in Chap. 11: Entner–Doudoroff pathway.

5 Improving Regeneration of NADPH

Availability of NADPH is a bottleneck in many biotransformation reactions involved in production of antibiotics, polymers, amino acids, alcohols, etc. Various approaches are being explored to engineer NADPH generating pathways. Lee et al. (2013) have reviewed the various strategies being adopted to improve NADPH re-generation:

NADPH is primarily generated by three main pathways:

1. Pentose phosphate pathway
2. Citric acid cycle
3. Transhydrogenases system.

1. **Pentose phosphate pathway**:
   The oxidative phase of PPP, catalyzed by glucose 6-phosphate dehydrogenase (G6PGH) and 6-phosphogluconate dehydrogenase (Gnd), is involved in

NADPH generation. Many studies have focused on modulation of PPP in *E. coli* to increase NADPH availability. The key approaches are summarized below:

a. Over-expression of genes for G6PGH and/or Gnd: G6PGH is more commonly over-expressed as this increases the flux toward Gnd reaction as well. Studies have shown that over-expression of G6PGH was three times more effective in NADPH regeneration as compared to over-expression of Gnd.
b. Re-directing metabolic flux from glycolysis to PPP:
   - Deletion or down-regulation of *pgi* gene encoding phosphoglucoisomerase that shunts glucose 6-phosphate toward glycolytic pathway was reported to increase NADPH dependent reactions. Down-regulation of *pgi* gene has been done by changing initiation codon from ATG to GTG (i.e., methionine to valine).
   - Deletion of phosphofructokinase genes (*pfkA* and *pfkB*) increased the metabolic flux toward PPP as fructose 6-phosphate is not converted to fructose 1,6-bisphosphate and can instead enter PPP by the action of phosphoglucoisomerase (*pgi*).
c. Expression of heterologous glucose dehydrogenase: Glucose dehydrogenase (GDH/G6PGH) oxidizes glucose to glucono 1,5-lactone with concomitant generation of NADPH. Glucono 1,5-lactone can then be converted to 6-phosphogluconate during the oxidative phase of PPP. Thus, heterologous GDH from various *Bacillus* sp. was over-expressed in *E. coli.* However, GDH uses glucose and not glucose 6-phosphate as a substrate (glucose enters as glucose 6-phosphate through PEP-PTS system which is the major uptake system in *E. coli*). Thus, efforts were made to increase direct uptake of glucose and to achieve this glucose facilitator gene (*glf*) from *Zymomonas mobilis* was coexpressed. This led to a substantial increase in biotransformation rates.
d. Deleting NADPH (or NADH) utilizing pathways: Deletion of genes involved in NADPH (or NADH) utilizing branches of glycolysis such as fermentation pathway (e.g., *adhE* encoding alcohol dehydrogenase and *ldh* encoding lactate dehydrogenase) or respiratory pathway (e.g., *ndh* encoding NADH dehydrogenase).

2. **Citric acid cycle**:
During the TCA cycle, isocitrate dehydrogenase is involved in NADPH generation. Further, action of the anapleurotic enzyme, malic enzyme also generates NADPH. Thus, both isocitrate dehydrogenase (*icd*) and malic enzyme (*maeB*) were over-expressed in *E. coli*. Results indicated that effect of over-expression of *icd* was at par with over-expression of G6PDH. Further, over-expression of *maeB* was detrimental to biotransformation rates. This could be due to activation of anapleurotic pathways at certain specific metabolic states only.
Thus, *icd* and *maeB* did not prove to be better options than G6PDH.

3. **Transhydrogenase system**:
In *E. coli*, NADPH can be regenerated by transhydrogenase system that includes two isoforms of transhydrogenase enzyme which can transfer electrons from NADH to NADPH and vice versa (Sauer et al. 2004):

- Membrane-bound transhydrogenase (PntAB) is energy-dependent and transfers hydrogen ion from NADH to $NADP^+$ to generate NADPH under conditions of low NADPH.
- Soluble transhydrogenase (UdhA) is energy-independent and transfers a hydrogen ion from NADPH to NAD^+ under conditions of high NADPH.

Over-expression of both PntAB and UdhA has been studied and both were reported to increase NADPH availability in *E. coli.*

Box 3: Balancing NADPH Over-Production with Carbon Flux

It should be noted that increasing NADPH production may not always lead to increased production of the desired biomolecule unless it is balanced with carbon flux into the required metabolic pathway such as EMP or PPP.

For example, over-production of the biopolymer polyhydroxybutyrate (PHB) requires not just increased NADPH production but also increased production of acetyl CoA, the precursor for PHB. This has been successfully achieved by over-expression of *tkt* gene (encoding for transketolase enzyme) that leads to accumulation of glyceraldehyde 3-phosphate which is subsequently converted to acetyl CoA (Lee et al. 2003).

6 Engineering *E. Coli* for Cometabolism of Glucose and Xylose to Yield Ethanol: A Case Study (Chiang et al. 2013)

Coutilization of xylose and glucose, if possible, would make simultaneous utilization of hexose and pentose generated from the hydrolysis of lignocellulosic wastes. Lignocellulosics are primarily made up of cellulose (source of hexose) and hemicelluloses (source of pentoses, largely xylose), besides lignin. The sugar mixture is prepared by delignification followed by hydrolysis.

Before going further, the first step is introduction of alcohol fermentation enzymes in *E. coli* since *E. coli* is a poor alcohol producer and also it produces alcohol from acetic acid. So, for direct conversion of pyruvate to alcohol, plasmid containing two genes, viz. pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhII*) from *Zymomonas mobilis,* is transformed in *E. coli* BL21 strain and named as BL-21-PA.

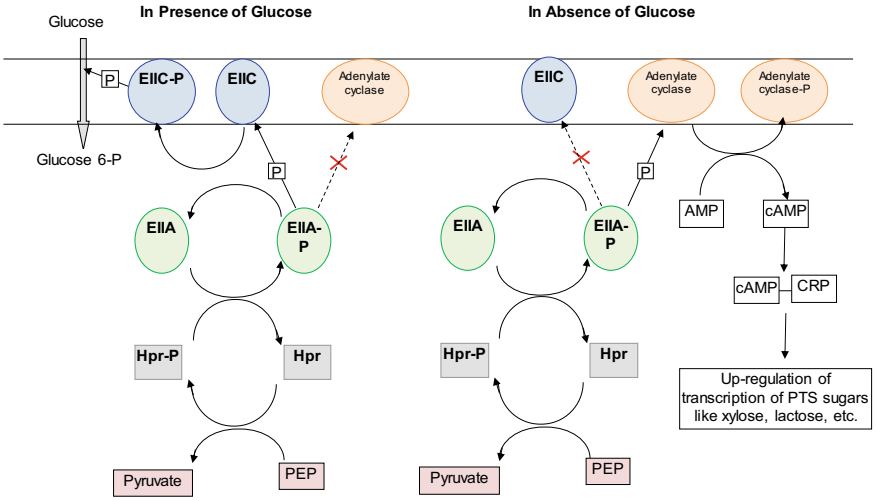


Fig. 5 Catabolite repression of PTS sugars in presence of glucose

This strain, when grown in the presence of both glucose and xylose, utilized all the glucose but very little xylose as xylose utilization is under catabolite repression.

Thus, a main problem of coutilization is the presence of catabolite repression. Due to this repression, the carbohydrates are used successively with glucose as the preferred sugar. Therefore, the organism growing on mixture of glucose and xylose would show diauxic growth curve, where first curve shows utilization of glucose followed by second curve concomitant with xylose utilization. Such catabolite repressions are due to depletion of cAMP-CRP complex which is a transcriptional activator for sugars other than glucose (Fig. 5).

Glucose is a PEP-PTS sugar and requires $EIIC^{gluc}$ (coded by *ptsG)* for its uptake. $EIIC^{gluc}$ is phosphorylated by $EIIA^{gluc}$ in the presence of glucose. On dephosphorylation, $EIIA^{gluc}$ lowers adenylate cyclase activity and hence cAMP. This in turn decreases the level of cAMP-CRP complex which is required for transcription of xylose operon.

Hence, the first hurdle in cometabolism of glucose and xylose is catabolite repression which needs to be de-regulated. For this, the strain BL-21-PA was further modified by deleting *ptsG* gene and termed as BL-G strain as suggested by a previous study (Chatterjee et al. 2001). Now, this strain utilized both the sugars with 60% of xylose being utilized. The alcohol yields were higher, but glucose utilization rate was lower showing that impairing *ptsG* gene slowed down glucose uptake and thus its utilization rates also decreased.

Glucose facilitation gene *glf* from *Z. mobilis* was known to function in *E. coli* (Parker et al. 1995). Therefore, *glf* gene was inserted in BL-G and consequently termed as BL-G2. Fermentation was studied, and it was observed that glucose utilization improved in the strain without affecting xylose utilization.

Xylose utilization rates can only be improved by working on pentose phosphate pathway enzymes, viz. ribulose 5-phosphate isomerase (*rpi*), ribulose 5-phosphate 3-epimerase (*rpe*), transketolase (*tktA*) and transaldolase (*talB*). Extra copies of *rpe- tktA and rpiA-talB* gene clusters were inserted with heterologous promoter in BL-G2 to generate a next strain named as BL-G3. This strain showed improved ethanol yields, but still whole of the xylose was not consumed. Also, ethanol had contaminating acids.

The above strain had improved glucose and xylose utilization with ethanol yields. However, ethanol had small amounts of organic acids such as acetate, lactate and succinate. To reduce the contamination and also improve metabolic flux to ethanol, various competing pathway genes, viz. *ldhA* (lactate dehydrogenase), *poxB* (pyruvate oxidase), *pta* (phosphate acetyl-transferase) and *frdA* (fumarate reductase), were all deleted to obtain next strain named BL-G4. Finally, both the sugars were simultaneously consumed with ethanol yields nearly 97% of theoretical yields.

Finally, *pdc* and *adhII* genes were integrated into the genome of BL-G4 to obtain a stable strain. This final strain which was plasmid-free could utilize both the sugars (30 g/L each) within the same time (16 h) with 27 g/L ethanol yields (Figs. 6 and 7).

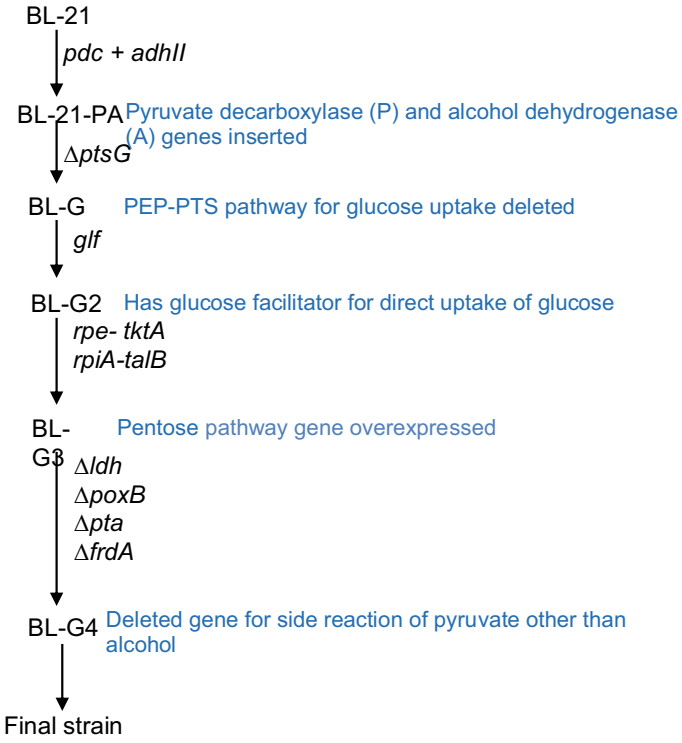


Fig. 6 Step-by-step *E. coli* strain engineering for coutilization of glucose and xylose for conversion to ethanol

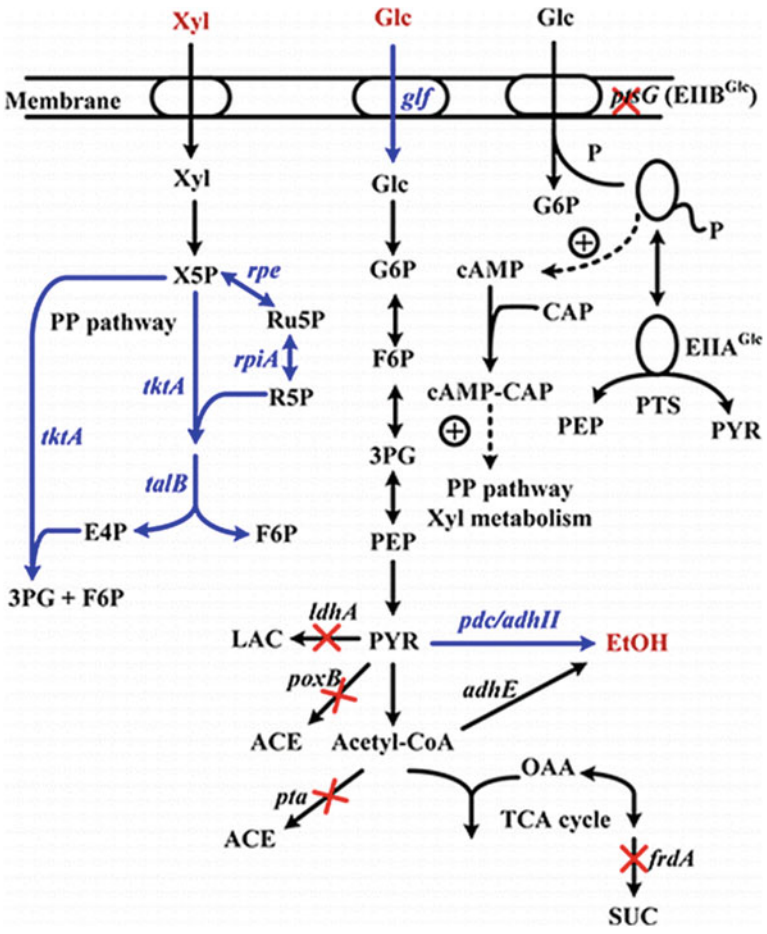


Fig. 7 Summary of the steps involved in metabolic engineering of *E. coli* for coutilization of glucose and xylose for conversion to ethanol. Blue indicates over-expression, and red cross indicates deletion. *Source* Chiang et al. (2013). With kind permission from American Chemical Society (ACS)

Summary

- Pentose phosphate pathway is ubiquitous and takes place in the cytosol.
- Pentose phosphate pathway (PPP) is an alternate pathway of glucose catabolism and a major source of the reductant, NADPH and pentose sugars for the biosynthesis of nucleotides.
- Pentose phosphate pathway is the main pathway for breakdown of pentose sugars.
- The pathway consists of an oxidative phase comprising of two irreversible oxidations and a non-oxidative phase comprising a series of reversible interconversions of sugar phosphates.

- Pentose phosphate pathway is a major catabolic pathway in bacteria lacking phosphofructokinase activity and also enzymes of Entner–Doudoroff pathway.
- Pentose phosphate pathway plays an important role in counteracting oxidative stress and maintaining redox homeostasis.
- All genes of pentose phosphate pathway are constitutive in expression except ribulose 5-phosphate isomerase B.
- It is interesting to note that the heterofermentative lactate fermentation produces lactate via a modified pentose phosphate pathway involving phosphoketolase enzyme.
- In archaebacteria, pentose degradation occurs via a modified Entner–Doudoroff pathway instead of the pentose phosphate pathway.

Questions

1. What is the role of pentose phosphate pathway in the cell?
2. Explain physiological conditions where pentose phosphate pathway would be linked to glycolysis and vice-versa.
3. Explain phosphoketolase pathway.
4. Draw the pentose phosphate pathway to show its anapleurotic role.
5. How $NADPH+H^+$ flux can be improved?
6. Pentose phosphate pathway plays an important role during oxidative stress. Explain.

References

Chatterjee, R., Millard, C. S., Champion, K., Clark, D. P., & Donnelly, M. I. (2001). Mutation of the *ptsG* Gene results in increased production of succinate in fermentation of glucose by *Escherichia coli*. *Applied and Environmental Microbiology, 67*(1), 148–154.

Chiang, C.-J., Lee, H. M., Guo, H. J., Wang, Z. W., Lin, L.-J., & Chao, Y.-P. (2013). Systematic approach to engineer escherichia coli pathways for co-utilization of a glucose-xylose mixture. *Journal of Agricultural and Food Chemistry, 61*(31), 7583–7590.

Kaur, P. K., Dinesh, N., Soumya, N., Babu, N. K., & Singh, S. (2012). Identification and characterization of a novel Ribose 5-phosphate isomerase B from *Leishmania donovani*. *Biochemical and Biophysical Research Communications, 421*(1), 51–56.

Lee, J. N., Shin, H. D., & Lee, Y. H. (2003). Metabolic engineering of pentose phosphate pathway in *Ralstonia eutropha* for enhanced biosynthesis of poly-beta-hydroxybutyrate. *Biotechnology Progress, 19,* 1444–1449.

Lee, W. H., Kim, M. D., Jin, Y. S., & Seo, J. H. (2013). Engineering of NADPH regenerators in *Escherichia coli* for enhanced biotransformation. *Applied Microbiology and Biotechnology, 97*(7), 2761–2772.

Loureiro, I., Faria, J., Clayton, C., Macedo-Ribeiro, S., Santarém, N., Roy, N., et al. (2015). Ribose 5-phosphate isomerase B knockdown compromises *Trypanosoma brucei* bloodstream form infectivity. *PLoS Neglected Tropical Diseases, 9*(1), e3430.

Parker, C., Barnell, W. O., Snoep, J. L., Ingram, L. O., & Conway, T. (1995). Characterization of the *Zymomonas mobilis* glucose facilitator gene product (*glf*) in recombinant *Escherichia coli*: examination of transport mechanism, kinetics and the role of glucokinase in glucose transport. *Molecular Microbiology, 15*(5), 795–802.

Sauer, U., Canonaco, F., Heri, S., Perrenoud, A., & Fischer, E. (2004). The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. *Journal of Biological Chemistry, 279*(8), 6613–6619.

Sprenger, G. A. (1995). Genetics of pentose-phosphate pathway enzymes of *Escherichia coli* K-12. *Archives of Microbiology, 164*(5), 324–330.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M., Campbell, K., Cheung, E., et al. (2015). The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biological Reviews of the Cambridge Philosophical Society, 90*(3), 927–963.

Wamelink, M. M. C., Struys, E. A., & Jakobs, C. (2008). The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: A review. *Journal of Inherited Metabolic Disease, 31*(6), 703–717.

Chapter 11
Entner–Doudoroff Pathway

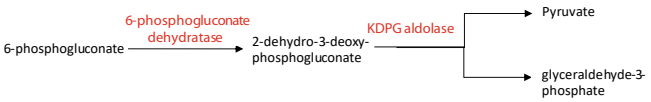
Rani Gupta and Namita Gupta

The Entner–Doudoroff (ED) pathway is one of three central metabolic pathways, in addition to Embden–Meyerhof–Parnas pathway (EMP) and pentose phosphate pathway (PPP). It is a fermentative pathway for glucose catabolism. It is by and large restricted to prokaryotes, both eubacteria and archae with few examples among eukaryotes, viz. *Aspergillus niger*, *Penicillium notatum* and *Entamoeba histolytica.* Evolutionarily, it seems to be more primitive than the EMP pathway. ED pathway can operate in a variety of modes ranging from linear to cyclic, inducible to constitutive or a modified non-phosphorylated variant (Conway, 1992).

1 Discovery of Entner–Doudoroff (ED) Pathway

The Entner–Doudoroff pathway was first discovered in *Pseudomonas saccharophila* in 1952 (Entner & Doudoroff, 1952). They reported evolution of C1-carbon of glucose entirely as CO_2, though the mechanism was not known at that time. Dinitrophenol poisoned cells (having decoupled electron transport chain) showed occurrence of C1-label of glucose in carboxyl group of pyruvate. In iodoacetate-poisoned cells (which is an inhibitor of 3-phosphoglyceraldehyde dehydrogenase), C1-label of glucose appeared in carboxyl groups of glyoxal and pyruvate. Same results were observed when gluconic acid was used in place of glucose. Later, the investigation of crude cell extracts led to the discovery of an enzyme that could cleave 6-phosphogluconic acid to pyruvate and glyceraldehyde-3-phosphate. It was speculated that 2-keto-3-deoxy-phosphogluconate (KDPG) was the likely intermediate which was subsequently crystallized from the enzyme preparation incubated with 6-phosphogluconate. Conversion of 6-phosphogluconate to pyruvate and glyceraldehyde-3-phosphate occurred in a two-step process via the action of the enzymes 6-phosphogluconate dehydratase and KDPG aldolase which were also purified and characterized.

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_11



2 Comparison Between EMP and ED Pathways

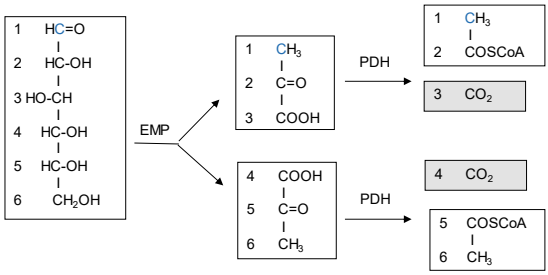
The overall schemes of the ED and EMP pathways are quite similar. In both the pathways, 6-carbon sugars are first primed by phosphorylation and then cleaved into two 3-carbon intermediates by aldolase enzymes. Further, metabolism of triosephosphate is common to both the pathways and leads to ATP generation by substrate-level phosphorylation. **The main demarcating reaction that differentiates between EMP pathway and ED pathway is the cleavage of 6-carbon intermediate by aldolase**.

In EMP pathway, fructose-1, 6-bisphosphate is cleaved into triose phosphate, viz. glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by the action of fructose bisphosphate aldolase. However, in Entner–Doudoroff pathway, 2-keto-3-deoxyphosphogluconate (KDPG) is cleaved into glyceraldehyde-3-phosphate and pyruvate by KDPG aldolase.

Thus, ED pathway has a net gain of one ATP by substrate-level phosphorylation as compared to gain of two ATP molecules in EMP pathway. Further, the total reducing equivalents generated by both the pathways is same. These reductants may lead to further energy gain depending on whether oxidative phosphorylation takes place or not (Fig. 1).

Radiorespirometry as a means to distinguish between EMP and ED pathways

EMP pathway—C1 carbon of glucose forms the methyl group of the first pyruvate molecule while the C4 carbon forms the carboxyl group of the second pyruvate molecule. Both the pyruvate molecules enter the TCA cycle. The C1 molecule is assimilated into various biosynthetic pathways, while the C4 molecule is released as CO_2 by the action of pyruvate dehydrogenase (PDH).



ED pathway—C1 carbon of glucose forms the carboxyl group of the first pyruvate molecule while C4 carbon forms the carboxyl group of the second pyruvate molecule.

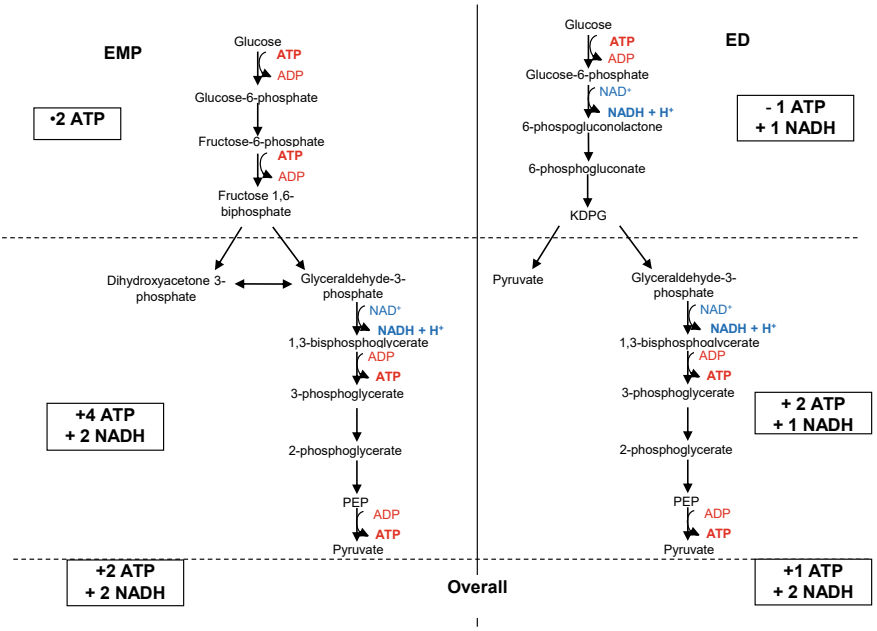
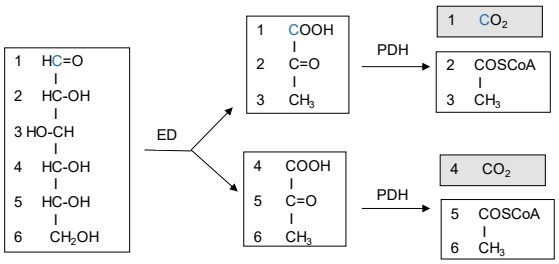


Fig. 1 Comparison between net energy and reductant gain in EMP and ED pathways

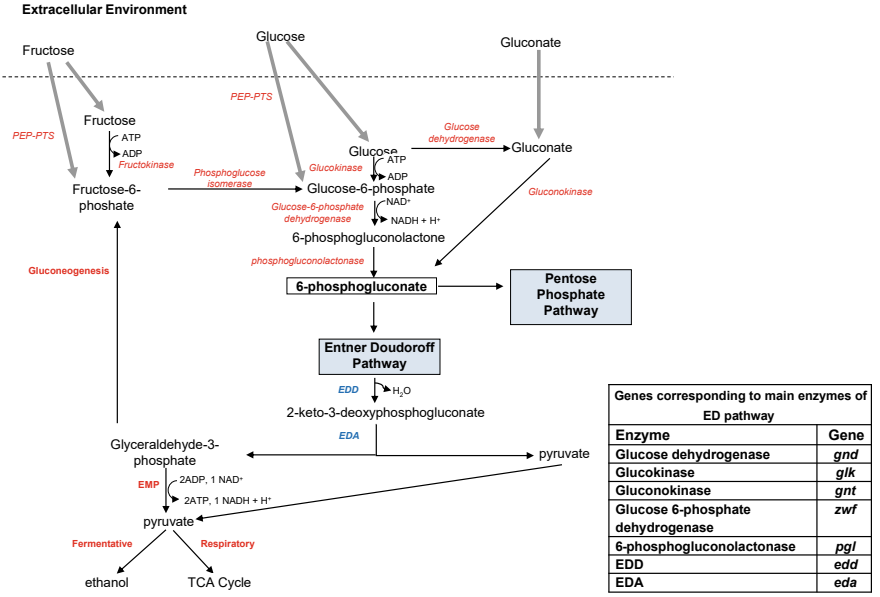
Both the pyruvate molecules can enter the TCA cycle. C1 and C4 carbons are both released as CO_2 by the action of pyruvate dehydrogenase.



Thus, if C1-labelled glucose is used, an early time course of appearance of labelled CO_2 serves as an indication of ED metabolism.

3 Key Reactions of ED Pathway

The ED pathway starts with the formation of 6-phosphogluconate which can enter either the ED pathway or the pentose phosphate pathway. On entering the ED



Genes corresponding to main enzymes of ED pathway	
Enzyme	**Gene**
Glucose dehydrogenase	*gnd*
Glucokinase	*glk*
Gluconokinase	*gnt*
Glucose 6-phosphate dehydrogenase	*zwf*
6-phosphogluconolactonase	*pgl*
EDD	*edd*
EDA	*eda*

Fig. 2 Representation of various steps/modes of Entner–Doudoroff pathway

pathway, 6-phosphogluconate is converted to KDPG which in turn is split into pyruvate and glyceraldehyde-3-phosphate (Fig. 2).

The first key enzyme which is unique to ED pathway is **6-phosphogluconate dehyratase** commonly known as Entner–Doudoroff dehydratase (EDD). It is encoded by *edd* gene. The enzyme catalyzes the dehydration of 6-phosphogluconate to form 2-keto3-deoxy-6-phosphogluconate (KDPG). The second key enzyme is KDPG aldolase, also known as Entner–Doudoroff aldolase (EDA). It is encoded by *eda* gene. It catalyzes aldol cleavage of KDPG to form glyceraldehyde-3-phosphate and pyruvate. KDPG aldolase is known as Entner–Doudoroff aldolase (EDA) and the gene coding is designated as *eda.* In contrast to EDD, EDA is a multifunctional enzyme found in a wide range of organisms. EDA can not only catalyze interconversion of KDPG with glyceraldehyde-3-phosphate and pyruvate, it is also capable of catalyzing interconversion of 2-ketohydroxyglutarate with pyruvate and glyoxylate, as well as β-oxidation of oxaloacetate where it is called KHG aldolase. In an interesting study, Gupta and Dekker (1984) showed involvement of KHG aldolase in complete mineralization of glyoxylate to carbon dioxide. The KHG aldolase catalyzes condensation of glyoxylate with pyruvate to KHG which is further metabolized by TCA cycle via α-ketoglutarate dehydrogenase, citrate synthase, malic enzymes, malate dehydrogenase followed by β-decarboxylation of oxaloacetate resulting in the formation of CO_2 and pyruvate.

4 Modes of ED Pathway

The Entner–Doudoroff pathway can operate in several modes (Conway, 1992). Provided below is a representation of the major variant modes of the ED pathway (Fig. 3).

Inducible linear pathway in enteric bacteria

In several bacteria, including enteric bacteria (such as *Escherichia coli*), EMP pathway and pentose phosphate pathway are the major pathways for carbohydrate metabolism. ED pathway is induced only when gluconate is available, and in such a condition, it becomes a major catabolic pathway for glucose oxidation (Peekhaus & Conway, 1998a.)

Thus, in the presence of gluconate, the enzymes of ED pathway, gluconate transport system and gluconokinase are induced simultaneously. Gluconate is transported in *E. coli* by active transport system and is phosphorylated to 6-phosphogluconate by gluconokinase, and further, the pathway is continued by EDD and EDA enzymes. This can occur both aerobically in conjunction with TCA or anaerobically in a fermentative mode wherein ethanol is produced via pyruvate conversion to acetyl CoA. Thus, in enteric bacteria during growth on gluconic acid, the major flow of carbon is via ED pathway with a minor flow through PPP (Fig. 4).

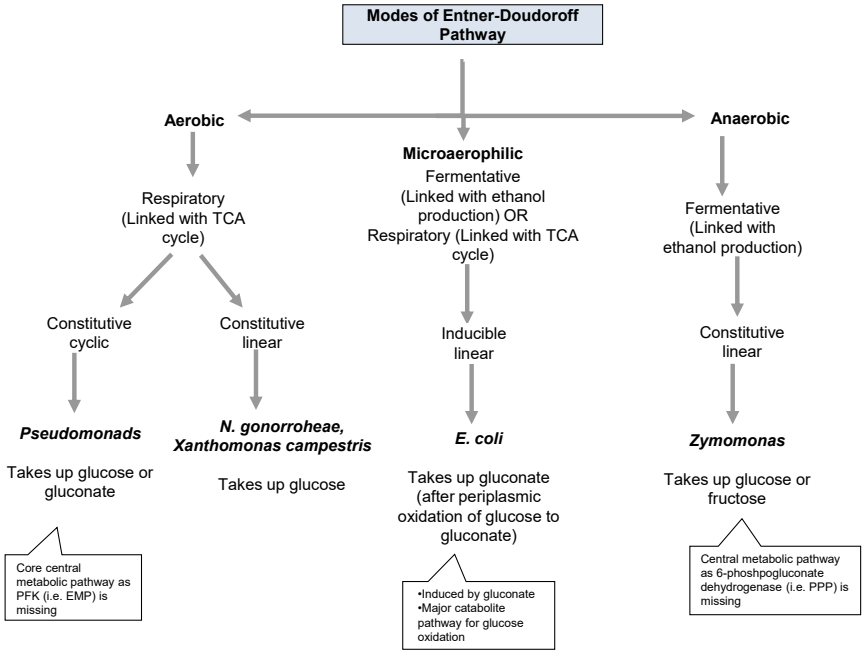


Fig. 3 Various modes of Entner–Doudoroff pathway

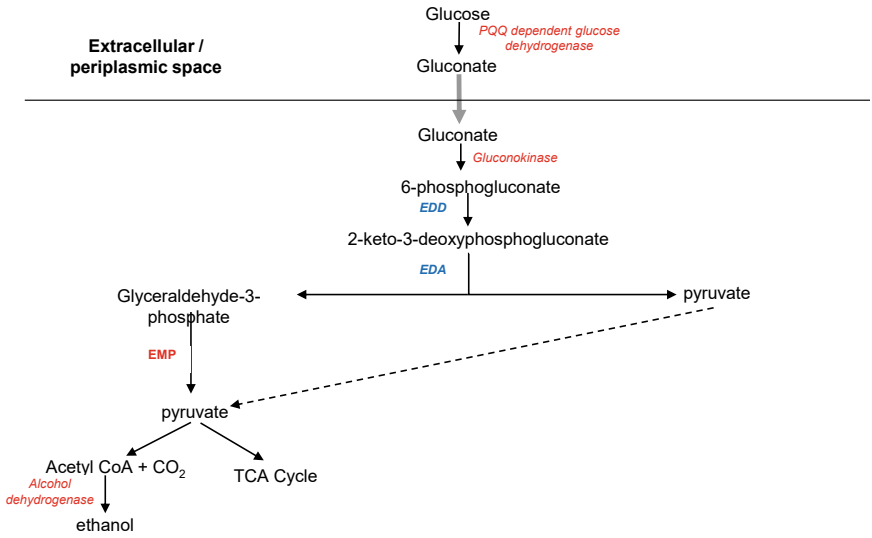


Fig. 4 Inducible linear ED pathway in *E. coli*

Advantages of ED pathway in *E. coli*: In *E. coli*, ED pathway operates in aerobic conditions and is stimulated by phosphate starvation, a condition generally prevalent in the aquatic and intestinal environment. Phosphate starvation induces PhoE porin and facilitates pyrroloquinoline quinone (PQQ) uptake from environment as wild-type *E. coli* does not synthesize PQQ. This stimulates glucose oxidation to gluconate by PQQ-dependent glucose dehydrogenase. Thus, ED pathway serves as an advantage for energy generation during carbon (in terms of glucose) and phosphate limitation and allows survival in extra-intestinal and aquatic environments. This is further supported by the fact that the presence of (PQQ) along with glucose results in higher growth yields than glucose alone.

Constitutive linear pathway in *Zymomonas mobilis*

Zymomonas mobilis is a bacterium which generally occurs in association with plants with high sugar content in their sap. The bacterium is able to use a narrow range of sugars. Glucose, fructose and sucrose are the only carbon as well as energy sources that support growth of *Z. mobilis.*

Z. mobilis is the only bacterium known to use ED pathway anaerobically, and this pathway is the major carbon catabolite route since the key enzyme for pentose phosphate pathway 6-phosphogluconate dehydrogenase is missing in *Z. mobilis.* ED pathway is expressed constitutively and operates in a linear fashion. All the metabolic intermediates are generated by transketolase/transaldolase system along with the incomplete TCA cycle.

Sugar uptake in the form of glucose and fructose is facilitated by high affinity diffusion followed by phosphorylation by ATP-dependent specific kinases, i.e., glucokinase/fructokinase. This is followed by the action of phosphoglucoisomerase to accumulate glucose-6-phosphate. Glucose-6-phosphate is further dehydrogenated to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. This hexose molecule is finally cleaved to pyruvate and a triose phosphate by the ED pathway enzymes, EDD and EDA.

The pyruvate thus generated is converted to acetaldehyde and CO_2 by non-oxidative decarboxylation by pyruvate decarboxylase using NADH + H^+ as reductant. Pyruvate decarboxylase of *Z. mobilis* is a unique enzyme as it is not found among prokaryotes but is common among ethanol fermenting yeasts. At the final step, ethanol is released by the action of alcohol dehydrogenase.

Glucose-6-phosphate dehydrogenase of *Z. mobilis* can use both NAD^+ and $NADP^+$ with approximately equal efficiency. This flexibility is advantageous as this is able to generate the reductant for assimilating reactions and also compensate for oxidation–reduction balance via NADH-dependent reduction of acetaldehyde by alcohol dehydrogenase (Fig. 5).

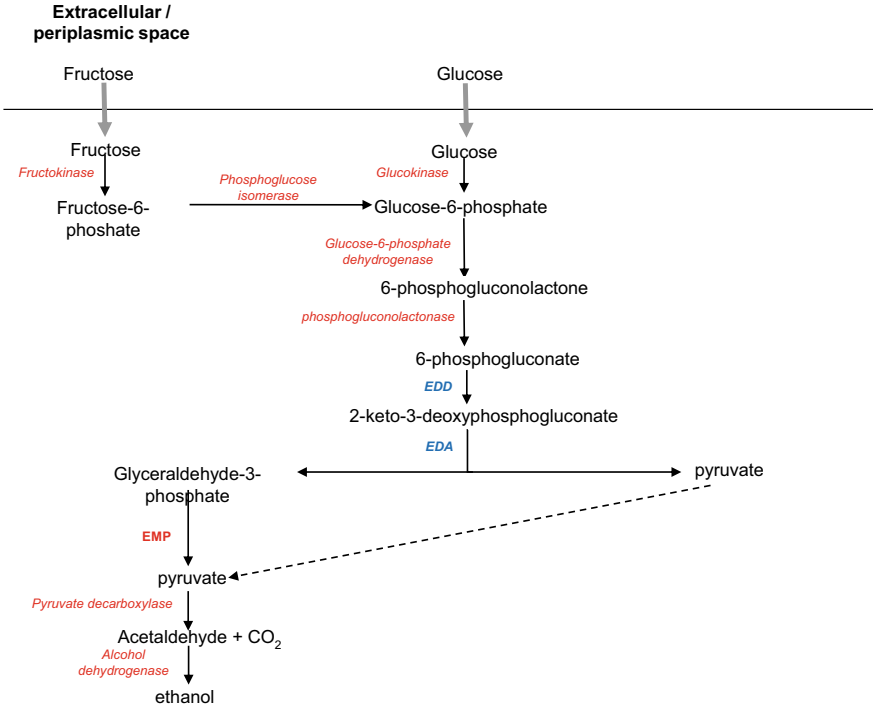


Fig. 5 Constitutive linear ED pathway in *Zymomonas mobilis*

In some organisms, such as *Neisseria gonorrhoeae*, the constitutive linear ED pathway may operate in conjunction with pentose phosphate pathway. *N. gonorrhoeae* utilizes only glucose as the carbon source under aerobic conditions. Acetate and CO_2 are the end products produced by the action of pyruvate dehydrogenase with acetyl CoA as an intermediate. This is also seen in *Xanthomonas campestris.*

Cyclic pathway in Pseudomonads

Pseudomonads are generally deficient in phosphofructokinase. Thus, EMP stage 1 is missing and glucose catabolism cannot occur via EMP pathway. The core central metabolic pathway is Entner–Doudoroff pathway as it has all the component enzymes including glucokinase, glucose-6-phosphate dehydrogenase, 6-phosphoglucolactonase, EDD and EDA enzymes. In addition to this, pseudomonads rapidly convert glucose to gluconate in the periplasm and are also able to utilize gluconate from the environment. Gluconate is phosphorylated directly to 6-phosphogluconate which then enters ED pathway. This gives them the advantage of rapidly utilizing glucose and out-competing other glucose-utilizing microorganisms.

Through the action of EDD and EDA, 6-phosphogluconate is converted to pyruvate and glyceraldehyde-3-phosphate. Like other ED pathway organisms, pyruvate directly enters the TCA cycle. On the other hand, glyceraldehyde-3-phosphate does not enter the later stages of EMP (the enzymes of which are available) as EMP is not a preferred pathway in pseudomonads. Normally, this may lead to accumulation of glyceraldehyde-3-phosphate. However, pseudomonads overcome this by adopting a cyclic mode wherein glyceraldehyde-3-phosphate is reutilized via gluconeogenic enzymes. Thus, the triose phosphate is recycled by transaldolase to form fructose-1,6-bisphosphate which gets converted to fructose-6-phosphate by fructose-1,6-bisphosphatase and fructose-6-phosphate isomerizes to glucose-6-phosphate to re-enter into ED pathway by glucose-6-phosphate dehydrogenase activity (Fig. 6).

Evidences in support of cyclic pathway

1. EDD mutants are unable to grow not only on glucose and gluconate (due to lack of ED pathway) but also fail to utilize those compounds which generate 3-carbon compounds such as glycerol (which generates dihydroxyacetone phosphate). This indicates that 3-carbon compounds are utilized via the ED pathway via the cyclic mode.
2. Mutants of phosphoglucoisomerase, glucose-6-phosphate dehydrogenase and fructose diphosphate aldolase fail to grow on glycerol (which generates dihydroxyacetone phosphate), fructose and mannitol (which are first converted to fructose-6-phosphate). This indicates that 3-carbon compounds enter the ED pathway via the cyclic mode. Further, 6-carbon compounds are also metabolized by ED pathway by conversion to glucose-6-phosphate and finally to 6-phosphogluconate.
3. In *Alcaligenes eutrophus* (which is related to pseudomonads), phosphoglycerate mutase mutants fail to grow on succinate but can grow on fructose. This indicates that phosphoglycerate mutase is required for succinate utilization via gluconeogenesis but not required for fructose metabolism as EMP does not take

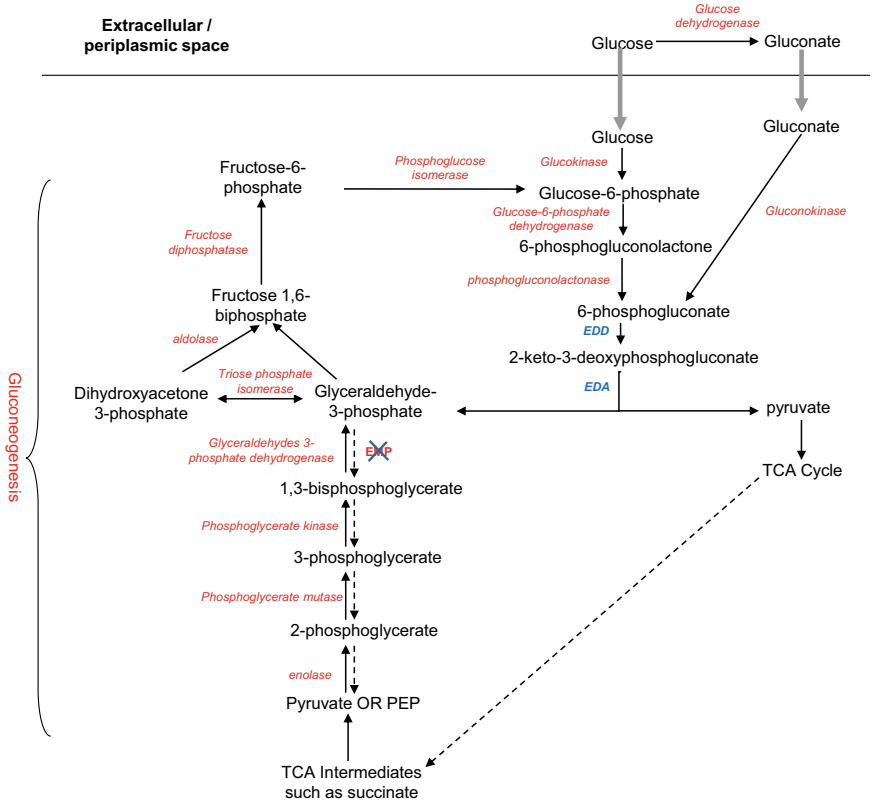


Fig. 6 Constitutive cyclic ED pathway in pseudomonads

place. Instead, fructose is utilized via ED pathway. In such situations, accumulation of glyceraldehyde-3-phosphate is expected. But, this does not happen as it is re-utilized via the cyclic mode.

Advantages of ED pathway in Pseudomonads: Pseudomonads preferably utilize the ED pathway over the EMP pathway as the ED pathway is important for generating redox equivalents (NADPH) that is required for counteracting oxidative stress encountered in the environmental niches normally inhabited by pseudomonads (Chavarría et al., 2013).

Exception: Non-phosphorylated ED pathway

Several organisms use a modified non-phosphorylated version of the ED pathway (Fig. 7) (Sato & Atomi, 2011).

Version 1: ED pathway in clostridia and certain archae is characterized by the lack of gluconoisomerase and EDD enzymes. The pathway is initiated on non-phosphorylated glucose which is converted to gluconate by glucose dehydrogenase. Gluconate is converted to 2-keto-3-deoxygluconate (KDG) by the action of gluconate

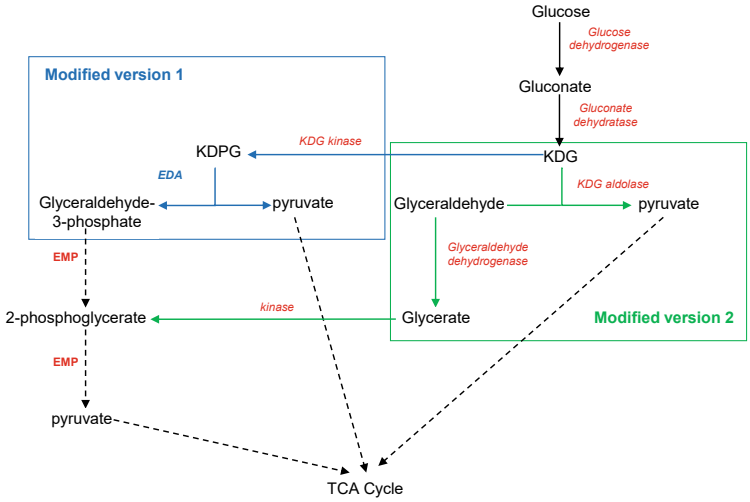


Fig. 7 Non-phosphorylated ED pathway

dehydrogenase and to KDPG by KDG kinase. KDPG is then cleaved by EDA to form glyceraldehyde-3-phsopahte and pyruvate.

Version 2: A second modification of this pathway has been reported from an extremophile, *Sulfolobus solfataricus.* Here, KDG is directly metabolized by KDG aldolase to glyceraldehyde and pyruvate. Non-phosphorylated glyceraldehyde is oxidized to glycerate by glyceraldehyde dehydrogenase which is then phosphorylated to 2-phosphoglycerate by a kinase.

Exception: Modified ED pathway for pentose degradation in archaea

Pentose degradation in archaea *Sulfolobus solfataricus* occur via the ED pathway as they do not have the pentose phosphate pathway. As discussed above, *Sulfolobus solfataricus* uses a non-phosphorylated version of the ED pathway for hexose degradation. It has been seen that the first and third enzymes of this ED pathway, viz. glucose dehydrogenase and 2-keto-3-deoxygluconate aldolase (KDG aldolase), have extended catalytic specificity toward 5-carbon sugars such as xylose and arabinose. However, a specific 5-carbon dehydratase is required for the second step as gluconate dehydratase is active on 6-carbon compounds only.

By the action of the above enzymes, 5-carbon sugars like arabinose and xylose are catabolized to pyruvate and glycolaldehyde. Pyruvate directly enters the citric acid cycle while glycolaldehyde is first converted to glycolate and then to glyoxylate. Glyoxylate can then enter the citric acid cycle via the action of malate synthase (Bräsen et al., 2014; Nunn et al., 2010) (Fig. 8). Other archaebacteria may have some other modifications to accomplish pentose degradation.

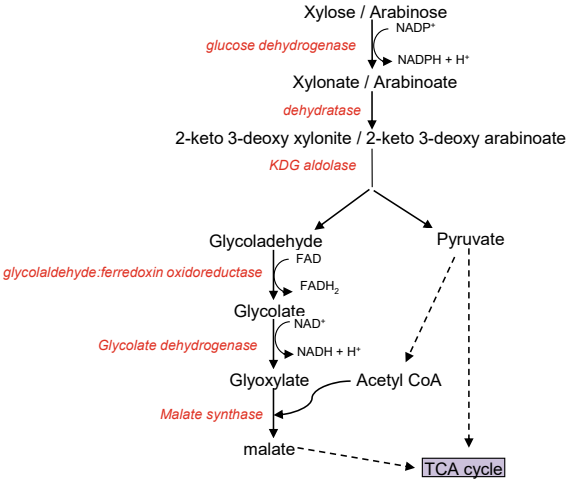


Fig. 8 Pentose degradation in archaea via modified ED pathway

5 Regulation of ED Pathway in *E. Coli*

ED pathway essentially refers to gluconate metabolism and is inducible by the presence of gluconate.

EDD and EDA enzymes—In *E. coli*, the two main enzymes of ED pathway, EDD and EDA, are enoded by *edd* and *eda* genes, respectively. These genes form part of a single operon (*edd-eda* operon) and are separated from each other by just 36 bp. This operon has multiple promoters (Table 1 and Fig. 9).

Table 1 Multiple promoters of ED operon along with their regulators

Promoter	Inducible /Constitutive	Inducer/Up-Regulator	Repressor
P1	Inducible	Gluconate	gntR
P2	Constitutive	glucuronate/galacturonate	kdgR
P3	Non-functional		
P4	Constitutive	PhoB-P (Phosphate starvation)	PhoB

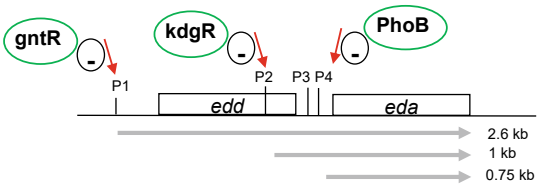


Fig. 9 *eda-edd* operon in *E. coli*

The first promoter is P1 that is located 109 bp upstream of the *edd* start codon and codes for a 2.6 kb long transcript that includes both *edd* and *eda gene* products. P1 is under the transcriptional regulation of GntR. GntR is coded by *gntR* and is expressed constitutively. GntR is a negative repressor for the *edd-eda* operon. In the presence of gluconate, GntR binding to this operon is inactivated and the promoter cotranscribes both *edd* and *eda*.

In addition, *eda* is under the control of three more promoters (P2, P3 and P4). P2 is located 353 bp upstream of *eda* start codon, within the coding sequence of *edd*. P2 leads to a transcript which is 1000 nucleotides long. P3 and P4 are very closely located. P3 seems to be non-functional. P4 is located 26 bp upstream of the *eda* start codon in the intergenic region between *edd* and *eda* and codes for a 750 nucleotides long transcript. Both P2 and P4 are constitutive in nature to ensure basal levels of EDA as EDA is involved in several metabolic pathways. Additionally, P2 is up-regulated by glucuronate/galacturonate levels and is under negative control by kdgR, a regulator of glucuronate and galacturonate metabolism. P4 is de-repressed under phosphate starvation conditions. In the presence of phosphate, the PhoB protein is in the form of a dephosphorylated monomer which binds to P4 promoter and represses it. Upon phosphate starvation, PhoB is phosphorylated (PhoB-P) and dimerization occurs. Dimerized PhoB-P is unable to bind to P4, and thus, de-repression occurs. It should be noted that this mechanism is unusual as generally phosphorylated PhoB (under phosphate starvation conditions) acts as a transcriptional activator (Murray & Conway, 2005).

Gluconate transport and phosphorylation—As discussed above, ED pathway in *E. coli* is induced by gluconate. Thus, the cells either take up gluconate present in extracellular environment or first convert extracellular glucose to gluconate and then take that up. Thus, gluconate metabolism requires gluconate transporters and kinases for its subsequent phosporylation to 6-phosphogluconate. These functions are provided by two systems: GntI and GntII. Of these, Gnt I is the main system while GntII is a subsidiary system (Peekhaus et al., 1997).

GntI comprises of three genes:

- *gntT* (high-affinity gluconate transporter) in monocistronic operon (*gntT* operon)
- *gntU* (low-affinity gluconate transporter) and *gntK* (thermoresistant gluconokinase) present in a polycistronic operon (*gntKU* operon)
- *gntR* is located upstream of *gntKU* and has its own promoter.

GntII comprises two genes:

- *gntS* (or *gntW*; high-affinity gluconate transporter) and *gntV* (thermolabile gluconokinase).

All these operons are negatively regulated by gntR. Further, *gntT* operon is up-regulated by cAMP-CRP system; i.e., it is catabolite repressible by glucose. In addition to glucose, gluconate also lowers cAMP-CRP levels in the same way as other non-PTS sugars like lactose by controlling the levels of CRP and thus leading to catabolite repression (Peekhaus and Conway, 1998b; Gómez et al., 2011). This is

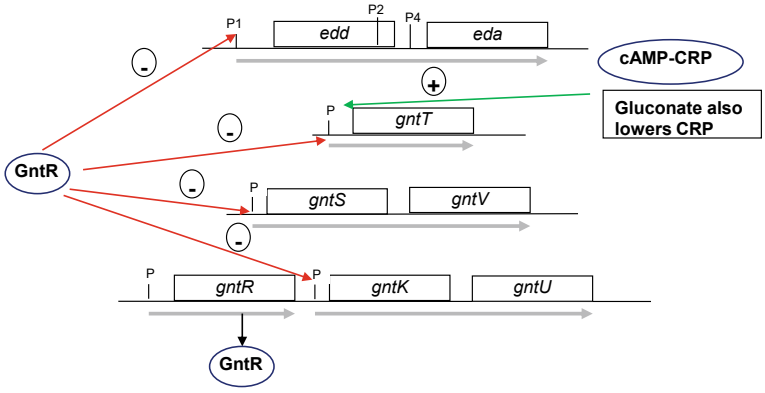


Fig. 10 Regulation of ED pathway in *E. coli*

favorable as high concentrations of gluconate will lead to accumulation of KDPG which is bacteriostatic at high levels.

All these operons together form the *gntR* regulon (Fig. 10).

6 Carbohydrate Metabolism in *Zymomonas Mobilis*, an Alternative Ethanol Producer, Using ED Pathway

Genome sequence of *Z. mobilis* ZM4 shows that it lacks 6-phosphofructokinase and most of the enzymes of PPP pathway. Entner–Doudoroff pathway is the only pathway of sugar fermentation.

Z. mobilis fetched the attention of microbiologists in 1912 as a causal organism for 'cider sickness' (spoiled fermented apple juice). This is a gram-negative anaerobic non-spore former, flagellated bacterium, isolated from fermenting sugars. The key interesting feature in comparison to yeasts is that it takes up glucose rapidly and ferments it to alcohol three-four times faster than yeast. Further, it has better ethanol tolerance. It does not need oxygen and grows in minimal medium without any organic compound requirement. It has high osmotolerance toward sugars but does not tolerate salt. In spite of many desirable features for alcohol fermentation, *Z. mobilis* has limited commercial applications in comparison to yeasts. A comprehensive review about *Z. mobilis* as a novel platform for production of biofuels is presented by He et al. (2014).

The detailed carbohydrate metabolism of *Z. mobilis* explains some of its limitations for large-scale alcohol fermentation. The key issue in any commercial venture is its economics which lies in the raw material cost, final product yield and downstream processing. With these facts in mind, the major limitation of *Z. mobilis* is:

1. Its capacity to use limited carbohydrate range. It can ferment only three sugars-glucose, fructose and sucrose.

2. Utilization pathways for all the three sugars have distinct features in terms of growth, byproduct formation and ethanol yields. The best sugar is glucose only, which is not commercially viable as agro-based raw materials do not have glucose content.

Comparative carbohydrate utilization pathways:

1. **Glucose**: Glucose is taken up by facilitated transport and phosphorylated by glucokinase. Following this, glucose-6-phosphate is fermented by ED pathway to PGA and pyruvate. PGA enters stage II of EMP and is converted to pyruvate. Pyruvate is decarboxylated to acetaldehyde by a unique enzyme, pyruvate decarboxylase. Acetaldehyde is then reduced to ethanol by two alcohol dehydrogenases, ADHI and ADHII. ADHII is ethanol tolerant. Further, pyruvate decarboxylase does not require thiamine pyrophosphate for its activity. Pyruvate decarboxylase is unique in *Z. mobilis* as it is not found among prokaryotes. The ethanol fermentation in bacteria generally is via acetyl CoA (Fig. 11).
   Z. mobilis oxidizes glucose exclusively by ED pathway which yields only 1 ATP in contrast to 2 ATP by EMP pathway. Still it outcompetes in the environment because of high levels of glycolytic and ethanol yielding enzymes which is nearly 50% of the mass of cytoplasm proteins.
2. **Fructose**: Fructose is also taken up by facilitated diffusion and enters ED pathway. However, in case of fructose, there is sufficient flow of carbon to byproduct generation, viz. mannitol, glycerol, dihydroxyacetone phosphate (major products) along with sorbitol, acetic acid, acetoin, acetaldehyde and lactic acid as minor products.

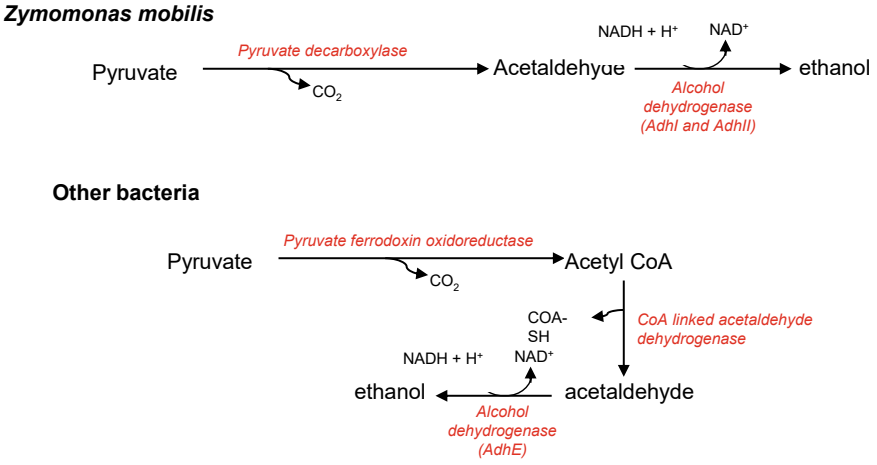


Fig. 11 Ethanol fermentation in *Zymomonas mobilis* Vs other bacteria

a. Mannitol dehydrogenase, a NADPH-dependent enzyme has high K_m for fructose. Therefore, it is active when bacterium is directly grown on fructose.

b. The above reaction depletes NADPH resulting in accumulation of acetaldehyde and in turn all other intermediates also build up.
c. Accumulation of PGA and DHP leads to activation of phosphatases, thereby yielding glycerol and dihydroxyacetone.
d. Acetaldehyde and dihydroxyacetone inhibit bacterial growth resulting in lower biomass and low ethanol yields along with byproducts.

3. **Sucrose**: Sucrose is another sugar which is broken down to glucose and fructose by levansucrase enzyme. However, this enzyme has transfructosylating activity that leads to formation of a low molecular weight polymer, levan, along with substantial amount of oligosaccharides. These products compete with ethanol fermentation. Another problem is that sucrose also leads to sorbitol formation by glucose–fructose oxidoreductase. Sorbitol cannot be utilized by the bacterium and thus accumulates in the medium.

Ethanol tolerance in *Z. mobilis*

Z. mobilis is tolerant to ethanol because of high content of hopanoids, functional analogs of sterols, found in yeast. However, sterol biosynthesis requires oxygen which hopanoids do not need. The content of hopanoids increases as a function of alcohol, which in turn decreases membrane fluidity and thus counteracts permeabilizing effects of ethanol.

7 Metabolic Fate of Pyruvate

At the final step of the glycolytic cycle and ED pathway, phosphoenol pyruvate is converted to pyruvate. Further, pentose phosphate pathway also links to glycolysis and generates pyruvate. Pyruvate can then enter various pathways depending on the available oxygen and energy conditions. The fate of pyruvate is also dependent on the type of organism. The major metabolic fates of pyruvate/pyruvate node are summarized in Fig. 12.

1. Decarboxylation of pyruvate to acetyl CoA
2. Carboxylation of pyruvate to oxaloacetate
3. Reduction of pyruvate to ethanol
4. Reduction of pyruvate to lactate
5. Oxidation of pyruvate to acetate.

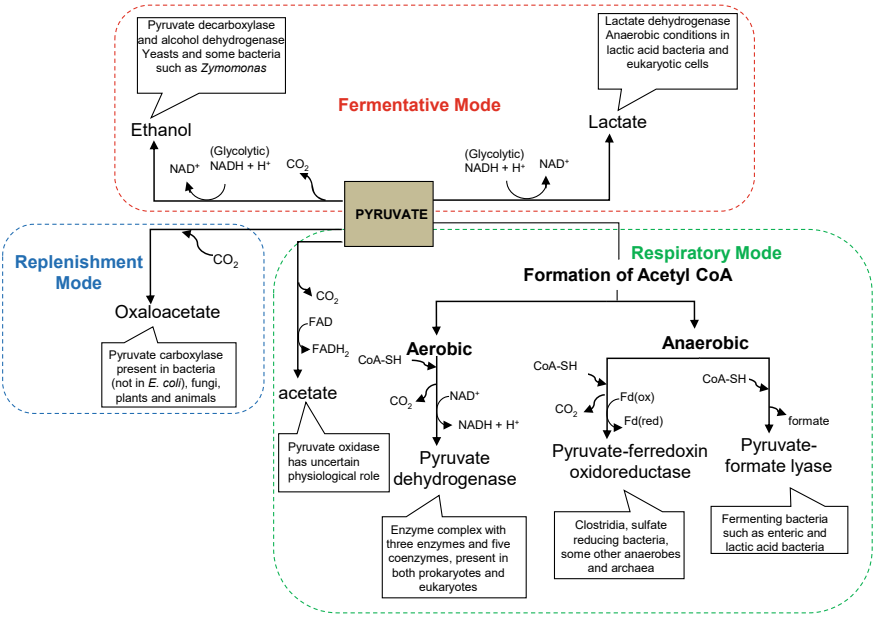


Fig. 12 Metabolic Fates of Pyruvate

1. **Decarboxylation of pyruvate to acetyl CoA**—Under respiratory conditions, pyruvate undergoes oxidative decarboxylation to form acetyl CoA which can then enter the citric acid cycle or the fatty acid biosynthetic pathways. In bacteria, there are three well-characterized systems for decarboxylation of pyruvate:

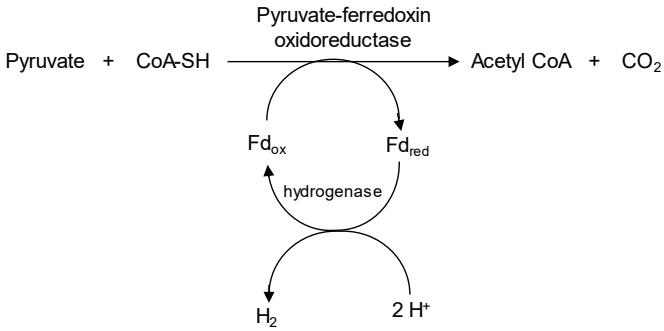
(a) **Pyruvate dehydrogenase complex**—During aerobic growth, the oxidation of pyruvate to acetyl CoA is catalyzed by the pyruvate dehydrogenase enzyme complex. This is present in both prokaryotes and eukaryotes where the reaction takes place in the mitochondrion.
The overall reaction is:

$$\text{pyruvate} + \text{CoA-SH} \xrightarrow[\text{NAD}^+ \quad \text{NADH} + \text{H}^+]{\text{Pyruvate dehydrogenase complex}} \text{Acetyl CoA} + \text{CO}_2$$

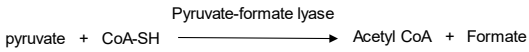
Most anaerobically growing bacteria use either pyruvate-ferredoxin oxidoreductase or pyruvate-formate lyase for oxidation of pyruvate to acetyl CoA.

(b) **Pyruvate-ferredoxin oxidoreductase**—This is found in clostridia, sulfate reducing bacteria, some other anaerobes and archaea. The reaction is similar to that catalyzed by pyruvate dehydrogenase complex with the difference being that the electron acceptor is ferrodoxin instead of NAD^+. This reaction is irreversible and is used for fixation of CO_2 in some anaerobic bacteria.

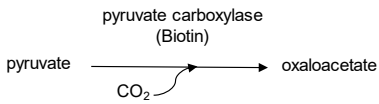
In fermenting bacteria, this reaction is coupled to hydrogenase enzyme that catalyzes electron transfer from ferrodoxin to H^+ leading to formation of hydrogen gas (that is produced during fermentations).



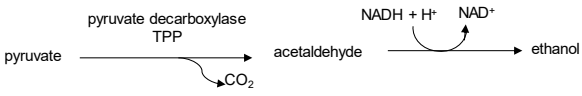
(c) **Pyruvate-formate lyase**—This is found in some fermenting bacteria such as the enteric bacteria and lactic acid bacteria. In this reaction, neither NADH nor reduced ferrodoxin is produced. Instead, the electrons remain with the carboxyl group, and thus, formate is formed instead of CO_2.



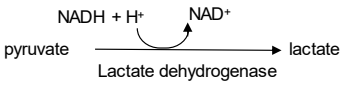
2. **Carboxylation of pyruvate to oxaloacetate**—The enzyme pyruvate carboxylase catalyzes this reaction which is biotin dependent. This reaction is irreversible and is mainly responsible for replenishment of oxaloacetate for continuity of citric acid cycle and also provides substrate for gluconeogenesis.



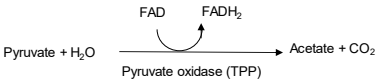
3. **Reduction of pyruvate to ethanol**—In fermentative microorganisms (mainly yeasts and some bacteria such as *Zymomonas*), pyruvate is converted to ethanol in two steps. In the first step, pyruvate is decarboxylated by pyruvate decarboxylase to form acetaldehyde using thiamine pyrophosphate as a coenzyme. Acetaldehyde is then converted to ethanol.



4. **Reduction of pyruvate to lactate**—In lactic acid bacteria and in eukaryotic cells, the final product of anaerobic glycolysis is lactate formed by reduction of pyruvate using lactate dehydrogenase. The reaction is reversible. Lactate formation is favored by elevated NADH/NAD^+ ratio.
   In exercising skeletal muscles in animals, there is NADH production which is in excess of the oxidative capacity of the respiratory chain. This leads to lactate formation which accumulates in exercising muscles causing cramps.



5. **Oxidation of pyruvate to acetate**—Under aerobic conditions, pyruvate is oxidatively decarboxylated to acetate by the action of a membrane-associated flavoprotein dehydrogenase known as pyruvate oxidase that feeds electrons into the *E. coli* aerobic respiratory chain. The enzyme is a dehydrogenase; however, it is named as pyruvate oxidase to differentiate it from the pyruvate dehydrogenase complex.
   Pyruvate oxidase is a tetramer of identical subunits wherein each subunit is linked to an FAD coenzyme. It also requires thiamin pyrophosphate (TPP) and Mg^{2+} as a cofactor. The enzyme is activated by the presence of lipids. It is a non-essential stationary phase enzyme with uncertain physiological role. However, it is important for aerobic growth in mutants lacking pyruvate dehydrogenase activity.



Summary

- The Entner–Doudoroff pathway is one of the three central metabolic pathways, in addition to Embden–Meyerhof–Parnas pathway and pentose phosphate pathway.
- It is a fermentative pathway for glucose catabolism and is by and large restricted to prokaryotes.
- The key enzymes unique to Entner–Doudoroff pathway are 6-phosphogluconate dehydratase, commonly known as Entner–Doudoroff dehydratase (EDD), and KDPG aldolase, commonly known as Entner–Doudoroff aldolase (EDA).
- In most organisms, this pathway operates in aerobic conditions, while in *Zymomonas mobilis,* it operates under anaerobic conditions.
- ED pathway has a lot of variants such as inducible linear pathway in *E. coli*, constitutive linear pathway in *Z. mobilis* and cyclic pathway in pseudomonads.
- Interestingly, a non-phosphorylated ED pathway also exists in clostridia and archaea.

- In *E. coli,* ED pathway is gluconate inducible and is stimulated by phosphate starvation. It allows survival in extra-intestinal and aquatic environments.
- ED pathway provides an environmental advantage to pseudomonads as well. Pseudomonads rapidly convert glucose to gluconate and outcompete other glucose-microorganisms.
- In *E. coli*, EDD and EDA are enoded by *edd* and *eda* genes that form part of a single operon (*edd-eda* operon). GntR is a negative repressor for the *edd-eda* operon.
- *edd* expression is inducible by gluconate.
- *eda* has constitutive basal levels and is up-regulated by glucuronate/galacturonate levels and de-repressed under phosphate starvation conditions.
- The end product of glycolysis, ED pathway and pentose phosphate pathway, i.e., pyruvate, can have various fates depending on the oxygen and energy conditions.

Questions

1. During glucose fermentation by Entner–Doudoroff pathway, if dinitrophenol is added, labelled C1 carbon would be recovered in which metabolite?
2. During glucose fermentation by Entner–Doudoroff pathway, if iodoacetate is added, labelled C1 carbon would be recovered in which metabolite?
3. To distinguish EMP pathway and ED pathway by respirometry, carbon dioxide evolution is observed from which of the labeled carbons?
4. From which of the carbon of glucose, evolution of labelled CO_2 is a sure test for operation of ED pathway alone?
5. Which is the first of the key enzymes of ED pathway?
6. Which enzyme of ED pathway is a multi-functional enzyme?
7. Which of the key enzymes of EMP pathway is missing in pseudomonads?
8. Which bacterium uses ED exclusively during anaerobic conditions?
9. Which enzyme is unique to *Zymomonas mobilis* and absent from other bacteria?
10. Match the following bacterial group with mode of ED pathway:

a. *E. coli*	a1. Non-phosphorylated ED
b. *Pseudomonas*	b1. Linear constitutive
c. *Zymomonas*	c1. Linear inducible
d. *Sulfolobus*	d1. Cyclic ED

11. Explain any two modes of Entner–Doudoroff pathway in brief.
12. What are the evidences of cyclic mode of ED pathway operation?
13. What are the advantages of ED pathway to bacteria?
14. Explain molecular regulation of ED enzymes in *E. coli.*
15. How archaea can use pentoses via ED pathway?
16. What are the possible fates of pyruvate formed from glucose oxidation?

References

Bräsen, C., Esser, D., Rauch, B., & Siebers, B. (2014). Carbohydrate metabolism in Archaea: Current insights into unusual enzymes and pathways and their regulation. *Microbiology and Molecular Biology Reviews, 78*(1), 89–175.

Chavarría, M., Nikel, P. I., Pérez-Pantoja, D., & de Lorenzo, V. (2013). The Entner-Doudoroff pathway empowers *Pseudomonas putida* KT2440 with a high tolerance to oxidative stress. *Environmental Microbiology, 15*(6), 1772–1785.

Conway, T. (1992). The Entner-Doudoroff pathway: History, physiology and molecular biology. *FEMS Microbiology Reviews, 103,* 1–28.

Entner, N., & Doudoroff, M. (1952). Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. *Journal of Biological Chemistry, 196,* 853–862.

Gómez, K. M., Rodríguez, A., Rodriguez, Y., Ramírez, A. H., & Istúriz, T. (2011). The subsidiary GntII system for gluconate metabolism in *Escherichia coli*: alternative induction of the *gntV* gene. *Biological Research, 44*(3), 269–275.

Gupta, S. C. & Dekker, E. E. (1984). Malyl-CoA formation in the NAD-, CoASH-, and alpha-ketoglutarate dehydrogenase-dependent oxidation of 2-keto-4-hydroxyglutarate. Possible coupled role of this reaction with 2-keto-4-hydroxyglutarate aldolase activity in a pyruvate-catalyzed cyclic oxidation of glyoxylate. *Journal of biological chemistry*, *259*(16), 10012-9.

He, M. X., Wu, B., Qin, H., Ruan, Z. Y., Tan, F. R., Wang, J. L., et al. (2014). *Zymomonas mobilis*: A novel platform for future biorefineries. *Biotechnology for Biofuels, 7*(1), 101.

Murray, E. L., & Conway, T. (2005). Multiple regulators control expression of the Entner-Doudoroff aldolase (Eda) of *Escherichia coli*. *Journal of Bacteriology, 187*(3), 991–1000.

Nunn, C. E., Johnsen, U., Schönheit, P., Fuhrer, T., Sauer, U., Hough, D. W., et al. (2010). Metabolism of pentose sugars in the hyperthermophilic archaea *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*. *Journal of Biological Chemistry, 285*(44), 33701–33709.

Peekhaus, N., & Conway, T. (1998a). What's for Dinner?: Entner-Doudoroff Metabolism in *Escherichia coli*. *Journal of Bacteriology, 180*(14), 3495–3502.

Peekhaus, N., & Conway, T. (1998b). Positive and negative transcriptional regulation of the *Escherichia coli* gluconate regulon gene *gntT* by GntR and the cyclic AMP (cAMP)-cAMP receptor protein complex. *Journal of Bacteriology, 180*(7), 1777–1785.

Peekhaus, N., Tong, S., Reizer, J., Saier, M. H., Jr., Murray, E., & Conway, T. (1997). Characterization of a novel transporter family that includes multiple *Escherichia coli* gluconate transporters and their homologues. *FEMS Microbiology Letters, 147*(2), 233–238.

Sato, T., & Atomi, H. (2011). Novel metabolic pathways in Archaea. *Current Opinions in Microbiology, 14*(3), 307–314.

Chapter 12
Tricarboxylic Acid Cycle

Rani Gupta and Namita Gupta

The central role of the **t**ri**c**arboxylic **a**cid cycle (also known as TCA cycle/citric acid cycle/Krebs cycle) is the oxidation of acetyl CoA (derived from metabolism of amino acids, fatty acids and carbohydrates) to CO_2 and H_2O with simultaneous conservation of energy as reduced coenzymes and eventually ATP. Intermediates of citric acid cycle also serve as starting points in a number of synthetic reactions. Oxaloacetate and α-ketoglutarate are involved in amino acid synthesis, succinyl CoA is used in heme synthesis, and citrate is the source of acetyl CoA used in synthesis of fats, lipids and some amino acids. In eukaryotes, citric acid cycle operates in the mitochondrial matrix.

1 Historical Background of TCA Cycle

In the early 1900s, various experiments established that anaerobic suspensions of minced animal tissue transfer hydrogen atoms from organic acids (namely fumarate, succinate, citrate and malate) to other reducible compounds such as methylene blue dye. In 1935, Albert Szent-Gyorgyi discovered that small amounts of these organic acids caused oxygen uptake by a tissue suspension far in excess of the oxygen required to completely oxidize them to CO_2 and H_2O. He concluded that the organic acids acted catalytically on the oxidation of glucose or other carbohydrates. He also showed that malonate which is an inhibitor of succinate dehydrogenase, inhibited oxygen utilization (respiration). In 1937, Hans Krebs postulated the citric acid cycle based on previous findings by Szent-Gyorgyi and his own findings that citrate catalytically stimulates respiration and is converted to α-ketoglutarate and then to succinate. Also, oxaloacetate is converted to citrate by addition of two carbons. The source of these two carbons was later identified to be acetyl CoA by works of Frtiz Lipman (1947) and Severo Ochoa (1952) (Table 1).

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_12

Table 1 History of TCA cycle

Year and Scientist	Event
1910–1920 Various scientists	• Anaerobic suspensions of minced animal tissue transfer hydrogen atoms from organic acids (namely fumarate, succinate, citrate and malate) to other reducible compounds such as methylene blue dye • Presence of oxygen in the same suspensions oxidized the organic acids to CO_2 and H_2O
1935 Albert Szent-Gyorgyi	• Small amounts of organic acids caused oxygen uptake by a tissue suspension far in excess of the oxygen required to completely oxidize them to CO_2 and H_2O, thus the organic acids acted catalytically on the oxidation of glucose or other carbohydrates • Malonate which is an inhibitor of succinate dehydrogenase, inhibited oxygen utilization (respiration). Thus, oxidation of succinate is an indispensable reaction in respiration
1937 Hans Krebs	• Organic acids oxaloacetate, α-ketoglutarate, isocitrate, *cis*-aconitate and citrate were also oxidized very rapidly and acted catalytically on the oxidation of pyruvate • Arranged the organic acids with catalytic potential in a chain: Citrate → *cis*-aconitate → isocitrate → α-ketoglutarate → succinate → fumarate → malate → oxaloacetate • Oxaloacetate is converted to citrate by addition of two carbons from an as yet unidentified source • Postulated the citric acid cycle
1947–1950 Fritz Lipmann	• Discovered coenzyme A (CoA), the coenzyme of acetylation • Acetate combines with CoA to form acetyl CoA, an active acetyl donor
1952 Severo Ochoa	• Acetyl CoA condenses with oxaloacetate to form citrate

2 Bridge Between Glycolysis and TCA Cycle—Pyruvate Dehydrogenase Complex

At the final step of the glycolytic cycle, phosphoenol pyruvate is converted to pyruvate. Under aerobic respiration, pyruvate is oxidized to acetyl CoA by the action of the pyruvate dehydrogenase enzyme complex. The acetyl CoA thus generated enters into the citric acid cycle. Thus, the pyruvate dehydrogenase enzyme complex acts as a bridge between the glycolytic pathway and the citric acid cycle.

Pyruvate dehydrogenase enzyme complex is present in both prokaryotes and eukaryotes where the reaction takes place in the mitochondrion. It is a multi-enzyme complex with an enormous size. The complex, a non-covalent assembly of three different enzymes, catalyzes five successive reactions involved in the conversion of pyruvate to acetyl CoA.

The three enzymes of the pyruvate dehydrogenase enzyme complex are pyruvate dehydrogenase (E1, also known as pyruvate decarboxylase), dihydrolipoate transacetylase (E2) and dihydrolipoate dehydrogenase (E3). In addition, the complex requires five coenzymes, namely thiamine pyrophosphate (TPP), FAD, lipoic acid, NAD + and coenzyme A. Each molecule of dihydrolipoate transacetylase contains

Table 2 Organization of pyruvate dehydrogenase complex in *E. coli*

Enzyme	Coenzyme (s)	No. of subunits
Pyruvate dehydrogenase (E1)	• Thiamine pyrophosphate (TPP)	24
Dihydrolipoate transacetylase (E2)	• Lipoic acid • Coenzyme A	24
Dihydrolipoate dehydrogenase (E3)	• FAD • NAD+	12

two molecules of lipoic acid attached by an amide bond between the carboxyl group at the end of lipoic acid's hydrocarbon chain and the terminal amino group of a lysine residue of the enzyme. TPP and FAD are catalyzing cofactors for pyruvate dehydrogenase and dihydrolipoate dehydrogenase, respectively, and are tightly bound to them.

The intermediates produced during the five successive reaction steps remain bound to the enzyme complex and are transferred from one active site to the other. The five-reaction sequence is thus an example of substrate channeling. The active sites of all the three enzymes are situated close to one another in such a way that the product of the first enzyme is the substrate for the second enzyme and is directly passed to the active site of the second enzyme.

Many molecules of each of these three enzymes are organized to form the enzyme complex. In gram-negative bacteria, pyruvate dehydrogenase complex has 60 subunits in three functional proteins (Table 2). In eukaryotes and gram-positive bacteria, it consists of 96 subunits in the three functional proteins. The pyruvate dehydrogenase complex is very large, and its size is several times bigger than a ribosome. This serves to channel the high pyruvate flux.

Pyruvate Dehydrogenase Complex in *E. coli*: In *E. coli*, the pyruvate dehydrogenase complex is 4.6 million Daltons in size and consists of 60 polypeptides (24 molecules of pyruvate dehydrogenase, 24 molecules of dihydrolipoate transacetylase, 12 molecules of dihydrolipoate dehydrogenase) (Fig. 1).

Reactions catalyzed by the pyruvate dehydrogenase complex:

- Reaction 1—Pyruvate is decarboxylated to form the key reactive intermediate, hydroxyethyl thiamine pyrophosphate (HETPP) using pyruvate dehydrogenase. Hydroxytheyl derivative is bound to the reactive carbon of TPP, the coenzyme for pyruvate dehydrogenase.
- Reaction 2—Oxidation of HETPP by transfer to the disulphide form of lipoic acid (bound to dihydrolipoate transacetylase) which itself is reduced to sulphydryl form. TPP is displaced, and the acetyl group is transferred to lipoic acid. This is also catalyzed by the action of pyruvate dehydrogenase.
- Reaction 3—Transacetylation reaction wherein the acetyl group, bound as a thioester to the side chain of lipoic acid is transferred to CoA-SH forming acetyl CoA and reduced lipoic acid. This reaction is catalyzed by dihydrolipoate transacetylase.

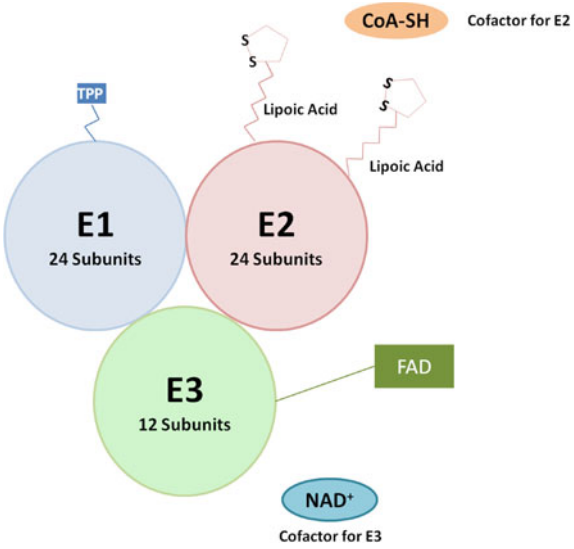


Fig. 1 Pyruvate dehydrogenase complex in *E. coli*

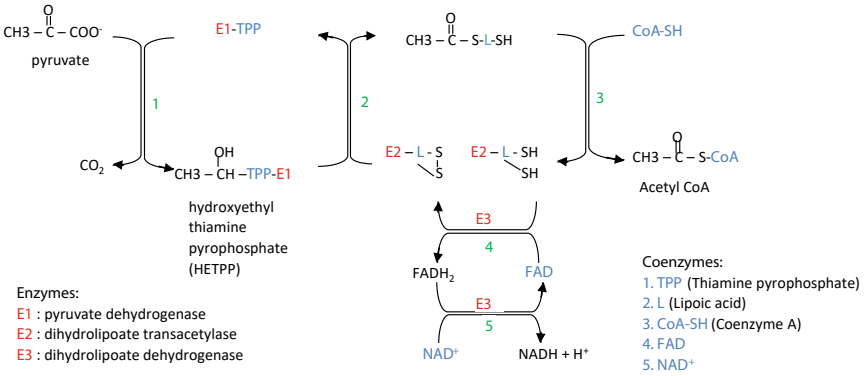


Fig. 2 Schematic representation of reactions catalyzed by pyruvate dehydrogenase complex

- Reaction 4—The reduced lipoic acid is reoxidized by FAD bound to dihydrolipoate dehydrogenase.
- Reaction 5—The reduced FAD bound to dihydrolipoate dehydrogenase is reoxidized by NAD^+. The reactions are summarized in Fig. 2.

Regulation of the pyruvate dehydrogenase complex: This reaction sequence is irreversible and regulated by several factors. In *E. coli*, pyruvate dehydrogenase complex is feedback inhibited by ATP, acetyl CoA and NADH. Further, PEP, AMP, NAD^+ and CoA-SH accumulate when very small amounts of acetate flows into the citric acid cycle and allosterically activates the enzyme complex. Thus, the enzyme

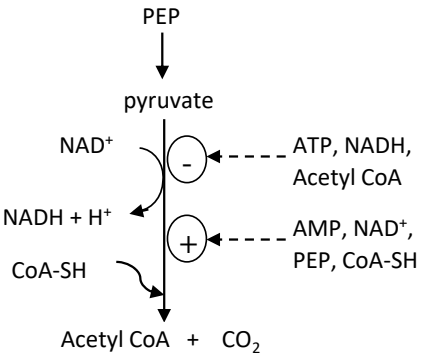


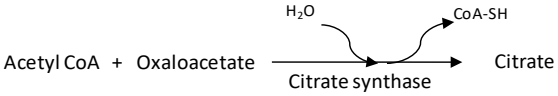
Fig. 3 Regulation of pyruvate dehydrogenase complex

complex is turned off when sufficient fuel is available (as acetyl CoA) and when the cell has high levels of energy charge and reducing equivalents. Conversely, high energy demands and requirement of greater flux of acetyl CoA into the citric acid cycle turn on the enzyme complex (Fig. 3).

3 Tricarboxylic Acid Pathway/Krebs's Cycle

Tricarboxylic acid cycle starts with the condensation of acetyl CoA (generated by reactions of the pyruvate dehydrogenase complex) and oxaloacetate to form citrate by citrate synthase. After citrate formation, the pathway proceeds in a cyclic manner to generate oxaloacetate back. During this cycle, two moles of CO_2, four moles of reductant (three NADH + H^+ and one $FADH_2$) and one GTP through substrate-level phosphorylation are generated (Fig. 4). Various steps are as follows:

1. Condensation of acetyl CoA and oxaloacetate to form citrate by citrate synthase.



- This reaction is irreversible and has equilibrium far in the direction of citrate synthesis.
- This reaction uses an intermediate of the TCA cycle (oxaloacetate) and produces another intermediate (citrate).
- The first substrate to bind to the enzyme, i.e., oxaloacetate induces a conformational change in the enzyme structure such that a binding site for the second substrate, acetyl CoA is created.

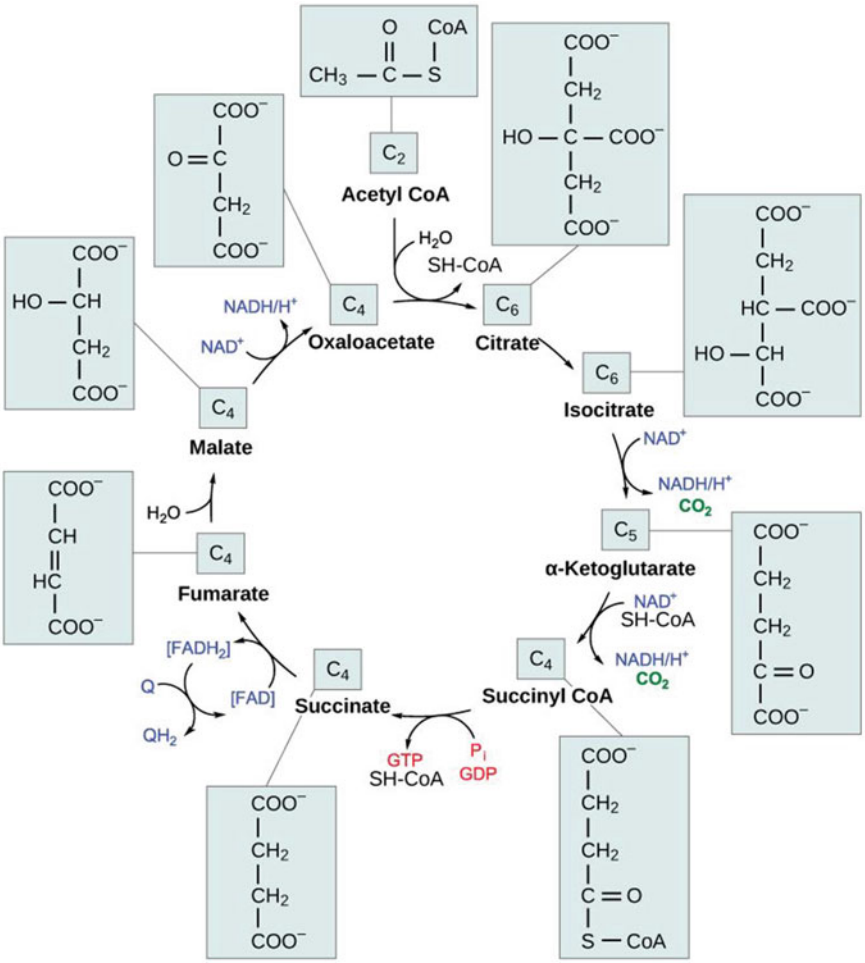
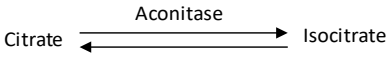


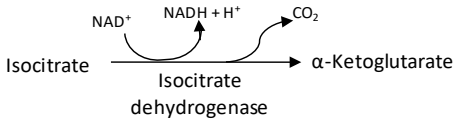
Fig. 4 Citric acid cycle. *Source* https://courses.lumenlearning.com. (Creative Commons Attribution License)

2. Citrate is isomerized to isocitrate by aconitase, also known as aconitate hydratase.



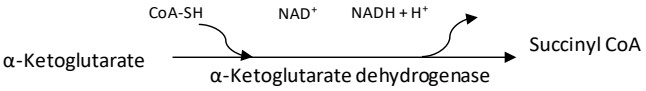
- This is a reversible reaction.
- Aconitase occurs both in mitochondrial and cytoplasmic form.

3. Decarboxylation and oxidation of isocitrate to α-ketoglutarate by isocitrate dehydrogenase.



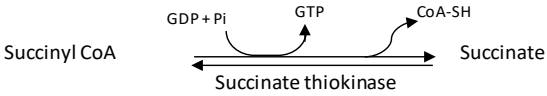
- This is a rate limiting step of the citric acid cycle.
- It is an irreversible reaction.
- Release of the first CO_2 and reductant ($NADH + H^+$) occurs.
- There are two different forms of isocitrate dehydrogenase in all cells, one requiring NAD^+ while the other requiring $NADP^+$ as electron acceptor. The overall reactions catalyzed by these two forms are identical. In eukaryotic cells, the NAD-dependent enzyme is present in the mitochondrial matrix and functions in the TCA cycle. The NADP-dependent enzyme is present both in cytosol and mitochondrial matrix and leads to generation of NADPH, which is essential for reductive anabolic reactions. In case the NADH pool within mitochondria is very high, citrate can be diverted out to the cytoplasm where it will be acted upon by cytosolic aconitase and isocitrate dehydrogenase to produce NADPH.

4. Oxidative decarboxylation of α-ketoglutarate by α-ketoglutarate dehydrogenase complex.



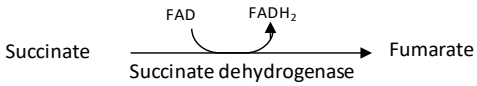
- This reaction is irreversible and has equilibrium far in the direction of succinyl CoA.
- Release of the second CO_2 and reductant ($NADH + H^+$) occurs
- Both the structure and function of α-ketoglutarate dehydrogenase complex closely resemble that of pyruvate dehydrogenase complex.
- It includes three enzymes, homologous to E1 (known as α-ketoglutarate dehydrogenase; E'1), E2 (known as dihydrolipolyl transsuccinylase or succinyl transferase; E'2) and E3 (dihydrolipoate dehydrogenase) of the pyruvate dehydrogenase complex. E3 of both the complexes is the same protein.
- This complex also requires five coenzymes, namely enzyme-bound TPP, bound lipoic acid, FAD, NAD^+ and coenzyme A.

5. Cleavage of succinyl CoA to succinic acid by succinate thiokinase, also called as succinyl CoA synthetase.



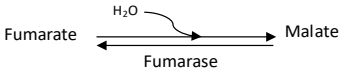
- It is a reversible reaction.
- It is the only reaction yielding high-energy GTP by substrate-level phosphorylation.

6. Succinate is oxidized to fumarate by succinate dehydrogenase.



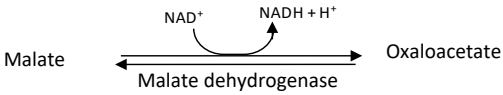
- It is an irreversible reaction.
- Release of the third reductant ($FADH_2$) occurs.
- In this reaction, the electron acceptor is FAD instead of NAD^+ as succinate does not have sufficient reducing power to reduce NAD^+.
- Malonate, an analog of succinate which is normally not present in cells, is a strong competitive inhibitor of succinate dehydrogenase. Its addition to mitochondria inhibits the activity of the citric acid cycle.

7. Fumarate is hydrated to malate by fumarase, also known as fumarate hydratase.



- It is a freely reversible reaction.

8. Malate is oxidized to oxaloacetate by malate dehydrogenase.



- This is a reversible reaction.
- Release of the fourth reductant ($NADH + H^+$) occurs.
- Malate dehydrogenase is present both in the mitochondrial matrix and the cytosol in case of eukaryotes.
- Another enzyme malate: Quinone oxidoreductase (Mqo) catalyzes the oxidation of malate to oxaloacetate by donating electrons to quinone. Both Mqo and malate dehydrogenase can be active in the cell at the same time. However, Mqo does not have a very a significant physiological role in malate oxidation.

Stoichiometry of Complete Oxidation of Glucose

Starting from acetyl CoA, the stoichiometry of the citric acid cycle is presented in Table 3:

Table 3 Stoichiometry of citric acid cycle

Reaction	No. of energy charge formed	No. of reducing equivalents formed
Isocitrate → α-ketoglutarate		1 NADH
α-ketoglutarate → succinyl-CoA		1 NADH
Succinyl-CoA → succinate	1 GTP (= 1 ATP)	
Succinate → fumarate		1 $FADH_2$
Malate → oxaloacetate		1 NADH
Total	1 ATP	3 NADH, 1 $FADH_2$
No. of ATP formed per TCA cycle	**10 ATP**	

Acetyl CoA + $2H_2O$ + $3NAD^+$ + FAD + GDP + Pi → $2CO_2$ + 3NADH + $3H^+$ + $FADH_2$ + GTP + CoA-SH

Further, during the electron transport cycle, 2.5 ATPs are produced per NADH and 1.5 ATPs are produced per $FADH_2$. Thus, one round of citric acid cycle yields 9 ATP and 1 GTP equivalent to 10 high-energy phosphates.

For complete oxidation of glucose, two rounds of TCA cycle are required. Thus, TCA cycle yields 20 ATP per glucose and total gain during complete oxidation via glycolysis till CO_2 and H_2O is 32 ATP (Table 4).

TCA cycle as an anabolic pathway

Intermediates of the TCA cycle act as precursors for various biosynthetic pathways such as synthesis of amino acids, heme, fats and lipids (Fig. 5). Oxaloacetate is used in synthesis of aspartate which in turn is involved in synthesis of five more amino acids. Succinyl CoA is utilized in the synthesis of lysine and methionine and synthesis

Table 4 Stoichiometry of complete oxidation of glucose via glycolytic pathway, pyruvate dehydrogenase complex reaction and citric acid cycle

Reaction	No. of energy charge formed	No. of reducing equivalents formed
Glycolytic pathway 1 Glucose → 2 Pyruvate	2 ATP	2 NADH
Pyruvate dehydrogenase complex 2 Pyruvate → 2 Acetyl CoA		2 NADH
Tricarboxylic acid cycle 2 acetyl CoA → 2 oxaloacetate	2 ATP	6 NADH, 2 $FADH_2$
Total	4 ATP	10 NADH, 2 $FADH_2$
No. of ATP formed per glucose	**32 ATP**	

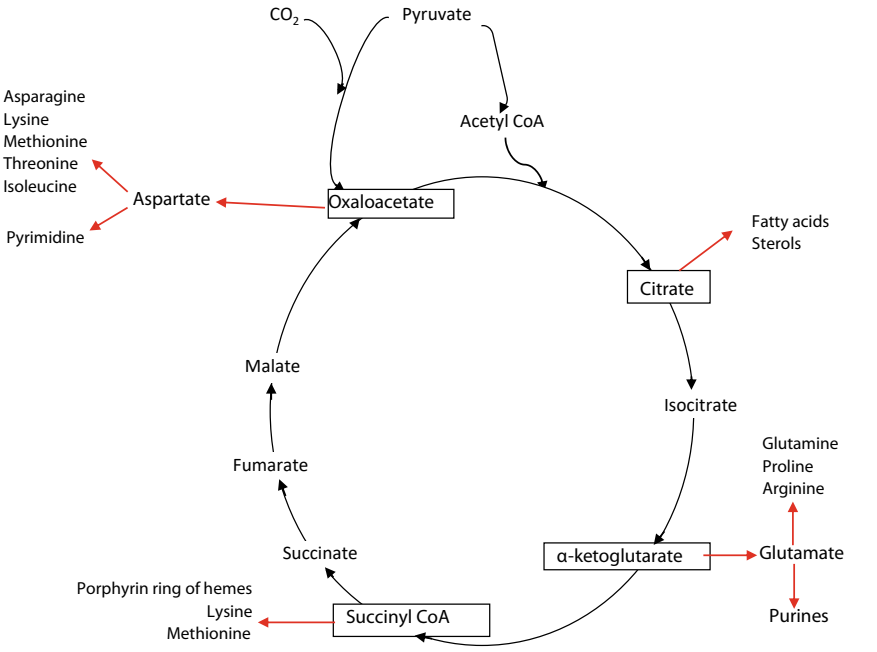


Fig. 5 Anabolic functions of the citric acid cycle

of tetrapyrroles present in cytochromes and chlorophylls. α-ketoglutarate is used for glutamate synthesis which is the precursor for three more amino acids. Fumarate also acts as the precursor for aspartate in some bacteria. Since the TCA cycle can function in a catabolic manner and also anabolically as a source of precursors for biosynthetic pathways, it is referred to as an amphibolic pathway.

Anapleurotic reactions of TCA cycle

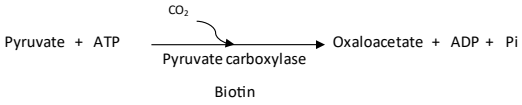
Intermediates of the cycle such as oxaloacetate, citrate, α-ketoglutarate and succinyl CoA act as precursors for various biosynthetic pathways are thus are constantly removed. The replenishment of these intermediates is important to maintain the cellular pool and continuity of the cycle. Cells have mechanisms of "refilling" the pools of citric acid cycle intermediates by pathways known as anapleurotic reactions. Under normal circumstances, there is a dynamic balance between the reactions involved in siphoning off the intermediates into other pathways and those responsible for their replenishment, such that the concentrations of these intermediates remain nearly constant.

Further, it is not necessary to replenish the intermediate that is being removed, as any intermediate can be replenished by feeding-in from any point in the cycle. Thus, cells need only one or two major anapleurotic reactions.

The continuity of the cycle is mostly maintained by maintaining the right levels of oxaloacetate. Oxaloacetate replenishment can occur by various pathways:

1. Sugars such as glucose are converted to pyruvate or phosphoenol pyruvate which can then be carboxylated to oxaloacetate via the action of enzymes pyruvate carboxylase and PEP carboxylase, respectively. Both these enzymes are widespread in bacteria. However, *E. coli* lacks pyruvate carboxylase. Further, PEP carboxylase is not found in fungi or animals.

   (a) **Pyruvate carboxylase**:
   Pyruvate carboxylase contains biotin as a coenzyme. It is an allosteric enzyme that is positively regulated by acetyl CoA (higher levels of acetyl CoA signify higher requirement of oxaloacetate). In some eukaryotes such as *Aspergillus niger*, pyruvate carboxylase can be present both in mitochondria and cytosol.



   (b) **PEP carboxylase**:
   In *E. coli*, PEP carboxylase is an allosteric enzyme. PEP carboxylase is negatively controlled by aspartate (which is formed from oxaloacetate and thus higher concentrations of aspartate signify higher concentrations of oxaloacetate) and positively controlled by acetyl CoA (higher concentration of acetyl CoA is a signal to produce higher amounts of oxaloacetate) (Fig. 6).

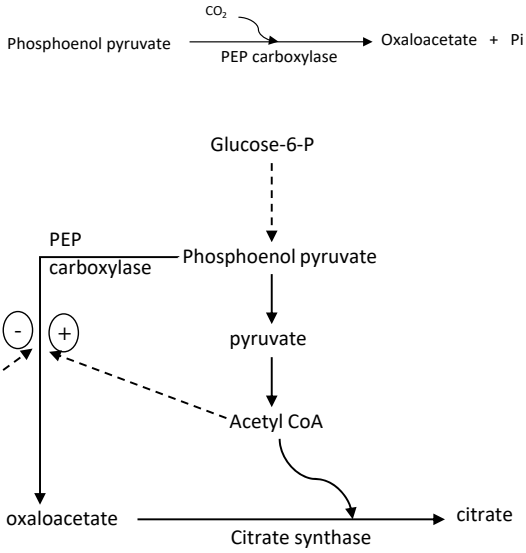
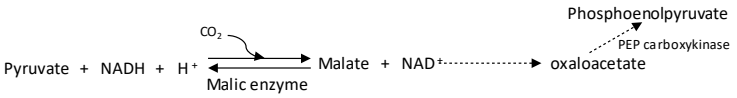


Fig. 6 Regulation of PEP carboxylase

2. Oxaloacetate is also replenished by the action of a third enzyme, commonly called malic enzyme. This enzyme catalyzes reductive carboxylation of pyruvate to malate which can then be oxidized to oxaloacetate.



This is a reversible reaction. Malic enzyme, together with PEP carboxykinase, is important for growth on citric acid intermediates such as succinate or malate.
3. In cases where there is excess availability of amino acids, they can be directly converted to oxaloacetate.
4. In cases, where organic acids such as fumarate, succinate and malate are available in excess, they can be directly oxidized to oxaloacetate in the standard citric acid cycle reaction.
5. The glyoxylate cycle can also contribute to replenishment of oxaloacetate by synthesis of malate.

Partitioning of Pyruvate between synthesis of acetyl CoA and oxaloacetate

Pyruvate has two major metabolic uses: oxidation of carbon via citric acid cycle for energy generation and providing precursors for biosynthetic pathways. Thus, pyruvate is partitioned between two main pathways—decarboxylation to acetyl CoA via activity of pyruvate dehydrogenase and carboxylation to oxaloacetate via activity of pyruvate carboxylase.

Acetyl CoA is a negative regulator for pyruvate dehydrogenase and a positive regulator for pyruvate carboxylase. Thus, in conditions where the intermediates of TCA cycle such as succinyl CoA or α-ketoglutarate are removed for biosynthesis, the levels of oxaloacetate would also decrease. Thus, the rate of citrate synthase reaction lowers and consequently acetyl CoA would accumulate. This leads to a decrease in activity of pyruvate dehydrogenase and increase in activity of pyruvate carboxylase thereby leading to increased production of oxaloacetate and continuation of TCA.

Regulation of TCA cycle

The regulation of the TCA cycle is largely governed by availability of substrate, energy charge and reducing equivalents in the cellular pool. Each of the three strongly exergonic steps in the cycle, those catalyzed by citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, and the reactions catalyzed by pyruvate dehydrogenase complex can become the rate-limiting step under some circumstances (Fig. 7).

Under condition of high energy charge of the cell, i.e., high ATP/ADP + AMP ratio, citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase are inhibited. In addition, pyruvate dehydrogenase complex that synthesizes acetyl CoA (which is required for the first reaction of TCA cycle) is also negatively regulated by ATP. This regulation ensures that the TCA cycle will not oxidize excessive amount

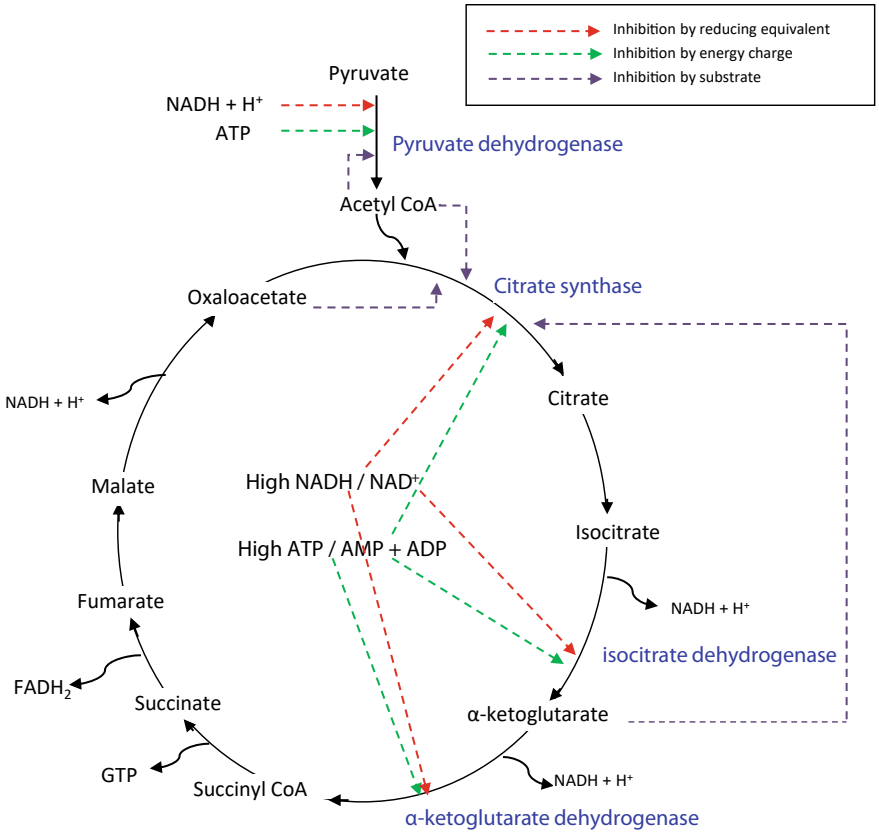


Fig. 7 Regulation of TCA Cycle

of pyruvate and acetyl CoA when ATP in the cell is plentiful. Conversely, when the energy charge in the cell is low, these enzymes are activated.

Availability of reducing equivalents, i.e., the ratio of NADH/NAD^+ is the other major control of the cycle. This is due to substrate inhibition by NADH of the enzymes that use NAD^+ as a substrate. This is desirable metabolically, since the operation of the cycle leads to an increase in this ratio. Electron transport and oxidative phosphorylation control the relative ratios of NAD^+ and NADH. Under condition of low oxygen, the electron transport system is inhibited and NADH accumulates. This in turn decreases NAD^+ availability for the citric acid cycle. Thus, the more "aerobic" the cellular environment is, the more the citric acid cycle runs. All the major checkpoints of the TCA cycle, i.e., activity of citrate synthase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and also pyruvate dehydrogenase, are inhibited by NADH. Further, all these enzymes are activated at low levels of the reducing equivalents.

Substrate availability is another limitation of the cycle. Pyruvate dehydrogenase can be allosterically inhibited by acetyl CoA. Citrate synthase is regulated by the availability of its substrates, acetyl CoA and oxaloacetate. Citrate synthase is also inhibited by α-ketoglutarate in facultative gram-negative bacteria such as *E. coli.*

In addition to the above regulatory mechanisms, isocitrate dehydrogenase is also controlled by a reversible phosphorylation and dephosphorylation mechanism which is catalyzed by a bifunctional kinase/phosphatase. Phosphorylation inactivates the enzymes while dephosphorylation activates the enzymes. Thus, isocitrate dehydrogenase is normally present in the dephosphorylated form. Regulation by kinase/phosphatase comes into play only in the presence of acetate as the carbon source and more details will be provided while studying the glyoxylate cycle in Chap. 13: Alternate Tricarboxylic acid cycle.

Shuttling of intermediates and reductants between cytosol and mitochondria in eukaryotes

The glucose oxidation pathways, viz. glycolysis and pentose phosphate pathway, occur in cytosol while citric acid cycle operates in the mitochondrial matrix in case of eukaryotes. In prokaryotes, there is no such compartmentalization.

Shuttling of intermediates—The glycolytic by-product pyruvate is produced in cytosol and transported to mitochondria before it is further processed. There it is degraded by pyruvate dehydrogenase to acetyl CoA and reductant and enters into the TCA cycle. At the same time, as a part of anapleurotic reaction, mitochondrial pyruvate carboxylase converts a part of pyruvate to oxaloacetate to continue the cycle. Citrate, the first product of the TCA cycle, is also required for biosynthesis and is transported out of mitochondria. In the cytosol, citrate is acted upon by ATP citrate lyase to generate acetyl CoA which is required for synthesis of fatty acids, phospholipids and biomass generation. The citrate export from mitochondria is compensated by oxaloacetate import back into the mitochondria in the form of malate. This is feasible by conversion of oxaloacetate to malate by cytosolic malate dehydrogenase. Malate enters the mitochondria and gets converted back to oxaloacetate (Fig. 8).

Shuttling of reductant—The transport of reductants from the cytosol to the mitochondrial matrix occurs via two shuttle pathways, namely the malate–aspartate shuttle and the glycerol phosphate shuttle (Fig. 8).

1. **Malate–aspartate shuttle**—In the first step, cytosolic malate dehydrogenase catalyzes the reaction of oxaloacetate and NADH + H^+ to produce malate and NAD^+. Thus, the reducing equivalents are attached to oxaloacetate to form malate. Malate thus produced is transported into the mitochondrial matrix. This import into the mitochondrial matrix is balanced by exporting out oxaloacetate formed by the action of mitochondrial malate dehydrogenase. During this reaction, NAD^+ is reduced to form NADH + H^+. Further, oxaloacetate cannot directly be transported into the cytosol and thus the export is in the form of aspartate. Mitochondrial aspartate aminotransferase converts oxaloacetate to aspartate using glutamate as an amino donor. In the reaction, glutamate is transformed into alpha-ketoglutarate. In the cytosol, aspartate is again converted

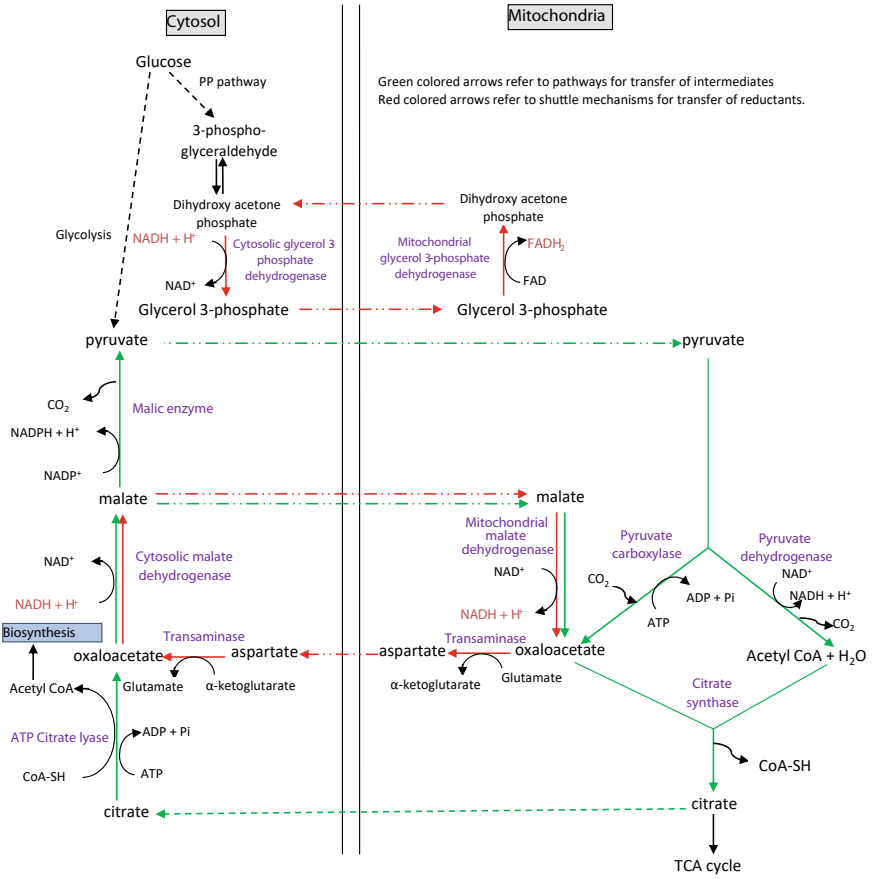


Fig. 8 Representation of shuttling of intermediates and reductants between cytosol and mitochondria in eukaryotes depicting malate–aspartate shuttle and glycerol phosphate shuttle

back to oxaloacetate by cytosolic aspartate aminotransferase by transferring the amino group to α-ketoglutarate to form glutamate. The net effect of the malate–aspartate shuttle is purely in terms of redox. NADH + H^+ in the cytosol is oxidized to NAD^+ and NAD^+ in the matrix is reduced to NADH + H^+.

2. **Glycerol phosphate shuttle**—The glycerol phosphate shuttle has a secondary role in transporting reducing equivalents. In the first step, cytosolic glycerol 3-phosphate dehydrogenase converts dihydroxyacetone phosphate (produced by isomerization of 3-phosphoglyceraldehyde) to glycerol 3-phosphate by oxidizing one molecule of NADH + H^+ to NAD^+. Glycerol 3-phosphate is transported into the mitochondrial matrix where it is converted back to dihydroxyacetone phosphate by mitochondrial glycerol-3-phosphate dehydrogenase, by reducing one molecule of FAD to $FADH_2$. The dihydroxyacetone phosphate is exported back to the cytosol to complete the carbon balance.

4 Citric Acid Production by *Aspergillus Niger*: An Overview

Citric acid is a primary metabolite, and its production is well regulated in organisms. Citrate synthase, the enzyme that catalyzes production of citric acid, is regulated by several factors including energy charge and reductant. Further, citrate is one of the key inhibitors of the glycolytic pathway. Under such a tight regulation scheme, it is surprising that any organism can over-produce citric acid. Various studies have shown that citric acid producing mutants have devised several control mechanisms and many biochemical events jointly contribute to the over-production (Papagianni, 2007; Netik et al., 1997).

The accumulation of citric acid is strongly influenced by the composition of the medium and physiological conditions. In *A. niger*, citric acid over-production is observed during the idiophase (stationery growth phase) and under specific conditions, viz. (i) acidic pH ranging from 1.6–2.2; (ii) high carbon source (sugar concentration 12–25% w/v); (iii) Mn^{2+} deficiency; and (iv) limited nitrogen source (high NH_4^+ concentration) (Table 5).

Glucose uptake—Under high sugar concentrations, a low-affinity glucose transporter is induced that provides the high flux of glucose required for citrate production. Some reports also suggest that simple diffusion which is not dependent on any transport systems is the primary mechanism for glucose uptake under these conditions.

Conversion of glucose to pyruvate—During glucose fermentation by the glycolytic pathway, the key regulatory enzyme phosphofructokinase I (PFK I) is inhibited by both ATP and citrate. At the stationery growth phase, citrate concentration in the cell is high even during slow growth because of high biomass. This would normally lead to inhibition of PFK I. For citric acid over-production, this inhibition is overcome by accumulation of various positive effectors of PFK I. Manganese is a known inhibitor for PFK I, and thus, the medium is made to be deficient in

Table 5 Functionalities of special medium components during citric acid over-production

Medium component	Functionality
High carbon source (high sugar)	High citrate, synthesis of fructose-2,6-bisphosphate which activates PFK I, glycerol accumulation which inhibits isocitrate dehydrogenase, represses synthesis of α-ketoglutarate dehydrogenase
Limited nitrogen source (high NH_4^+)	PFK I activation, represses synthesis of α-ketoglutarate dehydrogenase
Acidic pH	Facilitation of transport, inhibit production of other acids like oxalic acid
Mn^{2+} deficiency	PFK I activation, increase in intracellular NH_4^+ concentration, stimulates citrate efflux through active transport system (if required)

Mn^{2+}. Further, Mn^{2+} deficiency results in breakdown of cellular proteins which also contributes to an increase in intracellular concentration of ammonium. Ammonium ions further overcome citrate inhibition of PFK I. Furthermore, high sugar concentration leads to rapid production of fructose-2,6-bisphosphate, an activator of PFK I. These conditions lead to a flux through the glycolytic pathway and accumulation of pyruvate.

Citrate production—Some part of the accumulated pyruvate is transported to the mitochondrial matrix and enters TCA cycle as usual. High sugar concentration leads to accumulation of glycerol, an osmoprotectant which inhibits isocitrate dehydrogenase. On inhibition of isocitrate dehydrogenase, the unfavorable equilibrium of aconitase causes accumulation of large amounts of citrate rather than isocitrate. Moreover, the combination of high NH_4^+ concentration and high sugar concentration represses the synthesis of α-ketoglutarate dehydrogenase, thereby inhibiting citrate catabolism in the TCA cycle and favoring its overproduction. Thus, citric acid is an "overflow end product" due to high flux rates upstream and reduced flux rates downstream of the accumulation point.

Further, some part of the pyruvate accumulated due to the physiological conditions remain in the cytoplasm and is converted to oxaloacetate by cytosolic pyruvate carboxylase and then to malate by cytosolic malate dehydrogenase. Thus, the citrate accumulated in the mitochondrial matrix can be exported out to the cytoplasm in exchange for malate which is transported into the mitochondria.

Citrate export from mitochondria to cytosol—Citric acid is a tricarboxylic acid containing three carboxylic functional groups with three different values of pKa (3.1, 4.7 and 6.4). Thus, citric acid can occur in three ionization states depending upon the pH conditions. The normal ionization state of citrate at pH ~ 6 (prevalent in the cytosol) is $citrate^{2-}$ ($citrate^{3-}+H^+$). $Citrate^{3-}+H^+$ can be exchanged with malate (which is present as $malate^{2-}$ in the cytosol) via the tricarboxylate antiporter for delivery of citrate to the cytosol. The malate required for antiport exchange can be exported back to the cytosol via the dicarboxylate antiport, which accepts HPO_4^{2-}. This has a dual role since oxidative phosphorylation creates a continuous demand for phosphate in mitochondria which is supplied via this exchange.

The citrate produced in mitochondria is also exchanged for α-ketoglutarate via another mitochondrial organic acid transporter known as citrate–oxoglutarate shuttle protein (Kirimura et al., 2019). The exchange of citric acid for cytoplasmic malate and oxoglutarate/α-ketoglutarate serves a dual purpose of exporting produced citric acid from mitochondria and importing TCA cycle intermediates. The oxaloacetate, which is used up during citric acid formation, is regenerated from these imported TCA cycle intermediates, malate and α-ketoglutarate. This continues citric acid production even when the TCA cycle is slowed down at isocitrate and α-ketoglutarate step.

Citrate export from cytosol to extracellular medium—The export of citric acid out of cytoplasm occurs by ΔpH-driven H^+-symport-dependent system, active process and organic anion transporter.

There is a large pH gradient between the cytosol (which is at ~ pH 6) and the extracellular medium (which is at ~ pH 2). Thus, citric acid is present as citrate^{2-} in the cytosol while it is almost undissociated at the pH of the extracellular medium. As per Mattey (1977), the cell membrane is permeable to citrate^{2-} and citrate^{2-} is excreted by passive diffusion along a gradient of dissociated citric acid. To prevent uptake of these citrate^{2-} ions back in the cell, the extracellular pH is kept low. At low pH values, citrate^{2-} ions get protonated, become undissociated and cannot enter the cell by the diffusion process. Thus, low extracellular pH keeps the external citrate^{2-} concentrations at a negligible level and they cannot diffuse back into the cell. This is also the reason of different rates of citrate export observed at different external pH values.

Netik et al. (1997) demonstrated that citrate is excreted out of the cell by an active process requiring ATP. This export was also sensitive to metabolic inhibitors such as sodium azide and a proton conductor, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). This system is dependent on Mn^{2+} and this dependency could only partially be fulfilled by other divalent metal ions. Further, it was observed that citrate was not exported out unless the fungal mycelium was deficient in Mn^{2+} ions, whereas the uptake of citrate from the medium was only detectable upon pre-cultivation of *A. niger* in a medium supplemented with Mn^{2+} ions. Thus, it has been postulated that citrate efflux as well as uptake is reciprocally regulated by manganese ions. Thus, presence of Mn^{2+} stimulates citrate uptake and inhibits efflux while deficiency of Mn^{2+} inhibits uptake and stimulates efflux. Manganese ions are responsible for differential transcriptional regulation of the uptake, and the excretion system and carrier inversion occur depending upon the Mn^{2+} levels.

Thus, Mn^{2+} deficiency has a dual role in citric acid over-production conditions. As described above, the primary role is preventing inhibition of PFK I and increasing the intracellular NH_4^+ concentration. The secondary role is stimulating the citrate efflux by active transport system. It directly follows that it is vital to carefully select the ingredients of the medium as well as bioreactor material to ensure that even traces of manganese are not present which may affect citric acid yields during over-production conditions (Fig. 9).

Furthermore, various experiments have established that the active transport system is not the main means of citrate export under citrate over-production conditions. *A. niger* possesses the active transport mechanism but does not use it when the external pH is low. Protein CexA, a citrate-H^+ antiporter, is the main citric acid transporter in *A. niger* (Steiger et al., 2019). Its disruption completely abolishes citrate secretion into medium and reroutes the metabolism toward oxalic acid production. This cellular transport system is very important for *A. niger* cell as its over-expression leads to significant increase in citric acid secretion.

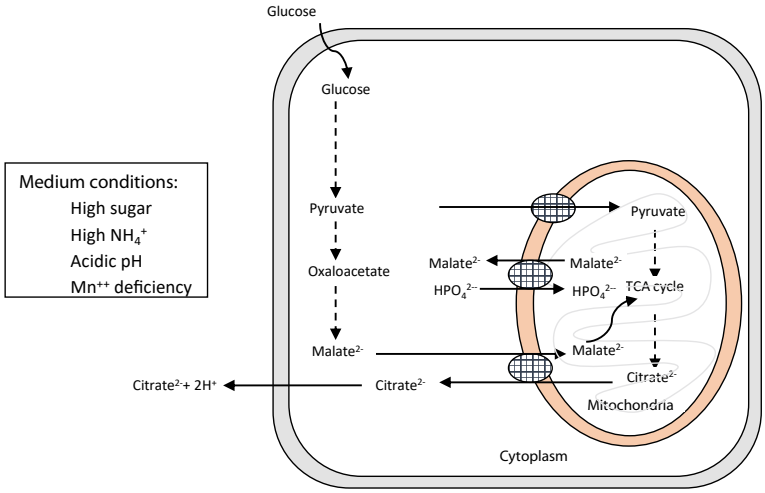


Fig. 9 Diagrammatic representation of citric acid over-production in *A. niger*

Summary

- Tricarboxylic acid cycle, commonly known as TCA cycle/Krebs cycle, is the oxidation of acetyl CoA to CO_2 and H_2O with generation of reductants.
- Hans Krebs in 1937 postulated the complete citric acid cycle based both on his own findings and some previous leads of Albert Szent-Gyorgyi and others.
- TCA cycle initiates with acetyl CoA generation from decarboxylation of pyruvate produced during glycolytic pathway.
- Decarboxylation of pyruvate may take place by pyruvate dehydrogenase complex in aerobic conditions in all organisms having aerobic metabolism.
- Pyruvate dehydrogenase complex is a multi-enzyme complex that uses a number of coenzymes, viz. TPP, lipoic acid, FAD, NAD^+ and CoA-SH.
- TCA starts with condensation of acetyl CoA and oxaloacetate to form citrate by citrate synthase.
- In a series of seven reactions, 6-carbon citric acid is broken down to 4-carbon oxaloacetate with generation of 2 mol of CO_2, 3 mol of NADH + H^+ and 1 mol $FADH_2$ along with one high-energy phosphate bond GTP.
- TCA cycle has a major anabolic role where precursors for amino acids, heme, fats and lipids pathways are generated.
- For continuity of the TCA cycle, the major anapleurotic reaction for generation of oxaloacetate is carboxylation of pyruvate or PEP by pyruvate carboxylase and/or PEP carboxylase.
- Partitioning of pyruvate between generation of acetyl CoA and oxaloacetate is largely regulated by acetyl CoA levels which is a positive regulator of pyruvate carboxylase.

- TCA cycle is regulated by availability of substrate, energy and reducing equivalents in cellular pool.

Questions

1. How pyruvate is oxidized to acetyl CoA in aerobic and anaerobic conditions?
2. Name the different coenzymes required by pyruvate dehydrogenase enzyme.
3. How many reductants are generated through one round of TCA cycle?
4. Name any one enzyme which catalyzes an anapleurotic reaction in TCA cycle.
5. How many ATPs are generated by one round of TCA?
6. Name any one inhibitor of TCA cycle.
7. Taking into account complete oxidation of one mole of glucose by EMP followed by TCA, what is the total ATP gain?
8. Write the pyruvate dehydrogenase reaction for oxidation of pyruvate to acetyl CoA.
9. How is oxaloacetate replenished during TCA cycle.
10. In a eukaryotic cell, how does the NADH produced during glycolytic cycle enters the mitochondria for electron transport?
11. Write different steps of TCA showing generation of reductant and high-energy phosphate.

References

Kirimura, K., Kobayashi, K., & Yoshioka, I. (2019). Decrease of citric acid produced by Aspergillus niger through disruption of the gene encoding a putative mitochondrial citrate-oxoglutarate shuttle protein. *Bioscience, Biotechnology, and Biochemistry, 83*(8), 1538–1546.

Mattey, M. (1977). Citrate regulation of citric acid production in *Aspergillus niger*. *FEMS Microbiology Letters, 2*(2), 71–74.

Netik, A., Torres, N. V., Riol, J. M. & Kubicek, C.P. (1997). Uptake and export of citric acid by *Aspergillus niger* is reciprocally regulated by manganese ions. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1326*(2), 287–294.

Papagianni, M. (2007). Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. *Biotechnology Advances, 25,* 244–263.

Steiger, M. G., Rassinger, A., Mattanovich, D., & Sauer, M. (2019). Engineering of the citrate exporter protein enables high citric acid production in *Aspergillus niger*. *Metabolic Engineering, 52,* 224–231.

Chapter 13
Alternate Tricarboxylic Acid Cycle

Rani Gupta and Namita Gupta

1 Distribution of TCA Cycle Among Bacteria Under Microaerophilic and Anaerobic Conditions

TCA cycle is widely present among all heterotrophs growing aerobically. In addition, TCA cycle is also observed in anaerobes belonging to group II sulfate reducers and certain archaea such as hyperthermophilic archaea that use sulfur or thiosulfate as terminal electron acceptor.

TCA cycle is an oxidative mode for complete oxidation of glucose. In the preceding chapter (Chap. 12: Tricarboxylic Acid Cycle), we have discussed details of TCA cycle as an oxidative pathway coupled to generation of reductant and energy. The studies on regulation of TCA cycle revealed that it is tightly regulated by reductant and energy charge of the cell. Since it is an oxidative mode of catabolism, it operates throughout in the presence of oxygen. However, in some facultative microorganisms, it runs partially with activation of some new enzyme activities and also down-regulation of existing enzymes under anaerobic or microaerophilic conditions.

In anaerobic or microaerophilic conditions, the levels of the enzyme α-ketoglutarate dehydrogenase are very low as it is repressed under anaerobic conditions. Under these conditions, an incomplete/ branched TCA cycle is operated wherein one branch is oxidative while the other branch is reductive in nature. This mode is observed in fermentative bacteria and facultative enteric bacteria such as *E. coli* that carry out anaerobic respiration with nitrate or any other inorganic electron acceptor. However, aerobic methylotrophs which grow on methane, methanol, etc., have the incomplete TCA cycle only as they lack α-ketoglutarate dehydrogenase. In contrast, some nitrate respirers like *Pseudomonas stutzeri* have complete oxidative TCA pathway even during denitrifying conditions. Similarly, *Paracoccus denitrificans* also has a complete oxidative TCA pathway when growing anaerobically using nitrate as electron acceptor.

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_13

Furthermore, some bacteria such as *E. coli* can grow on 2-carbon substrates, mainly acetate, by operating another variant of the TCA cycle known as the glyoxylate pathway. The glyoxylate pathway functions to conserve carbon by conserving CO_2 lost during tricarboxylic acid cycle.

2 Branched TCA

In anaerobic or microaerophilic conditions, the levels of the enzyme α-ketoglutarate dehydrogenase are very low. Thus, the TCA pathway is blocked at the step of conversion of α-ketoglutarate to succinyl CoA and cannot operate in the oxidative direction. However, at the same time, a new enzyme fumarate reductase is induced instead of succinate dehydrogenase. This leads to generation of succinyl CoA by reversing the reaction between oxaloacetate and succinate.

Thus, the TCA cycle is branched with one branch operating oxidatively to generate α-ketoglutarate while the other branch operating reductively to generate succinate. This pathway is referred to as an incomplete TCA cycle/branched TCA cycle (Fig. 1).

Conversion of pyruvate to acetyl CoA

Under anaerobic or microaerophilic conditions, pyruvate is converted to acetyl CoA by the enzyme pyruvate formate lyase (PFL) instead of the pyruvate dehydrogenase complex. The overall reaction is:

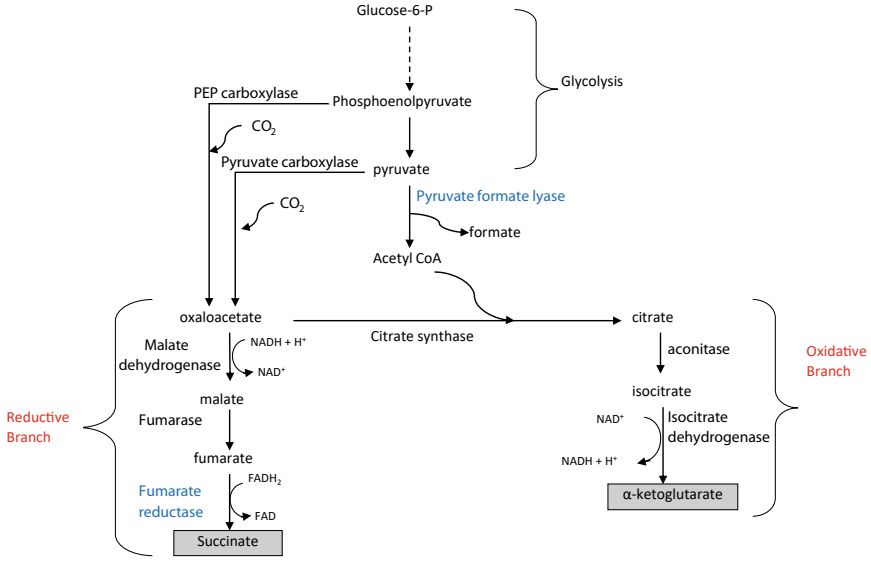


Fig. 1 Branched TCA cycle depicting oxidative and reductive branches

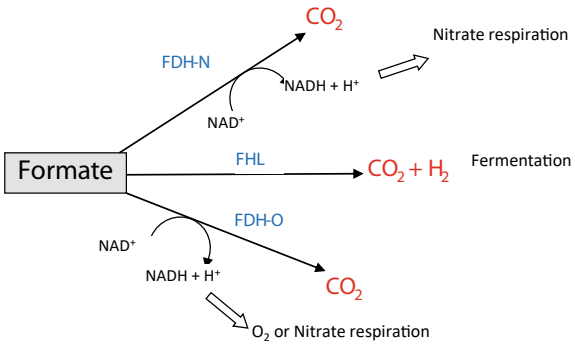


Fig. 2 Fate of formate under anaerobic/microaerophilic or fermentative conditions

$$\text{Pyruvate} + \text{CoA} - \text{SH} \xrightarrow{\text{Pyruvate} - \text{formate lyase}} \text{Acetyl CoA} + \text{Formate}$$

In the reaction catalyzed by pyruvate dehydrogenase complex, the reducing equivalents generated by the cleavage of pyruvate are transferred to NAD^+ to form NADH + H^+. In contrast, in the PFL reaction, the reducing equivalents remain with the product formate. Thus, this reaction is most suited for fermentative conditions wherein exogenous electro-acceptors are not available and maintenance of redox balance in the cell is most important.

Fate of formate

E. coli encodes three isozymes of formate dehydrogenase (FDH): FDH-N, FDH-O and FDH-H. The three isozymes are active under different conditions indicating that formate is metabolized by different pathways depending on the availability of exogenous electron acceptors (Fig. 2).

- FDH-N and FDH-O are operative under respiratory conditions and couple formate oxidation to CO_2 with nitrate or oxygen reduction, respectively. Though FDH-O is mostly induced under aerobic conditions, some reports suggest that it is also induced under nitrate respiring conditions (Sawers, 2005).
- The third isozyme FDH-H is operative under fermentative conditions and is responsible for dihydrogen generation by converting formate to CO_2 and hydrogen. FDH-H is part of the formate hydrogen lyase (FHL) complex which comprises seven polypeptides that majorly correspond to two enzymatic activities, formate dehydrogenase and hydrogenase (Sawers, 2005).

ATP generation

During the branched TCA cycle, substrate-level ATP gain by conversion of succinyl CoA to succinate is missing. Also, the reductive branch of the branched TCA cycle consumes reducing equivalents. Thus, the overall reducing equivalents generated during branched pathway are much lesser. The overall energy charge in the cell is

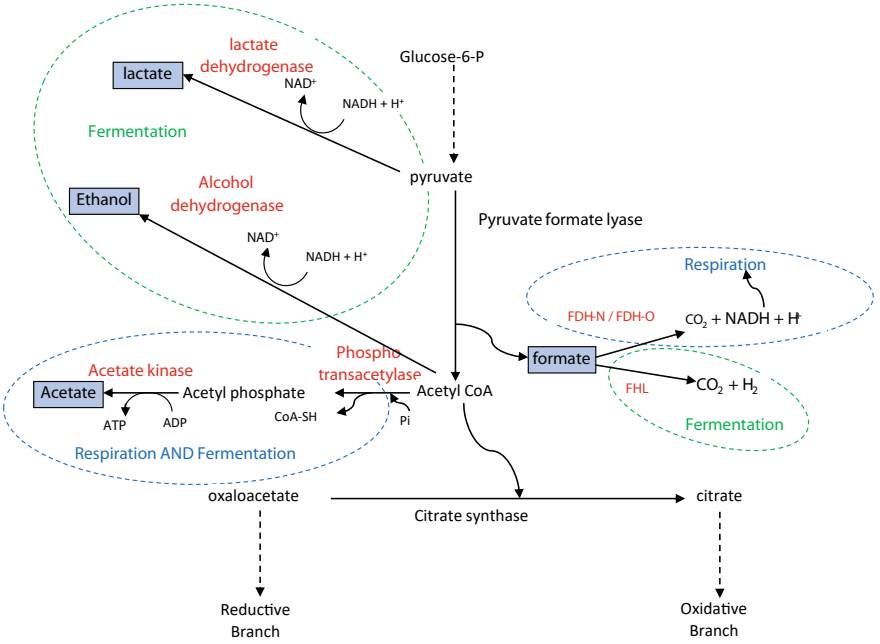
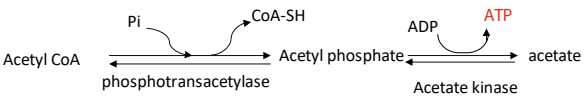


Fig. 3 Distinctive mode of NAD^+ generation under anaerobic/microaerophilic or fermentative conditions

lower under anaerobic or microaerophilic conditions as compared to aerobic conditions. To balance the cellular energy levels, ATP is produced via an alternative pathway wherein acetyl CoA is converted to acetate by the action of the enzymes phosphotransacetylase and acetate kinase. The two-step reaction is concomitant with the formation of ATP in a substrate-level phosphorylation. Thus, under these conditions, acetate is excreted as a major reduced end product (overflow metabolite).



Both these enzymes catalyze reversible reactions, and they may be used for conversion of acetate to acetyl CoA when the organism is growing on acetate.

Regeneration of NAD^+

Under fermentative conditions, the reducing equivalents generated via glycolysis and branched TCA cycle are not re-oxidized due to lack of terminal electron acceptors. Thus, the $NADH+H^+$ pool in the cell builds up and NAD^+ becomes scarce. Regeneration of NAD^+ is essential for continuity of these cycles. This is facilitated by two ways:

1. **Reduction of acetyl CoA to ethanol by the action of alcohol dehydrogenase** with concomitant generation of NAD^+. Thus, the reduction of acetyl CoA to ethanol sacrifices energy (which would have been produced by conversion of acetyl CoA to acetate) but consumes reducing equivalents. Thus, cell balances its requirements to regenerate NAD^+ with its energy requirements by modulating the generation of ethanol and acetate (Fig. 3).
   Alcohol dehydrogenase is encoded by the *adhE* gene and is a trifunctional enzyme harboring the functions: acetaldehyde-CoA dehydrogenase, alcohol dehydrogenase and pyruvate formate lyase deactivase. Of these, PFL deactivation has been studied only *in vitro* and it seems unlikely that this functionality is operative in *in vivo* conditions due to requirement of various cofactors such as $Fe^{2+,}$ NAD^+ and CoA-SH.
2. **Reduction of pyruvate to lactate by the action of NADH-dependent lactate dehydrogenase** with concomitant generation of NAD^+. This reaction consumes one NADH per pyruvate molecule. The cell modulates the generation of lactate as per its requirement to regenerate NAD^+. Lactate dehydrogenase is activated at low pH values (Fig. 3).

E. coli contains three lactate dehydrogenases. However, only one of them is responsible for fermentative conversion of pyruvate to lactate. The other two are membrane-bound flavoproteins which are described as lactate oxidases as they couple to the respiratory chain.

Thus, under anaerobic or microaerophilic respiratory conditions, the major overflow metabolites are acetate and formate (or CO_2). In contrast, under fermentative conditions, acetate, formate (or CO_2 and H2), ethanol and lactate are the major end products.

3 Glyoxylate Cycle

When *E. coli* is grown on acetate (2-carbon compound) as a source of carbon, the bacterium needs to conserve carbon and its breakdown to CO_2 via the TCA cycle is not ideal. Therefore, in such conditions, operation of an anapleurotic pathway is required. *E. coli* operates the glyoxylate pathway (or glyoxylate bypass; also called Krebs–Kornberg cycle) to grow on two carbon sources by bypassing carbon loss as CO_2. The glyoxylate cycle consists of six of the eight reactions of the citric acid cycle, and the two decarboxylation steps, isocitrate to α-ketoglutarate and α-ketoglutarate to succinate, are bypassed. The glyoxylate pathway involves two oxidative steps and hence is linked to electron transport and production of ATP (Fig. 4).

Glyoxylate pathway is catabolite repressible in the presence of easily metabolized carbon sources such as glucose and succinate. It is induced during growth on acetate or fatty acids as well as during exponential phase when acetate is accumulated.

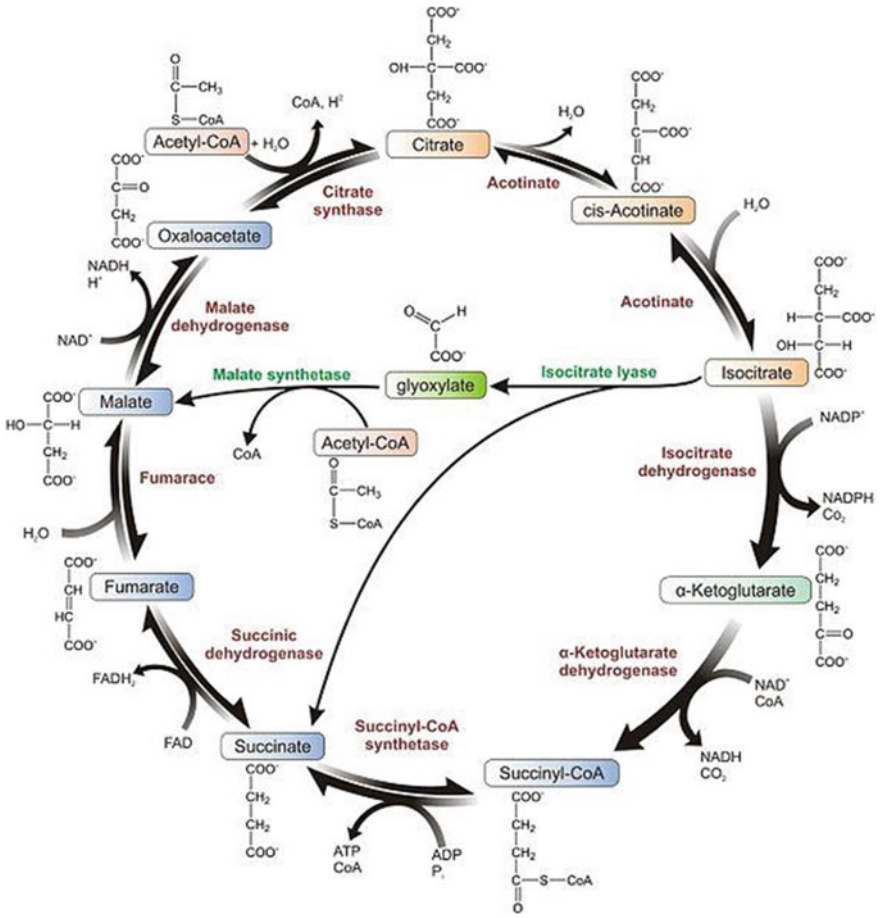


Fig. 4 Glyoxylate cycle. *Source* Wikimedia Commons (Creative Commons Attribution License)

Glyoxylate cycle was discovered by Hans A. Krebs and H.L. Kornberg in 1957 and thus called Krebs–Kornberg cycle. It is distributed among bacteria, protists, fungi and plants while absent from vertebrates (Kornberg, 1966).

It was observed that glyoxylate and succinate are formed in the cell extracts of *Pseudomonas aeruginosa* when only citrate or cis-aconitate was added. Later, it was established that the true substrate was isocitrate and not citrate or aconitate and the enzyme isocitrate lyase catalyzes the breakdown of isocitrate to succinate and glyoxylate, the first reaction of glyoxylate pathway. The second reaction of the bypass, discovered in *E. coli*, was the synthesis of malate by condensation of glyoxylate and acetyl CoA catalyzed by malate synthase.

$$\text{Acetyl CoA} + \text{Glyoxylate} \rightarrow \text{Malate}$$

The malate thus formed would complete the TCA pathway to form oxaloacetate by action of malate dehydrogenase. Thus, acetyl CoA results in net formation of C4 acid. The concerted action of both the enzymes was first demonstrated by Krebs and Kornberg in 1957.

Conversion of acetate to acetyl CoA

Before entering the glyoxylate cycle, acetate is first converted to acetyl CoA by the action of acetyl CoA synthetase (ACS enzyme or acetate CoA ligase). The reaction is as follows:

$$\text{Acetate} + \text{ATP} + \text{CoA} - \text{SH} \leftrightarrow \text{acetyl CoA} + \text{AMP} + \text{Pi}$$

However, this is not the only pathway for conversion of acetate to acetyl CoA. An alternative mode can be via the reversible action of the enzymes acetate kinase and phosphotransacetylase.

Partitioning of isocitrate dehydrogenase and isocitrate lyase activity

There was a general concept that TCA cycle or glyoxylate bypass is regulated in such a way that only one of them operates at time. However, through mutant study it has been established that citrate synthase mutants fail to grow on acetate suggesting that the first reaction of TCA cycle, i.e., citrate synthesis, is essential for acetate utilization as is for glucose metabolism. On the other hand, isocitrate lyase mutants failed to grow on acetate and could utilize all other carbons suggesting that isocitrate lyase activity is important for the bypass along with citrate synthase. It directly follows that citrate would be converted to isocitrate by aconitase enzyme and then isocitrate flux would be fractioned between isocitrate dehydrogenase and isocitrate lyase.

The partitioning of carbon flux in the form of isocitrate which is divided between TCA cycle and glyoxylate bypass is attained by regulation of isocitrate dehydrogenase (IDH) enzyme, which stands at the entry to the bypass. The IDH activity is regulated by reversible phosphorylation–dephosphorylation. This system represents the first example of such regulation of metabolic enzymes in prokaryotes. The demonstration of regulation by phosphorylation–dephosphorylation was made by Garnak and Reeves (1979); and Cozzone and El-Mansi (2005).

The Km of IDH for isocitrate is 1–2 μM while that for isocitrate lyase is 3 mM. IDH is regulated by phosphorylation (inactive) and dephosphorylation (active). When growing on more than two carbon sources, IDH is dephosphorylated and thus active and most of the carbon flux is directed toward TCA cycle. In the presence of 2-carbon substrates such as acetate, IDH is phosphorylated and becomes inactive. This leads to accumulation of isocitrate which can now be taken up by isocitrate lyase as it has low affinity for isocitrate. Thus, the isocitrate flux is shifted toward the glyoxylate bypass. Thus, isocitrate has a key role in controlling the phosphorylation state of IDH.

It directly follows that when a cell culture growing on acetate is switched to carbon sources such as glucose and pyruvate, IDH is immediately dephosphorylated. This in turn leads to decline of isocitrate concentration as it is now channelized through

the TCA pathway. Hence, mutants which fail to phosphorylate IDH cannot grow on acetate.

Phosphorylation and dephosphorylation of isocitrate dehydrogenase

The phosphorylation and dephosphorylation of IDH are by means of a bifunctional kinase–phosphatase called IDHKP. The bifunctional IDHKP was first discovered in *E. coli* by LaPorte and Koshland. The enzyme is a dimer having both kinase and phosphatase activities. Kinase activity of IDHKP is induced during growth on acetate and inhibited by isocitrate or other carbon sources which thus induce its phosphatase activity (Zheng and Jia, 2010).

The kinase transfers a phosphoryl group from ATP to a serine residue in IDH. IDH is phosphorylated at a site close to or at its NADP binding site. This leads to a complete loss of its activity probably because of the phosphorylated enzyme's inability to bind to coenzyme NADP. The phosphatase releases a Pi from phosphorylated IDH and requires a divalent metal ion and ADP/ATP for its activity.

Other than the levels of isocitrate, IDHKP activity is allosterically regulated by the concentrations of various other metabolites as described in Fig. 5.

- Phosphoenolpyruvate, pyruvate, α-ketoglutarate and 3-phosphoglycerate are all activators for phosphatase activity while inhibit the kinase activity. This is expected as all these compounds are precursors for biosynthetic reactions and high levels of these compounds would steer the carbon flux toward the TCA cycle.
- Low levels of AMP/ADP activate the phosphatase and inhibit the kinase.
- NADPH inhibits the phosphatase activity. This can be explained as feedback inhibition of IDH enzyme by an end product of the TCA cycle.

Box 1: Assimilation of Acetate by Alternate Glyoxylate Pathways in the Absence of Isocitrate Lyase (Ensign, 2006)

Some bacteria, for example, *Rhodobacter sphaeroides*, have been found to lack isocitrate lyase enzyme as well as its corresponding gene in the genome of the bacteria. In this case, the bacterium utilizes an **alternative glyoxylate pathway** for acetate assimilation wherein two C4 gluconeogenic precursors are formed using 3 molecules of acetyl CoA and 2 molecules of CO_2 in an overall reaction: **3 Acetyl CoA + 2 CO_2 → L-malate + succinate**.

Moreover, in some methylotrophs like *Methylobacterium extorquens*, acetate assimilation takes place in another distinct way which is defined as **glyoxylate regeneration cycle** with a net reaction of **2 Acetyl CoA → Succinate**.

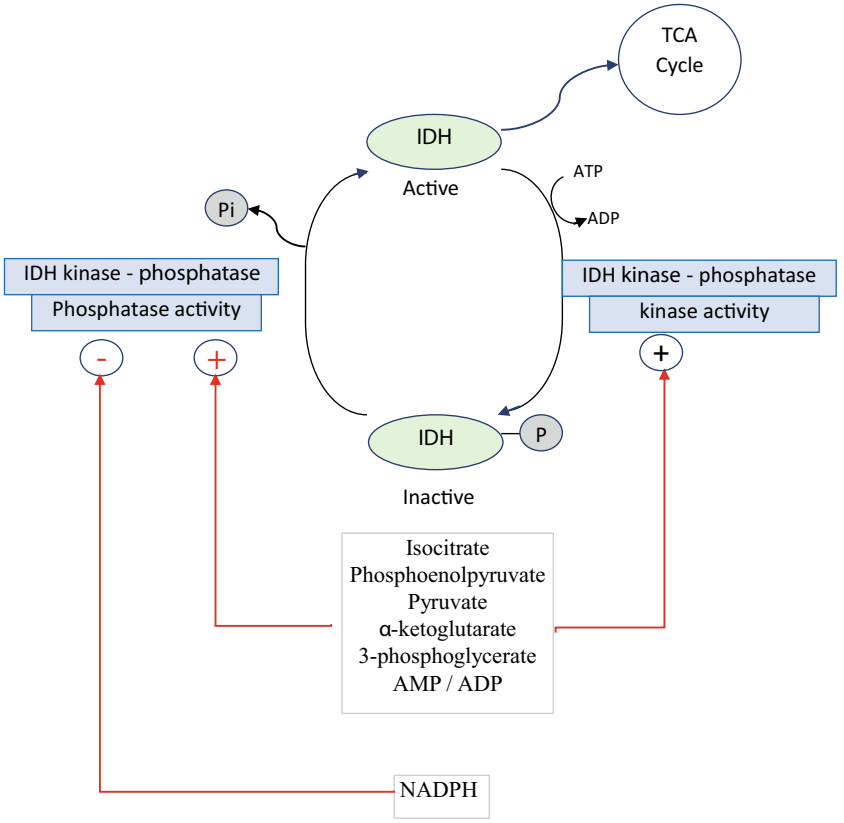


Fig. 5 Regulation of isocitrate dehydrogenase kinase–phosphatase (IDHKP) activity

4 Role of Glyoxylate Cycle in Gluconeogenesis

The glyoxylate pathway is generally regarded as restricted to microorganisms and higher plants and not in animals. Besides enabling microorganisms to grow on acetate, it also contributes to gluconeogenesis pathway during growth on lipids.

In eukaryotes, the glyoxylate cycle operates in peroxisomes, while in plants it occurs in special peroxisomes called glyoxysomes. Peroxisomes also harbor the enzymatic machinery for β-oxidation of fatty acids. Fatty acids stored in the lipid bodies enter the peroxisomes, wherein they undergo β-oxidation to generate acetyl CoA. Acetyl CoA enters the TCA to form isocitrate. Isocitrate is further metabolized by isocitrate lyase via glyoxylate pathway to form glyoxylate and succinate. The glyoxylate molecule accepts another acetyl CoA molecule to form malate and continues the cycle, while succinate becomes the starting point for various biosynthetic reactions. Succinate is transferred to mitochondria where it is converted by enzymes of the TCA cycle, first to fumarate and then to malate. Malate can diffuse

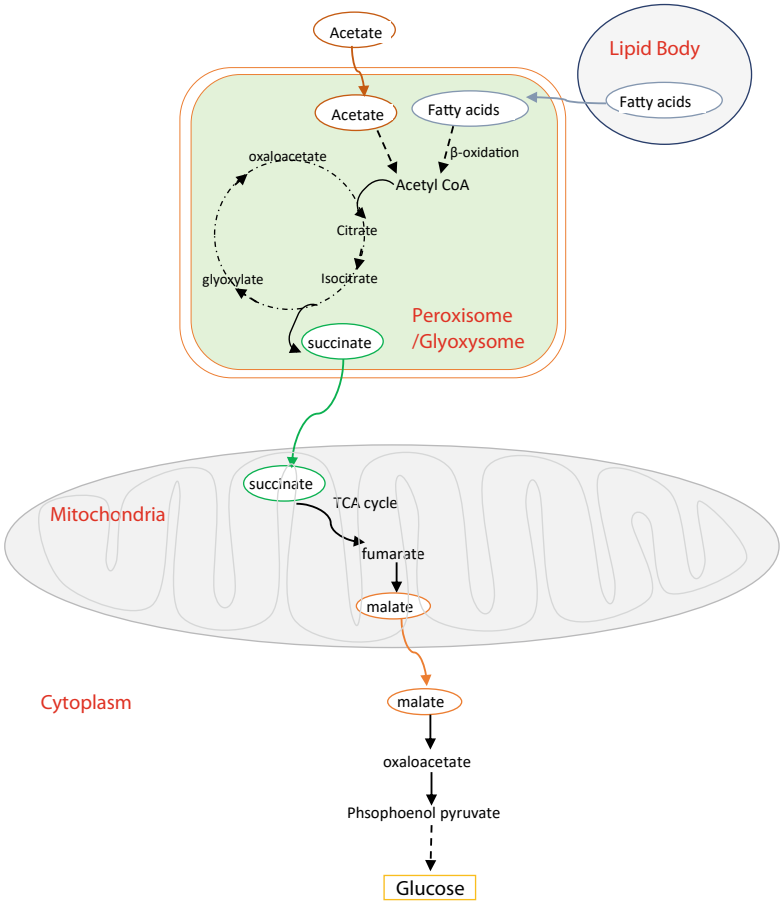


Fig. 6 Role of glyoxylate cycle in gluconeogenesis in eukaryotic cell

to the cytosol where it can be converted to phosphoenolpyruvate via the activity of PEP carboxykinase, the first enzyme in gluconeogenesis. Thus, the net result of the glyoxylate cycle is production of glucose from fatty acids. These events occur in the cytosol in prokaryotes (Fig. 6).

5 Glyoxylate Operon and Its Regulation

The glyoxylate pathway consists of three main enzymes, isocitrate lyase (encoded by *aceB*), malate synthase (encoded by *aceA*) and IDH kinase–phosphatase (encoded by *aceK*). These three genes are clustered together in the *aceBAK* operon which is expressed from a single promoter during growth on acetate or fatty acids. The operon

is under the transcriptional control of the *iclR* gene (having its own separate promoter) located downstream from *aceK*. IclR represses the expression of the *aceBAK* operon as well as its own gene *iclR* by binding to specific operator sequences (termed as IclR boxes) in the promoter regions. An operator sequence has been identified between −52 and −19 bases of *aceB* promoter which overlaps with the −35 region. Thus, binding of iclR to this sequence prevents binding of RNA polymerase to the promoter, thereby preventing transcription of the *aceBAK* genes. Another operator sequence has been identified between +14 and −21 bases of the *iclR* promoter which prevents transcription of the *iclR* gene (Cozzone, 1998; Sunnarborg et al., 1990).

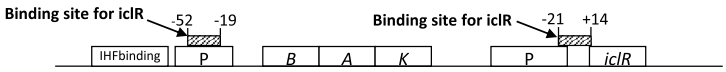
Under glyoxylate inducing conditions, i.e., presence of acetate or fatty acids, iclR does not bind to the *aceBAK* operon which can then be transcribed leading to the expression of the three enzymes. However, in the presence of other carbon sources such as glucose or pyruvate, iclR binds to the *aceBAK* operon and represses its transcription. Further, integration host factor (IHF) which has a binding site upstream of *aceBAK* also opposes iclR repression under inducing conditions, thereby amplifying the induction of *aceBAK* (Resnik et al., 1996).

The expression of *iclR* is also regulated by FadR, a regulator of the genes of fatty acid metabolism. FadR activates the transcription of *iclR* by binding just upstream of its promoter. FadR itself is inactivated during growth on fatty acids and thus prevents an increase in iclR during these conditions, thereby preventing repression of *aceBAK* operon. On all other carbon sources, FadR is activated and in turn activates expression of iclR which then represses the *aceBAK* operon. Thus, the response of *aceBAK* is indirectly mediated by FadR (Fig. 7).

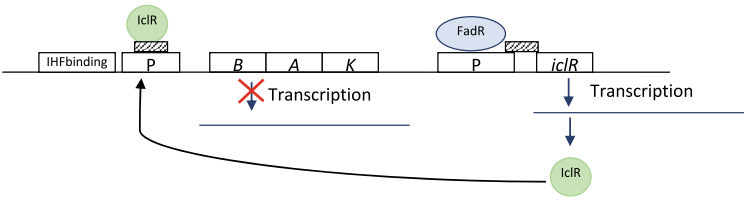
IclR also acts as a general feedback inhibitor of *iclR* protein maintaining constant expression under a variety of conditions. Unlike its behavior in regulating *aceBAK*, iclR repression of its own expression is not sensitive to carbon source. This may be due to the presence of IHF site on *aceBAK* and not on *iclR* (Gui et al., 1996a and b).

Further, a dual mode of iclR regulation by two effectors has been postulated. Pyruvate and glyoxylate act as antagonistic effectors of iclR by binding to the same site on iclR. On induction of the *aceBAK*, the flux through the glyoxylate cycle increases leading to accumulation of glyoxylate. Glyoxylate can bind to iclR, thereby preventing binding of iclR to *aceBAK* by favoring the inactive dimeric state of the protein. Thus, the presence of glyoxylate ensures that iclR cannot repress the operon (Lorca et al., 2007).

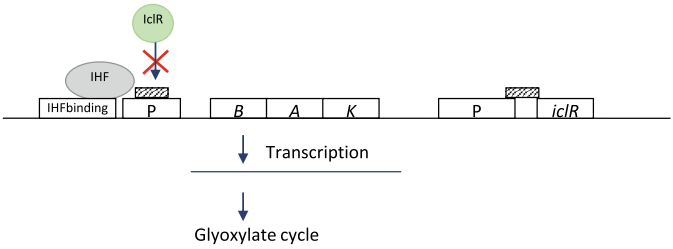
In contrast, the presence of pyruvate (due to its addition during growth on acetate or its production by way of gluconeogenesis) results in reduced carbon flux through glyoxylate pathway. This is due to the fact that pyruvate competes with glyoxylate for binding with iclR and changes its conformation to the active tetrameric form. This increases binding of iclR to *aceBAK* operon and shuts down the glyoxylate pathway (Fig. 8).



(a) Glyoxylate Operon



(b) Under glyoxylate repressing conditions (presence of glucose and other related carbon) – iclR binds to a site near -35 region thus preventing binding of RNA polymerase (FadR binds to *IclRp* and activates it)



(c) Under glyoxylate inducing conditions (absence of glucose and presence of fatty acids) – IHF imposes steric hindrance to binding of iclR (FadR is inactivated so IclR is not expressed in presence of fatty acid and IHF binding to glyoxylate operon causes stearic hindranvce for binding of IclR.

Fig. 7 Glyoxylate operon under repressive and inducible conditions

6 Metabolic Engineering of *E. Coli* for Over-production of Succinic Acid: An Overview

Succinic acid, also known as amber acid or butanedioic acid, is a C4-dicarboxylic acid. It is a key intermediate of the TCA cycle and an end product of anaerobic metabolism. It has a wide variety of applications in agricultural, food, pharmaceutical and chemical industries (Fig. 9).

Currently, succinic acid is primarily produced by a chemical process, in which petroleum-derived maleic anhydride is hydrogenated to succinic anhydride which in turn is hydrated to succinic acid. Only a small quantity of succinic acid is produced via microbial fermentation. Nevertheless, finite nature of petroleum resources, their increasing price and associated pollution problems have steered efforts toward developing biocatalysis as a viable and improved alternative.

Succinic acid is an essential metabolite found in all microbial, plant and animal cells. Fungi and bacteria have been largely exploited for its production. Fungi such as *A. niger*, *A. fumigatus*, *Lentinus degener*, *Paecilomyces variotii*, *Penicillium*

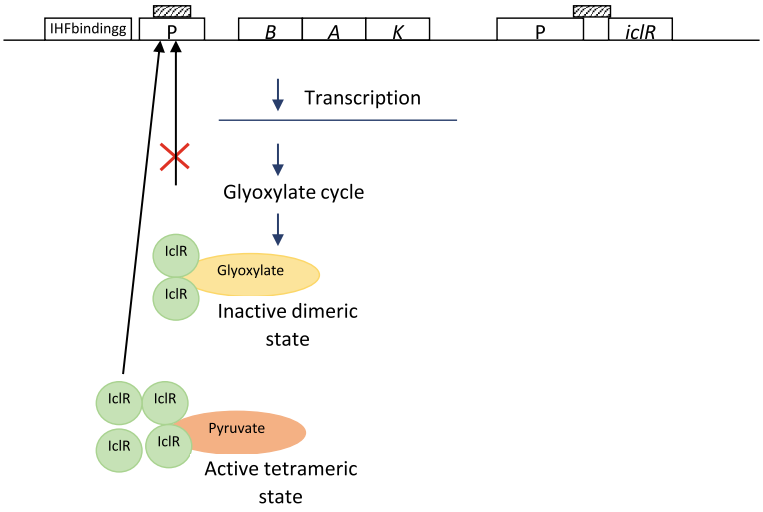


Fig. 8 Regulation of the glyoxylate operon by conformational state of IclR in the presence of pyruvate (active tetramer state) and glyoxylate (inactive dimer state)

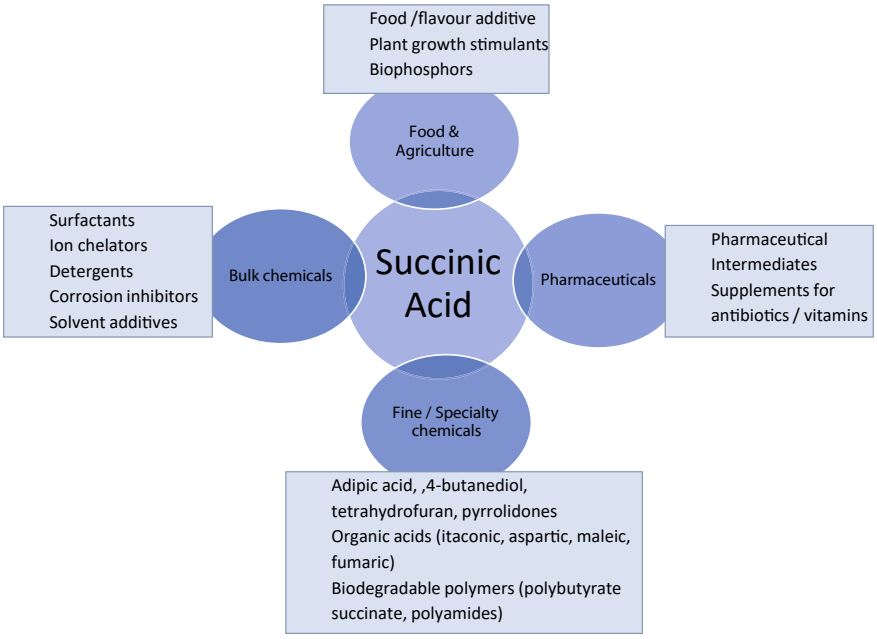


Fig. 9 Applications of succinic acid

veniferu, Byssochlamys nivea and *Saccharomyces cerevisiae* have been recognized as important succinic acid producers. It is also produced by various anaerobic and facultative anaerobic bacteria such as *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Corynebacterium glutamicum*, *Enterococcus faecalis*, *E. coli*, *Bacteroides* sp., *Pectinatus* sp., *Propionibacterium* sp., *Prevotella ruminicola*, *Ruminococcus flavefaciens*, *Succinomonas amylolytica* and *Succinivibrio dextrinisolvens.* Additionally, *Mannheimia succiniciproducens*, isolated from bovine rumen, has been recognized as one of the most promising succinic acid producers (Hong, 2007).

The biotechnological role of *E. coli* for production of various proteins and metabolites has been extended to succinic acid production as well. In *E. coli*, succinic acid can be produced via three different routes (Song and Lee, 2006):

1. As an intermediate in the oxidative TCA cycle
2. As an end product in anaerobic metabolism (reductive branch of branched TCA cycle)
3. As an end product in the aerobic glyoxylate cycle.

The three routes of succinic acid production are depicted in Fig. 10. The figure also provides the genes of the enzymes involved in the various important reactions.

1. **Oxidative TCA**: Of these three routes, oxidative TCA has not been explored to a very large extent. It is an aerobic pathway which would lead to high growth rates of the organism and thus an ideal environment for metabolite production. Still, this is not a pathway of choice for succinic acid production because of low conversion ratios attributed to loss of carbon via the two decarboxylation steps.
2. **Branched TCA**: In *E.* coli, the anaerobic pathway (branched TCA) has been explored to the maximum extent. Various strategies have been adopted to achieve over-production of succinic acid. All the strategies are centered around two main points:

    - **Oxaloacetate accumulation**—The accumulated oxaloacetate will be converted to succinate.
    - **Generation of reducing power—**Conversion of one mole of oxaloacetate to one mole of succinate uses two NADH. Thus, 4 mol of NADH is used per glucose during succinate production. However, glycolysis generates only two moles of NADH per mole of glucose. It directly follows that additional reducing equivalents need to be provided for sustained succinate production.

In addition, efforts have also been expended in improving organic acid tolerance of the strain. Further, anaerobic conditions lead to low ATP generation and thus low growth rate and biomass production. This has to be countered as well.

A. **Oxaloacetate accumulation—**This has been accomplished by the following ways:

i. Over-expression of key enzymes mainly:

- PEP carboxylase (homologous or heterologous)

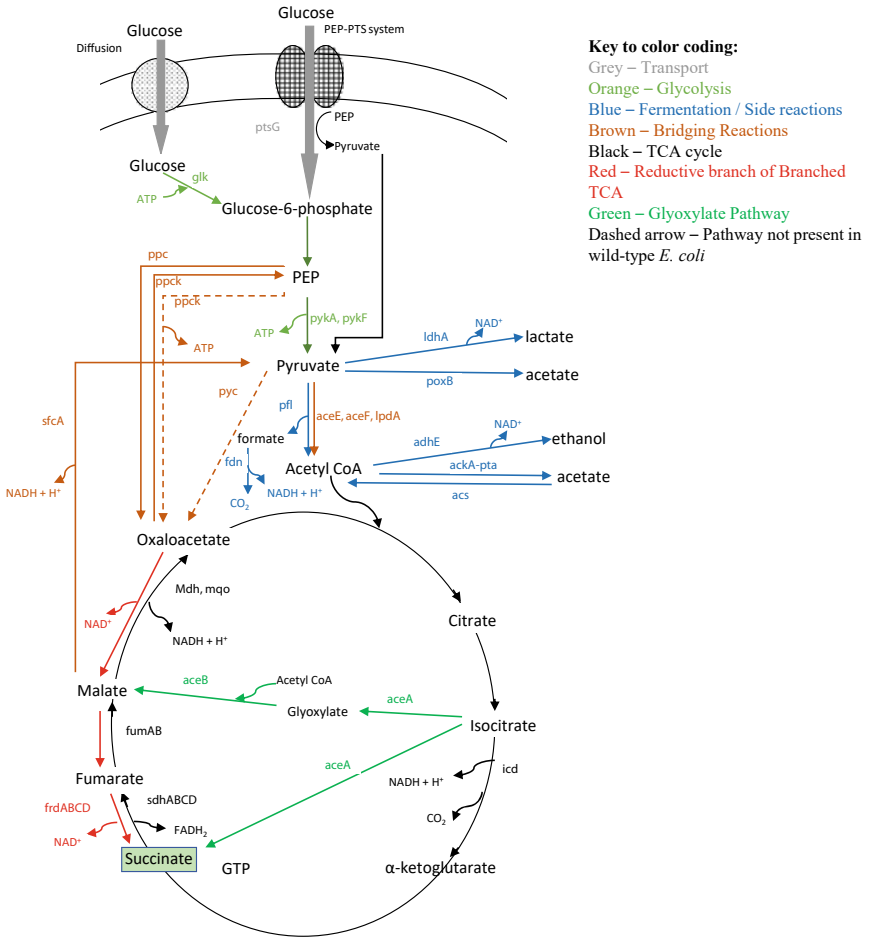


Fig. 10 Succinic acid production pathways in *E. coli*

- Pyruvate carboxylase (from some other organism as *E. coli* lacks pyruvate carboxylase)
- PEP carboxykinase (it is preferred over PEP carboxylase as it generates ATP, while PEP carboxylase releases inorganic phosphate).

ii. Deleting competing pathways mainly:

- Deleting PEP utilizing pathways such as PEP-PTS and gene for pyruvate kinase (*pykF*)
- Deleting pyruvate utilizing pathways such as activity of pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldhA*).

B. **Generation of reducing power—**This has been accomplished by the following ways:

i. Over-expression of key enzymes mainly:

- NADH forming pathway, i.e., activity of formate dehydrogenase (FDH) and malic enzyme.

ii. Deleting competing pathways mainly:

- Deleting NADH utilizing pathways such as activity of alcohol dehydrogenase (*adhE*) and lactate dehydrogenase (*ldhA*).

C. **Improving organic acid tolerance—**This has been accomplished by over-expression of:

- Efflux transporter
- Osmo-tolerant genes
- Organic acid tolerance genes.

D. **Improving growth rate under anaerobic conditions—**Anaerobic conditions lead to lower growth rates. To counter this, a 2-step approach has been followed wherein biomass buildup is done under aerobic conditions followed by succinate production under anaerobic conditions.

3. **Glyoxylate Cycle**: Glyoxylate cycle leads to direct conversion of isocitrate to succinate. Thus, it would lead to higher succinate yields due to bypassing the steps of carbon loss. However, glyoxylate pathway is repressed in the presence of glucose in *E. coli*. Therefore, metabolic engineering has been done to operate glyoxylate pathway in the presence of glucose. This has been accomplished by the following:

- Deleting *iclR* and making *aceBAK* constitutive
- Deleting competing enzymes, namely isocitrate dehydrogenase and succinate dehydrogenase.

Drawback of using glyoxylate Cycle—Isocitrate dehydrogenase has been deleted and thus α-ketoglutarate is not formed which is essential for cell growth **as** α-ketoglutarate is the precursor for glutamate. Thus, the external medium needs to be supplemented with glutamate.

4. **Simultaneous operation of oxidative and branched TCA cycle**—In recent times, a new strategy has been explored for succinate production wherein the oxidative TCA and branched TCA are used simultaneously. This is operated under aerobic conditions to improve growth rate. This has been facilitated by:

- Operating oxidative TCA by deleting succinate dehydrogenase to enhance succinate accumulation
- Operating branched TCA by derepressing fumarate reductase under aerobic conditions.

Summary

- TCA cycle operates in an oxidative mode during aerobic conditions.
- In facultative bacteria, it may be partially operative and is branched.
- Under anaerobic or microaerophilic conditions, the branched TCA operates with one branch operating oxidatively from citrate to α-ketoglutarate and the other reductively from oxaloacetate to succinyl CoA.
- In methylotrophs, partial TCA operates even in oxidative conditions as they lack α-ketoglutarate dehydrogenase.
- Some bacteria operate complete TCA even under denitrifying conditions, *viz. Pseudomonas*, *Paracoccus denitrificans.*
- During aerobic or microaerophilic conditions, pyruvate is converted to acetyl CoA by pyruvate formate lyase.
- Under such conditions, α-ketoglutarate dehydrogenase and succinate dehydrogenase activities are absent; instead fumarate reductase up-regulates.
- Formate generated by pyruvate formate lyase activity is further channelized by one of the three isozymes, FDH-N, FDH-O or FDH-H.
- Bacteria adopt either acetate generation or alcohol generation pathway depending upon demand for ATP or reductant, respectively.
- Glyoxylate pathway is marked by two key enzymes, *viz.* isocitrate lyase and malate synthetase.
- Glyoxylate consists of six out of eight reactions of TCA and serves as a bypass to conserve carbon.
- Glyoxylate is acetate activated in *E. coli* and is absent from animals.
- Glyoxylate operates in microbodies, peroxisomes or glyoxysomes in eukaryotes.
- Glyoxylate is regulated at isocitrate level by partition between isocitrate dehydrogenase (IDH) and isocitrate lyase (ICL).
- Isocitrate dehydrogenase is covalently regulated by phosphorylation and dephosphorylation by a bifunctional IDH kinase–phosphatase enzyme. Kinase activity is up-regulated by acetate, and phosphorylated IDH is inactive.
- The genes of the glyoxylate pathway are clustered together in the *aceBAK* operon which is under the transcriptional control of *iclR* gene.

Questions

1. State whether true or false:

   i. TCA cycle operates only under aerobic conditions.
   ii. Methylotrophs operate partial TCA cycle even under aerobic conditions.
   iii. Succinate dehydrogenase activity is an indicator of aerobic growth.
   iv. During anaerobic conditions, only three reactions of TCA operate oxidatively.
   v. All facultative bacteria operate branched TCA during anaerobic growth.

2. An *E. coli* mutant fails to grow on acetate but grew well in the presence of pyruvate. Which of the enzymes is possibly mutated and why do you think so?

3. Explain the fate of formate formed after pyruvate formate lyase activity.
4. How TCA is operated in E. coli anaerobic steady-state culture grown in the presence of NO^{3-} as terminal electron acceptor.
5. How acetyl CoA is partitioned between acetate fermentation and alcohol fermentation during anaerobic growth of *E. coli*?
6. Describe glyoxylate pathway of acetate assimilation.
7. How is isocitrate partitioned between isocitrate dehydrogenase and isocitrate lyase?
8. How is glyoxylate operon regulated?
9. Explain reductive reversal of TCA as an assimilatory pathway in photosynthetic bacteria.

References

Cozzone, A. J. (1998). Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. *Annual reviews of microbiology, 52,* 127–164.

Cozzone, A. J., & El-Mansi, M. (2005). Control of isocitrate dehydrogenase catalytic activity by protein phosphorylation in *Escherichia coli*. *Journal of Molecular Microbiology and Biotechnology, 9*(3–4), 132–146.

Ensign, S. A. (2006). Revisiting the glyoxylate cycle: Alternate pathways for microbial acetate assimilation. *Molecular Microbiology, 61*(2), 274–276.

Garnak, M., & Reeves, H. C. (1979). Phosphorylation of Isocitrate dehydrogenase of *Escherichia coli*. *Science, 203*(4385), 1111–1112.

Gui, L., Sunnarborg, A. & LaPorte, D. C. (1996a). Regulated expression of a repressor protein: FadR activates iclR. *Journal of Bacteriology*, *178*(15), 4704–4709.

Gui, L., Sunnarborg, A., Pan, B. & LaPorte, D. C. (1996b). Autoregulation of iclR, the gene encoding the repressor of the glyoxylate bypass operon. *Journal of Bacteriology*, *178*(1), 321–324.

Hong, S. H. (2007). Systems approaches to succinic acid-producing microorganisms. *Biotechnology and Bioprocess Engineering, 12*(2), 73–79.

Kornberg, H. L. (1966). The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochemical Journal, 99*(1), 1–11.

Lorca, G. L., Ezersky, A., Lunin, V. V., Walker, J. R., Altamentova, S., Evdokimova, E., et al. (2007). Glyoxylate and pyruvate are antagonistic effectors of the *Escherichia coli* IclR transcriptional regulator. *Journal of Biological Chemistry, 282*(22), 16476–16491.

Resnik, E., Pan, B., Ramani, N., Freundlich, M., & LaPorte, D. C. (1996). Integration host factor amplifies the induction of the aceBAK operon of *Escherichia coli* by relieving IclR repression. *Journal of Bacteriology, 178*(9), 2715–2717.

Sawers, M. (2005). Formate and its role in hydrogen production in *Escherichia coli*. *Biochemical Society Transactions, 33*(1), 42–46.

Song, H., & Lee, S. Y. (2006). Production of succinic acid by bacterial fermentation. *Enzyme and Microbial Technology, 39*(3), 352–361.

Sunnarborg, A., Klumpp, D., Chung, T., & LaPorte, D. C. (1990). Regulation of the glyoxylate bypass operon: Cloning and characterization of iclR. *Journal of Bacteriology, 172*(5), 2642–2649.

Zheng, J., & Jia, Z. (2010). Structure of the bifunctional isocitrate dehydrogenase kinase/phosphatase. *Nature, 465*(7300), 961–965.

Part V
Electron Transport and Energy Generation

Chapter 14
Electron Transport and Energy Generation

Rani Gupta, Namita Gupta, and Sudha Choudhary

During respiration, energy is generated by coupling flow of electrons in mitochondrial membrane in eukaryotes and cytosolic membrane in prokaryotes to create an electrochemical proton gradient which is harvested to synthesize ATP. Electrons flow from primary electron donor to terminal electron acceptor through a series of electron carriers constituting electron transport chain finally leads to ATP generation.

1 Primary Electron Donors

During aerobic growth, metabolism of glucose and other carbohydrates via EMP pathway and TCA cycle results in generation of ATP by substrate level phosphorylation and reducing equivalents in the form of NADH (or $FADH_2$). Other substrates are also incorporated in these pathways for subsequent degradation, for example lactate is converted to pyruvate by the action of aerobic lactate dehydrogenase. Thus, during aerobic growth, NADH (or $FADH_2$) acts as the primary electron donor in the electron transport chain. NADH is re-oxidized to NAD^+ by the activity of NADH dehydrogenase and two electrons are channeled into the electron transport chain by formation of reduced coenzymes (quinones). Other electron donors such as succinate, glycerol, lactate and fatty acids also couple electron flow to the quinone pool via their specific dehydrogenases (Table 1).

When oxygen is limiting or unavailable, many organisms such as *E. coli* can utilize alternative electron acceptors (such as nitrate, fumarate, TMAO, DMSO) as the respiratory substrates in place of oxygen. Under such conditions, in addition to NADH, some other electron donors, such as formate and their specific dehydrogenases play a more prominent role.

In all, *E. coli* consists of about 15 primary dehydrogenases including isozymes for some of the electron donors such as H_2, formate, NADH and glycerol 3-phosphate (Table 2). These isozymes are often produced under different conditions, such as changes in O_2 or nitrate supply. *E. coli* encodes two NADH dehydrogenases

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_14

Table 1 Electron donors and their dehydrogenases under aerobic conditions

Electron Donors	Enzyme
NADH	NADH dehydrogenase (*ndh*)
Succinate	Succinate dehydorgenase (*sdhCDAB*)
Glycerol 3-phosphate	Glycerol phosphate dehydrogenase (*glpD*)
Lactate	lactate dehydrogenase (*lctD*)

Table 2 Primary dehydrogenases of the respiratory chain in *E. coli* and their redox potential

Primary dehydrogenases	Redox potential (V)
Formate dehydrogenase N	−0.43
Formate dehydrogenase O	−0.43
Hydrogenase 1	−0.42
Hydrogenase 2	−0.42
NADH dehydrogenase I	−0.32
NADH dehydrogenase II	−0.32
Glycerol 3-phosphate dehydrogenase O	−0.19
Glycerol 3-phosphate dehydrogenase N	−0.19
D-lactate dehydrogenase	−0.19
L-lactate dehydrogenase	−0.19
Glucose dehydrogenase	−0.14
Succinate dehydrogenase	+0.03

(NADH:quinone oxidoreductases). NADH dehydrogenase II is encoded by *ndh* gene and is used primarily during aerobic and nitrate respiration. The alternative enzyme NADH dehydrogenase I, encoded by *nuo* gene, is used during fumarate respiration.

Electron carriers—The electron carriers are organized as membrane bound complexes that transfer electrons from electron donors at a low potential to acceptors at a higher potential. Four classes of electron carriers are involved in respiration:

- Flavoproteins
- Quinones
- Cytochromes
- Iron-Sulfur proteins.

Electrons can enter at any level, flavoproteins, quinones or cytochromes depending upon the potential of the donor. Some of the electron carriers carry both electrons and hydrogen such as flavoproteins and quinones while others such as iron-sulfur proteins and cytochromes carry only electrons.

Most of the electron carriers are proteins except quinones which are lipidic in nature. The proteins exist in the form of oxidoreductases that are multi-enzyme

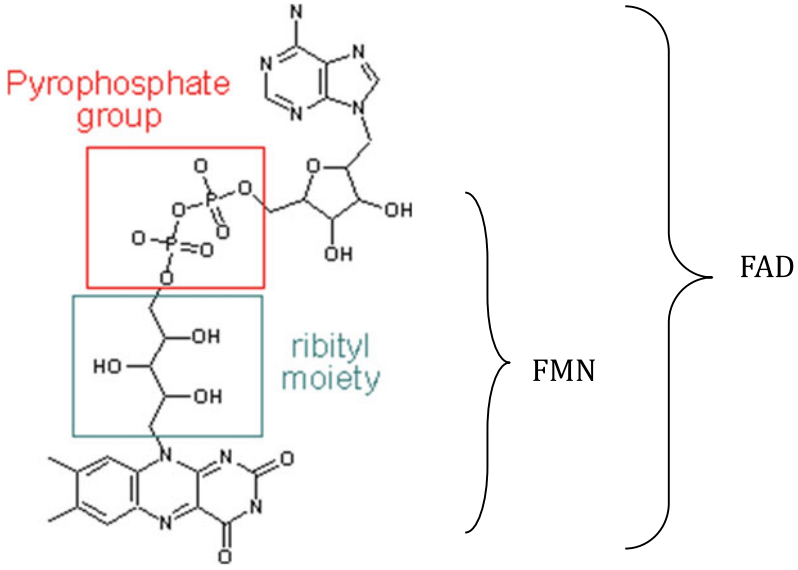


Fig. 1 Structure of FMN and FAD

complexes. These enzyme complexes contain a non-protein molecule called the prosthetic group which is responsible for carrying the electrons.

A. **Flavoproteins—**Flavoproteins (Fp) are proteins having a flavin molecule as their electron carrying prosthetic group. The flavin molecule can be either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). Flavins are synthesized from vitamin B2 (riboflavin). Phosphorylation of the ribityl C-5' hydroxyl group of riboflavin yields FMN while adenylation of FMN yields FAD (Fig. 1).

When flavins are reduced they carry 2H (i.e. two electrons and two hydrogen), one on each of the two-ring nitrogen. Thus, flavins are carriers of both hydrogen and electrons. Additionally, flavins can accept or donate electrons one at a time or two at a time. This is mediated by their one-electron reduction to semiquinone form or two electron reduction to the dihydroflavin form ($FADH_2$ and $FMNH_2$). An important manifestation of their redox versatility is that they serve as switch point from two-electron processes (predominant in cytosolic carbon metabolism) to one-electron processes (predominant in the membrane bound electron transfer chain). Biological electron acceptors or donors in flavin mediated reactions can be two-electron acceptors such as NAD^+ or $NADP^+$ or one-electron acceptors such as cytochromes (Fe^{2+}/Fe^{3+}) and quinones.

Flavins are very versatile redox coenzymes/prosthetic groups. Flavoproteins are dehydrogenases, oxidases and oxygenases that catalyze a variety of reactions in the cytoplasm and not just the ones associated with the electron transport chain in the membrane. FMN and FAD are functionally equivalent coenzymes and the one that is

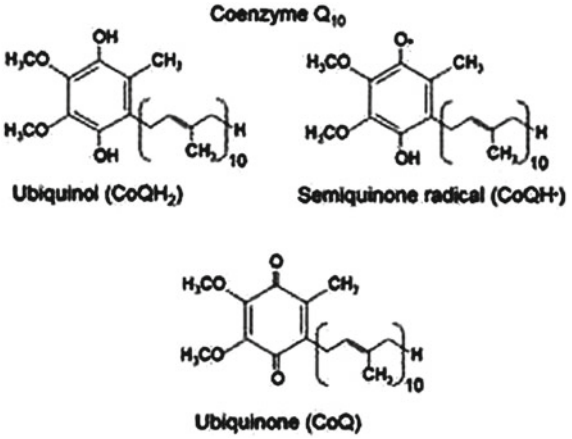


Fig. 2 Structure of Quinone and its reduced forms

involved with an enzyme is a matter of enzymatic binding specificity. For example, NADH dehydrogenase contains FMN while succinate dehydrogenase contains FAD.

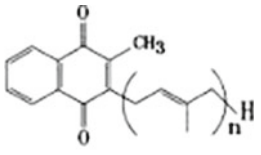
B. **Quinones—**Quinones are lipidic in nature, hence mobile in the lipid phase of the membrane and thus serve to carry electrons and hydrogens to and from the protein electron carriers (such as cytochromes and flavoproteins) that are stationery in the membrane. All quinones have hydrophobic isoprenoid side chains that contribute to their lipid solubility. Like flavoproteins, quinones can also accept/donate electrons one at a time or two at a time.

Mitochondrial membranes have ubiquinone (UQ), also known as coenzyme Q or coenzyme Q10, which is a benzoquinone with ten repeating isoprenoid units. Ubiquinone undergoes a two-electron reduction to the dihydroquinone (UQH_2) or a one-electron reduction to the semiquinone form (Fig. 2).

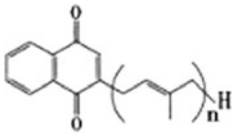
Prokaryotes generally have two types of quinones:

Ubiquinone (UQ) which is the same quinones found in mitochondria, though the number of isoprenoid units may vary from 4 to 10. For example, ubiquinone in *E. coli* has 8 isoprenoid units.

Menaquinone (MK or MQ) which are derivatives of Vitamin K. Menaquinones are naphthoquinones in which an additional benzene ring replaces the two methoxy groups present in ubiquinone. Like ubiquinones, menaquinones also have isoprenoid units varying from 4 to 10 in number. In *E. coli,* menaquinones have eight isoprenoid units.



Another class of quinone known as **demethylmenaquinone** (DMQ or DMK) have also been isolated from some bacteria such as *E. coli.* Demethylmenaquinone lacks the ring methyl substituent (C-2) of menaquinone and have isoprenoid side chains varying in length from one to nine units.



DMK

The three quinones differ in their electrode potential and are consequently responsible for carrying electrons under different conditions. Quinones function as carriers of reducing equivalents between the dehydrogenases and the various terminal enzyme complexes. In case of electron transport to oxygen, ubiquinone is required for activities of NADH, lactate, glycerol 3-phosphate and succinate dehydrogenases while menaquinone can only partly support activities of glycerol 3-phosphate dehydrogenase and lactate dehydrogenase. In case of nitrate respiration, activity of formate dehydrogenase can be supported by either ubiquinone or menaquinone while activities of glycerol 3-phosphate, lactate and NADH dehydrogenases showed patterns similar to that of aerobic respiration. During fumarate respiration, menaquinone is the predominant quinone

C. **Cytochromes—**Cytochromes are proteins that contain heme prosthetic groups. The heme iron is the electron carrier and is involved in one electron transfers involving the Fe^{2+} and Fe^{3+} oxidation states. Thus, cytochromes are one-electron carriers. Different types of cytochromes and their combination in mitochondrial membranes and in prokaryotes is presented in Table 3.
D. **Iron-Sulfur proteins (Fe-S proteins)—**These proteins have iron-sulfur clusters containing di-, tri-, and tetra iron sulfide linked centers in variable oxidation states. Fe-S proteins contain non-heme iron while the sulfur group can be either: (1) acid-labile sulfur wherein sulfide ion is attached to the iron by acid labile bonds i.e. on lowering the pH to 1, H_2S is released from the proteins or, (2) cysteine sulfur which is not acid labile.

In almost all Fe-S proteins, the Fe centers are tetrahedral and the terminal ligands are thiolate sulfur centers. The sulfide groups are either two- or three-coordinated. Three distinct kinds of Fe-S clusters are most common: [2Fe-2S], [4Fe-4S] and [3Fe-4S] clusters. Fe-S proteins cover a very wide range of redox potentials ranging from-400 mV to +350 mV. Thus, Fs-S proteins can carry out oxidation-reduction reaction at both low potential end and high potential end and are found at several locations of the electron transport chain.

Generally, Fe-S proteins contain the Fe-S clusters in a 1:1 ration i.e. one Fe-S cluster per protein. However, many proteins contain more than one Fe-S clusters

Table 3 Cytochromes and their combinations in mitochondrial and bacterial membranes

Mitochondrial membrane		
a and a_3	Cytochrome aa_3, cytochrome c oxidase	• Component of Complex IV of electron transport chain; electrons are delivered to the complex by soluble cytochrome c • Catalyses the oxidation of mitochondrial cytochrome c by O_2 • Enzyme complex also contains two copper centers • Also present in some bacteria such as *Paracoccus denitrificans*
b and c_1	Cytochrome bc_1	• Component of Complex III of electron transport chain
Bacterial cytoplasmic membrane		
b and d	Cytochrome bd, cytochrome d, cytochrome d oxidase	• Terminal ubiquinol oxidase, activated in conditions of limited oxygen supply
b and o	Cytochrome bo, cytochrome o, cytochrome bo_3, cytochrome o oxidase	• Terminal ubiquinol oxidase, activated in aerobic conditions with high oxygen tension

(such as in NADH: ubiquinone oxidoreductase of Complex I). The Fe-S clusters have different redox potentials and the electrons travel from one Fe-S cluster to the next having higher redox potential. Further, the electrons may not be localized on any one Fe atom and therefore the entire Fe-S cluster should be considered as carrying one electron irrespective of the number of Fe ions in the cluster.

2 Terminal Electron Acceptors

Mitochondria and aerobic bacteria only use oxygen as the terminal electron acceptor while facultative anaerobic bacteria such as *E. coli* are capable of using electron acceptors other than oxygen such as nitrate, fumarate, TMAO and DMSO under anaerobic conditions. These electron acceptors are used in a specific order or hierarchy based on their electrode potentials, more positive preferred first (Table 4).

Table 4 Terminal electron acceptors of *E. coli,* a facultative anaerobe

Electron acceptor	Redox potential (V)
Oxygen	+0.82
Nitrate	+0.42
DMSO	+0.16
TMAO	+ 0.13
Fumarate	+ 0.03

Box 1: Reduction Potentials

Reduction potential is a measure of the affinity of a substance for electrons in Volts. It reflects the ease with which a molecule can donate electrons and become oxidized or gain electrons and become reduced. Reduction potentials are expressed for pairs of molecules that are interconvertible by gain or loss of electrons. Hence, they are called as redox pairs. Thus NADH $+H^+$ and NAD^+ are a redox pair and O_2 and H_2O is another redox pair in aerobic electron transport chain. The redox pairs are analogous to acid -base pairs of BrØnsted acid. The reactions involving redox pairs are called redox reactions. A redox reaction can be divided into two half reactions/redox couples which can take place in separate compartments unlike acid base reactions. Hence for the redox reaction:

$NADH + H^+ + 1/2\,O_2 \leftrightharpoons NAD^+ + H_2O$, the two half reactions are:

$$NAD^+ + H^+ + 2e^- \leftrightharpoons NADH\left(E^{0\prime} = -0.32\right)$$

$$1/2\,O_2 + 2H^+ + 2e^- \leftrightharpoons H_2O\left(E^0 = +0.82\right)$$

By convention half reactions are written as reduction reactions with their respective standard reduction potentials, $E^{0\,\cdot}$ (refer to the table).

The two compartments in which two different redox reactions take place can be linked to form an electrochemical cell. Each compartment is called a half cell. When two half cells are connected through a wire, electrons are transferred from one half cell to another depending upon the reduction potentials of respective redox pairs. The flow of electrons is always from low reduction potential molecule to high reduction potential molecule.

The standard reduction potential is related to the free energy of the system by the expression $\Delta G^{0\prime} = -nF\Delta E^{0\prime}$.

Where $\Delta G^{0\,i}$s the standard free energy change of the reaction at pH 7,n is the number of electrons transferred, F is Faradays constant and has a value of 23.06 kcal/mol., the energy change when 1 mol of electrons falls through a redox potential of 1 V. $\Delta E^{0\prime}$ is the difference in the redox potentials of electron acceptor and electron donor species. It is clear from the foregoing account that $\Delta G^{0\prime}$ is directly proportional to the redox potential difference of the redox pairs involved. Three corollaries arise from the foregoing account:

(1) Positive $\Delta E^{0\,\cdot}$ indicates spontaneous reaction.

(2) Larger the difference in the redox potentials of redox couples greater would be the energy released. For example, oxidation of NADH $+H^+$ ($E^{0\prime} = -0.32$) by O_2 ($E^{0\prime} = +0.82$) would release more energy than its oxidation with nitrate (($E^{0\prime} = +0.42$) or DMSO ($E^{0\,\cdot} = +0.16$). The last two act as inorganic and organic terminal electron acceptors in anaerobic respirations.

(3) Oxygen is an ideal electron acceptor defining the highest limit of reduction potential for biological systems that makes aerobic respiration a highly efficient process.

Biologically important redox couples and reduction potentials

Redox couples	$E^{0'}$ (V)
$2H^+ + 2e^- \leftrightharpoons H_2$	− 0.42
Fd (Fe^{3+}) + $e^- \leftrightharpoons$ Fd (Fe^{2+})	− 0.42
NAD (P)$^+$ + H^+ + $2e^- \leftrightharpoons$ NAD (P)H	− 0.32
S + $2H^+$ + $2e^- \leftrightharpoons H_2S$	− 0.27
Pyruvate$^-$ + 2 H^+ + $2e^- \leftrightharpoons$ Lactate^{2-}	− 0.19
FAD + 2 H^+ + $2e^- \leftrightharpoons$ $FADH_2$	− 0.18
Fumarate^{2-} + $2H^+$ + $2e^- \leftrightharpoons$ Succinate^{2-}	0.03
Cytochrome b (Fe^{3+}) + $e^- \leftrightharpoons$ Cytochrome b (Fe^{2+})	0.08
Ubiquinone + 2 H^+ + $2e^- \leftrightharpoons$ Ubiquinol	0.10
Cytochrome c (Fe^{3+}) + $e^- \leftrightharpoons$ Cytochrome c (Fe^{2+})	0.25
Cytochrome a (Fe^{3+}) + $e^- \leftrightharpoons$ Cytochrome a (Fe^{2+})	0.29
Cytochrome a_3 (Fe^{3+}) + $e^- \leftrightharpoons$ Cytochrome a_3 (Fe^{2+})	0.35
NO_3^- + 2 H^+ + $2e^- \leftrightharpoons NO_2^-$ + H_2O	0.42
NO_2^- + 8 H^+ + $6e^- \leftrightharpoons NH_4^+$ + $2H_2O$	0.44
Fe^{3+} + $e^- \leftrightharpoons Fe^{2+}$	0.77
½ O_2 +2 H^+ +$2e^- \leftrightharpoons H_2O$	0.82

Note Half reactions are written as Oxidant +e $-\leftrightharpoons$ *Reductant.*

The reduction potential of free iron (Fe) is different from that of bound iron of heme (cytochromes) and nonheme (Ferredoxins) iron proteins. This indicates that the protein components of redox-active centres modulate their reduction potentials.

Hypothetically the electrons can move from donor at one position to acceptor at next lower position in this redox tower which, however, is limited by availability of enzymes in the cell.

3 Electron Transport Complex

The electrons removed from the cell's metabolism during oxidation of substrates are fed to an electron transport chain which in eukaryotes is present on the invaginations of inner mitochondrial membrane called cristae and in prokaryotes on the cell membrane. The construction of the electron transport chain (ETC) is such that it

allows controlled release of potential energy of the electrons so that most of it can be conserved and used to synthesize ATP. It requires large multienzyme, supramolecular complexes which direct the electrons to terminal electron acceptors. The electron carriers in the mitochondrion are organized into four submitochondrial complexes referred as Complex I, II, III, IV and coordinate with a fifth complex that synthesizes ATP (Table 5). In aerobic respiration, electrons travel from NADPH to oxygen and during this flow protons are also ejected unidirectionally to intramembrane space to build up proton gradient to generate ATP. In this context, amongst the four electron transport complexes, there are three electron transport driven proton pumps: Complex I, III and IV (Fig. 3).

All electron transporters except the quinones are present as integral membrane proteins. The latter is a lipid polyisoprenoid. The one present in mitochondrion is called Ubiquinone for its ubiquitous occurrence. It has ten isoprene units hence the name CoQ_{10} and a commonly found bacterial species is Menaquinone (MK) with eight isoprene units. The number of isoprene units, however can vary.

Complex I: NADH-Q Reductase

NADH-Q reductase (complex I) of the mitochondrion is a **L-shaped** large multisubunit complex with a molecular weight of one million Dalton. It is the largest of

Table 5 Electron transport complexes/proteins of mitochondrial electron transport chain

Enzyme complex	Mass (kDa)	Number of subunits	Prosthetic group(s)	Electron donor	Terminal Electron acceptor of the complex	Redox potential/span (for complex) (V)
Complex I NADH Q Reductase	1000	45 (14 in bacteria)	FMN, Fe-S centres (2Fe2S,4Fe-4S)	NADH + H^+	Q	−0.315 to −0.150
Complex II Succinate Q reductase	140	4	FAD, Fe-S centres (2Fe-2S,4Fe-4S, 3Fe-4S)	$FADH_2$	Q	−0.040 to −0.080
Quinone	0.864	1	–	Fe-S centre	Cyt b/c_1	0.045
Complex III Ubiquinone:cyt c oxidoreductase	250	11	Hemes, Fe-S centres (2Fe2S, Rieske iron-sulfur protein)	QH_2	Cyt c	0.030 to 0.215
Cytochrome c	13	1	Heme	Cyt c_1	Heme of complex IV	0.235
Complex IV Cytochrome oxidase	204	13(3−4 in bacteria)	Hemes, Cu_A, Cu_B	Cyt c	Oxygen	0.210 to 0.385*

** The redox potential of terminal electron acceptor molecular oxygen is a high value with positive magnitude of 0.815 and is the major driving force for electron transport in the cell*

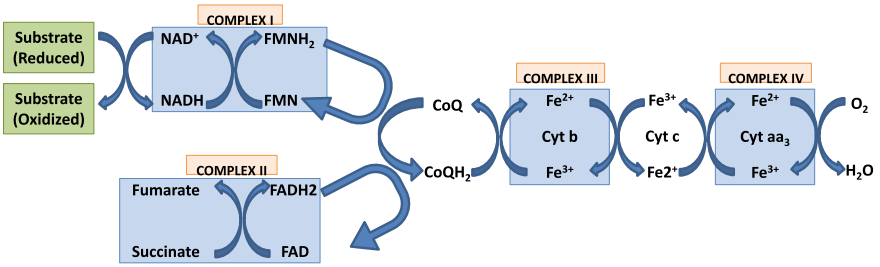


Fig. 3 Reactions carried out by complex I, Complex II, Complex III and complex IV during electron transport

all respiratory complexes. The complex isolated from bovine heart mitochondria, and a number of aerobic and anaerobic microorganisms including yeasts, and filamentous fungi has provided an insight into the mechanism of redox reactions and coupled energy transduction occurring through it. In the mitochondrion, it comprises of approximately 44 subunits of which at least seven are encoded by mitochondrial DNA (hence its role is implied in a number of neurogenerative diseases). Of these, 14 subunits form the core of the complex and are similar from human to bacteria. These are concerned with energy transduction and are present in the **horizontal arm** of the complex.

The mechanisms of electron coupling and H^+ transfer are spatially separated by about 100 A^0. The energy is released in two portions. While a small amount is released upon reduction of terminal Fe-S cluster, most of it is released upon reduction of quinone. The transfer of electron from NADH to Fe-S cluster via FMN initiates a cascade of changes in the membrane embedded portion of the complex eventually recuing ubiquinone (Fig. 4). The reduced UQ (UQH_2) diffuses to Complex

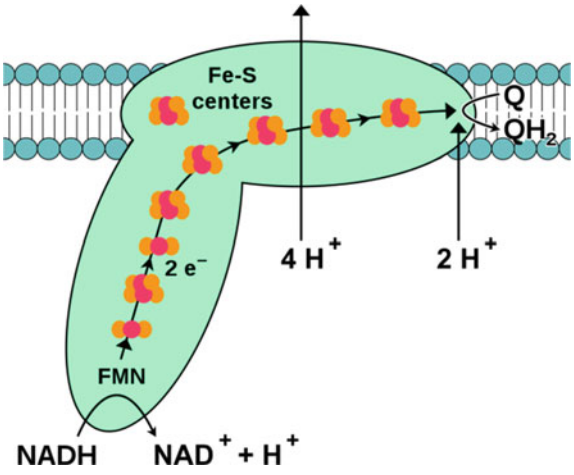


Fig. 4 Electron transport through Complex I: NADH-Q Reductase.*Source* Wikipedia. (Public Domain)

III. Here the protons have been acquired from the matrix which will be released to the cytoplasmic side. The vectorial movement of protons have significance for ATP synthesis.

Complex I: **Succinate-Q reductase**

Complex II of the electron transport chain, succinate Q reductase, catalyzes the transfer of electrons from $FADH_2$ to complex III, i.e. cytochrome reductase complex of the mitochondrion. The multi-subunit assembly is a heterotetrameric protein consisting of

i. a catalytic subunit
ii. an electron transporting subunit
iii. a heterodimer anchor.

The catalytic subunit comprises succinate dehydrogenase which is the only enzyme of TCA cycle embedded in the membrane. This catalyzes conversion of succinate to fumarate and uses FAD for reduction instead of NAD^+ unlike all other TCA cycle enzymes. It performs the dual function of oxidizing succinate to fumarate and catalyzing the transfer of electrons from $FADH_2$ to quinone (Fig. 5). Among electron transporters also, it is unique as it is the only submitochondrial complex which does not pump protons and no subunit is encoded by mitochondrial genome. It is a heterodimer with a larger subunit towards mitochondrial matrix which is succinate oxidation site and has 4 Fe centres and a FAD binding site. The smaller subunit is also a heterodimer anchor having 2 Fe centres with electron potential of 0 V and 3 Fe centre with positive potential. This complex also has small polypeptides which interact with ubiquinone (Zubay, 1989).

Complex III: Cytochrome bc_1 complex

Complex III or cytochrome bc_1 complex of the mitochondrial electron transport complex is a homodimer, with each monomer consisting of eleven subunits. The

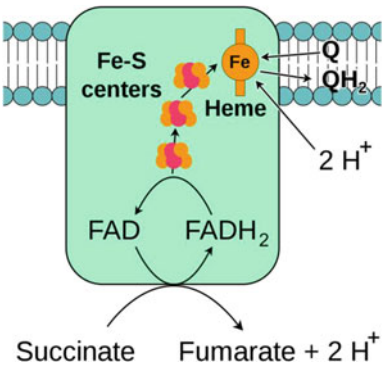


Fig. 5 Electron transport through Complex II: Succinate-Q Reductase*Source* Wikipedia. (Public Domain)

mass of one monomer being 240 kD. The complex has three different types of redox active centers: they are cyt b, cyt c_1 and 2 Fe-2S center (Reiske protein). Reiske iron sulfur center is different from Complex I's iron sulfur clusters in that the iron atoms in this are coordinated by histidine residues instead of cysteine. This arrangement is beneficial as it stabilizes the complex in its reduced form and results in raising its redox potential so as to enable it to accept electrons from quinol readily. The cytochrome b is of two types: cyt b_H (electron potential 0.05 V) and cyt b_L (electron potential 0.0.3 V). Here, the terms H and L refer to high and low potential, respectively. The mitochondrial complex contains eight additional subunits which contribute to assemblage and stabilization of the complex. They, however, are not involved with electron transport. The cytochrome c1 is reduced on inner side of inner membrane towards inter membrane space.

Electron transport through Complex III—Q cycle

Q-cycle describes the sequential reduction and oxidation of coenzyme Q10 between quinone and quinol which results in net movement of protons across mitochondrial inner membrane (or lipid bilayer in case of prokaryotes). The Q-cycle was first proposed by Peter D. Mitchell though it is presently modified to explain how complex III contributes to development of proton gradient across membrane.

Q-cycle presumes that complex III has two sites: P site facing inside and N site facing outside. P site interacts with QH_2 while N site binds to UQ (or UQ^-). QH_2 (ubiquinol) binds to P site via His182 of Rieske Fe-S protein and to Glu272 of cyt b and two protons ($2\ H^+$) are ejected into intramembrane space. QH_2 gets converted to semiquinone (Q) as it remains in interaction with heme of cyt b transmembrane protein. After acquiring electron from ubiquinol, Rieske Fe-S protein migrates to cytochrome c_1 subunit reducing its bound heme which in turn reduces cyt c bound outside the complex.

The other electron which was transferred to cyt b heme gets transferred to other heme of cyt b on other side where it is donated to ubiquinone reducing it to semiquinone. The reaction continues to stabilize semiquinone and for this, a second Q-cycle operates reducing semiquinone to ubiquinol via cyt b and by acquiring two protons. (Palsdottir et al., 2003).

Finally, the Q-cycle results in four protons in transmembrane space: two from matrix and two from reduction of two molecules of cyt c. The reduced cyt c is re-oxidized by complex IV and ubiquinol generated at N site can be reused (Fig. 6).

The high potential transport of electron takes the route:

$$QH_2 \rightarrow \text{Reiske center} \rightarrow \text{cyt}\,c_1 \rightarrow \text{cyt}\,c$$

The reaction for the two Q half cycles to frame Q cycle is as follows:

$$QH_2 + Q + \text{cyt}\,c_{(ox)} \rightarrow Q +^{\square} Q^- 2\,H^+_{(IMS)} + \text{cyt}\,c_{(red)}$$

$$QH_2 +^{\square} Q^- + 2\,H^+_{(matrix)} + \text{cyt}\,c_{(ox)} \rightarrow Q + QH_2 + 2\,H^+_{(IMS)} + \text{cyt}\,c_{(red)}$$

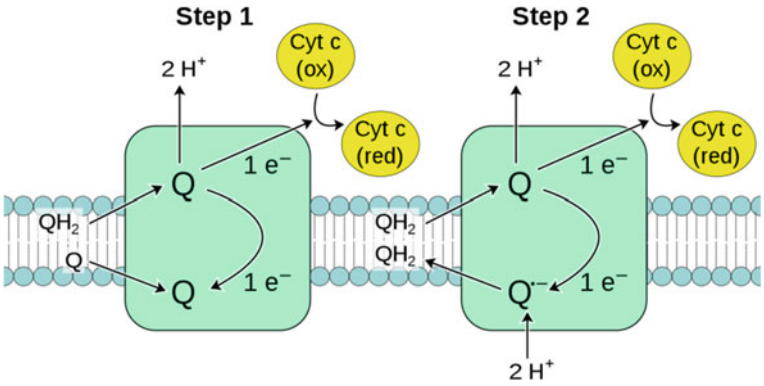


Fig. 6 Electron transport through Complex III: Cytochrome bc_1 complex showing Q cycle*Source* Wikipedia. (Public Domain)

Complex IV: Cytochrome oxidase/Terminal Oxidases

Complex IV or cytochrome oxidase is the terminal oxidase of the ETC which delivers the electrons to final electron acceptor i.e. molecular oxygen in mitochondria and organisms living in aerobic conditions. It catalyzes the transfer of electrons from cyt c to dioxygen thereby coupling the oxidation of one electron donor substrate to four electron reduction of oxygen to form water. There are three families of terminal oxidase. Of these, heme copper reductases are the most extensively studied and are necessary for aerobic cellular respiration (Fig. 7).

1. Heme—copper super family

They are characterized by the presence of a low spin heme and a binuclear center consisting of high spin heme and Cu_B where reduction of oxygen takes place. Except

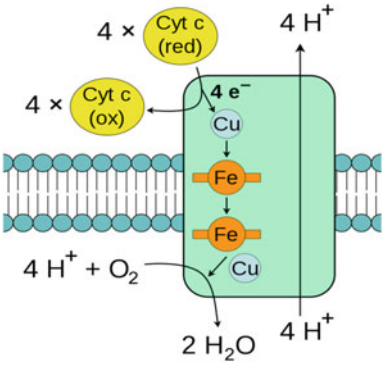


Fig. 7 Electron transport through Complex IV: Cytochrome oxidase/Terminal Oxidases *Source* Wikipedia. (Public Domain)

for this common feature, the superfamily is otherwise quite diverse in terms of subunit composition, heme types (a_3, o_3, or b_3 type), electron donor (cyt c or quinol), and variable affinity for oxygen. Based on structural analyses three subfamilies are recognized within this group. Subfamily A in prokaryotic and eukaryotic domains, both cyt c as well as quinol oxidases are found in it. The second subfamily B includes oxygen reductases distributed among prokaryotes, Gram positive bacteria, purple bacteria, archaea including extremophiles like *Thermus thermophilus*. These are ba_3 type of oxidases. Subfamily C is the second largest oxidase family of cyt cbb3 type, found in many pathogenic and symbiotic bacteria these oxygenases have five to eight-fold high affinity for oxygen.

2. Alternative Oxidase (AOX) family

While all other respiratory oxidases couple oxygen reduction with ATP synthesis, these enzymes are an exception. They occur in prokaryotes, protists, fungi, and mitochondria of plants and animal species, engage quinol oxidases as electron donor and contain a non-heme di-iron carboxylate for oxygen reduction. The enzyme catalyzes oxidation of quinol directly with oxygen reduction. This bypassing of complex III and IV results in lowered ATP yields. Moreover, if transfer of electrons is from complex II to AOX then electron flow is totally uncoupled from ATP synthesis. Due to non-involvement of cytochrome oxidase it is cyanide insensitive respiration.

3. Cytochrome bd family

This family comprises of quinol oxidases found in a number of prokaryotes including a wide variety of pathogens and help them live in microaerobic conditions. They bear no sequence homologies with the other two families and are lacking in copper and non heme iron proteins, generate proton motive force by charge separation without pumping protons and therefore have half the value of proton electron ratio (H^+/e^-) compared to the heme copper oxidases. Apart from contributing to PMF, bd oxidases perform a number of other functions. Their oxygen scavenging ability supports nitrogenase activity in N-fixers. With high affinity for O_2, they facilitate pathogenic and commensal bacteria to inhabit O_2 poor environment, all the more reason for their occurrence in bacterial pathogens causing tuberculosis, meningitis, *pneumonia*, sepsis, and bacillary dysentery. There is, in fact, a direct correlation between the virulence of the pathogen and the level of oxidase. Sufficient data exists to prove that bd type oxidases allow bacteria to live at low O_2 concentration(microaerobic), in the presence of poisons such as H_2O_2, CN^- (Cyanide Insensitive Oxidase, CIO), and tolerate stressful conditions like high temperature, hydrostatic pressure, alkalization of the medium.

These oxidases are more significant because they are restricted to prokaryotes and among them common in human pathogens. Therefore, these enzymes are of interest as drug target.

4 Bacterial Electron Transport: A General Account

Bacteria are highly versatile organisms inhabiting diverse habitats ranging from aerobic through microaerobic to anaerobic environments in terms of oxygen availability, an important factor in respiration. Accordingly, the organisms are aerobic, microaerophilic or anaerobic.

Most fungi, protists and aerobic bacteria use oxygen as final electron acceptor. *Micrococcus luteus* is a prototype of this group, which has been extensively studied for its electron transport. Many enteric bacteria like *E. coli*, and *Enterococcus,* employ nitrate and perform nitrate respiration. Others like *Paracoccus denitrificans*, *Bacillus licheniformis*, *Pseudomonas stutzeri*, *Alcaligenes faecalis*, prefer oxygen respiration if possible, however, when it is insufficient or absent altogether these bacteria reduce nitrate to molecular nitrogen and become a part of denitrification. They are facultative anaerobes. Obligate anaerobes like methanogens reduce CO_2 to CH_4 and perform carbonate respiration. Sulfate respiration is a property of sulfate reducing bacteria which produce hydrogen sulfide as the end product of sulfate reduction e.g. *Desulfovibrio*, *Desulfobacter*. Hydrogen sulfide which is produced on a very large scale by these organisms either combines with metals to form metal sulfides or used by other organisms in chemolithotrophic oxidation and recycled.

It is, therefore, not unusual to find large variety in the redox carrier composition of electron transport chains of different bacterial species since external terminal acceptors have varying redox potentials. A large amount of electron acceptor is reduced and the reduced product like nitrogen or hydrogen sulfide or methane are excreted from the cell. Variation in electron transport chain composition also exists within the same species growing under different conditions. Interspecies variations usually involve replacement of one electron transporter by another, e.g. replacement of coenzyme Q by menaquinone in *Micrococcus luteus* or total absence of a specific type of electron carrier for example a cyt of c type is absent in *E. coli* (Table 6). Intraspecies

Table 6 Redox carriers of some select species of bacteria under aerobic conditions showing sequence of electron carriers

Direction of electron flow							
Micrococcus luteus	NADH	Fp.Fe-S	MQ	cyt b	cyt c cyt c	cyt aa_3 (cyt o)	O_2
Azotobacter vinelandii	NADH	Fp.Fe-S	Q	cyt b cyt b	cyt c	cyt aa_1 (cyt d)	O_2
Methylophilus methylotrophus	NADH	Fp.Fe-S	Q (PQQ)	cyt b	Cyt c	Cyt aa_3 (cyt o)	O_2
Pseudomonas fluorescens	NADH	Fp.Fe-S	Q	cyt b	Cyt c	Cyt o	O_2

Brackets indicate carriers with low activity. They are actually operative at low oxygen tensions. PQQ (Pyrroloquinoline quinone) is associated with methanol or methylamine oxidation and is not a part of NADH respiratory chain, however, it reduces oxygen by transfer of electrons via *cyt o. Fp stands for flavoprotein.*

variations occur because of changes in the growth environment such as limited availability of nutrients most commonly oxygen or sulfur or iron. Oxygen limited growth is generally accompanied with increased synthesis of alternate terminal oxidases which have higher affinities for it such as cyt o or cyt d. Growth under sulfur and iron limited conditions results in decreased levels of iron-sulfur proteins and cytochromes (White, 2000).

Thus, we observe that cytochromes electron transport chain with variable composition is a characteristic feature of bacteria, however, the basic plan remains same as with mitochondrion. Dehydrogenases and oxidases connected by quinones and/or cytochromes accomplish electron transport. In bacteria capable of reducing inorganic ions other than oxygen during anaerobic respiration, these enzymes are called as reductases rather than oxidases. In addition to variations in redox carrier composition, considerable variations exist in the sequence of these carriers in the electron transport chain.

All aerobic respiratory chains of bacteria exhibit branching which generally exists at quinone (menaquinone) or cyt c level. The branching at primary dehydrogenase level enables bacteria to utilize a wide range of substrates and at terminal oxidase level gives them an ecological advantage. This metabolic flexibility is probably the reason for ubiquitous occurrence of bacteria.

The spatial organization of redox carriers has important bearing on proton translocation. The highest quotients exhibited when the respiratory chain consists of NADH oxidoreductase, a cyt c and a cyt aa_3 are $H^+/O = 8$ for the oxidation of NAD linked substrates. Examples are P. *denitrificans* and *B. stearothermophilus*. In contrast, the lowest quotients (a value of 4) is obtained with *E. coli*, *B. subtilis* whose electron transport chains lack some of these features like proton pumping site. Replacement of endogenous substrates by flavin linked substrates further reduces it. In other words, P:O ratio (the number of ATPs made for every two electrons transfer to oxygen)—a standard expression for evaluating the output of electron transport varies from 0.67 to 2.7 per NADH for *E. coli* with different modules of electron transport. This value is considerably lower than mitochondrial which is 2 and 3 for $FADH_2$ and NADH oxidation, respectively. Variable ATP yields provide bacteria with leverage in adjusting the proton motive force generated during respiration. Since a large PMF can reverse the electron flow and slow down oxidation of dehydrogenase and quinol, the organisms divert the electrons along the routes that bypass the coupling sites.

Bacterial electron transporters are present on the cell membrane and are much simpler protein aggregates than the mitochondrial complexes that are indispensable for e^- transport and H^+ translocation. In bacteria and archaea, they seem to contain only the functional cores of their corresponding eukaryotic counterparts.

5 Branched Electron Transport Chain in *E. coli*

E. coli is a heterotrophic, fermentative, facultative anaerobe which is found in the lower gut of warm-blooded animals, a habitat characterized by very low concentration

or absence of oxygen. It is capable of living outside the body for a limited period of time aerobically. The organism meets these demands by switching its metabolism from aerobic to anaerobic or fermentative mode. This switch over becomes possible because of a branched electron transport chain with variable composition engaging different electron donors and acceptors enabling the organism to adapt to a wide range of environmental conditions.

The respiratory chain dehydrogenases of *E. coli* are linked by quinones to terminal oxidases or reductases. Three types of quinones are present in *E. coli*. These are ubiquinone (UQ), menaquinone (MQ) and demethylmenaquinone. While UQ is most suitable for aerobic ETC; MQ and DMQ are involved in anaerobic ETC. The relative concentrations of these three quinones vary with oxygen concentration. The aerobic electron transport chain of *E. coli* comprises of three major primary dehydrogenases. These are succinate:quinone oxidoreductase (*sdh* gene product), Type I NADH:quinone oxidoreductase (*nuo* gene product) and Type II NADH:quinone oxidoreductase (*ndh* gene product), three ubiquinol:oxygen oxidoreductases and cyt bo_3 (with two hemes b and o, is also called as cyt b) and cyt bd complex (with hemes b and d, is also called as cyt d).When oxygen levels are high, cyt b complex encoded by *cyoABCDE* operon dominates and at low oxygen tensions cyt d encoded by *cyoAB* operon is the dominant form. Cyt d with high affinity for oxygen (low K_m) works efficiently at low concentration of oxygen. Although both the oxidases have coupling sites, between the two, cyt o is more efficient as it is a proton pump. During the oxidation of quinol, it moves two protons across the membrane compared to one proton by cyt d (Fig. 8).

E. coli grows in the absence of oxygen with nitrate (nitrate respiration) or fumarate (fumarate respiration) as the terminal electron acceptors and reduces them to nitrite/ammonia and succinate, respectively. Transition from aerobic to anaerobic environment results in the repression of aerobic pathways and induction of anaerobic enzymes. One dehydrogenase complex is replaced by another and an oxidase is replaced by a reductase and accordingly the required respiratory module is plugged in.

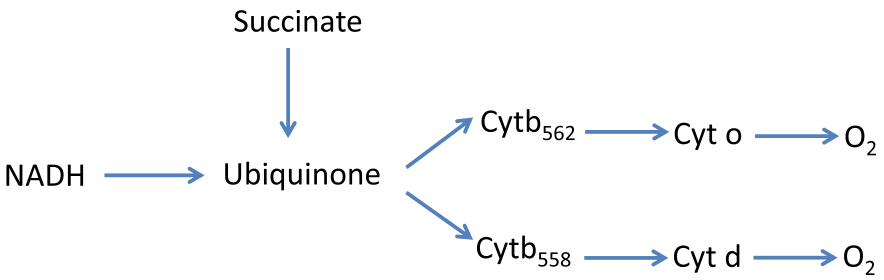


Fig. 8 Aerobic respiratory chain of *E. coli* showing two cytochrome complexes: cyt bo complex operates at higher concentrations of oxygen and is a proton pump, Cyt bd complex functions at low oxygen tension

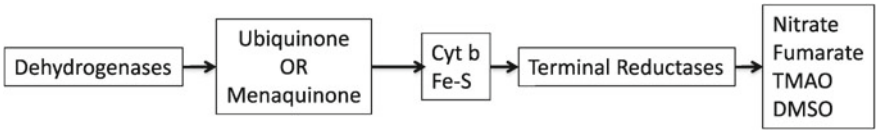


Fig. 9 Anaerobic respiratory chain of *E. coli*

During nitrate respiration, the bacterium synthesizes nitrate and nitrite reductases. The anaerobic electron transport chain consists of NADH dehydrogenase, a menaquinone/ubiquinone and a terminal reductase. Both types of NADH dehydrogenases have been implicated in nitrate respiration. With type I, however, there are two coupling sites—one at dehydrogenase level and the other at the time of quinol oxidation. As such nitrate reductase does not seem to be a proton pump. When both oxygen and nitrate are unavailable the cells synthesize fumarate reductase enzyme (Fig. 9) encoded by *frd*ABCD. There are different terminal reductases. For example for reduction of fumarate (+0.033 V), menaquinone (−0.074 V) is more suitable than UQ (+0.011 V). Nitrate can be reduced by either of them. Each reductase seems to be a complex of several proteins and prosthetic groups.

Since fumarate reductase, like nitrate reductase, is unlikely to be a proton pump it should employ type I NADH dehydrogenase which is a coupling site also. Other terminal reductases used by *E. coli* are DMSO reductase (*dms*ABC gene product), an enzyme with broad specificity that can reduce other sulphoxides and amine-N-oxides. When TMAO is present, it is reduced by one of the two TMAO reductases.

In all, *E. coli* has about 10 terminal reductases including isozymes for oxidases and nitrate and nitrite reductases (Table 7).

Table 7 Terminal Oxidases/Reductases of the respiratory chain in *E. coli*

S. No.	Enzyme	Redox potential (V)	Gene
1	Quinol oxidase bo_3	+0.82	*cyoABCDE*
2	Quinol oxidase *bd*	+0.82	*cydAB*
3	Nitrate reductase A	+0.42	*narGHJI*
4	Nitrate reductase Z	+0.42	*narZYWV*
5	Nitrate reductase, periplasmic	+0.42	*napFDAGHBC*
6	Nitrite reductase, periplasmic	+0.36	*nrfABCDEFG*
7	DMSO/TMAO reductase	+0.16	*dmsABC*
8	TMAO reductase	+0.13	*torCAD*
9	Fumarate reductase	+0.03	*frdABCD*

6 Oxidative Phosphorylation

Oxidative phosphorylation is the synthesis of ATP from ADP and Pi at the expense of energy yielded by electron transport to oxygen in aerobes and other inorganic ions in anaerobes. In eukaryotic cells, this redox mediated process occurs in the matrix of the mitochondrion while in bacteria it occurs in cytoplasm (a similar photoredox phenomenon occurs in the stroma of the chloroplasts during photosynthesis). A number of experiments with reconstituted vesicles obtained from mitoplasts (inner mitochondrial membrane with matrix) and spheroplasts—the wall less bacteria have been performed. The reconstitution experiments unequivocally proved that the inner membrane of the mitochondrion is the site of electron transport chain and the membrane spheres are the enzyme required for coupled phosphorylation. Efraim Racker called these as coupling **f**actors and they were known as $\mathbf{F_1}$ ATPase, a property consistent with F type ATPases (Racker, 1970). All these experiments led to the origin of different theories for the mechanism of ATP synthesis. Mitchell's hypothesis called **Chemiosmotic Hypothesis** has found wide acceptance as the theory of oxidative phosphorylation. Mitchell has been conferred the Nobel Prize in 1978 for his contribution to understanding the mechanism of ATP synthesis during aerobic respiration.

Mechanisms of Proton Transport and ATP synthesis

Three mechanisms have been proposed by different workers for proton translocation; however, the most accepted is Mitchell hypothesis as stated above. This is based on few assumptions where key one is vectorial flow of protons across the membrane. The main components of complete electron transport which generates pmf for the synthesis of ATP are Q cycle and Proton Pumps.

Proton Motive Force (pmf) is the free energy stored in all the gradient of H^+ has and has two components, a membrane potential (ψ) or charge gradient and a chemical gradient or pH. The former is due to the membrane being positively charged on the outside compared to inside and unequal distribution of H^+ creates a ΔpH. Mathematically,

$$\text{PMF} = \Delta\text{pH} + \Delta\psi$$

It has been observed that during electron transport, the pH outside is 1.4 units lower than inside with outside being positive and a membrane potential of 0.14 V is generated across the membrane. This PMF corresponds to a free energy of 5.2 kcal/mole of protons.

Use of antibodies directed against electron transporting proteins have revealed a vectorial distribution of electron and hydrogen carriers across the mitochondrial inner membrane thereby lending support to the hypothesis that protons are absorbed from the matrix side and released on the cytoplasmic side.

With the use of compounds that increase the permeability of the membrane to protons, by dissipating the proton gradient without affecting electron transport, effectively uncouples electron transport chain from oxidative phosphorylation. This shows that electron transport chain is an active proton generating mechanism across an intact membrane and that F_oF_1 ATP synthase uses this gradient for ATP synthesis.

Proton pumps catalyze the electrogenic transfer of H^+ across the membrane. Mitochondrial complex I and IV contribute to pmf by pumping protons. Mitochondrial NADH dehydrogenase, as also NDH-1 of bacteria, translocate 4 H^+ per NADH oxidized. Cytochrome oxidase complex which is formed of cytochromes only extrudes protons when oxidized by oxygen. Electrostatic changes in the cytochrome oxidase probably lead to proton movements as has been observed with NADH Q reductase (complex I).

ATP synthesis is catalyzed by the enzyme ATP synthase which constitutes complex V of the electron transport chain. This enzyme utilizes the energy of pmf generated by electron transport. According to Mitchell hypothesis, during electron transport there is vectorial flow of electrons and protons are ejected towards cytoplasmic side of inner membrane of mitochondria which re-enter through channel of complex V i.e. ATP synthase. Consequently, ATP is synthesized at F_1 domain of F_0F_1 particles by complexing ADP with Pi towards matrix side of mitochondria. Finally, proton gradient and pH is dissipated.

7 Uncouplers and Inhibitors of Electron Transport and Oxidative Phosphorylation

The flow of electrons in electron transport chain can be inhibited by several compounds which inhibit electron flow by interaction with specific electron transport complex (Table 8). They have been extremely useful in dissecting out the electron transport chain and revealing the sequence of electron carriers. Administering any one such inhibitor at a time followed by spectrophotometry makes it possible to measure the relative amounts of oxidized and reduced forms of electron carriers. Another approach is to allow oxygen to an anaerobic suspension of mitochondria in the presence of excess of substrate. All carriers are fully reduced under anaerobic conditions. When oxygen is introduced, the carriers become oxidized in the following order: cyt aa_3, followed by cyt c then cyt b and lastly NADH.

The electron transport in mitochondrion is tightly coupled to oxidative phosphorylation by a proton gradient across an impermeable membrane. The proton channels exist only in F_1F_0 ATP synthase. There is another class of inhibitors which dissipate proton gradient across the membrane; these are called uncouplers and ionophores.

Compounds like 2,4-dinitrophenol (DNP), tetrachlorosalicylanilide (TCS) and carboxylcyanide-p trifluoromethoxyphenylhydrazone (FCCP) have been found to uncouple the two processes. They act by dissipating the proton gradient, thus pmf cannot be established and ATP synthesis is impaired. DNP is the classical uncoupling

Table 8 Respiratory chain complex inhibitors

Inhibitor	Action
Inhibitors of Complex I	
Rotenone	Block transfer of e^- from NADH to UQH_2
Amytal	Blocks electron transport through Fe-S clusters
Inhibitors of Complex III	
Antimycin	Binds to N site of complex III and inhibits transfer of electron from heme b_H to b_L
Myxothiazol Stigmatellin	It binds at P site and Inhibits transfer of electron from QH_2 to Rieske Fe-S centre
Propylhexadrine	Inhibits cytochrome c reductase
Inhibitors of Complex IV	
Propylhexadrine	Inhibits cytochrome c reductase
Cyanide Azide Carbon monoxide	Binds to cytochrome c oxidase. Higher conc. of oxygen is needed to alleviate inhibition. Cyanide is a non-competitive inhibitor binding to partially reduced enzyme
Nitric oxide H_2S	Inhibits cytochrome oxidase activity by binding to regulatory site. High levels of ATP inhibit cytochrome oxidase activity allosterically

agent introduced by Loomis & Lipmann (1948). Certain other compounds called ionophores like valinomycin and gramicidin act as inhibitors in the presence of monovalent cations. The ions which are not able to cross the membrane on their own form lipid soluble complexes with these compounds.

Oligomycin, rutamycin and dicyclohexylcarbodiimide (DCCD) are also inhibitors of oxidative phosphorylation but differ from uncouplers in that they prevent oxygen uptake as well as ATP synthesis by inhibiting ATPase enzyme.

Summary

- During respiration, energy is generated by coupling flow of electrons in mitochondrial membrane in eukaryotes and cytosolic membrane in prokaryotes to create an electrochemical proton gradient which is harvested to synthesize ATP.
- In all, *E. coli* consists of about 15 primary dehydrogenases including isozymes for some of the electron donors such as H_2, formate, NADH and glycerol 3-phosphate.
- Four classes of electron carriers are involved in respiration: Flavoproteins, quninones, cytochromes and Iron-Sulfur proteins.
- Electrons can enter at the level of flavoproteins, quinones or cytochromes depending upon the potential of the donor.
- Some of the electron carriers carry both electrons and hydrogen such as flavoproteins and quinones while others such as iron-sulfur proteins and cytochromes carry only electrons.
- All electron carriers are proteins except quinones which are lipidic in nature and serve to carry electrons and hydrogens

- Mitochondria use only oxygen as the terminal electron acceptor and have terminal oxidases to reduce it to H_2O. Aerobic bacteria also use oxygen as the terminal electron acceptor and have terminal oxidases for its reduction to H_2O.
- Facultative anaerobic bacteria such as *E.* coli, on the other hand, are capable of using electron acceptors other than oxygen such as nitrate, fumarate, TMAO and DMSO under anaerobic conditions.
- The electron carriers are organized into four sub mitochondrial complexes, Complex I, II, III, IV and coordinate with complex V which synthesizes ATP.
- Complex 1 NADH dehydrogenase Q reductase transfers electrons to complex II of the electron transport chain, succinate Q reductase which catalyzes the transfer of electrons from $FADH_2$ to complex III i.e. cytochrome reductase complex.
- The complex III has three different types of redox active centers. They are cyt b, cyt c_1 and 2 Fe-2S center (Reiske protein).
- The Q cycle was first proposed by P. Mitchell and is the most accepted H^+ translocation mechanism by complex III
- Complex IV or cytochrome oxidase is the terminal oxidase of the ETC which delivers the electrons to final electron acceptor i.e. molecular oxygen in mitochondria and organisms living in aerobic conditions.
- All aerobic respiratory chains of bacteria exhibit branching which generally exists a at quinone.
- Oxidative phosphorylation is the synthesis of ATP from ADP and Pi at the expense of energy yielded by electron transport to oxygen in aerobic organisms and other inorganic ions in anaerobes.
- Mitchell's chemiosmotic hypothesis is the most accepted theory of oxidative phosphorylation. This is based on key assumption that there is vectorial flow of electrons across the membrane.
- The main components of complete electron transport which generates pmf for the synthesis of ATP are Q cycle and Proton Pumps
- Uncouplers like 2,4-dinitrophenol (DNP), tetrachlorosalicylanilide (TCS) and carboxylcyanide-p trifluoromethoxyphenylhydrazone (FCCP) uncouple ETC and ATP synthesis by dissipating the proton gradient. Ionophores like Valinomycin and Gramicidine also function similarly but in presence of ions.
- Oligomycin, rutamycin and dicyclohexylcarbodiimide (DCCD) are also inhibitors of oxidative phosphorylation but differ from uncouplers as they prevent oxygen uptake as well as ATP synthesis.

Questions

1. Name any two primary electron donors of aerobic respiratory electron chain.
2. What are different electron carriers? How are quinones different from all other electron carriers?
3. What are different types of quinones present in *E coli?*
4. What are different electron transport complexes?
5. Describe Q cycle.

6. Diagrammatically represent branched electron transport of *E coli* operated during aerobic and microaerophilic growth,
7. Explain Chemiosmotic Hypothesis of oxidative phosphorylation.
8. Differentiate between uncoupler and ionophore with suitable examples.
9. Name the inhibitors that inhibit flow of electron through different electron transport complexes.
10. Name any two ATPase inhibitors.

References

Loomis, W. F., & Lipmann, F. (1948). Reversible inhibition of the coupling between phosphorylation and oxidation. *Journal of Biological Chemistry, 173*(2), 807–808.

Palsdottir, H., Lojero, C. G., Trumpower, B. L., & Hunte, C. (2003). Structure of the yeast cytochrome bc1 complex with a hydroxyquinone anion Qo site inhibitor bound. *Journal of Biological Chemistry, 278*(33), 31303–31311.

Racker, E. (1970). The two faces of the inner mitochondrial membrane. *Essays in Biochemistry, 6,* 1.

White, D. (2000). *Physiology and biochemistry of prokaryotes.* Oxford University Press.

Zubay, G. (1989). *Biochemistry* 2nd edn. Macmillan.

Part VI
Metabolic Diversity of Carbon and Energy Source

Chapter 15
Nutritional Diversity Amongst Bacteria: Chemolithotrophy and Phototrophy

Rani Gupta, Namita Gupta, and Sunita Aggarwal

1 Nutritional Diversity Amongst Bacteria

All living organisms have two basic requirements to survive and grow: (a) energy in the form of ATP; and (b) cell metabolites or cell carbon. Figure 1 shows the classification of living organisms in terms of energy and carbon sources.

Prokaryotes can use a wide variety of compounds (both organic and inorganic) as their source of carbon and energy. Many species are unique in having one compound serving as their source of carbon while a different compound serving as the source of energy. This variability leads to a wide range of metabolic diversity in bacteria. Thus, based on their source of energy, bacteria can be either chemotrophs or phototrophs.

- Chemotrophs—Organic or inorganic compounds are oxidized and the electrons released are fed into the electron transport chain and travel to the terminal electron acceptor thereby generating the proton-motive force. Depending on the terminal electron acceptor, the energy generating pathways can be aerobic (oxygen is the terminal electron acceptor) or anaerobic (nitrate, sulfate, fumarate, etc. act as terminal electron acceptor). Figure 2 shows the classification of chemotrophic organisms in terms of energy and carbon sources.
  In most prokaryotes and all eukaryotes (other than plants and algae), the same compound acts as the source of carbon and energy. Energy is generated by a chemical reaction involving oxidation of organic compounds; such organisms are known as chemoheterotrophs. Chemolithotrophs are those microorganisms that obtain their energy from oxidation of inorganic electron donors such as hydrogen, carbon monoxide, ammonia, nitrite, sulphite, iron etc. In chemolithoautotrophs, the source of carbon is CO_2. In chemolithoheterotrophs, source is carbon is organic and they are also known as mixotrophs.
- Phototrophs—Phototrophs are organisms that carry out photon capture to acquire energy. Phototrophs use the electron transport chains to establish an electro-chemical gradient which leads to ATP generation. Most of the phototrophs are photoautotrophic, capable of utilizing CO_2 as the sole carbon source. Some

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_15

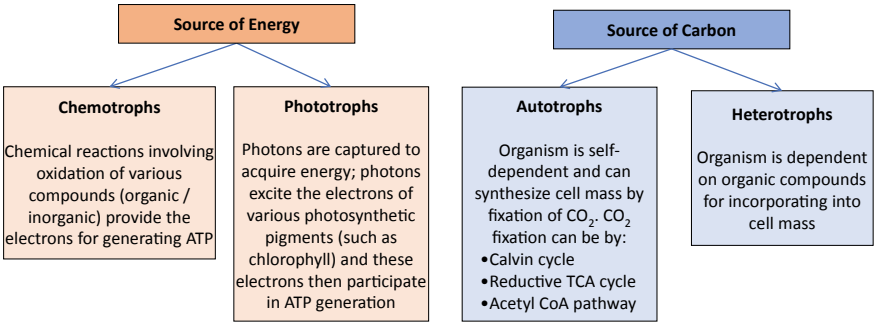


Fig. 1 Classification of living organisms in terms of energy and carbon sources

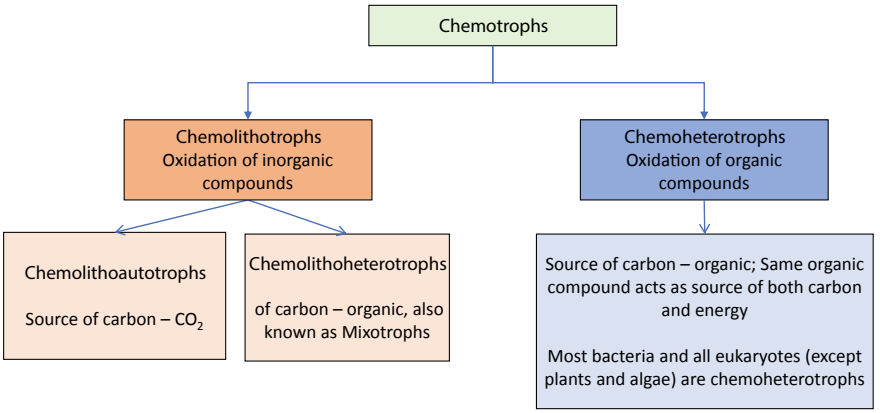


Fig. 2 Classification of chemotrophic organisms in terms of energy and carbon sources

are photoheterotrophs and use organic carbon. Depending on the photosynthetic system, light energy can do one or both of the following:

- In all photosynthetic systems, light energy can drive the phosphorylation of ADP to form ATP
- In some photosynthetic systems, light energy can also drive the transfer of electrons to generate the reducing power NADPH.

2 Chemolithotrophy

Chemolithotrophs are those prokaryotes which use a variety of inorganic compounds as electron donors to generate proton motive force (PMF) and hence ATP. Chemolithotrophy was first characterized by Russian microbiologist Sergei Winogradsky. During electron transport, depending upon the redox potential of each of the inorganic compounds, reductant is generated either directly as in case of compounds with more electronegative potential than NAD^+/NADH such as carbon dioxide and

Table 1 Redox potential of inorganic electron donors used by chemolithotrophs

Redox couple	E^0 (V)
CO_2/CO	−0.54
$2H^+/H_2$	−0.41
NAD(P)H/NAD(P)	−0.32
H_2S/S	−0.25
SO_3^{2-}/S^0	+0.05
SO_4^{2-}/SO_3^{2-}	+0.17
NO_2^-/NO_3^-	+0.42
NH_4^+/NO_2^-	+0.44
Fe^{3+}/Fe^{2+}	+0.78
H_2O/O_2	+0.86

hydrogen or through reverse electron flow in case of compounds with more electropositive potential than NAD^+/NADH. This is called reverse electron transport as it involves transfer of electrons uphill for generation of NADH which requires consumption of energy from proton motive force. Hence, chemolithotrophs are generally slow growers as they consume energy during uphill flow of electrons. Redox potential of various inorganic compounds is listed in Table 1.

The energy and reductant generated by oxidation of inorganic compounds is utilized during fixation of CO_2 by calvin cycle, reductive TCA or acetyl CoA pathways by chemolithoautotrophs. As majority of chemolithotrophs are autotrophs, hence the terms chemolithoautotrophs and chemolithotrophs are interchangeably used. Some are facultative chemoautotrophs and can grow heterotrophically utilizing various organic carbon sources including all the bacterial hydrogen oxidizers, some sulfur oxidizing thiobacilli, and some thermophilic iron oxidizing bacteria. For example, *Alcaligenes eutrophus* is a hydrogen oxidizing bacteria and can grow on fumarate. Chemolithotrophs are by and large aerobes using oxygen as terminal electron acceptor, however few can be facultative using nitrate or nitrite as the electron acceptor or they can be obligate anaerobes with H_2 or CO_2 as the electron acceptor.

Grouping of chemolithotrophs

The requirement of chemolithotrophs is simple but they are metabolically very diverse and complex, as they synthesize most of their biomolecules utilizing energy generated from inorganic compounds. On the basis of the source of inorganic compounds used, the chemolithotrophs have been placed into different groups:

1. Hydrogen oxidizers
2. Carbon monoxide oxidizers
3. Ammonia (NH_3) oxidizers
4. Nitrite oxidizers
5. Sulphur bacteria
6. Iron Bacteria.

3 Hydrogen Oxidizers

The bacteria using hydrogen as electron donor are spread in both bacterial and archaeal domains. These bacteria oxidize hydrogen by hydrogenases to release electron and proton. These bacteria are also called Knallgas bacteria, which means oxyhydrogen. There are two types of hydrogenases with different redox potentials. One hydrogenase is soluble and is more electronegative to NAD^+ and directly generates reductant NADH. Examples of organisms having soluble hydrogenases are *Nocardia opaca*, *N. autotrophic* etc. The other is particulate or membrane bound hydrogenase which is more electropositive to NAD^+ and transfers its electrons to cytochrome via ubiquinone to generate ATP by oxidative phosphorylation (Fig. 3) and reductant is generated by reverse electron transport. Examples of organisms having only membrane bound hydrogenases are *Paracoccus denitrificans*, *Aquaspirillum autotrophicum* etc. Some organisms have both soluble and membrane found hydrogenases such as *Alcaligenes eutrophus*, *Pseudomonas saccharophila* etc. The soluble hydrogenases have low affinity for substrate while particulate one has high affinity. Hence the bacteria having membrane bound hydrogenases oxidize hydrogen at low concentrations. An exception is found in the bacterium *Hydrogenovibrio marinus* where particulate hydrogenase is NAD^+ dependent (Kim & Geoffrey 2008).

Most of the hydrogen oxidizers are aerobic and H_2 is oxidized by O_2 leading to formation of a proton motive force and ATP generation. The overall reaction is highly exergonic.

$$H_2 + 1/2\,O_2 \rightarrow H_2O$$

Hydrogen oxidizers are amongst the fastest growing chemolithotrophs due to the high difference in redox potentials of the $2H^+/H_2$ and O_2/H_2O redox couples.

Anaerobic Hydrogen Oxidizers—These include strict anaerobes that reduce nitrate/sulphate/carbon dioxide through electrons released from hydrogen. These

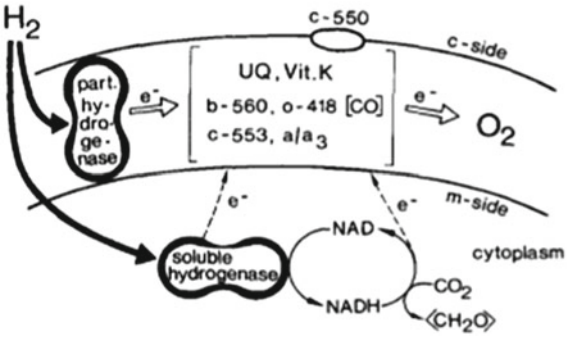


Fig. 3 Hydrogen oxidation in bacteria in presence of soluble and particulate hydergenase *Source* Gottschalk (1986). With kind permission from Springer Nature

Table 2 Anaerobic Hydrogen Oxidizers along with their electron donors and electron acceptors

Organism	Electron donor	Electron acceptor
Thermovibrio ammonificans	H_2	NO_3^- (converted to NH_4^+)
T. ruber	H_2	S^0 (converted to H_2S)
Caminibacter	H_2	$O_2/NO_3^-/S^0$
Hydrogenomonas	H_2	$O_2/NO_3^-/S^0$
Balnearium	H_2	NO_3^-
Ferroglobus	H_2	NO_3^-
Dechloromonas	H_2	NO_3^-
Paracoccus denitrificans	H_2	NO_3^-

bacteria comprise group of sulphate reducers, methanogens and acetogens. *Hydrogenomonas thermophile* is a microaerophilic hydrogen oxidizer, which uses oxygen, nitrate or elemental sulphur as the electron acceptor. Few anaerobic hydrogen oxidizers are listed in Table 2.

This is to remember that hydrogen oxidizing bacteria do not produce hydrogen. Therefore, these hydrogenases, both particulate and soluble, are together clubbed as uptake hydrogenases as they take up hydrogen but do not produce hydrogen. On the other hand, fermentative bacteria possess hydrogenases which use H^+/protons to reduce them to produce H_2. These hydrogenases are called evolution or production hydrogenases.

$$2H^+ + 2e^- (\text{reduced ferredoxin}) \rightarrow H_2$$

These two types of hydrogenases, uptake and evolution, are further demarcated on the basis of their metal centres where Ni-Fe centres are present in uptake hydrogenases and Fe–Fe centres are found in evolution hydrogenases. Apart from these two, another hydrogenase with only one Fe centre is present exclusively in methanogenic archaea (Fig. 4).

4 Carbon Monoxide Oxidizers (Carboxydobacteria)

These are also facultative chemolithotrophs that utilize carbon monoxide (CO) for energy production. Most of the carboxydobacteria are gram-negative and use only carbon monoxide like *Pseudomonas carboxydovorans* except for *Alcaligenes carboxydus* that can use hydrogen as well.

$$CO + 1/2O_2 \rightarrow CO_2$$

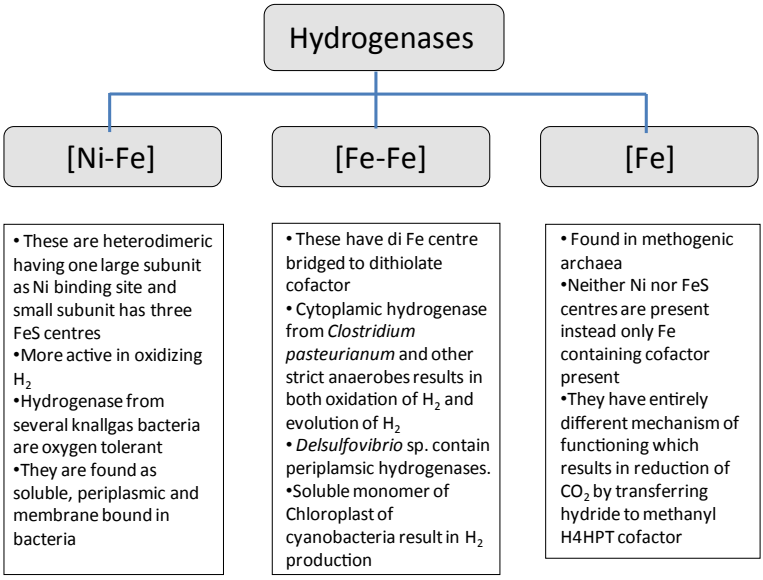


Fig. 4 Classification of hydrogenases based on their metallic centers

These organisms possess the enzyme carbon monoxide oxidase to oxidize CO to CO_2 and to release electrons. Carbon monoxide oxidase is a molybdoprotein and like hydrogenase enzymes contain Fe-S centre and FAD. Production of NADH occurs through reverse electron transfer as it occurs in hydrogen oxidizers.

To avoid toxicity of CO, these organisms possess branched respiratory chain, in which one of the branches is insensitive to CO. As redox potential of CO/CO_2 is very low, the enzyme CO oxidase is coupled to carrier molecules of ETC in cell membrane.

Some exceptions are *Mycobacterium tuberculosis* that can use CO as sole source of carbon and energy (Park et al., 2003) and *Pseudomonas carboxydoflava* that uses CO anaerobically in presence of nitrate as terminal electron acceptor (Frunzke & Meyer, 1990).

5 Ammonia Oxidizers

Ammonia oxidizers are species of *Nitrosomonas*, *Nitrosococcus, Nitrosospira* and *Nitrosoglobus.* These organisms are aerobic so final electron acceptor is oxygen. These organisms have two enzymes for oxidation of ammonia, namely ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). Firstly, ammonia is oxidised to hydroxylamine by AMO in presence of oxygen that takes place in cytoplasm. The enzyme ammonia monooxygenase accepts two elec-

trons from cytochrome P_{460} that acts as co-substrate required by ammonia monooxygenase and hydroxylamine reduction is coupled to Cyt 554 and Cyt 552 reduction (Yamanaka and Shinra 1974).

The electron transport is well-studied in *Nitrosomonas*. In *N. europaea*, four electrons released by HAO are channelled to ETS through cytochrome 552 where two electrons are channelled back to AMO and 1.6 are passed to O_2 and 0.35 are used to reduce $NADP^+$ through reverse electron flow (Whittaker et al., 2000; Beaumont et al., 2002) as shown in Fig. 5.

The overall reaction is:

$$NH_4^+ + 1\,1/2O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

First step: Oxidation of ammonia to hydroxylamine catalysed by ammonia monooxygenase (AMO)

$$2H^+ + NH3 + 2e^- + O_2 \rightarrow NH_2OH + H_2O$$

Second step: Oxidation of hydroxylamine to nitrite catalysed by the enzyme hydroxylamine oxidoreductase (HAO)

$$NH_2OH + H_2O \rightarrow HONO + 4e^- + 4H^+$$

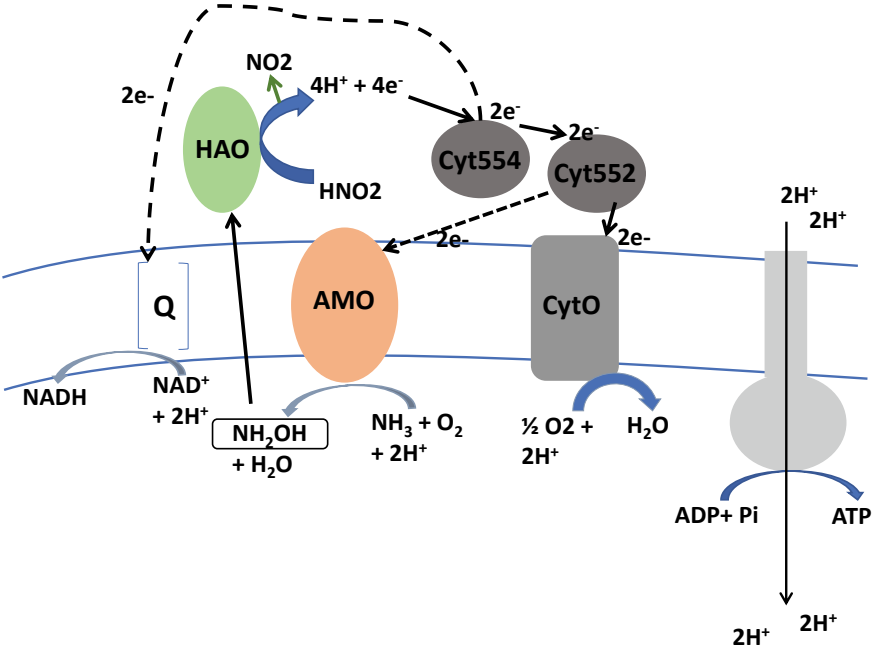


Fig. 5 Diagrammatic representation of electron transport in ammonia oxidising bacterium *Nirosomanas europaea*

Box 1: Ammonia Oxidizing Bacteria (AOB) and Ammonia Oxidising Archaea (AOA)
Recent molecular biology studies have shown that ammonia monooxygenase (*amoA* gene) is found in large numbers in archaea proving that archaea has capacity for NH_3 oxidation. Ammonia oxidizing archaea have been classified in a separate phylum thaumarchaeota which were initially considered to be crenarchaeotes. Further, a number of studies show an abundance of AOA not only in extreme environment but in soil as well indicating that AOA are a main player in global nitrogen cycle. The conventional notion that it is conducted only by AOB is no longer true. AOA can help in developing novel processes under extreme environmental conditions like low temperature and low O_2. AOB and AOA together can also be exploited to remove nitrogen from wastewater (He et al., 2018; Yin et al., 2018).

Annamox: **Anaerobic ammonia oxidizers**

Planctomycetes are specialized chemolithoautotrophs that carry out anaerobic oxidation of ammonia to generate N_2 using nitrite as an electron acceptor and the process is called as annamox. It is a significant part of biogeochemical nitrogen cycling. The most unusual features of annamox bacteria are their intracellular compartmentalization resembling eukaryotes. The annamox cell contains a unique internal compartment named annamoxosome, surrounded by a curved bilayer membrane, which is huge in size and occupies a lot of volume. This compartment is proposed to be analogous to eukaryotic mitochondrion and it is the site where annamox reaction happens. The annamox membrane contains an ATP synthase enzyme which leads to ATP synthesis due to generation of proton motive force across the membrane mediated via flow of electrons through cytochrome *c*. The annamox planctomycetes inhabit both fresh water and marine habitats and are major contributors to the global nitrogen cycle. The annamox bacteria are also employed in waste water treatment successfully for removal of ammonium. In 2002, the first annamox wastewater treatment plant was established in Netherlands for removal of ammonia from wastewater (Remy et al., 2016; Driessen et al., 2012)

6 Nitrite Oxidizers

Oxidation of ammonia by *Nitrosomonas* yields nitrite which is further oxidised to nitrate by another group of microbes belonging to genera *Nitrococcus*, *Nitrobacter*, *Nitrospira,* etc. They are aerobic obligate chemolithotrophics, with the exception of *Nitrobacter*, which is a facultative autotroph. The oxidation of nitrite is catalyzed

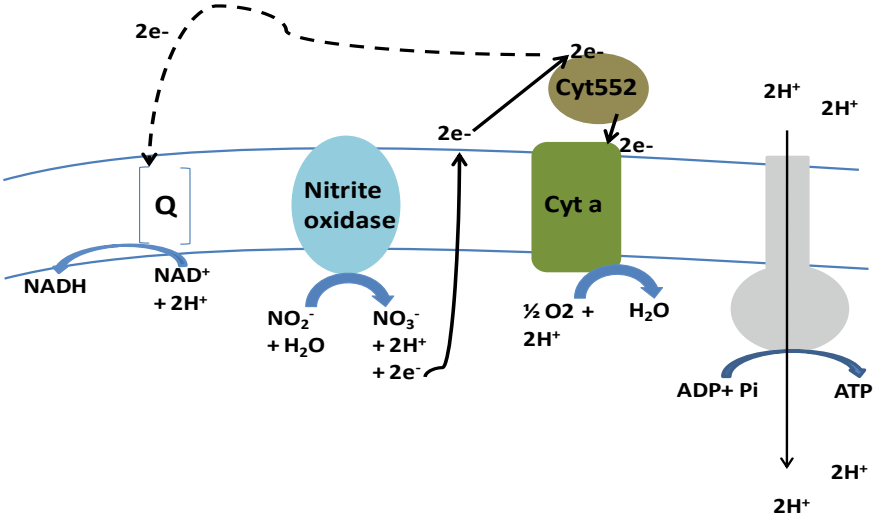


Fig. 6 Diagrammatic representation of electron flow during nitrite oxidation

by membrane bound enzyme nitrite oxidoreductase (nitrite oxidase) which is a molybdenum containing FeS protein. This first reduces cytochrome 552 followed by cytochrome oxidase (cytochrome a) which reduces oxygen to water (Fig. 6). Due to the high potential of the $N0_3^-/NO_2^-$ couple, only small amounts of energy are available and thus the growth yields of nitrite oxidizers are relatively low.

Commamox: Complete Ammonia Oxidizers

Complete oxidation of ammonia to nitrate in a single organism is called Commamox. Complete ammonia oxidation was observed in two species of *Nitrospira* that codes all enzymes necessary for ammonia oxidation via nitrite to nitrate (van Kessel et al., 2015). The AMO enzyme of these bacteria was found to be phylogenetically different from *Nitrosomonas* but similar to earlier described methane monooxygenase. Before the discovery of *Nitrospira* sp., it was accepted that nitrification occurred in two steps catalysed by two groups of bacteria.

7 Sulphur Bacteria

Bacteria/archaea which can oxidize sulfur compounds like hydrogen sulfide, sulfur (S^0), thiosulfate, thiocyanate, polythionate and sulphite and use them as energy source are grouped as sulfur chemolithotrophs. These prokaryotes are colorless as against phototrophic sulfur utilizers which are all pigmented. Sulfur oxidation pathway and enzymes involved in the pathway are highly diverse and after oxidation, oxidized

sulfur is deposited either intracellularly as in *Beggiatoa* or extracellularly as in *Thiobacillus* (Shively, 1974; Beard et al., 2011).

Sulphur bacteria are a physiologically heterogeneous group. Most of the sulphur bacteria are obligate chemolithotrophs e.g. *Thiomicrospira* or facultative chemolithotrophs e.g. *Thiobacillus*, *Sulfolobus*, *Acidianus*. Others are facultative heterotrophs (*Paracoccus versutus*) or mixotrophs (*Beggiatoa*). *Thiobacillus denitrificans* (H_2S, S^o, $S_2O_3{}^{2-}$) is a facultative anaerobe that can use $NO_3{}^-$ as electron acceptor when grown anaerobically. *Thiovulum* (H_2S, S^o) is a tubeworm symbiont that is isolated from deep-sea vents and secretes H_2SO_4. *Beggiatoa* is present in cool neutral freshwater and sulfide springs and yields S granules. Oxidation of inorganic sulphur compounds is one of the important energy-yielding reactions in many extreme environments like acidic mine drainage, hyperalkaline lakes, metal-rich marine geothermal fields, craters of volcanoes, volcanic hot springs, submarine hydrothermal vents, etc.

These bacteria oxidize a variety of reduced S compounds like sulphide, sulphite, thiosulphite or elemental sulphur to sulphate to generate energy. Of these, sulphide is a better electron donor and allows transport of more protons across the cell membrane. S oxidation results in S as cytoplasmic granules or secretion as acid (H_2SO_4).

$$S^{2-} + 2O_2 \rightarrow SO_4^{2-} + 2H^+$$
$$HS^- + 1/2\,O_2 + H^+ \rightarrow S^0 + H_2O$$
$$S^0 + 1\,1/2\,O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$$
$$S_2O_3^{2-} + H_2O + 2O_2 \rightarrow 2SO_4^{2-} + 2H^+$$
$$S^- \text{or } S^o \rightarrow H_2SO_4$$

Example includes *Acidothiobacillus thiooxidans* (S^o), *Thiomonas intermedia* ($S_2O_3{}^{2-}$), *Thiothrix* (H_2S), *Thiobacillus thioparus* (H_2S, sulfides, S^o, S_2O_3), *Thermothrix* (H_2S, $S_2O_3{}^-$), *Thiosphaera* (H_2S, $S_2O_3{}^{2-}$, H_2), *Acidothiobacillus ferrooxidans* (S^o, metal sulfide, Fe^{2+}), *Paracoccus pentotrophus (thiosulphate).*

Other S oxidizers such as *Sulfurimonas* and *Thioalkalispira* can carry out chemolithotrophic denitrification dependent sulfur oxidation of certain reduced sulfur compounds to sulfate in association with denitrification. The reaction equations are:

$$5S^o + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+$$
$$5S_2O_3^{2-} + 8NO_3^- + H_2O \rightarrow 10SO_4^{2-} + 4N_2 + 2H^+$$
$$5HS^- + 8NO_3^- + 3H^+ \rightarrow 5SO_4^{2-} + 4N_2 + 4H_2O$$

Oxidation of sulphur compounds occurs in stages. In the oxidation process, elemental sulphur is used as polysulphide. Sulphide is first oxidized to polysulphide S using glutathione (GSH). Polysulfide is then first oxidized to $SO_3{}^{2-}$ and further to $SO_4{}^{2-}$ using sulfur oxidase (membrane bound molybdoprotein) and sulfite

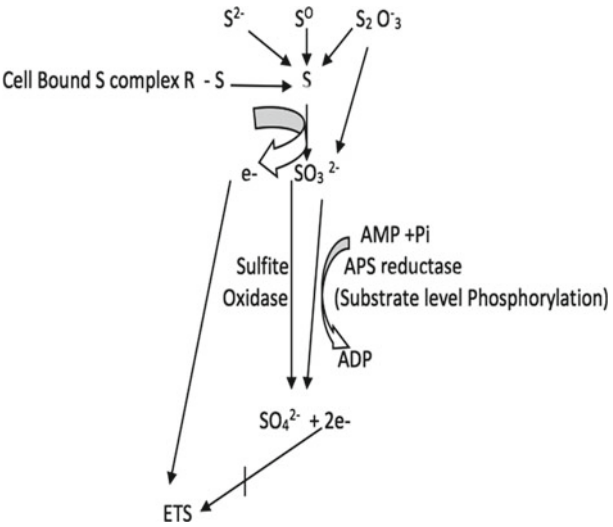


Fig. 7 Schematic representation of sulphur oxidation *Adapted from Gottschalk, (*1986). With kind permission from Springer Nature

oxidase enzymes. Conversion of SO_3^{2-} to SO_4^{2-} can also be carried by another set of enzymes: APS reductase and ADP sulfurylases which convert SO_3^{2-} first to APS and then to SO_4^{2-} and ADP (reversal of the APS reductase system). The process is accompanied by substrate level phosphorylation (AMP to ADP).

Different enzyme systems like rhodanase, thiosulphate reducing enzyme, thiosulfate oxidizing multienzyme complex acts on thiosulphate to produce different products i.e. elemental S & sulfite, sulphite & sulfide and SO_4^{2-}, respectively.

Electrons released during oxidation are transported to respiratory chain either through cytochrome c (aerobic thiobacilli e.g. *T. thiooxidans*) or flavoproteins (facultative anaerobe e.g. *T. denitrificans*). Because of the presence of additional protein cytochrome b between flavoprotein and cytochrome c, the growth yield of *T. denitrificans* is much higher than *T. thiooxidans.*

Reverse electron transfer oxidizes NAD^+ while proton motive force established through electron transfer is utilized for ATP synthesis (Fig. 7).

8 Iron Bacteria or Iron Oxidisers

Iron bacteria carry out oxidation of ferrous (Fe^{2+}) for proton scavenging. In these bacteria, the energy is obtained by converting ferrous (Fe^{2+}) to ferric (Fe^{3+}) ion (Fig. 8). The reaction is:

$$Fe_2^+ + 1/2O_2 + H^+ \rightarrow Fe_3^+ + 1/2H_2O$$

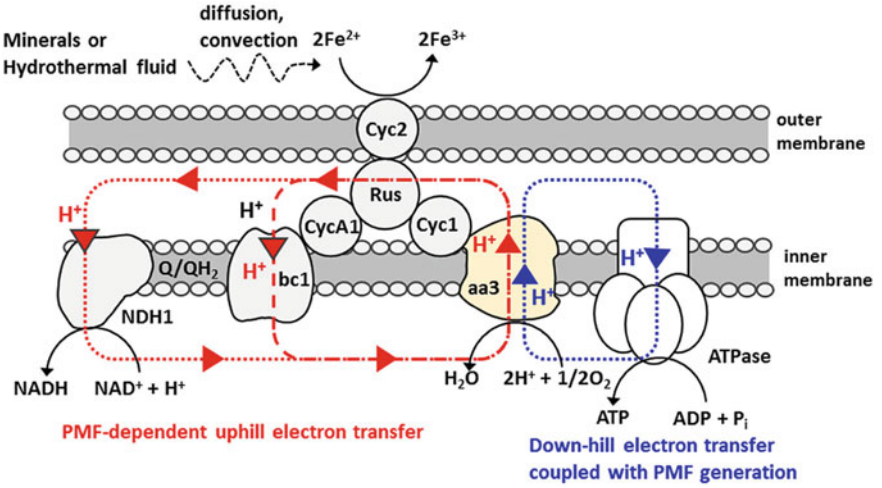


Fig. 8 Electron transport and proton mobilization during of Fe(II) oxidation in *Acidithiobacillus ferrooxidans Source* Ishii et al., (2015). (Creative Commons Attribution license)

However, bioenergetics is not much in favor of this reaction because of positive standard reduction potential. Unlike other groups, the reaction is possible only in acidic conditions as free energy change in alkaline and neutral pH is not sufficient to couple it to ATP production. These organisms grow at acidic pH of 2 while cell's internal pH is 6. The utilization of proton in energy production via ATP synthase helps in preventing acidifications of cell by proton flowing that may otherwise occur because of difference in pH. Also, organisms should use ferrous (Fe^{2+}) quickly before oxygen oxidizes it spontaneously.

Oxidation of ferrous to ferric occurs extracellularly by a complex of Fe^{3+} present in outer membrane. Electrons released are transferred through cytochrome c to rusticyanin in periplasm, then to cytochrome oxidase (cytochrome a_1) at the surface of cell membrane which donates to electron oxygen in the cytoplasm (Fig. 8). The free energy change during ferrous oxidation is very small as redox potential differences between Fe^{+3}/Fe^{+2} (0.078 V) and O_2/H_2O (0.86 V) is very little. For the same reason, they are the slowest growers among all chemolithotrophs. However, since these bacteria grow in acidic conditions, protons from periplasm are pumped to terminal cytochrome oxidases and proton consumption results in generation of PMF (Ishii et al., 2015).

Some bacteria e.g. *Acidithiobacillus ferrooxidans* can utilize both reduced sulphur (S^o, H_2S) and ferrous. *Gallionella ferruginea* (circumneutral pH conditions) can also result in oxidation of ferrous to ferric. Some strict chemolithotrophs like *Leptospirillum ferrooxidans* or *Ferroplasma* sp. are capable of using ferrous ion as energy source.

Iron oxidisers are commonly present in geographical deposits of iron-sulphide minerals and can result in acid mine drainage from pyritic ores. Sulphur in pyrites is

oxidized to sulphuric acid. An archaeon *Ferroglobus placidus* oxidises ferrous under anaerobic conditions in presence of nitrate as electron acceptor (Kim & Gadd, 2008).

T. ferrooxidans and *T. thiooxidans* are important in leaching of metal ores. Iron pyrite (FeS_2) is oxidized to ferric sulphate and sulphuric acid whereas iron-copper pyrite (Chalcopyrite $CuFeS_{2)}$ is oxidized to copper sulphate and ferric sulphate. Copper can be isolated from copper sulphate. Ferric sulphate can be involved in leaching uranium from insoluble uranium dioxide to soluble uranyl cations at pH of 2 to 3. Equations are:

$$4FeS_2 + 2H_2O + 15O_2 \rightarrow 4Fe^{3+} + 8SO_4^- + 4H^+ \text{(Leaching of iron from pyrite)}$$

$$UO_2 + Fe_2(SO_4)_3 \rightarrow (UO_2)SO_4 + 2Fe\,SO_4 \quad \text{(Leaching of Uranium)}.$$

9 Economic Importance of Chemolithotrophs

Chemolithotrophs play an important role in the geochemical cycles of elements like sulfur, nitrogen, and carbon. Anammox organisms can be used to industrially to remove nitrogen from wastewater treatment processes. Hydrogen oxidation is important for hydrogen-based biofuel synthesis. [Ni Fe] hydrogenase from *Ralstonia eutropha* is a promising candidate as it is oxygen tolerant and can be grown heterotrophically. Hydrogenase enzyme is present at anode for H_2 oxidation. Chemolithotrophic sulfur-oxidizing bacteria are used in remediation of H_2S produced during various industrial activities and *Thiobacillus ferrooxidans* has been utilized in bioleaching for removal of H_2S from sour gases. Chemolithotrophs play an important part in biogeochemical cycles of metals such as iron, manganese and arsenic. These results in acid mine drainage, an environmental problem. Some chemolithotrophs e.g. *Acidithiobacillus ferrooxidans*, generate sulfuric acid by utilising ferrous, sulfide and oxygen (O_2) in the mining area that enter the environment and change the pH of groundwater and streams. Such activities also occur at other places like rocky beds of glaciers and soil though at a low level. They are also exploited in developing microbial fuel cells to generate electricity.

10 Phototrophy

Photosynthesis is an autotrophic process in which carbon dioxide is converted to complex organic compounds. It is a multistep pathway that requires a lot of input in the form of energy and reductants. The light mediated reaction leading to the synthesis of sugar is called photosynthesis. One third of CO_2 fixation that occurs on the earth's surface takes place in oceans and is carried out by microorganisms. Several

groups of bacteria are capable of photosynthesis and they are thus 'photoautotrophs'. These bacteria have been useful in the study of photosynthesis as their photosynthetic apparatus is much simpler than that of plants. Although there are important differences between photosynthesis of bacteria and plants, the photochemical reactions that capture the energy of light are basically the same. The major difference lies in the fact that plant photosynthesis evolves oxygen while bacterial does not. In plants, overall photosynthesis reaction can be written as:

$$CO_2 + H_2O + \text{Light} \rightarrow CH_2O + O_2$$

In bacteria, water is replaced by any other electron donors, inorganic or organic, so that the overall reaction becomes:

$$CO_2 + H_2A \rightarrow (CH_2O)_x + A$$

Cornelis Bernardus van Niel, in 1930, recognized that bacteria have light mediated energy yielding metabolism without evolution of oxygen. Thus, bacterial photosynthesis is called as anoxygenic photosynthesis in contrast to oxygenic photosynthesis found in plants and algae. Absence of oxygen evolution was the major reason for the delayed discovery of photosynthesis in bacteria. Around 1885, Theodor Wilhelm Engelmann had suggested that purple bacteria show photosynthetic response and their growth is favoured in presence of light indicating their photosynthetic nature. However, he did not receive much support on this observation due to absence of oxygen. Similarly, Sergei Winogradsky also demonstrated that some purple bacteria can oxidize H_2S to sulphate with transient accumulation of elemental sulfur. Later, in 1905, Hans Molisch observed that purple bacteria can grow, either in the light or in the dark, in complex organic media and do not oxidize H_2S. All these observations were finally concluded after C. B. Van Niel's report of bacterial photosynthesis without evolution of oxygen came up.

11 Diversity of Photosynthetic Bacteria

Photosynthesis requires light-sensitive pigments, chlorophyll (Chl) in plants, algae and cyanobacteria and bacteriochlorophyll (BChl) in purple and green bacteria. Light energy excites electrons of the photosynthetic pigment which then takes part in photophosphorylation to generate ATP. An electron donor (generally inorganic but in some cases organic) then donates an electron to the photosynthetic pigment. When the electron donor is inorganic electron donor (e.g. H_2O, H_2, H_2S), the organism is also known as photolithotroph. Photosynthesis as carried out by green plants, algae and cyanobacteria, is accompanied by the evolution of oxygen (oxygenic photosynthesis) and they use H_2O as the source of electrons. Photosynthesis carried out by phototrophic bacteria is anoxygenic. They use reduced sulfur compounds, molecular hydrogen or organic substrates as the source of electrons (Fig. 9).

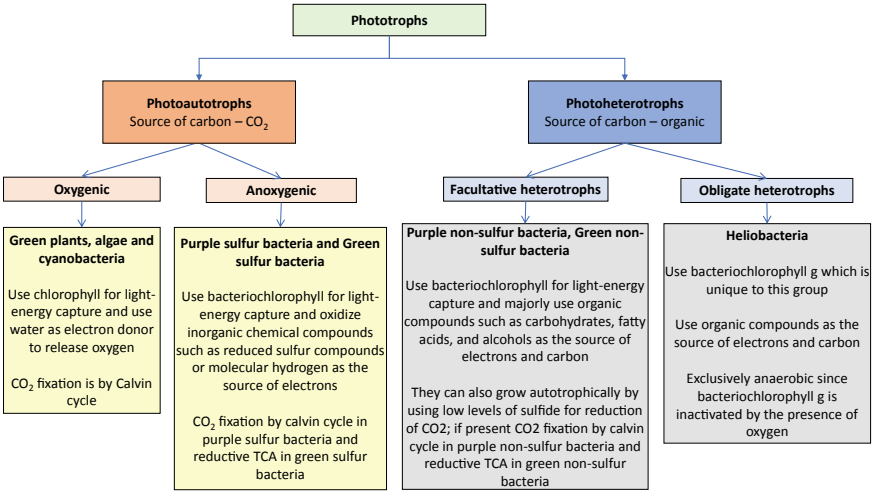


Fig. 9 Classification of phototrophic organisms in terms of energy and carbon sources

Thus, there are three major groups of bacteria which derive energy from light including: Aerobic bacteria, anaerobic bacteria and Halobacteria.

- Aerobic bacteria are cyanobacteria, also known as blue green algae, and have similar mechanism of photosynthesis as observed in plants and algae. Photosynthesis is associated with oxygen evolution and generates both ATP and NADPH as a result of photophosphorylation. There are two reaction centres present in these bacteria.
- Anaerobic bacteria comprise four major groups of bacteria called purple non-sulfur bacteria (Rhodospirillaceae), purple sulfur bacteria (Chromatiaceae), green sulfur bacteria (Chlorobiaceae) and green non-sulfur bacteria (Chloroflexaceae). Photosynthesis in these bacteria is different from aerobic bacteria as it is not accompanied by oxygen evolution and there is a single reaction centre, coupled to only ATP generation. Reducing power is generated in a separate reaction. Another group of anaerobic photosynthetic bacteria is heliobacteria.
- Halobacteria comprise aerobic halophiles classified as archaea and they generate ATP utilizing light induced proton pump. Major pigment involved in this process is bacteriorhodopsin while chlorophylls are absent (For other details, refer Chap.1: Diversity of Prokaryotes).

Based on the evolution of oxygen as a by-product of photosynthesis, we can thus say that there are two types of organisms: oxygenic phototrophs with oxygen evolution during photosynthesis and anoxygenic phototrophs where oxygen is not evolved (Table 3).

Table 3 A comparison between oxygenic and anoxygenic photosynthesis

Anoxygenic photosynthesis	Oxygenic photosynthesis
• During this process, no oxygen is evolved and thus there is no photolysis of water	• Oxygen is one of the products which is formed as a result of photolysis of water
• Electron donors may be organic compounds, a reduced sulfur compound or hydrogen	• Water is the electron donor
• Anoxygenic phototrophs grow phototrophically only under anaerobic conditions because pigment synthesis is repressed by oxygen	• Oxygenic phototrophs grow aerobically
• These bacteria prefer diffused light as they use infrared portion of the spectrum for photosynthesis	• These bacteria use visible spectrum
• Many anoxygenic bacteria grow phototrophically with CO_2 as carbon source while some are photoheterotrophs. Also, white light is energy source and organic carbon is carbon source.	• All are photoautotrophs with CO_2 as carbon source
• Examples include: purple sulfur bacteria, green sulfur bacteria, purple non-sulfur bacteria, green non-sulfur bacteria and heliobacteria.	• Examples include: green plants, algae and cyanobacteria

12 Classification of Phototrophic Bacteria

1. **Purple sulfur bacteria**

They grow phototrophically in anaerobic environments using hydrogen sulfide as the electron donor and CO_2 as the carbon source. Their natural habitats are freshwater lakes and ponds or marine waters. They oxidize sulfide to elemental sulfur that accumulates intracellularly as granules. The sulfur is further oxidized to sulfuric acid. The overall reaction can be represented as:

$$2CO_2 + H_2S + H_2O \rightarrow 2(CHO) + H_2SO_4$$

Some purple bacteria can grow photoheterotrophically utilizing acetate and pyruvate as carbon source, and few others grow chemolithotrophically in darkness with thiosulphate as electron donor. Examples of genera included in this family are: *Chromatium, Thiocapsa, Thiocystis, Thiococcus.*

2. **Purple non-sulfur bacteria**

These bacteria have been called "non-sulfur" because it was originally thought that they were unable to use sulfide as an electron donor for reduction of CO_2. However, sulfide can be used by most species, although the levels of sulfide utilized well by purple sulfur bacteria are toxic to most purple non-sulfur bacteria. Some purple

non-sulfur bacteria are anaerobic, can grow even in dark using fermentative pathway, while others are aerobic and can also grow in dark through respiration but use organic and inorganic compounds as electron donors to oxygen (chemolithotrophic growth). Thus, they are photoheterotrophs and can be isolated by enriching with an organic compound. Another noteworthy character is that they mostly are N_2 fixers also. Thus, they can grow with N_2 as sole nitrogen source. Examples of genera included in this family are: *Rhodospirillum, Rhodopseudomonas, Rhodobacter, Rhodovibrio.*

3. **Green sulfur bacteria**

They are strictly anaerobic and obligately phototrophic, being unable to carry out respiratory metabolism in dark. Most green sulfur bacteria can assimilate simple organic substances for phototrophic growth, provided that a reduced sulfur compound is present as a sulfur source. Organic compounds that can be used by them are acetate, propionate, pyruvate and lactate. Granules of sulfur are deposited outside the cells, never within the cells, unlike the purple sulfur bacteria. Their light harvesting pigments are located in special inclusion bodies called chlorosomes. Examples of genera included in this family are: *Chlorobium* and *Prosthecochloris.*

4. **Green non-sulfur bacteria**

They are also called green gliding bacteria. They are mainly photoorganotrophic as purple non-sulfur bacteria. They grow both photoheterotrophically and photoautotrophically with either H_2 or H_2S as the electron donor or chemoheterotrophically in the dark. Example of genus included in this family is: *Chloroflexus.* They are also known as filamentous green anoxygenic phototrophs (FAP).

5. **Heliobacteria**

Heliobacteria are a unique group of photosynthetic bacteria that have bacteriochlorophyll g and bacteriochlorophyll a is absent. This group consists of three genera: *Heliobacterium, Heliophilum* and *Heliobacillus.* They are strictly anaerobic and heterotrophic. Although physiologically similar to non-sulphur purple bacteria, heliobacteria are unable to grow by respiratory means as is the case in non-sulphur purple bacteria.

13 Photosynthetic Pigments

Each photosynthetic bacterial group has its distinct combination of photosynthetic pigments. Distribution of different bacteriochlorophylls among these groups indicate that bacteriochlorophylls a and b are present in purple bacteria while bacteriochlorophyll c, d or e along with small amount of bacteriochlorophyll a are present in green bacteria. Heliobacteria are unique in possessing only bacteriochlorophyll g.

The color of the bacterium is due to the characteristic carotenoid present in them. However, frequently purple phototrophic bacteria may not be purple but rather brown,

pink, brown-red or purple-violet. Also, most of the green bacteria are actually brown in color due to their complement of carotenoids. Thus, color may not be good indicator for isolation and identification as green or purple bacteria.

Chlorophyll

Chlorophylls are basically cyclic tetrapyrroles and are derived biosynthetically from protoporphyrin 1X with central atoms as magnesium. The basic structure of chlorophyll is shown in Fig. 10. In case of cyanobacteria and plants, the two major chlorophylls Chl a and Chl b, have one of the pyrrole rings (ring iv) reduced by addition of two hydrogens while in Chl a and Chl b which occurs in purple and green bacteria, two of the rings are reduced (rings ii and iv). In addition to porphyrin ring, chlorophyll has a propionyl side chain of ring iv which is esterified with long chain isoprenoid alcohol; chlorophylls a and b contain phytol while bacteriochlorophylls a and b have either phytol, geranylgeranyl or farnesyl depending on the species of bacteria. In vivo, chlorophyll is found non-covalently bound to membrane proteins. In spite of the chemical differences, the absorption spectrum of bacteriochlorophylls present in green bacteria, i.e. c, d and e closely resemble that of Chl a while BChl a and BChl b are 100 nm farther from red region thus falling in the infra-red zone (Fig. 11).

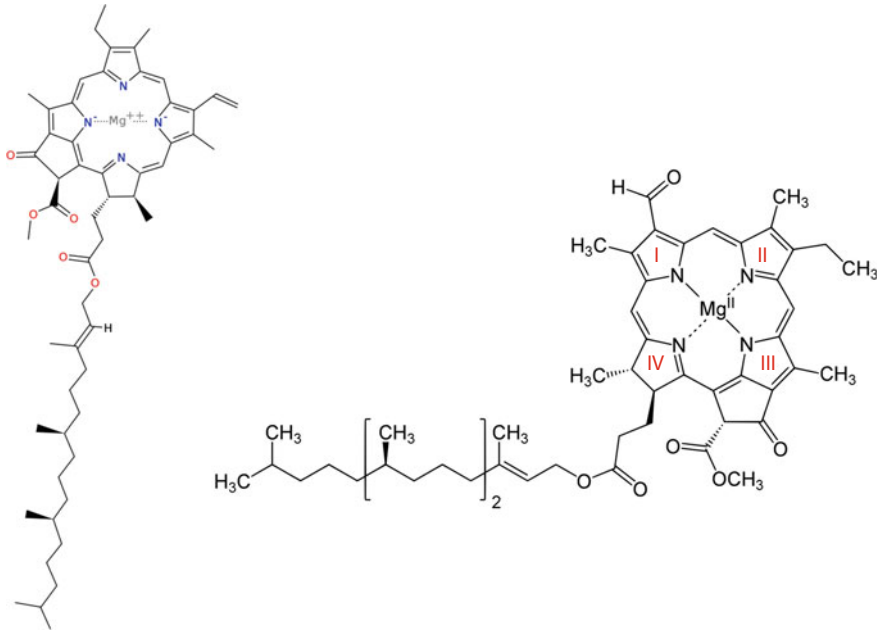
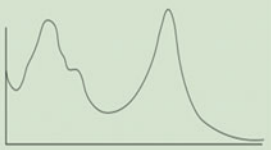


Fig. 10 Basic structure of cholorophylls *Source* Wikipedia. (Public Domain)

Class of phototrophy	Source of reducing power	Photopigments	Cellular absorption spectra	Number of photosystems
Photoautotrophy: Light energy absorption + CO_2 fixed for biomass				
Purple sulfur e.g. *Chromatium*	Photolysis of H_2S	BChl a & b; carotenoid accessory pigments		1
Green sulfur e.g. *Chlorobium*	Photolysis of H_2S	BChl a & b; carotenoid accessory pigments		1
Cyanobacteria e.g. *Anabaena*	Photolysis of H_2O	Chl a & b: carotenoids & phycobilin accessory pigments		2

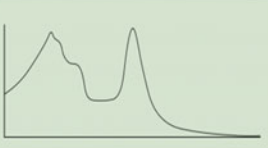
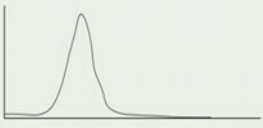
Class of phototrophy	Source of reducing power	Photopigments	Cellular absorption spectra	Number of photosystems
Photoheterotrophy: Light energy absorption + organic nutrients converted to biomass				
Purple nonsulfur e.g. *Rhodospirillum*	Photolysis of CHO	BChl a & b; carotenoid accessory pigments		1
Green nonsulfur e.g. *Chloroflexus*	Photolysis of CHO	BChl a & b; carotenoid accessory pigments		1
Retinal-based proton pumps: Bacteriorhodopsin (e.g. *Halobacetrium salinarum*) & proteorhodopsin (e.g. *Pelagibacter ubique*	Heterotrophic metabolism	Retinal in bacteriorhodopsin (halophilic archaea) and proteorhodopsin (marine proteobacterial) proteins		N/A

Fig. 11 A detailed comparison of photosynthetic pigments, reductants and type of photosynthetic process among different group of bacteria *Source* Keenleyside (2019). (Creative Commons Attribution License)

Carotenoids

The most widespread accessory pigments are the carotenoids which are always present in phototrophic organisms. These are hydrophobic pigments (isoprenoids) firmly embedded in the membrane. Carotenoids are yellow, red, brown or green and absorb light in the blue region of the spectrum. Carotenoids are closely associated with chlorophylls or bacteriochlorophylls in the photosynthetic membrane. They function as light harvesting pigments and transfer energy to the reaction centre and this energy can be used for photophosphorylation by pigments present at the reaction centre. Carotenoids also serve as photoprotective agents protecting chlorophyll against photo-oxidation.

Phycobilins and Phycobilisomes

Cyanobacteria and red algae contain phycobiliproteins as their main light-harvesting pigments. Phycobiliproteins consist of open-chain tetrapyrroles coupled to proteins. There are three classes of phycobiliproteins: phycoerythrin (absorbs maximally at 550 nm), phycocyanin (absorbs maximally at 620 nm) and allophycocyanin (absorbs at about 650 nm). Phycobiliproteins occur as high molecular weight aggregates, called phycobilisomes, attached to the photosynthetic membranes. Phycobiliproteins are arranged in layers around each other with phycoerythrin on the outside, phycocyanin in the middle and allophycocyanin in the inside closest to the reaction centre. The phycobilisome thus yields very efficient energy transfer from the biliprotein complex to chlorophyll present in the reaction centre.

Photosynthetic pigments in all photosynthetic organisms are assembled as light harvesting pigments and reaction center pigments. The purpose of light harvesting system is to harvest as much as energy as it can from the light source available and consolidate on its reaction center. Therefore, light harvesting centers are composed largely of accessory pigments which absorb high energy, shorter wavelengths. Light energy is captured by antenna pigments comprising of both chlorophyll/bacteriochlorophyll and accessory pigments allowing harvest of light energy over a broad spectrum of wavelength. These harvesting antenna pigments form pigment-protein complexes which are termed as the light harvesting system (LHS). The excited antenna pigment molecules temporarily stay in excited state till their energy is transferred to reaction center which is largely composed of chlorophylls/bacteriochlorophylls only. These ‘Reaction Centres (RC)’ are also pigment protein complexes embedded in lipid bilayer. A pair of Chls/Bchls of the RC sets the photochemical reaction by triggering charge separation across the membrane. The charge separation initiates a series of electron transfer reactions coupled with proton translocation leading finally to generation of proton motive force. The pmf is ultimately used to generate ATP. The whole process of generating ATP as a result of absorption of light energy is called photophosphorylation.

The phenomenon of photosynthetic photophosphorylation was discovered by Arnon in 1954 using isolated spinach chloroplast wherein it was observed that the same chloroplast which fixed CO_2 also produced ATP in presence of light (without

the participation of respiration) (Arnon, 1959) . It was demarcated from other phosphorylations viz. substrate level phosphorylation and oxidative phosphorylation on the basis that:

1. It occurs in chloroplast lamellae without addition of any other enzyme system
2. Only light is required as external energy source
3. It is not linked to oxygen utilization or its evolution.

14 Oxygenic Photosynthesis

The photosynthetic apparatus of cyanobacteria and algae comprises of two distinct but interconnected photochemical reactions. One light reaction (Photosystem I; PS I) covers a span of approximately from +400 mV to −600 mV and resembles the photosystem of green bacteria. The second system (PS II), however, pushes electrons up a reaction center as far positive as from +850 to 0 mV. It is this system that allows the first step in oxygenic photosynthesis, the splitting of water into oxygen and hydrogen atoms.

The chlorophyll a of the photosystem II (P680) absorbs a quantum of light near 680 nm, becomes energized and reduces an acceptor molecule, pheophytin. Having lost an electron, the redox potential of P680 is very electropositive sufficient to replace the lost electron with one from water. From pheophytin, the electron travels through several membrane carriers including quinones, cytochromes and a copper-containing protein called plastocyanin.

Plastocyanin donated electrons to photosystem I (P700) which has previously absorbed light quanta. Form here, the electrons travel to several carriers like chlorophyll a, phytoquinone, iron-sulfur centers and an iron-sulfur protein, ferredoxin. Ferredoxin in turn reduces $NADP^+$. Thus, the two light reactions in series energize electron flow from H_2O to $NADP^+$. This is called non-cyclic electron flow because electrons never return to the reaction center.

During transfer of electrons from the acceptor in PS II to the reaction center chlorophyll in PS I, electron transport occurs in a thermodynamically favorable direction. This generates a proton-motive force from which ATP can be produced. This is called non-cyclic photophosphorylation. However, ATP can also be produced by cyclic photophosphorylation involving only PS I. This occurs when electrons flow from ferredoxin to cytochrome *bf* complex from where they return to P700. This flow creates a membrane potential and synthesis of ATP (Fig. 12).

From the forgoing discussion, it is clear that light is first captured by light harvesting pigments which transfer energy through resonance to the reaction center from where the photochemical reaction is initiated.

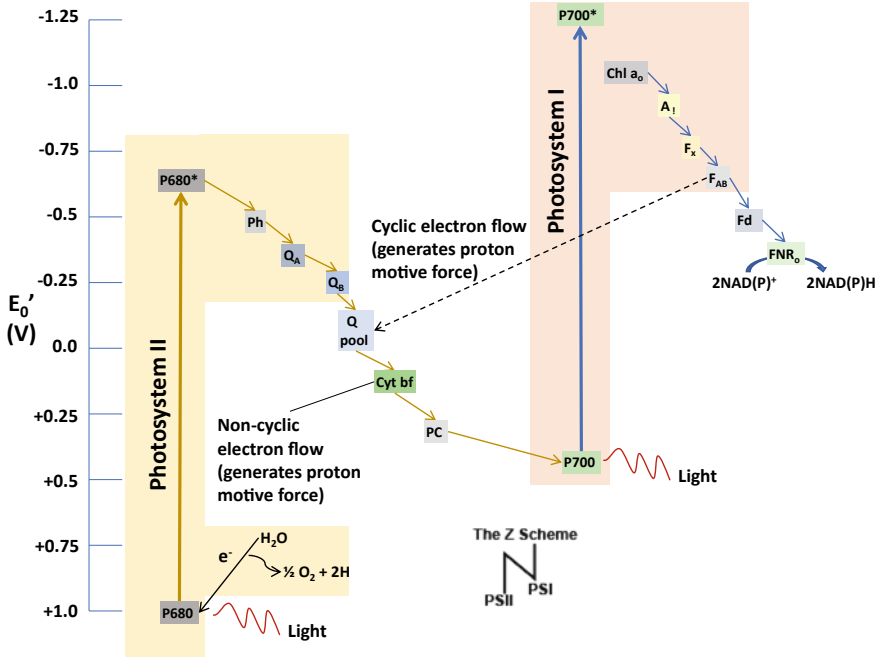


Fig. 12 Scheme for electron flow in cyanobacteria

15 Anoxygenic Photosynthesis

The major components of photophosphorylation system constitutes light harvesting antenna pigments (LHC) and reaction centre which has key chlorophyll associated with a series of electron carriers to generate proton motive forces as electron travels downhill. Here, photophosphorylation will be described in brief in three different groups of phototrophs.

Purple photosynthetic bacteria

LHC of purple photosynthetic bacteria consists of 2 complexes LH2 and LH1 (antenna pigments). Of these, LH2 is called peripheral complex as it spans the membrane and forms both pigment-pigment and pigment-protein complex. It has two polypeptides α and β which interact with 3 BChl and 1 carotenoid. LH1 is a core complex which is associated with reaction centre.

The energy travels from several LH2 complexes to LH1 and then to reaction centre which results in charge separation. Therefore, there is every chance of back flow of energy if reaction centre is charged. This back flow needs to be dissipated in a harmless manner wherein carotenoids play an important role in protecting BChl against photo-oxidation. Besides this, carotenoids also expand the spectrum of light absorption.

- The amount of LH2 varies with environmental conditions such as light intensity and oxygen availability. The three BChls are present as one BChl 800 and two Bchl 850 molecules which strongly coupled to each other.
- The amount of LH1 to RC is fixed as it combines to form LH1-RC super complex. At the core of each reaction centre, there are two dimers each of BChl and BChl phaeophytin (without Mg^{2+}). The BChl of LH1 is BChl 875.

Reaction Centre

The reaction centre of *R. sphaeroides* comprises of three polypeptides, ten cofactors, four BChl, two phaeophytins, two ubiquinones, a carotenoid and a non heme iron. BChl, BPhe and Ub are arranged within a protein scaffold spanning the membrane.

Energy flow from LH2 to LH1-RC

LH2 complex has carotenoids along with BChls which absorb within 400–500 nm spectral region. Carotenoids absorb light energy and transfer energy to BChl 800 and BChl 850 of LH2. The bacteriochlorophylls may get excited to higher energy levels to either high intensities or slow transfer due to the situation when RC is already charged. Under such situation, the excited BChl (referred as $BChl^{*}$) can transfer energy to oxygen which can get converted to superoxide radical leading to photodamage. However, when carotenoids interact with BChl triplet state, they get excited to carotenoids triplet which dissipates as heat.

Energy absorbed by BChl 800 must be transferred to BChl 850 which transfers it to core complex having BChl 875 wherein finally it is trapped by the reaction centre. Here it leads to charge separation. It is noteworthy that there is no charge separation in antenna pigments and there is mere energy transfer from one molecule to other by resonance. So a range of transfers occur one after the other before it reaches LH1.

Mechanism of photophosphorylation

Purple bacteria grow both as photoheterotrophs and photoautotrophs. Therefore, they show cyclic and non-cyclic photophosphorylation during heterotrophic and autotrophic growth, respectively. The energy transfer steps are as follows:

$$\mathbf{LH2 \rightarrow LH1 \rightarrow RC}$$

Reaction centre has two dimers each of BChl 875 and BChl Phaeophytin (without Mg^{2+}; BChl Ph). The BChl 875 is excited and reaches $BChl\ 875^{+}$/BChl 875 state. It then donates electron to BChl Ph making it $BChl\ Ph^{-}$/BChl Ph. The charge separation sets up electron flow to existing ubiquinone (Ub) which gains electron. Another similar cycle is set-up to provide one more electron to Ub converting it to as Ubiquinol which accepts protons from the cytoplasm to become UbH_2. Next, electron travels to Cyt bc complex with ejection of protons and generation of pmf which is linked to ATP formation. Electron is then donated to mobile Cyt c_2 which gives electron back to RC completing cyclic photophosphorylation in a heterotroph.

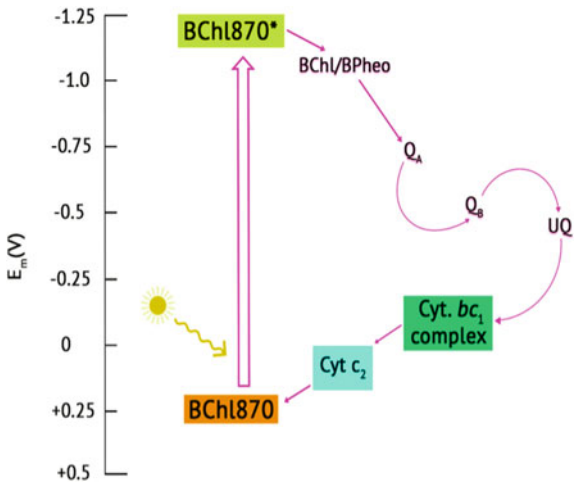


Fig. 13 Cyclic and Noncyclic photophosphorylation in Purple Sulphur Bacteria *Source* Keenleyside (2019). (Creative Commons Attribution License)

The redox potential of RC in purple bacteria is −1.0 V after excitation which is insufficient to reduce NAD(P) with redox potential of −0.32 V. Therefore, to reduce NAD(P), the bacterium should employ reverse electron flow. There has to be an external source of electron to feed excited chlorophyll. Here it is H_2S or elemental sulphur or sulphur by-products. Hence, purple bacteria use cyclic photophosphorylation to generate ATP while growing on organic carbon and use non-cyclic pathway on sulphur compound wherein they reduce NAD/NADP by reverse electron flow (Fig. 13).

Green bacteria

The organization of the photosynthetic apparatus in the two families of green bacteria is different. Here, the light harvesting centers are present in special organelles called chlorosomes. Light is absorbed by these pigmented organelles and transferred to the reaction centers that are situated in the cytoplasmic membrane.

The reaction centers of green sulfur bacteria are similar to the reaction centers I of cyanobacteria and chloroplasts. The photochemically active pair of bacteriochlorophyll molecules resides in a reaction center called P840. The excited state of the reaction center bacteriochlorophylls resides at a significantly more negative redox potential. The significance of this is that unlike purple bacteria, where the first stable acceptor molecule (quinone) has an E_0' of about 0 V, the acceptors of green bacteria have a much more electronegative E_0', sufficient to reduce NAD^+ to NADH directly (via a ferredoxin protein) without the necessity for reverse electron flow. Here, the electron donor is a reduced inorganic sulfur compound which is oxidized by cytochrome c_{555} (the reductant for P840), which feeds electrons into the reaction center.

Alternatively, the electrons can also flow in a cyclic manner. Here, the electron flows from the primary acceptor back to the reaction center via menaquinone and a bc_1 complex creating a pH gradient. The mechanism of proton extraction is analogous to the one in purple bacteria.

LHC of Green bacteria and other filamentous green anoxygenic phototrophs (FAP)

The antenna pigments of these bacteria differ from that of purple sulphur bacteria in the sense that they are not only super antennas due to large size but are also not complexed with proteins. The super antennas are present as a complex structure (a kind of organelle) called as chlorosomes. These complex light harvesting systems have been developed to capture as much light as it can since these bacteria thrive very deep up to 100 m depth in the black sea where there is very low light intensity. The chlorosomes contain as many as 2,50,000 chlorophyll molecules. These are ellipsoidal bodies varying from 100-200 nm length, 50-100 nm width and 15-30 nm height in Green sulphur bacteria. However, their size can vary from species to species. These are supramolecular antenna systems having thousands of BChl c/d/e molecules enclosed by a single membrane and a base plate. They are not enclosed in protein scaffold. The energy flow occurs via a small protein CsmA embedded in the base plate to the reaction center (Fig. 14).

They are present in green sulphur bacteria (phylum Chlorobia) and in some filamentous anoxygenic phototrophs (FAPs) of Phylum Chloroflexi and a newly discovered aerobic phototroph *Candidatus* Chloracedobacterium thermo-phylum of phylum Acidobacteria (Bryant et al., 2007). *Chlorobaculum tepidum*, also known as *Chlorobium*, is considered as a model organism for the group.

Chlorosomes are attached to reaction center via FMO protein (Fenna-Matthews-Olson protein) and a base plate having CsmA protein. However, Chloroflexi lack

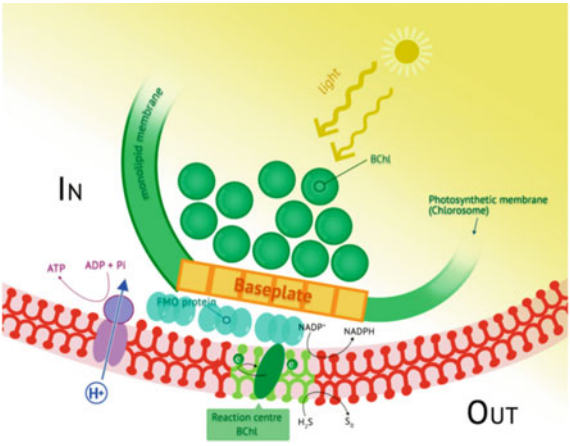


Fig. 14 Structural organization of Chlorosome of a green sulphur bacterium *Source* Keenleyside (2019). (Creative Commons Attribution License)

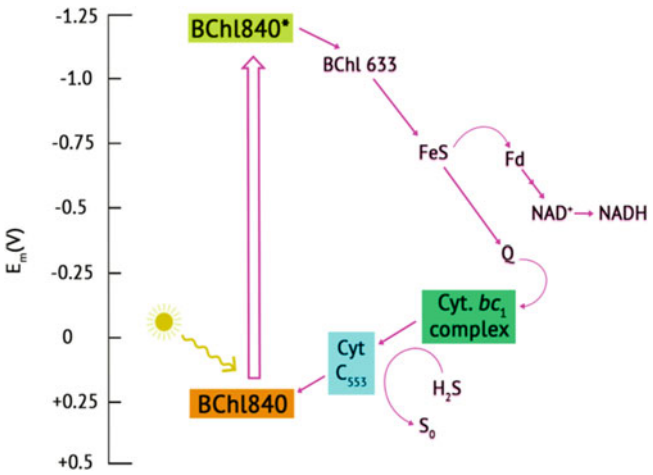


Fig. 15 Cyclic/non-cyclic photophosphorylation in green sulfur bacteria *Source* Keenleyside (2019). (Creative Commons Attribution License)

FMO protein and instead use other protein complexes. Composition of chlorosomes is mostly BChl c/d/e, small amount of carotenoids and quinones surrounded by lipid monolayer. The monolayers can contain up to 11 different proteins. The pigments self-assemble in lamellar structures of 10-30 nm wide without protein scaffold. For energy transfer, FMO protein link chromosome to reactive centre. It is a trimeric protein. Each subunit contains eight BChl a molecules bound to protein scaffold via Mg^{++} ion forming a simple protein-pigment complex. Reaction centre is equivalent to PS1 in plants and cyanobacteria. Green sulphur bacteria use sulfide, hydrogen or ferrous as electron donors.

Mechanism of photophosphorylation

Green bacteria exhibit both cyclic and non-cyclic photophosphorylation. Unlike purple bacteria where quinone is the first reductant at potential 0.0 V, here the first reductant is Fe-S protein at reduction potential −0.6 V which is much more electronegative than NADH. Here, there is direct reduction of NAD^+ to NADH and H^+ through ferredoxin or reduced ferredoxin. It is similar to oxygenic phototrophy where both ATP and NADPH are generated during light reaction. The reaction centre has P840 as BChl a (Fig. 15).

16 C1 Metabolism

C1 carbon metabolism essentially relates to utilization of C1 carbon in any of the inorganic or organic forms e.g. CO_2, CH_4, CH_3OH, CH_3NH_2 and utilize it in autotrophic or heterotrophic mode. Of these, carbon dioxide is used by autotrophs to fix it either in

the form of sugars or acetyl CoA to acetic acid and methane while methane, methanol or methylamine is utilized as source of energy by methylotrophs or methanotrophs. Firstly, CO_2 assimilation will be discussed here.

Carbon dioxide assimilation

Carbon dioxide is largely fixed by Calvin-Benson cycle as sugar-starch or by reductive TCA cycle followed by gluconeogenesis by autotrophs. Acetogens and methanogens fix it first as acetyl CoA. The following pathways of CO_2 fixation will be described:

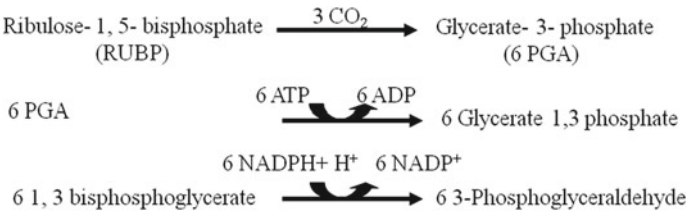
1. Calvin cycle
2. Reductive TCA
3. Acetyl CoA to acetic acid.

Calvin-Benson cycle: This pathway is also called reductive pentose cycle as it involves most of the reactions similar to pentose pathway. This is a dark reaction of CO_2 fixation among phototrophs including purple bacteria, cyanobacteria, plants and algae. This utilizes products of photophosphorylation i.e. ATP and NADH + H^+. The pathway was constructed by Melvin Calvin and his coworkers after a series of studies involving radioautographic analysis of radiolabeled carbon. These experiments showed that PGA is the first stable product of CO_2 fixation and RUBP (Ribulose 1,5 bis-phosphate) is the acceptor of CO_2. The enzyme responsible for CO_2 fixation is ribulose biphosphate carboxylase (Rubisco) (Fig. 16).

The whole Calvin cycle can be divided into three stages:

1. Carbon dioxide fixation
2. Sugar rearrangements
3. Regeneration of acceptor

1. Fixation of carbon dioxide catalyzed by Rubisco enzyme



Ribulose 1, 5-bisphosphate gets converted to 2 mol of 3-phosphoglycerate after carboxylation catalyzed by ribulose 1,5-bisphosphate carboxylase. PGA is then first phosphorylated to glycerate 1,3-bisphosphate before getting reduced to 3-phosphoglyceraldehyde. These reactions utilize by-products of light reaction, ATP and NADPH + H^+ (Note that reductant here is NADPH + H^+ in contrast to similar reaction in glycolysis where reductant is NAD and NADH catalyzed by GAPDH). This reaction is pentose reductive carboxylation.

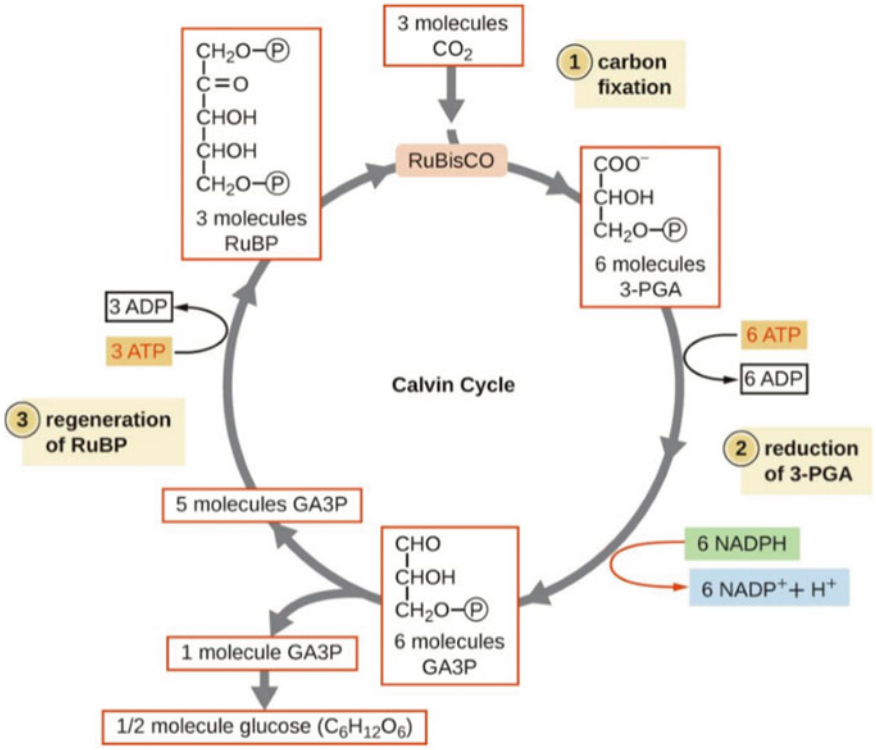
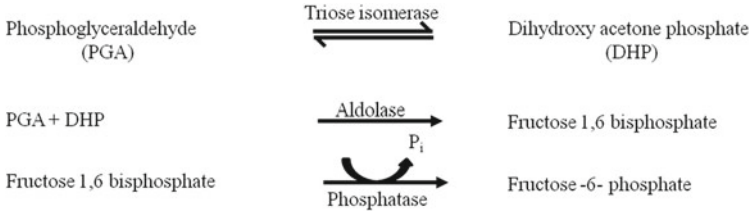
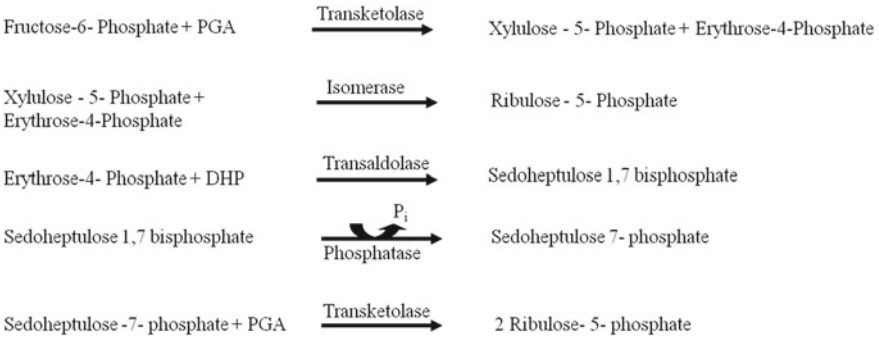


Fig. 16 Calvin- Benson cycle for fixation of carbon dioxide *Source* Keenleyside (2019). (Creative Commons Attribution License)

2. Sugar Rearrangement



Subsequent reaction by transketolases and transaldolases yield 3 mol of Ribulose-5-phosphate.



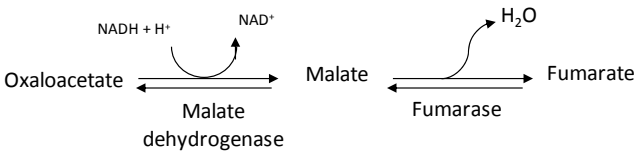
3. Regeneration of acceptor

3 mol of Ribulose monophosphate are phosphorylated in presence of Ribulose 5-phosphatase enzyme to acceptor ribulose 1,5-bisphosphate.

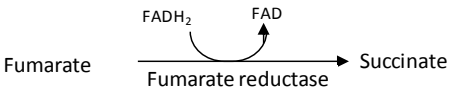
Reductive TCA: The reductive TCA cycle is likely to be the earliest autotrophic pathway that evolved. This was first discovered in green sulfur bacterium *Chlorobium limicola.* Later, this pathway was confirmed in many anaerobic and microaerobic bacteria such as sulfate-reducing *Desulfobacter hydrogenophilus*, various proteobacteria and species of Aquificaceae such as *Aquifex pyrophilus* and *Hydrogenobacter thermophilus.* The pathway is also present in archaea (Hugler et al., 2007).

The cycle requires three new enzymes, namely fumarate reductase, α-ketoglutarate synthase (α-ketoglutarate ferredoxin oxidoreductase) and ATP dependent citrate lyase (Fig. 17). The overall reaction uses four moles of carbon dioxide to generate one mole of oxaloacetate. In this pathway, the following reactions take place:

1. Oxaloacetate is converted to malate and then to fumarate by reverse reaction of malate dehydrogenase and fumarase



2. Fumarate is reduced to succinate by FAD dependent fumarate reductase.



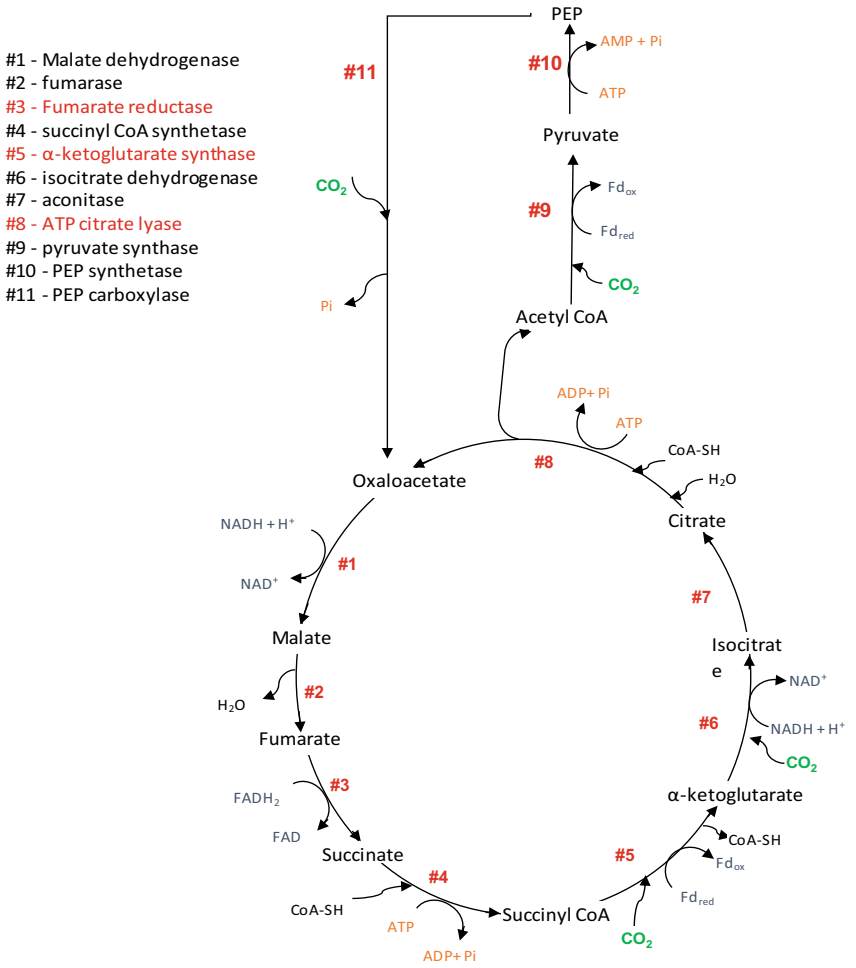
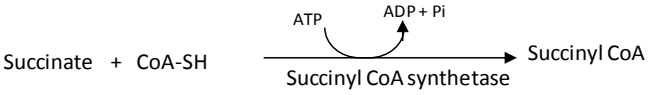
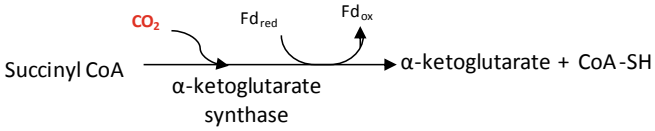


Fig. 17 Schematic representation of reductive TCA cycle

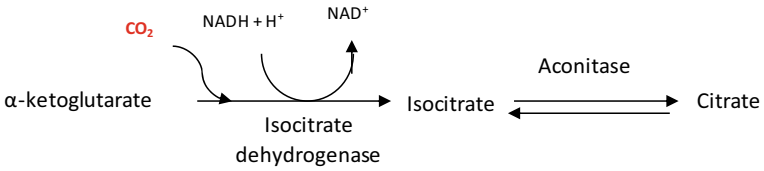
3. Succinate is converted to succinyl CoA by succinyl CoA synthetase (succinate thiokinase).



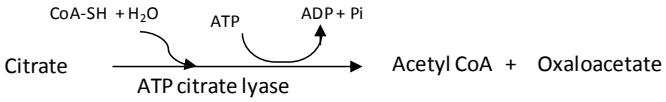
4. Succinyl CoA is converted to α-ketoglutarate by carboxylation through α-ketoglutarate synthase (α-ketoglutarate ferredoxin oxidoreductase).



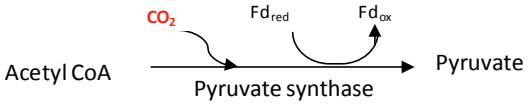
5. α-ketoglutarate is carboxylated to isocitrate and it then isomerized to citrate.



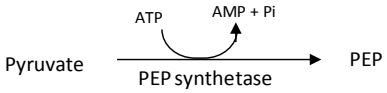
6. Finally, ATP citrate lyase yields acetyl CoA and one mole of oxaloacetate.



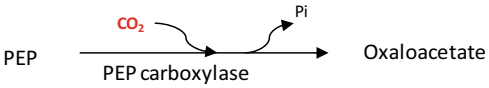
7. Acetyl CoA is carboxylated to pyruvate by pyruvate synthase using reduced ferredoxin as the reductant.



8. Pyruvate gets phosphorylated to PEP by PEP synthetase.



9. Phosphoenol pyruvate is carboxylated to oxaloacetate by PEP carboxylase



Ferredoxin is used as the reductant as it has more reducing potential than both NADH and $FADH_2$.

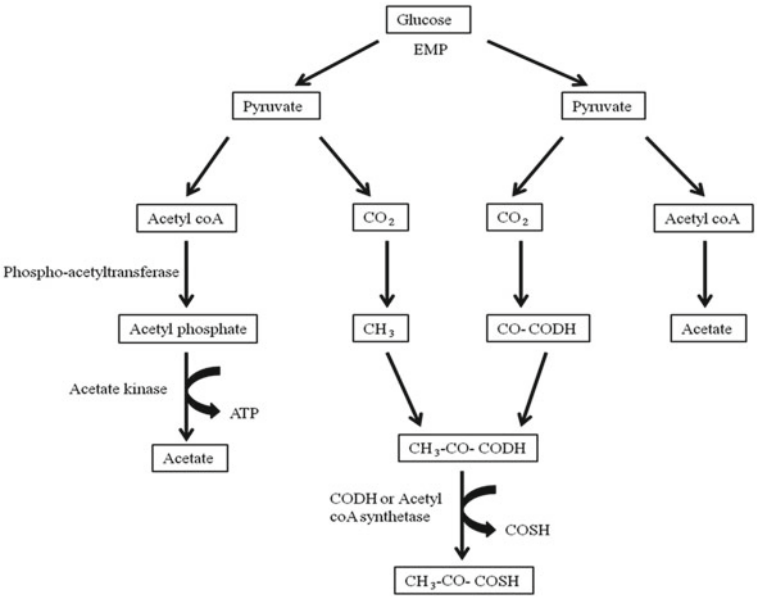


Fig. 18 Acetate generation during heterotrophic growth

Acetyl CoA pathway: This pathway operates in bacteria and archaea i.e. acetogens and methanogens. It is not a cyclic pathway. This pathway operates both in heterotrophs and autotrophs. Difference between the two is that in heterotrophs, carbon dioxide is obtained when pyruvate is decarboxylated to acetyl CoA during glycolysis. In autotrophs, on the other hand, reductant is hydrogen and hydrogenases catalyze the release of H^+ ions for reduction. From acetyl CoA, acetate is formed by phosphotransacetylase and acetate kinase activity (Fig. 18).

Summary

- All living organisms have two basic requirements to survive and grow: energy in the form of ATP and cell metabolites or cell carbon.
- Bacteria can use a wide variety of compounds (both organic and inorganic) as their source of carbon and energy. Many species are unique in having one compound serving as their source of carbon while a different compound serving as the source of energy. Based on their source of energy, bacteria can be either chemotrophs or phototrophs.
- In chemotrophs, organic or inorganic compounds are oxidized and the electrons released are fed into the electron transport chain. Depending on the terminal electron acceptor, the energy generating pathways can be aerobic (oxygen is the terminal electron acceptor) or anaerobic (nitrate, sulfate, fumarate, etc. act as terminal electron acceptor).

- Phototrophs carry out photon capture to acquire energy. Most phototrophs are photoautotrophic, capable of utilizing CO_2 as the sole carbon source. Some are photoheterotrophs and use organic carbon. Plants, algae and cyanobacteria are photoautotrophs wherein CO_2 is fixed to form the cellular mass and light energy acts as the source of energy.
- In most prokaryotes and all eukaryotes (other than plants and algae), the same compound acts as the source of carbon and energy. Energy is generated by a chemical reaction involving oxidation of organic compounds; such organisms are known as chemoheterotrophs.
- Chemolithotrophs are those microorganisms that obtain their energy from oxidation of inorganic electron donors such as hydrogen, carbon monoxide, ammonia, nitrite, sulphite, iron etc. In chemolithoautotrophs, the source of carbon is CO_2. In chemolithoheterotrophs, source is carbon is organic and they are also known as mixotrophs.
- In chemolithotrophs, reductants are generally generated either directly or through reverse electron transport.
- Hydrogen oxidisers possess soluble & particulate hydrogenases grouped into three classes based on the metal ions in their active sites- [Ni-Fe], [Fe–Fe], [Fe].
- Carboxydobacteria utilise CO as source of electron oxidising it to CO_2.
- Ammonia oxidisers that completely oxidise ammonia to monooxygenase and hydroxylamine oxidoreductases, possessed by ammonia oxidisers, carry out ammonia oxidation. Organisms that carry out complete oxidation of ammonia to nitrate in a single step are classified as commamox (e.g. *Nitrospira*).
- Bacteria carrying out anaerobic oxidation of ammonia (annamox) play an important role in global nitrogen cycle and have been employed successfully in wastewater treatment.
- Sulphur bacteria obtain their energy by oxidation of variety of reduced sulphur compounds and the oxidised compounds maybe deposited intracellularly or extracellularly. Some S oxidisers carry out denitrification dependent S oxidation (*Sulfurimonas*).
- In iron bacteria, oxidation of ferrous ion to ferric ions releases energy. However, due to positive standard reduction potential, the oxidation of iron ions is feasible only under acidic conditions.
- Chemolithotrophs are major contributors in global geochemical cycles of elements and of certain metals, however, chemolithotrophic activity leading to acid mine drainage is a major environment concern.
- Chemolithotrophs find various industrial applications such as in developing microbial fuel cells in generating electricity.
- Photosynthesis is an autotrophic process in which carbon dioxide is converted to complex organic compounds. It is a multistep pathway requiring energy and reductants.
- C.B. Van Niel, in 1930, recognized that bacteria have light mediated energy yielding metabolism without evolution of oxygen. Thus, bacterial photosynthesis is called as anoxygenic photosynthesis in contrast to oxygenic photosynthesis found in plants and algae.

- There are three major groups of bacteria which derive energy from light including: aerobic bacteria (cyanobacteria also known as blue green algae), anaerobic bacteria and halobacteria.
- Anaerobic bacteria comprise four major groups of bacteria called purple non-sulfur bacteria (Rhodospirillaceae), purple sulfur bacteria (Chromatiaceae), green sulfur bacteria (Chlorobiaceae) and green non-sulfur bacteria (Chloroflexaceae).
- Purple sulfur bacteria grow phototrophically in anaerobic environments using hydrogen sulfide as the electron donor and CO2 as the carbon source
- Purple non-sulfur bacteria have been called "non-sulfur" because it was originally thought that they were unable to use sulfide as an electron donor for reduction of CO_2.
- Green sulphur bacteria can assimilate simple organic substance for phototrophic growth, provided that a reduced sulphur compound is present as a sulphur source. Organic compounds that can be used by them are acetate, propionate, pyruvate and lactate.
- Green non-sulphur bacteria are also called green gliding bacteria. They are mainly photoorganotrophic as purple non-sulphur bacteria. They grow as photo-heterotrophs, photoautotrophs and chemoheterotrophs in the dark.
- Distribution of different bacteriochlorophylls among these groups indicate that bacteriochlorophylls a and b are present in purple bacteria while bacteriochloro-phylls c, d or e along with small amount of bacteriochlorophyll a are present in green bacteria. Heliobacteria is unique in possessing only bacteriochlorophyll g and bacteriochlorophyll a is absent
- The photosynthetic apparatus of cyanobacteria and algae comprises of two distinct but interconnected photochemical systems.
- The whole process of generating ATP as a result of absorption of light energy is called photophosphorylation.
- The phenomenon of photosynthetic photophosphorylation was discovered by Arnon in 1954 using isolated spinach chloroplast.
- Purple bacteria show cyclic and non-cyclic photophosphorylation during heterotrophic and autotrophic growth, respectively.
- The organization of the photosynthetic apparatus in the two families of green bacteria is different. Here, the light harvesting centers are present in special organelles called chlorosomes. Light is absorbed by these pigmented organelles and transferred to the reaction centers that are situated in the cytoplasmic membrane.
- Green bacteria exhibit both cyclic and non-cyclic photophosphorylation. Unlike purple bacteria where quinone is first reductant at potential 0.0 V, here the first reductant is Fe-S protein at reduction potential −0.6 V which is much more electronegative than NADH.
- There is direct reduction of NAD^+ to NADH and H^+ through ferredoxin or reduced ferredoxin. Here it is similar to oxygenic phototrophy where both ATP and NADPH are generated during light reaction. The reaction centre has P840 as Bchl a.

- Carbon dioxide is largely fixed by Calvin-Benson cycle as sugar-starch or by reductive TCA cycle followed by gluconeogenesis by autotrophs. Acetogens and methanogens fix it first as acetyl COA which is then converted to either acetate or methane.

Questions

1. Define chemolithotrophs. List their different groups.
2. What is meant by reverse electron transport?
3. Differentiate between soluble and membrane bound hydrogenases.
4. Discuss type of hydrogenases based on metal ions in their active site.
5. What are uptake hydrogenases? How are different from evolution ones?
6. Give examples of two anaerobic hydrogen oxidisers.
7. Name the enzymes involved in ammonia oxidation.
8. What is commonox? Explain by giving an example.
9. Explain briefly anaerobic ammonia oxidation.
10. Discuss in brief the oxidation of S compounds by sulphur oxidisers.
11. Why iron oxidisers are slow growers?
12. Diagrammatically explain electron transfer and proton mobilisation in iron oxidisers.
13. Write about economic importance of chemolithotrophs.
14. What is the contribution of C.B. Van Niel to the discovery of bacterial photosynthesis?
15. Differentiate between anoxygenic and oxygenic photosynthesis.
16. What are different groups of photosynthetic bacteria? Compare their pigment distribution and electron donor and CO_2 fixation pathway.
17. Compare photophosphorylation in purple-sulphur bacteria and green-sulphur bacteria.
18. Describe Z-scheme with reference to photophosphorylation in cyanobacteria.
19. Write a note on CO_2 fixation by Calvin cycle.
20. What are different CO_2 fixation steps during CO_2 fixation by reductive TCA pathway in green sulphur bacteria?

References

Arnon, D. I. (1959). Conversion of light into chemical energy in photosynthesis. *Nature, 184*(4679), 10–21.

Beard, S. D., Paradela, A. D., Albar, J. P. D., & Jerez, C. A. D. (2011). Growth of Acidithiobacillus ferrooxidans ATCC 23270 in thiosulfate under oxygen-limiting conditions generates extracellular sulfur globules by means of a secreted tetrathionate hydrolase. *Frontiers in Microbiology, 2,* 79.

Beaumont, H. J., Hommes, N. G., Sayavedra-Soto, L. A., Arp, D. J., Arciero, D. M., Hooper, A. B., et al. (2002). Nitrite reductase of Nitrosomonas europaea is not essential for production of gaseous nitrogen oxides and confers tolerance to nitrite. *Journal of Bacteriology, 184*(9), 2557–2560.

Bryant, D. A., Costas, A. M. G., Maresca, J. A., Chew, A. G. M., Klatt, C. G., Bateson, M. M., et al. (2007). Candidatus Chloracidobacterium thermophilum: an aerobic phototrophic acidobacterium. *Science, 317*(5837), 523–526.

Driessen, W., Ettinger van, M., Remy, M., Hendrickx, T., & Kruit, J. (2012). The Anammox Process, design considerations and operation experience. In *17th European biosolids and organic resources conference.* Aqua Enviro.

Frunzke, K., & Meyer, O. (1990). Nitrate respiration, denitrification, and utilization of nitrogen sources by aerobic carbon monoxide-oxidizing bacteria. *Archives of Microbiology, 154*(2), 168–174.

Gottschalk, G. (1986). Regulation of bacterial metabolism. In *Bacterial metabolism* (pp. 178–207). Springer.

He, H., Zhen, Y., Mi, T., Fu, L., & Yu, Z. (2018). Ammonia-oxidizing Archaea and Bacteria differentially contribute to ammonia oxidation in sediments from adjacent waters of Rushan Bay, China. *Frontiers in Microbiology, 9,* 116.

Hügler, M., Huber, H., Molyneaux, S. J., Vetriani, C., & Sievert, S. M. (2007). Autotrophic CO2 fixation via the reductive tricarboxylic acid cycle in different lineages within the phylum Aquificae: evidence for two ways of citrate cleavage. *Environmental Microbiology, 9*(1), 81–92.

Ishii, T., Kawaichi, S., Nakagawa, H., Hashimoto, K., & Nakamura, R. (2015). From chemolithoautotrophs to electrolithoautotrophs: CO2 fixation by Fe (II)-oxidizing bacteria coupled with direct uptake of electrons from solid electron sources. *Frontiers in Microbiology, 6,* 994.

Keenleyside, W. (2019). Microbiology: Canadian Edition.

Kim, B. H., & Gadd, G. M. (2008). *Prokaryotic metabolism and physiology*. Cambridge University Press.

Kim, B. H., & Geoffrey, G. M. (2008). *Bacterial physiology and metabolism.* Cambridge University Press.

Park, S. W., Hwang, E. H., Park, H., Kim, J. A., Heo, J., Lee, K. H., et al. (2003). Growth of mycobacteria on carbon monoxide and methanol. *Journal of Bacteriology, 185*(1), 142–147.

Remy, M., Hendrickx, T., & Haarhuis, R. (2016, Sept). Over a decade of experience with the ANAMMOX® reactor start-up and long-term performance. In *Proceedings of the 89th annual water environment federation technical exhibition and conference* (pp. 24–28).

Shively, J. M. (1974). Inclusion bodies of prokaryotes. *Annual Reviews in Microbiology, 28*(1), 167–188.

van Kessel, M. A., Speth, D. R., Albertsen, M., Nielsen, P. H., den Camp, H. J. O., Kartal, B., et al. (2015). Complete nitrification by a single microorganism. *Nature, 528*(7583), 555–559.

Whittaker, M., Bergmann, D., Arciero, D., & Hooper, A. B. (2000). Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium Nitrosomonas europaea. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1459*(2–3), 346–355.

Yamanaka, T., & Shinra, M. (1974). Cytochrome c-552 and cytochrome c-554 derived from Nitrosomonas europaea. *The Journal of Biochemistry, 75*(6), 1265–1273.

Yin, Z., Bi, X., & Xu, C. (2018). Ammonia-oxidizing archaea (AOA) play with ammonia-oxidizing bacteria (AOB) in nitrogen removal from wastewater. *Archaea*, *2018.*

Part VII
Nitrogen Metabolism

Chapter 16
Nitrogen Assimilation and Dissimilation

Rani Gupta and Namita Gupta

1 Nitrogen Cycle and Nitrogen Assimilation: An Overview

Nitrogen is an important element of Earth's atmosphere and exists in nature in a variety of forms viz. nitrogen gas (N_2), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), nitric oxide (NO) or organic nitrogen. Nitrogen cycle transforms nitrogen from one form to another through various biological and physical processes. The major steps involved in the cycling of nitrogen are nitrogen fixation, ammonification, nitrification, denitrification and anammox (Table 1 and Fig. 1).

Atmospheric molecular nitrogen (N_2) can be fixed by few microorganisms. The conversion of N_2 to ammonia (NH_3) is referred to as nitrogen fixation. This can be done by either free living, non-symbiotic bacteria such as *Azotobacter, Clostridium* or symbiotic bacteria such as *Rhizobium* in association with legumes. The enzyme catalysing nitrogen fixation is nitrogenase.

Complex organic nitrogen consisting of proteins, nucleic acids and amino sugars are present in the soil in the form of plant and animal wastes. These cannot be used by plants as nutrients directly and thus proteins are converted to smaller peptides by proteinases and further to amino acids by peptidases. These amino acids are then broken down either by decarboxylation or deamination and finally ammonia is released. This process is called ammonification. It can be carried out by various bacteria such as *Clostridium, Proteus*, *Pseudomonas* and *Bacillus.*

Ammonia is converted to nitrate in a two step process,wherein ammonia is first oxidised to nitrite and then to ammonia by ammonia oxidizing bacteria such as *Nitrosomonas, Nitrosococcus, Nitrosovibrio* followed by nitrite-oxidizing bacteria such as *Nitrobacter* and *Nitrospira.* The whole process is called nitrification.

The cycle is completed by conversion of nitrates to free molecular nitrogen by the process of **denitrification**. Nitrate is converted to nitrite followed by nitric oxide, nitrous oxide and finally molecular nitrogen. This can be carried out by *Pseudomonas,*

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_16

Table 1 Major steps involved in nitrogen cycle

Process	Reaction	Micro-organisms involved
Nitrogen fixation	$N_2 + 8H^+ \rightarrow 2NH_3 + H_2$	Free-living (*Azotobacter, Clostridium*) Symbiotic (*Rhizobium*)
Ammonification	Organic N $\rightarrow$ NH_4^+	*Clostridium, Proteus, Pseudomonas*, *Bacillus*
Nitrification	$NH_4^+ \rightarrow NO_2^-$ $NH_4^+ \rightarrow NO_3^-$	*Nitrosomonas, Nitrosococcus* *Nitrobacter, Nitrospina*
Denitrification	$NO_3^- \rightarrow N_2$	*Pseudomonas, Clostridium, Alcaligenes*
Anammox	NO_2^- /$NH_3 \rightarrow 2\ N_2$	*Brocadia, Anammoxoglobus*

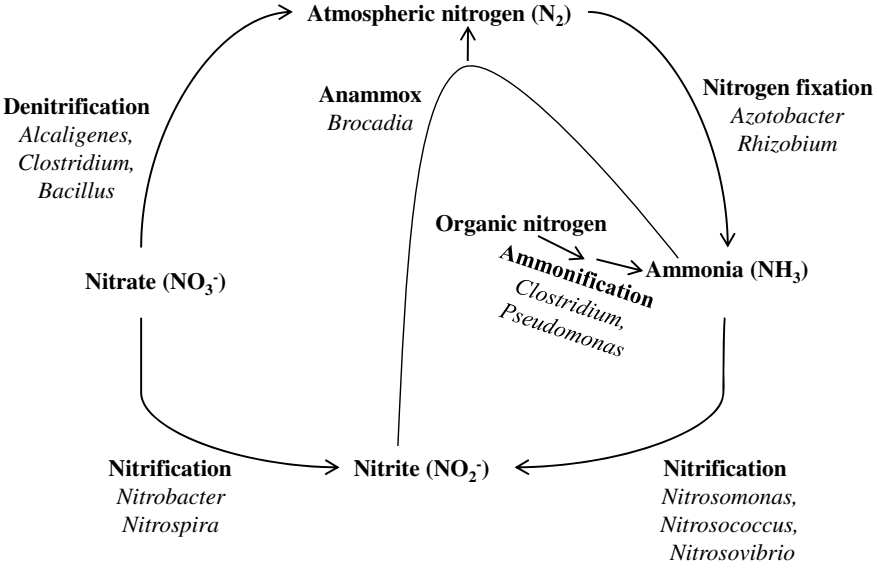


Fig. 1 Simplified nitrogen cycle

Clostridium, Alcaligenes. It can be considered as an undesirable process as it leads to loss of fixed nitrogen from the soil and hence decline in nutrients for plant growth.

Anammox or anaerobic ammonium oxidation is the process of conversion of nitrite and ammonium to molecular nitrogen. It can be carried out by members of phylum Planctomycetes e.g. *Candidatus* Brocadia anammoxidans, *Candidatus* Annamoxoglobus. It is a major step of nitrogen conversion in oceans.

Nitrogen Assimilation

Nitrogen is an important constituent of bacterial growth medium and may be provided in the form of proteins, protein hydrolysates, urea, nitrates or ammonium compound.

The proteins and protein hydrolysates are broken down to small oligo-peptides by extracellular and cell bound proteases. The peptides are transported inside the bacterial cell cytoplasm by specific transporters. These peptides are either utilized as such or broken down further to amino acids which are incorporated into proteins. On the other hand, inorganic nitrogen sources are mostly utilized in the form of ammonium ions and incorporated into keto acids to form amino acids. Urea is acted upon by urease to form ammonia and carbon dioxide while nitrates are reduced to ammonium ions by assimilatory nitrate reductases. Thus, nitrogen is utilized in the form of ammonium ions to form amino acids or directly as amino acids obtained by breakdown of proteins (Fig. 2). Thus, micro-organisms can use both inorganic and organic form of nitrogen by virtue of presence of both assimilatory and dissimilatory enzymes (Table 2).

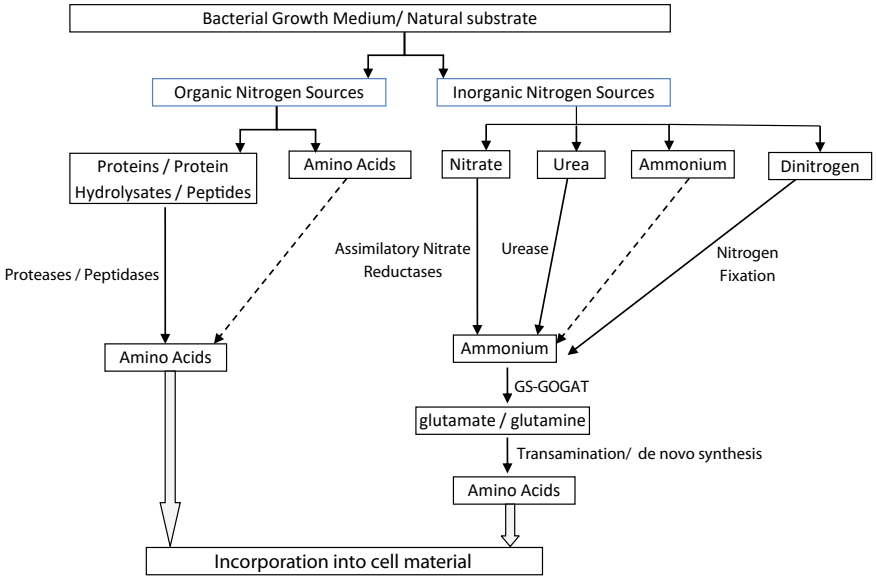


Fig. 2 Nitrogen assimilation into cellular material

Table 2 Assimilation of different nitrogen sources and enzymes involved

Nitrogen source	Enzyme /Type of reaction	Fate
NO_3^-	Nitrate reductase	NH_4^+
NH_4^+	Glutamate dehydrogenase, reductive amination	Glutamate
Amino acids	Transaminases	Nitrogen transferred to keto acids for other amino acids
Proteins	Proteases	Peptides assimilated
Urea	Urease	NH_3 assimilated

Bacteria can assimilate inorganic nitrogen NO_3^-, NH_4^+ and urea to complex nitrogen compounds, amino acids and other derivatives. The proteins and amino acids are also utilized as a part of protein turn-over such as shifting from vegetative to sporulating phase and vice versa, besides being directly utilized as nitrogen sources. Therefore, nitrogen metabolism of micro-organisms can be divided under the following headings:

- Assimilation/utilization of inorganic nitrogen NO_3^-, NH_4^+ and urea
- De novo biosynthesis of amino acids
- Utilization of amino acids both as carbon and nitrogen source

Box 1: Essential Amino Acids

- Amino acids which are not synthesized de novo by the organism and thus obtained from diet are called essential amino acids.
- The following nine amino acids are essential in human.
  - Phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine (i.e. F V T W M L I K H)

Non-protein amino acids

- These amino acids, although never found in proteins, perform several important biological functions.
- Classified as alpha and non-alpha amino acids:

Alpha amino acids	Non-alpha amino acids
Ornithine	Beta-alanine
Citruline	Beta-aminoisobutyric acid
Arginosuccinic acid	Gamma-aminobutyric acid (GABA)
Thyroxine	Aminolevulinic acid (ALA)
Triodothyroxine	Taurine
S-Adenosylmethionine	4-aminobenzoic acid (PABA)
Homocysteine	
3,4-Dihydroxy phenylalanine (DOPA)	
Creatinine	
Ovathiol	
Azaserine	

2 Assimilation/Utilization of Inorganic Nitrogen NO_3^- and NH_4^+

***Nitrate assimilation*—**Nitrate is the preferential source of nitrogen for fungi and yeasts while bacteria prefer NH_4^+. Nitrate ions are utilized by their reduction to NH_4^+ by the nitrate reductases.

$$NO_3^- \rightarrow NH_4^+$$

The ammonia thus produced is then assimilated in the form of glutamate or glutamine which will be studied later. These are called assimilatory nitrate reductases that are independent of oxygen.

Another group of enzymes are the dissimilatory nitrate reductases that are more common in bacteria. They are inducible enzymes that participate in nitrate respiration using NO_3^- as terminal electron acceptor. The end product is again NH_4^+. However, both these routes have a very distinct regulation. The assimilatory reduction is independent of oxygen but is highly sensitive to ammonium availability while the respiratory reduction is repressed in oxygen but insensitive to the availability of ammonia or any other nitrogen source (Table 3). Dissimilatory nitrate reduction takes place under strict anaerobic conditions, where sufficient reductant is available. It is preferred over denitrification as it is a way to optimally use available oxidant (NO_3^-) to generate NAD^+ to sustain growth. Electrons required for reduction to NH_4^+ are six as against just two or three electrons required in denitrification of nitrite to N_2O/N_2 (Mohan & Cole 2007).

Denitrification also uses dissimilatory nitrate reductases wherein the following reaction takes place:

$$NO_3^- \rightarrow N_2$$

Some common examples of denitrifiers are bacterial species belonging to *Pseudomonas, Clostridium* and *Alcaligenes.*

Ammonia assimilation—This is the simplest assimilatory form of nitrogen used by bacteria. Ammonia is fixed in different amino acids during their biosynthesis as bacteria synthesize all twenty amino acids, besides certain non-protein amino acids. The carbon skeleton of amino acids is obtained from glycolysis and TCA cycle intermediates as all amino acids are aminated keto acids where α-carbon has

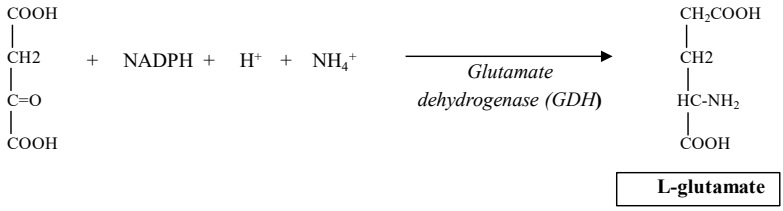
Table 3 Comparison between assimilatory and dissimilatory nitrate reductases

Assimilatory nitrate reductases	Dissimilatory nitrate reductases
Independent of oxygen	Hypoxia activated
NO_3^- independent	NO_3^-/NO_2^- induced
NH_3 repressed	NH_3 independent

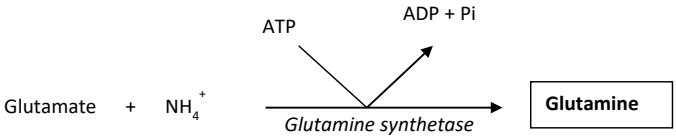
both carboxyl and amino group. The first amino acid synthesized during growth on inorganic nitrogen is glutamate and glutamine. These amino acids then serve as nitrogen source in the cell for synthesis of various biomolecules such as nucleic acids, co-enzymes, amino sugars and amino acids.

Glutamate and Glutamine Biosynthesis—Glutamate is the first amino acid to be synthesized by reductive amination of α-ketoglutarate by glutamate dehydrogenase during growth of bacterium on inorganic ammonium compounds. The enzyme uses NADPH as reductant in *E. coli*, however in some organisms, both NAD^+/NADH and $NADP^+$/NADPH dependent glutamate dehydrogenase exist. Further, it is important to know that this enzyme has higher K_m for NH_4^+ (1.1 mM), so excess availability of NH_4^+ salts is important. However, when ammonium concentrations are low, glutamate is converted to glutamine by glutamine synthetase in presence of ATP since it has lower K_m for NH_4^+ (0.2 mM).

1. **Reductive amination of α-ketoglutarate by glutamate dehydrogenase (High concentration of NH_4^+)**

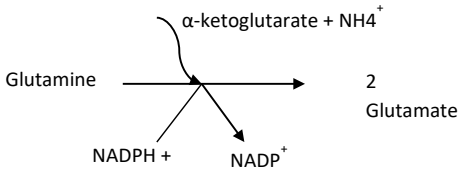


2. **Conversion of NH_4^+ to amide group of glutamine by GS-GOGAT pathway (Low concentration of NH_4^+which is mostly found within cell cytoplasm)**



3. **Amination of α-ketoglutarate by amide group of L-glutamine by glutamine:2- oxoglutarate amidotransferase (GOGAT) or glutamate synthase**

When low concentration of NH_4^+ is present, besides the reaction by glutamine synthetase, the amination of α-ketoglutarate is also catalyzed by glutamate synthase enzyme. The reductant in bacteria is NADPH + H^+ while in fungi it may also be NADH + H^+.



Regulation of glutamine synthetase

Glutamine synthetase (GS) is a key enzyme which regulates the flow of nitrogen and is tightly regulated by the availability of nitrogen. The regulation of GS in *E. coli* is well studied and it has been shown by Stadtman & Ginsberg (1974) that GS is composed of twelve identical subunits of 50,000 molecular weight and the subunits form a dimer of hexameric rings. The enzyme shows feedback inhibition by several nitrogenous compounds. It is also regulated by covalent adenylation and deadenylation of subunits at tyrosine group of each of the twelve subunits. As the adenylation progresses, the enzyme accordingly slows down with complete inhibition when all twelve subunits are adenylated (Jiang et al., 1998; Stadtman 2004).

When nitrogen is in excess as signaled by high glutamine to α-ketoglutarate ratio, the enzyme is adenylated. Further, upon nitrogen limitation as sensed by high α-ketoglutarate or high α-ketoglutarate: glutamine ratio, the enzyme is deadenylated or activated. Both of these reactions involve a cascade of regulatory interactions involving other regulatory proteins. Thus, GS has 12 sites for AMP molecules and on the basis of nitrogen concentration (low or high) AMP molecules are removed or added, respectively, one by one by repeated reactions of deadenylation or adenylation and all 12 AMPs are not removed from GS in one go. The cycle is repeated and fully active GS is obtained on complete deadenylation if low nitrogen persists for a long time.

This regulation involves two main enzymes: uridylyltransferase (UTase) and adenylyltransferase (ATase) along with a regulatory protein PII. PII is generally uridylated and exists as PII-UMP. PII can be uridylated or deuridylated with 4 UMP. Uridylyltransferase has both uridylating and deuridylating activities. Further, adenylyltransferase enzyme interacts with PII or PII-4UMP and acquires adenylation activity or deadenylation activity, respectively. Adenylyltransferase also has a binding site for glutamine synthetase. Depending on the nitrogen condition, adenylyl transferase adenylates or deadenylates glutamine synthetase thereby rendering it inactive or active, respectively (Fig. 3).

Regulation during high nitrogen or high glutamine: α-ketoglutarate ratio: During high nitrogen concentration, uridylyl removing enzyme removes UMP from PII. Adenylyltransferase then binds to PII and transfers an adenyl group (from ATP) to glutamine synthase. Thus, glutamine synthase becomes adenylated and gets inactivated.

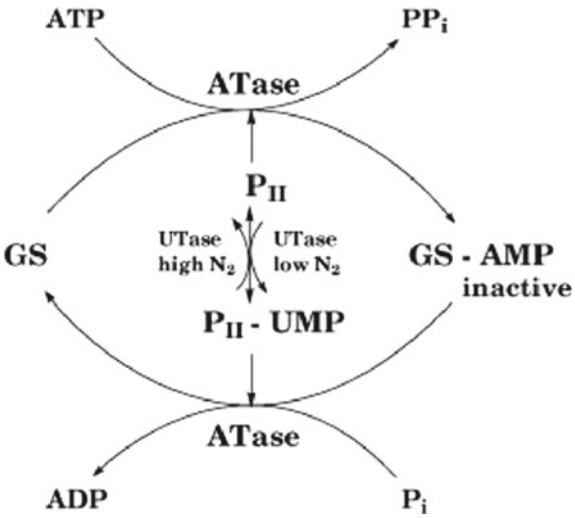


Fig. 3 Glutamine synthetase regulation depending on nitrogen concentration *Source* Wikipedia (Creative Commons Attribution license)

Regulation during low nitrogen concentration: On the contrary, at low nitrogen concentrations or at high α-ketoglutarate:glutamine ratio, uridylyltransferase becomes active and it uridylates PII to PII-4UMP. Now, adenylyl transferase enzyme combines with PII-4UMP and deadenylates glutamine synthase.

Inhibition of Glutamine synthetase (GS): Methionine sulfoximine (MSX), Phosphorus containing analogues of glutamic acid, bisphosphonates and several other inhibitors have been developed to inhibit GS activity. These have been proven to be effective against tuberculosis with high selectivity and also against several other pathogenic bacteria. It was also shown that GS inhibitors are also useful in cancer therapy (Eisenberg et al., 2000, Berlicki 2008).

3 De Novo Biosynthesis of Amino Acids

Bacteria are unique since they can synthesize all twenty amino acids. However, higher eukaryotes have biosynthetic pathways for selected amino acids. These amino acids that have to be supplemented via diet are known as essential amino acids such as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Bacterial mutants which require incorporation of amino acids in medium are called auxotrophic mutants.

Amino acids are carboxylated α-amines and can be derived by direct reductive amination of keto group or by transamination from an amino acid. All the amino acids are synthesized as a branch point from a few intermediates in the central metabolic pathways, glycolysis, pentose phosphate pathway and TCA cycle. Hence, the carbon

skeleton for all the amino acids is provided by carbon metabolic pathways. Glucose provides the carbon intermediates and ammonium serves as nitrogen for synthesis of all the twenty amino acids in bacteria. On the basis of various intermediate precursors of amino acids, the various biosynthetic pathways can be divided into six different families (Fig. 4).

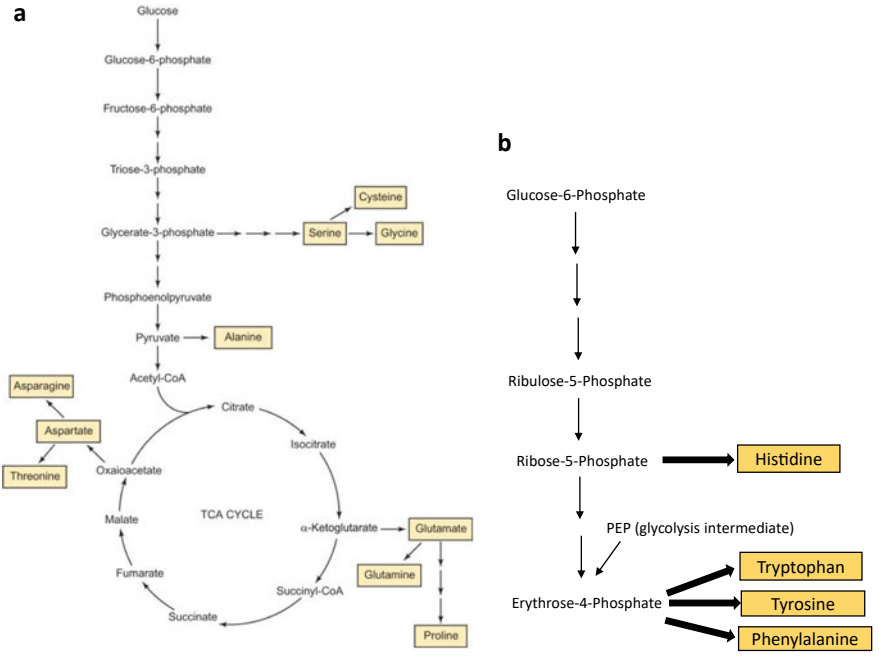


Fig. 4 **a** Precursors from glycolysis pathway and Citric acid cycle used for de novo biosynthesis of amino acids *Source* Litwack (2018). With kind permission from Elsevier A. **b** Precursors from pentose phosphate pathway used for de novo biosynthesis of amino acids

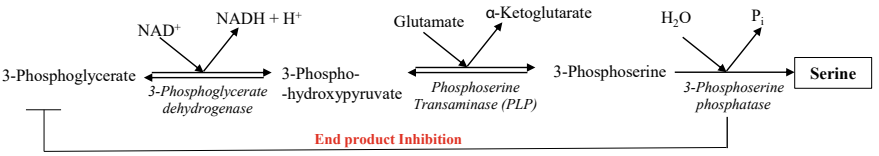


Fig. 5 Biosynthesis of serine from 3-phosphoglycerate

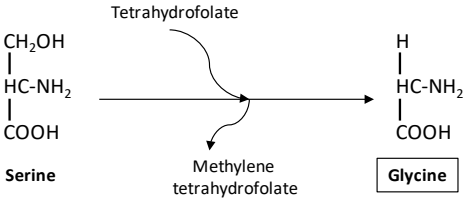
A. Amino Acids Synthesized using Precursors from Glycolytic Pathway

1. Biosynthesis of serine family amino acids: L-serine, L-glycine and L-cysteine

Serine family amino acids are serine, glycine and cysteine for which the carbon skeleton is 3-phosphoglycerate from the glycolytic pathway. These amino acids are not only required for protein synthesis but also serve several other functions. Serine to glycine conversion serves as one-carbon donor in many reactions used in purine thymine and methionine synthesis (Box 2). Serine is directly incorporated in phospholipids and tryptophan. Sulphur in the form of sulphide is first added to cysteine from where it is transferred to methionine and other sulphur containing compounds.

Conversion of 3-phosphoglycerate to serine—Serine is synthesized in three steps, oxidation by NAD linked dehydrogenase followed by transamination by phosphor serine-glutamate transaminase and dephosphorylation of phosphoserine by phosphatase yielding serine (Fig. 5). The NAD linked dehydrogenase undergoes end product inhibition by serine. This phosphorylated pathway is common to most microorganisms and plants. However, a reversible non-phosphorylated synthesis from glycine is reported in some microorganisms as a part of catabolic reactions.

Conversion of serine to glycine—Conversion of serine to glycine requires a complex single step reaction catalyzed by serine hydroxyl methyl transferase. The reaction yields formaldehyde which is transferred to tetrahydrofolate factor. This reaction is hence important for supply of one carbon moiety to other biochemical reactions.

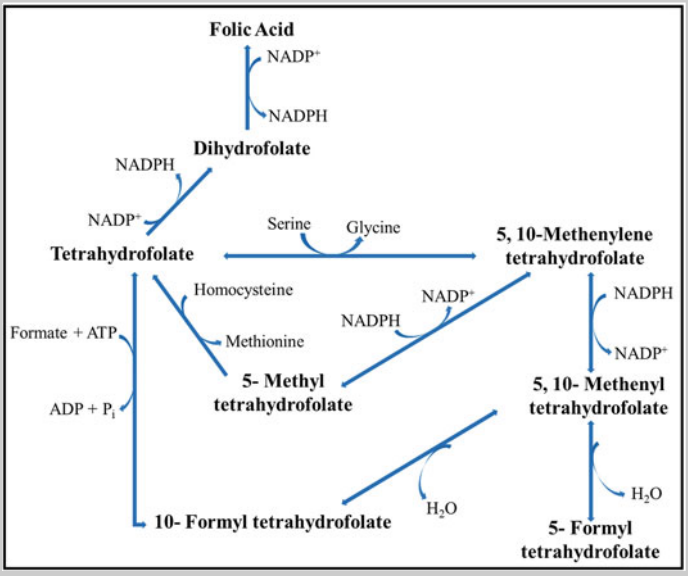


Box 2: One-Carbon Transfer

A number of biochemical pathways require transfer of a single carbon moiety mediated by a special set of molecules. The reactions mediated by these molecules are collectively termed as 'one-carbon metabolism' because the common mechanism between them is the transfer of one-carbon group. Existence of such specialized molecules specifically for transfer of one-carbon group is important due to highly volatile nature of these groups. They need to be attached to something for stability and are transferred in a conjugated form. There are three fundamental compounds to which these volatile one carbon atom groups are conjugated for mediating transfer: Tetrahydrofolate (THF),

S-adenosylmethionine (SAM) and Cobalamin (Vitamin B12). Of these, THF acts as a cofactor in enzymatic reactions, SAM is the principal methyl donor and cobalamin mediates methylation and rearrangement reactions.

Tetrahydrofolate—It is the most versatile of the one-carbon donors during various pathways and is in fact the reduced form of vitamin folic acid (folate). Chemically, folic acid is pteroyl-glutamic acid made of pteridine nucleus, p-amino benzoic acid (PABA) and glutamic acid. Microorganisms and plants can synthesize folate using PABA but humans obtain it from dietary sources as they are not capable of synthesizing it. Folate is further converted to THF by the enzyme Dihydrofolate reductase (DHFR). In cell, THF exists in three oxidation states with each state mediating certain biochemical reactions. These states are shown **below** and their occurrence overall allow THF to carry a variety of 1-carbon groups. Different oxidation states of THF act as important coenzymes functioning in different pathways to carry one-carbon moiety.



Oxidation states of Tetrahydrofolate

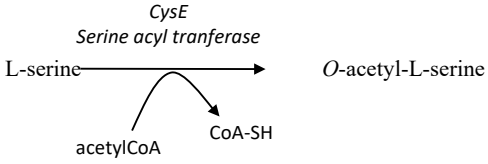
Amino acids are the ultimate initial donors of one carbon moiety; these include serine, glycine, histidine and tryptophan. Of these, mostly serine donates C1 unit to THF. This C1 unit is donated as formaldehyde to THF, converting it to intermediate 5,10-methylene THF and is itself converted to glycine. This 5,10-methylene THF is utilized in pyrimidine biosynthesis where it donates methyl group to dUMP (deoxyuridylate monophosphate) converting it to Thymidylate monophosphate (TMP). After donating methyl group to

dUMP, it is consequently converted to Dihydrofolate which is reconverted to THF by DHFR. 5,10-methylene THF is also oxidized to 5,10-methenyl THF which is further oxidized 10-formyl THF parallelly generating reductants. These highly oxidized states of THF are utilized in purine biosynthesis and formyl THF is also required in synthesizing formyl methionine tRNA during protein synthesis in prokaryotes and mitochondria. 5,10-methylene THF is further reduced to 5-methyl THF by the enzyme Methylene Tetrahydrofolate reductase (MTHFR) utilizing NADPH. This 5-methyl THF is utilized in converting homocysteine to methionine.

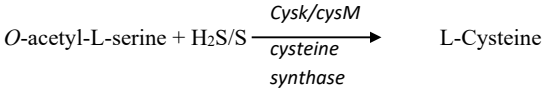
S-adenosylmethionine—SAM is synthesized from essential amino acid methionine and the reaction is catalyzed by methionine adenosyltransferase utilizing three high-energy phosphate bonds. SAM is particularly important because it is capable of donating active methyl (CH_3) group to a wide range of acceptors including phospholipids and nucleic acids. SAM is utilized in the synthesis of cysteine, however bacteria can also synthesize cysteine directly from serine.

Cobalamin—Vitamin B12 or cobalamin is a very essential component required as a cofactor in certain biochemical reactions. It consists of a central corrin ring containing a cobalt atom, an adenosyl moiety and a nucleotide tip. Cobalamin functions in one-carbon transfer due to various oxidation states of central cobalt atom and its ability to form a cobalt-carbon linkage. It is required as a cofactor for a highly conserved enzyme methionine synthetase. This enzyme catalyzes transfer of a methyl group from methyltetrahydrofolate to homocysteine in the final step during methionine synthesis.

Conversion of serine to cysteine—Cysteine is synthesized by a two-step pathway from L-serine involving two enzymes, serine acyl transferase (*cysE*) and cysteine synthase (cys*K*/cys*M*). Serine acetyltransferase (*cysE*) carries out the first step of cysteine biosynthesis (Kredich & Tomkins, 1966) and it is inhibited by cysteine.



Cysteine synthase B (cys*K*/cys*M*) catalyzes formation of L-cysteine from *O*-acetyl-L-serine and sulfide.



There are two isozymes and both can do reverse reaction to release sulfur from cysteine. Cysteine synthase B can use thiosulphate as well. The enzyme is also called *O*-acetyl-serine sulhydrylase. For cysteine biosynthesis, sulfide is provided to the cell through a number of reactions catalyzed by different enzymes during sulfate reduction to S/H_2S (Fig. 6). The reactions are as follows:

i. SO_4^{-2} is transported intracellularly using ABC transporter (*cysZ*)*
ii. Activation of SO_4^{-2} (present in the medium) by ATP to Adenosine-5-pyrophosphosulphate (APS) by ATP sulfurylase
iii. Further activation required in *E. coli* and certain other bacteria is 3'phosphorylation by APS kinase to 3-phosphoadenosine-5-pyrophosphosulphate (PAPS)
iv. Reduction of sulphonyl moiety of APS/PAPS to sulphite occurs by reduced thioredoxin/glutathione and results in SO_3^{2-} and oxidized acceptor
v. SO_3^{2-} is reduced to S^{-2} by sulphite reductase having both heme and FeS center.

**In prokaryotes, different types of transport proteins are involved in uptake of sulfate intracellularly. These include sulfate permeases (CysZ), ABC sulfate transporter complex (CysTWA/SulT), sulfate and proton/sodium symporter (SulP), sulfate and solute exchangers and the phosphate transporters (CysP).*

2. Biosynthesis of Aromatic Family of Amino acids: L-tryptophan, L-tyrosine and L-phenylalanine

The aromatic amino acids are all synthesized by a common pathway called shikimate pathway. The pathway is a key to supply intermediates for the synthesis of vitamins E and K, folic acid, ubiquinone and plastoquinones and also certain siderophores viz. enterochelin. The branch point is at chorismate and that is why aromatic amino acid pathway is also called chorismate pathway. This pathway can be studied in two steps:

1. Synthesis of chorismate
2. Branching of chorismate to individual amino acids, phenylalanine, tyrosine and tryptophan.

Synthesis of chorismate: Synthesis of chorismate (Fig. 7) involves a series of steps . The steps are described below:

1. It begins with condensation of pyruvate enol phosphate (PEP) and erythrose-4-phosphate, intermediates of glycolysis and pentose phosphate pathways respectively, to form 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAHP) catalyzed by the enzyme DAHP synthase. This enzyme occurs in three isoforms *aroF*, *aroH*, and *aroG*, each specific for tyrosine, tryptophan and phenylalanine, respectively. Each isoform is subjected to feedback inhibition by particular amino acid.
2. Next step is the formation of 3-Dehyroquinate catalyzed by 3-Dehyroquinate synthase (*aroB*).

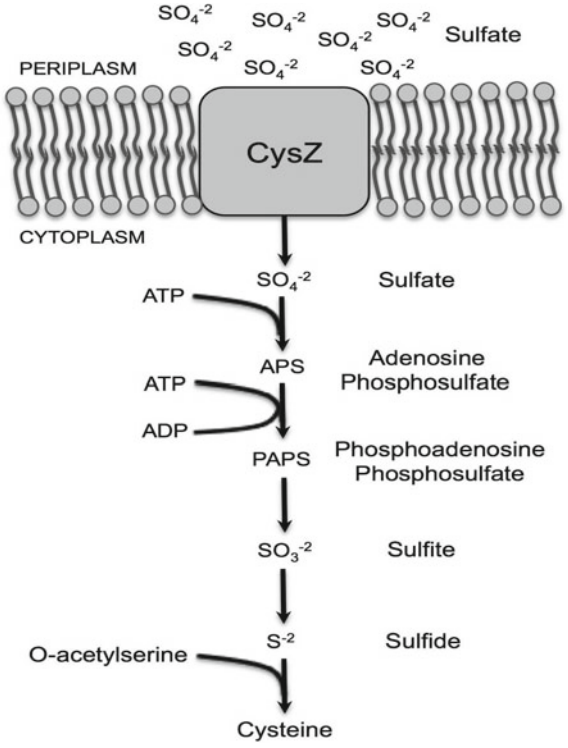


Fig. 6 Source of sulfur for cysteine synthesis *Source* Sanghai et al. (2018). (Creative Commons Attribution license)

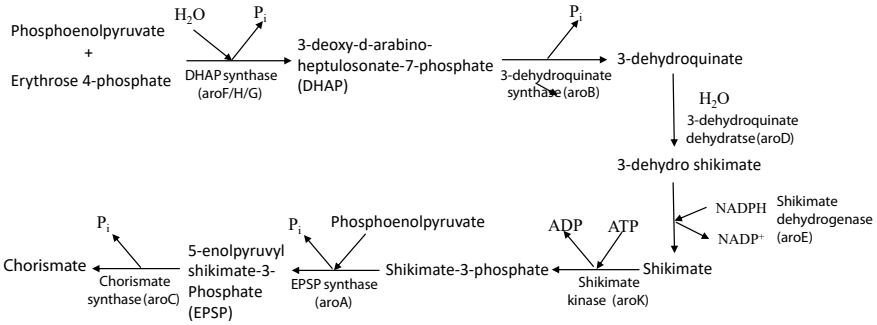


Fig. 7 Shikimate pathway for synthesis of chorismate, source for aromatic amino acid synthesis

3. 3-Dehyroquinate is then converted to 3-Dehyroshikimate by 3-Dehyroquinate dehydratase (*aroD)*
4. 3-Dehyroshikimate is converted to shikimate by the enzyme shikimate dehydrogenase (*aroE).*
5. Then Shikimate-3-phosphate is formed from shikimate catalyzed by shikimate kinase (*aroK* or *aroL*).
6. Shikimate-3-phosphate combines with PEP to form 5-enolpyruvyl-shikimate-3-phosphate (EPSP) catalyzed by EPSP synthase (*aroA*).
7. Finally chorismate is formed from EPSP which is catalyzed by the enzyme chorismate synthase (*aroC*).

Chorismate to phenylalanine and tyrosine: Chorismate is then used as a precursor for synthesis of the aromatic amino acids tryptophan, tyrosine and phenylalanine. First chorismate gets isomerized by a mutase to prephenate which branches to two amino acids—by dehydratase and transamination to phenylalanine and through dehydrogenation and transamination to tyrosine (Fig. 8).

Chorismate to Tryptophan: The tryptophan biosynthetic pathway is the most studied. The arrangement of genes and the distribution of enzyme activities per protein may vary among different bacteria but the overall pathway is same. Tryptophan synthesis also branches from chorismate but it is a different branch than that of phenylalanine and tyrosine. The first specific reaction is glutamate dependent conversion of chorismate to anthranilate. The amination of chorismate may also happen in presence of high concentration of ammonia. All anthranilate synthases have two catalytic components—glutamate amidotransferase activity and chorismate to anthranilate activity.

The second step is transfer of ribose phosphate from phosphoribosyl-1-pyrophosphate (PRPP) by anthranilate phosphoribosyl transferase to form 5-phosphoribosyl-1-anthranilate. Subsequently, phophoribosyl anthranilate isomerase converts ribosyl to ribulosyl form and the product is finally cyclized to indole glycerol phosphate by indole glycerol phosphate synthase. Final reaction is catalyzed by tryptophan synthase by removal of glyceraldehyde-3-phosphate and the enzyme bound indole gets condensed with serine (Fig. 9).

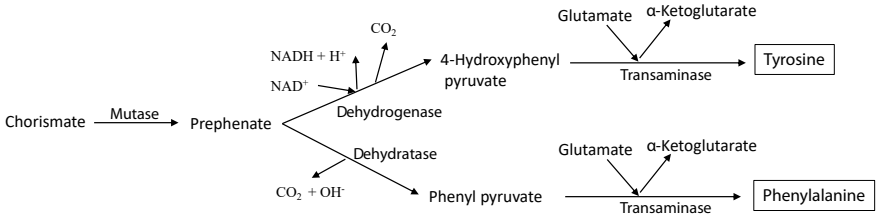


Fig. 8 Chorismate pathway for synthesis of tyrosine and phenylalanine

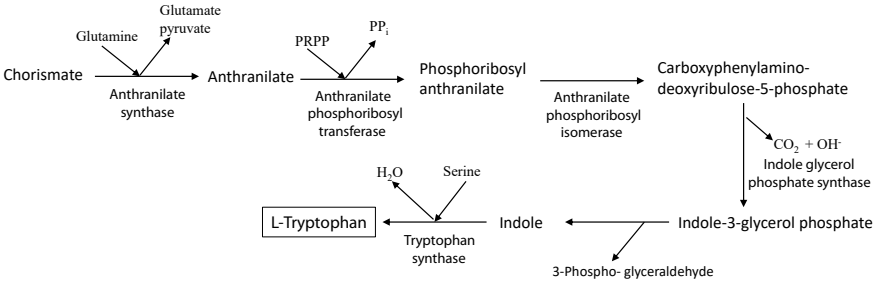


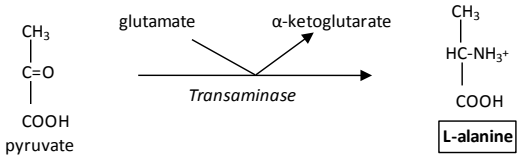
Fig. 9 Synthesis of tryptophan from Chorismate

Thus, five enzyme activities are required for tryptophan synthesis from chorismate:

1. Anthranilate synthase
2. Anthranilate phosphoribosyl transferase
3. Anthranilate phosphoribosyl isomerase
4. Indole glycerol phosphate synthase
5. Tryptophan synthase.

3. Biosynthesis of Amino acids of Pyruvate Family: L-alanine, L-valine, L-leucine and L-isoleucine

L-alanine is synthesized by simple transamination of pyruvate by glutamate. The alanine pool in a cell is always sufficient unless nitrogen supply is limited. Since the transaminations are reversible reactions, it does not affect the pyruvate pool.



L-valine is synthesized using two molecules of pyruvate which undergo a series of reaction to finally form valine. Valine biosynthesis pathway shares four enzymes with isoleucine biosynthetic pathway which is also synthesized from pyruvate in combination with 2-oxobutanoate. This came into light from the study of *Neurospora* and *E. coli* mutants which were altered in single gene but required both isoleucine and valine. The observation was contradictory to one gene-one enzyme hypothesis. Later studies indicated that a single enzyme catalyzed reaction of both the pathways. 2-oxobutanoate for synthesis of isoleucine is provided by threonine following a series of steps. L-leucine is formed by methyl addition to α-ketoglutarate from acetyl CoA followed by transamination by glutamate (Fig. 10).

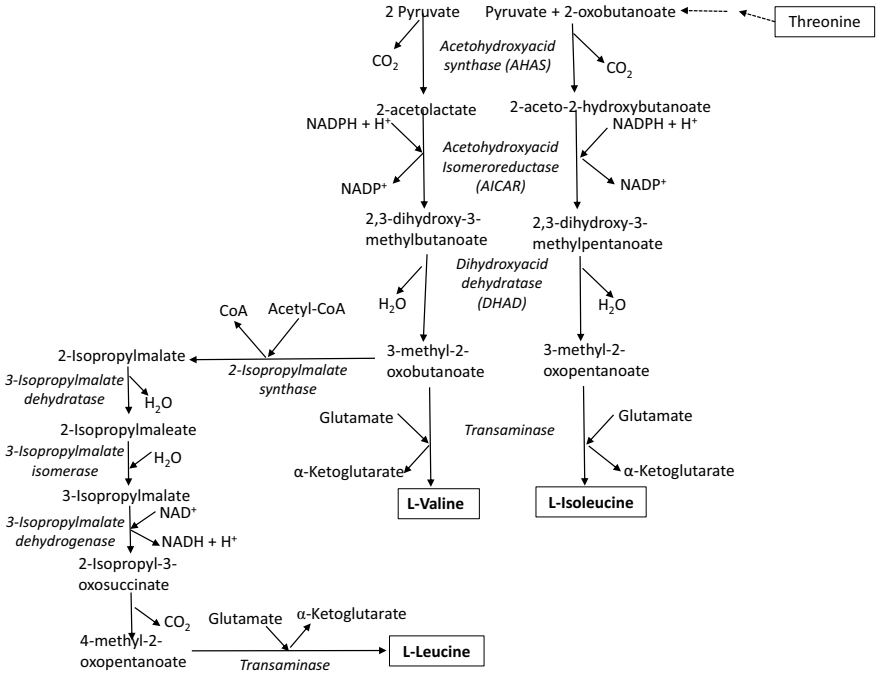


Fig. 10 Synthesis of valine, leucine and isoleucine from pyruvate

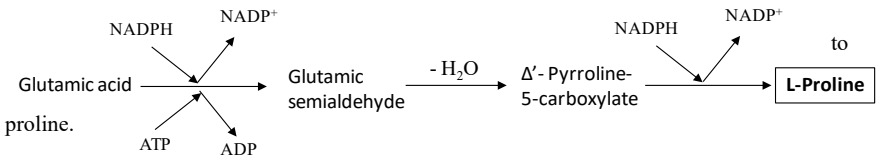
B. Amino Acids Synthesized using Precursors from Citric Acid Cycle

1. Biosynthesis of Glutamate Family: L-glutamate, L-glutamine, L-proline, L-arginine

All the amino acids of the glutamate family are synthesized from glutamate such as glutamine, proline and arginine. However, in fungi lysine is also a part of this family as the precursor for lysine synthesis is α-ketoglutarate.

Glutamate and Glutamine Biosynthesis: This has already been discussed under ammonia assimilation.

Synthesis of proline from glutamate: Three reactions namely activation (kinase), reduction of γ-carboxyl group followed by spontaneous cyclization leads to Δ^2-pyrroline-5-carboxylate which reduces to proline.



Synthesis of arginine from glutamate: This also requires activation and reduction of γ-carboxylate. However, in this reaction the α-carbon is first protected by acetylation thereby preventing ring closure and then synthesis occurs via L-ornithine. Three amino groups are added from transamination by glutamate, transcarbamylase by carbamoyl phosphate and Mg^{2+} dependent synthase using aspartate (Fig. 11)

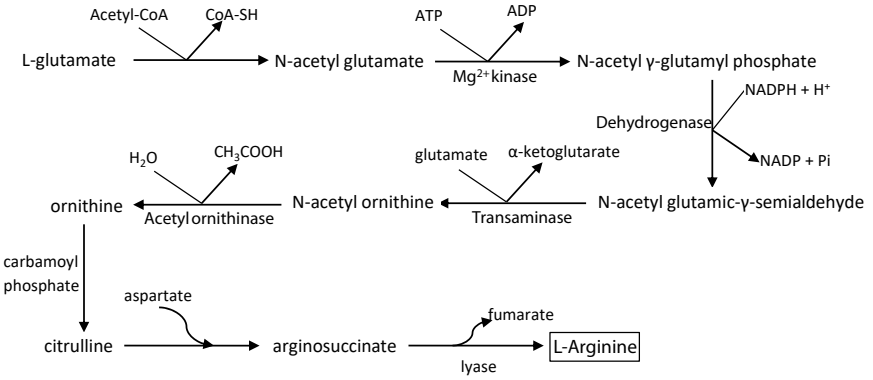


Fig. 11 Biosynthesis of arginine from glutamate

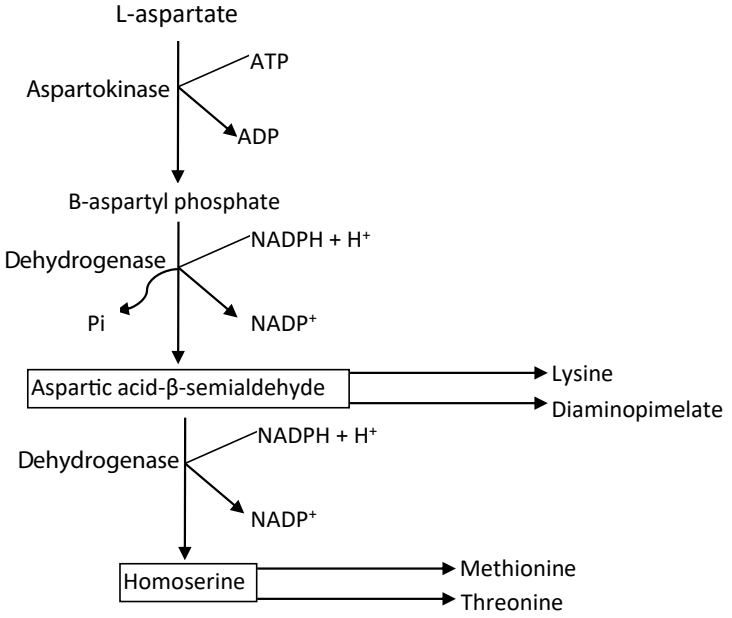
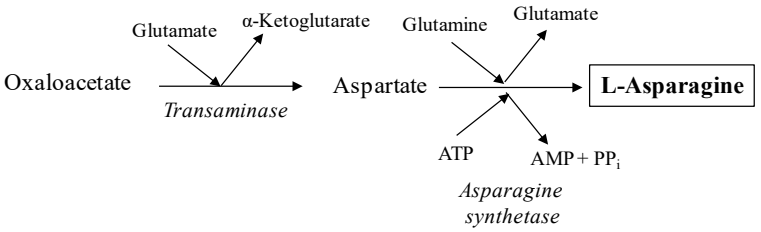


Fig. 12 Synthesis of aspartic acid-β-semialdehyde and L-homoserine from aspartate for synthesis of lysine, methionine and threonine respectively

2. Biosynthesis of Aspartate Family: L-aspartate, L-asparagine, L-methionine, L-threonine, and L-lysine

Aspartate and asparagine biosynthesis: Aspartate family amino acids are formed from oxaloacetate and aspartic acid is the first amino acid formed by transamination reaction with glutamate as amino donor. The enzyme in *E. coli* is aspartate-glutamate transaminase. Asparagine synthesis is an ATP dependent transfer of amide group from glutamate to β-carboxyl of aspartate by enzyme asparagine synthase. The reaction is similar to glutamate and glutamine synthesis from α-ketoglutarate to glutamate to glutamine. However, the main difference is that this reaction involves β-aspartyl adenylation while glutamine synthesis has γ-glutamyl enzyme complex.



Biosynthesis of L-lysine, L-methionine and L-threonine: L-aspartate is converted to these three amino acids by reduction of β-carboxylate group. The reduction of this group first requires activation of L-aspartate to β-aspartyl phosphate by an enzyme aspartokinase. This is then reduced to L-aspartic acid-β-semialdehyde. This is the branch point for lysine biosynthesis in bacteria. There is another reduction of L-aspartic acid-β-semialdehyde to hydroxyl by homoserine dehydrogenase forming L-homoserine (Fig. 12) (Pittard & Yang, 2008).

Regulation of aspartokinase enzyme: Two kinds of regulations are often observed:

i. One aspartokinase enzyme but multivate regulation i.e. it requires more than one amino acid for its inhibition.

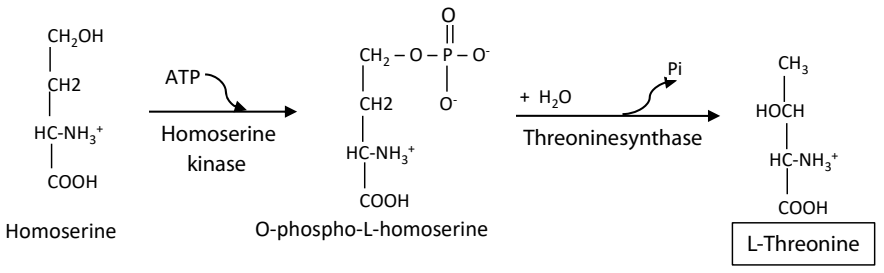


Fig. 13 Biosynthesis of threonine from homoserine

ii. Multiple aspartokinase each inhibited by a specific amino acid as in enteric bacteria. *E. coli* has three aspartokinases, I, II and III inhibited by threonine, methionine and lysine, respectively.

Homoserine to threonine: Threonine is synthesized from homoserine following two steps catalyzed by homoserine kinase and threonine synthase (Fig. 13). As it can be observed, synthesis of threonine would require shift of hydroxyl group to β-position from γ-position. This is facilitated by first activation of γ group by phosphorylation to form o-phospho-L-homoserine. The reaction is inhibited by threonine. This is followed by pyridoxal-dependent threonine synthase dephosphorylation reaction.

Homoserine to Methionine: Methionine is an important amino acid as it is the first amino acid to be incorporated during protein translation. It is considered as a hydrophobic amino acid like other hydrophobic amino acids but presently its role in stabilization of proteins by S/π interactions has been elucidated (Valley et al., 2012). It is also a sulfur containing amino acid like cysteine and is the only amino acid to have thioether linkage. It occurs largely as S-adenosyl methionine (SAM) and carries out intracellular methylations.

In *E. coli*, there are four genes *metA*, *metB*, *metC* and *metE/metH* which carry our methionine biosynthesis from homoserine. Homoserine is derived from aspartate. The key steps in synthesis of methionine require acylation, sulfurylation and methylation of sulfuhydryl groups (Fig. 14) (Ferla & Patrick, 2014).

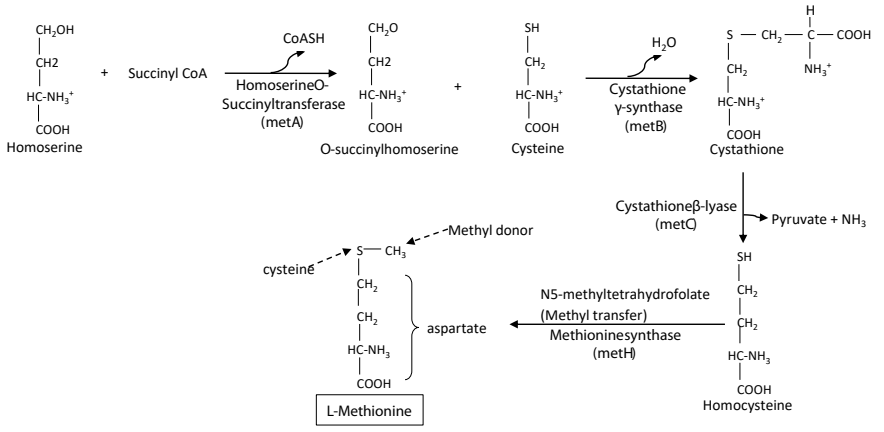


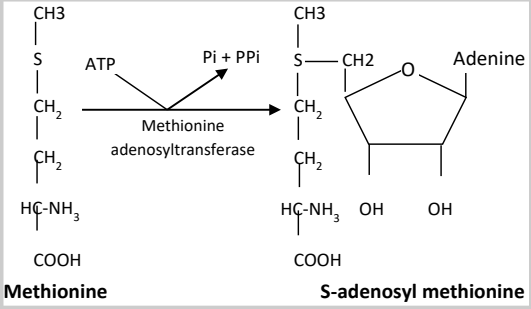
Fig. 14 Methionine biosynthesis from homoserine

i. The acylation moiety in *E. coli* is succinyl CoA and its transfer to γ-hydroxyl group of homoserine is catalyzed by homoserine-O-succinyltransferase (*metA*).
ii. Next step is sulfurylation where cysteine is the sulfur donor and the reaction is catalyzed first by cystathione γ-synthase (*metB*) for cysteine transfer followed by its lysis through action of another enzyme cystathione lyase (*metC*) with release of pyruvate and ammonia (Clausen et al., 1996).
iii. The third and the final step is the synthesis of methionine by transfer of methyl from 5-methyl-THF or 5-methyl-tetrahydropteroyl tri-glutamate catalyzed by methionine synthase (*metE/H*). *metH* is cobalamine dependent and uses 5-methyl-THF and *metE* is cobalamine independent and uses 5-methyl-tertahydropteroyl tri-glutamate (Whitfield et al., 1970).

Activation step of homoserine before sulfurylation has some variations among bacteria. In *Thermotoga maritima*, *Bacillus cereus* and *Agrobacterium tumefaciens*, activation *O*-acetyl group is used for acylation rather than *O*-succinyl group catalyzed by *O*-acetyl transferases (*metX*). In another bacterium *Haliangium ochraceum*, both *metA* and *metX* genes are absent, instead it possesses another gene *thrB*, homoserine kinase which yields *O*-phosphohomoserine by phosphorylation. *O*-phosphohomoserine is also the activated form of homoserine in plants for methionine and threonine biosynthesis.

After homoserine activation, direct sulfurylation has also been observed in many bacteria which involves replacement of *O*-acetyl or *O*-succinyl group from activated homoserine with free hydrogen sulfide, yielding homocysteine in a single step. This step is mediated by two enzymes *O*-acetylhomoserine thiolase and *O*-succinylhomoserine thiolase encoded by genes *metY* and *metZ* respectively. In addition, extremophiles like *Thermus thermophilus*, also carry out direct sulfurylation when they encounter H_2S in environment.

Box 3: Biosynthesis of S-Adenosyl Methionine



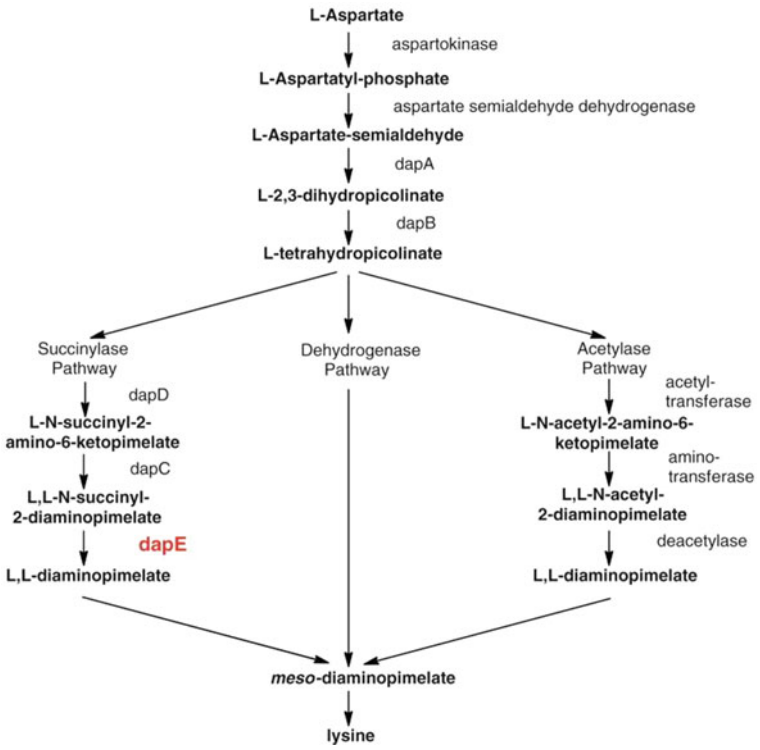


Fig. 15 Schematic diagram of L-lysine biosynthesis in bacteria from aspartic acid *Source* Gillner et al. (2013). With kind permission from Springer Nature

Lysine biosynthesis: Lysine is an essential amino acid which is not synthesized by humans but it is synthesized by lysine biosynthetic pathway in most bacteria from aspartic acid. The pathway has a branch point for the synthesis of dipicolinate (spore constituent) and diaminopimelate (cell wall constituent) and lysine is also an important constituent of cell wall of gram-positive bacteria. Aspartate is first converted to tetrahydrodipicolinate and from this point lysine biosynthesis can occur by three different pathways viz. succinylase pathway, dehydrogenase pathway and acetylase pathway which have varied distribution among bacteria (Fig. 15). The succinylase pathway is the main pathway employed by most bacteria involving steps of dehydrogenation, succinyltransferase activity followed by glutamate transamination and finally desuccinylase activity which leads to the formation of diaminopimelate. Its epimerization and finally decarboxylation leads to formation of lysine. Lysine biosynthesis is regulated by end product inhibition of aspartokinase, the first enzyme in the synthesis. It has been shown that in succinylase pathway, DapE encoded desuccinylase enzyme is inevitable for the survival of organisms as reported in case of *Helicobacter pylori* and *Mycobacterium smegmatis* (Gillner et al., 2013). The second pathway is the most energy intensive pathway and is limited to only a few *Bacillus*

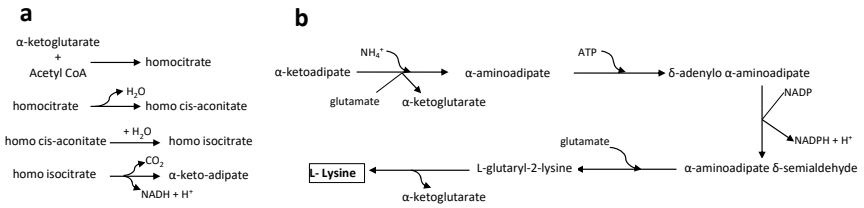
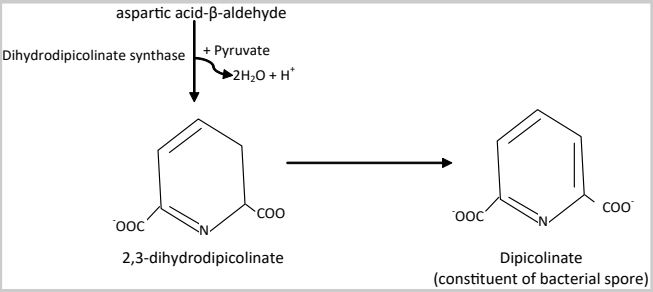


Fig. 16 Lysine biosynthesis in fungi through α-ketoadipate **a** Conversion of α-ketoglutarate to α-ketoadipate; **b** Conversion of α-ketoadipate to Lysine

species. It leads to diaminopimelate synthesis in one step. The third pathway is also very limiting and occurs in few *Bacillus* species only.

Box 4: Conversion of Aspartic Acid-β-Aldehyde to Dipicolinate



In this, aspartylsemialdehyde is cyclized to 2,3-dihydrodipicolinate by dihydrodipicolinate synthase in presence of pyruvate. The enzyme is inhibited by lysine. 2,3-dihydrodipicolinic acid is oxidized to dipicolinate in spore formers. In some bacteria, the enzyme is desensitized to lysine inhibition at the onset of sporulation

Lysine biosynthesis in fungi: Lysine biosynthesis in fungi and yeasts belongs to α-ketoglutarate family in contrast to bacterial pathway which is a part of aspartate family. Lysine biosynthetic pathway intermediates in fungi are important for β-lactam antibiotic synthesis while bacterial pathway is important for cell wall constituents. The lysine biosynthetic pathway can be studied in two steps:

i. α-ketoglutarate → α-ketoadipate: The pathway starts with condensation of α-ketoglutarate with acetyl CoA to form homocitrate. From homocitrate, it goes through a series of reactions to α-ketoadipate similar to TCA cycle where citrate gets converted to α-ketoglutarate. Further, homcitrate formation is subjected to end product inhibition (Fig. 16a).

ii. α-ketoadipate → Lysine: α-ketoadipate is then converted to lysine by transamination at both α-keto group and at γ-carboxyl group. This is facilitated by direct transamination at α-position and reduction of γ-carboxyl through adenylation followed by transamination using glutamate (Fig. 16b).

Box 5: Lysine biosynthetic pathway and β-lactam antibiotics

Penicillin and cephalosporins are synthesized by a branch from lysine pathway. Likewise, inhibition of production of these antibiotics is observed during excess lysine supply. The major intermediate required for these antibiotic peptide synthesis is L-α-aminoadipate which condenses with L-cysteine and L-valine to form tripeptide ACV [δ-(L- α-aminoadipyl)-L-cyteinyl-D-valine] by an ATP dependent ACV synthetase (Fazius et al., 2013).

C. **Amino Acids Synthesized using Precursors from Pentose Phosphate Pathway**

1. **Biosynthesis of Histidine**

The precursor of histidine in both plants and bacteria is phosphoribosyl pyrophosphate (PRPP). Histidine biosynthesis has been studied in only a few bacteria. Here again, number of activities per protein may vary among different bacteria, yeasts and fungi. However, the pathway remains the same. Histidine biosynthesis has been well-studied in *E. coli* which involves 10 steps (16.17) (Winkler & Ramos- Montanez, 2009). The steps are as follows:

i. The pathway starts with the transfer of N1 and C2 of adenine moiety of ATP to ribose of PRPP to form phosphoribosyl-ATP which is catalyzed by the enzyme ATP phosphoribosyl transferase (HisG).
ii. Second step involves conversion of phosphoribosyl-ATP to phosphoribosyl-AMP by the release of pyrophosphate (PPi) by the enzyme phosphoribosyl-ATP pyrophosphohydrolase (HisE). This step is irreversible.
iii. Phosphoribosyl-AMP is then converted to phosphoribosyl formimino-AICAR-phosphate by the enzyme phosphoribosyl AMP cyclohydrolase (HisI).
iv. In the next step catalyzed by the enzyme phosphoribosyl formimino-AICAR-phosphate isomerase (HisA), phosphoribosyl formimino-AICAR-phosphate is isomerized to form phosphoribulosyl formimino-AICAR-phosphate.
v. Phosphoribulosyl formimino-AICAR-phosphate is then acted upon by two enzymes to form D-erythro-imidazole-glycerol phosphate. The first enzyme involved is glutamine amidotransferase (HisH) which transfers ammonia group from glutamine and transfers it to phosphoribulosyl formimino-AICAR-phosphate and releases glutamate. The intermediate is then used as a substrate by the enzyme imidazole-glycerol-phosphate synthase cyclase

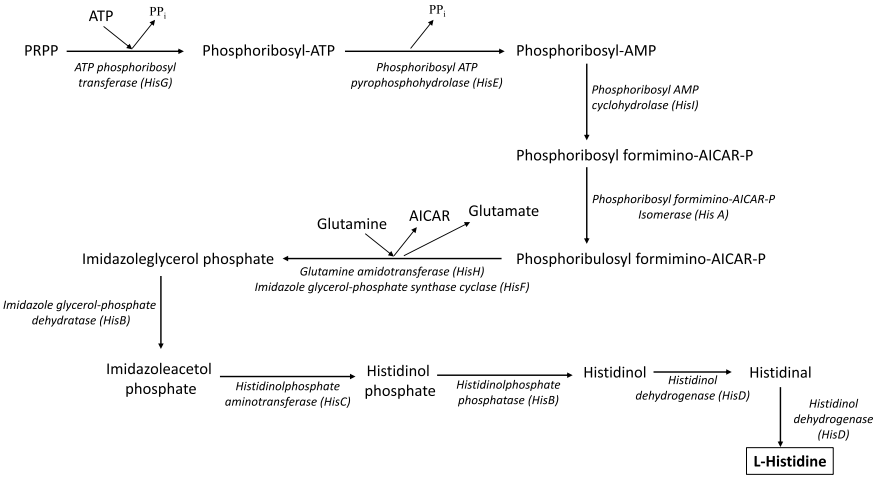


Fig. 17 Histidine biosynthesis from precursors of pentose phosphate pathway

(HisF) to form D-erythro-imidazole-glycerol phosphate. AICAR is removed at this step.

vi. D-erythro-imidazole-glycerol phosphate is then converted to imidazole-acetol phosphate by the enzyme imidazole-glycerol-phosphate dehydratase (HisB) by the release of water molecule.

vii. Histidinol phosphate is then formed from imidazole-acetol phosphate by the action of the enzyme histidinol phosphate aminotransferase (HisC).

viii. Then histidinol is formed by removal of phosphate group from histidinol phosphate by the enzyme histidinol phosphate phosphatase (HisB).

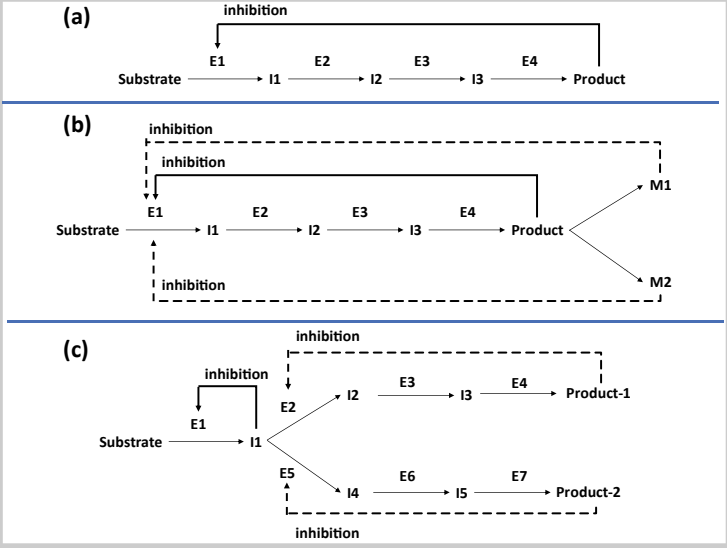
ix. Histidinol then undergoes oxidation to form histidinal (an amino aldehyde) and finally histidinal is converted to histidine by another oxidation reaction. Both these steps are catalyzed by the action of a single enzyme histidinol dehydrogenase enzyme (HisD) (Fig. 17).

Box 6: Regulation of Amino Acid Biosynthesis

Amino acid biosynthesis can be regulated by three different mechanisms as follows:

- **Feedback inhibition**: Inhibition of the irreversible reaction of the biosynthetic pathway, which is usually catalyzed by an allosteric enzyme, by end product of the pathway is known as feedback inhibition. It is the most responsive regulation of amino acid synthesis. The most popular example of feedback inhibition is isoleucine biosynthesis where isoleucine inhibits the very first enzyme, threonine deaminase (Umbarger, 1978).

- **Concerted inhibition**: In this case, multiple products of the pathway or other products obtained from the amino acid synthesized, negatively modulates the allosteric enzyme activity in a concerted manner and the overall effect is more than the simple additive effect. Glutamine synthesis is an example of concerted inhibition. Six products derived from glutamine serve as negative feedback modulators of glutamine synthetase enzyme (Stadtman, 2004).
- **Sequential feedback inhibition**: This kind of inhibition is observed in case of biosynthesis of aromatic amino acids which initially proceeds with the synthesis of common intermediate, for example chorismite, which later branched off for the synthesis of respective amino acids. The common intermediate can inhibit the first step of their synthesis, but the final amino acid products can only inhibit the branching step that leads to their own synthesis (Nester & Jensen, 1966).



Types of regulation of amino acid biosynthesis; **a** feedback inhibition; **b** concerted inhibition; and **c** sequential feedback inhibition (E: enzyme; I: intermediates; M: metabolites)

Box 7: Strategy for Amino Acid Over-Production

Biosynthetic pathways of amino acids and their respective regulations help us to decide the strategy for its over-production. For every amino acid a different strategical approach should be followed. However, following are the key points which must be kept in mind during strain engineering for over-production of a particular amino acid:

- Overcome feedback inhibition—Identify mutants that are not inhibited by substrate analogs
- Auxotrophic mutants of other branches in a branched pathway
- Over-expression of enzyme involved in biosynthesis
- Improve flux towards the biosynthetic pathway
- Improve efflux/export
- Strain selection by comparative pathway analysis.

Example of strain improvement for L-lysine over-production

Strategy I: To select mutants which lack feed-back repression/inhibition

- Select mutants in presence of lysine substrate analogue S-(2-aminoethyl)-L-cystein (AEC)
- This analog is able to support only those mutants where aspartokinase regulatory site does not bind to lysine or binds with low affinity

Strategy II: Selecting auxotrophic mutants of other branches in a branched pathway

- Both lysine and alanine production require pyruvate—Alanine auxotrophs are lysine over-producers
- Homoserine auxotrophs
- Threonine-methionine double auxotrophs.

Strategy III: Over-expression of enzymes involved in lysine biosynthesis

- Several lysine biosynthetic enzymes can be expressed in high copy number plasmid
- Strain with plasmid carrying dihydrodipicolinate synthase shows improved yields.

Strategy IV: Improving flux towards the biosynthetic pathway

A. **Improving oxaloacetate availability**:

- Biotin stimulates the activities of both pyruvate carboxylase and PEP carboxylase
- Increase in oxaloacetate flux leads to higher lysine concentration.

B. **Diverting L-aspartyl semialdehyde flux towards lysine**:

- Dihydrodipicolinate synthase changes flux of semialdehyde towards synthesis of lysine and thus compete with homoserine dehydrogenase for L-aspartyl semialdehyde
- Homoserine dehydrogenase is feedback inhibited by threonine and it has high affinity for semialdehyde
- In response to threonine, dehydrogenase activity would decrease making semialdehyde flux available for synthase.

C. **Improving flux through ammonium pimelate dehydrogenase in *Corynebacterium glutamicum***

- This is the shortest route to lysine involving just one step instead of four in succinylase route
- The flux through this branch is nitrogen dependent
- Diaminopimelate dehydrogenase has weak affinity for ammonium because of which productivity decreases as the process is prolonged
- This can be overcome by maintaining NH_4^+ concentration throughout fermentation
- *C. glutamicum* has a specific lysine exporter (lysE carrier protein)
- Secondary transport mediated via electrochemical proton potential and lysine gradient
- LysE has low affinity (Km of 20 mM) for lysine; thus, lysine is exported only when it is accumulated
- Export potential of over-producing strain was found to be much higher than wildtype indicating simultaneous mutation in the exporter as well
- LysG is the positive regulator of *LysE* expression; *lysG* encodes for a LysR-type transcriptional regulator.

Strategy VI: Strain selection by comparative pathway analysis

- *E. coli* has a very complicated regulatory mechanism for these amino acids
  - Each product inhibits/represses the first enzyme of the pathway, aspartokinase
  - Each product also regulates first enzyme of its specific branch.
- It is very difficult to produce any one amino acid in excess of others using *E. coli*
- Reason for such tight regulation is that *E. coli* inhabits gut and is subjected to large fluctuations in the availability of amino acid pool
- For over-production, bacterial strains with simpler regulations are preferred
- Such strains are abundant in environments where they do not encounter variable amino acid levels. They constantly live in poor amino acid environment
- Example—Soil bacterium *Corynebacterium glutamicum.*

4 Amino Acid Utilisation

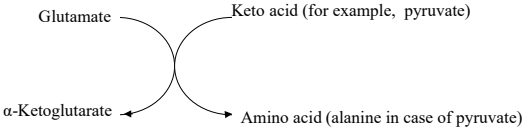
The amino acids are units of proteins and source of nitrogen. Thus, in presence of surplus carbon, amino acids serve as nitrogen source either as such by getting incorporated directly into proteins or by donating ammonium nitrogen to synthesize newer amino acids during a reaction called transamination. However, at time they serve as source of: (a). both carbon and nitrogen; (b). methyl donor; (c). donor of sulphydryl group; (d). may also have a function during pH homeostasis. All these functions are mediated via a set of different reactions:

1. Decarboxylation leading to amines which raises pH and serves to regulate pH during acidic conditions.
2. Deamination leading to free ammonia and keto acids serving both as source of carbon and nitrogen.
3. Desulfhydrase releasing H_2S and NH_3 both serving as sources of carbon, nitrogen and sulphur.
4. Stickland reaction where amino acids function as source of energy restricted to some members of clostridia.

Amino acid catabolism: The various reactions of amino acids involving any of the groups require pyridoxal 5'-phosphate (PLP), a form of vitamin B6, as coenzyme. The coenzyme PLP is very versatile and is utilized with a variety of enzymes to catalyze various distinct chemical reactions. This is assumed that appropriate alignment of PLP on to apoenzyme facilitates the breakdown of C-X bond during a large number of different reactions such as transamination, α/β decarboxylation, aldol cleavage, β/γ displacement, β/γ elimination and also racemization of L to D and vice versa.

Nitrogen removal from amino acids: Amino acids are source of nitrogen and release it in the form of NH_4^+ ions. There are two types of reactions. The first type is transfer of NH_4^+ to another keto group to form another amino acid through transamination. Another reaction is oxidative deamination and non-oxidative deamination which truly results in catabolism of amino acids.

Transamination—This is the most widely present method of nitrogen removal and generation of amino acids from keto-acid intermediates of carbon catabolic pathways. The reaction is mediated by transaminases which have pyridoxal 5'-phosphate as the coenzyme and is specific for amino acids. Most important transaminations are through glutamate.



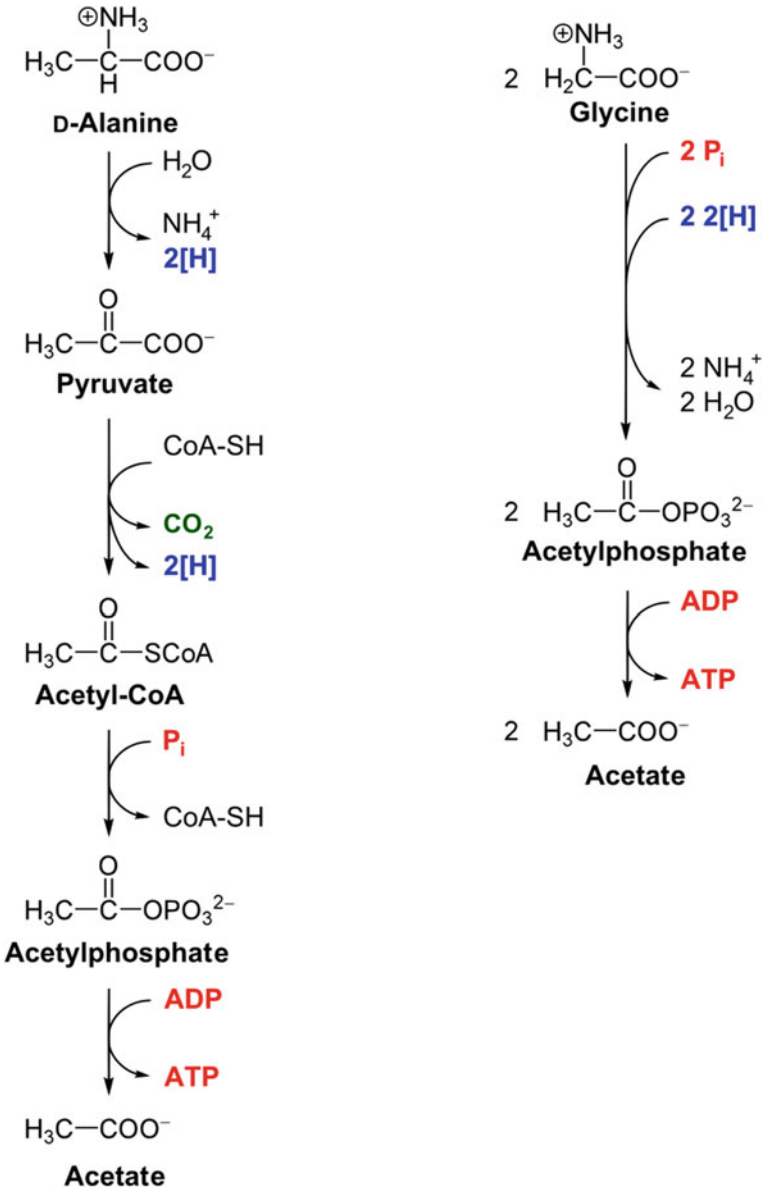
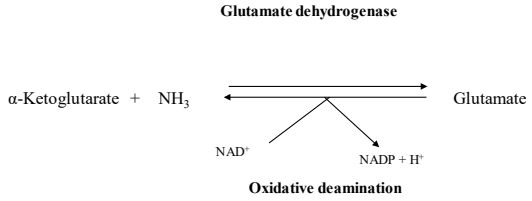
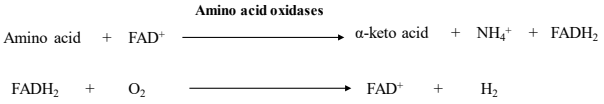


Fig. 18 Stickland reaction showing alanine as electron donor and glycine as electron acceptor *Source* Wikipedia (Public Domain)

Oxidative deamination – Oxidative deamination results in net loss or release of NH_4^+ and it is a reversal of glutamate dehydrogenase reaction. However, it uses NAD^+ as reductant while reductive deamination uses $NADP^+$ as reductant.



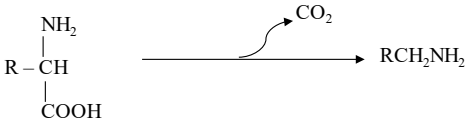
This may also occur by amino acid oxidases which use FAD^+/FMN^+ as reductant. This is also called aerobic deamination and occurs in two steps. In the first step $FADH_2$ is generated and in the second step reductant transfers hydrogen either to another reductant or to O_2.



Amino acid oxidases are non-specific and both D- and L- oxidases are found.

Non-oxidative deamination – Non-oxidative deaminases are specific to their amino acid substrates. There is no requirement of reductant, instead they require pyridoxal 5'-phosphate as coenzyme e.g. aspartase deaminates aspartic acid to fumarate and ammonia. Serine and threonine deaminases release water along with NH_3. Cysteinde sulfhydrase is another deaminase which releases both H_2S and NH_3. Phenylalanine deaminases result in trans-cinnamic acid and NH_3.

Decarboxylation of amino acids: Amino acid decarboxylases are substrate specific and are generally inducible by acidic pH. In such conditions, the removal of CO_2 results in accumulation of amino acids as amines which raises the pH. The decarboxylase reactions are irreversible reactions and are critical to synthesis of polyamines. Amino acid decarboxylases also use pyridoxal 5'-phosphate as coenzyme.



Stickland reaction: Stickland demonstrated that in presence of certain bacterial suspensions there was coupled deamination in presence of a donor and an acceptor amino acid (Stickland, 1934). Before that, he had already reported that certain amino acids e.g. alanine, valine, leucine and isoleucine can reduce certain redox dyes viz. methylene blue, brilliant cresyl blue etc. while amino acids like glycine,

proline, hydroxyproline and ornithine could reoxidise reduced dyes in presence of the same bacterial suspension. Stickland reaction is restricted to anaerobic bacteria and proteinogenic clostridia. Glycine-alanine pair is the best studied example of stickland reaction (Nisman, 1954) (Fig. 18).

Summary

- Nitrogen is an important element of Earth's atmosphere and exists in various organic and inorganic forms viz. nitrogen gas (N_2), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), nitric oxide (NO) or organic nitrogen.
- The major steps involved in the cycling of nitrogen catalyzed by different microbes are nitrogen fixation, ammonification, nitrification, denitrification and anammox.
- Ammonification is breakdown of proteins to amino acids and then to ammonia which is further converted to nitrate in nitrification process.
- Anaerobic conversion of nitrite and ammonium to molecular nitrogen is anammox.
- Microorganisms can use both inorganic and organic form of nitrogen by virtue of presence of both assimilatory and dissimilatory enzymes.
- Bacteria assimilate inorganic nitrogen NO_3^-, $NH_4^+$and urea to complex nitrogen compounds, amino acids and other derivatives.
- Assimilatory reduction is independent of oxygen but is highly sensitive to ammonium availability while the respiratory reduction is repressed in oxygen but insensitive to the availability of ammonia or any other nitrogen source.
- The first amino to be synthesized are glutamate and glutamine by respective activity of glutamate dehydrogenase glutamine synthase during growth on inorganic nitrogen.
- Glutamine synthase is tightly regulated both at transcriptional and translational level by an accessory protein PII.
- Bacteria are unique since they can synthesize all twenty amino acids de novo.
- The carbon skeleton for all the amino acids is provided by carbon metabolic pathways. Glucose provides the carbon intermediates and ammonium serves as nitrogen for synthesis of all the twenty amino acids in bacteria. On the basis of various intermediate precursors, there are 6 different families of amino acids. Three families (serine, aromatic and pyruvate) are derived from glycolysis pathway, two families (glutamate and aspartate) are derived from citric acid cycle and one family (Histidine) is derived from pentose phosphate pathway.
- Biosynthesis of serine family amino acids L-serine, L-glycine and L-cysteine via glycolytic pathway uses 3-phosphoglycerate as carbon skeleton.
- Biosynthesis of Aromatic Family of Amino acids: L-tryptophan, L-tyrosine and L-phenylalanine takes place via shikimate pathway.
- Lysine biosynthesis in fungi and yeasts belongs to α-ketoglutarate family in contrast to bacterial pathway which is a part of aspartate family.

- Lysine biosynthetic pathway intermediates in fungi are important for β-lactam antibiotic synthesis while bacterial pathway is important for synthesis of cell wall constituents (diaminopimelate) and spores (dipicolinate).
- All the pathways are similar in bacteria and fungi except lysine where oxaloacetate is precursor in bacteria in place of α-ketoglutarate in fungi.
- Methionine is synthesized from homoserine and is mostly present in the form of cofactor SAM which helps in methylation reactions intracellularly.
- Amino acid synthesis in *E. coli* is strictly regulated by various regulation mechanisms such as feedback inhibition, concerted inhibition and sequential feedback inhibition.
- The amino acids are units of proteins and source of nitrogen.
- They serve as source of both carbon and nitrogen, methyl donor, donor of sulphydryl group and may also have a function during pH homeostasis. All these functions are mediated via a set of different reaction such as decarboxylation, deamination, desulfhydrylation and Stickland reaction.
- The various reactions of amino acids involve pyridoxal-5-phosphate as co-enzyme.
- Stickland reaction is restricted to anaerobic clostridia and proteogenic bacteria wherein amino acids act as both donor and acceptor and are used as source of energy.

Questions

1. In which form is sulfur added to the amino acid cysteine?
2. Which reaction is catalyzed by serine hydroxyl methyl transferase and what is its importance?
3. What are essential amino acids? Name few of them.
4. What are non-proteogenic amino acids?
5. Which is the simplest assimilatory form of nitrogen?
6. What is the effect of nitrogen concentration on the regulation of the enzyme glutamine synthetase?
7. Which are the six families of amino acid biosynthesis? Describe the basis of their division
8. Which amino acid biosynthetic pathway is involved in the synthesis of beta-lactam antibiotics?
9. Which amino acid is majorly present inside the cell as cofactor SAM?
10. Which co-enzyme is used in amino acid catabolism?
11. Which amino acid catabolic reaction is restricted to anaerobic bacteria?

References

Berlicki, L. (2008). Inhibitors of glutamine synthetase and their potential application in medicine. *Mini Reviews in Medicinal Chemistry., 8*(9), 869–878.

Clausen, T., Huber, R., Laber, B., Pohlenz, H. D., & Messerschmidt, A. (1996). Crystal structure of the pyridoxal-5′-phosphate dependent cystathionine β-lyase from Escherichia coliat 1.83 Å. *Journal of Molecular Biology, 262*(2), 202–224.

Eisenberg, D., Gill, H. S., Pfluegl, G. M., & Rotstein, S. H. (2000). Structure–function relationships of glutamine synthetases. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1477*(1–2), 122–145.

Fazius, F., Zaehle, C., & Brock, M. (2013). Lysine biosynthesis in microbes: Relevance as drug target and prospects for β-lactam antibiotics production. *Applied Microbiology and Biotechnology, 97*(9), 3763–3772.

Ferla, M. P., & Patrick, W. M. (2014). Bacterial methionine biosynthesis. *Microbiology, 160*(8), 1571–1584.

Gillner, D. M., Becker, D. P., & Holz, R. C. (2013). Lysine biosynthesis in bacteria: A metallodesuccinylase as a potential antimicrobial target. *JBIC Journal of Biological Inorganic Chemistry, 18*(2), 155–163.

Jiang, P., Peliska, J. A., & Ninfa, A. J. (1998). The regulation of *Escherichia coli* glutamine synthetase revisited: Role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state. *Biochemistry, 37*(37), 12802–12810.

Kredich, N. M., & Tomkins, G. M. (1966). The enzymic synthesis of L-cysteine in Escherichia coli and Salmonella typhimurium. *Journal of Biological Chemistry, 241*(21), 4955–4965.

Litwack, G. (2018). Chapter 4—Proteins. In *Human* biochemistry (pp. 63–94). Academic Press.

Mohan, S. B., & Cole, J. A. (2007). The dissimilatory reduction of nitrate to ammonia by anaerobic bacteria. In *Biology of the nitrogen cycle* (pp. 93-106). Elsevier.

Nester, E. W., & Jensen, R. A. (1966). Control of aromatic acid biosynthesis in Bacillus subtilis: Sequential feedback inhibition. *Journal of Bacteriology, 91*(4), 1594–1598.

Nisman, B. (1954). The stickland reaction. *Bacteriological Reviews, 18*(1), 16.

Pittard, J., & Yang, J. (2008). Biosynthesis of the aromatic amino acids. *EcoSal Plus*, *3*(1).

Sanghai, Z. A., Liu, Q., Clarke, O. B., Belcher-Dufrisne, M., Wiriyasermkul, P., Giese, M. H., et al. (2018). Structure-based analysis of CysZ-mediated cellular uptake of sulfate. *Elife, 7,* e27829.

Stadtman, E. R. (2004). Regulation of glutamine synthetase activity. *EcoSal Plus, 1*(1).

Stadtman, E. R., & Ginsburg, A. (1974). 24. The Glutamine synthetase of *Escherichia coli*: Structure and control. In *The enzymes* (Vol. 10, pp. 755–807). Academic Press.

Stickland, L. H. (1934). Studies in the metabolism of the strict anaerobes (genus *Clostridium*): The chemical reactions by which Cl. sporogenes obtains its energy. *Biochemical Journal*, *28*(5), 1746.

Umbarger, H. E. (1978). Amino acid biosynthesis and its regulation. *Annual Review of Biochemistry, 47*(1), 533–606.

Valley, C. C., Cembran, A., Perlmutter, J. D., Lewis, A. K., Labello, N. P., Gao, J., et al. (2012). The methionine-aromatic motif plays a unique role in stabilizing protein structure. *Journal of Biological Chemistry, 287*(42), 34979–34991.

Whitfield, C. D., Steers, E. J., & Weissbach, H. (1970). Purification and properties of 5-methyltetrahydropteroyltriglutamate-homocysteine transmethylase. *Journal of Biological Chemistry, 245*(2), 390–401.

Winkler, M. E., & Ramos-Montanez, S. (2009). Biosynthesis of histidine. *EcoSal Plus*, *3*(2).

Chapter 17
Glutathione and Polyamines in Bacteria

Rani Gupta and Namita Gupta

1 Diversity of Low Molecular Weight Thiols in Bacteria

Low molecular weight thiols are important in cellular chemistry in all living organisms. Most ubiquitous is glutathione or L-γ-glutamyl-L-cysteinyl-glycine, a non-ribosomal peptide. Glutathione is present in cytosol in the range between 0.1–10 mM. Among prokaryotes, gram-negative bacteria like *E. coli* have glutathione as the major thiol while some bacteria, archaea and fungi have other low molecular weight thiols. In addition to glutathione, some common cellular thiols like cysteine, homocysteine, lipoic acid, coenzyme A also function as redox thiols (Poole, 2015). However, some organism specific thiols are also reported (Newton et al., 2009; Fahey, 2013; Van Laer et al., 2013; Bartsch et al., 1996) like bacillithiol, mycothiol as mentioned in Fig. 1 and Table 1.

Glutathione is an important redox regulator of the cell and is known to be helpful in combating various environmental stresses including oxidative stress, osmotic shock, acidity and toxicity caused by methylglyoxal and chlorine compounds. It also helps in maintaining potassium ion concentration inside the cell (Masip et al., 2006).

2 Structure of Glutathione

Glutathione is a tripeptide formed by glutamine, cysteine, and glycine glutamine and cysteine residues are joined by a gamma bond and an usual alpha-peptide bond between cysteine and glycine residues (Fig. 1). This feature protects glutathione from degradation by general proteases/peptidases present inside the cell. However, glutathione can be cleaved by a special peptidase that is gamma-glutamyl transpeptidase. The cysteinyl-sulfhydryl group present in glutathione serves as an electron donor making glutathione a strong reducing agent (Smirnova & Oktyabrsky, 2005).

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_17

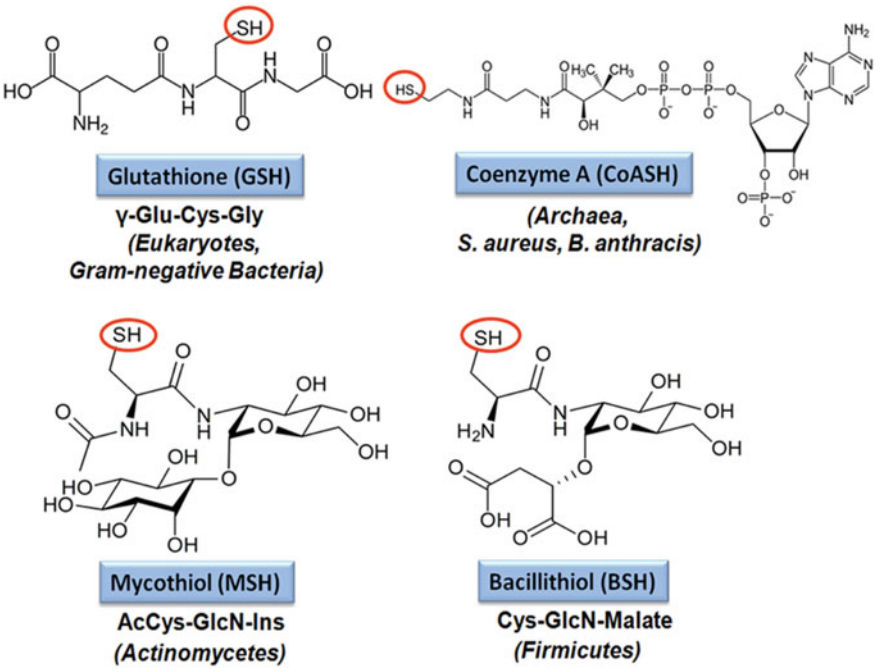


Fig. 1 Structures of low molecular weight thiols present in bacteria. *Source* Loi et al. (2015). (Creative Commons Attribution License)

Table 1 Distribution of low molecular weight thiols among bacteria

Organism specific low molecular weight thiols	Distribution among bacteria
Bacillithiol	Firmicutes
Mycothiol	Actinobacteria
γ-glutamyl-cysteine	Halobacteria and lactic acid bacteria
Ergothioneine	Mycobacteria
Coenzyme M and B	Methanogenic bacteria

3 Glutathione Biosynthesis and Transmembrane Cycling

This tripeptide is synthesized in two steps inside the bacterial cell using two enzymes viz. gamma-glutamyl-cysteine synthetase (γ-GCS; product of *gshA* gene) and glutathione synthetase (GSHS; product of *gshB* gene) by the following reactions respectively:

$$\mathrm{L-Glu + L-Cys + ATP} \xrightleftharpoons{\gamma-\mathrm{GCS}} \gamma-\mathrm{Glu-Cys + ADP + Pi} \qquad (1)$$

$$\gamma - \text{Glu} - \text{Cys} + \text{Gly} + \text{ATP} \xrightleftharpoons{\text{GSHS}} \gamma - \text{Glu} - \text{Cys} - \text{Gly} + \text{ADP} + \text{Pi} \quad (2)$$

In the first reaction, catalysed by γ-GCS enzyme, ATP hydrolysis is accompanied with the formation of peptide bond between the γ-COOH group of glutamate and the α-NH_2 group of cysteine (1), resulting in the formation of a **γ-peptide bond**. This is followed by the hydrolysis of one more ATP molecule along with the formation of one more peptide bond that is catalysed by GSHS enzyme. In this reaction, an **α-peptide bond** forms between the α-COOH group of cysteine in γ-glutamyl-cysteine and the α-NH_2 group of glycine (2).

Under normal physiological conditions, γ-GCS enzyme is feedback inhibited by glutathione and hence the reaction catalysed by γ-GCS enzyme is the rate limiting step in the synthesis of glutathione. Glutathione production can also be regulated by the availability of precursor amino acids and by controlling the expression of γ-GCS at genetic level. Moreover, with respect to the availability of precursor amino acids, limited concentrations of cysteine inside the cell exert an effect on the rate of glutathione synthesis (Murata & Kimura, 1990). Though feedback inhibition and cysteine concentration majorly control glutathione levels, cells also control glutathione pool by its degradation mediated by gamma-glutamyl transpeptidase (GGT) enzyme.

In *E. coli*, GGT enzyme is present in the periplasmic space. Thus, the bacterial cell needs to translocate glutathione to the periplasm for its degradation and the only known exporter of glutathione to the periplasmic space is an ATP-binding-cassette type transporter CydDC (Pittman et al., 2005). GGT enzyme catalyses the cleavage of gamma-peptide bond present between glutamate and cysteine residues of glutathione molecule. GGT forms an acyl enzyme intermediate with the glutamyl moiety while L-cysteinyl-glycine leaves the active site of the enzyme. The latter is further cleaved by dipeptidase cysteinylglycinase (CGase) and the amino acids, cysteine and glycine thus released are absorbed. The recycled and internalized cysteine and glycine residues are then used by the cell either for glutathione synthesis or protein synthesis. On the other hand, GGT also transfers the γ-glutamyl moiety of glutathione to an amino acid or water molecule forming a gamma-glutamyl amino acid (γ-Glu-AA) or glutamic acid, respectively, which are also absorbed by the cell. Therefore, GGT is an important enzyme for glutathione cycling and amino acid uptake (Fig. 2) (Suzuki et al., 1993, 1999).

Besides this, extracellular glutathione is also transported into the periplasm where it is either degraded by GGT enzyme or imported into the cytoplasm via ATP-binding cassette transporter YliABCD coded by *yliA, -B, -C*, and *-D* genes in *E. coli*. Here, YliA codes for ATP-binding protein; YliB codes for a periplasmic binding protein; and YliC and -D code for plasma membrane components (Suzuki et al., 2005).

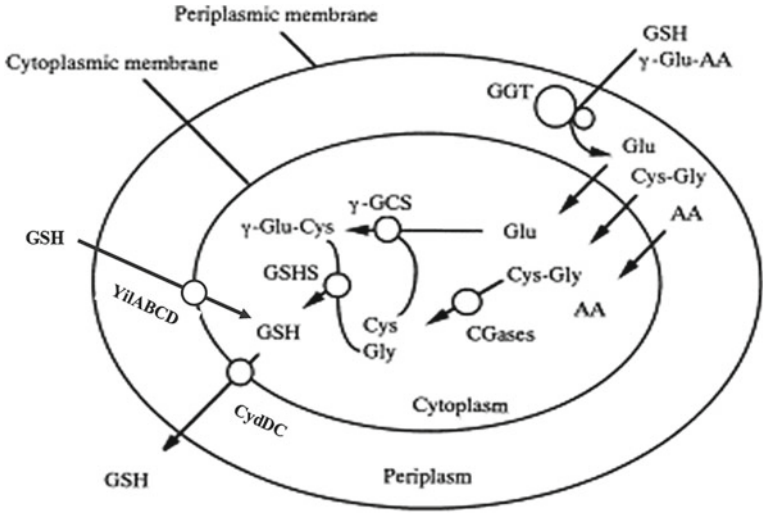


Fig. 2 Glutathione transmembrane cycling in *E. coli.* (CydDC: GSH exporter; YliABCD: exporter of GSH; GGT: gamma-glutamyl transpeptidase; GSHS: Glutathione synthetase; γ-GCS: gamma-glutamyl cysteine synthetase). *Source* Suzuki et al. (1999). With kind permission from Elsevier

Box 1: Modulation of Bacterial Virulence by Host Glutathione Apart from transmembrane cycling of glutathione, some intracellular bacterial pathogens utilize host glutathione for its own physiological purposes. On infecting the host cytosol, pathogenic bacteria like *Listeria* and *Burkholderia* uptake glutathione from host cytosol by means of direct import via. ABC transporter or through degradation pathway using GGT enzyme. In fact, it has been shown that host glutathione serves as a modulator of virulent genes or switches on the virulence factors of the pathogen. Thus, both activation of virulence factors and depletion of host glutathione pool by intracellular pathogens result in chronic infections (Ku & Gan, 2019).

4 Glutathione Homeostasis

Glutathione is present in two major forms inside the cell, the reduced form i.e., glutathione (GSH) itself and the oxidized form glutathione disulphide (GSSG). Apart from these, glutathione also occurs as mixed disulfides, mainly GSS-protein, and with low molecular weight SH-compounds such as cysteine, α-pantetheine, and coenzyme A (CoA). Ratio of GSH:GSSG inside the cell determines the redox potential of the

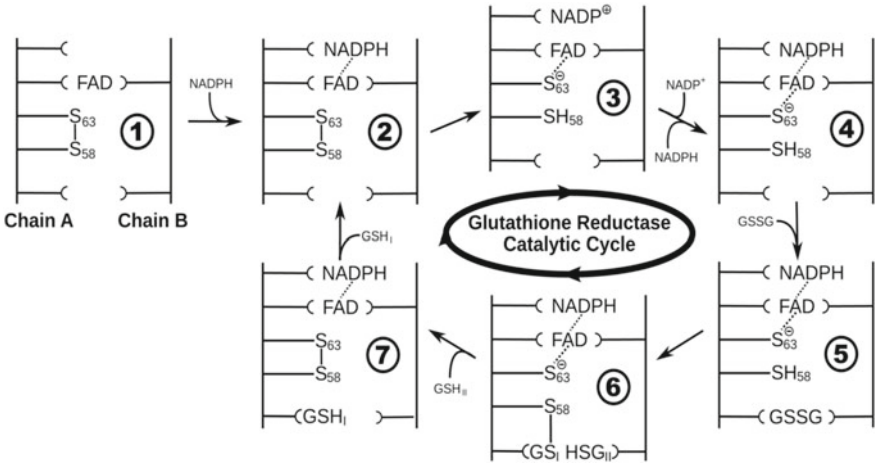


Fig. 3 Mechanism of glutathione reductase enzyme. *Source* Wikipedia. (Creative Commons Attribution License)

cell; a ratio of around 200 corresponds to a redox potential of −240 mV at pH 7 and 25 °C in an experimentally grown *E.coli* in LB media.

The GSH:GSSG ratio is controlled in the cytoplasm through reduction of GSSG to GSH which is catalyzed by glutathione reductase (GOR), a FAD dependent enzyme. GOR is a homodimeric enzyme with each subunit containing four domains viz. a FAD-binding domain, an NADPH-binding domain, a central domain and an interface domain. The active site is located at the dimeric interface and thus both the subunits are essential for an active enzyme (Bashir et al., 1995). GOR catalyzes the transfer of reduced equivalents from NADPH to GSSG and thus reduces GSSG to GSH, as shown in the reaction equation below and in Fig. 3.

$$\mathrm{NADPH} + \mathrm{GSSG} + \mathrm{H+} \rightarrow 2\mathrm{GSH} + \mathrm{NADP}$$

Glutathione reductase catalyses a multi-step reaction depicted below in 7 steps. GOR enzyme is first reduced by NADPH forming a semiquinone of FAD, a sulfur radical and a thiol (step 1-3). Next, reduced GOR reacts with GSSG and results in the formation of a molecule of GSH (reduced glutathione) and red-GOR-SG complex (step 4-6). Finally, an electron rearrangement results in splitting off second molecule of GSH from red-GOR-SG complex and oxidized GOR is restored (step 7) (Massey & Williams 1965; Berkholz et al., 2008).

5 Glutathione for Reduction and Glutathionylation of Proteins

Reduction of proteins: A variety of thiol-redox enzymes containing an active site sequence motif CXXC (thioredoxin motif) perform reversible reductions of disulfide bonds in proteins. In *E. coli*, two independent pathways, viz. thioredoxin and glutaredoxin pathways, exist which initiate cascade of reductions utilizing cellular reductants NADPH and in turn reducing proteins (Fig. 4).

Thioredoxin receives electrons directly from thioredoxin reductase and use them for reducing protein disulphide (Fig. 4a). However, in case of glutaredoxin pathway, glutathione acts as reducing agent that reduces oxidized glutaredoxins. The oxidized glutathione gets back to its reduced form with the help of glutathione disulphide reductase enzyme (Fig. 4b) (Fernandes & Holmgren, 2004). The reduction reactions are observed during ribonucleotide reductase activity and in reducing FNR regulator (fumarate-nitrate reduction regulator) (Tran et al., 2000).

Glutathionylation of proteins: Protein glutathionylation involves the formation of disulphide bond between the cysteine residue of protein and glutathione resulting in the formation of mixed-disulphides. In *E. coli*, mixed-disulfides account for approximately 2% of the total glutathione content in the cell. Protein glutathionylation is one of the mechanisms of the cell to protect over-oxidation of reactive thiols of proteins under oxidative stress. If under oxidative stress, cysteine residue of protein gets oxidized to sulfinic acid (Cys-SO2H). Then they cannot be reduced by glutaredoxin or thioredoxin pathways operating in a cell, and thus the protein becomes permanently inactive. Glutathionylation not only protects the proteins from degradation but has been reported to alter the function of certain bacterial proteins involved in sulphur metabolism, examples are methionine synthase and PAPS reductase (Dalle-Donne et al., 2009).

6 Role of Glutathione During Environmental Stress and Chemical Toxicity

Oxidation stress: Glutathione acts as an anti-oxidant during oxidative stress. Glutathione deficient (*gshA* mutant) *E. coli* cells are more sensitive towards H_2O_2 during stationary phase as compared to wild-type. GSH indirectly helps in glutaredoxin-1 mediated reduction during peroxide stress (Aslund & Beckwith, 1999; Eser et al., 2009).

The oxidative stress created by superoxides formed by autooxidation of dihydromenaquinone is also controlled by glutathione in the periplasm as in such cases an increased export of glutathione in the periplasmic compartment has been observed (Korshunov & Imlay, 2006; Smirnova et al., 2012).

Osmotic stress: Glutathione is involved in regulating K^+ efflux channels as it inhibits potassium export channels KefB and KefC (Booth et al., 1996). Besides this,

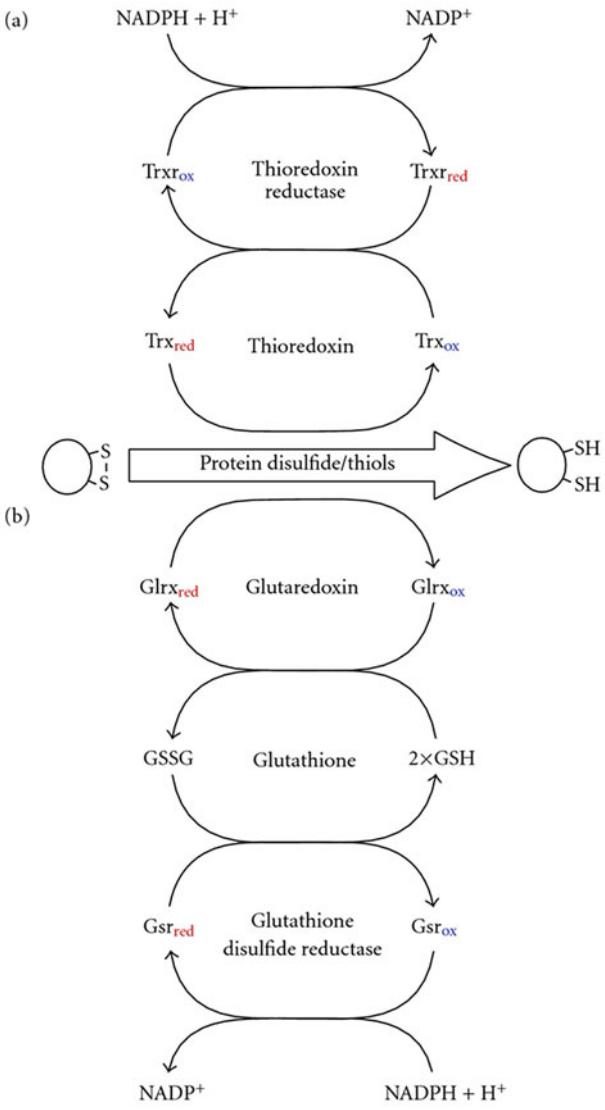


Fig. 4 Glutathione-glutaredoxin and thioredoxin redox regulation systems. *Source* Johnston and Ebert (2012). (Creative Commons Attribution License)

it also provides anti-oxidant activity as during osmotic stress, reactions characteristic of oxidative stress occur in bacteria (Smirnova et al., 2012).

Low pH: pH homeostasis in bacterial cells are suggested to be dependent on Na^+ and K^+ transport. In *E. coli*, the potassium export channels KefB and KefC are inhibited by GSH and in the absence of GSH, K^+ leaks out of the cell. Thus, glutathione plays an important role by regulating the K^+ ion efflux from the cell

and hence helps in regulating pH inside the cell. High intracellular K^+ concentration protects the cell from acidic pH (Booth et al., 1996).

Protection from methylglyoxal: Toxic methylglyoxal that is formed by methylglyoxal pathway operating during phosphate starvation is eliminated from the cell by glyoxalase I and II enzymes with the help of glutathione by converting it to D-lactate. As these reactions reduce the intracellular glutathione pool, K^+ ions start leaking out of the cell as a result of activation of KefB and KefC ion channels, which are otherwise inhibited in the presence of glutathione. Efflux of K^+ ions is accompanied with the influx of protons which decreases the intracellular pH of the cell and low pH is reported to protect the cell from methylglyoxal toxicity (Ferguson & Booth, 1998).

Protection against chlorinated compounds and xenobiotics: In *E. coli*, glutathione conjugates directly and spontaneously (not mediated by enzymes) with toxic hypochlorous acid (HOCl) and monochloroamine (NH2Cl) compounds or enzymatically with xenobiotics and several hydrocarbon compounds via enzymatic reaction catalysed by glutathione S-transferases (Allocati et al., 2009). Glutathione conjugates thus formed are non-toxic and can be removed from the environment (Chesney et al., 1996).

7 Distribution of Common and Unusual Polyamines in Bacteria

Polyamine, as the name suggests, are organic molecules that contain two or more quaternary nitrogen. They are basically polycationic aliphatic hydrocarbons that are present ubiquitously in all living forms ranging from prokaryotes to eukaryotes. They are essential for both growth and multiplication of these organisms (Cohen, 1998). Polyamines have net positive charge at physiological pH and thus interact with negatively charged moieties in the cellular environment such as negatively charged nucleic acid, polymers and proteins through electrostatic bridging (Shah & Swiatlo, 2008). These interactions resulted in variety of effects on nucleic acid biosynthesis and metabolism, in vivo. They have been reported to affect biofilm formation, bacteriocin and toxin production in micro-organisms. Polyamines have also been implicated in survival under adverse conditions as they protect against oxidative stress and acidic stress. Looking into all these aspects they have been linked to microbial pathogenesis. Thus, polyamines concentration is stringently controlled by coordinated regulation of their uptake, biosynthesis and degradation.

Common and unusual polyamines

Putrescine (1, 4-diaminobutane), cadaverine (1, 5-diaminopentane), spermidine and spermine are the four major polyamines in bacteria. The first two are simple diamines while the other two are tri- and tetra- amines, respectively (Fig. 5). Their presence and relative concentrations vary from organism to organism. In fact, some species like *Halobacterium* and *Methanobacterium* lack any of these polyamines and their

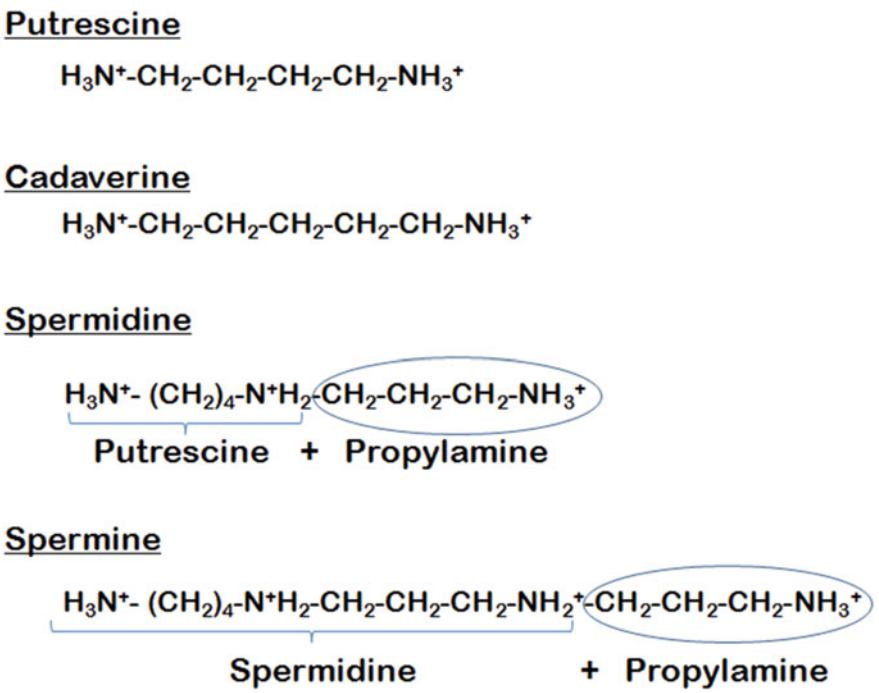


Fig. 5 Major polyamines

functions might be taken over by some other basic substances (Kneifel et al., 1986). Putrescine is the predominant polyamine in *E. coli* followed by spermidine.

Besides the four polyamines listed above, there are many unusual longer- or branched-chain polyamines found in various other bacteria and extremophiles (Fig. 6). Homospermidine or homospermine have one extra carbon than normal spermidine or spermine while norspermidine or norspermine have one carbon less respectively, in the aliphatic hydrocarbon chain. Homospermidine is found in many photosynthetic bacteria, for example *Rhodopseudomonas*, *Rhodospirinum* and *Chromatium.* Also, homospermidine is the major polyamine found in nitrogen fixing cyanobacteria (*Anabaena, Nostoc* and *Tolypothrix*) (Hamana & Matsuzaki, 1985).

Thermophilic bacteria contain an array of 16 different polyamines including norspermidine and norspermine, which have been reported to be crucial for their survival in extreme conditions. They also have longer chain and branched chain polyamines which are known to stabilize DNA in harsh environment and thus help in multiplication of the cells. The tetraamine tetrakis(3-aminopropyl)ammonium, and long linear polyamine caldohexamine, are two major polyamines present in *Thermus thermophilus* (Oshima, 2007).

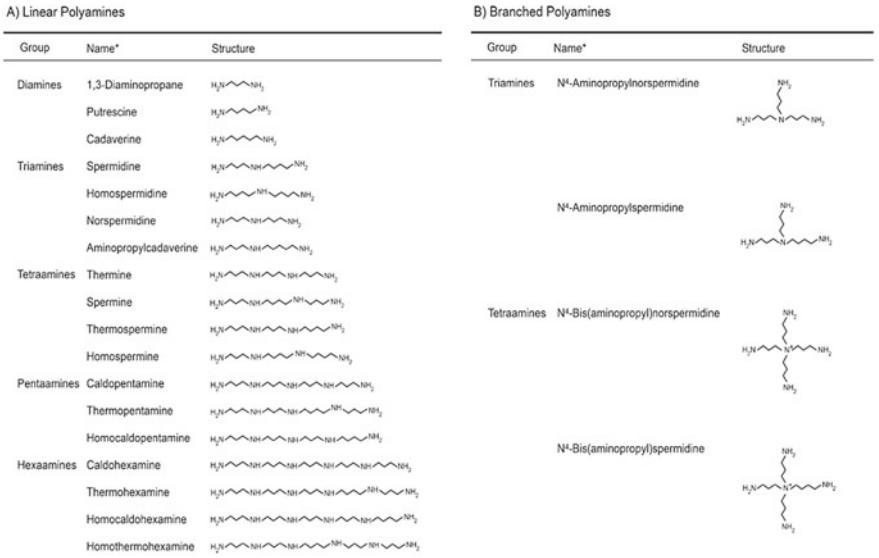


Fig. 6 Common and unique polyamines in bacteria, archaea and extremophiles. *Source* Schneider and Wendisch (2011). With kind permission from Springer Nature

8 Putrescine and Cadaverine Biosynthesis

Polyamines are synthesized by decarboxylation of amino acids and then further modifications may result into functional polyamines. The simplest polyamine is putrescine and is synthesized in majority of prokaryotes by two pathways:

1. Decarboxylation of ornithine; ornithine decarboxylase pathway (ODC pathway)
2. Decarboxylation of arginine; arginine decarboxylase pathway (ADC pathway)

ODC pathway: Ornithine is decarboxylated by ornithine decarboxylase enzyme resulting in putrescine (Fig. 7). The enzyme is encoded by gene *speC*. The enzyme ODC is regulated by multiple mechanisms which are as follows:

1. Feedback inhibition by putrescine and spermidine (Morris & Fillingame, 1974; Cohen, 1998)

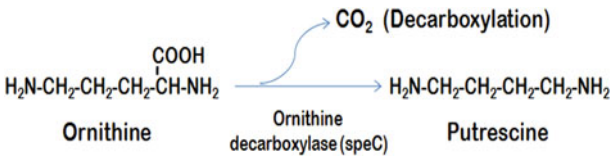


Fig. 7 Ornithine decarboxylase pathway

2. Transcriptional regulation by cAMP and cAMP repressor protein complex
3. An antizyme is also reported to decrease ODC activity in bacteria
4. GTP binding activates ODC activity.

ADC pathway: This pathway involves two steps—firstly decarboxylation of arginine to agmatine by arginine decarboxylase (coded by *speA* gene) takes place and secondly urea removal from agmatine by agmatine ureohydrolase, coded by *speB* gene, results in putrescine (Fig. 8). However, in *Pseudomonas aeruginosa,* conversion of agmatine to putrescine takes place via N- carbamoylputrescine (Nakada & Itoh, 2003).

In a similar manner, **cadaverine** is synthesized by decarboxylation of lysine (Fig. 9). The gene encoding lysine decarboxylase in *E. coli* is *cadA*.

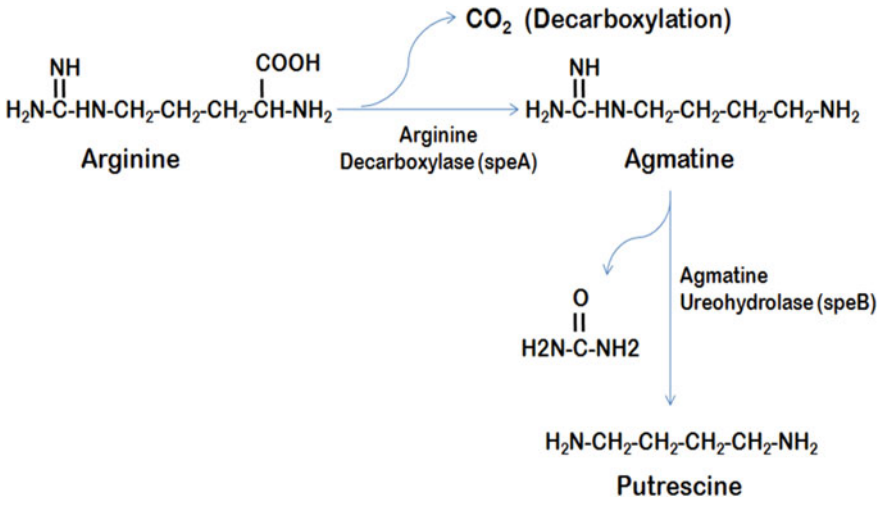


Fig. 8 Arginine decarboxylase pathway

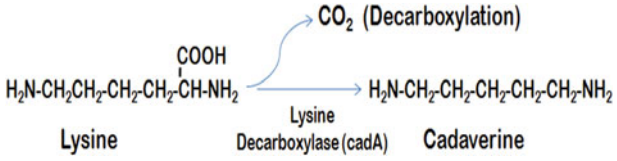


Fig. 9 Cadaverine biosynthesis

9 Spermidine and Spermine Biosynthesis

Spermidine is synthesized by transfer of a propylamine moiety to putrescine. Hence, generally spermidine levels are higher than putrescine and thus always relative ratio of putrescine to spermidine is meaningful rather than their absolute concentrations in cell.

In *E. coli*, key molecule for transfer of polyamine is SAM (S-adenosyl methionine) which is synthesized by conversion of methionine by S-methionine adenosyl transferase, coded by *metK*. Susequently, S-adenosylmethionine is decarboxylated by SAM decarboxylase enzyme which is coded by *speD* gene. Finally, spermidine synthase (SpeE) transfers propylamine from decarboxylated SAM to putrescine in order to generate spermidine (Fig. 10, Tabor et al., 1961).

Spermidine synthesis is regulated by feedback inhibition as spermidine inhibits SAM decarboxylase enzyme. However, deletion of *speE* and *speD* genes does not affect the growth of *E. coli* showing that spemidine is not an essential polyamine in case of *E. coli*.

Spermine synthesis is initiated by transfer of propylamine to spermidine in a similar manner as in spermidine synthesis. The only difference is that here propylamine is transferred to spermidine by spermine synthase. Further, in *E. coli*, it

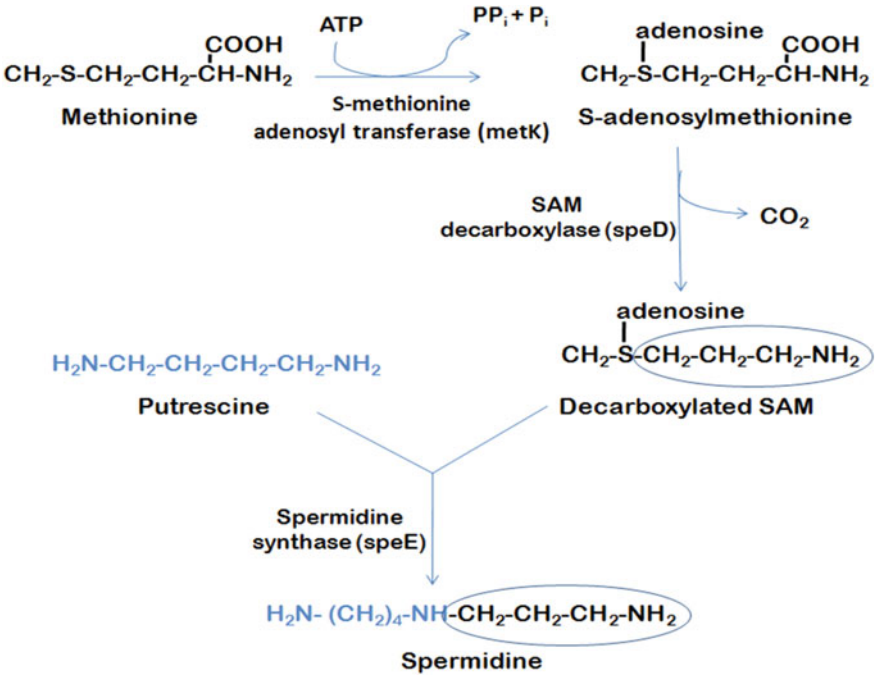


Fig. 10 Spermidine biosynthesis

was observed that spermine synthases is enhanced in absence of putrescine because in speA, speB and speC mutant, spermine (tetra-amine) was found to accumulate. Otherwise, the spermine levels are found to be insignificant.

10 Polyamines Transport and Their Role in Cell Physiology

Polyamines transport

In *E. coli*, there exists two ABC transporters specific for putrescine and spermidine. Additional anti-porters PotE and CadB allow exchange of putrescine/ornithine and lysine/cadaverine, respectively. Uni-porters for putrescine and cadaverine with preference for putrescine followed by spermidine and spermine are also described (Kashiwagi et al., 1986).

Polyamine transporter consists of four genes coded by *potABCD* for spermidine and *potFGHI* for putrescine. These operons consist of ATP binding proteins PotA and PotG, periplasmic substrate binding proteins PotD and PotF and transmembrane alpha-helix channels coded by PotH and PotI, respectively.

Intracellular high levels of polyamines decrease the exogenous uptake by inhibiting ATPase activity of PotA.

Anti-porter PotE and CadB have 12 transmembrane helices with both N and C terminal located in the cytoplasmic side (Fig. 11). These transporters are unique as they mediate both uptake and excretion of respective polyamine. Besides, PotE extrudes putrescine for ornithine uptake while CadB exchanges cadaverine for lysine uptake. CadB is the component of cadBA operon of which cadA codes for lysine

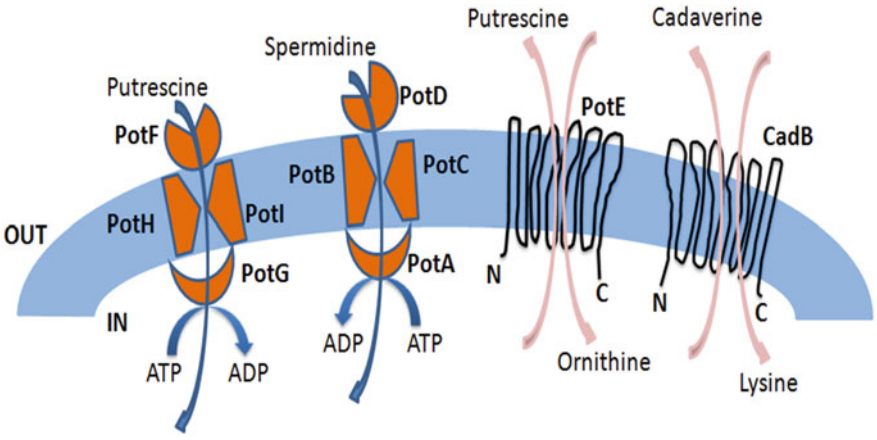


Fig. 11 Polyamine transport

decarboxylase. This operon is activated by acidic pH which up-regulates lysine decarboxylase and cadaverine synthase by cadA. The cadaverine thus formed is exchanged for lysine by CadB and pH is neutralized (Neely & Olson, 1996).

Role of polyamines in gene regulation

Intracellularly, polyamines have been mostly found to be complexed with RNA molecules as RNA-polyamine complex, stabilizing generally unstable double-stranded RNA structure (Shah & Swiatlo, 2008). Such association of polyamines point towards their importance in transcription and translation mechanisms. Polyamines also interact with 30S ribosomal subunit and are known to increase the fidelity of translation (Ito & Igarashi, 1986). They have been known to regulate translation of some major proteins, in vivo and in vitro. The genes coding for such proteins are collectively termed as 'polyamine modulon' (Igarashi & Kashiwagi, 2015). Further in *E. coli*, polyamine modulon regulates proteins involved in transcription, translation, nutrient transport, cell viability and signal transduction (Gevrekci, 2017). It has been reported that polyamines directly regulate the translation of some transcription factors which further regulate the expression of other genes. Polyamines regulate translation by facilitating formation of initiation complex even when the initiation codon is far from the Shine-Dalgarno sequence. Examples include OppA, a periplasmic substrate-binding protein in *E. coli*, transcription regulator, FecI, of an iron transporter operon and a transcriptional regulator of rRNA. Polyamines are also known to enhance the efficiency of codons UUG or GUG as initiation codons allowing UUG- or GUG- dependent fMet-tRNA binding to mRNA-ribosome complex (mRNA transcribed by genes from 'polyamine modulon' pool). Polyamines also stimulate read-through amber codon UAG and also allow a +1 frameshift at the UGA codon. In *Thermus thermophilus*, it was reported that at high temperatures of above 65 °C, spermine stimulated incorporation of phenylalanine while in absence of spermine the reaction stopped earlier reflecting that in this organism, polyamines play an indispensable role in efficient translation at higher temperatures (Ono-Iwashita et al., 1975).

Although polyamines are associated to play role in essential cellular processes, their intracellular levels are maintained by feedback regulation of ornithine decarboxylase (ODC) through its antizymes as ODC inhibitor. The first antizyme was identified in *Schizosaccharomyces pombe* and named SPA (*S. pombe* ODC antizyme) and its deletion caused 40 times more intracellular accumulation of putrescine. Antizyme-like regulator proteins have also been identified in *E. coli* (Ivanov et al., 1998) and *Selenomonas ruminantium* (Yamaguchi et al., 2002). In archaea *Sulfolobus acidocaldaricus*, polyamine spermidine has also been implicated in hypusination of translation factor eIF5A. Hypusination is an essential post-translational modification in which a lysine residue is converted to an unusual amino acid hypusine which converts the inactive eIF5A to its active form. Spermidine is involved in the initial step of hypusination and inhibition of the process using spermidine homologue leads to cell cycle arrest in *S. acidocaldaricus* (Jansson et al., 2000).

Role of polyamines during physiological stress

Oxidative stress: Polyamines offer better survival during oxidative stress by differential expression of protective enzyme superoxide dismutase or by directly acting as free radical scavangers through regulation of "sox" gene. In *Vibrio vulnificus*, soxR regulates the cadaverine operon. SoxR binds to cadBA promoter and increases the synthesis of cadaverine which scavenges superoxide radicals (Kim et al., 2006). Likewise in pneumococci, transcription of potD, a polyamine transporter, increases as a response to oxidative stress, temperature stress and choline limitation. Enhanced polyamine uptake protects pneomococci during stress conditions (Shah & Swiatlo, 2008).

Acidic stress: Intestinal pathogens and commensals encounter acidic conditions under nutrient limitation. Polyamines seem to be the key mediators during acidic conditions. The effect is by induction of amino acid decarboxylases which can increase the pH by increasing amine concentration. It has been reported that in *E. coli*, glutamate decarboxylases are induced by polyamine and in a polyamine deficient mutant expression of glutamate decarboxylase is severely affected. Similarly, lysine improves survival of *E. coli* in Pi starved cells by inducing expression of cadaverine operon switching on the synthesis of lysine decarboxylase along with glutamate decarboxylase (Moreau, 2007).

Polyamines in biofilm formation and virulent phenotype

Biofilm formation is crucial to microbial pathogenesis. It has been observed that putrescine mutant (speA and speC) of *Yersinia pestis* failed to produce detectable biofilm which can be restored by exogenous supplementation of putrescine (Patel et al., 2006). In *Vibrio cholera*, increasing norspermidine concentration in the environment activates biofilm formation (Wotanis et al., 2017). Transposon inactivation of speAB genes led to the loss of swarming phenotype of *Proteus mirabilis* (this phenotype is associated with virulence) which was regained by exogenous putrescine (Sturgill & Rather, 2004).

Toxin immunity

Polyamine deficient mutant of *E. coli* showed to produce lower levels of ColE7 which was restored by exogenous supply of polyamines. ColE7 operon is an SOS response regulon, which encodes for bacteriocin ColE7 (Pan et al., 2006). In wild type, polyamines reduce expression of genes required for colicin uptake. This suggests that polyamines upregulate colicin production and at the same time down regulate its uptake. Role of polyamines in pathogenesis is multifacet as it helps not only in eliciting virulence factors but also help the pathogen to strive through adverse host environment such as oxidative stress, acid stress and also nutrient starvation. Further, polyamine transport appears necessary for multiplication of pathogens and pneumococcal virulence. This was taken as a clue for testing immune response in mouse against PotD. PotD immunization not only elicited antibody but also reduced bacterial colonization. Similar results has also been reported in case of *Burkholderia pseudomallei* (Ware et al., 2006).

11 Polyamine Inhibitors and Disease Prevention

Polyamines have been well demonstrated to play an important role in virulence and pathogenesis including invasion, transmission and biofilm formation (Shah & Swiatlo, 2008). They have been reported to promote the synthesis of Type III secretion system in *Salmonella typhimurium* aiding in its invasion (Ibarra & Steele-Mortimer, 2009). In *Yersinia pestis*, polyamines were reported to play a crucial role in transmission and biofilm formation. A polyamine transporter PotD has been found to be important for pathogenecity caused by *Streptococcus pneumoniae* (Polissi et al., 1998; Ware et al., 2006). Further, high polyamine concentration is known to be a distinctive feature of rapidly growing cells.

The main target for inhibiting any biosynthetic pathway is always the enzymes involved. Therefore, one of the first rationally designed inhibitor, DFMO (α-Difluoromethylornithin) is against orinithin decarboxylase (ODC) (Fig. 12). Inhibition of ODC results in low levels of putrescine and spermidine in the cell but it has lesser effect on spermine levels. As spermine can retroconvert to spermidine and putrescine, DFMO inhibition results in cytostatic effect in vivo rather than cytotoxic effect on cancer cells. However, DFMO showed greater potential against parasitic infections caused by *Trypanosoma brucei* and other related species and proved to be antiprotozoal (Bacchi et al., 1980).

Also, DFMO has been used as antifungal agent in order to protect plants from many of the fungal infections like rust of french beans caused by *Uromyces phaseoli* and barley caused by *Erysiphe graminis*. Higher plants have both ODC and ADC pathways to synthesize putrescine, whereas pathogenic fungi are largely limited to ODC pathway for putrescine synthesis. Thus, when fungus infected plants were treated with DFMO, the inhibitor restricts the ODC pathway in both plant as well as in fungal pathogen. However, plant growth and development do not get hampered as plants have ADC pathway as an alternative to synthesize putrescine while there is no such alternative available to fungal pathogen. Therefore, DFMO ceases growth and differentiation of fungus in plants and subsequently cures plant

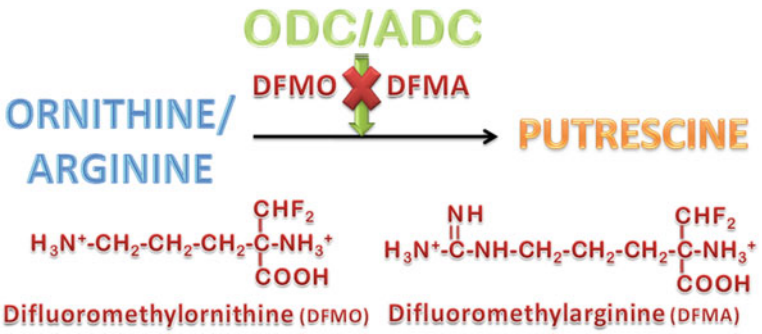


Fig. 12 DFMO/DFMA inhibition

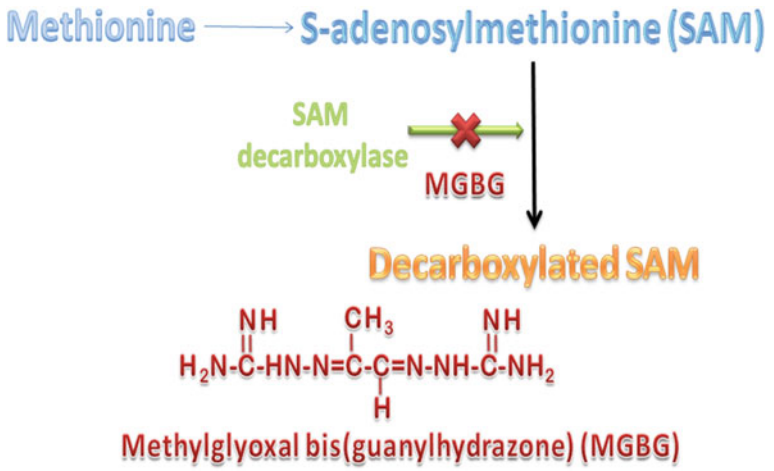


Fig. 13 MGBG inhibition

from the infection. Likewise, arginine decarboxylase activity can be inhibited by DFMA (Difluoromethylarginine) (Gruhn & Boyle, 1991).

The next important inhibitor is methylglyoxal bis(guanylhydrazone) or MGBG. It is similar in structure to spermidine and therefore thought to bind to DNA molecules, but the interaction is weaker as MGBG is not fully protonated at physiological pH. It is a competitive inhibitor of SAM decarboxylase (Fig. 13) and thus acts by depleting spermidine and spermine concentrations in the cell (Porter et al., 1980). MGBG was the first polyamine inhibitor which demonstrated antiviral properties. The drug has been shown to inhibit the replication of poliovirus type I, Echo virus b and coxsackie B3 virus in human amnion cells (Regenass et al., 1992).

12 Industrial Application of Polyamines and Overproduction Strategies for Cadaverin Synthesis

Bio-based polyamides are polymers of polyamines cross-linked with carboxy group provided by acids like adipic acid (Schneider & Wendisch, 2011). Amino group from polyamine and carboxy group from acid form an amide-link through a condensation reaction and provides a polymer that can be commercially used in many areas like textile, automobiles etc. In this respect cadaverine cross-linked polyamides have been reported as eco-friendly substitutes for petroleum-based products (Ma et al., 2017).

In order to commercialize bio-based polyamides and to harvest their potential at industrial scale, over-production of polyamines like putrescine and cadaverine on industrial scale has to be carried out to provide raw material for bio-based polyamides.

For this purpose, scientists have worked on microbial strains that have been metabolically engineered for over-production of amino acids like lysine. In bacteria like *E. coli*, bio-synthesis of cadaverine takes place through decarboxylation of lysine and thus to over-produce it, metabolic flux must be oriented towards the bio-synthesis of lysine.

Taking an example of over-production of cadaverine in *E. coli*, let's see how a bacterial strain can be engineered metabolically. In *E. coli*, cadaverine is synthesized from L-lysine through a decarboxylation reaction. Decarboxylation of L-lysine is catalysed by lysine decarboxylase enzyme and in *E. coli* two isozymes of lysine decarboxylase exist. One is a product of *cadA* gene which is an acid-inducible gene while the other one is a product of a constitutive gene *ldcC*. Therefore, over-expression of lysine decarboxylase enzyme must result in over-production of cadaverine. However, out of the two isozymes, over-expression of LdcC has been shown to be beneficial than over-expression of CadA (Kind et al., 2010).

Further, over-production of L-lysine is crucial for obtaining high yield of cadaverine. In *E. coli,* L-lysine is synthesized by ten enzymatically catalysed steps initiating from the TCA-cycle intermediate oxaloacetate and lysine biosynthesis in *E. coli* is feedback inhibited at aspartokinase step (LysC).

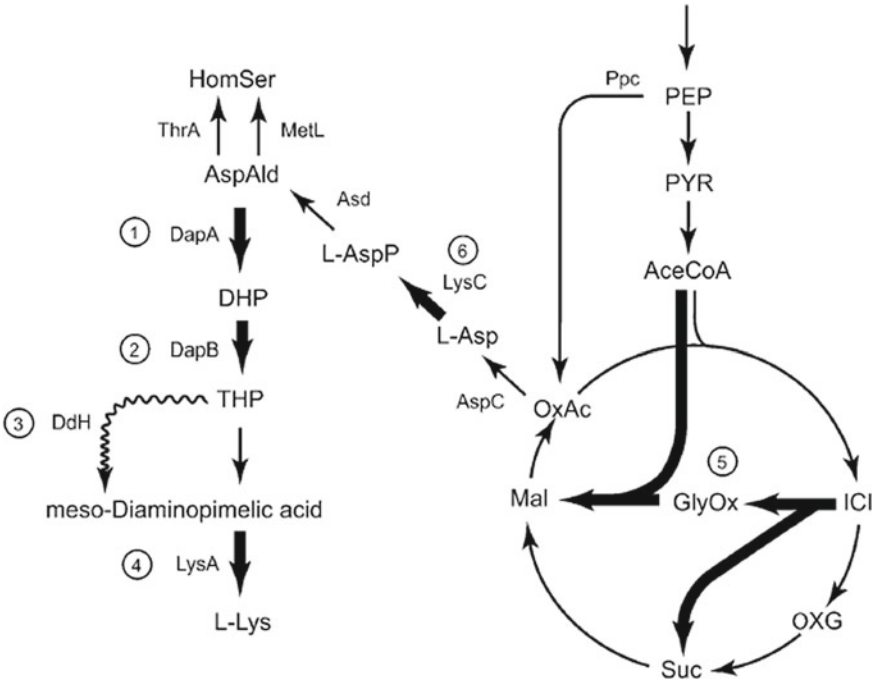


Fig. 14 Targets for metabolic engineering of *E. coli* for lysine over-production *Source* Schneider and Wendisch, (2011). With kind permission from Springer Nature

Following are some strategies which scientists have employed to develop a lysine over-producing *E. coli* strain and the corresponding targets have been numbered in the Fig. 14:

1. Over-expression of DapA (Dihydropicolinate synthase) (Step1), DapB (Step 2) (Dihydropicolinate reductase), LysA (Step 4) (diaminopimelic acid decarboxylase) by promoter exchange to enhance the flux of aspartic acid towards lysine.
2. Over-expression of LysC (Step 6) or using a feedback insensitive LysC variant to result in lysine over-production without any inhibition.
3. Heterologous expression of DdH (Step 3) (diaminopimelate dehydrogenase) from *Corynebacterium glutamicum* to enhance the conversion of tetrahydropicolinic (THP) to meso-Diaminopimelic acid.

However, most of the above-mentioned mutations did not result in enhanced production of L-lysine which could be due to limiting oxaloacetate supply from TCA cycle. Thus, another engineering step was incorporated for increasing oxaloacetate level by increasing the activity of glyoxylate shunt (step 5) and also overexpressing PEP carboxylase (Qian et al., 2011). Simultaneously, the PEP utilizing steps was deleted and PEP-PTS was substituted by glucose facilitator.

Summary

- Glutathione or L-γ-glutamyl-L-cysteinyl-glycine is a non-ribosomal thiol tripeptide ubiquitously present in both prokaryotes and eukaryotes.
- Glutathione is present in gram-negative bacteria like *E. coli* while absent in gram-positive bacteria and archaea with some exceptions like *Streptococcus* and *Enterococcus* species and green sulphur bacteria.
- Some of the bacterial species that lack glutathione have other thiols like bacillithiols in *Bacillus* species, glutathione amide in anaerobic sulphur bacteria, γ-glutamylcysteine in aerobic phototrophic halobacteria and mycothiol in actinomycetes.
- Glutathione is a tripeptide of glutamine, cysteine, and glycine.
- Glutathione can be cleaved by a special peptidase that is gamma-glutamyl transpeptidase.
- The sulfhydryl amino acid cysteine makes glutathione a reducing agent by providing free-SH group.
- Glutathione is synthesized in two steps inside the bacterial cell using two enzymes viz. gamma-glutamyl-cysteine synthetase and glutathione synthetase.
- Gamma-glutamyl transpeptidase hydrolyses glutathione and in *E. coli* it is located in periplasm.
- Glutathione is the key-redox regulator of the cell.
- Glutathione is present in two major forms inside the cell, the reduced form i.e., glutathione (GSH) itself and the oxidized form, glutathione disulphide (GSSG).
- Glutathione also occurs as mixed disulfides, mainly GSS-protein inside the cell.
- Glutathione acts as an anti-oxidant during oxidative stress.

- Glutathione is involved in regulating K^+ efflux channels, thus has role in maintaining turgor tension and in regulating pH inside the cell.
- Toxic methylglyoxal, that is formed by methylglyoxal pathway, is eliminated from the cell by glyoxalase I and II enzyme with the help of glutathione.
- In *E. coli*, glutathione reacts directly and spontaneously with toxic hypochlorous acid (HOCl) and monochloroamine (NH_2Cl) compounds to produce less harmful substances.
- Glutathione acts as reducing agent in glutaredoxin pathway.
- Glutathione also reduces FNR (fumarate nitrate reductase), an oxygen sensor regulator.
- Protein glutathionylation involves the formation of disulphide bond between the cysteine residue of protein and glutathione resulting in the formation of mixed-disulphides.
- Polyamines are organic molecules that contain two or more quaternary nitrogen. They are polycationic aliphatic hydrocarbons that are present ubiquitously in all living forms.
- Putrescine (1, 4-diaminobutane), cadaverine (1, 5-diaminopentane), spermidine and spermine are the four major polyamines in bacteria.
- Some species like *Halobacterium* and *Methanobacterium* lack any of these polyamines and their functions might be taken over by some other basic substances.
- Putrescine and spermidine are major polyamines in bacteria, while cadaverin is least prevalent in bacterial species with no traces found in *E. coli*.
- Thermophilic bacteria contain an array of 16 different polyamines including norspermidine and norspermine.
- Tetra-amine tetra-kis(3-aminopropyl)ammonium, and long linear polyamine caldohexamine, are two major polyamines present in *Thermus thermophilus.*
- Polyamines are synthesized by decarboxylation of amino acids via. ODC and ADC pathways.
- Cadaverine is synthesized by decarboxylation of lysine.
- Spermidine is synthesized by transfer of a propylamine moiety to putrescine via. S-adenosyl methionine.
- In *E. coli*, two ABC transporters specific for putrescine and spermidine exist.
- Anti-porters PotE and CadB are also present which allow exchange of putrescine/ornithine and lysine/cadaverine respectively.
- Uni-porters for putrescine and cadaverine uptake with preference for putrescine followed by spermidine and spermine are also present.
- Intracellularly, polyamines have been mostly found to be complexed with RNA molecules, thus stabilizing generally unstable double-stranded RNA structure.
- Polyamines also interact with ribosomes and areknown to increase the fidelity of translation.
- Survival of *E. coli* is increased in Pi starved cells by addition of lysine which induces expression of cadaverine operon.

- DFMO and DFMA are two potential inhibitors of putrescine pathway as they are substrate analogues of ornithine decarboxylase and arginine decarboxylase, respectively.
- DFMO have been used as antifungal agent in order to protect plants from many of the fungal infections like rust of french beans caused by *Uromyces phaseoli* and barley caused by *Erysiphe graminis*.
- MGBG is a competitive inhibitor of SAM decarboxylase and thus acts by depleting spermidine and spermine concentrations in the cell.
- Polyamines are biotechnologically important for the synthesis of polyamides that are source for bio-plastic. They can be overproduced by metabolically engineering respective amino acid pathway followed by decarboxylation.

Questions

- What is glutathione? Draw its structure.
- What are alternate thiols in microbes where glutathione is absent? Name at least two.
- Which enzyme can degrade glutathione?
- Draw glutathione biosynthesis steps as found in *E. coli*.
- Explain how glutathione can facilitate amino acid uptake?
- How glutathione protects against oxidative stress and osmotic stress?
- What is glutathionylation of proteins? What is its significance?
- What are polyamines? Name at least two common polyamines.
- Draw biosynthetic pathway of putrescine from ornithine.
- What is ADC and ODC pathway?
- Explain role of S-adenosyl methionine in the synthesis of polyamines.
- Name any two physiological roles of cellular polyamine level which aids in survival of microbes.
- Expand: DFMO, DFMA and MGBG; and also add one line on their importance.

References

Allocati, N., Federici, L., Masulli, M., & Di Ilio, C. (2009). Glutathione transferases in bacteria. *The FEBS Journal, 276*(1), 58–75.

Åslund, F., & Beckwith, J. (1999). Bridge over troubled waters: Sensing stress by disulfide bond formation. *Cell, 96*(6), 751–753.

Bacchi, C. J., Nathan, H. C., Hutner, S. H., McCann, P. P., & Sjoerdsma, A. (1980). Polyamine metabolism: A potential therapeutic target in trypanosomes. *Science, 210*(4467), 332–334.

Bartsch, R. G., Newton, G. L., Sherrill, C., & Fahey, R. C. (1996). Glutathione amide and its perthiol in anaerobic sulfur bacteria. *Journal of Bacteriology, 178*(15), 4742–4746.

Bashir, A., Perham, R. N., Scrutton, N. S., & Berry, A. (1995). Altering kinetic mechanism and enzyme stability by mutagenesis of the dimer interface of glutathione reductase. *Biochemical Journal, 312*(2), 527–533.

Berkholz, D. S., Faber, H. R., Savvides, S. N., & Karplus, P. A. (2008). Catalytic cycle of human glutathione reductase near 1 Å resolution. *Journal of Molecular Biology, 382*(2), 371–384.

Booth, I. R., Jones, M. A., McLaggan, D., Nikolaev, Y., Ness, L. S., Wood, C. M., et al. (1996). Bacterial ion channels. In W. N. Konings, H. R. Kaback, & J. S. Lolkema (Eds.), *Transport processes in eukaryotic and prokaryotic organisms* (Vol. 2, pp. 693–729). Elsevier Press.

Chesney, J. A., Eaton, J. W., & Mahoney, J. R. (1996). Bacterial glutathione: A sacrificial defense against chlorine compounds. *Journal of Bacteriology, 178*(7), 2131–2135.

Cohen, S. S. (1998). *Guide to the Polyamines*. Oxford University Press.

Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., & Milzani, A. (2009). Protein S-glutathionylation: A regulatory device from bacteria to humans. *Trends in Biochemical Sciences, 34*(2), 85–96.

Eser, M., Masip, L., Kadokura, H., Georgiou, G., & Beckwith, J. (2009). Disulfide bond formation by exported glutaredoxin indicates glutathione's presence in the *E. coli* periplasm. *Proceedings of the National Academy of Sciences*, *106*(5), 1572–1577.

Fahey, R. C. (2013). Glutathione analogs in prokaryotes. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1830*(5), 3182–3198.

Ferguson, G. P., & Booth, I. R. (1998). Importance of glutathione for growth and survival of Escherichia coli cells: Detoxification of methylglyoxal and maintenance of intracellular K+. *Journal of Bacteriology, 180*(16), 4314–4318.

Fernandes, A. P., & Holmgren, A. (2004). Glutaredoxins: Glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxidants & Redox Signaling, 6*(1), 63–74.

Gevrekci, A. Ö. (2017). The roles of polyamines in microorganisms. *World Journal of Microbiology & Biotechnology, 33*(11), 204.

Gruhn, C. M., & Boyle, S. M. (1991). Biochemical and morphological effects of polyamine biosynthesis inhibitors on Trichophyton and Microsporum. *Journal of Medical and Veterinary Mycology, 29*(2), 63–72.

Hamana, K., & Matsuzaki, S. (1985). Distribution of polyamines in prokaryotes, algae, plants and fungi. In K. Imahori, F. Suzuki, O. Suzuki, & U. Bachrach (Eds.), *Polyamines: Basic and clinical aspects* (pp. 105–118). Netherlands: VNU Science press.

Ibarra, J. A., & Steele-Mortimer, O. (2009). Salmonella–the ultimate insider. Salmonella virulence factors that modulate intracellular survival. *Cellular microbiology*, *11*(11), 1579–1586.

Igarashi, K., & Kashiwagi, K. (2015). Modulation of protein synthesis by polyamines. *IUBMB Life, 67*(3), 160–169.

Ito, K., & Igarashi, K. (1986). The increase by spermidine of fidelity of protamine synthesis in a wheat-germ cell-free system. *European Journal of Biochemistry, 156*(3), 505–510.

Ivanov, I. P., Gesteland, R. F., & Atkins, J. F. (1998). Does antizyme exist in *Escherichia coli*? *Molecular Microbiology, 29*(6), 1521–1522.

Jansson, B. M., Malandrin, L., & Johansson, H. E. (2000). Cell cycle arrest in archaea by the hypusination inhibitor N 1-guanyl-1, 7-diaminoheptane. *Journal of Bacteriology, 182*(4), 1158–1161.

Johnston, A. D., & Ebert, P. R. (2012). The redox system in C. elegans, a phylogenetic approach. *Journal of Toxicology*, *2012*.

Kashiwagi, K. E. I. K. O., Kobayashi, H. I. R. O. S. H. I., & Igarashi, K. A. Z. U. E. I. (1986). Apparently unidirectional polyamine transport by proton motive force in polyamine-deficient Escherichia coli. *Journal of Bacteriology, 165*(3), 972–977.

Kim, J. S., Choi, S. H., & Lee, J. K. (2006). Lysine decarboxylase expression by Vibrio vulnificus is induced by SoxR in response to superoxide stress. *Journal of Bacteriology, 188*(24), 8586–8592.

Kind, S., Jeong, W. K., Schröder, H., & Wittmann, C. (2010). Systems-wide metabolic pathway engineering in Corynebacterium glutamicum for bio-based production of diaminopentane. *Metabolic Engineering, 12*(4), 341–351.

Kneifel, H., Stetter, K. O., Andreesen, J. R., Wiegel, J., König, H., & Schoberth, S. M. (1986). Distribution of polyamines in representative species of archaebacteria. *Systematic and Applied Microbiology, 7*(2–3), 241–245.

Korshunov, S., & Imlay, J. A. (2006). Detection and quantification of superoxide formed within the periplasm of Escherichia coli. *Journal of Bacteriology, 188*(17), 6326–6334.

Ku, J. W., & Gan, Y. H. (2019). Modulation of bacterial virulence and fitness by host glutathione. *Current Opinion in Microbiology, 47,* 8–13.

Loi, V. V., Rossius, M., & Antelmann, H. (2015). Redox regulation by reversible protein S-thiolation in bacteria. *Frontiers in Microbiology, 6,* 187.

Ma, W., Chen, K., Li, Y., Hao, N., Wang, X., & Ouyang, P. (2017). Advances in cadaverine bacterial production and its applications. *Engineering, 3*(3), 308–317.

Masip, L., Veeravalli, K., & Georgiou, G. (2006). The many faces of glutathione in bacteria. *Antioxidants & Redox Signaling, 8*(5–6), 753–762.

Massey, V., & Williams, C. H. (1965). On the reaction mechanism of yeast glutathione reductase. *Journal of Biological Chemistry, 240*(11), 4470–4480.

Moreau, P. L. (2007). The lysine decarboxylase CadA protects Escherichia coli starved of phosphate against fermentation acids. *Journal of Bacteriology, 189*(6), 2249–2261.

Morris, D. R., & Fillingame, R. H. (1974). Regulation of amino acid decarboxylation. *Annual Review of Biochemistry, 43*(1), 303–321.

Murata, K., & Kimura, A. (1990). Overproduction of glutathione and its derivatives by genetically engineered microbial cells. *Biotechnology Advances, 8*(1), 59–96.

Nakada, Y., & Itoh, Y. (2003). Identification of the putrescine biosynthetic genes in Pseudomonas aeruginosa and characterization of agmatine deiminase and N-carbamoylputrescine amidohydrolase of the arginine decarboxylase pathway. *Microbiology, 149*(3), 707–714.

Neely, M. N., & Olson, E. R. (1996). Kinetics of expression of the Escherichia coli cad operon as a function of pH and lysine. *Journal of Bacteriology, 178*(18), 5522–5528.

Newton, G. L., Rawat, M., La Clair, J. J., Jothivasan, V. K., Budiarto, T., Hamilton, C. J., et al. (2009). Bacillithiol is an antioxidant thiol produced in Bacilli. *Nature Chemical Biology, 5*(9), 625–627.

Ono-Iwashita, Y., Oshima, T., & Imahori, K. (1975). In vitro protein synthesis at elevated temperature by an extract of an extreme thermophile. Effects of polyamines on the polyuridylic acid-directed reaction. *Archives of Biochemistry and Biophysics*, *171*(2), 490.

Oshima, T. (2007). Unique polyamines produced by an extreme thermophile, thermus thermophilus. *Amino Acids, 33*(2), 367–372.

Pan, Y. H., Liao, C. C., Kuo, C. C., Duan, K. J., Liang, P. H., Yuan, H. S., et al. (2006). The critical roles of polyamines in regulating ColE7 production and restricting ColE7 uptake of the colicin-producing Escherichia coli. *Journal of Biological Chemistry, 281*(19), 13083–13091.

Patel, C. N., Wortham, B. W., Lines, J. L., Fetherston, J. D., Perry, R. D., & Oliveira, M. A. (2006). Polyamines are essential for the formation of plague biofilm. *Journal of Bacteriology, 188*(7), 2355–2363.

Pittman, M. S., Robinson, H. C., & Poole, R. K. (2005). A bacterial glutathione transporter (Escherichia coli CydDC) exports reductant to the periplasm. *Journal of Biological Chemistry, 280*(37), 32254–32261.

Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottl, H., Ferrari, L., et al. (1998). Large-Scale Identification of Virulence Genes from Streptococcus pneumoniae. *Infection and Immunity, 66*(12), 5620–5629.

Poole, L. B. (2015). The basics of thiols and cysteines in redox biology and chemistry. *Free Radical Biology and Medicine, 80,* 148–157.

Porter, C. W., Dworaczyk, D., Ganis, B., & Weiser, M. M. (1980). Polyamines and biosynthetic enzymes in the rat intestinal mucosa and the influence of methylglyoxal-bis (guanylhydrazone). *Cancer Research, 40*(7), 2330–2335.

Qian, Z. G., Xia, X. X., & Lee, S. Y. (2011). Metabolic engineering of Escherichia coli for the production of cadaverine: A five carbon diamine. *Biotechnology and Bioengineering, 108*(1), 93–103.

Regenass, U., Caravatti, G., Mett, H., Stanek, J., Schneider, P., Müller, M., et al. (1992). New S-adenosylmethionine decarboxylase inhibitors with potent antitumor activity. *Cancer Research, 52*(17), 4712–4718.

Schneider, J., & Wendisch, V. F. (2011). Biotechnological production of polyamines by bacteria: Recent achievements and future perspectives. *Applied Microbiology and Biotechnology, 91*(1), 17–30.

Shah, P., & Swiatlo, E. (2008). A multifaceted role for polyamines in bacterial pathogens. *Molecular Microbiology, 68*(1), 4–16.

Smirnova, G. V., & Oktyabrsky, O. N. (2005). Glutathione in bacteria. *Biochemistry (Moscow), 70*(11), 1199–1211.

Smirnova, G., Muzyka, N., & Oktyabrsky, O. (2012). Transmembrane glutathione cycling in growing Escherichia coli cells. *Microbiological Research, 167*(3), 166–172.

Sturgill, G., & Rather, P. N. (2004). Evidence that putrescine acts as an extracellular signal required for swarming in Proteus mirabilis. *Molecular Microbiology, 51*(2), 437–446.

Suzuki, H., Hashimoto, W., & Kumagai, H. (1993). Escherichia coli K-12 can utilize an exogenous gamma-glutamyl peptide as an amino acid source, for which gamma-glutamyltranspeptidase is essential. *Journal of Bacteriology, 175*(18), 6038–6040.

Suzuki, H., Hashimoto, W., & Kumagai, H. (1999). Glutathione metabolism in Escherichia coli. *Journal of Molecular Catalysis. B, Enzymatic, 6*(3), 175–184.

Suzuki, H., Koyanagi, T., Izuka, S., Onishi, A., & Kumagai, H. (2005). The yliA,-B,-C, and-D genes of Escherichia coli K-12 encode a novel glutathione importer with an ATP-binding cassette. *Journal of Bacteriology, 187*(17), 5861–5867.

Tabor, H., Tabor, C. W., & Rosenthal, S. M. (1961). The biochemistry of the polyamines: spermidine and spermine. *Annual Review of Biochemistry, 30*(1), 579–604.

Tran, Q. H., Arras, T., Becker, S., Holighaus, G., Ohlberger, G., & Unden, G. (2000). Role of glutathione in the formation of the active form of the oxygen sensor FNR ([4Fe-4S]· FNR) and in the control of FNR function. *European Journal of Biochemistry, 267*(15), 4817–4824.

Van Laer, K., Hamilton, C. J., & Messens, J. (2013). Low-molecular-weight thiols in thiol–disulfide exchange. *Antioxidants & Redox Signaling, 18*(13), 1642–1653.

Ware, D., Jiang, Y., Lin, W., & Swiatlo, E. (2006). Involvement of potD in Streptococcus pneumoniae polyamine transport and pathogenesis. *Infection and Immunity, 74*(1), 352–361.

Wotanis, C. K., Brennan III, W. P., Angotti, A. D., Villa, E. A., Zayner, J. P., Mozina, A. N., & Karatan, E., et al. (2017). Relative contributions of norspermidine synthesis and signaling pathways to the regulation of Vibrio cholerae biofilm formation. *PloS One*, *12*(10).

Yamaguchi, Y., Takatsuka, Y., & Kamio, Y. (2002). Identification of a 22-kDa protein required for the degradation of *Selenomonas ruminantium* lysine decarboxylase by ATP-dependent protease. *Bioscience, Biotechnology, and Biochemistry, 66*(6), 1431–1434.

Part VIII
Lipid Metabolism and Nucleotide Biosynthesis

Chapter 18
Lipid Biosynthesis and Degradation

Rani Gupta and Namita Gupta

1 Distribution of Lipids in Microbes and Their Biological Significance

Lipids are found in various forms in microbes, and each class of microorganisms has a distinct and characteristic lipid composition. They are mainly associated with membranous structures of both bacteria and archaea which has been described in great detail in Chap. 2: Prokaryotic Cell Structure. In case of gram-positive bacteria and archaea, lipids are present in the cytoplasmic membrane, whereas in gram-negative bacteria, lipids are an integral part of the outer membrane as well along with cytoplasmic membrane. Some photosynthetic bacteria also possess lipids in the intracytoplasmic membranes. Apart from this, there are other smaller molecules of lipids which are involved significantly in various cellular processes such as being an electron carrier, enzyme cofactor and light-absorbing pigment.

Few of the important biological functions of lipids are listed below:

1. **Membrane structure**: Membrane lipids constitute a major structural component of biological membranes. They are amphipathic molecules containing both hydrophilic heads and hydrophobic tails forming bilayers. The five major types of membrane or structural lipids include glycerophospholipids, galactolipids and sulfolipids, archaeal lipids, sphingolipids and sterols. The amount and distribution of each lipid varies in different classes of organisms.
2. **Signaling**: Lipids play a vital role in cell signaling. For instance, ceramide derived sphingolipids are involved in the regulation of calcium mobilization, cell growth and apoptosis. Prostaglandins are another type of fatty acid derived eicosanoids that are involved in inflammation and immunity.
3. **Virulence factors**: One of the detrimental functions of lipids is that they act as virulence factors for certain microorganisms. For example: Lipid A, present on the outer surface of gram-negative bacteria, functions as an endotoxin and is responsible for symptoms associated with infection by gram-negative bacteria such as fever, inflammation and blood clotting.

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_18

4. **Other functions**: Isoprene-based lipids such as vitamin A, D, E and K, also known as fat soluble vitamins, are commonly found in liver and fatty tissues of the body and exhibit diverse range of functions. They act as cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors for proteins, chaperone to help membrane proteins fold, emulsifying agents in digestive tract and hormones.

2 Bacterial Lipids

Cytoplasmic membranes of bacteria act as permeability barriers to polar solutes that are present in an aqueous environment. This characteristic is attributed to the presence of amphipathic molecules called phospholipids or phosphoglycerides. Common phospholipids found in membranes of most bacteria are phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and phosphatidic acid. Monounsaturated fatty acids and ring-containing fatty acids impart flexibility and fluidity to cell membranes, and their ratio varies in response to environmental conditions to maintain membrane fluidity and permeability. Generally, bacterial membranes do not have sterols; however, many contain sterol-like molecules called hopanoids which also influence the permeability of the membrane.

Gram-positive and negative bacteria vary in their lipid composition. Cell surface of gram-positive bacteria contains lipoteichoic acid and glycosyldiglycerides. Glycosyldiglycerides contain glucose, galactose or mannose. Lipoteichoic acid is the major component of the cell wall. Its composition varies among different genera of gram-positive bacteria. It may contain long chains of ribitol or glycerol phosphate. It is anchored to the cell membrane via diacylglycerol.

Outer surface of gram-negative bacteria has lipopolysaccharide (LPS) which is absent in gram-positive bacteria. LPS is also known as lipoglycan and endotoxin. It consists of a lipid, core polysaccharides and O-antigen joined by a covalent bond. A highly conserved component of the LPS is lipid A. Lipid A is a phosphorylated glucosamine disaccharide with multiple fatty acids which anchor the LPS into the bacterial membrane. This lipid A domain is responsible for the toxicity of gram-negative bacteria. The low molecular weight form of bacterial LPS is called lipooligosaccharide (LOS). It lacks the O-antigen and contains only lipid A and oligosaccharide core. It is present in *Neisseria* and *Haemophilus* species and plays an important role in their pathogenesis. One such lipid antigen is cardiolipin (diphosphatidylglycerol) which is an important part of the membrane of *Haemophilus parainfluenzae*.

Some bacteria have a unique lipid composition. The most prominent examples are provided below:

1. **Anaerobic bacteria harboring plasmalogens in their lipids**: Genera of anaerobic bacteria are uniquely enriched in ether lipids called plasmalogens. Plasmalogens have ether linkage between fatty acid and C-1 of glycerol. Strict anaerobes from the genera *Clostridium*, *Desulfovibrio*, *Propionibacterium*, *Treponema* and *Bacteroides* all contain plasmalogens. The membranes of halophiles also contain a large proportion of these ether lipids. The major components of the plasmalogens of *Clostridium butyricum* are saturated C_{16} and C_{18} fatty acid (16:0 and 18:0), monounsaturated C_{16} and C_{18} fatty acid (16:1 and 18:1), C_{17} and C_{19} cyclopropane fatty acid (17:cyc and 19:cyc).
2. **Mycoplasmas with sterols and glycolipids**: Mycoplasmas are some of the smallest organisms known. These bacteria lack cell wall and resemble protoplasts. However, they are quite resistant to osmotic stress due to the presence of sterols in their cytoplasmic membranes. Because of this, many mycoplasmas require sterols in their growth media. Some mycoplasmas also contain lipoglycans, long-chain heteropolysaccharides covalently linked to membrane lipids. These lipoglycans help in stabilizing the cytoplasmic membranes and also aid in attachment to host cell surface receptors.
3. **Mycobacteria with mycolic acids**: *Mycobacterium* contains unique lipids called mycolic acids which are a group of complex branched-chain hydroxylated lipids covalently bound to the cell wall peptidoglycan. The cell surface has a waxy consistency and does not stain well with gram's stain. Instead, *Mycobacterium* can be stained by Ziehl–Neelsen stain, in which the 'acid-fast' mycobacteria are stained bright red and stand out clearly against a blue background. These bacteria are termed 'acid fast' because they are able to resist decolorization with acid alcohol. *M. tuberculosis* is characterized by formation of long cord-like structures due to the presence of a glycolipid called the cord factor on the cell surface which leads to side-to-side aggregation and intertwining of long chains of bacteria. Cord factor is a trehalose dimycolate, i.e., it contains two molecules of mycolic acid attached to trehalose. The side chain composition of cord factor can slightly vary from $C_{24}H_{49}$ in case of human *M. tuberculosis* to $C_{22}H_{45}$ in avian *M. tuberculosis*.
4. **Bacteria with sphingolipids**: Bacteroidetes are one of the few groups of bacteria containing sphingolipids that are common in mammalian tissues. Synthesis of sphingolipids is seen in various genera of this phylum such as *Bacteroides*, *Flectobacillus*, *Prevotella*, *Porphyromonas* and *Sphingobacterium.*
5. **Corynemycolenic acid in *Corynebacterium***: Corynemycolenic acid is a unique fatty acid found in *Corynebacterium* species.
6. ***Deinococcus radiotolerans*with palmitoleic acid**: The organism is a thermophile that has plasma membrane with large amounts of palmitoleic acid rather than phosphatidylglycerol phospholipids.
7. **Hyperthermophilic bacteria with ether lipids**: Thermodesulfobacteria are hyperthermophilic bacteria with optimal growth temperatures above 80 °C. *Thermodesulfobacterium*, an obligate anaerobe, is a thermophilic sulfate reducer which is characterized by the presence of ether-linked lipids, a hallmark

of archaea. In contrast to archaea, however, the glycerol side chains of the ether-linked lipids in *Thermodesulfobacterium* are not phytanyl groups but are composed of a unique C17 hydrocarbon along with some fatty acids.

3 Archaeal Lipids

Archaeal domain is composed of diverse group of organisms that can live and thrive under extreme environmental conditions, for example, high temperatures, high salt concentrations and acidic hot springs.

In contrast to eubacteria which have ester linked glycerol lipids, archaebacteria have ether-linked membrane lipids, as also discussed in Chap. 2: Prokaryotic Cell Structure. The hydrophobic core of their membrane contains long-chain (32 carbons) branched hydrocarbons that are linked to glycerol moiety. Phytanyl diethers in which two C_{20} phytanyl chains are ether linked to glycerol can be regarded as universal components in archaeal cell membrane. In most of the archaea such as methanogenic, thermophilic and thermoacidophilic archaea, these diphytanyl groups are ether linked to two glycerol molecules. Exception to these structures are present in haloalkalophilic archaea which have diethers with a C_{20} phytanyl and a C_{25} sesterterpanyl chains and some with two C_{25} sesterterpanyl chains, in addition to the usual diethers. Longer acyl chains and dual head groups can replace two normal phospholipids forming a monolayer instead of a bilayer. These are twice the length of phospholipids and sphingolipids and span the width of the surface membrane.

Some examples of archaea with unique arrangement of lipids are as follows:

1. Thermophilic methanogen, *Methanocaldococcus jannaschii*, contains a diether possessing a C_{40} biphytanyl macrocyclic loop as a major component.
2. *Methanopyrus*, another hyperthermophilic methanogen, contains membrane lipids not found in any other known organism. The ether-linked lipids of *Methanopyrus* are an unsaturated form of the otherwise saturated dibiphytanyl tetraethers found in all other hyperthermophilic archaea. These unusual lipids may help in membrane stabilization at the extremely high growth temperatures.
3. *Picrophilus* is an extreme acidophile that grows optimally at pH 0.7 and can also grow at pH 0. Its cytoplasmic membrane is characterized by an unusual lipid arrangement that forms a highly acid-impermeable membrane at low pH values.
4. Thermoplasmas are thermophilic and extremely acidophilic. They lack cell walls and have evolved a unique cytoplasmic membrane structure to withstand stress due to low pH and high temperature. Their membranes contain a lipopolysaccharide-like material called lipoglycan which consists of a tetraether lipid monolayer membrane with mannose and glucose. Lipoglycans constitute a major portion of the total lipids. Their cell membranes are strengthened by large quantities of caldarchaeol, lipid containing polysaccharides.

4 Biosynthesis of Lipids

Fatty acids are the basic components of lipids. Thus, fatty acids are first synthesized and are then condensed to form different types of lipids as per the cellular requirement. Fatty acid biosynthesis is well studied in prokaryotes, eukaryotes and animals. Fatty acid synthesis occurs as a result of surplus carbon which is accumulated in the form of storage lipids that act as energy reservoirs. Further, it is a crucial pathway for the synthesis of phospholipids which need to be synthesized de novo in bacteria during growth.

Fatty acids are carboxylated acyl chains that grow by condensation of 2-carbon units each time, i.e., acetyl CoA. In fact, if one looks closely at saturated fatty acids, we will find they are generally even numbered. The acetyl CoA units are condensed and subsequently reduced to grow as a fatty acid chain. Thus, the basic requirement of fatty acid synthesis is the availability of acetyl CoA and reductant NADPH. Fatty acid biosynthesis occurs in cytosol, and acetyl CoA should be either generated in the cytosol or transported to the cytosol, as is the case in eukaryotes.

1. **Source of acetyl CoA**—The major source of acetyl CoA is pathways of carbohydrate oxidation viz. glycolysis and TCA cycle or pathways of protein oxidation, i.e., degradation of amino acids. In prokaryotes, all these reactions take place in the cytosol, and thus, they are directly utilized for fatty acid synthesis if they are in surplus. However, in eukaryotes, pyruvate oxidation to acetyl CoA via the action of pyruvate dehydrogenase complex takes place in the mitochondria. Additionally, mitochondria are the sites for generation of acetyl CoA via other metabolic routes also viz. amino acid degradation and fatty acid degradation. Thus, acetyl CoA needs to be transported out from mitochondria in eukaryotes. Acetyl CoA cannot cross the mitochondrial membrane and can only be transported out of the mitochondrial matrix as citrate. Citrate transport is only possible when there is accumulation of citrate as a result of low isocitrate dehydrogenase activities which happens when ATP is high or cell has high energy charge. The citrate is transported out and is cleaved in cytosol by ATP-**citrate lyase** to generate oxaloacetate and acetyl CoA with the consumption of one mole of ATP. Oxaloacetate generated in the cytosol returns back to mitochondria either in the form of malate or pyruvate as a result of malate dehydrogenase and malic enzyme activities, respectively (Fig. 1).
2. **Source of NADPH**—The main sources of reductant are glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of the pentose phosphate pathway. Further, in eukaryotes, malic enzyme also generates NADPH.
3. **Fatty acid synthesis**—Fatty acid synthesis in bacteria and eukaryotes is by and large similar with differences in the final product. Bacteria generally synthesize short chain length saturated fatty acids, are rich in monoenoic fatty acids and lack polyunsaturated fatty acids. Also, there are few other modifications that lead to the synthesis of hydroxylated fatty acids, branched fatty acids, etc. In bacteria, unsaturation of fatty acids takes place during the process when fatty acids are being elongated, while in eukaryotes, unsaturation is an independent event and

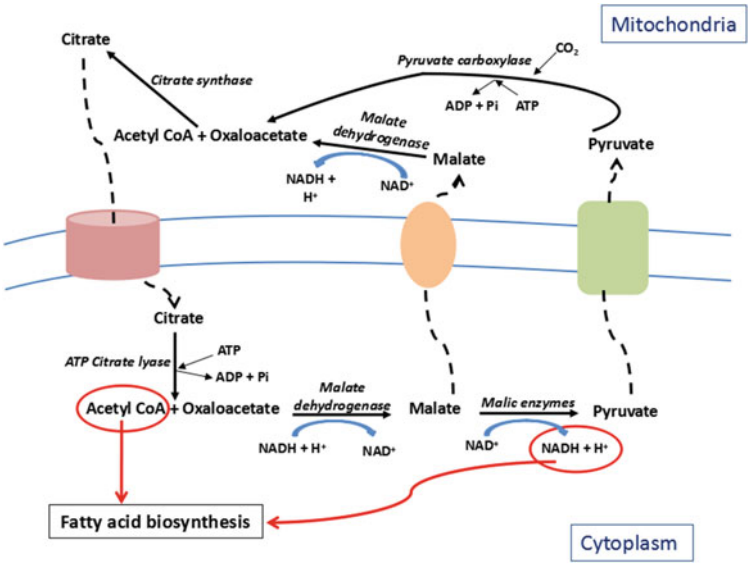


Fig. 1 Acetyl CoA transport in eukaryotes

takes place once synthesis of saturated fatty acid is complete. Fatty acid biosynthesis requires at least eight serial reactions to yield the first product (4-carbon butyryl-S-ACP) and requires the concerted activities of several enzymes which form part of the fatty acid synthase (FAS) complex. Prokaryotes have FAS Type II, while eukaryotes have FAS Type I. Although reactions catalyzed by Type I and Type II fatty acid synthases are similar, they have distinct structural differences. In eukaryotes, fatty acid synthase is in the form of a multi-enzyme complex, while it exists as easily dissociable subunits in prokaryotes such as in *Escherichia coli*. The differences between the two classes of enzymes have been listed in Table 1. These unique differences between Type I and II synthases make them prospective drug targets, and inhibitors to Type II synthases can act as antibiotics. An important difference between prokaryotic and eukaryotic biosynthesis is the requirement of small acyl carrier protein (ACP) in bacteria which ensures mobility of fatty acyl chain from one enzyme to another till completion, since fatty acid synthase is a loose complex in prokaryotes. However, in eukaryotes, ACP is not required. Instead, a molecule of 4′-phosphopantetheine covalently binds to a domain present in the multi-enzyme FAS complex of eukaryotes.

Various steps involved in synthesis of saturated fatty acids

1. Activation of acetyl CoA to malonyl CoA
2. Transacylation of acetyl CoA and malonyl CoA to ACP
3. Initiation by condensation of acetyl CoA and malonyl CoA

4. Reduction and dehydration of β-keto group
5. Elongation of fatty acid chain length
6. Termination and release of product.

Step 1—Activation of acetyl CoA to malonyl CoA

A fatty acid is a polymer of acetyl CoA; however, one molecule of acetyl CoA cannot directly bind to another molecule of acetyl CoA. For this binding, it is important to activate the methyl group of acetyl CoA. This activation is by carboxylation of the methyl group to form malonyl CoA. This is the first committed step of fatty acid synthesis and involves biotin-dependent **acetyl CoA carboxylase (ACC)**. ACC is not a dedicated enzyme of this pathway as it is required in several other reactions such as during synthesis of polyketides. Acetyl CoA carboxylase enzyme incorporates inorganic carbonate to activate methyl end of acetyl CoA. ACC carboxylase of *E. coli* comprises three enzymes: biotin carboxylase (two subunits of 49,000 Da each); biotin carboxyl carrier protein (22,500 Da) and carboxyltransferase (130,000 Da). The reaction is catalyzed in three steps as depicted in Fig. 2.

1. The first reaction is carried out by biotin carboxylase (BC) and involves the ATP-dependent carboxylation of biotin present in biotin carboxyl carrier protein (BCCP) with bicarbonate serving as the source of CO_2.
2. The second reaction is catalyzed by carboxyltransferase (CT) and involves the transfer of the carboxyl group of biotin to acetyl CoA to form malonyl CoA.

Step 2—Transacylation of acetyl CoA and malonyl CoA to acyl carrier protein

The sequence of reactions after carboxylation of acetyl CoA is catalyzed by various subunits of fatty acid synthase Type I (FAS I) in eukaryotes and Type II (FAS II) in prokaryotes. The key feature of fatty acid biosynthesis is that all the intermediates are bound to a small acidic highly soluble protein called acyl carrier protein (ACP).

Table 1 Differences between Type I and Type II fatty acid synthases

Fatty acid synthases	Type I synthase (FAS I)	Type II synthase (FAS II)
Occurrence	Eukaryotes, CMN group of bacteria (Corynebacterium, Mycobacterium, Nocardia)	Prokaryotes
Organization of enzymes	Integrated	Discrete
Enzymes	Multifunctional enzymes	Monofunctional enzymes
Enzyme complex	Tight	Loose
Requirement for ACP	Not always	Yes
Examples	FAS complex in yeasts is a multi-enzyme complex and exists as a dimer of two giant polypeptides that cannot be dissociated	*E. coli* FAS is a soluble complex formed by seven individual enzyme components which can be isolated separately

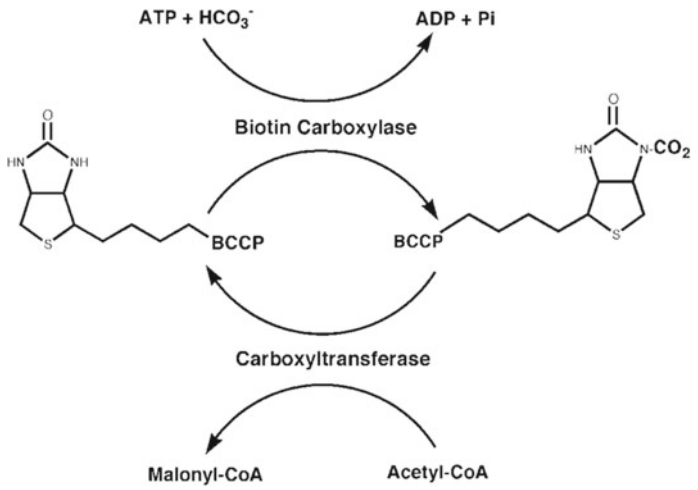


Fig. 2 Reaction mechanism of bacterial acetyl CoA carboxylase. *Source* Salie and Thelen (2016). With kind permission from Elsevier

ACP is the most abundant protein of *E. coli* and constitutes around 0.25% of all soluble proteins. ACP is an absolute requirement of *E. coli*, and its deletion markedly affects bacterial growth. The transfer of acyl group to ACP is facilitated by 4′-phosphopantetheinyl transferase which transfers acyl-4′-phosphopantetheine (4′-PP) moieties to apo-ACP releasing 3′,5′-ADP from CoA.

A molecule of acetyl CoA is transferred to SH of ACP to form acetyl-S-ACP, and likewise a molecule of malonyl CoA is transferred to SH of ACP to form malonyl-S-ACP. These reactions are catalyzed by acetyl CoA-ACP transacylase and malonyl CoA-ACP transacylase, respectively.

$$\text{Acetyl CoA} + \text{ACP-SH} \rightarrow \text{Acetyl-S-ACP} + \text{CoA}$$

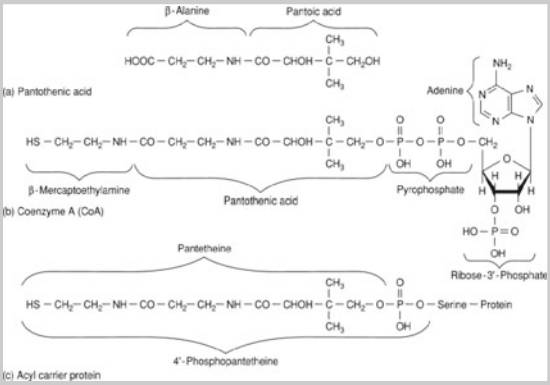
$$\text{Malonyl CoA} + \text{ACP-SH} \rightarrow \text{Malonyl-S-ACP} + \text{CoA}$$

In *E. coli*, the transacylation is carried out by β-ketoacyl-ACP synthase that carries out the following reaction. Thus, in *E. coli*, acetyl CoA is added without getting transferred to ACP protein.

$$\text{Acetyl CoA} + \text{Malonyl-S-ACP} \rightarrow \text{Acetoacetyl-S-ACP} + \text{CoA} + CO_2$$

Box 1: ACP and CoA Have Similar Components

Acyl carrier protein is covalently attached by hydroxyl of serine to phosphopantothenic acid which has mercaptoethylamine attached to it to ensure thioester formation with fatty acid. Pantothenate ensures movement of growing chain. It is similar to CoA in structure where adenosine diphosphate is replaced by acyl carrier protein.



Structure of **a** pantothenic acid; **b** coenzyme A (CoA); and **c** acyl carrier protein (ACP). *Source* Ball (2003). With kind permission from Elsevier

Step 3—Condensation of acetyl-S-ACP and malonyl-S-ACP

This is the first step in biosynthesis of fatty acid chain length once the precursors are available. The acetyl-S-ACP and malonyl-S-ACP condense to form 4-C acetoacetyl-S-ACP or β-ketoacyl-S-ACP by the enzyme β-ketoacyl-ACP synthase III (encoded by the *FabH* gene). Here, methylene of malonyl functions as a nucleophile and displaces ACP from acetyl-ACP. Note that in *E. coli*, this condensation has already taken place at Step 2 itself. The reaction is accomplished by release of energy and the removal of carboxylate as CO_2 (Fig. 3).

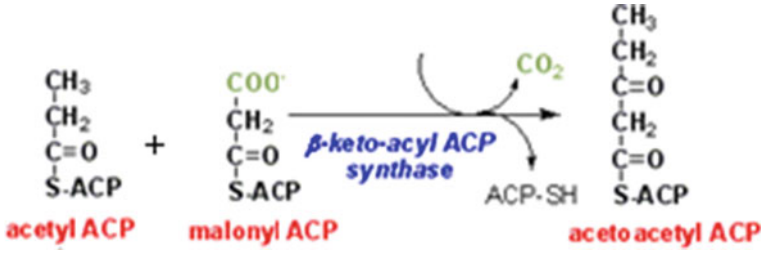


Fig. 3 Condensation of acetyl-S-ACP and malonyl-S-ACP

It is interesting to note that branched-chain fatty acids are pre-destined in the first step itself as in the cases where branched fatty acids are synthesized along with straight chain fatty acids, the first carbon in place of acetyl CoA is replaced by isovaleryl CoA, isobutyryl CoA, 2-methyl butyryl CoA and so on. There are homologues of FabH in *Bacillus subtilis* which accepts the branched acetyl CoA, while *E. coli* FabH does not accept branched acetyl CoA but can accept propionyl CoA giving generation of odd-numbered fatty acids.

Step 4—Conversion of β-ketoacyl-ACP to saturated 4 carbon acyl-ACP

After condensation of 2C units, a set of three reactions convert β-ketoacyl-ACP to saturated 4 carbon acyl-ACP by a series of reduction, dehydration and reduction. The β-keto group is reduced at the expense of NADPH in presence of β-ketoacyl-S-ACP reductase (coded by *FabG* gene). The enzyme shows activity with different chain length ketoacyl-ACPs with more preference for longer chains. It does not accept NADH + H^+ at all. Over-expression of *FabG* leads to enhanced synthesis of C14 and C16 fatty acyl-ACPs.

Reaction 1: β-ketoacyl-ACP reductase (FabG) reduces keto group to alcohol by using NADPH as reductant (Fig. 4).

Next step in fatty acid synthesis is dehydration of hydroxylated acyl chain leading to enoyl synthesis and release of water molecules. This reaction is catalyzed by FabZ (β-hydroxyacyl-ACP dehydratase I).

Reaction 2: β-hydroxyacyl-ACP dehydratase I (FabZ) dehydrates β-hydroxyacetyl-ACP to generate double bond (Fig. 5).

Enoyl-ACP reductase I (encoded by *FabI*) activity is the last step in the completion of fatty acid chain. It catalyzes reduction of enoylacyl-ACP at the expense of NADPH + H^+, and it is highly selective enzyme and has no other homologues. Therefore, it is an essential enzyme if cultures are not fed with fatty acids. This enzyme is the determining step and is responsible for completion of synthesis.

Reaction 3: Enoyl-ACP reductase I (FabI) reduces the double bond by using another molecule of reductant to form saturated 4C acyl chain, i.e., Butyryl-S-ACP (Fig. 6).

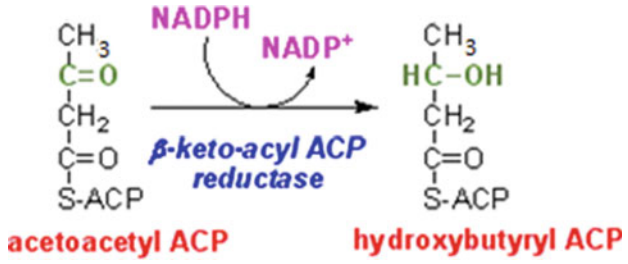


Fig. 4 Reduction of acetoacetyl ACP

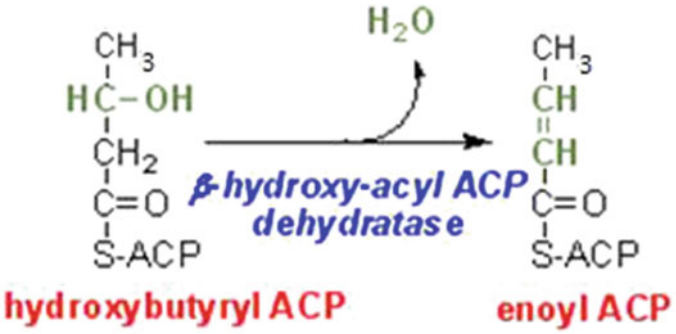


Fig. 5 Synthesis of enoyl-ACP

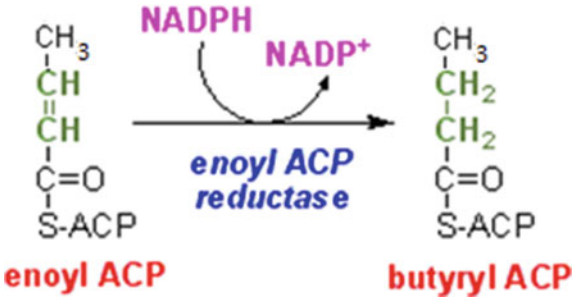


Fig. 6 Reduction of enoyl-ACP to butyryl-S-ACP

Finally, as a result of six reactions, the first product, butyryl-S-ACP (4-C), is generated.

Chain elongation

Chain elongation takes place by condensation of malonyl-ACP with 4C butyryl-S-ACP with liberation of CO_2. Thus, the fatty acid chain is elongated by two carbons each time via the six recurring reactions until the 16-carbon palmitic acid is produced. This is followed by release of ACP thioester facilitated by palmitoyl thioesterase. This process is the same in prokaryotes and eukaryotes with the difference being in the FAS enzyme complex. Thus, to generate a 16-carbon fatty acid (palmitic acid), 1 acetyl and 7 malonyl are utilized, and two carbons of acetyl form methylene of fatty acid (Fig. 7).

Stoichiometry of fatty acid biosynthesis for a 16C palmitic acid

For synthesis of one mole of palmitic acid, we would require 1 mol of acetyl CoA and 7 mol of malonyl CoA, i.e., expenditure of 7 ATP (during conversion of acetyl CoA to malonyl CoA) and 14 NADPH (7 rounds of chain elongation each requiring

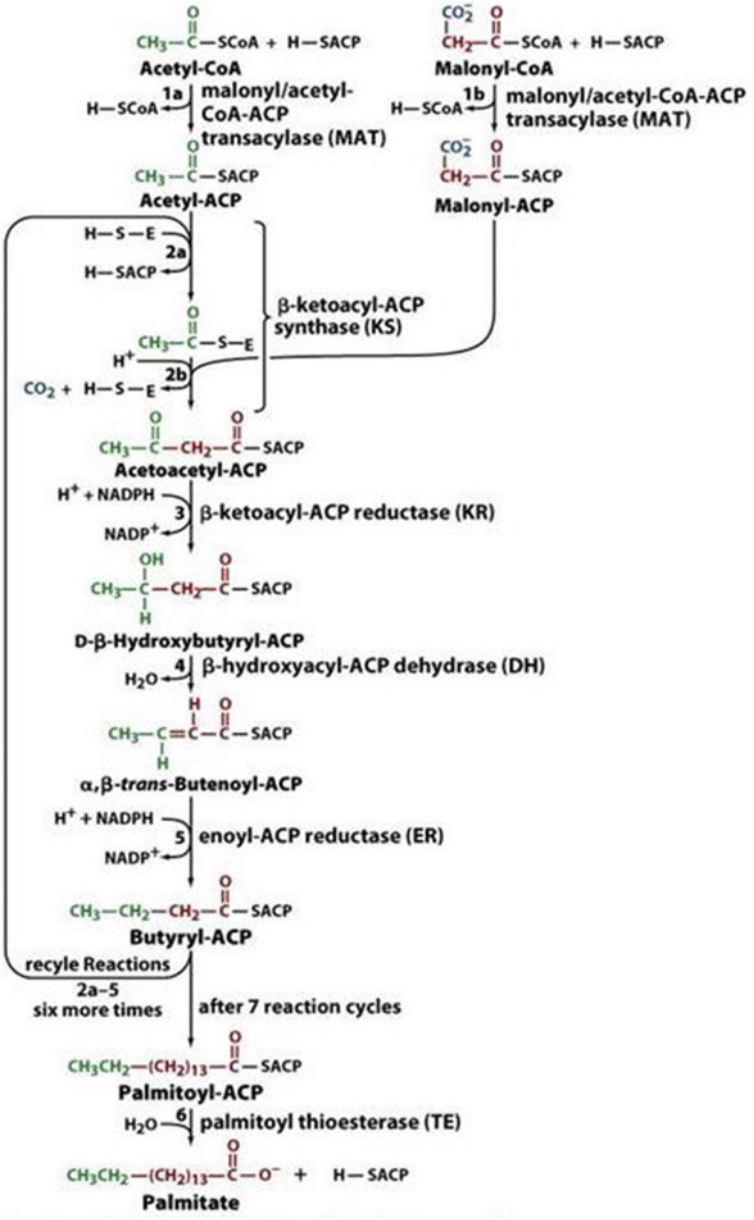


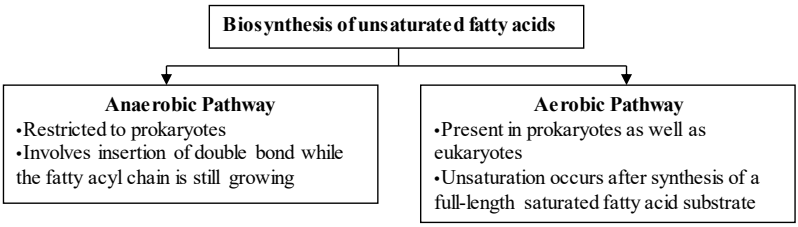
Fig. 7 Chain elongation reaction sequence for fatty acid biosynthesis. *Source* Voet et al. (2016). With kind permission from John Wiley & Sons

two reductions). Overall, fatty acid synthesis is a very energy intensive process, and thus, lipids are sinks of energy and reductants.

4. Biosynthesis of unsaturated fatty acids

Unsaturated fatty acids are important for maintenance of membranes. Bacteria generally have monosaturated fatty acids, while polyunsaturated fatty acids are common in eukaryotes. The unsaturation of fatty acids can be both anaerobic and aerobic pathway.

Anaerobic pathway is restricted to prokaryotes, while aerobic pathway is present in both prokaryotes and eukaryotes.



Aerobic pathway for unsaturated fatty acids

The aerobic pathway for unsaturation of fatty acid is widespread among all eukaryotes and some prokaryotes. This pathway uses special desaturases, called oxidases, which introduce unsaturation in saturated fatty acid substrates. These desaturases use O_2 and NADH for reduction and release of water molecule. The electrons from fatty acyl derivative and two electrons from reductant combine with O_2 to form two molecules of H_2O. Desaturases are specific for the double bond they induce in the substrate. Bacteria produce oleic acid (C-18 with a double bond between C9 and C10) by this pathway (using C18 saturated stearic acid) (Fig. 8). The reducing equivalents are

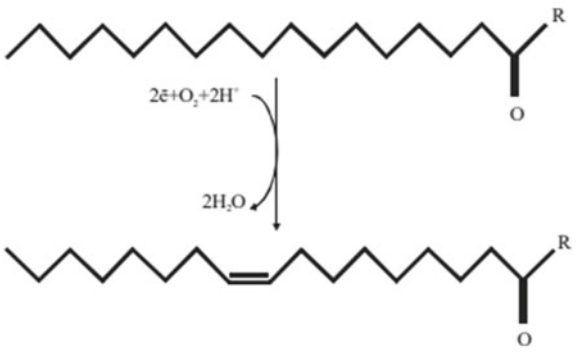


Fig. 8 Desaturation reaction catalyzed by the aerobic desaturase enzyme. *Source* Garba et al. (2017). (Creative Commons License)

derived either from cytochrome b5 (plants, fungi and animals) or from ferredoxins (plant plastids and bacteria) (Garba et al., 2017).

Box 2: Regulation of Fatty Acid Desaturases by Two-Component System

Bacteria like *Bacillus subtilis* are poikilothermic organisms, and when they are exposed to suboptimal growth temperatures, their membrane lipids become more rigid, leading to subnormal functioning of cellular activities (Phadtare, 2004). Bacteria can adapt to such conditions by altering the composition of their membranes. In case of low temperature, bacteria start increasing the proportion of unsaturated fatty acids (UFAs) in their membrane so that membrane lipid fluidity can be restored to some extent (Mansilla et al., 2004; Phadtare, 2004). For this purpose, bacteria utilize their desaturase enzymes which catalyze the synthesis of UFAs.

But how these desaturase enzymes come to know when there is a need?

To signal desaturase enzyme over-expression, a two-component system comes into action during changes in temperature conditions. For example, *Bacillus subtilisin* has a two-component system known as DesK/DesR function to switch on/off the gene coding for desaturase enzyme Δ5 desaturase (Aguilar et al., 2001). Δ5 desaturase is a product of *des* gene which is regulated by DesK/DesR system. Here, DesK is a sensor kinase and contains five transmembrane helices and a long cytoplasmic C-terminal tail, which harbors the kinase domain, DesKC, while DesR is the response regulator. Phosphorylation of DesR by DesK kinase activity leads to the formation of a stable tetramer which binds to *des* promotor and activates transcription (Cybulski et al., 2004).

Anaerobic pathway for unsaturated fatty acids

The anaerobic pathway is utilized by many bacteria for synthesizing unsaturated fatty acids. This pathway is well understood in *E. coli* and was first put forth in 1960. This pathway does not utilize oxygen and is dependent on enzymes to insert the double bond before elongation utilizing the normal fatty acid synthesis machinery. Thus, unsaturation occurs simultaneously as the fatty acyl chain is still growing. A special desaturase **β-ketoacyl-ACP synthase II (FabA) (Fab A; β-hydroxydecanoyl-ACP dehydratase)** competes with hydroxydecanoyl-ACP dehydratase and carries out dehydration of β-hydroxydecanoyl-ACP (10-carbon) to an intermediate trans α, β-decanoyl-ACP which is then isomerized to cis-β,γ-decenoyl ACP. The cis double bond acyl ACP is not a substrate for enoyl-ACP reductase and thus cannot be reduced. It can only be elongated further by Fab B elongase **(β-ketoacyl-ACP synthase)** leading to an unsaturated fatty acid (Fig. 9). This pathway is used for synthesis of two important monoenoic acids, palmitoleic acids (C16 Δ^9-hexadecenoic acid) and cis-vaccenic acid (C18 Δ^{11}-octadecenoic acid) (Feng & Cronan, 2011). The reasons for abundance of palmitate rather than stearate in *E. coli* are:

1. No β-ketoacyl-ACP synthase uses C_{18} effectively in *E. coli.*
2. β-ketoacyl-ACP synthase converts C16 Δ^9 to C18 Δ^{11} which is most abundant in *E. coli.*
3. Rate of unsaturation is also determined by the rate at which acyl ACPs are used for phospholipid synthesis.
4. It is also regulated by temperature.

5. **Synthesis of triacylglycerides from fatty acids**

To synthesis triacylglycerides, we need a glycerol backbone which is provided either by glycolysis or as a breakdown product of other lipids, etc. Triacylglyceride biosynthesis needs two precursor molecules viz. glycerol-3-phosphate and fatty acyl CoA. Glycerol-3-phosphate can be obtained by catalytic action of NAD^+-linked glycerol-3-phosphate dehydrogenase on dihydroxyacetone phosphate (DHAP). Lysophosphatidic acid can be obtained as a product either from acyltransferase reaction using glycerol-3-phosphate or directly from acyltransferase and reduction reactions using dihydroxyacetone phosphate. The resulting lysophosphatidic acid undergoes another acyltransferase reaction to form phosphatidic acid which is further catalyzed by phosphatidic acid phosphatase and acyltransferase to form triacylglycerol (Fig. 10).

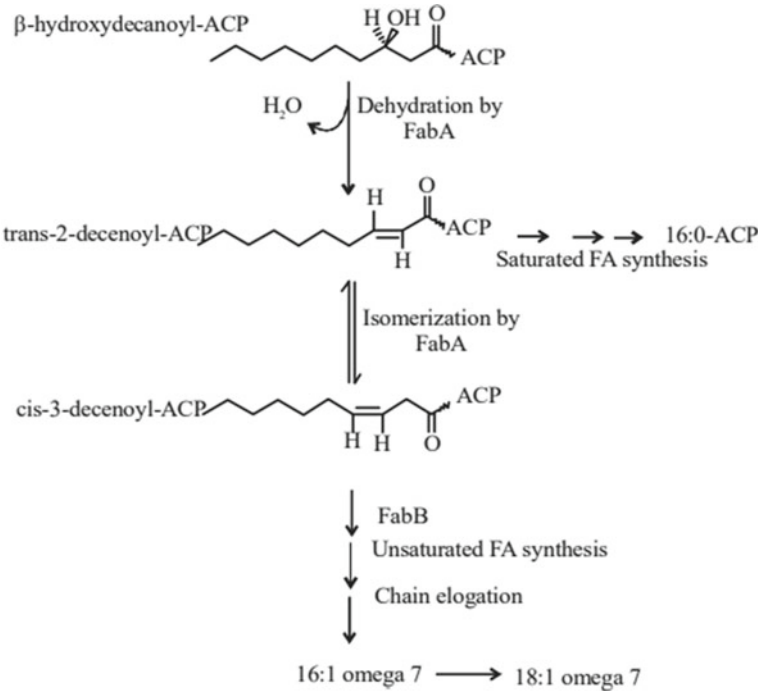


Fig. 9 Anaerobic pathway for biosynthesis of unsaturated fatty acids. *Source* Garba et al. (2017). (Creative Commons License)

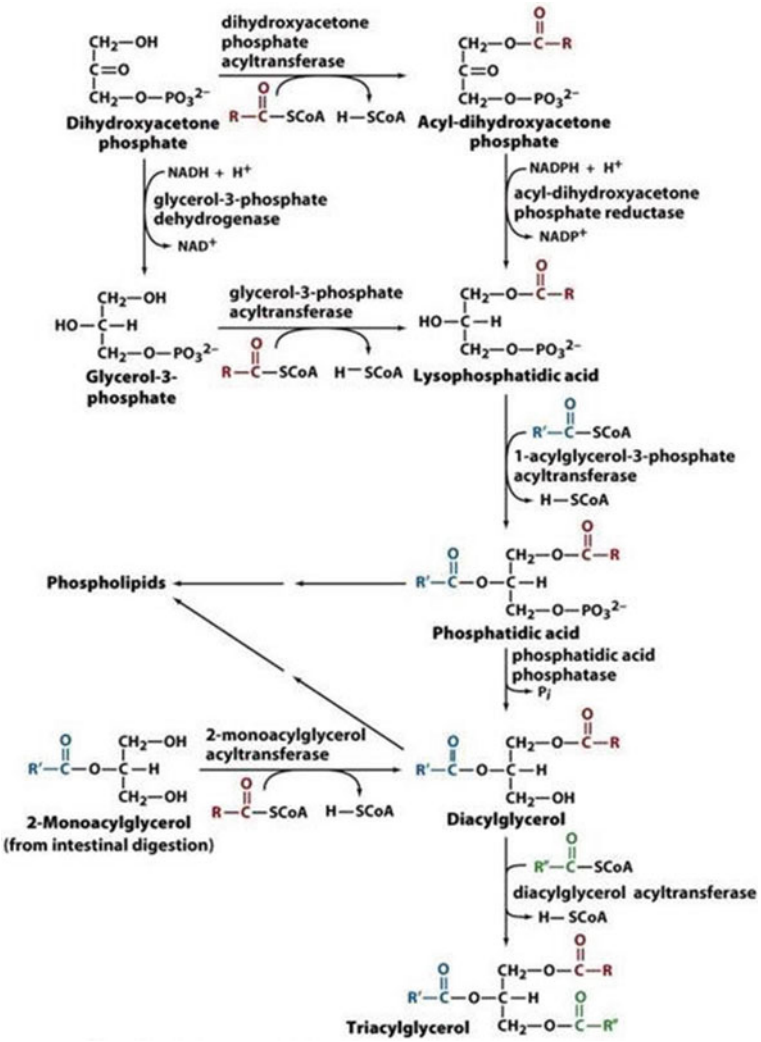


Fig. 10 Reaction pathway for triacylglycerol biosynthesis. *Source* Voet et al. (2016). With kind permission from John Wiley & Sons

6. Synthesis of phosphoglycerides

Biosynthesis of phosphoglycerides initiates with dihydroxyacetone phosphate (DHAP) by following various enzymatic steps. It requires cytidine triphosphate to synthesize CDP-diacylglycerol.

- **Glycerol phosphate dehydrogenase**: Reduction of DHAP to glycerol phosphate by the enzyme glycerol phosphate dehydrogenase.
- **G3P acyl transferases**: The transfer to glycerol phosphate of fatty acids from fatty acyl-S-ACP resulting in the formation of phosphatidic acid. The reactions are catalyzed by membrane-bound enzymes called G3P acyl transferases.
- **CDP-diglyceride synthase**: The phosphate on phosphatidic acid reacts with cytidine triphosphate (CTP) and displaces PPi to form CDP-diacylglycerol. This reaction is catalyzed by CDP-diglyceride synthase.

Following the synthesis of CDP-diacylglycerol, the pathway can take either of two directions depending on the phosphoglyceride being synthesized. Finally, synthesis of various phospholipids, e.g., phosphatidylserine, phosphatidylethanolamine and others is by displacement of CDP.

7. **Biosynthesis of archaeal lipids**

Archaeal lipids have long-chain alcohols called isopranyl alcohols and are also unique as they show ether bond rather than an ester bond. Here also, glycerol backbone is synthesized from either glycerol-3-phosphate or DHAP. However, alcohol is believed to be derived from geranylgeranyl pyrophosphate through mevalonic acid pathway which is widespread among bacteria and higher organisms (Jain et al., 2014).

5 Lipid Accumulation in Yeasts

It is well known that under nitrogen depleted conditions, few eukaryotic microorganisms can accumulate triacylglycerides as storage lipids as much as 80% of their dry weight. In such conditions, the overall synthesis of nucleic acids and proteins is much reduced. Lipid accumulating microbes are called oleaginous species. Non-oleaginous microbes hardly produce 7–8% lipid under such conditions. Under similar conditions, bacteria accumulate polyhydroxybutyrates as storage lipids.

The initial interest in lipid accumulation organisms has been due to the fact that composition of some lipids was found to be rich in polyunsaturated fatty acids which find applications as food and dietary supplements. This gave rise to the term ‘single cell oils’ for oils from microorganisms. Major source is mucorales like *Mucor circinelloides*, *Mortierella* species producing linolenic and arachidonic acid rich lipids, respectively. Among yeasts, major lipid accumulators are *Rhodotorula* species, *Lipomyces starkyi*, *Cryptococcus curvatus*, etc., which can accumulate upto 70% triglycerides. The lipid accumulation by fungi and yeasts has been known for over hundred years, but the biochemical basis for such accumulation has been understood only during the last two decades or so.

Biochemistry of lipid accumulation

Insights into biochemical basis of lipid accumulation in oleaginous yeast were obtained by a comparative study of an oleaginous yeast *Candida* 107 and a non-oleaginous yeast *Candida utilis* by Botham and Ratledge (1979). They observed no distinct difference in uptake of glucose or in lipid turnover. Further, even acetyl CoA carboxylase, a key enzyme for fatty acid synthesis, was biochemically similar in both the species except that in *Candida* 107, it was 40% activated in presence of citrate.

The key factor for lipid accumulation is nutrient depletion. Depletion of nitrogen is more important even when carbon in the form of glucose is still available. The unbalanced growth with very high C:N ratio shifts metabolism toward storage. Glucose metabolism shifts toward increased fatty acid synthesis, and protein synthesis stops due to nitrogen starvation. A stage is reached when there is no cell proliferation, and lipids are stored within the existing cells. However, this mode of metabolic shift is common to both oleaginous and non-oleaginous organisms. Therefore, it is interesting to understand the key regulatory features of oleaginous organisms that are responsible for over-synthesis of lipids. The differences need to be looked in terms of availability of precursors required for fatty acid synthesis (acetyl CoA and reductant NADPH).

1. **The first metabolic shift on nitrogen starvation is the activation of AMP deaminase** which is a key activity in oleaginous microbes that triggers lipid accumulation. It transiently supplies NH_4^+ by cleaving AMP as follows:

$$\text{AMP} \xrightarrow[\text{AMP deaminase}]{} \text{IMP} + NH_4^+$$

   - With decrease in AMP, there is sudden change in oxygen consumption and CO_2 output.
   - In oleaginous microbes, major changes are observed in isocitrate dehydrogenase activity which is inhibited as it is AMP dependent. The decrease in isocitrate dehydrogenase activity leads to accumulation of isocitrate, and an equilibrium between citrate and isocitrate is in favor of citrate via aconitase.
   - In non-oleaginous ones, there is no such dependence of isocitrate dehydrogenase on AMP.

2. **Citrate is transported out of mitochondria** via malate/citrate transporters, and citrate reaches cytoplasm.

   - Citrate is cleaved to acetyl CoA by ATP: citrate lyase (ACL) which is generally absent from non-oleaginous microbes.

$$\text{citrate} \xrightarrow[\text{CoA-SH}]{\text{ATP} \quad \text{ADP} + \text{Pi}} \text{acetyl CoA} + \text{oxaloacetate}$$

- Oxaloacetate is converted back to malate by cytoplasmic malate dehydrogenase which balances citrate efflux from mitochondria.
- There is a strong relationship between ACL activity and lipid accumulation.
- There are few exceptional yeasts which have ATP: citrate lyase activity but they do not accumulate lipid.
- Level of ACL also does not correlate with extent of lipid accumulation suggesting that ACL is an absolutely important enzyme for lipid accumulation but is not solely responsible for it.

3. **Supply of reductant**: Fatty acids are highly reduced biomolecules, and thus, the reductant NADPH is essential for their synthesis.

- The major route of NADPH generation is via malic enzymes which convert malate to pyruvate in cytoplasm.

$$\text{malate} + \text{NADP}^{+} \longrightarrow \text{pyruvate} + \text{NADPH} + \text{H}^{+}$$

- Malic enzymes have been found in majority of oleaginous microorganisms except a few organisms such as *Lipomyces* sp. and some *Candida* sp. where there are alternate modes of NADPH generation via cytosolic NADPH-dependent isocitrate dehydrogenase.
- It is important to note that there no such pool of NADPH is available in oleaginous microbes.

6 Fatty Acid Biosynthesis Pathway Enzymes as Drug Targets

Fatty acid biosynthesis is carried out by several enzymes which are essential for bacterial viability and growth. Thus, these enzymes have been explored as drug target sites. Structural availability has made it possible to design drugs. Following are some of the most potential inhibitors of fatty acid synthesis pathway which target different enzymes of the pathway. A comprehensive review on this has been presented by Heath et al. (2002) (Fig. 11).

1. Reaction intermediate analog BP1 (inhibitor of acetyl CoA carboxylase): It is a synthetic reaction intermediate analogue derivative of biotin that inhibits the biotin carboxylase subunit (AccC) of acetyl CoA carboxylase (Blanchard et al., 1999).
2. Moiramide B and its derivatives (inhibitor of acetyl CoA carboxylase): It is a broad-spectrum natural pseudopeptide pyrrolidinedione antibiotic that targets the carboxyltransferase reaction of acetyl CoA carboxylase enzyme with a competitive inhibition pattern versus malonyl CoA (Ki value = 5 nM) (Freiberg et al., 2004, 2006).

3. Methyl-CoA disulfide (inhibitor of FabH): It is a mechanism-based inhibitor of β-Ketoacyl-ACP-synthase III (FabH) that irreversibly inactivates the catalytic cysteine of the enzyme which is responsible for acyl transfer (Alhamadsheh et al., 2007).
4. Cerulenin (inhibitor of FabB/FabF): It is a hydrophobic epoxide that targets the hydrophobic groove of the acyl site and reacts covalently with the active site cysteine of β-ketoacyl-ACP-synthase I (FabB) or β-ketoacyl-ACP-synthase II (FabF) and block the interaction with malonyl CoA (Omura, 1976).
5. Thiolactomycin (inhibitor of FabB, FabF and FabH): It is also a natural product that irreversibly inhibits FabB, FabF and FabH, condensing enzyme of fatty acid synthesis pathway. It acts by binding malonyl-ACP site of the enzymes (Price et al., 2001).
6. 3-decynoyl-N-acetylcysteamine (NAC) (inhibitor of FabA): It is a mechanism-based suicide inhibitor of β-Hydroxydecanoyl-ACP dehydratase/isomerase (FabA) (Helmkamp et al., 1968).
7. Triclosan (inhibitor of FabI): It is a chlorinated *bis*-phenol that reversibly inhibits trans-2-enoyl-ACP reductase I (FabI) enzyme. It binds to enzyme-cofactor complex and forms a stable ternary complex of triclosan-NAD^+-FabI (Ward et al., 1999).
8. Diazaborine (inhibitor of FabI): Forms covalent adducts with NAD^+ 2′-ribose hydroxyl group of FabI, and this complex formation leads to formation of the substrate-binding loop that covers the active site in the ternary complex (Grassberger et al., 1984).
9. Isoniazid (inhibitor of InhA) analogue of FabI in *E coli*: It is a prodrug that needs activation by mycobacterial catalase-peroxidase and inhibits *M. tuberculosis* InhA enzyme that performs same function as FabI in *E. coli*. It reacts irreversibly with NAD^+ (Quemard et al., 1995).

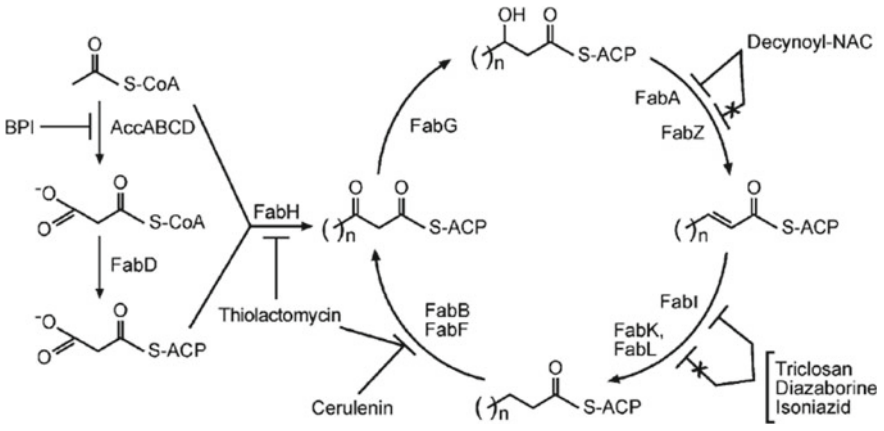


Fig. 11 Inhibitors of fatty acid synthesis pathway. *Source* Heath et al. (2002). With kind permission from Springer Nature

7 Lipid Degradation

Microorganisms can utilize a variety of lipids such as triglycerides, waxes, hydrocarbons as a source of energy and carbon. However, microorganisms use these only in absence of other readily assimilable carbon sources such as sugars. Thus, lipid degradation is usually under catabolite repression. For the uptake of lipids, microorganisms have devised various methods such as production of surfactants which emulsify these compounds and form micelles that facilitate transport inside the cells. Besides this, microorganisms also produce cell bound and extracellular esterases and lipases that cleave the lipids into simpler glycerides and fatty acids that can be easily taken up by the cell. Glycerol is metabolized by EMP pathway, while fatty acids are degraded to CO_2 and H_2O.

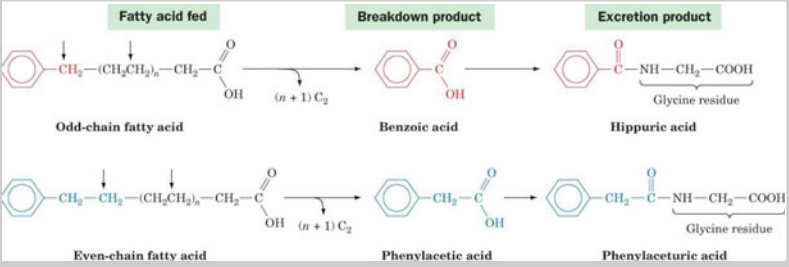
Overall, lipid degradation is a major source of energy gain and biomass production in absence of easily assimilable sugars. Fatty acid oxidation results in production of acetyl CoA, which is finally oxidized by TCA cycle and releases energy through generation of reductants.

Fatty Acid Degradation—Major source of fatty acids is lipids available in the environment or fed in the culture medium. The lipids in the form of triacylglycerides and phospholipids are degraded by lipases and phospholipases, respectively, and fatty acids are released which are oxidized to release energy and also to produce glucose by gluconeogenetic pathway. There are three types of fatty acid oxidation, i.e., alpha, beta and omega depending on the position of carbon where cleavage occurs.

- **Alpha oxidation** occurs at the alpha carbon. It is a minor pathway for fatty acid oxidation and occurs in two cases: either when beta carbon is blocked as in phytanic acid or in case of lipids with long-chain fatty acids. The process involves oxidation of alpha carbon by hydroxylation and final release of CO_2 from terminal carboxyl end and thus shortening acyl chain by one carbon each time. Final acyl chain will be degraded by beta-oxidation.
- **Beta-oxidation** occurs at the carbon that is beta to the carboxyl carbon. Beta-oxidation is the most common and ubiquitous in occurrence. This pathway will be studied in more details in the later part of this chapter.
- **Omega oxidation** occurs at methyl end in case of medium and long-chain fatty acids. This pathway involves an oxidase that uses molecular oxygen, and both an alcohol and aldehyde dehydrogenase to produce a molecule with a carboxyl group at each end. The net result is dicarboxylic acids which are then subjected to beta-oxidation. This pathway is present in animals only.

Box 3: Oxidation of Fatty Acids—History

It was clearly established in 1954 that fatty acid oxidation occurs by β-oxidation reaction that cleaves a 2-C acetyl CoA at every cycle. Release of the energy of fatty acids is accomplished by oxidation of acetyl CoA by the TCA cycle. However, German scientist Franz Knoop reported for the first time that fatty acid oxidation is a removal of two carbon at a time (Knoop, 1904). A series of experiments were conducted wherein dogs were fed fatty acyl CoA in which the methyl group was replaced by phenyl group and their urine was subsequently analyzed. It was observed that dogs fed even numbered fatty acyl CoA produced phenylacetic acid (benzene plus two carbon acid) which was excreted as its glycine conjugate phenaceturic acid. On the other hand, dogs fed with odd number fatty acyl CoA produced benzoic acid (benzene plus one carbon acid) which was excreted as its glycine conjugate hippuric acid.



Experiment conducted by Franz Knoop. *Source* Voet et al. (2016). With kind permission from John Wiley & Sons.

From this, he concluded that the metabolism of fatty acids proceeds by the successive removal of two carbon fragments. The remaining fatty acid chain had to contain a carboxylic acid. Later, Feodor Lynen reported that acetyl CoA is the active acetate form present in fatty acid oxidation in yeasts (Lynen et al., 1951). At the same time, it was established that fatty acyl CoA formation is the first step in oxidation of fatty acids, and it is a *β*-oxidation pathway. Its connection to the respiratory chain for energy generation was reported by Ruzicka and Beinert (1977).

β-oxidation pathway of fatty acid—β-oxidation occurs by cleavage at β-position releasing 2C and acetyl CoA each time. Most bacteria generally utilize fatty acid after prolonged lag phase as fatty acid oxidation is an inducible pathway and oxidation enzymes are subject to catabolite repression. The different steps involved in complete oxidation of fatty acids seem to be reversal of fatty acid biosynthetic pathway. However, it is not a true reversal of the synthetic pathway. The enzymes involved in degradation are different than biosynthetic pathway, and also, the sites

Table 2 Differences between β-oxidation and biosynthesis of fatty acids

β-oxidation	Fatty acid synthesis
Site of β-oxidation is mitochondria and peroxisomes (in eukaryotes)	Biosynthesis takes place in cytoplasm
Reductant in β-oxidation is NADH	Reductant in biosynthesis is NADPH
Coenzyme A is required	Acyl carrier protein (ACP) is required
β-oxidation requires activation of fatty acids to fatty acyl CoA oxidation	Biosynthesis requires carboxylation of acetyl CoA to malonyl CoA synthesis

of biosynthesis and degradation are different in eukaryotes. The differences between the two pathways are presented in Table 2.

Steps in fatty acid degradation—Fatty acid degradation broadly takes place in four stages:

- Stage I: Activation of fatty acids to fatty acyl CoA
- Stage II: Transport of fatty acyl CoA from cytosol to mitochondria (in case of eukaryotes)
- Stage III: Beta-oxidation of fatty acids
- Stage IV: Complete oxidation of acetyl CoA/propionyl CoA.

Stage I: Activation of fatty acids to fatty acyl CoA

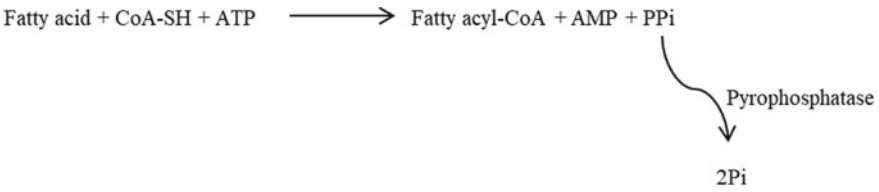
Fatty acid degradation is preceded by its transport across cytoplasmic membrane wherein long-chain fatty acids are transported by facilitated diffusion, while short and medium chain length are transported by simple diffusion in bacteria. However, OmpF porin mediated transport of short chain fatty acids is also reported. Long-chain fatty acid transport is facilitated by FadL protein and accompanied by its activation at inner membrane by the enzyme acyl CoA synthetase (FadD). Long-chain acyl CoA thioesters subsequently bind to FadR which in turn fails to bind to promoters of Fab and Fad operons. The result is that genes involved in fatty acid synthesis are no longer expressed while fatty acid degrading genes are de-repressed (Black & DiRusso, 1994).

The first committed step in fatty acid degradation is the activation of fatty acids to fatty acyl CoA by a reaction catalyzed by fatty acyl CoA synthetase (FACS; also known as fatty acyl CoA ligase or thiokinase) utilizing energy of two phosphate bonds. FACS catalyzes a two-step process that proceeds through the hydrolysis of ATP to yield pyrophosphate.

1. Formation of an adenylated intermediate—This step involves the linking of the carboxyl group of the fatty acid through an acyl bond to the phosphoryl group of AMP.
   Fatty acid + ATP → Fatty acyl-AMP + PPi
2. Formation of fatty acyl CoA—The fatty acyl group is transferred to the sulfhydryl group of coenzyme A, and AMP is released.

Fatty acyl-AMP + CoA → Fatty acyl-CoA + AMP

The pyrophosphate produced is subsequently degraded to inorganic phosphate by inorganic pyrophosphatase and this cleavage helps to drive the acylation reaction to completion. The overall reaction is as follows:



Stage II: Transport of fatty acyl CoA from cytosol to mitochondria (in case of eukaryotes)

Fatty acid activation occurs in the cytosol, but they are oxidized inside the mitochondrion in case of eukaryotes. Thus, the fatty acyl CoA is transported across the inner mitochondrial membrane which generally depends on the fatty acid chain length. Short and mid-length fatty acids can enter the mitochondria through simple diffusion. In such cases, activation takes place inside the mitochondria. The transport is mediated by converting acyl CoA to acyl-carnitine conjugates by the activity of carnitine acyltransferase I located on cytosolic faces of outer membrane. Then, these conjugates are shuttled through inner membrane by specific translocase wherein acyl-carnitine is converted back to carnitine and acyl CoA by another enzyme carnitine acyltransferase II. Carnitine is transported back to cytosol, and acyl CoA reaches the mitochondria (Fig. 12).

However, the membrane acts as a barrier for long-chain fatty acids. The cell has special transporters for their uptake where two classes of proteins have been identified (carnitine acyltransferase I and II). Acyl CoA is transesterified to carnitine by carnitine acyltransferase I, located on the cytosolic faces of the outer and inner mitochondrial membranes. Acyl-carnitine is then shuttled inside by a carnitine-acyl-carnitine translocase, as a carnitine is shuttled outside. Acyl-carnitine is converted back to acyl CoA by carnitine acyltransferase II, located on the interior face of the inner mitochondrial membrane. The liberated carnitine is shuttled back to the cytosol, as an acyl-carnitine is shuttled into the matrix.

Stage III: Beta-oxidation of fatty acids

Following steps are involved in beta-oxidation of fatty acids (Fig. 13).

1. Dehydrogenation of fatty acyl CoA by acyl CoA dehydrogenase to form enoyl CoA and $FADH_2$.
2. Hydration of enoyl CoA to 3-hydroxyacyl CoA by enoyl CoA hydrase in presence of H_2O.
3. Reduction of 3 hydroxyacyl CoA by hydroxyl acyl CoA dehydrogenase to β-keto acyl CoA and generation of NADH + H^+.

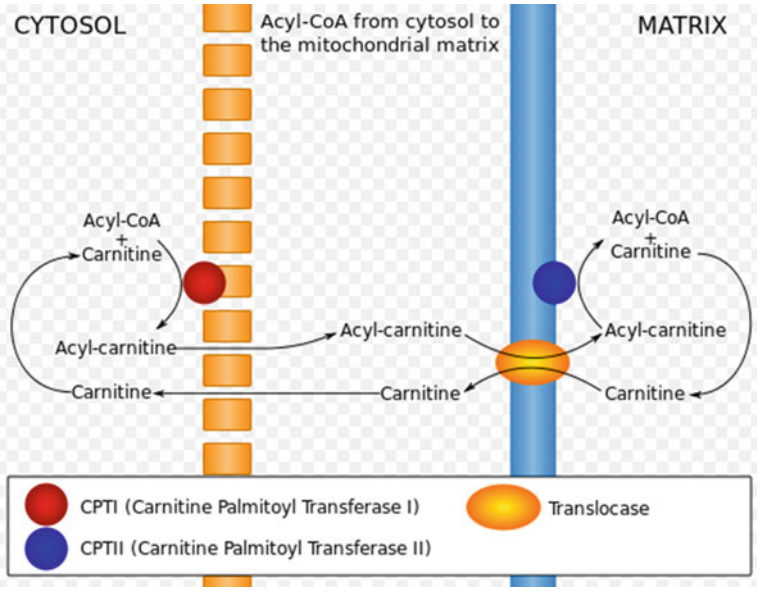


Fig. 12 Transport of fatty acyl CoA from cytosol to mitochondria through carnitine shuttle. *Source* Wikipedia (Creative Commons License)

4. Finally, thiolase cleaves 2-carbon acetyl CoA and 2-carbon-less fatty acyl CoA.

The overall strategy of beta-oxidation is to create a carbonyl group on the β-C. The first three reactions do that while the fourth cleaves the 'β-keto ester'. The final products after one cycle of beta-oxidation are an acetyl CoA and a fatty acid two carbons shorter. The series of 1–4 steps are repeated again and again until the entire chain is cleaved into acetyl CoA units. For example, in case of 16 carbon palmitoyl CoA, seven rounds of steps 1–4 will be carried out to release 8 acetyl CoAs. Concomitantly, one molecule of $FADH_2$ and NADH are formed. Thus, the final product of fatty acids with even numbered fatty acids will be acetyl CoA. However, in case of odd-numbered fatty acid chain length, the end product is propionyl CoA.

Stage IV: Complete oxidation of acetyl CoA/propionyl CoA

Acetyl CoA: Complete oxidation of acetyl CoA takes place through TCA. In case of bacteria, it takes place in the cytosol itself. However, in eukaryotes, it needs to be transported to the mitochondria either through special exporters or through glyoxylate cycle intermediate, i.e., malate which is again oxidized by TCA.

Propionyl CoA: Propionyl CoA, which is generated after degradation of odd-numbered fatty acids, is majorly processed via modified TCA cycle (methylcitrate pathway) or acrylate pathway in bacteria (Fig. 14 and Table 3).

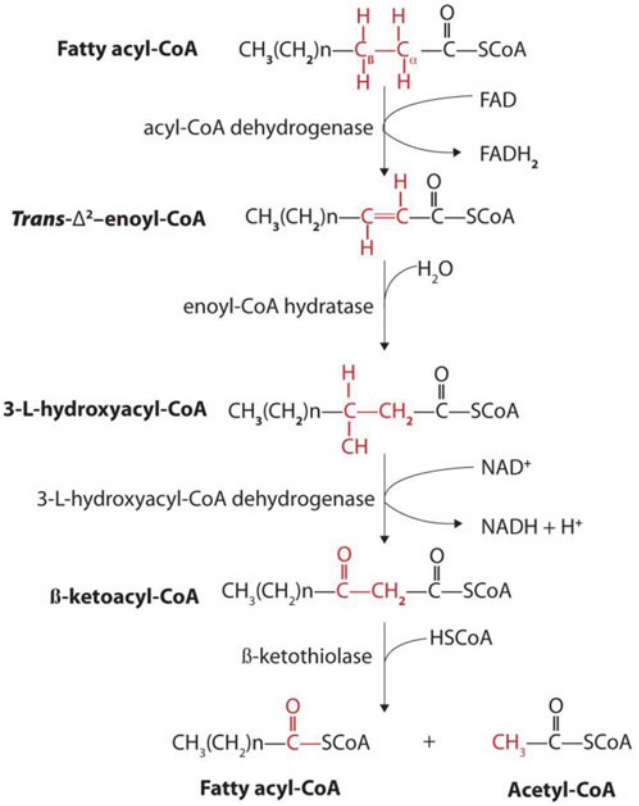


Fig. 13 Reactions involved in beta-oxidation of fatty acids

8 Regulation of Fatty Acid Degradation in *E. coli*

Fatty acid degradation in *E. coli* is regulated by several physiological parameters such as carbon sources, oxygen levels, osmotic conditions and also nitrogen levels in the medium. It is exhaustively reviewed by Jimenez-Diaz et al. (2019).

In *E. coli*, fatty acid uptake by membrane-bound FadL protein is accompanied by its activation at inner membrane by FadD enzyme where fatty acids are converted to fatty acyl CoA with conversion of ATP to AMP. Immediately upon activation, long-chain acyl CoA binds to repressor FadR which in turn de-represses fatty acid degradation pathway. Further, FadR is a multifunctional global transcriptional regulator which positively regulates fatty acid biosynthesis and IclR gene, a repressor of glyoxylate operon. Therefore, fatty acids complexed FadR fails to bind to these promoters, and thus, fatty acid synthesis operon and synthesis of repressor of glyoxylate pathway IclR protein are suppressed .

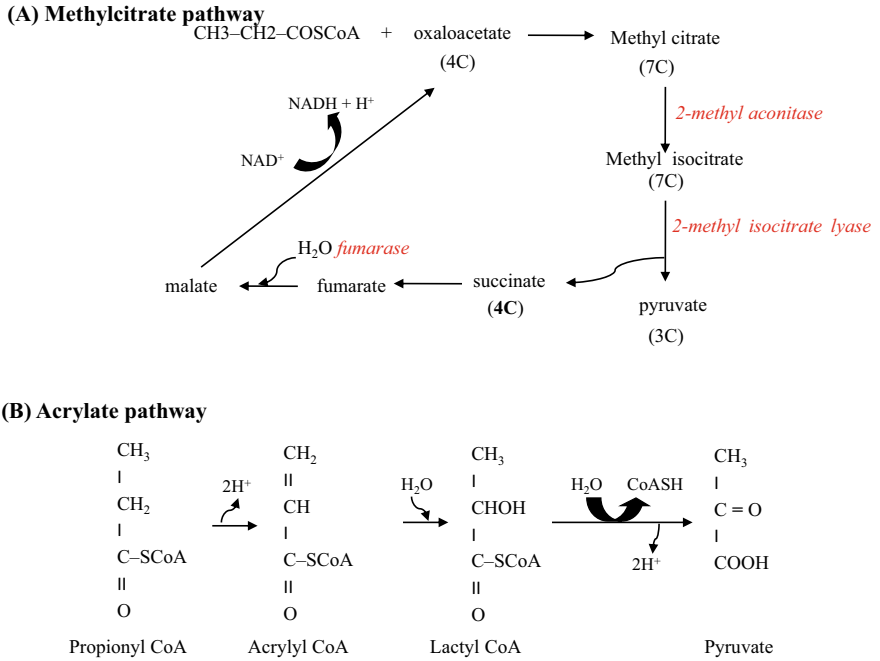


Fig. 14 Degradation of Propionyl CoA via **A** Methylcitrate pathway and **B** Acrylate pathway

Table 3 Stoichiometry for oxidation of a 16-carbon fatty acid (palmitic acid)

Step	Reaction	Energy gain
Activation of fatty acids to fatty acyl CoA	Palmitic acid + ATP + CoA-SH → palmitoyl CoA + 2Pi + AMP	−2 ATP
Beta-oxidation	Palmitoyl CoA + $7FAD^+$ + $7NAD^+$ + $7H_2O$ → 8 acetyl CoA + $7FADH_2$ + 7NADH + $7H^+$	
Complete oxidation of acetyl CoA by TCA	8 acetyl CoA → 8 rounds of TCA cycle (10 ATP per round)	+80 ATP
Oxidative phosphorylation	$7FADH_2$ (each $FADH_2$ yields 1.5 ATP) 7NADH + $7H^+$(each NADH yields 2.5 ATP)	+10.5 ATP +17.5 ATP
	Net gain	106 ATP

Thus, FadR has three main functions when bound to their respective promoters, namely, it represses fatty acid degradation; activates fatty acid biosynthesis; and activates expression of IclR which represses glyoxylate shunt (Fig. 15).

Uptake of fatty acid, its conversion to fatty acyl CoA and binding to FadR repressor causes conformational changes in FadR repressor and leads to its dissociation from

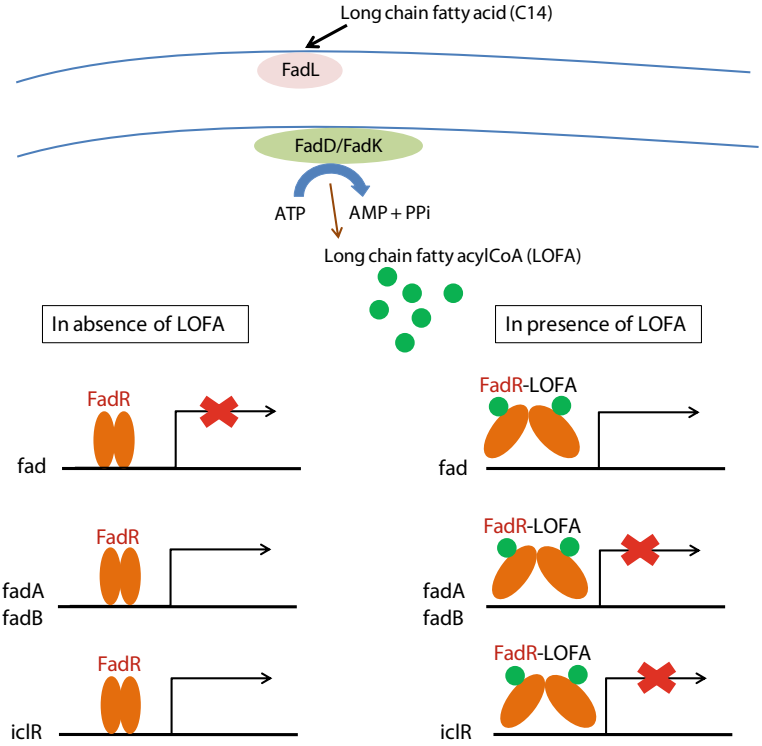


Fig. 15 Regulation of fatty acid metabolism by FadR repressor in absence and presence of fatty acyl CoA

the promoter regions of the three operons. The consequences of dissociation of FadR are as follows:

i. FadR is a repressor of fatty acid degradation operon (Fad operon), and its dissociation results in de-repression of fatty acid degradation.
ii. FadR is positive regulator of fatty acid synthesis, and its dissociation results in suppression of fatty acid synthesis.
iii. FadR is a repressor of isocitrate lyase repressor (IclR), and its dissociation leads to no synthesis of repressor of isocitrate lyase and thus de-repression of glyoxylate pathway.

Positive regulation of fatty acid degradation by catabolite repression—Fatty acid degradation enzymes show cAMP-dependent catabolite repression in presence of glucose. The expression of the fatty acid degradation enzymes is positively controlled by the cAMP-CRP complex.

Regulation under anoxic conditions—Under low oxygen conditions, fatty acid degradation is regulated by ArcA/B two-component system wherein autophosphorylation of sensor kinase under reduced oxygen concentration causes ArcA to phosphorylate and ArcA-P suppresses Fad promoter.

Regulation under high osmotic stress—Fatty acid degradation is also repressed during high osmotic condition through EnzV-OmpR two-component signaling wherein OmpR-P binds upstream to FadL promoter and inhibits it thus inhibiting the fatty acid uptake step.

Summary

- Various forms of lipids are found in microbes, and each class of microorganisms has a distinct and characteristic lipid composition.
- Lipids are an integral part of various organisms where they play important biological roles such as major structural component of biological membranes, role in cell signaling and virulence factors.
- Cytoplasmic membranes of bacteria act as permeability barriers to polar solutes that are present in an aqueous environment.
- Gram-positive and negative bacteria vary in their lipid composition. Cell surface of gram-positive bacteria contains lipoteichoic acid and glycosyldiglycerides.
- Outer surface of gram-negative bacteria has lipopolysaccharide (LPS) which is absent in gram-positive bacteria.
- In *Neisseria* and *Haemophilus* species, low molecular weight form of bacterial LPS called as lipooligosaccharide (LOS) is present. It lacks the O-antigen and contains only lipid A and oligosaccharide core and plays an important role in their pathogenesis.
- Genera of anaerobic bacteria are uniquely enriched in ether lipids called plasmalogens.
- Mycoplasmas, the smallest organisms, lack cell wall and resemble protoplasts. However, they are quite resistant to osmotic stress due to the presence of sterols in their cytoplasmic membranes.
- *Mycobacterium* contains unique lipids called mycolic acids which are a group of complex branched-chain hydroxylated lipids covalently bound to the cell wall peptidoglycan.
- The thermophilic bacteria *Thermodesulfobacterium*, an obligate anaerobe, is a thermophilic sulfate reducer which is characterized by the presence of ether-linked lipids, a hallmark of archaea. In contrast to archaea, however, the glycerol side chains of the ether-linked lipids in *Thermodesulfobacterium* are not phytanyl groups but are composed of a unique C17 hydrocarbon along with some fatty acids.
- Archaea have ether-linked membrane lipids in contrast to eubacteria which have ester linked glycerol lipids. Archaeal lipids have long-chain alcohols called isopranyl alcohols.
- Fatty acid biosynthesis occurs in cytosol, and acetyl CoA should be either generated in the cytosol or transported to the cytosol as is the case in eukaryotes.

- The major source of acetyl CoA is pathways of carbohydrate oxidation viz. glycolysis and TCA cycle or pathways of protein oxidation, i.e., degradation of amino acids.
- In eukaryotes, acetyl CoA can only be transported out of the mitochondrial matrix under conditions of high oxaloacetate as citrate by the action of the enzyme citrate synthase.
- The main source of reductant is glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of the pentose phosphate pathway. Further, in eukaryotes, the malic enzyme also generates NADPH.
- Various steps involved in synthesis of saturated fatty acids include: Activation of acetyl CoA to malonyl CoA, transacylation of acetyl CoA and malonyl CoA to ACP, initiation by condensation of acetyl CoA and malonyl CoA, reduction and dehydration of β-keto group, elongation of fatty acid chain length, termination and release of product.
- For synthesis of one mole of palmitic acid, 1 mol of acetyl CoA and 7 mol of malonyl CoA are required, i.e., expenditure of 7 ATP and 14 NADPH.
- Unsaturated fatty acids are important for maintenance of membranes. The unsaturation of fatty acids can be both anaerobic and aerobic pathway.
- Anaerobic pathway is restricted to prokaryotes, while aerobic pathway is present in both prokaryotes and eukaryotes.
- To synthesis triacylglycerides, a glycerol backbone is needed which is provided either by glycolysis or as a breakdown product of other lipids.
- Under nitrogen depleted conditions, few eukaryotic microorganisms can accumulate triacylglycerides as storage lipids as much as 80% of their dry weight.
- The first metabolic shift on nitrogen starvation is the activation of AMP deaminase which is the key activity in oleaginous microbes that triggers lipid accumulation. It transiently supplies NH_4^+.
- In oleaginous microbes, major changes are observed in isocitrate dehydrogenase activity which is inhibited as it is AMP dependent.
- In non-oleaginous organisms, there is no such dependence of isocitrate dehydrogenase on AMP.
- Microorganisms can utilize a variety of lipids such as triglycerides, waxes, hydrocarbons as a source of energy and carbon. However, microorganisms use these only in absence of other readily assimilable carbon sources such as sugars.
- There are three types of fatty acid oxidation, i.e., alpha, beta and omega depending on the position of carbon where cleavage occurs.
- Fatty acid degradation in *E. coli* is regulated by several physiological parameters such as carbon sources, oxygen levels, osmotic conditions and also nitrogen levels in the medium.
- Long-chain acyl CoA binds to FadR and de-represses fatty acid degradation pathway. FadR is a multifunctional global transcriptional regulator and also represses fatty acid biosynthesis.

Questions

1. Name any prokaryote (among bacteria or archaea) which has the following characteristic lipid:

   i. Plasmalogens
   ii. Mycolic acid
   iii. Cardiolipins or low molecular weight bacterial LPS called LOS
   iv. Bacterial group having sterols in their plasma membrane
   v. Cord factor
   vi. Sphingolipids
   vii. Corynemycolenic acid
   viii. Lipoglycans (Caldarchaeol)
   ix. Glycerol Dialkyl Glycerol Tetraethers (GDGTs)
   x. Unsaturated form of tetraethers

2. What is the source of acetyl CoA and reductant for the biosynthesis of fatty acids?
3. Differentiate between Type I and Type II fatty acid synthases.
4. Explain the reaction for the conversion of acetyl CoA to malonyl CoA.
5. Write a note on anaerobic and aerobic conversion of unsaturated fatty acid from saturated fatty acid.
6. Explain major biochemical difference between oleaginous and non-oleaginous yeast.
7. Explain β-oxidation pathway of fatty acid degradation and calculate energy gain during oxidation of one mole of palmitic acid.
8. Explain the role of FadR, a multifunctional global transcription factor, in regulating fatty acid metabolism.

References

Aguilar, P. S., Hernandez-Arriaga, A. M., Cybulski, L. E., Erazo, A. C., & de Mendoza, D. (2001). Molecular basis of thermosensing: A two-component signal transduction thermometer in *Bacillus subtilis*. *The EMBO Journal, 20*(7), 1681–1691.

Alhamadsheh, M. M., Musayev, F., Komissarov, A. A., Sachdeva, S., Wright, H. T., Scarsdale, N., Florova, G., & Reynolds, K. A. (2007). Alkyl-CoA disulfides as inhibitors and mechanistic probes for FabH enzymes. *Chemistry & Biology, 14*(5), 513–524.

Ball, G. F. M. (2003). Pantothenic acid—properties and determination. In B. Caballero (Ed.), *Encyclopedia of food sciences and nutrition* (2nd ed., pp. 4332–4338). Academic Press.

Black, P. N., & DiRusso, C. C. (1994). Molecular and biochemical analyses of fatty acid transport, metabolism, and gene regulation in *Escherichia coli*. *Biochimica et Biophysica Acta, L. Lipids and Lipid Metabolism, 1210*(2), 123–145.

Blanchard, C. Z., Amspacher, D., Strongin, R., & Waldrop, G. L. (1999). Inhibition of biotin carboxylase by a reaction intermediate analog: Implications for the kinetic mechanism. *Biochemical and Biophysical Research Communications, 266*(2), 466–471.

Botham, P. A., & Ratledge, C. (1979). A biochemical explanation for lipid accumulation in Candida 107 and other oleaginous micro-organisms. *Microbiology, 114*(2), 361–375.

Cybulski, L. E., Del Solar, G., Craig, P. O., Espinosa, M., & De Mendoza, D. (2004). *Bacillus subtilis* DesR functions as a phosphorylation-activated switch to control membrane lipid fluidity. *Journal of Biological Chemistry, 279*(38), 39340–39347.

Feng, Y., & Cronan, J. E. (2011). Complex binding of the FabR repressor of bacterial unsaturated fatty acid biosynthesis to its cognate promoters. *Molecular Microbiology, 80*(1), 195–218.

Freiberg, C., Brunner, N. A., Schiffer, G., Lampe, T., Pohlmann, J., Brands, M., Raabe, M., Häbich, D., & Ziegelbauer, K. (2004). Identification and characterization of the first class of potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity. *Journal of Biological Chemistry, 279*(25), 26066–26073.

Freiberg, C., Pohlmann, J., Nell, P. G., Endermann, R., Schuhmacher, J., Newton, B., Otteneder, M., Lampe, T., Häbich, D., & Ziegelbauer, K. (2006). Novel bacterial acetyl coenzyme A carboxylase inhibitors with antibiotic efficacy in vivo. *Antimicrobial Agents and Chemotherapy, 50*(8), 2707–2712.

Garba, L., Shukuri Mo, M., Nurbaya Os, S., & Noor Zalih, R. (2017). Review on fatty acid desaturases and their roles in temperature acclimatisation. *Journal of Applied Sciences, 17*, 282–295.

Grassberger, M. A., Turnowsky, F., & Hildebrandt, J. (1984). Preparation and antibacterial activities of new 1, 2, 3-diazaborine derivatives and analogs. *Journal of Medicinal Chemistry, 27*(8), 947–953.

Heath, R., White, S., & Rock, C. (2002). Inhibitors of fatty acid synthesis as antimicrobial chemotherapeutics. *Applied Microbiology and Biotechnology, 58*(6), 695–703.

Helmkamp, G. M., Rando, R. R., Brock, D. J. H., & Bloch, K. (1968). β-hydroxydecanoyl thioester dehydrase specificity of substrates and acetylenic inhibitors. *Journal of Biological Chemistry, 243*(12), 3229–3231.

Jain, S., Caforio, A., & Driessen, A. J. (2014). Biosynthesis of archaeal membrane ether lipids. *Frontiers in Microbiology, 5,* 641.

Jimenez-Diaz, L., Caballero, A., & Segura, A. (2019). Regulation of fatty acids degradation in bacteria. In F. Rojo (Ed.), *Aerobic utilization of hydrocarbons, oils, and lipids. Handbook of hydrocarbon and lipid microbiology* (pp. 751–771). Springer.

Knoop, F. (1904). Der Abbau aromatischer Fettsauren im Tierkorper. *Beitraege zur Chemischen Physiologie und Pathologie, 6,* 150–162.

Lynen, F., Reichert, E., & Rueff, L. (1951). Zum biologischen Abbau der Essigsäure VI. "Aktivierte Essigsäure", ihre Isolierung aus Hefe und ihre chemische Natur. *Justus Liebigs Annalen der Chemie, 574*(1), 1–32.

Mansilla, M. C., Cybulski, L. E., Albanesi, D., & de Mendoza, D. (2004). Control of membrane lipid fluidity by molecular thermosensors. *Journal of Bacteriology, 186*(20), 6681–6688.

Omura, S. (1976). The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. *Bacteriological Reviews, 40*(3), 681.

Phadtare, S. (2004). Recent developments in bacterial cold-shock response. *Current Issues in Molecular Biology, 6*(2), 125–136.

Price, A. C., Choi, K. H., Heath, R. J., Li, Z., White, S. W., & Rock, C. O. (2001). Inhibition of β-ketoacyl-acyl carrier protein synthases by thiolactomycin and cerulenin structure and mechanism. *Journal of Biological Chemistry, 276*(9), 6551–6559.

Quemard, A., Sacchettini, J. C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W. R., Jr., & Blanchard, J. S. (1995). Enzymic characterization of the target for isoniazid in *Mycobacterium tuberculosis. Biochemistry, 34*(26), 8235–8241.

Ruzicka, F. J., & Beinert, H. (1977). A new iron-sulfur flavoprotein of the respiratory chain. *Journal of Biological Chemistry, 252,* 8440–8445.

Salie, M. J., & Thelen, J. J. (2016). Regulation and structure of the heteromeric acetyl-CoA carboxylase. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1861*(9), 1207–1213.

Voet, D., Voet, J. G., & Pratt, C. W. (2016). *Fundamentals of biochemistry: Life at the molecular level*. Wiley.

Ward, W. H., Holdgate, G. A., Rowsell, S., McLean, E. G., Pauptit, R. A., Clayton, E., Nichols, W. W., Colls, J. G., Minshull, C. A., Jude, D. A., Mistry, A., Timms, D., Camble, R., Hales, N. J., Britton, C. J., & Taylor, I. W. F. (1999). Kinetic and structural characteristics of the inhibition of enoyl (acyl carrier protein) reductase by triclosan. *Biochemistry, 38*(38), 12514–12525.

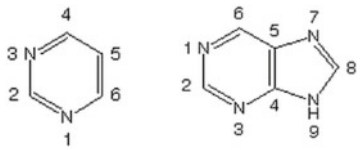
Chapter 19
Nucleotide Biosynthesis and Regulation

Rani Gupta and Namita Gupta

Bacteria are fast-growing organisms that need to rapidly synthesize nucleotides to sustain growth. This is true for cancer cells as well. This in is a striking contrast to humans and animals where rapidly multiplying cells are localized such as gonads. Rapidly multiplying cells in these organisms constitute abnormal cancerous growth. Thus, nucleotide synthesis is a good target for anti-cancer/antibacterial strategies. Nucleotides are either synthesized by de novo or by salvage pathway using by-products of nucleic acid degradation.

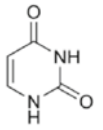
1 Purines and Pyrimidines

Purines and pyrimidines are the parent compounds of the two types of nitrogen-containing heterocyclic aromatic compounds. Pyrimidines are six-membered heterocyclic rings containing two nitrogen atoms. Purines, on the other hand, are bicyclic compounds of a six- and five-membered heterocyclic rings fused together at carbons 4 and 5. A purine is essentially a pyrimidine ring fused to an imidazole ring and contains four nitrogen atoms.

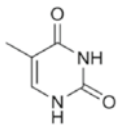


Pyrimidines that occur in RNA are cytosine and uracil while pyrimidines in DNA are cytosine and thymine (in place of uracil).

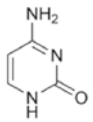
R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_19



Uracil
(2,4-dioxo
pyrimidine)

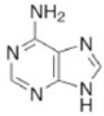


Thymine
(2,4-dioxo-5-methyl
pyrimidine; methylated
uracil)

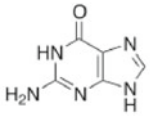


Cytosine
(2-oxo-4-amino
pyrimidine)

Adenine and guanine are the principal purines of both DNA and RNA.

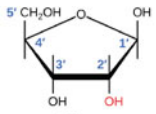


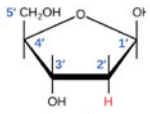
Adenine
(6-amino purine)

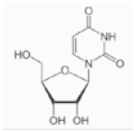


Guanine
(2-amino-6-oxopurine)

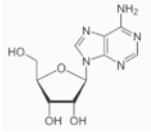
Nucleosides: Purine or pyrimidine bases are linked to pentoses, namely D-ribofuranose (D-ribose; in RNA) or 2-deoxy-D-ribofuranose (2′-Deoxyribose; in DNA) by N-glycosidic linkage at C1′ carbon of the sugar with N1 atom in purine and N9 atom in pyrimidines.





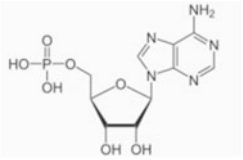


Uridine
(a pyrimidine
nucleoside)



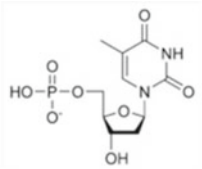
Adenosine
(a purine
nucleoside)

Nucleotides: Nucleosides are generally phosphorylated at the 5′ end of the molecule through esterification. The phosphates can be mono-, di- or triphosphates.



Adenosine monophosphate (AMP)

(a ribonucleotide)



2′-Deoxythymidine monophosphate (dTMP)

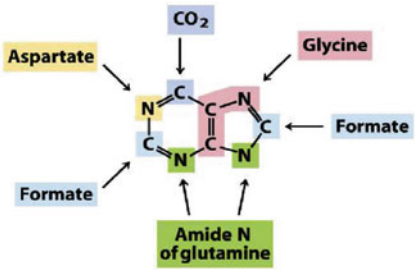
(a deoxyribonucleotide)

Nucleotide biosynthetic pathways are of two types

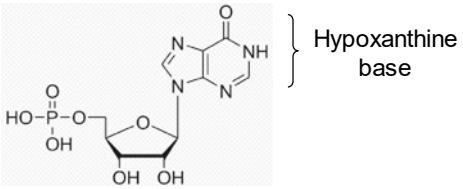
- **De novo pathway**—It is the pathway to synthesize purine and pyrimidine nucleotides from low-molecular-weight precursors. De novo pathways are almost identical in all organisms.
- **Salvage pathways**—It is a pathway to synthesize nucleotides from nucleosides or bases that become available through the diet or from degradation of nucleic acids.

2 Synthesis of Purine Ribonucleotides

John Buchanan obtained the first clues of the de novo process in 1948. In purines, two glutamine, one glycine and one aspartate are required. In addition, two methylation and one carboxylation steps are involved. Various precursors are shown below:

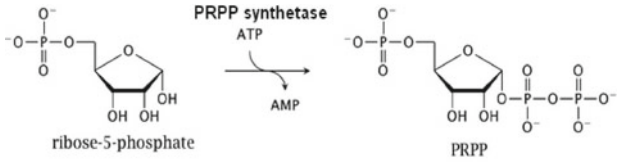


The biosynthetic pathway was elucidated by John Buchanan and G. Robert Greenberg where first purine derivative is inosine monophosphate (IMP). Subsequently, AMP and GMP are synthesized via separate pathways.

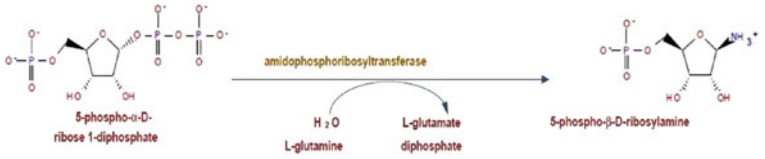


Biosynthesis of IMP

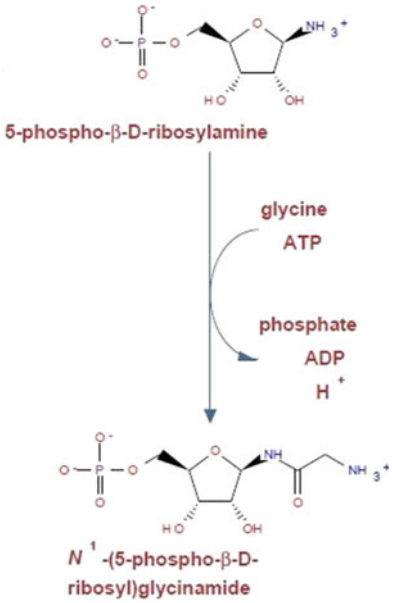
1. **Activation of ribose-5-phosphate**: Ribose-5-phosphate, which is a key intermediate of pentose phosphate pathway, is activated by PRPP synthetase which results in phosphorylation at C1 position to form PRPP, 5-phosphoribosyl 1-pyrophosphate (5-phospho-α-D-ribose 1-diphosphate).



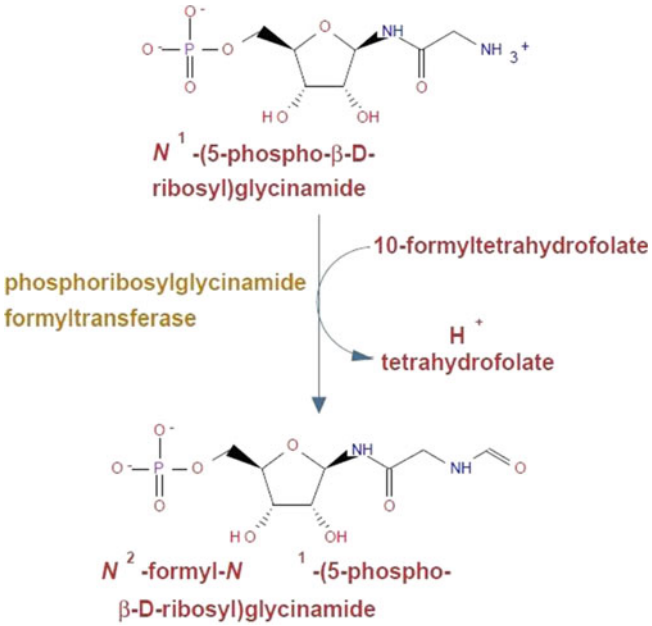
2. **Acquisition of purine atom N9**: PRPP's pyrophosphate group is displaced by amide nitrogen of glutamine to yield 5-phospho-β-ribosylamine (PRA). This is the first committed step toward de novo purine synthesis. This also generates flux since PPi is released. The enzyme catalyzing this reaction, i.e., amidophosphoryl transferase enzyme is regulated by feedback inhibition by purine nucleotides.



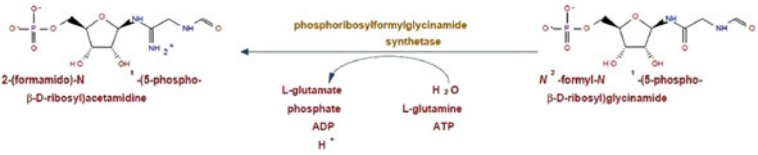
3. **Acquisition of carbon atom at 4 and 5 positions and nitrogen at 7 position**: This is the step where more than one atom of the ring is synthesized. Here, a molecule of glycine is added in a reaction catalyzed by GAR synthetase to generate N1-(5-Phospho-β-D-ribosyl)glycinamide also known as glycinamide ribotide (GAR). The reaction is reversible though it involves hydrolysis of ATP to ADP + Pi.



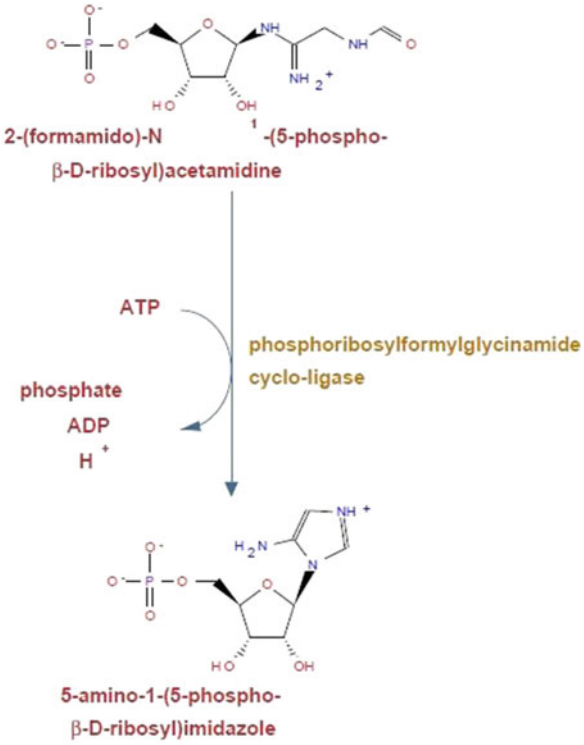
4. **Acquisition of purine atom C8**: Amino group of GAR is formylated by GAR transformylase or phosphoribosyl glycinamide formyltransferase to yield N^2-formyl-N^1-(5-phospho-β-D-ribosyl)glycinamide (also known as formyl glycinamide ribotide; FGAR). The formyl donor in this reaction is N^{10}-formyl tetrahydrofolate (N^{10}-formyl-THF). This reaction is inhibited by sulfonamide.



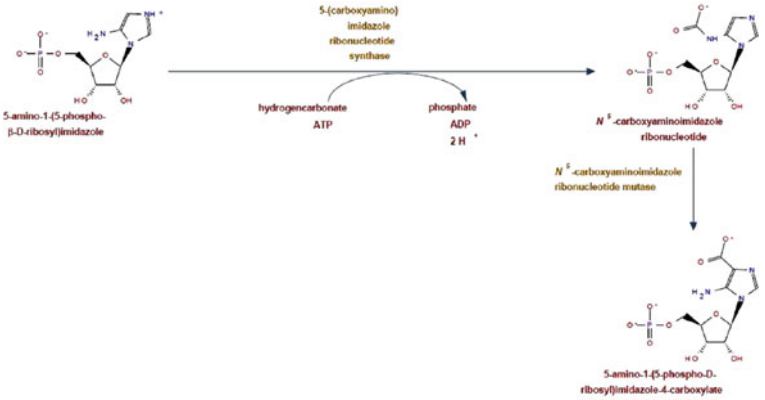
5. **Acquisition of purine atom N3**: Second glutamine donates amino group to the growing purine ring to form 2-(formamido)-N^1-(5-phospho-β-D-ribosyl)acetamidine (also known as formyl glycinamidine ribonucleotide; FGAM) in presence of enzyme phosphoribosyl formyl glycinamide synthetase (FGAM synthetase) with coupled hydrolysis of ATP to ADP + Pi.



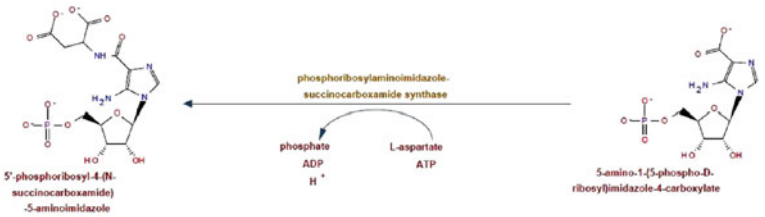
6. **Formation of purine imidazole ring**: The purine imidazole ring closes to yield 5-amino-1-(5-phospho-β-D-ribosyl)imidazole (also known as 5-amino imidazole ribotide; AIR) in a reaction catalyzed by AIR synthetase (also known as FGAM cyclo-ligase) in presence of ATP.



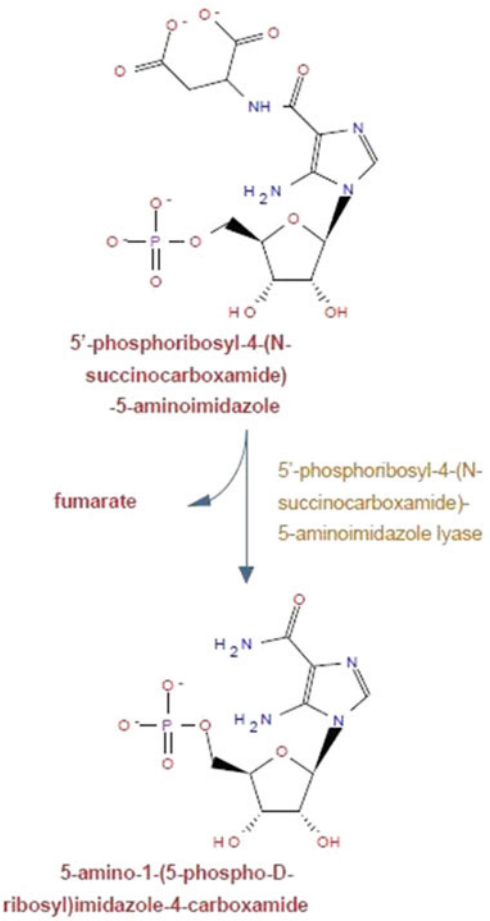
7. **Acquisition of purine atom C6**: C6 atom is introduced by HCO_3^- (CO_2) yielding N^5-carboxyamino imidazole ribonucleotide (CAIR). This reaction is catalyzed by CAIR synthase and involves ATP hydrolysis. CAIR is then converted to 5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxylate by the enzyme CAIR mutase.



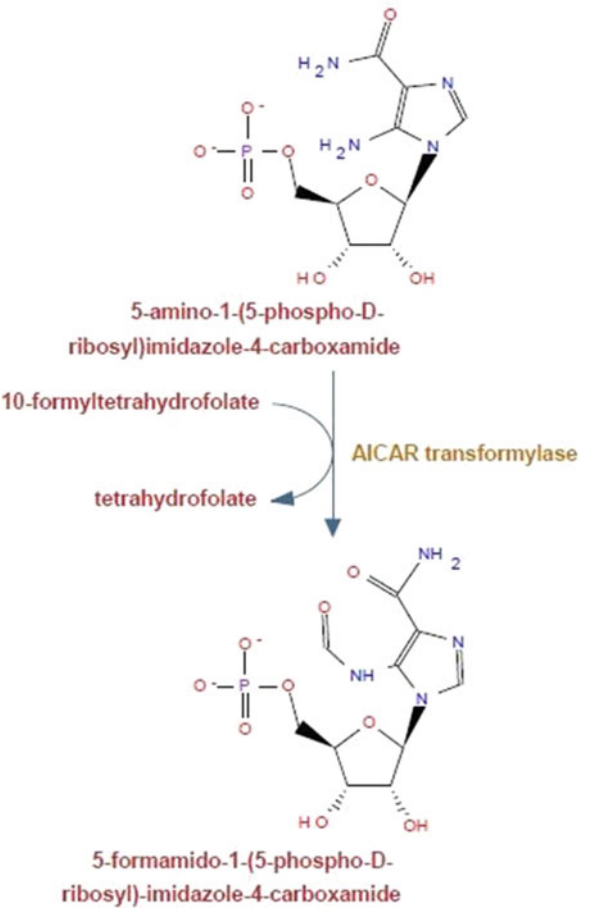
8. **Acquisition of purine atom N1**: Aspartate then contributes N1 nitrogen in condensation reaction yielding 5-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole (SAICAR) catalyzed by SAICAR synthase. This reaction is also coupled by ATP hydrolysis.



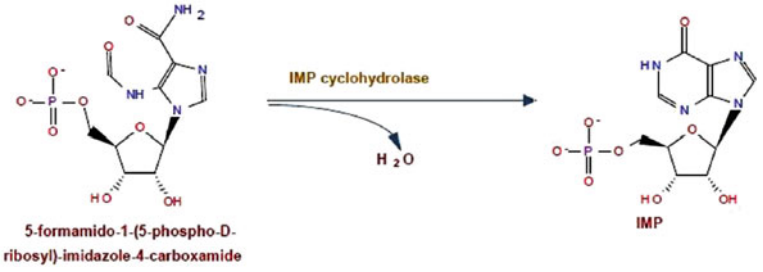
9. **Elimination of fumarate**: SAICAR is then converted to 5-amino-1-(5-phospho-D-robosyl) imidazole-4-carboxamide (AICAR) in a reaction catalyzed by SAICAR lyase. During this conversion, a molecule of fumarate is released.



10. **Acquisition of purine atom C2**: AICAR transformylase catalyzes methylation at C2 position through formylation by N^{10}-formyl-tetrahydrofolate yielding 5-formamido-1-(5-phospho-D-ribosyl)-imidazole-4-caroxamide (FAICAR).



11. **Cyclization to form IMP**: IMP cyclohydrolase (also known as IMP synthase) then cyclizes FAICAR yielding a closed-ring product IMP. No ATP is used in this reaction.



All the pathways representing synthesis of IMP taken from ecocyc.org. With kind permission from SRI Intrenational.

ATP consumption: Six ATPs are required in the purine biosynthetic pathway from ribose-5-phosphate to IMP. They are required in the following steps:

- Step 1—Ribose 5-phosphate PRPP
- Step 3—β-5-phosphoribosylamine glycinamide ribotide
- Step 5—formyl glycinamide ribotide formyl glycinamidine ribotidine
- Step 6—formyl glycinamidine ribotidine 5-amino imidazole ribotide
- Step 7—5-amino imidazole ribotide carboxyamino imidazole ribotide
- Step 8—carboxyamino imidazole ribotide amino imidazole-4-(N-succinylo carboxamide) ribotide.

In total, 6 ATPs and energy equivalent to 7 phosphates are utilized since PRPP synthesis uses energy of two phosphate bonds.

Synthesis of AMP and GMP

AMP and GMP are synthesized from IMP. GTP is used to make AMP while ATP is used to make GMP (Fig. 1).

In the branch leading to AMP, the 6-O group of inosine is displaced by aspartate in a GTP-dependent reaction catalyzed by adenylosuccinate synthetase. Subsequently, the 4-carbon skeleton of aspartate is non-hydrolytically removed as fumarate by the action of the enzyme adenylosuccinase. The amino group is retained as the 6-amino group of AMP.

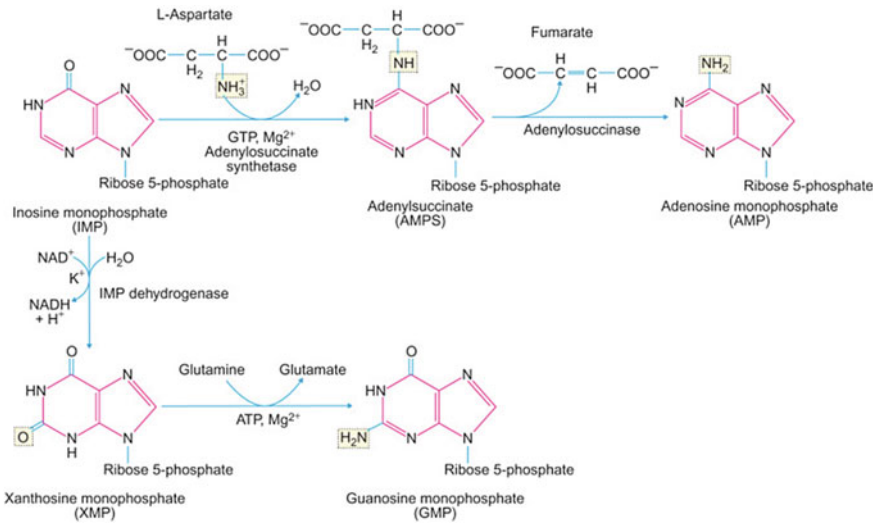
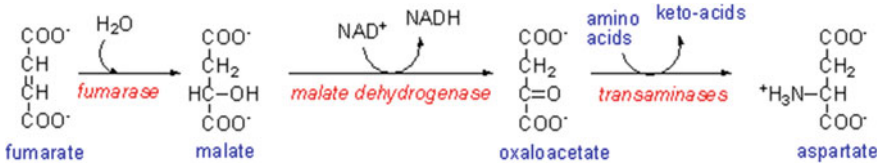


Fig. 1 Biosynthesis of AMP and GMP from inosine monophosphate. *Source* Bhagavan and Ha (2015). With kind permission from Elsevier

In the branch leading to GMP, C-2 of purine ring is oxidized by the enzyme IMP dehydrogenase followed by replacement of the oxygen on C-2 with an amino group by glutamine that yields GMP in presence of enzyme GMP synthetase.

Fumarate recycling to aspartate

The synthesis of 5-aminoimidazole-4-carboxamide ribotide (AICAR) from carboxyamino imidazole ribotide (CAIR) and AMP from IMP have the net effect of deaminating aspartate to fumarate. Fumarate can be recycled back to aspartate via the following path:



This regenerates not only aspartate but also aids in replenishment of ATP via generation of the reductant $NADH^{+}$.

3 Regulation of Purine De Novo Synthesis

The rate limiting steps in purine biosynthesis are at the first two steps catalyzed by Ribose 5′-phosphate pyrophosphokinase (or PRPP synthetase) and glutamine phosphoribosyl pyrophosphate amidotransferase (or glutamine PRPP amidotransferase). The first step catalyzed by PRPP synthetase (E1) is feedback inhibited by purine nucleotides. The second step catalyzed by glutamine PRPP amidotransferase (E2) is also feedback inhibited by purine nucleotides. Glutamine PRPP amidotransferase enzyme has two allosteric sites, one where AMP, ADP and ATP bind and the second where GMP, GDP and GTP bind. This enzyme is also activated by PRPP. Further, the two branches of the pathway leading to synthesis of AMP and GMP from IMP are also regulated by relative levels of AMP and GMP where AMP branch is inhibited by AMP while GMP branch, IMP dehydrogenase (E4), is inhibited by GMP (Fig. 2).

4 Synthesis of Pyrimidine Ribonucleotides

In contrast to the purine rings that are assembled on phosphoribose, the pyrimidine bases are synthesized first as orotic acid (uracil-6-carboxylic acid), and then get attached to ribose phosphate. Orotic acid is then converted to the nucleotide orotidine monophosphate (OMP). In pyrimidines:

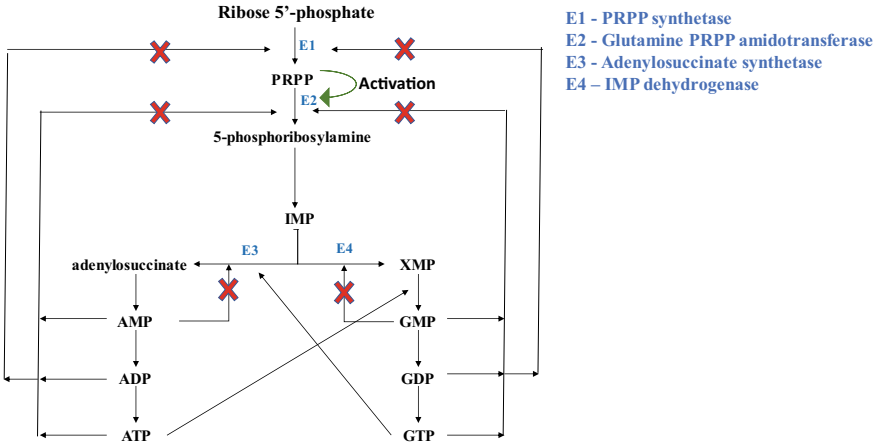
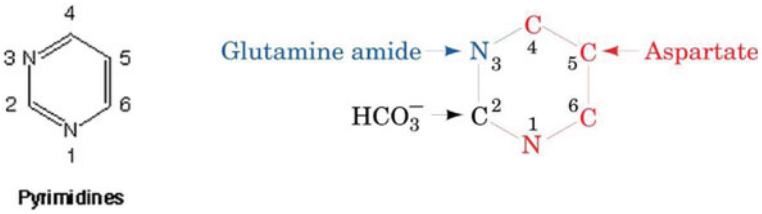


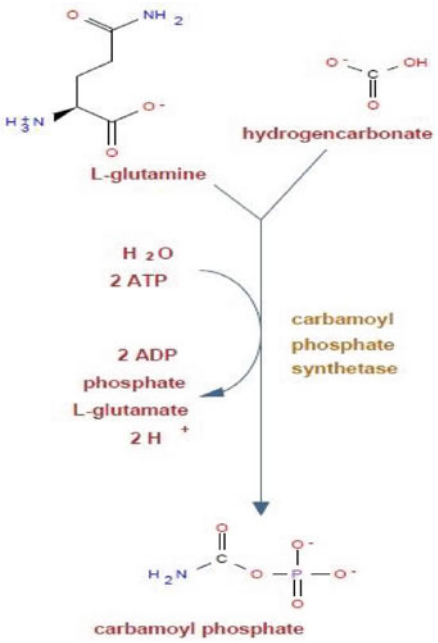
Fig. 2 Feedback inhibition, feed-forward activation and cross-regulation of purine biosynthetic pathway

- N1, C4, C5 and C6 come from aspartic acid
- C2 is from CO_2
- N3 is derived from glutamine.

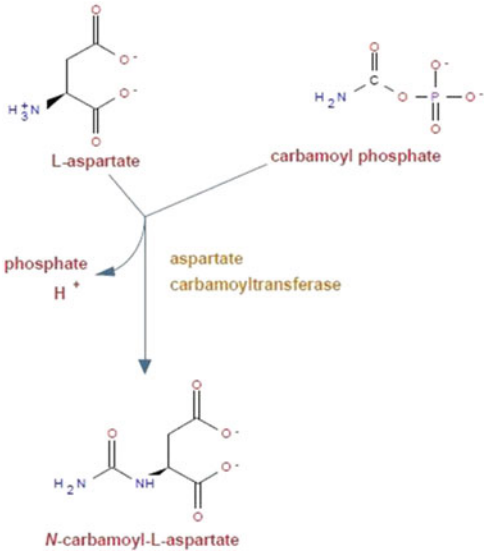


Steps in the synthesis of uridine monophosphate (UMP)

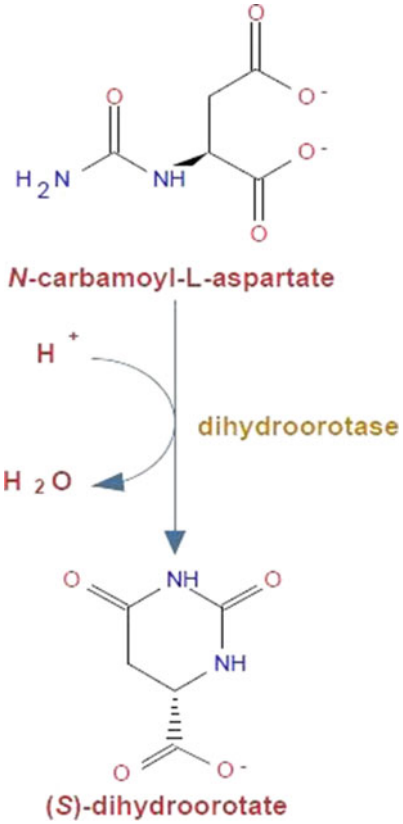
1. **Synthesis of carbamoyl phosphate**: The first reaction of the pathway involves HCO_3^- and amide nitrogen of glutamine and is catalyzed by cytosolic enzyme carbamoyl phosphate synthetase (CPS II). The reaction consumes 2 ATPs wherein the first ATP provides PO_4^{2-} group while the second ATP energizes the reaction.



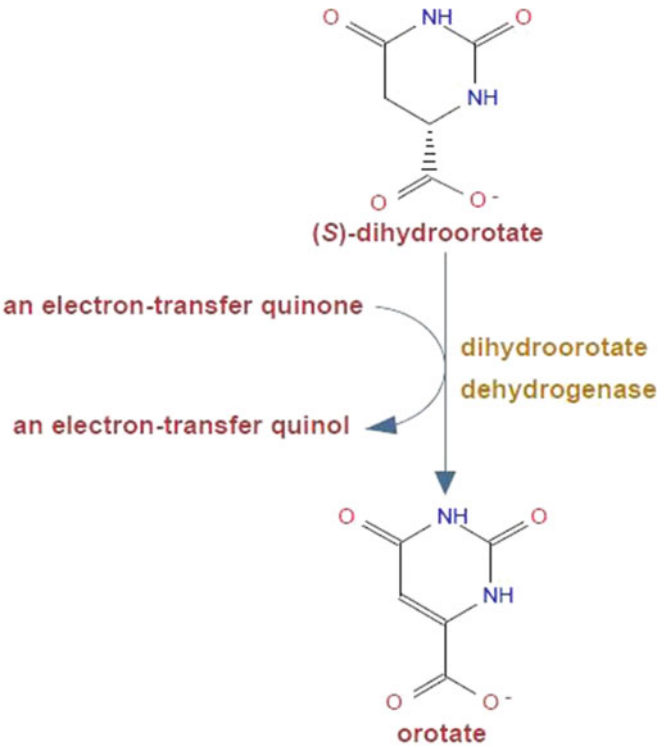
2. **Synthesis of carbamoyl aspartate**: Carbamoyl-P is condensed with aspartate to form carbamoyl aspartate in presence of aspartate transcarbamylase (ATCase). This is the flux generating step of the pathway. This reaction occurs without the need for ATP as carbamoyl-P is intrinsically activated.



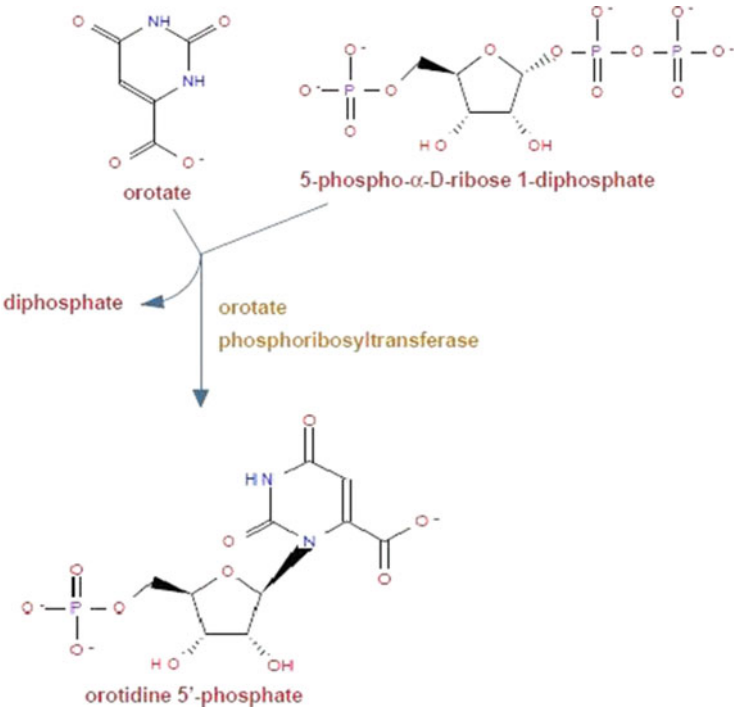
3. **Ring closure to form dihydroorotate**: This step was elucidated by Arthur Kornberg. This step is catalyzed by zinc metalloenzyme dihydroorotase wherein N-carbamoyl-L-aspartate is converted to dihydroorotate.



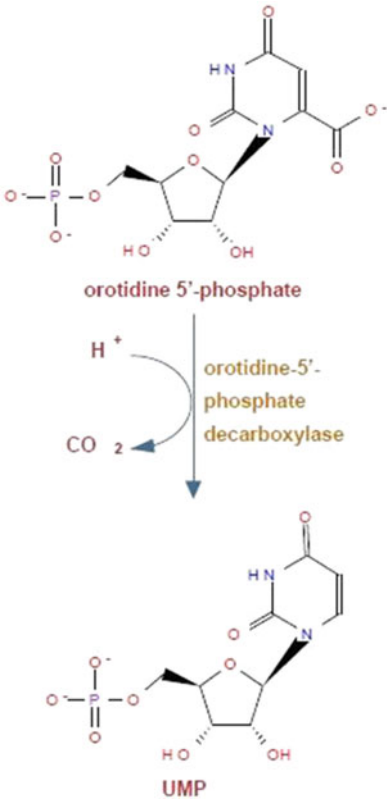
4. **Oxidation of dihydroorotate**: Dihydroorotate is oxidized to orotate by the action of the enzyme dihydroorotate dehydrogenase.



5. **Acquisition of ribose-5 moiety**: Orotate is phosphoribosylated to form OMP, orotidine-5-phosphate (orotidylate). This step is catalyzed by the enzyme orotate phosphoribosyltransferase (O-PRT).



6. **Decarboxylation of OMP to form UMP (uridylate)**: This step is catalyzed by the enzyme OMP decarboxylase (also known as UMP synthetase).

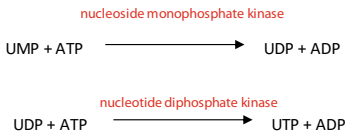


All the pathways representing synthesis of UMP taken from ecocyc.org. With kind permission from SRI Intrenational.

ATP consumption: Four ATPs are required in the UMP biosynthetic pathway. These are required in the following steps:

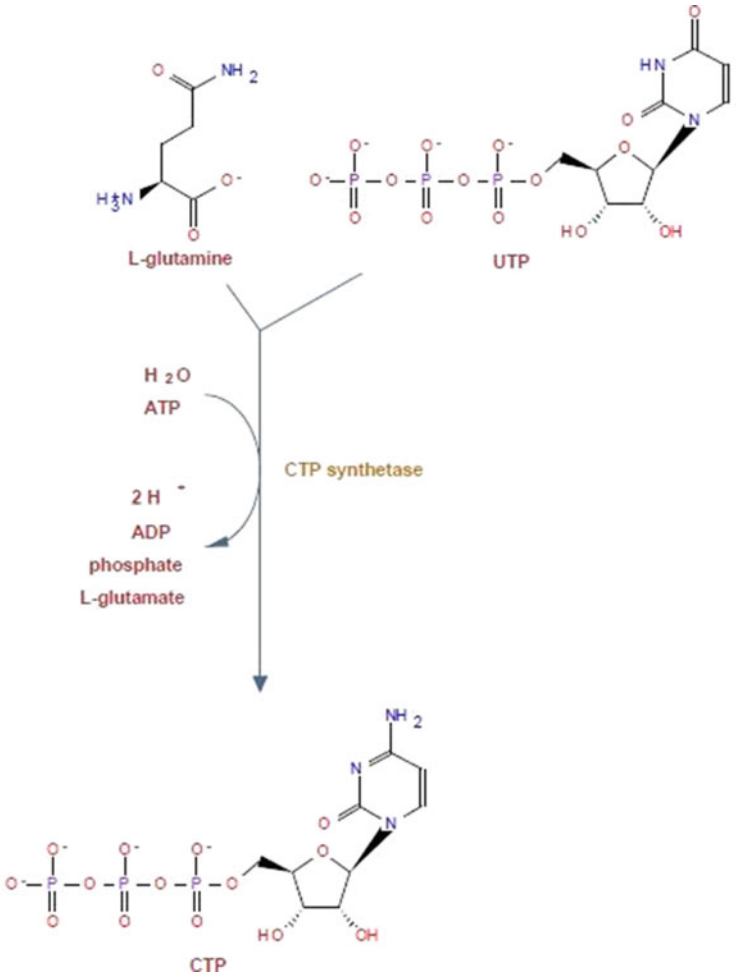
- Step 1—Glutamine carbamoyl phosphate (2ATPs)
- Step 5—Orotate + PRPP OMP (2 high-energy phosphate bonds used in synthesis of PRPP).

Phosphorylation of UMP: UMP is phosphorylated to form UDP via an ATP-dependent nucleoside monophosphate kinase. UDP is then converted to UTP by nucleoside diphosphate kinase.



Synthesis of CTP

CTP is produced by replacing the 4-keto group of UTP with NH_2 donated by L-glutamine. This reaction is catalyzed by CTP synthetase.



The pathways representing synthesis of CTP taken from ecocyc.org. With kind permission from SRI Intrenational.

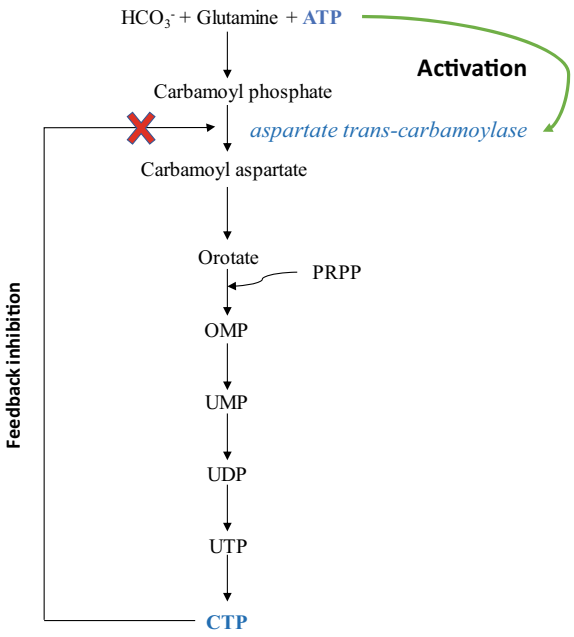


Fig. 3 Regulation of pyrimidine biosynthesis in *E. coli*

5 Regulation of Pyrimidine Synthesis in *E. coli*

In *Escherichia coli*, pyrimidine biosynthesis is regulated by feedback inhibition of aspartate trans-carbamoylase (ATCase) by the end product, CTP while it is activated by ATP (Fig. 3).

6 Synthesis of Deoxyribonucleotides

The ribonucleotides are converted to deoxyribonucleotides by ribonucleotide reductase which uses nucleotide diphosphates. All the four ribonucleotide diphosphates (ADP, CDP, GDP and UDP) are reduced at the 2′ position of the ribose sugar by ribonucleotide reductase (RNR) to form the corresponding deoxyribonucleoside diphosphates (dADP, CDP, dGDP and dUDP). The deoxyribonucleotide diphosphates (dNDPs) thus produced are then phosphorylated to deoxyribonucleotide triphosphates (dNTPs) by ATP-dependent nucleotide diphosphate kinases. It should be noted that uridine nucleotides do not occur in DNA; however, dUDP is still formed because it acts as the precursor for dTTP.

Ribonucleotide reduction by the enzyme ribonucleotide reductase (RNR) involves a cascade of reductions with reduced thioredoxin/glutaredoxin as the immediate

source of reducing equivalents and NADPH as the final source. Both thioredoxin and glutaredoxin have reactive Cys-sulfhydryl groups which serve as primary electron donors. The enzymes thioredoxin reductase or glutathione reductase then regenerate the reactive-SH pair of thioredoxin and glutaredoxin, respectively, using NADPH as the electron donor (Fig. 4).

Ribonucleotide reductase (RNR)

Ribonucleotide reductases (RNR) have been classified into three different classes: I, II and III, on the basis of their structure and mechanism of action. Based on the type of metal center required to generate the protein radical, class I RNR is further distributed in three sub-classes, viz. Ia, Ib and Ic (Table 1).

Usually, bacteria harbor more than one type of RNR, thus enabling them to survive under different conditions, as class II and III RNRs enable survival under anaerobic conditions while class I RNR is required for survival in aerobic conditions. *Pseudomonas aeruginosa* codes all three type of RNRs (type Ia, II and III). However,

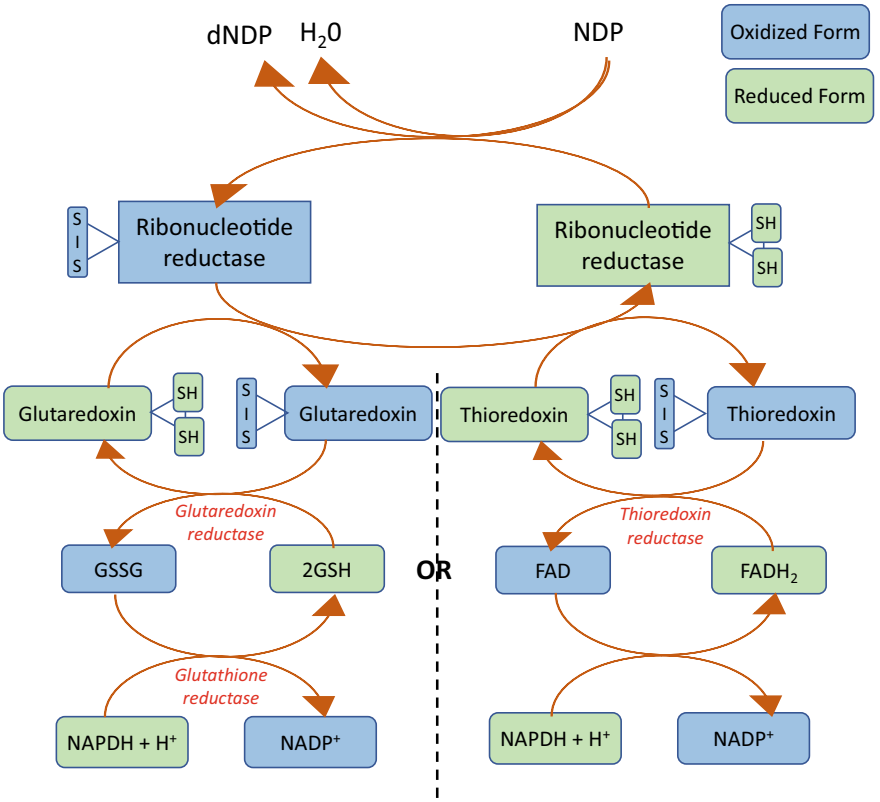


Fig. 4 Ribonucleotide reductase reaction showing cascade of reductions

Table 1 Different classes of ribonucleotide reductase (RNR)

	Class I	Class II	Class III
Subunit organization	Comprises of two proteins arranged in α2 + β2 or αn + βm form	Comprises of single α-chain polypeptide	Comprises of two homodimers α2 + β2
Function of subunits	α-subunit: catalytic site and allosteric regulation sites (specificity and activity); β-subunit: metallocofactor binding site	Contain catalytic site and allosteric specificity site but lack allosteric activity site; use S-adenosylcobalamine in place of β-subunit	α-subunit: catalytic site and allosteric regulation sites (specificity and activity); β-subunit: metallocofactor binding site (β-subunit need SAM binding to FeS center for radical generation)
Metallocofactor used	Ia: di-iron center (FeIII-O-FeIII); Ib: di-manganese center (MnIII-O-MnIII); Ic: MnIV-O-FeIII center	Cobalt (Co)	FeII-SII
Requirement for radical generation	Aerobic condition	Oxygen independent	Anaerobic condition
Reductants used	Thioredoxin/glutaredoxin	Thioredoxin	Formate
Distribution	Eukaryotes, eubacteria and archaea	Eubacteria and archaea	Eubacteria and archaea

bacteria like *Bacillus subtilis* which an obligate aerobe, codes for only one class of RNR, i.e., class Ib as they always require oxygen for their survival (Torrents, 2014).

In spite of a very low sequence homology among different classes of RNRs, they share more or less similar structural features and have two subunits. The center of the R1 protein (or α-subunit) contains allosteric regulation sites both for substrate specificity and for activity along with the catalytic site. Substrate specificity and activity effectors such as dNTPs and NTP bind to allosteric sites and modulates the catalytic activity of the RNR enzyme. On the other hand, R2 protein, i.e., β-subunit of RNR contains the metal cofactor binding site which is used to generate protein radical for reduction reaction (Fig. 5).

RNR is a complex enzyme and has various different kinds of inhibitors which act on different sites of the enzyme and inhibit it with different modes of action (Fig. 6). The first type of inhibitors are substrate analogs or inactivators of sulphydryl group which bind to catalytic site or allosteric site. The second group of inhibitors are the metal chelators or radical scavengers that interact with the β-subunit of the enzyme. The third group is antisense RNA molecules that bind to mRNA encoding the components of the enzyme (Torrents, 2014).

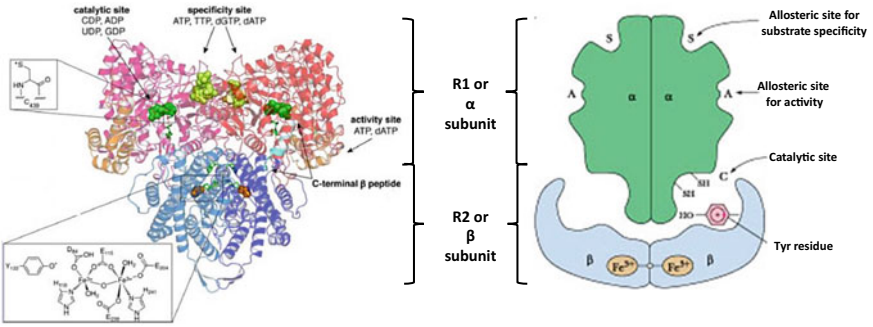


Fig. 5 Subunit organization of RNR and positioning of allosteric and catalytic sites. *Source* Minnihan et al. (2013). With kind permission from American Chemical Society (ACS)

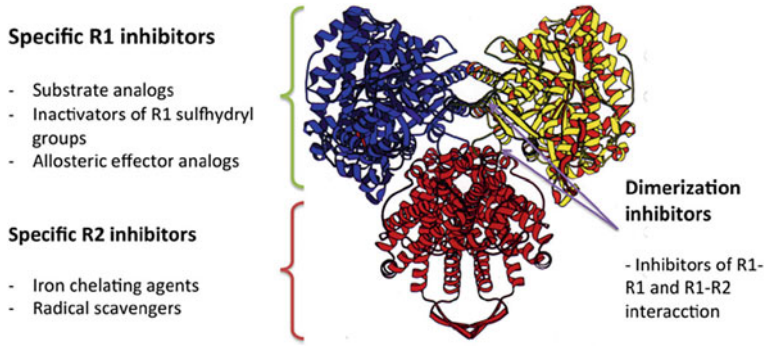


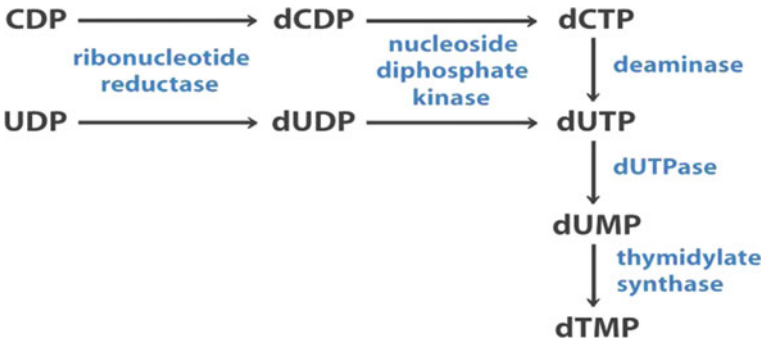
Fig. 6 Mode of action of different RNR inhibitors. *Source* Torrents (2014). (Creative Commons Attribution License)

Formation of dTMP from dUMP

Thymine containing nucleotides are built in a complex way to prevent accumulation of dUTP at a very high concentration so as to prevent dUTP incorporation into DNA by mistake. In *E. coli*, there are two pathways leading to dTTP:

- dCDP is converted to dCTP which is then deaminated to form dUTP
- dUDP is converted to dUTP.

Subsequently, dUTP is degraded by the highly active dUTPase to dUMP and PPi. UMP is then methylated at the 5′-position to form TMP by the enzyme thymidylate synthase (TS) which uses N^5, N^{10}-methylene tetrahydrofolate as the methyl donor.



Regeneration of N^5, N^{10}-methylene THF—In the reaction catalyzed by the enzyme thymidylate synthase, N^5, N^{10}-methylene THF is oxidized to DHF. DHF is then recycled to N^5, N^{10}-methylene THF through two sequential reactions:

- DHF is first reduced by dihydrofolate reductase (DHFR) to THF using NADPH.
- In the next step, a hydroxyl methyl group of serine is transferred to THF by the enzyme serine hydroxymethyl transferase, leading to formation of N^5, N^{10}-methylene THF and glycine (Fig. 7). The differences between purine and pyrimidine synthesis have been summarized in Table 2.

7 Inhibitors of Nucleotide Biosynthesis

The most important inhibitors of nucleotide biosynthesis can be classified into the following types:

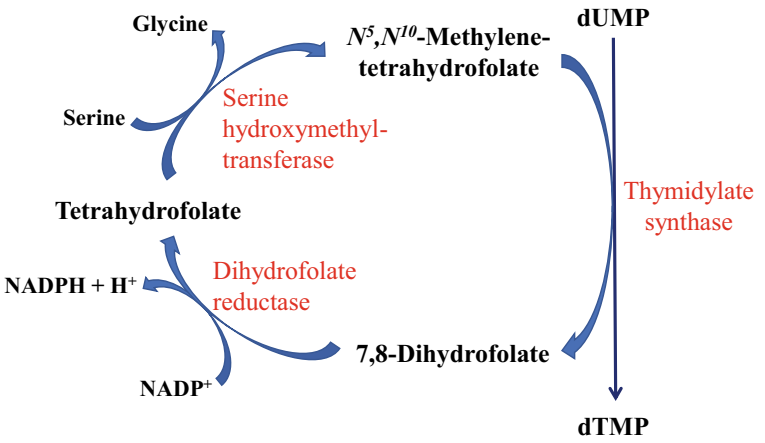


Fig. 7 Regeneration of N^5, N^{10}-methylene THF during the synthesis of dTMP

Table 2 Difference between purine and pyrimidine biosynthesis

	Purine	Pyrimidine
When and how the ring is added	PRPP is the precursor on which the ring is added	The ring is made first, then PRPP is added
No. of enzymatic steps	10 reactions to generate the first ribonucleotide (IMP)	6 reactions to generate the first ribonucleotide (orotidine monophosphate)
Which nucleotide is synthesized first	IMP is synthesized which is the precursor for both AMP and GMP	UMP is synthesized first and the other pyrimidines are synthesized from UMP; TMP is formed only from dUMP
Flux generating step	PRPP β-5-phosphoribosylamine	Carbamoyl phosphate carbamoyl aspartate
ATP consumption	Higher	Lower

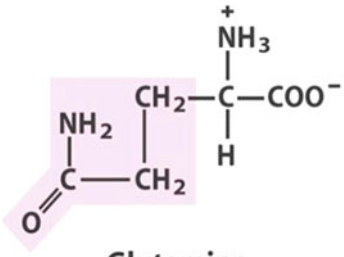
- Nucleotide analogs
- Precursor/substrate analogs
- Inhibitors of folic acid pathway.

Nucleotide analogs

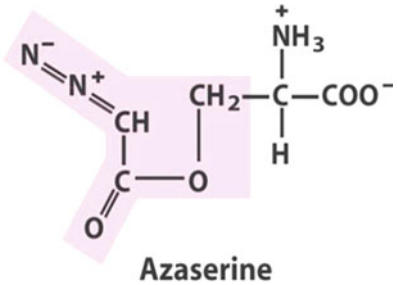
5-fluorodeoxyuridylate (FdUMP) is an analog of dUMP. It is an irreversible inhibitor of thymidylate synthase. Like dUMP, it binds to thymidylate synthase and to THF is permanently immobilized as the enzyme-FdUMP-THF ternary complex. Such inhibitors are called **mechanism-based inhibitors** (alternatively called **suicide substrates** because they cause the enzyme to commit suicide). FdUMP is recognized as an anti-tumor agent as its presence limits a steady supply of dTMP to rapidly multiplying cancer cells.

Precursor/substrate analogs

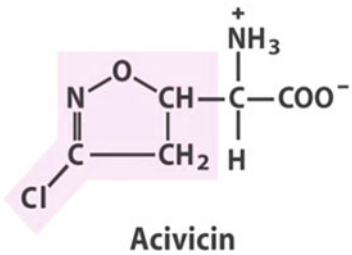
Reactions involving glutamine as a substrate are inhibited by glutamine analogs azaserine and acivicin.



Glutamine



Azaserine



Acivicin

Inhibitors of folic acid pathway

Purine biosynthesis is dependent on folic acid compounds at two steps:

- Step 4—Formylation of glycinamide ribotide (GAR) to form formyl glycinamide ribotide (FGAR) using N^{10}-formyl tetrahydrofolate
- Step 10—Formylation of 5-aminoimidazole-4-carboxamide ribotide (AICAR) to form 5-formylaminoimidazole-4-carboxamide ribotide (FAICAR) using N^{10}-formyl-THF.

dTMP biosynthesis from dUMP is also dependent on N^5, N^{10}-methylene THF which is used as a methyl donor.

Folic acid pathway can be inhibited by various compounds:

1. Compounds such as methotrexate (amethopterin), aminopterin and trimethopterin are DHF analogs that competitively bind to the enzyme DHFR. Inhibition of DHFR prevents reduction of DHF to THF, and thus, thymidylate synthesis is inhibited. This also indirectly blocks purine biosynthesis because of THF depletion. DHFR, therefore, provides an attractive target for chemotherapy.
2. Bacteria synthesize folic acid de novo from precursors, including p-aminobenzoic acid (PABA). Sulfa drugs, or sulfonamides are structural analogs of PABA and competitively inhibit bacterial synthesis of folic acid. In contrast, animals obtain folic acid through their diets and are less susceptible to sulfonamides.

8 Nucleotide Salvage Pathway

This pathway utilizes intermediates generated during degradative pathways for nucleotides, RNA and DNA. This pathway helps in conserving energy specially in higher organisms.

Salvage pathway for purine nucleotides: Phosphoribosyltransferases are the key enzymes of this pathway (Fig. 8). Adenine phosphoribosyltransferase (APRTase) for AMP formation and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) forms IMP from hypoxanthine and GMP from guanine.

$$\text{Base} + \text{PRPP} \rightarrow \text{nucleoside-5}'\text{-phosphate} + \text{PPi}$$

Salvage pathway for pyrimidine nucleotides: Uracil/thymine can be converted to uridine and thymidine by respective phosphorylases which will attach to either ribose or deoxyribose PRPP. Later on, these are converted to respective triphosphates (UTP/TTP) by kinases. Cytidine or deoxycytidine can be either directly phosphorylated to CTP or dCTP by respective kinases or they can first deaminated to uridine and deoxyuridine and enter the uracil salvage pathway (Fig. 9).

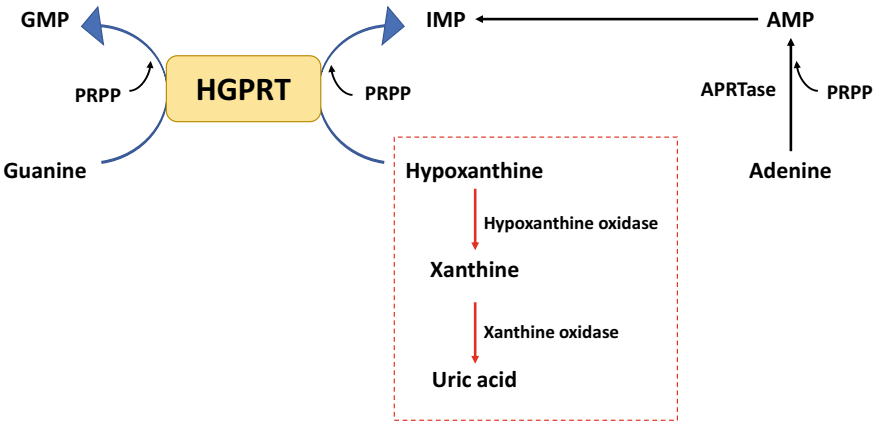


Fig. 8 Salvage pathway for purine nucleotides. Defect in HGPRT leads to uric acid accumulation (red box)

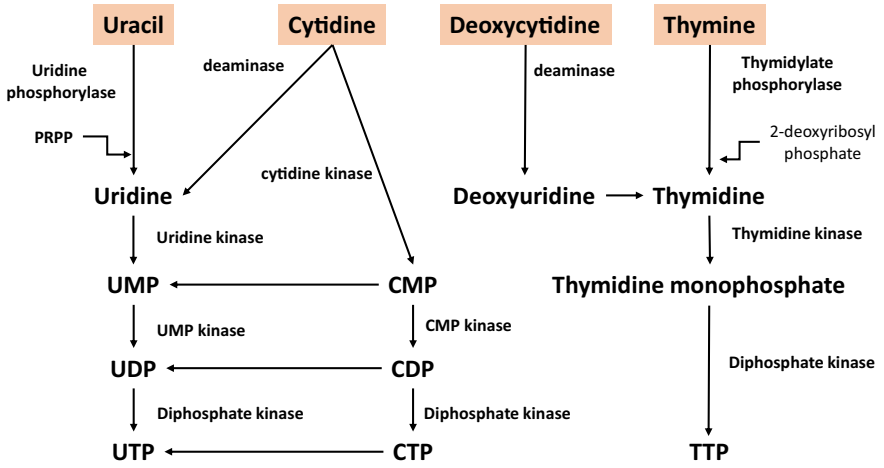


Fig. 9 Salvage pathway for pyrimidine nucleotides

Box 1: Biotechnological Applications of Nucleosides (Mikhailopulo & Miroshnikov, 2010)

Large-scale production of nucleosides and nucleotides is garnering continuous interest because of their applications in a wide variety of fields such as:

(i) **Synthesis of oligonucleotides such as short-interfering RNA (siRNA), microRNA (miRNA), etc**.

(ii) **Use of structural analogs of nucleosides as anticancer and antiviral drugs**—A large variety of naturally occurring analogs of nucleosides have been isolated. Detailed study of these compounds has provided useful clues for carrying out known and novel enzymatic biotransformations for synthesis of new nucleoside analogs as potential anticancer and antiviral drugs. Some of compounds that are now routinely used in biomedical applications include 6-mercaptopurine, thioguanine, 5-fluorouracil, thymidine analogs (such as 2′-deoxy-5-iodouridine, idoxuridine, iduviran, 2′-deoxy-5-fluorouridine, floxuridine, 3′-deoxy-3′-fluorothymidine, alovudine, 3′-deoxy-3′-azidothymidine, zidovudine), β-Darabinofuranosyl nucleosides, analogs having modified sugar moiety (Aciclovir, ACV, Zovirax, Gancyclovir, DHPG, Cytovene, Buciclovir, etc.).

(iii) **Use of nucleotides as umami flavor enhancers**—L-glutamate was identified as the main umami tastant in traditional Japanese cuisine prepared by dipping dried kelp into boiling water. Later, other umami compounds were found in bonito and shiitake mushrooms and identified to be IMP and GMP, respectively. Now, XMP is also recognized as an umami tastant. Use of these nucleotides as flavor enhancers for

meat, poultry, fish, vegetables and other prepared foods is now widely practiced and is approved by FAO and WHO.

Traditionally, various chemical reactions have been used for production of these compounds. However, biological methods including over-production in recombinant hosts and biotransformations employing specific enzymes are proving to be highly effective and promising technologies. Some of the processes employed for this are listed below:

- Yeast RNA is hydrolyzed using nucleases to generate AMP and GMP which are then deaminated to form IMP
- Inosine and guanosine produced by *B. subtilis* mutants are phosphorylated by chemical processes
- Sugar is directly fermented to IMP by mutants of *C. glutamicum*
- Guanine is converted to GMP using salvage pathway of *Brevibacterium ammoniagenes*.

9 Diseases Related to Nucleotide Biosynthetic Pathways

Lesch-Nyhan syndrome and hyperuricemia: This disease is caused by severe HGPRT deficiency. HGPRT deficiency leads to accumulation of uric acid leading to hyperuricemia. Defects in this enzyme fail to convert guanine and hypoxanthine bases to GMP and IMP, respectively, which causes an increase in the concentration of the formers and are thus degraded by xanthine oxidase to build up uric acid.

Box 2: Hyperuricemia-Preventing Probiotics (Li et al., 2014)

Lactic acid can be utilized to lower uric acid as they produce nucleosidases. These enzymes are widely found in plants and microorganisms but absent in mammals. Mammalian intestine epithelial cells and bacteria compete for nucleosides present in food, though some get absorbed the intestinal epithelium cells and convert to uric acid, but the concentration of uric acid decreased to a greater extent in presence of probiotic bacteria. Therefore, probiotics containing lactic acid bacteria capable of degrading nucleosides can be used to treat hyperuricemia in patients at early stages.

Muscle cramps—These are caused by a genetic deficiency in the enzyme AMP deaminase (also known as myoadenylate deaminase, which converts AMP to IMP). Increased muscular activity requires energy which is generated by activation of citric acid cycle. Citric acid cycle is activated by an increase in its intermediates, but the only intermediate that muscle tissues can generate is fumarate. Without sufficient

AMP deaminase, sufficient fumarate is not generated. In such conditions, muscles fatigue easily and exercising results in cramps.

Orotic aciduria—This condition is characterized by increased excretion of orotic acid in urine due to defective enzymes orotate phosphoribosyl transferase and orotate monophosphate decarboxylase that together catalyze the following reaction:

$$\text{Orotate} \rightarrow \text{OMP} \rightarrow \text{UMP}$$

This leads to growth retardation and anemia. The treatment involves administering uridine and/or cytidine that are phosphorylated to UMP and CMP. UMP and CMP inhibit carbamoyl phosphate synthetase II thereby decreasing production of orotic acid.

Summary

- Purines and pyrimidines are bases for nucleotide synthesis.
- Bacteria are fast-growing organisms that need to rapidly synthesize nucleotides to sustain growth. This is true for cancer cells as well. Thus, nucleotide synthesis is a good target for anti-cancer/antibacterial strategies.
- Nucleotide biosynthetic pathways are of two types, viz. (i) de novo pathway and (ii) salvage pathway.
- For purine nucleotide biosynthesis, phosphoribosyl pyrophosphate (PRPP) acts as a precursor on which a ring is added and after ten reaction steps the first ribonucleotide, inosine monophosphate (IMP), is generated which is used to synthesize AMP and GMP.
- Pyrimidine nucleotides are synthesized by adding PRPP on pyrimidine ring which then undergoes six consecutive reactions to form orotidine monophosphate, the first ribonucleotide in pyrimidine biosynthesis.
- UMP is synthesized first which leads to the synthesis of other pyrimidine nucleotides.
- Nucleotide biosynthesis is regulated by feedback inhibition, feed-forward activation as well as by cross-regulation.
- Nucleotide analogs, precursor/substrate analogs and inhibitors of folic acid pathway can inhibit nucleotide biosynthesis.

Questions

1. Which is not a catabolic product of purine:

    a. IMP
    b. Xanthine
    c. Hypoxanthine
    d. Cytidine

2. Purine ribosyl transferases catalyze:

a. Purine bases to nucleosides
b. Purine bases to monophosphate nucleotides
c. Purine bases to diphosphate nucleotides
d. Purine bases to triphosphate nucleotides

3. Draw purine ring and label precursors of C and N groups.
4. Differentiate between purine and pyrimidine biosynthesis.
5. Purine nucleotide levels are cross-regulated. Explain.
6. Ribonucleotide reductase reaction involves cascade of sulphydryl reduction. Explain.
7. Folate pathway is major target for regulating nucleotide biosynthesis. Explain.
8. What is salvage pathway?
9. Why it is necessary to keep uridine nucleotide levels low in a cell? How it is maintained?

References

Bhagavan, N. V., & Ha, C. E. (2015). *Essentials of medical biochemistry: With clinical cases* (2nd ed.). Academic Press.

Li, M., Yang, D., Mei, L., Yuan, L., Xie, A., & Yuan, J. (2014). Screening and characterization of purine nucleoside degrading lactic acid bacteria isolated from Chinese sauerkraut and evaluation of the serum uric acid lowering effect in hyperuricemic rats. *PLoS ONE, 9*(9), e105577.

Mikhailopulo, I. A., & Miroshnikov, A. I. (2010). New trends in nucleoside biotechnology. *Acta Naturae, 2*(2(5)).

Minnihan, E. C., Nocera, D. G., & Stubbe, J. (2013). Reversible, long-range radical transfer in *E. coli* class Ia ribonucleotide reductase. *Accounts of Chemical Research, 46*(11), 2524–2535.

Torrents, E. (2014). Ribonucleotide reductases: Essential enzymes for bacterial life. *Frontiers in Cellular and Infection Microbiology, 4,* 52.

Part IX
Response to Environmental Signalling

Chapter 20
Two-Component Systems

Rani Gupta and Namita Gupta

Two-component system of signaling environmental stimuli is ubiquitous in occurrence among all three domains of life, i.e., bacteria, eukarya and archaea. However, they are more abundant among prokaryotes. Primarily, two-component systems comprise a sensor kinase (SK) which receives stimuli and gets autophosphorylated and a response regulator (RR) which in turn is activated and has a DNA binding domain to regulate gene expression. The generalized phosphotransfer reaction requires divalent Mg^{2+} and ATP.

Sensor kinases are defined by a conserved amino acid sequence of nearly 200 amino acids with a conserved histidine residue at C-terminal that is phosphorylated in response to stimulus. N-terminal domain is variable as it responds to different signals. They are also called as histidine kinases (HK).

Response Regulator is defined by a conserved amino terminal domain of nearly 100 amino acids which interacts with C-terminal of histidine kinase at an aspartate residue.

1 Diversity of Two-Component Systems in Three Domains of Life

There is a large diversity of two-component systems of signaling in prokaryotes with as many as 30 histidine kinases and 32 response regulators in *Escherichia coli* and 80 two-component proteins in *Synechocystis* sp. In eukaryotes, one or two histidine kinases have been reported that are related to osmoregulation. Apart from these, two-component systems are also reported from plants, *Arabidopsis thaliana* and tomato, where they are involved in ethylene-induced fruit ripening. Some examples of distribution of two-component systems in different domains are tabulated in Table 1 (Stock et al., 2000).

Examples of some of the best studied two-component systems along with the signals perceived by them are provided in Table 2.

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_20

Table 1 Distribution of two-component systems in eubacteria, archaea and eukaryotes

Organism	No. of two-component systems present	
Prokaryotes		
Mycoplasma genitalium	0	
Escherichia coli	30	
Bacillus subtilis	70	
Haemophilus influenzae	9	
Helicobacter pylori	11	
Thermotoga maritima	19	
Archaea		
Methanobacterium thermoautotrophicum	24	
Methanococcus jannaschii	0	
Eukaryotes		
Saccharomyces cerevisiae	1	Involved in osmoregulation
Neurospora crassa	2	
Candida albicans	2	
Aspergillus nidulans	2	

Table 2 Examples of two-component systems with their functions and signal molecules

S. No.	Two-component system (HK/RR)	Functionality	Signal perceived	Organism
1	ArcB/ArcA	Oxygen and redox sensor	Quniones (reflecting redox state)	*E. coli*
2	BvgS/BvgA	Virulence	Temperature, sulfate ions, nicotinic acid	*Bordetella pertussis*
3	CheA/CheY	Chemotaxis	Chemo-attractants such as serine and aspartate	*E. coli*
4	CitA/CitB	Transport and anaerobic metabolism of citrate	Citrate	*E. coli*
5	DesR/DesK	Lipid modification	Temperature	*B. subtilis*
6	EnvZ/OmpR	Osmolarity	–	*E. coli*
7	FixL/FixJ	Nitrogen fixation	O_2, CO, NO	*Rhizobium meliloti*

(continued)

Table 2 (continued)

S. No.	Two-component system (HK/RR)	Functionality	Signal perceived	Organism
8	KinB/Spo0F	Sporulation	Maybe ATP	*B. subtilis*
9	LuxQ/LuxO	Quorum sensing	AI-2	*Vibrio fischeri*
10	NtrB/NtrC	Nitrogen utilization	α-ketoglutarate, glutamine	*E. coli*, *Salmonella enterica*, *Klebsiella pneumoniae*
11	NarX/NarL	Nitrate and nitrite metabolism	Nitrate/nitrite	*E. coli*
12	PhoQ/PhoP	Virulence, adaptation to low Mg^{2+} environments	Mg^{2+} and Ca^{2+}	*S. enterica*
13	PhoR/PhoB	Transport and assimilation of inorganic phosphate	Inorganic phosphate	*E. coli*
14	TodS/TodT	Degradation of benzene derivatives	Monoaromatic compounds	*Pseudomonas putida*

2 Types of Two-Component Systems

Two-component systems can be simple or complex (Attwood, 2013):

- **Simple system**
  In the simplest system, extracellular signals such as small molecule ligands and ions are detected by the periplasmic sensor domain of the histidine kinase (at its N-terminal) which modulates the catalytic activity of the cytoplasmic autokinase domain and promotes ATP-dependent autophosphorylation of a conserved histidine residue at its C-terminal. The phosphate is subsequently transferred to a conserved aspartate of the cognate response regulator (at its regulator side) through a phosphotransfer mechanism, and the activity of the response regulator is modulated in turn. The effector domain of the response regulator then binds to DNA and controls gene transcription, and thus allows cellular adaptation to an environmental signal. The type form is the EnvZ-OmpR osmosensor system of *E. coli* (Fig. 1) (Chang & Stewart, 1998; Mattison & Kenney, 2002).

- **Complex system**
  In complex systems, either a single histidine kinase activates multiple response regulators or multiple histidine kinases regulate single response regulator. Examples for this include:

  1. **Chemotaxis system in *E. coli***: Here, a single histidine kinase CheA phosphorylates two response regulators (CheB and CheY). A cell surface transmembrane receptor protein (called chemoreceptors or MCP) detects the stimulus,

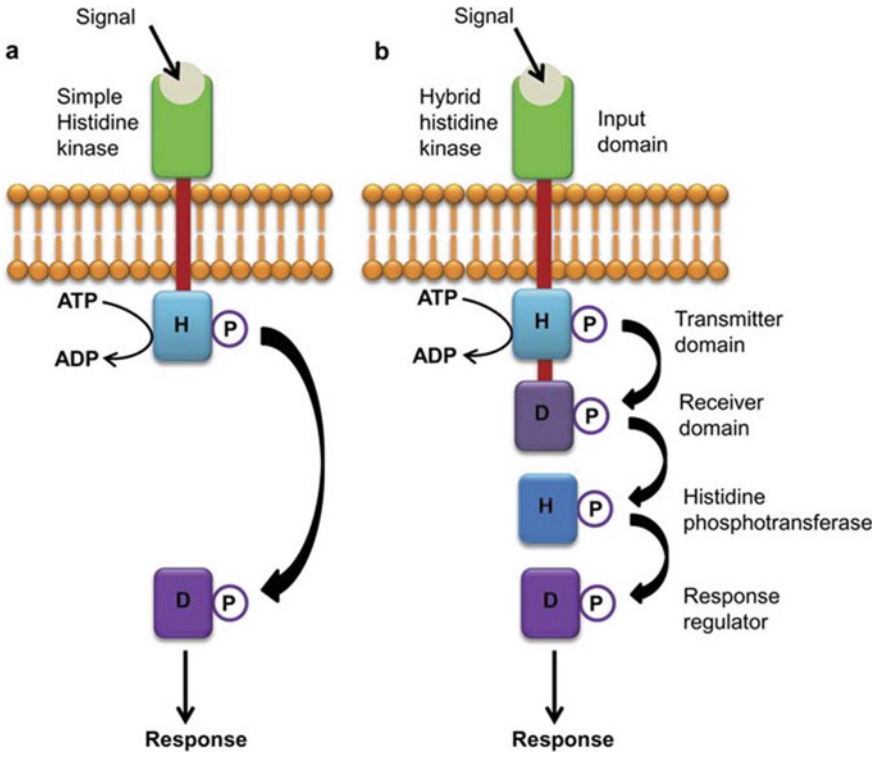


Fig. 1 Mechanism of simple (**a**) and complex (**b**) two-component system. H-histidine; D-aspartate; P-phosphoryl group and arrows indicate transfer of phosphoryl group during phosphorylation events. *Source* Sharan et al. (2017). (Creative Commons attribution license)

and this information is conveyed to the histidine kinase, CheA, via protein–protein interaction. CheA then phosphorylates either of the two response regulators, CheB or CheY. CheB promotes sensory adaptation, while CheY promotes changes in swimming direction (Fig. 2a).

2. ***E. coli* Pho system**: It is responsible for controlling gene expression in response to phosphate availability. A transport protein senses the phosphate concentration and transfers this information to the histidine kinases, PhoR and CreC, which in turn phosphorylate the response regulator PhoB (Fig. 2b).
3. **ArcAB system**: It regulates expression of various genes in response to the oxygen and redox conditions. The sensor kinase ArcB perceives stimulus and undergoes a His-Asp-His phoshphotransfer and then phosphorylates the response regulator ArcA (Fig. 2c).
4. **Nitrate/nitrite regulation system in *E. coli***: Here, two sensor kinases (NarX and NarQ) phosphorylate two response regulators (NarL and NarP) (Fig. 2d) (Chang & Stewart, 1998).

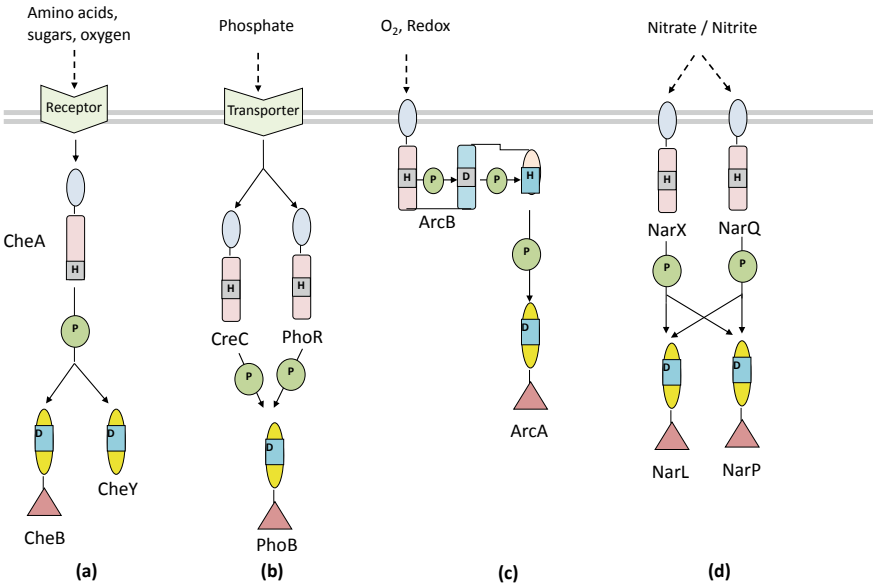


Fig. 2 Mechanisms involved in complex two-component systems in *E. coli* **a** chemotaxis system; **b** Pho system; **c** ArcB/ArcA system; **d** nitrate/nitrite regulation system

3 Sensor Kinases

Sensor kinases have an N-terminal input domain (usually periplasmic) that senses a specific stimulus, e.g., by binding or reacting with a signaling molecule or by interaction with a physical stimulus. The information is transduced through intramolecular changes resulting in activation of the cytoplasmic transmitter domain by autophosphorylation of a conserved amino acid which is usually histidine. Many eukaryotic systems predominantly have Ser/Thr/Tyr phosphorylation instead of histidine phosphorylation. However, there are examples where both phosphorylating systems have been found to function in both prokaryotes and eukaryotes. The difference is that Ser/Thr/Tyr kinases create phosphoesters whereas histidine kinases create phosphoamidates.

The nature of histidine kinase sensor proteins is such that the catalytic kinase domain and other cytoplasmic elements are conserved whereas the sensor domains are modular and highly variable in sequence to facilitate perception of diverse stimuli. The conserved kinase site gets autophosphorylated in the presence of ATP at a conserved histidine residue. Mostly, sensor kinases are known to function in a dimeric state where phosphorylation of a monomer catalyzes the subsequent phosphorylation of a second monomer. Subsequently, the phosphoryl group is transferred from histidine kinases to an aspartyl residue of the cognate response regulators. Thus, histidine kinases regulate the functioning of the two-component system by phosphorylation of its response regulator. Further, regulation is tightly operated by certain HKs which

have phosphatase activity as well and can dephosphorylate their cognate RR. This helps in fast switching on/off system for genes which need to be shut down rapidly.

Histidine kinases range from <40 to >200 kDa; the larger histidine kinases may have multiple functionality unique domains. The sensor kinases interact with their specific sensor molecules and also with nucleotides such as ATP which is used for phosphorylation. The affinity for sensor molecules varies with the sensor kinase, and it is in turn related to physiological regulation. Sensor kinases bind to ATP and ADP with almost similar affinity over a variety of sensor kinases.

Types of histidine kinases

Histidine kinases are divided into two classes comprising simple or orthodox and hybrid kinases:

- **Simple Histidine Kinases**: This class is exemplified by the most common EnvZ protein sensor kinase for osmosensing and controlling differential porin expression. The sensor kinase has a periplasmic N-terminal sensing domain and a C-terminal cytosolic domain, interconnected by two transmembrane domains (TM1 and TM2). There may also be multiple transmembrane domains, e.g., FixL for nitrogen fixation. There are cases where these sensor kinases lack the transmembrane domain and are not membrane bound such as chemotaxis kinase CheA and nitrogen regulatory kinase NtrB.
- **Hybrid Histidine Kinases**: The hybrid kinases are quite complex and are found in some prokaryotes but in all eukaryotic systems. These kinases have multiple phosphor-donor and phosphor-transfer sites. The best example is ArcB which

Table 3 Domain diversity among histidine kinases

Histidine kinase	Autokinase domain	Additional cytoplasmic domains	Periplasmic domains	Transmembrane domain
DesK	1	–	✗	✓
EnvZ	1	HAMP-1	✗	✓
FixL	1	PAS-1	✗	✓
NtrB	1	PAS-1	✗	✗
CitA	1	PAS-1	PAS-1	✓
PhoQ	1	HAMP-1	PAS-1	✓
LuxQ	1	RRR-1	LuxQ	✓
KinA	1	PAS-3	✗	✗
ArcB	1	PAS-1, RRR-1, Hpt-1	✗	✓
CheA	1	Hpt-1, CheY binding-1, CheW-1	✗	✗
TodS	2	PAS-2, RRR-1	✗	✗

has a N-terminal transmembrane domain followed by His-kinase domain, then response regulator-type aspartic phosphorylating domain (RRR) and a secondary histidine phosphorylating domain called HPt domain. It has been estimated that nearly 20% of all histidine kinases are encoded as hybrid proteins, containing additional RRR or HPt domains. Another example is TodS (sensor for degradation of benzene derivatives) which has two autophosphorylation domains, while citA (sensor for transport and metabolism of citrate) has two sensor domains each in periplasm and cytoplasm, and kinase has three PAS domains (phosphor-relay sensor kinases). Some of the sensor kinases are known to exist in dimer form as an active conformation. In these cases, switching from monomer to dimer is a way of de-repression of two-component systems, viz. CheA/CheY and PhoR/PhoB (Table 3) (Krell et al., 2010).

Structure of sensor kinases

The prototypical sensor kinase contains a N-terminal periplasmic sensor domain and a C-terminal cytoplasmic transmitter domain. The periplasmic sensor domain is flanked on either side by transmembrane helices (TM1 and TM2) wherein the second transmembrane helix (TM1) connects the sensor domain with the transmitter domain. The transmitter domain contains a sequence with the conserved histidine residue for autophosphorylation (H box) and ends with the highly conserved autokinase (or catalytic) domain. The domain with the conserved His residue typically contains two α-helices (X box), which serve as a dimerization domain (dimerization and histidine phosphotransfer) or Histidine kinase A domain. The catalytic domain contains conserved sequences. This domain catalyzes autophosphorylation of the histidine kinase (HK) by phosphoryl transfer from ATP to HisKA domain (Mascher et al., 2006). The response regulators then catalyze their own phosphorylation, with the phosphoryl-HK as the phosphodonor (Fig. 3).

However, a large number of sensor kinases depict numerous variations of this standard prototype. For example, approximately 83% sensor kinases have transmembrane regions while the remaining are located in the cytosol. Many sensor kinases contain some additional domains which can be either signal transduction domains

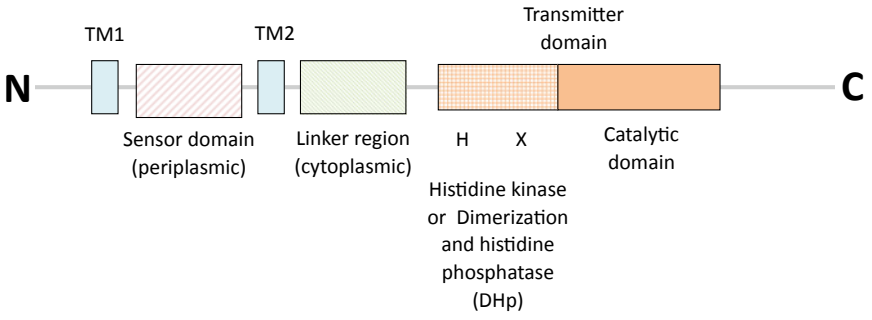


Fig. 3 Domain organization of simple histidine protein kinase

or sensory input domains. Some of these domains such as HAMP or PAS domain may be linker regions that exist between the second transmembrane region and the transmitter domain, while others may be additional phosphorylation domains that exist downstream of the transmitter domain. Some of these are described below:

1. **PAS domains**: These are important signaling modules that monitor changes in light, redox potential, oxygen, small ligands and overall energy level of a cell. Unlike most other sensor modules, PAS domains are located in the cytosol. PAS domains have been identified in various proteins such as histidine and serine/threonine kinases, chemoreceptors and other types of proteins in all branches of the phylogenetic tree. PAS domains are structurally conserved, 100- to 120-residue-long and are composed of a central, five-stranded anti-parallel β sheet flanked by α helices. PAS is an acronym formed from the names of the three proteins in which such sequences were first recognized: *Drosophila* period clock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT) and *Drosophila* single-minded protein (SIM). PAS domains are found almost exclusively in sensors of two-component regulatory systems in case of prokaryotes and archaea (Taylor & Zhulin, 1999).
2. **HAMP domain**: HAMP domain signaling motifs are so-called because they are found in histidine kinases (HKs), adenylyl cyclases, methyl-accepting chemotaxis proteins (MCPs), and some phosphatases. They are important linker domains for signal transduction from periplasmic input to cytoplasmic kinase domain. In membrane-associated proteins, HAMP domains usually lie near the cytoplasmic side of the membrane, where they convert transmembrane and intracellular sensory inputs to output response signals. HAMP motifs are defined by a few conserved residues, by characteristic hydrophobic heptad repeats and by predicted helical and non-helical secondary-structure elements. HAMP subunits are ∼50 residues long, with two predicted amphiphilic (AS1 and AS2) helices joined by a non-helical connector. Two alternative HAMP structures have been described, HAMP(A) which is a tightly packed, parallel, four-helix bundle and HAMP(B) which is a more loosely packed bundle with an altered AS2/AS2′ packing arrangement.
   Stimulus-induced conformational changes probably modulate HAMP signaling by shifting the relative stabilities of these opposing structural states, HAMP(A) and HAMP(B). These changes in turn modulate output signals by altering structural interactions between output helices through heptad repeat stutters that produce packing phase clashes. Output helices that are too tightly or too loosely packed most likely produce kinase-off output states, whereas kinase-on states require an intermediate range of HAMP stabilities and dynamic behaviors (Parkinson, 2010).
3. **RRR**: This is the response regulator-type receiver domain which is an additional phosphorylation domain present in hybrid kinases. This comprises receiver domains typical for response regulators with a conserved aspartate residue for phosphorylation.

4. **HPt domain**: This is the ***H***istidine ***p***hospho***t***ransfer domain which contains an active histidine residue that mediates the phosphotransfer reaction between the RRR domain and the terminal response regulator. HPt domain was first discovered in the *E. coli* ArcB sensor.

It should be noted that in addition to the above, many sensor kinases possess various other domains such as CACHE, CHASE, GAF, PBP, TarH. (West & Stock, 2001; Krell et al., 2010).

Activity of sensor kinases

Sensor kinases perform three activities: (1) autophosphorylation; (2) transphosphorylation of its cognate response regulator; and (3) dephosphorylation of response regulator. The first two activities are invariably present throughout, while the third activity is selectively present, e.g., vancomycin two-component system.

I. **Autophosphorylation**: The autokinase domain of the histidine kinase catalyzes an ATP-dependent transautophosphorylation reaction in which one subunit of the histidine kinase dimer phosphorylates a specific His residue within the other subunit. The resultant phosphoimidazole is chemically ideal for donating a phosphoryl group to an Asp side chain of a downstream response regulator.

II. **Transphosphorylation**: The basic mode of transphosphorylation comprises autophosphorylation of sensor kinase and subsequent direct phosphorylation of response regulator by protein–protein interaction between sensor kinase domain and response regulator domain (as in the PhoQ/PhoP system). However, a large number of sensor kinases are known where transphosphorylation is a multi-step phenomenon before the response regulator is phosphorylated (Krell et al., 2010). These multi-steps may have multiple autokinase domains, or it may have multiple phosphorelay mechanism. Multiple autokinases are present in TodS/TodT two-component system wherein both the autokinases have similar activities but only the N-terminal domain is stimulated by signal binding. Phospho-relay involves an autokinase domain, a RRR domain and subsequently an HPT domain prior to the phosphorylation of the response regulator. Many two-component systems have the phosphorelay mechanism such as KinA/Spo0F/Spo0B/Spo0A system and ArcAB system. However, in all cases, the chemistry of transfer is conserved and consists of His → Asp → His → Asp. Of all, KinA/Spo0F/Spo0B/Spo0A system for phosphorelay is unique as it requires four additional proteins which are phosphorylated before finally response regulator is phosphorylated. These additional proteins are called connectors. These will be dealt in more detail while dealing with signaling during sporulation and competence in *B. subtilis*.

III. **Dephosphorylation**: The response regulator is dephosphorylated to set the system back to the prestimulus state. The phosphatase responsible for this can be an intrinsic property of the kinase toward the regulator or of the response regulator (autophosphatase). However, external phosphatases are also common. Additionally, some histidine kinases show autophosphatase activity

toward their own His-phosphate group. Some response regulators also catalyze significant back transfer of the phosphate group to the corresponding His kinase (Stock et al., 2000; Mitrophanov & Groisman, 2008).

4 Mechanism of Signal Sensing by Sensor Kinases

The signal perception can be grouped into three classes: (1) sensing at sensor domain; (2) sensing at transmembrane domain; and (3) sensing at auxiliary proteins. This is well reviewed by Krell et al. (2010) and Mascher et al. (2006).

1. **Sensing at sensor domain**: The most frequently found sensor domain at N-terminal is PAS domain which is diverse in sequence but conserved in topology, being a α/β fold. PAS domain has been shown to have a cavity called signal recognition site. The ligands which show in vivo effect were able to autophosphorylate sensor kinase in vitro and the ligand which did not show any in vivo activity failed to autophosphorylate the sensor kinase. This suggests that ligand binding triggers further event of autophosphorylation. Besides this, there are some other modes of sensing at the sensor domain as described below:

    - *Sensing by cofactor containing PAS domain*: PAS domain can perceive signal by cofactor containing PAS domain. The cofactor binding PAS domain was first identified in response to changes in internal oxygen concentration, redox potential and light. Major cofactors involved are FAD, FMN and heme bound to central cavity. The oxygen sensing sensor kinase FixL has transmembrane domain, a PAS domain and C-terminal autokinase domain. It does not have any periplasmic domain as it senses internal redox. PAS domain has ferrous heme molecule where oxygen binds at distal end. The oxygen binding site is surrounded by hydrophobic residue which restricts oxygen access to other sites. Oxygen binding brings conformational change leading to kinase inactivation. The structural analysis of ligand bound and ligand free PAS domain shows that upon oxygen binding, a loop proximal to heme site called FG gets displaced, in turn inactivates kinase. There are several sensor kinases which sense cellular redox and can mediate through even more than one PAS (Taylor & Zhulin, 1999; Dupré et al., 2013).
    - *Sensing through membrane interface*: Another mode of sensing is through membrane interface and is exemplified by PhoQ sensor kinase. Initial studies had shown that PhoQ/PhoP (involved in *Salmonella* to induce S genes required for survival)-mediated transcription is repressed by bivalent cations. Later, it was shown that high concentration of cations does not affect autokinase activity but stimulates its phosphatase activity which dephosphorylates response regulator and stops transcription. The structure of PhoQ bound to Ca^{++} ions shows that the acidic patch of PAS domain surface facing the membrane gets neutralized by cations and the repulsive force between membrane and PAS domain decreases. It contracts the PAS domain bringing

it closer to membrane. This structural change finally changes conformation of PhoQ, and its phosphatase activity is switched on. On the other hand, antimicrobials activate PhoQ by displacing metal cations and relieving it from the membrane and again the conformational changes take place in sensor kinase which is activated.

- *Sensing through signal-mediated modulation of kinase oligomeric state by disulfide bond formation*: Another mode is the signal-mediated modulation of kinase oligomeric state by disulfide bond formation. This is best exemplified by ArcB/ArcA two-component system. Under aerobic conditions, the ArcB/ArcA system is switched off as ArcB kinase is inactive, and it exhibits phosphatase activity and dephosphorylates ArcA, and thus, transcription is switched off. The signal perceived by ArcB is ubiquinone. The molecular mechanism regulating kinase and phosphatase activity of ArcB has been studied. The ArcB PAS domain has two cysteine residues, and ArcB exhibits kinase activity when these cysteines (C180 and C241) are reduced and ArcB exists in monomeric form. When cysteine C180 is oxidized, dimerization takes place and kinase activity is reduced by 15%. However, when both the cysteines are oxidized and two S–S bonds are generated, there is complete loss of kinase activity. Thus, ArcB PAS dimerization leads to loss of kinase activity. The oxidation of PAS domain is a result of reduced ubiquinone availability which is in turn an indicator of available oxygen. Thus, under aerobic conditions, there is more UbH_2 and ArcB gets dimerized resulting in loss of kinase activity and concomitant dephosphorylation of ArcA response regulator and finally transcription is inhibited.

2. **Sensing at transmembrane domain**: The discovery of sensor kinases lacking sensor domain led to indications that there are alternate sites as well. This is best exemplified by DesK/DesR two-component system which senses temperature and regulates temperature-dependent lipid synthesis via *des* genes encoding $\Delta 5$-lipid desaturase. DesK sensor kinase has both autophosphorylation and phosphatase activity. At 37 °C, DesK has primarily phosphatase activity and DeR is dephosphorylated and desaturase transcription is repressed. However, at lower temperatures, the reverse happens. Here, there is no outer sensor domain and it contains five transmembrane regions. The temperature is sensed at transmembrane region which is passed on further and it is actually temperature-dependent fluidity of membrane lipids.
3. **Indirect sensing by signal perception at accessory proteins**: In these cases, signal is perceived by additional proteins that transmit it to sensor kinases. Thus, such systems are generally called **three-component systems**. Few well-studied examples of accessory proteins are periplasmic accessory protein involved in luminescence (LuxP), methyl-accepting chemotaxis proteins involved in chemotaxis and cytosolic accessory protein in nitrogen regulation (PII).

    - *Periplasmic accessory protein LuxP*: Lux system is a quorum-sensing system that regulates luminescence gene expression in response to population density and host association in gram-negative bacteria. The quorum-sensing

signal is AI-2 which is at a low concentration at low cell densities. Lux system has a transmembrane sensor kinase protein LuxQ. In the absence of AI-2 (i.e., at low cell density), LuxQ has autokinase activity and gets phosphorylated. The phosphoryl group is then transferred to the LuxQ receiver domain, then to the phosphotransferase protein LuxU and finally to the transcriptional regulator LuxO.

Binding of AI-2 occurs at the LuxP sensor protein, which forms a complex with the periplasmic domain of LuxQ. Binding of AI-2 causes a conformational change within LuxP. This conformational change stabilizes a quaternary arrangement in which two LuxP/LuxQ monomers are asymmetrically associated. This asymmetric structure represses the kinase activity of LuxQ, thereby triggering the transition into quorum-sensing mode.

- *Transmembrane accessory proteins MCP*: Methyl-accepting chemotaxis proteins (MCPs) are the predominant chemoreceptors in bacteria. MCPs are transmembrane receptors comprising a periplasmic ligand binding site (LBD) and a cytoplasmic signaling domain. The cytoplasmic signaling domain interacts with the histidine kinase CheA. On signal binding, the periplasmic LBD generates a stimulus that is transmitted across the membrane and modulates autophosphorylation of the sensor kinase (CheA). The Tar chemoreceptor is the best example of MCP. It has two transmembrane regions and a LBD that forms a four-helix bundle. The final α-helix of the LBD extends into the membrane forming a transmembrane region. Binding of aspartate at the LBD causes a shift in the final α-helix, and thus, ligand binding involves a piston-type shift of this transmembrane helix. This is the stimulus that alters CheA autophosphorylation.

  Results have suggested that the molecular mechanism of MCP transmembrane signaling is similar to that of sensor kinases. This was seen in case of NarX sensor domain where ligand (nitrate) binding induced piston-type displacement between the N- and C-terminal helices of the periplasmic domain that was proposed to trigger transmembrane signaling.
- *Cytosolic accessory protein PII*: The synthesis of many enzymes required for nitrogen assimilation in enterobacteria is regulated at the level of gene transcription by a nitrogen regulation (Ntr) system. The glnALG operon belonging to Ntr regulon contains glnA, the structural gene for glutamine synthetase (GS) and glnL and glnG, the structural genes for sensor kinase NRII (NtrB) and response regulator NRI (NtrC), respectively. Glutamine synthetase is capable of assimilating nitrogen from poor nitrogen sources in absence of ammonia.

PII, a cytosolic accessory protein, regulates the activities of NtrB sensor kinase toward its response regulator NtrC. NtrB sensor kinase has a periplasmic PAS domain for which no function has been assigned, and NtrB regulation is mediated indirectly by a cytoplasmic protein PII.

In the presence of high nitrogen, PII binds to NtrB and activates its phosphatase activity, and consequently, NtrC is dephosphorylated and transcription of Ntr operon

genes is switched off. On the other hand, in low nitrogen condition, PII exists either as PII-UMP or as PII-α-ketoglutarate. In both cases, availability of PII is depleted and NtrB exhibits autokinase activity and NtrC is phosphorylated to activate transcription of Ntr operon which is required during ammonia depletion (Lee et al., 2000).

5 Response Regulators

Response regulators are defined by a conserved amino terminal regulatory/receiver domain of nearly 100 amino acids which interacts with C-terminal of histidine kinase and catalyzes phosphorylation at an aspartyl residue. On its C-terminal, it has a variable DNA-binding domain or effector domain. Response regulators thus function as phosphorylation-regulated switches that couple a wide variety of cellular behaviors to environmental cues (Gao et al., 2007).

The regulatory domains of response regulators have three activities:

1. They interact with phosphorylated histidine kinases and catalyze transfer of a phosphoryl group to one of their own Asp residues. They can also get phosphorylated by other phosphorylated compounds; acetyl phosphate, carbamoyl phosphate, imidazole phosphate and phosphoamidate suggesting that it can function in the absence of histidine kinases. Though histidine kinases or HPt domains are not required for catalysis, rates of phosphotransfer from these phosphor His-containing proteins are much greater than from small molecules.
2. They catalyze autodephosphorylation (phosphatase activity) thereby limiting the lifetime of active response regulator. The phosphatase activity varies greatly among different response regulators with half-lives ranging from seconds to hours. However, the lifetimes of different response regulators appear well correlated with their physiological functions and other regulatory strategies of the system.
3. They regulate the activities of their associated effector domains in a phosphorylation-dependent manner.

Effector domain

Effector domains have DNA-binding activity and function to activate and/or repress transcription of specific genes. Based on the type of effector domains, response regulators are classified into three major sub-families:

OmpR: It is the largest sub-family of response regulators. OmpR functions both as an activator and as repressor for the *ompC* and *ompF* genes that encode outer membrane porin proteins. OmpR dimers bind to F and C boxes preceding porin genes. It exists in different binding conformations when bound to *ompF* and *ompC* (Mattison & Kenney, 2002). **It is a winged helix transcription factor**. PhoB is another important member of this sub-family. There is lot of structural similarity among this sub-family; still, these response regulators interact differently with RNA

polymerase as OmpR interacts with α-subunit of RNA polymerase, while PhoB interacts with σ^{70}-subunit (Itou & Tanaka, 2001; Kenney, 2002).

NarL: NarL is a transcription factor that activates/represses genes during nitrate and nitrite metabolism. This sub-family has a typical **helix turn helix motif** which allows specific interaction between recognition helix and bases in the NarL recognition heptameric sequences present on the DNA. Another important response regulator under this family is FixJ (Maris et al., 2002, 2005).

NtrC: It is the most functionally complex sub-family of response regulators. NtrC is a transcriptional enhancer, and its DNA binding effector domain has **ATPase domain along with helix turn helix domain**. It functions as a dimer and oligomerizes to octamer on phosphorylation. On oligomerization, ATP hydrolysis is stimulated which activates transcription (Lee et al., 2000).

Box 1: Bioinformatic Analysis of TCS

The increasing number of annotated bacterial genomes has resulted in compilation of signal transduction proteins in databases. The availability of large sequence databases has led to the development of new approaches for their analyses. Some of the important databases are:

- Microbial Signal Transduction (MiST) database uses a domain-based approach for identification of signal transduction molecules and represents a comprehensive electronic catalog of signaling components in microbial genomes (https://mistdb.com/).
- Another database (https://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html) classifies bacterial RR proteins according to their domain architectures.
- Prokaryotic two-component Systems (P2CS) database provides detailed annotation of each TCS gene including family classification, sequence features and functional domains (https://www.p2cs.org/).

Summary

- Two-component systems of signaling environmental stimuli are ubiquitous in occurrence among all three domains of life, i.e., bacteria, eukarya and archaea.
- Two-component systems comprise a sensor kinase (SK) which receives stimuli and gets autophosphorylated and a response regulator (RR) which regulates gene expression.
- Sensor kinases, also known as histidine kinases, have a N-terminal sensor domain (usually periplasmic) that senses a specific stimulus and a conserved histidine residue at C-terminal (autokinase domain) that is autophosphorylated in the presence of ATP in response to stimulus.

- Response regulators have a conserved aspartate residue at the N-terminal (regulatory domain) which interacts with C-terminal of histidine kinase and a DNA binding or effector domain at its C-terminal.
- Two-component systems can be simple or complex.
- In simple two-component systems, the stimulus is detected by the periplasmic sensor domain of the histidine kinase which activates its autokinase and promotes autophosphorylation of the conserved histidine residue. The phosphate is then transferred to a conserved aspartate of the response regulator, and its effector domain can then bind to DNA and control gene transcription. EnvZ-OmpR osmosensor system of *E. coli* is an example of a simple two-component system.
- In complex two-component systems, either a single histidine kinase activates multiple response regulators or multiple histidine kinases regulate single response regulator. Examples for this include the chemotaxis system, Pho system, ArcAB system and nitrate/nitrite regulation system in *E. coli.*
- In sensor kinases, the catalytic kinase domain and other cytoplasmic elements are conserved whereas the sensor domains are highly variable in sequence to facilitate perception of diverse stimuli.
- Mostly, sensor kinases are known to function in a dimeric state where phosphorylation of a monomer catalyzes the subsequent phosphorylation of a second monomer.
- Histidine kinases can be simple or hybrid kinases.
- Simple sensor kinases have a periplasmic N-terminal sensing domain and a C-terminal cytosolic domain, interconnected by two transmembrane domains. Examples are EnvZ and FixL sensor kinases.
- Hybrid kinases have multiple phospho donor and phospho transfer sites by having additional domains such as response regulator-type aspartic phosphorylating domain (RRR), secondary histidine phosphorylating domain (HPt) or additional sensor or autokinase domains. Examples are ArcB and TodS sensor kinases.
- Sensor kinases perform three activities—autophosphorylation, transphosphorylation of its cognate response regulator and dephosphorylation of response regulator.
- Transphosphorylation comprises autophosphorylation of sensor kinase and subsequent direct phosphorylation of response regulator. In a large number of sensor kinases, transphosphorylation is a multi-step phenomenon involving multiple autokinase domains or multiple phosphorelay mechanism.
- Signal perception by sensor kinases can be at the sensor domain, at the transmembrane domain or indirect sensing through auxiliary proteins.
- Regulatory domains of response regulators exist in two conformations, inactive and active, wherein the active conformation is stabilized by phosphorylation. Phosphorylation leads to repositioning of N- and C-terminal domains to facilitate binding of effector domain to the DNA.
- Based on the type of effector domains, response regulators are classified into three major sub-families, i.e., OmpR, NarL and NtrC.

Questions

1. Answer in one line:
   i. What is two-component system of signaling? Is it present throughout living organisms?
   ii. How is a sensor kinase different from typical protein kinases found in animal kingdom?
   iii. What is the distinctive characteristic of a sensor kinase and a response regulator?
   iv. Give any one example each of simple and complex two-component systems.
2. Differentiate between simple and hybrid sensor kinases.
3. Expand the following domain nomenclature:
   i. PAS ii. HAMP iii. RRR iv. HPt
4. What are the activities of a sensor kinase?
5. Write a detailed note on signaling mechanism.
6. What are response regulators?
7. What are the characteristic features of a response regulator?
8. What are the major families of response regulators?

References

Attwood, P. V. (2013). Histidine kinases from bacteria to humans. *Biochemical Society Transactions, 41*(4), 1023–1028.

Chang, C., & Stewart, R. C. (1998). The two-component system: Regulation of diverse signaling pathways in prokaryotes and eukaryotes. *Plant Physiology, 117*(3), 723–731.

Dupré, E., Wohlkonig, A., Herrou, J., Locht, C., Jacob-Dubuisson, F., & Antoine, R. (2013). Characterization of the PAS domain in the sensor-kinase BvgS: Mechanical role in signal transmission. *BMC Microbiology, 13*(1), 172.

Gao, R., Mack, T. R., & Stock, A. M. (2007). Bacterial response regulators: Versatile regulatory strategies from common domains. *Trends in Biochemical Sciences, 32*(5), 225–234.

Itou, H., & Tanaka, I. (2001). The OmpR-family of proteins: Insight into the tertiary structure and functions of two-component regulator proteins. *The Journal of Biochemistry, 129*(3), 343–350.

Kenney, L. J. (2002). Structure/function relationships in OmpR and other winged-helix transcription factors. *Current Opinion in Microbiology, 5*(2), 135–141.

Krell, T., Lacal, J., Busch, A., Silva-Jiménez, H., Guazzaroni, M. E., & Ramos, J. L. (2010). Bacterial sensor kinases: Diversity in the recognition of environmental signals. *Annual Review of Microbiology, 64,* 539–559.

Lee, J., Owens, J. T., Hwang, I., Meares, C., & Kustu, S. (2000). Phosphorylation-induced signal propagation in the response regulator NtrC. *Journal of Bacteriology, 182*(18), 5188–5195.

Maris, A. E., Kaczor-Grzeskowiak, M., Ma, Z., Kopka, M. L., Gunsalus, R. P., & Dickerson, R. E. (2005). Primary and secondary modes of DNA recognition by the NarL two-component response regulator. *Biochemistry, 44*(44), 14538–14552.

Maris, A. E., Sawaya, M. R., Kaczor-Grzeskowiak, M., Jarvis, M. R., Bearson, S. M., Kopka, M. L., Schröder, I., Gunsalus, R. P., & Dickerson, R. E. (2002). Dimerization allows DNA target site recognition by the NarL response regulator. *Nature Structural Biology, 9*(10), 771–778.

Mascher, T., Helmann, J. D., & Unden, G. (2006). Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiology and Molecular Biology Reviews, 70*(4), 910–938.

Mattison, K., & Kenney, L. J. (2002). Phosphorylation alters the interaction of the response regulator OmpR with its sensor kinase EnvZ. *Journal of Biological Chemistry, 277*(13), 11143–11148.

Mitrophanov, A. Y., & Groisman, E. A. (2008). Signal integration in bacterial two-component regulatory systems. *Genes & Development, 22*(19), 2601–2611.

Parkinson, J. S. (2010). Signaling mechanisms of HAMP domains in chemoreceptors and sensor kinases. *Annual Review of Microbiology, 64,* 101–122.

Sharan, A., Soni, P., Nongpiur, R. C., Singla-Pareek, S. L., & Pareek, A. (2017). Mapping the 'Two-component system' network in rice. *Scientific Reports, 7*(1), 1–13.

Stock, A. M., Robinson, V. L., & Goudreau, P. N. (2000). Two-component signal transduction. *Annual Review of Biochemistry, 69*(1), 183–215.

Taylor, B. L., & Zhulin, I. B. (1999). PAS domains: Internal sensors of oxygen, redox potential, and light. *Microbiology and Molecular Biology Reviews, 63*(2), 479–506.

West, A. H., & Stock, A. M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends in Biochemical Sciences, 26*(6), 369–376.

Chapter 21
Bacterial Response to Oxygen Availability

Rani Gupta and Namita Gupta

1 Introduction to Oxygen Sensors in *E. coli*

Bacteria respond to oxygen availability, and this is well documented among facultative anaerobes which can switch over to fermentative mode or to other modifications to immediately adopt to hypoxia or anoxia when encountered with anaerobic or microaerophilic conditions. Such adaptations may be short term/transient or long term/persistent. The short-term adaptations may occur in a fraction of seconds and include movement toward conducive environment (aerotaxis) or covalent modification of proteins or some other transient modifications. On the other hand, long-term adaptations are delayed responses and largely include metabolic shifts by switching on and off of a variety of operons. All these adaptations are a result of signal transduction which need not be always a two-component system.

There are a variety of oxygen sensors through which a cell responds to oxygen availability. At a time, a complex response is often observed as a result of a single factor as sensors have functional diversity. However, these sensors may respond to different levels of hypoxia or superoxia. In *E. coli*, as many as eight sensors are known which respond to different levels of anaerobiosis. Some respond with fast reactions while others are known to bring out delayed catabolic changes. All oxygen sensors are classified on the basis of the physiological functions they carry out. They respond to oxygen availability and switch on a series of responses ranging from fast protective/defensive action to altered catabolic activity to develop a balance between oxygen availability and oxygen demand. The eight sensory proteins are tabulated in Table 1 (Iuchi & Weiner, 1996).

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_21

Table 1 Features of the oxygen sensors in *E. coli*

Name of sensor	Function	Role (protective/catabolic)	Action
Rapid response sensors			
Aer	Aerotaxis	Protective role	Rapid movement toward microenvironment optimal for growth
OxyR SoxRS	Superoxide dismutase and catalase activity	ROS protective role	Scavenge ROS in the form of hydrogen peroxide and superoxide radicals
Hmp	Soluble flavohemoglobin	Protective role	Soluble flavohemoglobin traps the available oxygen
Dsb	Disulfide bond formation	Protective role	Protects periplasmic proteins by rapid oxidative folding of the proteins
Dos	Direct oxygen sensor	Protective role	Phosphodiesterase activity toward cyclic-bis diguanylic acid
Delayed response sensors			
Fnr	Formate nitrate reductase	Catabolic role	Global regulators Major sensors responsible for controlling catabolic gene expression in response to oxygen availability Regulate ~120 proteins
ArcAB	Anoxic respiratory control	Catabolic role	

2 Rapid Response Sensors

Rapid response sensors respond with fast reactions to changing oxygen levels. The various rapid response sensors are as follows:

1. **Aer system**: Aer, a major aero-taxis transducer in *E. coli*, is a member of the methyl-accepting chemotaxis protein (MCP) family of chemoreceptors and mediates robust aerotactic responses by rapid movement of bacterium toward microenvironment optimal for growth. Aer is a transmembrane receptor protein and has a cytoplasmic N-terminal sensing domain (PAS) that senses the oxygen status through redox changes in a FAD prosthetic group and modulates the activities of motility sensor kinase CheA (a histidine kinase).

2. **SoxRS and OxyR systems**: The first and foremost protection is against reactive oxygen radicals that an organism faces in normal growth times as well as aerobic conditions. At all times, the reactive oxygen radicals (ROS) in the form of hydrogen peroxide and superoxide radicals are scavenged by the activities of various enzymes modulated by SoxRS and OxyR sensors.

   - **SoxRS**: *SoxR* and *SoxS* genes activate the transcription of ~10 proteins, such as superoxide dismutase (SodA), endonuclease IV, glucose 6-phosphate dehydrogenase, fumarase C and NADH: ferrodoxin oxidoreductase, that protect the cell from various types of damage (Touati, 2000).
     These proteins do not belong to the family of two-component systems. However, their activation requires two steps where SoxR functions as the sensor while SoxS directly controls target gene expression.
   - **OxyR system**: OxyR protein is a transcription factor that activates the expression of a subset of peroxide stress-inducible genes, most of which are involved in defense systems against oxidative stress including catalase I, alkylhydroperoxide reductase, glutathione reductase and glutaredoxin (Lee et al., 2004).

3. **Hmp**: The *hmp* gene encodes a soluble flavohemoglobin having protective response against NO and functions both as a reductant and as an oxidase. These flavohemoglobins have a two-domain structure formed by a globin-like domain (containing a single B-type heme) and a $NADP^+$: ferrodoxin oxidoreductase-like domain (harboring a FAD moiety and a NAD(P)H binding motif). Their expression is induced in the presence of reactive nitrogen and oxygen species. They function as NO (nitric oxide) scavengers and detoxify NO in an aerobic process, termed nitric oxide-dioxygenase reaction, which protects the host from various noxious nitrogen compounds. They have alkylhydroperoxide reductant activity and lipid-binding property and hence are probably involved in repair of membrane lipid damage, caused by oxidative and nitrosative stress (Cruz-Ramos et al., 2002).
4. **Dsb proteins**—These are disulfide bond-forming enzymes used by gram-negative bacteria such as *E. coli* to ensure disulfide bond formation between the correct pairs of cysteines in a large number of proteins exported to the periplasmic space. Disulfide bonds contribute to the stability, activity and folding of the proteins. The Dsb proteins are, in most cases, oxidoreductases belonging to the thioredoxin superfamily that have an active site containing a CXXC motif (cysteines separated by two amino acids) embedded in a thioredoxin fold. Five Dsb proteins, namely DsbA, DsbB, DsbC, DsbG and DsbD, have been identified (Kadokura & Beckwith, 2010).

   - **DsbA**: It is a small monomeric periplasmic protein (21 kDa) having a redox active sequence, Cys30-Pro31-His32-Cys33, embedded in a thioredoxin fold. It was the first protein shown to be involved in disulphide bond

formation in the bacterial periplasm. In the absence of DsbA, many cell envelope proteins of *E. coli* acquire their disulfides much more slowly or fail to acquire them.

- **DsbB**: It is a membrane protein that maintains DsbA in its oxidized active state. DsbB (20 kDa) is an inner membrane protein that spans the membrane four times. It has two pairs of essential cysteine residues, one pair located in each periplasmic domain. In the oxidized state of DsbB, the cysteines form disulfide bonds. DsbB re-oxidizes DsbA protein in a way that electrons from DsbA flow sequentially to the first and second cysteine pairs of DsbB and from there to quinones in the respiratory chain.
- **DsbC**: DsbC is a periplasmic disulfide bond isomerase. It is a homodimeric molecule **constituted by 23 kDa monomers. It has a** N-terminal dimerization domain connected by a linker α-helix. DsbC repairs proteins with incorrect disulfide bonds and ensures their correct folding.
- **DsbG**: It is a 26 kDa monomeric periplasmic thiol-oxidoreductase homologous to DsbC. DsbG functions by dimerization with its N-terminal dimerization domain and its C-terminal thioredoxin domain connected by a linker helix.
- **DsbD**: It is membrane bound largest among Dsb family protein with a molecular mass of 59 kDa. It has three distinct domains: an N-terminal periplasmic domain with an immunoglobulin-like fold, a hydrophobic core domain with eight transmembrane segments and a C-terminal periplasmic domain with a thioredoxin-like fold. DsbD functions to maintain both DsbC and DsbG in the reduced (active) state and represents a two-electron transporter (Kadokura & Beckwith, 2010; Collet & Bardwell, 2002).

5. **DosP**: Direct oxygen sensor (DosP) of *E. coli* has a phosphodiesterase activity that catalyses linearization of cyclic-di-GMP which is a secondary messenger in *E. coli* and regulates a large number of physiological functions, for example, motility, virulence, biofilm formation, etc. DosP has an N-terminal heme bound PAS domain for binding to oxygen and C-terminal phosphodiesterase activity which is highly enhanced when oxygen is bound to N-terminal. When oxygen is dissociated from heme, phosphodiesterase activity of DosP is inhibited which results in building up of cyclic-di-GMP that in turn promotes biofilm formation (Sasakura et al., 2002).

3 Delayed Response Sensors

These bring out delayed catabolic changes. These include the Fnr and ArcAB sensors which regulate catabolic pathways at both kinetic level and at transcriptional level. As many as 120 proteins are known to be affected and their expression changes in response to switching from aerobic to microaerophilic conditions. These two global regulators are major sensors responsible for controlling catabolic gene expression in response to oxygen availability in *E. coli.*

ArcAB two component system

ArcAB system, previously known as aerobic respiratory control/anaerobic respiratory control, and now accepted as **anoxia respiratory control** system of signal transduction, is a two-component system. In this, ArcB is a histidine kinase and ArcA is its cognate response regulator. Apart from catabolic regulation, it has a number of pleiotropic effects on unrelated cellular functions, viz. expression of F plasmid transfer genes and Xer-based recombination of *psi* site required for stable plasmid inheritance. ArcAB represents a global regulator for both up-regulation and down-regulation of key enzymes of TCA cycle, glyoxylate shunt, fatty acid degradation and also cytochrome oxidases.

ArcB: ArcB is a 82 kDa protein constitutively expressed under both aerobic and anaerobic conditions. It is a multi-domain sensor kinase anchored as a membrane protein by transmembrane N-terminal domain bridged by 17 amino acid short periplasmic domain. Toward the cytoplamic side, there is a leucine zipper and a PAS domain connected to His-292 phosphorylation site in the transmitter domain. ArcB is a tripartite sensor kinase and thus has additional histidine phosphotransfer domain (Hpt) His-717 at C terminal besides a receiver domain Asp-576 (Fig. 1) (Kwon et al., 2000).

ArcA: ArcA is a cognate response regulator for ArcB sensor kinase. It is a 29 kDa cytoplasmic protein having N-terminal receiver phosphorylating site at Asp-54 and a C-terminal DNA-binding site as helix-turn-helix (HTH) DNA-binding domain.

Phospho-relay mechanism: Upon receiving stimulus, ArcB autophosphorylates and subsequently phosphorylates ArcA. In this reference, earlier it was believed that direct phosphorylation of ArcA can result both by primary sensory site His292 or through Hpt after complete phospho-relay to Hpt via its internal receiver domain (Fig. 2).

However, recently it has been clearly demonstrated that it is only through phospho-relay in vivo via ArcBHis292-ArcBAsp576-ArcBHis717 → ArcAAsp54.

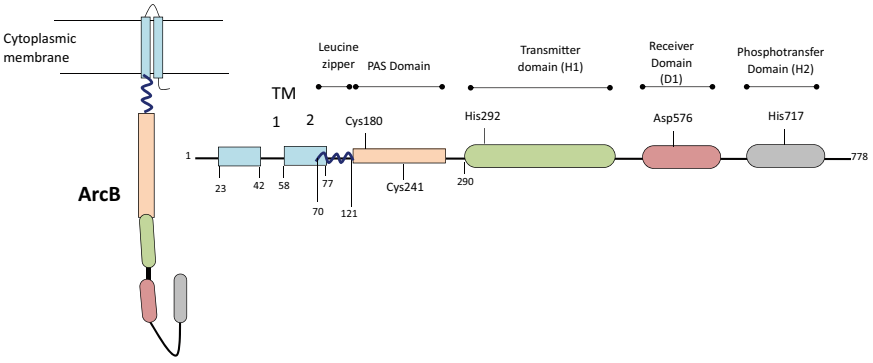


Fig. 1 Schematic representation of domain organization of ArcB in *E. coli*

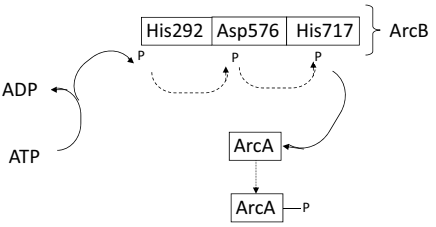


Fig. 2 Phospho-relay mechanism in ArcAB

This has been proved by studying phosphorylation of ArcA from expression of *cydA* (cytochrome d oxidase subunit 1) and *lldP* (permease of lactate dehydrogenase operon) and analyzed by *CydA-LacZ* and *LldP-LacZ* fusion proteins. *cydA* is up-regulated and *lldP* is down-regulated in ArcAB system upon exposure to anaerobic conditions. In this experiment, it was clearly shown that all three domains of ArcB are essential for phosphorylation of ArcA, and transcription of both *cydA* and *lldP* is affected in absence of C domain of ArcB. Hence, this complex phospho-relay is the sole mechanism of phosphorylation of ArcA.

Transmembrane domain—TM1 and TM2: ArcB is anchored in the cytoplasmic membrane by two transmembrane domains (TM1 and TM2) along with a short 17 amino acid periplasmic bridge. In an experiment to study importance of this domain in signal transduction, it was found that on complete deletion of this domain by replacing it with subunit of maltose permease (MalF), ArcB failed to phosphorylate ArcA while deletion of only one transmembrane domain had minor effect on *cydA-lacZ* expression. Further, when ArcB had a truncated transmembrane domain and became completely cytoplasmic, its kinase activity for ArcA phosphorylation became constitutive. A latest study also showed that on partially deleting TM domain and releasing ArcB in cytoplasm, ArcB had wild-type nature toward expression of *sdh* (succinate dehydrogenase which is repressed by ArcAB) as studied by expression of *Sdh-LacZ* fusion proteins. These results confirm that signal is perceived at the cytoplasmic end and not externally at periplasmic end.

Significance of leucine zipper of ArcB in signal transduction

Leucine zipper motif is characterized by typical coiled coil structure which is formed by interaction of helices of monomers. The motif has four contiguous heptad repeats ($LX_6LX_6LX_6LX_6$). In each of these heptad repeats donated as abcdefg, leucine is present in position "d". Such leucine zipper motifs are known to be involved in homo- or heterodimerization. The hydrophobic faces of the helices from two monomers face each other to give a coiled coil dimeric structure (Fig. 3).

Presence of leucine zipper indicates ArcB function through homo-dimerization. ArcB function has been known to work through reduction of disulfide linkages of cytoplasmic redox active cystein residues of ArcB. This redox reaction is possible only through dimerization of ArcB which is only facilitated by proximity of two cysteins. The presence of leucine zipper linker further strengthens the existence of

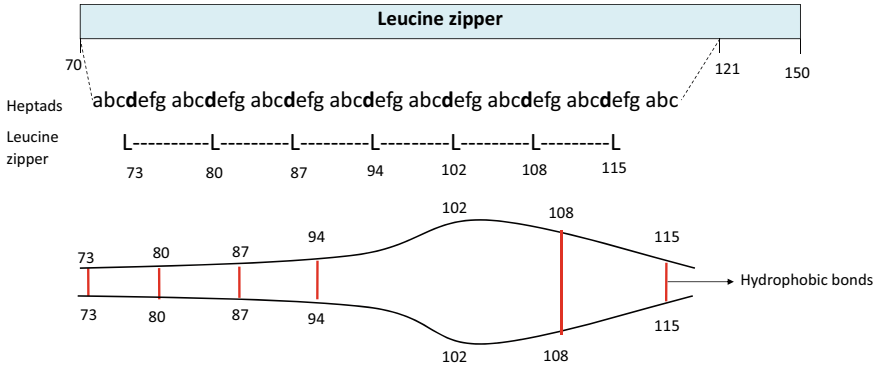


Fig. 3 Diagrammatic representation of leucine zipper in ArcB of *E. coli*

ArcB as dimer as these motifs have long been known to promote homo or hetero-dimerization of various proteins.

Leucine zipper of ArcB of *E. coli* starts from amino acid 70 upto amino acid 150 where there are seven conserved leucine residues at positions 73, 80, 87, 94, 102, 108 and 115. The dimeric coil shows proximities of six leucine residues; L73, L80, L87, L94, L108 and L115 while L102 was found free.

Functionality of these leucine residues was checked by mutating each one to valine which will not disturb the coil structure. The leucine mutants produced three phenotypes:

- Semi-constitutive: retaining kinase activity only and no phosphatase activity;
- Complete loss of ArcB function;
- Kinase activity was lost without any effect on phosphatase activity.

The paired mutants: L73 and L80; L87 and L94; L108 and L115, belong to the respective three phenotypes. Another interesting observation was that the phenotype was dominant negative phenotype in presence of $ArcB^+$ complementation. Such a result is possible only if the conformation of mutant is fixed after L to V mutation as kinase or phosphatase conformation. Further, the mutant which lost activity (L87 and L94) and did not recover in $ArcB^+$ strain is possible by heterodimer formation in mutant. This study clearly indicates that the leucine zipper conformation of its coiled coil fold is an integral part of ArcB signaling (Oreza et al., 2012).

Periplasmic domain

ArcB sensor kinase is unique as it has a very small periplasmic sequence of about 17 amino acids. This is in contrast to most of the sensory kinases which have a characteristic periplasmic domain to perceive signal. The experiments for localizing the role of transmembrane domain have clearly shown that this domain serves only as an anchor to keep ArcB in the membrane close to the signal specially reduced ubiquinones/menaquinones. However, the catalytic function of ArcB is not lost even if it is liberated in the cytoplasm by completely removing the periplasmic domain

by genetic deletions. This further suggests that a signal can be perceived at cytosolic end (Kwon et al., 2000).

Mechanism of catalytic function of sensor kinase ArcB at the cytosolic domain

The mechanism of catalytic function of sensor kinase ArcB was explored by developing *Tar-ArcB* hybrids. The hybrid did not show aspartate activation, a feature of Tar periplasmic B and also the hybrid was constitutive both in aerobic and anaerobic conditions, showed autokinase activity (checked by *LldP-LacZ* fusion protein which is repressed in anaerobic conditions) and *lldP-lacZ* was repressed in all conditions. This shows that Tar fusion led to a conformation which led to a certain orientation of cytoplasmic domain which perceives redox signal. Thus, the periplasmic domain is of no use to ArcB sensor kinase, and it does not have piston-like movement of transmembrane domain as in Tar kinase.

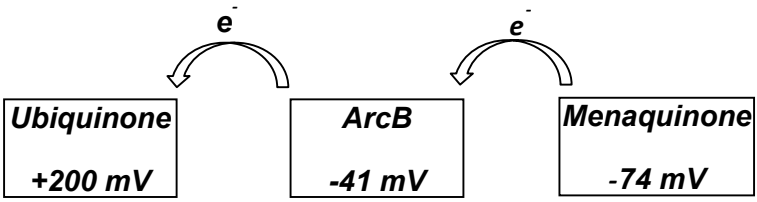
ArcB is a homodimer and is stabilized by disulphide bonds. It functions by intermolecular phosphoryl group transfer from one subunit to other. Finally, it was shown that rotational movement at the cytoplasmic end of one monomer relative to other on signal perception is facilitated by reduction and oxidation of disulphide bond between the homodimer (Kwon et al., 2003).

ArcB senses oxygen availability through redox state of cell

ArcB does not sense oxygen directly as during in vitro experimentation it was found that products such as lactate, acetate, NADH which accumulate during anaerobic conditions promoted phosphorylation of ArcA. Further, level of succinate dehydrogenase as studied by *sdh-lacZ* fusion was found to vary with the electron acceptor present, i.e., it was highest in oxygen followed by nitrate and fumarate. This also suggests that possibly ArcB senses the reduced electron acceptors. This was proved experimentally where *cyo* and *cyd* deletion mutants were used. It was observed that by deleting terminal oxidases, the expression of *Cyd-LacZ* increased and *Cyo-LacZ* was lowered even in aerobic conditions. This indicated that in absence of terminal oxidases, the cell sensed the conditions to be anaerobic even in presence of oxygen. This clearly supports that accumulation of reducing equivalents are being sensed as indictor of anaerobic conditions and not oxygen per se.

The terminal oxidase mutants have constitutive ArcB activity, and down-regulation of *lldp-lacZ* was obtained even in aerobic conditions. This further necessitates identification of one real redox regulator. In this respect, it was known that ubiquinones are negative regulator of ArcB kinase activity as it promoted oxidation to two cystein residues responsible for intermolecular disulfide bond formation. However, role of ubiquinone as ArcB kinase silencer under aerobic conditions was long disputed. Recently, Alvarez et al. (2013) addressed this issue by studying expression in a *ubiCA* mutant carrying *cydA-lacZ* fusion operon. No decrease in reporter gene was observed in the mutant while in the wild strain shifting from anaerobic to aerobic conditions led to a rapid decrease in expression. The mutant expression was restored by cloning low-copy *ubiCA* gene confirming role of ubiquinone in silencing ArcB kinase activity. This was further strengthened by repeating the experiment in another *E. coli* strain and a different fusion protein *Sdh-LacZ* where expression is

down-regulated in stimulated condition. Here also, in a ubiquinone mutant while moving from anaerobic to aerobic conditions, the repression was not overcome, emphasizing that ArcB kinase activity is not silenced in absence of ubiquinone (Malpica et al., 2004; Bekker et al., 2010).



Ubiquinone can easily serve as an oxidant according to redox potential difference also. The redox potential for ubiquinone is +200 mV and that for ArcB is −41 mV. On the other hand, menaquinone may transfer electron to ArcB as menaquinones have much lower redox potential of −74 mV. Therefore, it is suggested that ubiquinones serve as silencer when shifted from anaerobic to aerobic conditions by oxidizing cysteins, while menaquinone can serve as activator by donating electrons during shift from aerobic to anaerobic conditions. Based on these assumptions, it can be put forth that ArcB has affinity for both ubiquinone and menaquinone and thus is regulated by UB/MQ ratio under variety of oxygen availability conditions ranging from aerobic to microaerophilic to anaerobic (Fig. 4) (Alexeeva et al., 2003).

FNR (fumarate–nitrate reductase) activator

Fnr gene was discovered to be necessary for the anaerobic induction of fumarate, nitrate and nitrite reductases. FNR was recognized to be responsible for inducing the expression of many genes in response to anaerobiosis. With few exceptions, FNR serves as a transcription activator.

The FNR protein is best characterized in *E. coli*, but its homologs are widely conserved among bacteria. In *E. coli*, FNR controls a large regulon of 300 genes that are involved in anaerobic metabolism (Spiro & Guest, 1990; Sawers et al., 1997).

In *E. coli*, FNR protein has been isolated in a monomeric form (molecular weight: 30,000 Da). It is expressed constitutively; but is active only in anaerobic conditions. The cellular concentration of FNR is similar under both anaerobic and aerobic growths, but its activity is regulated directly by oxygen. Thus, in *E. coli*, the FNR expression is regulated at post-transcriptional level as *fnr* transcript is similar in both aerobic and anaerobic conditions. However, in *Bacillus subtilis*, it is regulated at transcriptional level. Further, *Fnr* gene in *E. coli* is subject to negative autoregulation during anaerobic growth.

FNR is related to the *E. coli* cAMP receptor protein (CRP). Apart from a cysteine-rich N-terminal domain, the primary structure of FNR is highly homologous to the structure of CRP and both FNR and CRP are founder members of a well-characterized global family of structurally related transcription factors in bacteria (Sawers et al., 1997). Proteins of the CRP/FNR superfamily contain:

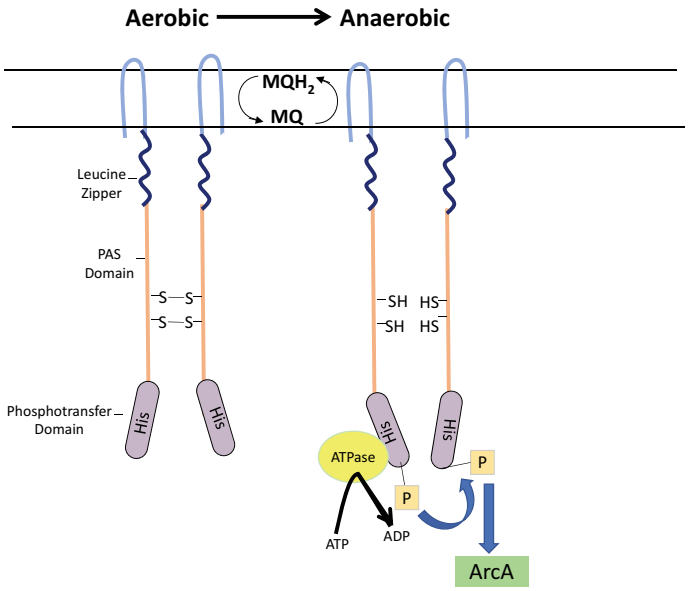


Fig. 4 Simplified mechanism of autophosphorylation of ArcAB sensor kinase under shift from aerobic to anaerobic conditions

- N-terminal sensory domain which is a ligand-binding domain for small molecules (e.g., cAMP, NO, or O_2) that has an extension of 29 amino acids containing three cysteins (C20, C23 and C29);
- C-terminal DNA-binding domain having a helix-turn-helix motif for DNA binding;
- Dimerization domain between N-terminal and C-terminal.

FNR is a Fe-S protein. The Fe-S cluster plays a key role in sensing anaerobiosis. The protein acts in different states: oxidized (2Fe-2S) form which is inactive and reduced (4Fe-4S) form which is active. The formation of the active form (4Fe-4S) involves four conserved cysteine residues. These include the three essential cysteines at the N-terminal which bind the (4Fe-4S) cluster along with C122 (Kiley & Beinert, 1998).

On exposure to anaerobic conditions, FNR acquires a $(4Fe\text{-}4S)^{-2}$ cluster that causes a conformational change and dimerization of the protein that causes it to become activated. On exposure to oxygen, FNR is inactivated due to the disassembly of the dimers and the oxidation of the $(4Fe\text{-}4S)^{-2}$ clusters to $(2Fe\text{-}2S)^{-2}$ clusters. On prolonged oxygen exposure, the $(2Fe\text{-}2S)^{-2}$ cluster is destroyed and apo-FNR, which lacks an Fe-S cluster, is the primary form of FNR under aerobiosis. Thus, a reversible conversion of FNR protein from reduced dimer to oxidized monomer and even to an apo-protein occurs on exposure to oxygen. Thus, in aerobic conditions, FNR protein is present as oxidized monomer (2Fe-2S) or under superoxia even as apo-protein which is inactive and does not bind to DNA (Unden & Trageser, 1991;

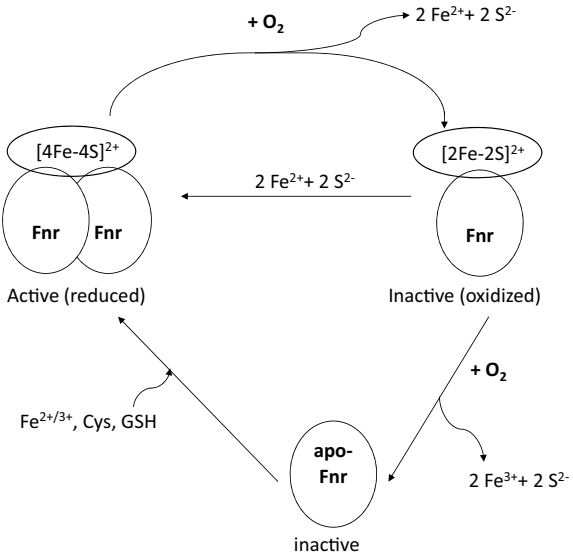


Fig. 5 Diagrammatic representation of reversible oxidation and reduction of Fnr in response to availability of oxygen

Kiley & Beinert, 1998). The Fe-S cluster can react with various cellular reductants, such as glutathione or thiol proteins (Fig. 5).

4 ArcAB- and FNR-Regulated Carbon Metabolism in *E. coli* in Response to Oxygen Availability

E. coli is a facultative anaerobe and resides as a free-living bacterium in microaerophilic gut environment. In such environments, *E. coli* maintain its growth by switching from aerobic to anaerobic and finally to facultative mode of glucose oxidation. In this way, the bacterium is able to generate intermediate reductant and ATP for its multiplication and survival. There are three modes of glucose catabolism: (1). Complete TCA cycle operative in fully aerobic conditions (Fig. 6a); (2) Branched TCA cycle in microaerophilic conditions (Fig. 6b); and (3) Fermentation during anaerobic conditions (Fig. 6c–e).

Thus, the bacteria can switch from aerobic respiration to anaerobic respiration and then to fermentation. The first change is with respect to pyruvate flux movement which goes through pyruvate dehydrogenase complex in aerobic and pyruvate formate lyase in anaerobic respiration and fermentative conditions. The metabolic switch with reference to oxygen availability is studied by transcript analysis of an anaerobic steady-state culture on introducing oxygen. The major changes in central metabolic enzymes were observed when transcripts for TCA cycle including pyruvate

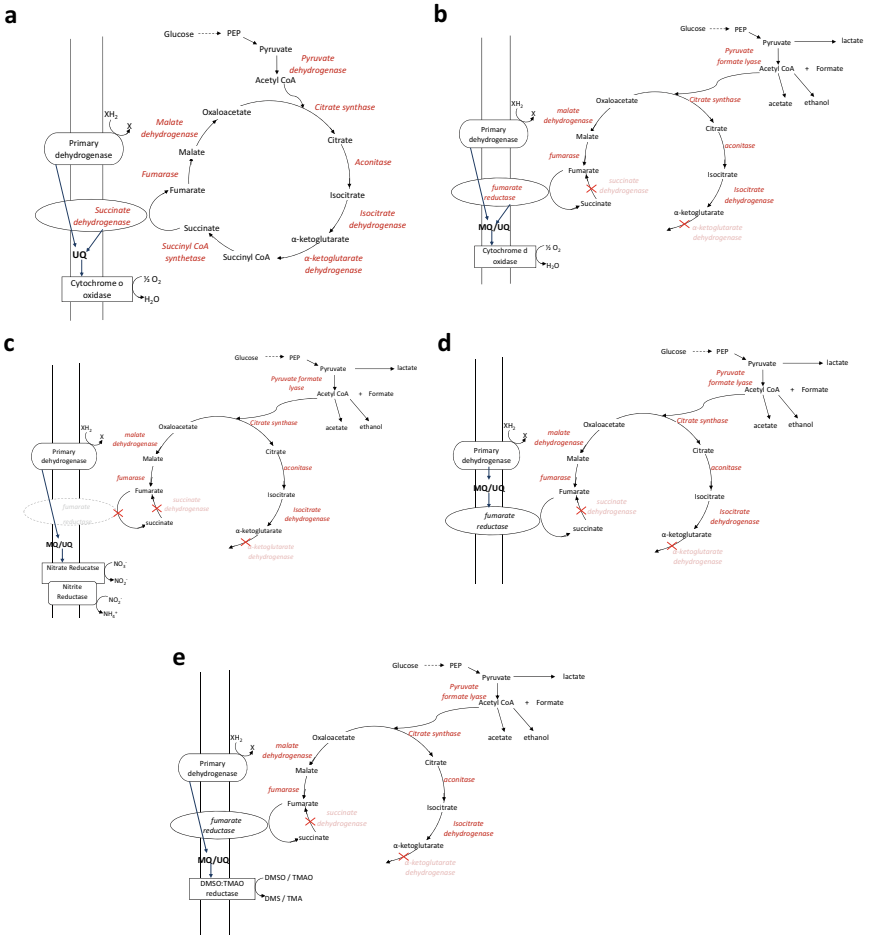


Fig. 6 Comparison of carbon metabolism and respiratory systems in *E. coli*. **a** 100% aerobic; **b** microaerophilic; **c** anaerobic (with nitrate as terminal electron acceptor); **d** anaerobic (with fumarate as terminal electron acceptor); **e** anaerobic (with DMSO/TMAO as terminal electron acceptor)

dehydrogenase, citrate synthase, aconitase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, NADH dehydrogenase II and cytochrome o oxidase increased while those of pyruvate formate lyase, hydrogenase, alcohol dehydrogenase, terminal reductases and cytochrome d oxidase decreased. Noticeable was induction of pyruvate formate lyase repair protein indicating microaerobic conditions (Unden & Bongaerts, 1997).

On exposure to aerobic conditions, the anaerobic metabolites alcohol, acetate and succinate decreased. However, acetate concentration was not much affected till fully aerobic condition is reached. Extracellular fumarate was also detected indicative

of limiting fumarase activity. Further, there was a transient excretion of pyruvate immediately after introduction of oxygen to anaerobic steady state culture which later decreased. This transient excretion of pyruvate suggests that on exposure to aerobic condition, pyruvate formate lyase activity immediately stopped while activation of pyruvate dehydrogenase complex takes time to achieve maxima; hence, the glycolytic flux is more than pyruvate channeling further and thus overflow was observed (Partridge et al., 2006).

These metabolites are regulated by ArcAB regulator in both aerobic and anaerobic conditions, and the role of ArcAB is permanent during microaerophilic conditions.

The detailed role of ArcAB in regulating catabolic enzymes was investigated through *arcA* mutants. A strong relationship was found between NADH/NAD^+ ratio and acetate production under microaerophilic conditions in this mutant. It was found that acetate overflow (occurs when NADH/$NAD^+ \geq 0.06$) could be delayed with concomitant heterologous expression of NADH oxidase which decreased the ratio of NADH/NAD^+. Further, *arcA* mutants showed activation of TCA cycle (which is repressed by phosphorylated ArcA in the presence of high NADH/NAD^+). Deletion did not have any effect on activities of pyruvate dehydrogenase and pyruvate formate lyase in fully aerobic or anaerobic conditions. However, under microaerophilic conditions, major changes in the mutant were observed with respect to increased growth and altered flux between cytochrome o and cytochrome d with unaltered preference for cytochrome o even in oxygen-restricted state. The NADH/NAD^+ levels also increased in an *arcA* mutant under microaerophilic conditions and were similar to wild type in both aerobic and anaerobic conditions. This clearly proves that ArcAB is a regulator under microaerophilic conditions rather than in complete anaerobic conditions (Alexeeva et al., 2003).

In an ArcA deletion mutant, the following was observed:

a. There was activation of TCA cycle enzymes (KDH) which is otherwise repressed during anaerobic conditions.
b. Pyruvate dehydrogenase and pyruvate formate lyase were unaltered both in aerobic and anaerobic conditions.
c. Increased respiration and altered flux of cytochrome o- and d-terminal oxidases with unaltered preference of cytochrome-o was observed even in oxygen restricted conditions.

Besides this, there is another global regulator FNR which is not a two-component system and regulates particularly during anaerobic conditions when there are alternate electron acceptors are present in place of oxygen. FNR up-regulates most of the enzyme activities which are up-regulated by ArcAB except cytochrome-d oxidase. Instead, it up-regulates alternate terminal oxidases for nitrate, DMSO, TMAO, etc.

Thus, major differences between ArcAB and FNR regulation are up-regulation of terminal oxidases in presence of oxygen limited conditions or anaerobic conditions.

Besides many regulatory steps in TCA cycle and electron transport, major regulation is by pyruvate dehydrogenase complex through its repression and de-repression following introduction of oxygen to anaerobic steady state cultures.

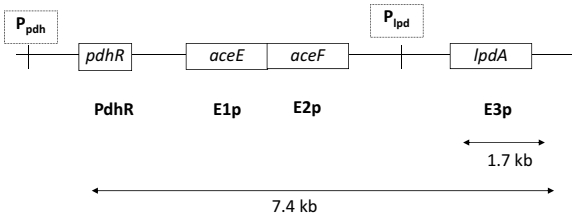


Fig. 7 Schematic diagram of *pdh* operon

Regulation of pyruvate dehydrogenase complex

***pdh* operon**: *pdh* operon codes for three enzyme activities: E1p (pyruvate dehyrogenase); E2p (dihydrolipoate transacetylase) and E3p (dihydrolipoate dehydrogenase) coded by (*pdhR—aceE—aceF—lpdA*) operon containing two promoters P_{pdh} and P_{lpd} (Fig. 7).

ArcA regulates P_{1pd} promoter coding for E3p for pyruvate dehydrogenase (PDH) complex and α-ketoglutarate dehydrogenase (oxoglutarate dehydrogenase; ODH) complex.

P_{pdh} promoter is negatively regulated by PdhR repressor which binds in absence of pyruvate. *lpdA* gene product E3p protein is also a part of ODH. E3 subunit in both is expressed from P_{pdh} promoter and also P_{lpd} promoter. For synthesis of PDH complex, it is expressed along with *aceEF* genes, while for ODH complex, it is coded by P_{lpd} promoter which is also regulated with *sucA* and *sucB* genes coding for succinate dehydrogenase. LacZ fusion studies have shown that P_{pdh} is unaffected, while P_{lpd} activities are repressed during anaerobic conditions. This was substantiated by ArcA deletion mutants where it was observed that there was de-repression of P_{lpd} promoter in anaerobic conditions resulting in active α-ketoglutarate dehydrogenase.

Cunningham et al. (1998) reported a single binding site for ArcA-P in P_{lpd} coding for E3, common to both PDH and ODH. This suggests that during anaerobic conditions or microaerobic conditions, ArcA-P blocks transcription through P_{lpd} i.e. *lpdA* gene synthesis. Additionally, it was found that *sdh* promoter is also regulated by ArcA-P. These studies clearly showed that P_{pdh} activities are not regulated by ArcA and during microaerobic condition or anaerobic condition, α-ketoglutarate dehydrogenase and succinate dehydrogenase are co-regulated by ArcA-P.

Pyruvate dehydrogenase regulator-mediated regulation of NADH dehydrogenase and cytochrome-o operon

Cytochrome-o and NADH dehydrogenase II promoter has binding site for FNR and ArcA-P. In addition to these transcription factors, the promoter also binds to CRP and PdhR; hence, its regulation is quite complex. The repressor effect of FNR and ArcA-P is under anaerobic condition, while the repressor effect of PdhR is not significant under aerobic or anaerobic condition but is reduced in presence of excess pyruvate.

NADH dehydrogenase II is used by *E. coli* both in presence of O_2 and NO_3^-. NADH dehydrogenase II is also down-regulated by FNR and ArcA under anaerobic condition and also regulated negatively both in aerobic and anaerobic conditions, but

it depends on pyruvate concentration. If pyruvate is low, then PdhR is free to repress (Ogasawara et al., 2007).

Pyruvate formate lyase regulation under anaerobic conditions by ArcA and FNR

The *pfl* operon is known to be constitutive for the expression of *focA* and *pfl* genes. The two promoters P6 and P7 are known to be regulated by ArcA-P and FNR transcription factors. These promoters have non-additive effects on pyruvate formate lyase (PFL) expression. Presence of promoter P6 is necessary for anaerobic expression of P7 promoter. The anaerobically inducible *pfl* operon has a 494 bp regulatory sequence encompassing P6 and P7 promoters for binding to ArcA-P and FNR. Thus, PFL activity is up-regulated during anaerobic conditions. In addition to transcriptional regulation, PFL is also modified post-translationally as active and inactive form. The active form is a homodimer of PFL activating enzyme I and formate acetyltransferase I. The active site has an oxygen labile glycyl 734 residue. In presence of oxygen, some of its activity can be restored by a PFL repair enzyme *Yfid* gene product which repairs it when exposed to oxygen (anaerobic to aerobic shifts) (Reyes-Ramírez & Sawers, 2006).

Yfid protein, an activator of pyruvate formate lyase, is up-regulated by FNR in a *arcA* mutant

Yfid is expressed under microaerobic condition in *arcA* mutant where PFL is much higher than wild type. Yfid is regulated by FNR, and its up-regulation in *arcA* mutant suggests FNR up-regulation as well.

Box 1: Experimental Proof for Redox as Signal of Anaerobiosis

The ArcB protein does not respond to oxygen per se, but it is actually the redox state of the cell such as the reduced form of an electron transport carrier (flavoprotein, quinone, cytochrome) or NADH or some metabolic intermediate that might accumulate anaerobically.

This was tested by generating deletion mutants of genes coding for cytochrome o, *cyo*, and cytochrome d *cyd*. Deletion of *cyo* and *cyd* would inhibit the electron transport at the terminal step leading to accumulation of reduced forms of electron carrier and metabolites that are formed in absence of terminal electron acceptor. The mutants were transformed with plasmids having fusion constructs of reporter gene *lacZ*.

For this, *cyo-lacZ* and *cyd-lacZ* fusions were constructed to monitor the expression of *cyo* and *cyd* genes. Under aerobic conditions, when both *cyo* and *cyd* were deleted, *cyo-lacZ* expression decreased while *cyd-lacZ* expression increased as is the case when conditions are anaerobic.

Thus, reduced forms of electron transport carrier may act as a signal for ArcB which then activates ArcA. ArcB also responds to metabolites that accumulate under low oxygen conditions such as pyruvate, acetate and lactate.

Summary

- Microbes respond to a variety of environmental conditions by adopting metabolic pathway suited to the existing physiological conditions.
- One of the well-studied environmental factors for bacterial growth is availability of oxygen. Bacteria respond to hypoxia or anoxia by making adaptations that may be short term/transient or long term/persistent.
- *E. coli* has about eight oxygen sensors, some of which respond with fast reactions while others bring about delayed catabolic changes.
- Aer, SoxRS, OxyR, Dsb and Hmp are the rapid response sensors, while ArcAB and Fnr are delayed response sensors.
- Aer, a major oxygen-sensing transducer in *E. coli*, is a member of the methyl-accepting chemotaxis protein (MCP) family of chemoreceptors and mediates aerotactic responses.
- SoxRS and OxyR sensors modulate the activities of various enzymes that scavenge the reactive oxygen radicals (ROS) in the form of hydrogen peroxide and superoxide radicals.
- Dsb proteins ensure disulfide bond formation between the correct pairs of cysteines in various proteins and thus help in maintaining the correct structures.
- Hmp protein is a soluble flavohemoglobin that functions as NO (nitric oxide) scavenger and detoxifies NO.
- Dos is a heme-based oxygen sensor protein displaying phosphodiesterase activity toward cyclic-bis diguanylic acid in response to oxygen availability.
- ArcAB and Fnr regulate catabolic pathways at both kinetic level and at transcriptional level.
- ArcAB is a two-component system wherein ArcB is the histidine kinase and ArcA is its cognate response regulator.
- ArcB is a multi-domain sensor kinase that is constitutively expressed under both aerobic and anaerobic conditions.
- ArcB senses oxygen availability through redox state of the cell.
- ArcB has affinity for both ubiquinone and menaquinone and is regulated by their ratio under a variety of oxygen availability conditions.
- Fnr transcriptional activator is not a two-component system. It is a Fe-S protein that is expressed constitutively but is activated only under anaerobic conditions.
- There are three modes of glucose catabolism: (i) complete TCA cycle in fully aerobic conditions; (ii) branched TCA cycle in microaerophilic conditions; and (iii) fermentation during anaerobic conditions.
- Pyruvate flux movement goes through pyruvate dehydrogenase complex in aerobic and pyruvate formate lyase in anaerobic respiration and fermentative conditions.
- Metabolites like acetate, alcohol, succinate, etc. are regulated by ArcAB regulator in both aerobic and anaerobic conditions.
- Another global regulator FNR regulates particularly during anaerobic conditions when there are alternate electron acceptors present in place of oxygen.
- FNR up-regulates alternate terminal oxidases for nitrate, DMSO, TMAO, etc.

- ArcA regulates P1pd promoter coding for E3p for pyruvate dehydrogenase (PDH) complex and α-ketoglutarate dehydrogenase.
- Ppdh promoter is negatively regulated by PdhR repressor which binds in absence of pyruvate.
- Cytochrome-o and NADH dehydrogenase II promoter has binding site for FNR and ArcA-P also binds to CRP and PdhR; hence, its regulation is quite complex.
- The two promoters P6 and P7 of *pfl* operon are known to be regulated by ArcA-P and FNR transcription factors.
- PFL is also modified post-translationally; the active form is a homodimer of PFL activating enzyme I and formate acetyltransferase I.
- Yfid repairs PFL when exposed to oxygen.

Questions

1. Describe briefly five oxygen sensors present in *E. coli.*
2. What is aerotaxis in *E. coli*?
3. Give examples of some rapid responses to anaerobiosis in *E. coli.*
4. Explain role of DOS and HAMP sensors during oxygen tension.
5. Describe the domain organization of ArcB and the significance of the various domains.
6. Explain briefly the phospho-relay mechanism of ArcAB.
7. How does ArcB sense oxygen availability?
8. Write a short note on the Fnr activator protein.
9. What are the key changes in the transcript levels of TCA enzymes when oxygen is introduced to anaerobically grown steady-state culture of *E. coli*?
10. What is the sequence of preferred electron acceptors after oxygen in *E. coli* among nitrate, fumarate and DMSO and why?
11. Which enzymes in TCA cycle are regulated by ArcAB system in microaerophilic condition in *E. coli*?
12. On introduction of oxygen to an anaerobically grown *E. coli* culture, there is a transient secretion of pyruvate. Explain.
13. How is pyruvate flux mobilized in aerobic, microaerophilic and anaerobic conditions in *E. coli*?
14. Explain pyruvate formate lyase regulation under anaerobic condition by ArcA and FNR.

References

Alexeeva, S., Hellingwerf, K. J., & de Mattos, M. J. T. (2003). Requirement of ArcA for redox regulation in *Escherichia coli* under microaerobic but not anaerobic or aerobic conditions. *Journal of Bacteriology, 185*(1), 204–209.

Alvarez, A. F., Rodriguez, C., & Georgellis, D. (2013). Ubiquinone and menaquinone electron carriers represent the yin and yang in the redox regulation of the ArcB sensor kinase. *Journal of Bacteriology, 195*(13), 3054–3061.

Bekker, M., Alexeeva, S., Laan, W., Sawers, G., De Mattos, J. T., & Hellingwerf, K. (2010). The ArcBA two-component system of *Escherichia coli* is regulated by the redox state of both the ubiquinone and the menaquinone pool. *Journal of Bacteriology, 192*(3), 746–754.

Collet, J. F., & Bardwell, J. C. (2002). Oxidative protein folding in bacteria. *Molecular Microbiology, 44*(1), 1–8.

Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M. N., Scott, C., Thomson, A. J., Green, J., & Poole, R. K. (2002). NO sensing by FNR: Regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. *The EMBO Journal*, *21*(13), 3235–3244.

Cunningham, L., Georgellis, D., Green, J., & Guest, J. R. (1998). Co-regulation of lipoamide dehydrogenase and 2-oxoglutarate dehydrogenase synthesis in *Escherichia coli*: Characterisation of an ArcA binding site in the lpd promoter. *FEMS Microbiology Letters, 169*(2), 403–408.

Iuchi, S., & Weiner, L. (1996). Cellular and molecular physiology of *Escherichia coli* in the adaptation to aerobic environments. *The Journal of Biochemistry, 120*(6), 1055–1063.

Kadokura, H., & Beckwith, J. (2010). Mechanisms of oxidative protein folding in the bacterial cell envelope. *Antioxidants & Redox Signaling, 13*(8), 1231–1246.

Kiley, P. J., & Beinert, H. (1998). Oxygen sensing by the global regulator, FNR: The role of the iron-sulfur cluster. *FEMS Microbiology Reviews, 22*(5), 341–352.

Kwon, O., Georgellis, D., & Lin, E. C. (2003). Rotational on-off switching of a hybrid membrane sensor kinase Tar-ArcB in *Escherichia coli*. *Journal of Biological Chemistry, 278*(15), 13192–13195.

Kwon, O., Georgellis, D., Lynch, A. S., Boyd, D., & Lin, E. C. C. (2000). The ArcB sensor kinase of *Escherichia coli*: Genetic exploration of the transmembrane region. *Journal of Bacteriology, 182*(10), 2960–2966.

Lee, C., Lee, S. M., Mukhopadhyay, P., Kim, S. J., Lee, S. C., Ahn, W. S., Yu, M.-H., Storz, G., & Ryu, S. E. (2004). Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. *Nature Structural & Molecular Biology, 11*(12), 1179–1185.

Malpica, R., Franco, B., Rodriguez, C., Kwon, O., & Georgellis, D. (2004). Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. *Proceedings of the National Academy of Sciences, 101*(36), 13318–13323.

Ogasawara, H., Ishida, Y., Yamada, K., Yamamoto, K., & Ishihama, A. (2007). PdhR (pyruvate dehydrogenase complex regulator) controls the respiratory electron transport system in *Escherichia coli*. *Journal of Bacteriology, 189*(15), 5534–5541.

Oreza, L. A. N., Alvarez, A. F., Arias-Olguín, I. I., Larios, A. T., & Georgellis, D. (2012). The ArcB leucine zipper domain is required for proper ArcB signaling. *PLoS ONE, 7*(5), e38187.

Partridge, J. D., Scott, C., Tang, Y., Poole, R. K., & Green, J. (2006). *Escherichia coli* transcriptome dynamics during the transition from anaerobic to aerobic conditions. *Journal of Biological Chemistry, 281*(38), 27806–27815.

Reyes-Ramírez, F., & Sawers, R. G. (2006). Aerobic activation of transcription of the anaerobically inducible *Escherichia coli* focA-pfl operon by fumarate nitrate regulator. *FEMS Microbiology Letters, 255*(2), 262–267.

Sasakura, Y., Hirata, S., Sugiyama, S., Suzuki, S., Taguchi, S., Watanabe, M., Matsui, T., Sagami, I., & Shimizu, T. (2002). Characterization of a direct oxygen sensor heme protein from *Escherichia coli*. Effects of the heme redox states and mutations at the heme-binding site on catalysis and structure. *Journal of Biological Chemistry, 277*(26), 23821–23827.

Sawers, G., Kaiser, M., Sirko, A., & Freundlich, M. (1997). Transcriptional activation by FNR and CRP: Reciprocity of binding-site recognition. *Molecular Microbiology, 23*(4), 835–845.

Spiro, S., & Guest, J. R. (1990). FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. *FEMS Microbiology Reviews, 6*(4), 399–428.

Touati, D. (2000). Sensing and protecting against superoxide stress in *Escherichia coli*—How many ways are there to trigger soxRS response? *Redox Report, 5*(5), 287–293.

Unden, G., & Bongaerts, J. (1997). Alternative respiratory pathways of *Escherichia coli*: Energetics and transcriptional regulation in response to electron acceptors. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1320*(3), 217–234.

Unden, G., & Trageser, M. (1991). Oxygen regulated gene expression in *Escherichia coli*: Control of anaerobic respiration by the FNR protein. *Antonie van Leeuwenhoek, 59*(2), 65–76.

Chapter 22
Regulation of Anaerobic Respiration

Rani Gupta and Namita Gupta

1 Anaerobic Respiration: An Introduction

E. coli is a facultative anaerobe and as described earlier can survive through a wide range of oxygen availability conditions. It can shift from aerobic to microaerophilic to fermentation mode of growth and metabolism depending upon the availability of oxygen, i.e., from >5 mbar to 1–5 mbar to <1 mbar. It is also capable of growing under anaerobic conditions by using electron acceptors other than oxygen such as nitrate, fumarate, TMAO and DMSO. The preference is based on the redox potential in the order nitrate, TMAO, DMSO and fumarate.

Oxygen is most preferred and represses all anaerobic respiration pathways. The most favorable terminal acceptor after oxygen is nitrate. On availability of nitrate, the bacterium adopts anaerobic mode of respiration using formate as electron donor for reduction of nitrate. The alternate respiratory gene expression is controlled by FNR sensor which in turn switches on a cascade of signal transduction for de-repression/induction of nitrate operon. Further, in presence of nitrate, other anaerobic pathways (for respiration of fumarate, TMAO and DMSO) are repressed.

2 Nitrate Respiration

Bacteria which use NO_3^- as respiratory terminal acceptor generally adopt a short route to reduction of nitrate to ammonia via nitrite and do not denitrify nitrate to N_2. This short route to direct formation of ammonia from nitrate is a feature often met with commensal bacteria without exception. Further, this anaerobic nitrate reduction is favoured via carbon rich environment in absence of oxygen. The respiratory reduction is repressed in oxygen but not affected by ammonia or any other nitrogen source. On the other hand, assimilatory reduction is independent of oxygen but is highly sensitive to ammonium availability (Fig. 1 and Table 1).

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_22

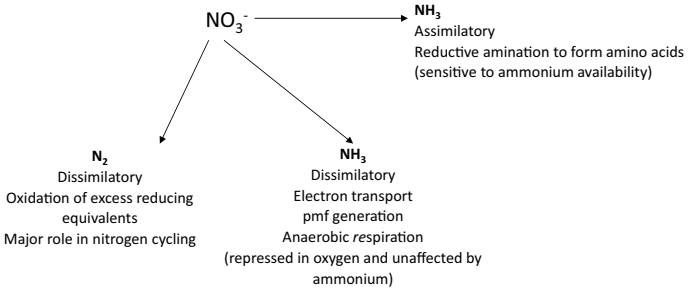


Fig. 1 Assimilatory and dissimilatory conditions

Table 1 Assimilatory versus dissimilatory nitrate reductases

Assimilatory nitrate reductases	Dissimilatory nitrate reductases
Independent of oxygen	Hypoxia activated
NO_3^- independent	NO_3^-/NO_2^- induced
NH_3 repressed	NH_3 independent
$NO_3^- \rightarrow NH_3$	$NO_3^- \rightarrow NH_3$ $NO_3^- \rightarrow N_2$
Cytoplasmic activity	Periplasmic/cytoplasmic activity
Uptake is must	Uptake needed for cytoplasmic nitrate reductase and not for periplasmic nitrate reductase

3 Nitrate Reductases

There are three different operons which regulate nitrate utilization in *E. coli* under different physiological conditions:

1. ***NarGHJI* (*NarG*) operon**: This is the most extensively studied operon. It codes for a major inducible respiratory nitrate reductase NarG which is bound to the cytoplasmic membrane and has a cytoplasmic catalytic center. The enzyme expression is induced during anaerobic growth through the mediation of FNR protein and presence of nitrate through Nar regulatory system. The enzyme oxidizes quinols and reduces NO_3^- to NO_2^- by two electron reduction and generates pmf for anaerobic respiration.
   The enzyme complex has three subunits:

   a. NarG catalytic α subunit (139 kDa) and contains a molybdopterin cofactor
   b. NarH soluble β subunit (58 kDa) and contains four [4Fe–4S] centers; these Fe-S centers are responsible for reduction of NO_3^- to NO_2^-
   c. NarI γ subunit (24 kDa) and contains two b-type hemes.

   α and β subunits are on the cytoplasmic side and are bound to a membrane-bound γ subunit which is cytochrome b-type heme protein. The electrons supplied by

quinol reduction to the cytochrome b-like γ subunit are transferred to the Fe-S centers of β subunit and finally to the molybdenum cofactor bound to the α subunit where actual nitrate reduction takes place. Thus, various substrates such as formate, lactate, glycerol get oxidized to generate NADH which in turn reduce quinones. Protein NarJ is not associated with the complex, rather it is involved in assembly and stabilization of α, β dimer prior to its interaction with γ subunit. The enzyme activity is inhibited by azide.

2. ***NapFDAGHBC* (*NapF*) operon**: It codes for a periplasmic cytochrome c linked nitrate reductase. It is induced by anaerobiosis through the mediation of FNR protein and stimulated by both NO_3^- and NO_2^- through Nar regulatory system. The Nap enzyme functions when concentrations of nitrate are too low to support respiration by membrane-bound nitrate reductase. In *Paracoccus*, it is synthesized during aerobic growth irrespective of added NO_3^-.
NapFDAGHBC operon is a seven gene operon wherein:

   - *napA* encodes the catalytic subunit NapA which is a molybdoprotein
   - *napB* encodes a di-heme cytochrome c
   - *napC* encodes a membrane-bound tetraheme cytochrome c protein, which passes electrons either from NapGH or directly from the quinone pool to NapB
   - *napD* encodes a protein required for the post-translational assembly of the molybdoprotein NapA
   - *napF*, *napG* and *napH* encode iron–sulfur proteins and are not required for Nap activity; NapG and NapH facilitate electron transfer from ubiquinol via NapC to NapAB.

   NapA is a 90 kDa protein having (4Fe–4S) cluster in addition to a molybdenum cofactor. It is coded along with di-heme cytochrome c NapB. NapD is a cytoplasmic protein which interacts with NapA and is transported to periplasm as NapDA complex. NapA oxidizes quinols and reduces NO_3^- to NO_2^- by two electron reduction. Therefore, this enzyme is responsible for nitrate dissimilation. However, this quinol oxidation is not coupled to proton translocation. Exception to this is NapA from *Paracoccus pantotrophus* where it functions as respiratory enzyme coupled to NADH dehydrogenase1 (Dow et al., 2014).
   The physiological role of Nap is that of mediating anaerobic respiration at the expense of low concentrations of nitrate. Nap has a significantly higher affinity for nitrate than NarG and is thus able to exploit the low concentrations of nitrate occurring in the natural environment of *E. coli.*
   Under nitrate respiratory conditions, nitrate reduction to nitrite is followed by periplasmic nitrite reduction to NH_3 through a six electron transfer probably with NO and NH_2OH as bound intermediates by the action of periplasmic nitrite reductase NrfA. Here, formate acts as an electron donor, and formate oxidation is coupled to generation of pmf.
   Further, activity of NapABC is not sensitive to azide (Stewart et al., 2002).

3. ***NarZYWV* (*NarZ*) operon**: It codes for an assimilatory nitrate reductase NarZ which is cytoplasmic in localization. NarZ is believed to be constitutively

Table 2 Comparison of the three nitrate reductases: NapF, NarG and NarZ

Features	NapF	NarG	NarZ
Operon	*NapFDAGHBC*	*NarGHJI*	*NarZYWV*
Location	Periplasmic	Cytoplasmic, membrane bound	Cytoplasmic
Role	Dissimilatory	Dissimilatory	Assimilatory
Involved in	Nitrogen cycling, Nitrate respiration	Nitrate respiration along with energy generation	Nitrogen cycling
Regulation	Anaerobically induced by FNR and stimulated by both NO_3^- and NO_2^- through Nar regulatory system	Induced anaerobically by FNR protein and presence of nitrate through Nar regulatory system	Constitutively expressed at relatively low levels. Negatively controlled by FNR

expressed at relatively low levels. The expression of the genes of the *NarZ operon* appears to be negatively controlled by the FNR protein in anaerobiosis. Similar to NarG*HIJ*, NarZYWV is also composed of three subunits:

a. NarZ which is the catalytic α subunit (140 kDa) and contains a molybdopterin cofactor
b. NarY which is the β subunit (60 kDa) and contains the Fe-S centers
c. NarV which is the γ subunit (26 kDa) and is the heme-iron center.

Further, protein NarW is not associated with the complex and seems to be involved in assembly of the other three subunits of the complex.
Different cofactors and other features of the three nitrate reductases are tabulated in Table 2.

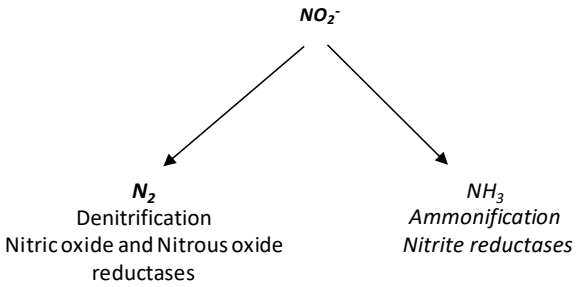
Among the three nitrate operons, only two have significant role—one is *NapF* coding for periplasmic nitrate reductase NapA for which nitrate uptake is not mandatory, and the other is NarG, a cytoplasmic membrane-bound nitrate reductase for which nitrate uptake must take place. Both operons are inducible under anaerobic conditions and are linked to electron transport chain, thus are respiratory. This is in contrast to the third constitutive operon *NarZWV* which is of little significance because of low activity and can be classified as coding for assimilatory nitrate reductase. Since NarZ activity is too low, it is often said that *E. coli* is a poor nitrate assimilator.

4 Nitrite Reductases

Nitrite reduction will vary depending upon whether bacterium can catalyze denitrification to N_2 or to ammonia as observed in commensal bacteria. It is rare for any bacterial species to have both pathways for denitrification and ammonia formation (Bonnefoy & Demoss, 1994).

These two types of reductions are carried out by distinct nitrite reductases and are synthesized generally during anaerobic conditions.

- Denitrifying enzymes are located in periplasm which reduce nitrite to nitric oxide and then to nitrous oxide and nitrogen by successive activities of nitric oxide and nitrous oxide reductases.
- Ammonifying nitrite reductases are localized both in periplasm and cytoplasm as in *E. coli*.
  - The cytoplasmic nitrite reductase (NirBD) helps in rapid detoxification of nitrite generated by membrane-bound nitrate reductase (NarG).
  - There is another nitrite reductase NrfA located in periplasm of gram-negative bacteria. The enzyme is a terminal component of respiratory electron transport where nitrite is reduced via formate reduction, thus the nomenclature Nrf (nitrite reduction via formate). Thus, Nrf activity is closely coordinated with periplasmic nitrate reductase NapA for conversion of nitrate to ammonia in periplasm.



1. ***NirBDC* (*NirB*) operon**: It codes for the cytoplasmic NADH-nitrite reductase, which detoxifies the nitrite formed as the product of nitrate reduction. It is a siroheme-containing enzyme that uses NADH as an electron donor to reduce nitrite in the cytoplasm. Transcription of the *nirBDC* operon is driven from a single promoter, and expression is activated by two environmental signals: absence of oxygen and presence of nitrite or nitrate ions.
   NirBDC operon codes for:
   - NirBD nitrite reductase is formed of two subunits nirB and nirD and has siroheme as a cofactor. The enzyme is soluble and uses NADH as electron donor to reduce nitrite in cytoplasm.
   - NirC is the nitrite transporter.
   - *CysG* gene, located downstream, is cotranscribed with the *nirBDC* operon. The product of the *cysG* gene, siroheme synthase, catalyzes the synthesis of a heme cofactor, siroheme, which is employed by the nitrite reductase enzyme in the nitrite reduction process.
2. ***NrfABCDEFG* (*NrfA*) operon**: It codes for a membrane-bound periplasmic respiratory nitrite reductase complex that is coupled to formate oxidation via quinoles and thus helps in generation of pmf. The pathway requires at least two

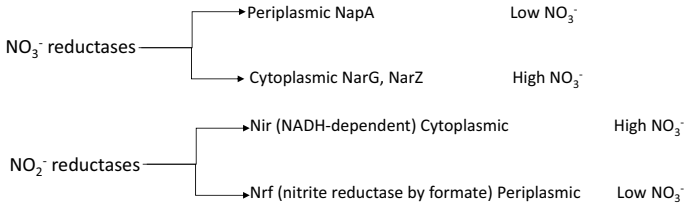


Fig. 2 Differential expression of nitrate and nitrite reductases

c-type cytochrome proteins. It is a seven gene operon, at least five of which are essential for formate-dependent nitrite reduction to ammonia. Expression of the *nrfA* operon shows FNR-dependent anaerobic induction, nitrite induction and nitrate repression (Clarke et al., 2008).

Nap and *Nrf* code for periplasmic nitrate and nitrite reductases, while *NarG* and *Nir* are cytoplasmic nitrate and nitrite reductases. They are expressed differentially—periplasmic at low concentration of nitrate while cytoplasmic at high concentration of nitrate (Fig. 2). It is significant to note that nitrate concentration in body fluid can induce only periplasmic enzymes. However, in the oral cavity where nitrate is higher, cytoplasmic enzymes accumulate.

5 Nitrate and Nitrite Transport in *E. Coli*

There are membrane transporters present for transport of NO_3^- and NO_2^- anions; NarK, NarU and NirC.

- NarK is involved in both nitrate and nitrite uptake; it also extrudes NO_2^-. It is a part of *NarK* operon (having sites for fnr and NarL binding) and is induced under anaerobic conditions in presence of nitrate.
- NarU is an antiporter importing NO_3^- and expelling NO_2^-. This may be part of constitutive nitrate operon *NarZYWV* (assimilatory nitrate reductase).
- NirC is involved only in nitrite transport, and it is a part of the *nirBDC* operon encoding cytoplasmic nitrite reductase.

Both NarK and NarU are NO_3^-/NO_2^- antiporters; when nitrate is available, it is taken up by NarK/NarU and reduced to nitrite.

Strains expressing only one of the transporters from NarK, NarU and NirC were grown anaerobically with NO_3^-. Maximum NO_3^- uptake and reduction was seen in the strain expressing NarK as compared to the one expressing NarU. Further, NirC strain was unable to transport NO_3^-, while it showed highest uptake and reduction of NO_2^- (Jia & Cole, 2005).

NarK was expressed in exponential phase, while NarU increased during stationary phase. However, even then at stationary phase, NarK concentration was 1000 times more than NarU. NarU increase during stationary phase suggests that it may be a

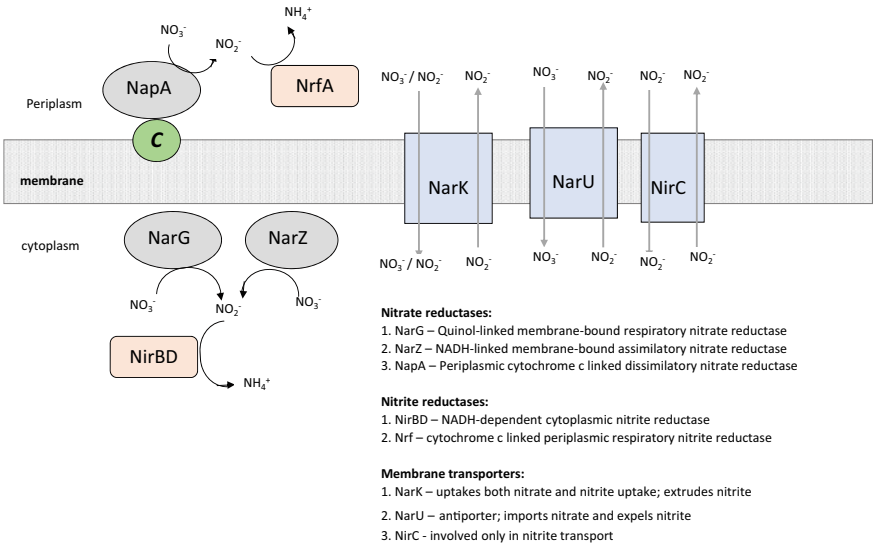


Fig. 3 Nitrate and nitrite transport and reduction in *E. coli*

part of constitutive nitrate operon *NarZYWV*. Further, the transport studies suggest that both NarK and NarU are NO_3^-/NO_2^- antiporter. Thus, when nitrate is available, it is taken up by NarK/NarU and reduced to nitrite, and some of it is expelled and taken up by NirC for reduction to ammonia (Fig. 3).

6 Two-Component System for NO_3^-/NO_2^- Sensing

Nitrate reduction enzyme activation is a complex two-component system having two sensor kinases NarX and NarQ and two response regulators NarL and NarP. These sensor kinases cross react with both response regulators. The system has histidyl-aspartyl phosphorelay mechanism. NarX and NarQ sensors respond to nitrate and/or nitrite (Lee et al., 1999; Fig. 4).

The two-component system is similar to the sensor regulator transcription regulators DegS-DegU (which controls extracellular enzyme synthesis in *Bacillus subtilis*) and UhpB-UhpA (which controls hexose phosphate uptake in *E. coli*).

In *E. coli*, *NarX* and *NarQ* genes are paralogues, i.e., they diverged following gene duplication. However, they play subtly different roles in nitrate- and nitrite-mediated gene expression. NarQ recognizes both ligands equally well, whereas NarX discriminates between the two and recognizes nitrate only. The strains carrying double mutations for *NarX* and *NarQ* were devoid of nitrate regulation, but single mutants were much like the wild strain. Both the sensors are alike in one aspect of NO_3^- sensing and NarL phosphorylation.

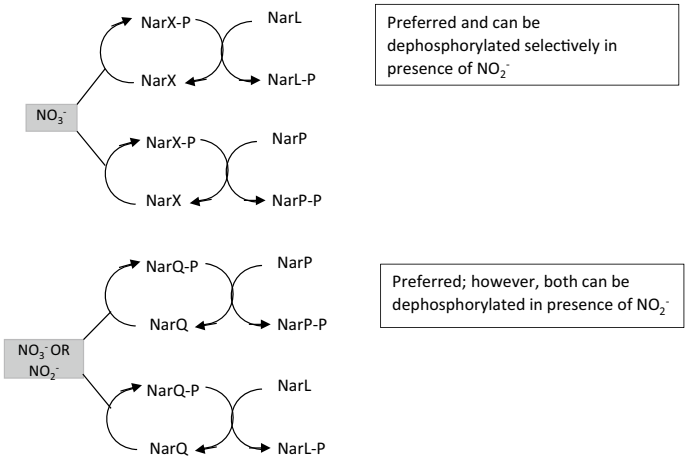


Fig. 4 Phosphoryl transfer to response regulators NarL and NarP results in activation or repression of target operon transcription

Mechanism of nitrate and nitrite signal transduction

The cognate partner for NarX sensor kinase is NarL, while that for NarQ sensor kinase is NarP. NarX senses only nitrate, while NarQ senses both nitrate and nitrite. NarX show high preference for NarL, whereas NarQ is more generalized for interaction with either NarP or NarL. *narXL* operon expression is unaffected by anaerobiosis, whereas expression of *narP* and *narQ* is induced approximately four-fold. Further, FNR and ArcA proteins are not required for this anaerobic induction (Jones et al., 2011).

On receiving the signal, i.e., in the presence of nitrate, NarX can phosphorylate both its cognate partner NarL and its cross-regulation partner, NarP. However, NarX is less effective than NarQ for NarP phosphorylation. However, on removal of the signal (in the presence of nitrite), it can dephosphorylate NarL only by activation of its phosphatase activity. Thus, nitrate and nitrite have a differential effect on NarX activity. NarQ, on the other hand, can phosphorylate and dephosphorylate both NarL and NarP. Thus, the NarX/NarL couple is responsible for the differential response to nitrate and nitrite (Noriega et al., 2010).

Both NarL and NarP regulate the expression of nitrate and nitrite reductases and also repress other anaerobic respiratory systems like fumarate reductase Frd or genes involved in fermentation. Phosphorylated NarL (NarL-P) activates the gene for respiratory nitrate reductase (*narG*) while inhibits the gene for respiratory nitrite reductase (*nrfA*). In contrast, *nrfA* is activated by NarP-P. Thus, high nitrate leads to accumulation of NarL-P which stimulates expression of *narG,* while nitrite leads to dephosphorylation of NarL, thereby depressing *NarG*. On the other hand, *nrfA* is repressed by nitrate and not by nitrite by lowering the amounts of NarL-P and

increasing NarP-P. Both nitrate and nitrite induce the synthesis of cytoplasmic nitrite reductase (*nirB*) which helps in preventing the accumulation of toxic nitrite in the cytoplasm.

7 Coregulation of Formate Dehydrogenase in Presence of Nitrate, Nitrite and Formate

There are three formate dehydrognases in *E. coli* coded by three different operons, *fdnGHI*, *fdoGHI* and *fdhF*. The formate oxidation is important for supplying electrons to nitrate or any other electron acceptor during anaerobic respiration (Overton et al., 2006).

a. **Formate dehydrogenase N (fdh-N)** is a membrane-bound formate dehydrogenase coded by *fdnGHI*. The activity of this enzyme is maximal when nitrate is present, and it is thus called fdh- N. This enzyme functions along with membrane-bound nitrate reductase NarG for transfer of electrons from formate to nitrate via NarG nitrate reductase (Jormakka et al., 2002).

   Reaction: Formate + NAD^+ à CO_2 + NADH + H^+

b. **Formate dehydrogenase H (fdh-H)** is encoded by *fdhF* operon and is either loosely held to cytoplasmic membrane or located in the cytoplasm. The enzyme is a part of formate hydrogen lyase and is thus called fdh-h. Formate hydrogen lyase consists of two enzymes, formate dehydrogenase H and hydrogenase 3. Fdh-H is a 80 kDa solenopolypeptide. It is anaerobically induced but also requires presence of formate. The promoter is nitrate suppressed and formate activated (Wang & Gunsalus, 2003).

c. **Formate dehydrogenase O (fdh-O)** is also a membrane-bound enzyme similar to fdh-N and is coded by *fdoGHI*. It is always present in low concentrations independent of oxygen and nitrate. It is considered to play a minor role between aerobic to anaerobic switch till the time full activity of anaerobic enzyme is obtained.

   Reaction: Formate + NAD^+ à CO_2 + NADH + H^+.

8 Fumarate Respiration

During anaerobic respiration, *E. coli* can use fumarate as a terminal electron acceptor by the action of the enzyme fumarate reductase (encoded by *frdABCD* operon) which reduces fumarate to succinate. It is a membrane-bound complex composed of four non-identical polypeptides:

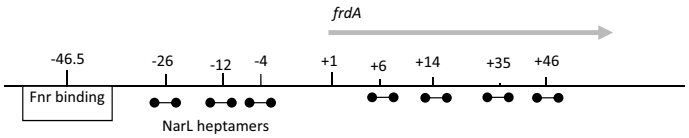


Fig. 5 Schematic diagram of *frd* operon showing NarL heptamers

i. FrdA is 69 kDa protein that contains a covalently bound FAD.
ii. FrdB (27 kDa) contains the Fe-S centers of the enzyme.
iii. FrdC (15 kDa) and FrdD (13 kDa) are integral membrane proteins that bind the catalytic FrdAB domain to the inner side of the cytoplasmic membrane.

NarL-mediated repression of the frdA promoter is achieved by NarL binding over a large region centered near the transcription start site and including the FNR site. The operon contains seven NarL-binding heptamers located around the transcriptional start site (+1) preventing the RNA polymerase from binding, thereby repressing the expression (Fig. 5; Jones & Gunsalus, 1987).

9 DMSO and TMAO Respiration

DMSO and TMAO are frequently found in aquatic environments. DMSO is formed from degradation of phytoplankton, microbial oxidation of DMS and photooxidation of DMS in water surface layers and atmosphere. TMAO is also found in marine biota as an osmolyte.

E. coli can use -*S*- and *N*- oxides such as DMSO and TMAO as terminal electron acceptors during anaerobic respiration by the action of the enzymes DMSO reductase and TMAO reductase, respectively. Both these are molybdoenzymes containing a pterin molybdenum cofactor (McCrindle et al., 2005).

Box 1: Symbiotic Role of Nitrate-Reducing Bacteria

Dietary nitrate has since long been associated with the development of cancer and methaemoglobinaemia. This deleterious effect is primarily due to reduction of nitrate to nitrite by facultative anaerobic bacteria. Nitrite enhances generation of carcinogenic N-nitrosamines that have been implicated in gastric and bladder cancer, while methaemoglobinaemia is caused by oxidation of hemoglobin by nitrite or nitric oxide to an abnormal form (methaemoglobin) that cannot bind or transport oxygen. As a consequence, there has been a great degree of research and effort to reduce exposure to this anion.

In striking contrast to this viewpoint, recent evidence has suggested that nitrite is converted to reactive nitrogen intermediates (RNIs; including nitric

oxide, NO) that are important for host defense and maintenance of homeostasis. Generation of RNIs by this pathway seems to complement production of RNIs by nitric oxide synthase (NOS) in white blood cells. Thus, nitrate-reducing bacteria play symbiotic role. This point has been highlighted in detail by Lundberg et al. (2004) in their review article entitled 'Nitrate, bacteria and human health' (Nature Reviews Microbiology, 2, 593–602), a summary of which is provided below.

Generating RNIs from nitrite

In addition to *N*-nitrosamines, various RNIs (such as $NO^{\cdot}$, $NO_2^{\cdot}$, N_2O_3, N_2O_4, NO_2^-, *S*-nitrosothiols, $ONOO^-$ and NO_3^-) are also generated in vivo from salivary nitrite on acidification. Many of these RNIs have biological effects such as role in host defense.

Antimicrobial effects of RNI—Under acidic conditions, nitrite is protonated to nitrous acid (HNO_2), which further decomposes to a variety of RNIs. These RNIs have antimicrobial activity against many bacteria and fungi. Nitrogen oxides act on multiple cellular targets such as DNA, cell-surface and intracellular proteins. Different nitrogen oxides have different effects on pathogens. For example, Mycobacterium tuberculosis is sensitive to NO but not other nitrogen oxides such as S-nitrosothiols and peroxynitrite. In contrast, *E. coli* is sensitive to other nitrogen oxides but not NO.

Bacterial protection against RNIs—Bacteria, both in the human body and outside, have evolved various mechanisms to respond to environmental stress such as reactive nitrogen (and oxygen) species. The defense mechanism involved in resistance against ROIs and RNIs overlaps to some extent, but there are specific pathways as well. Pathways for resistance to RNIs include indirect pathways such as inhibition of uptake or production of RNIs, repair of RNI-dependent damage and scavenging and detoxification of RNIs and related products. The well-studied enzymes involved in detoxifixation of RNIs include nitrite and NO reductases and also peroxynitrite and hydroxylamine reductases. However, lesser information is available on mechanism of detoxification of other RNIs.

Beneficial effects of acidified nitrite

- ***Killing ingested pathogens***—Dietary nitrate has a role in protection against ingested pathogens such as *E. coli*, *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Enteritidis. In a nitrate-free diet, concentration of salivary nitrite is not sufficient to kill these pathogens. However, on ingesting a nitrate-rich diet, enough nitrite is produced to kill these pathogens.
- ***Gastric mucosal integrity***—NO has been implicated in improving gastric mucosal blood flow. Further, NO can travel across biological membranes,

and NO-donating drugs are gastroprotective. Thus, it has been proposed that nitrite-derived NO is involved in regulating gastric mucosal blood flow. In various studies, it was shown that mucosal blood flow and mucus secretions increased after luminal application of nitrite-rich saliva. In contrast, saliva from a fasting individual had no effect. Other experiments have shown that dietary nitrate has gastroprotective role due to NO production in stomach. This protective role was abolished on removing oral microflora by antibiotic treatment. Thus, dietary nitrate has gastroprotective role which is mediated by reduction nitrate to nitrite by oral microflora.

- ***Oral cavity***—In addition to acidification of salivary nitrite inside the stomach, acidification can occur in the oral cavity also. This leads to generation of RNIs within the oral cavity which helps in protecting against dental caries.
- ***Skin***—Human skin continually produces NO which is mostly attributed to the action of nitric oxide synthase present in dermal vascular endothelial cells. However, evidence has suggested that skin bacteria produce NO by reduction of sweat nitrate to nitrite and its conversion to NO by acidic pH of skin. The concentration of nitrogen oxides thus produced helps in suppressing the growth of fungal skin pathogens.
- ***Urine***—Most plasma nitrate is excreted in the urine; however, since urine is sterile, the concentrations of nitrite are very low. During a lower-urinary-tract infection, bacteria can produce nitrite from nitrate; however, it is not further reduced to NO as the pH is high. However, if the pH is decreased, large amounts of RNIs are formed. Studies have shown that all urinary pathogens (E. coli, Pseudomonas aeruginosa and Staphylococcus saprophyticus) are sensitive to acidified nitrite. Thus, urinary infections may be treated by ingesting ascorbic acid (Vitamin C) that will lower the urine pH leading to generation of bactericidal nitrogen oxide.
- ***Nitrite reduction to NO in the systemic circulation***—NO can be generated from nitrite systemically in tissues with a low pH value, for example, ischaemic tissues. Additionally, various mammalian enzymes have been implicated in conversion of nitrite to NO. Experiments have shown that nitrite can dilate blood vessels through conversion to NO and thus nitrite may be an important vascular storage pool of NO. It was also seen that nitrite levels in plasma increased significantly after ingestion of nitrate and this increase was due to action of salivary oral flora. Thus, commensal oral flora may also be responsible for systemic effects such as regulation of vascular tone, platelet function and leukocyte adhesion.

Thus, a number of beneficial effects of nitrite are now being discovered and could have therapeutic implications. This certainly reverses the long-held view of nitrate-reducing bacteria as being only harmful.

Box 2: Nitrate-Reducing Bacteria for Controlling Souring in Oil Reservoirs

Oil reservoir souring refers to the generation of hydrogen sulfide (H_2S) by sulfate-reducing bacteria (SRB), a group of anaerobic bacteria that respire sulfate and produce sulfide while oxidizing diverse electron donors. Souring is common during secondary oil recovery during which water/seawater is flooded downhole to sweep the remaining oil, thereby increasing the oil production levels from an oil field. The sulfate-reducing bacteria reduce sulfate in the injection water leading to increased sulfide concentrations in the water, oil and gas. Industrial problems associated with souring are infrastructure corrosion, reservoir plugging, health and safety issues. Sulfide production also increases the sulfur content of the crude oil which decreases its value and increases refining costs.

Microbial souring is mostly controlled post-production by various physical and chemical treatments that help in minimizing leaks due to corrosion, odor complaints and cleanup. These treatments include use of non-metallic pipes or polymer coatings inside pipelines, sulfide scavengers and vapor recovery units to remove H_2S gas. Biocides are also used for inhibiting microbial growth and metabolism.

An effective method for in situ removal involves continuous nitrate injection that changes the microbial community from mainly SRB to one enriched in nitrate-reducing bacteria (NRB). NRB includes nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) that can oxidize H_2S directly and heterotrophic NRB (hNRB) which compete with SRB for degradable organic electron donors. NRB also inhibits SRB by production of nitrite, formed during the nitrate reduction pathway.

hNRB—Microbial competition between hNRB and SRP for available electron donor is the predominant mechanism of controlling souring. The main electron donors (called oil organics) are degradable hydrocarbons (alkanes and monoaromatics like toluene) and volatile fatty acids (VFAs: acetate, butyrate and propionate). hNRB competitively inhibits SRP by the following mechanisms:

- hNRB outgrows SRP because nitrate/nitrite reduction along with oxidation of oil organics yields more energy than sulfate reduction
- In many SRB, high nitrate concentrations competitively inhibit the enzyme dissimilatory sulfite reductase (Dsr) that catalyzes conversion of sulfite to sulfide. Dsr has a strong affinity for nitrite and slowly reduces it to ammonium. Thus, nitrate can shift the metabolism of some SRP from sulfate reduction to ammonification, thereby decreasing sulfide generation.

However, hNRB can be inhibited by high concentrations of sulfide, so this phenomenon may not work in very sour oil fields. Also, greater concentrations of nitrate are required for continual hNRB activity.

NR-SOB—NR-SOB oxidizes the existing sulfide to sulfate and elemental sulfur by using nitrate as the electron acceptor. Thus, they decrease the concentration of sulfide. This is dependent upon the availability of sufficient amounts of electron donors (sulfide and organic carbon) and acceptors (nitrate or nitrite). NR-SOB is generally autotrophic, and thus, they do not compete with SRP for organic carbon sources. Examples of NR-SOB include Epsilonproteobacterium *Thiomicrospira* sp. strain CVO, reclassified as *Sulfurimonas denitrificans* and *Arcobacter* sp. FWKO_B.

Some hNRB such as *Sulfurospirillum* sp. strain NO2B can also function as NR-SOB by oxidizing elemental sulfur. Thus, they can use either mechanism for controlling souring depending on the geochemical conditions.

Summary

- Facultative anaerobic bacteria such as *E. coli* are capable of growing under anaerobic conditions by using electron acceptors other than oxygen such as nitrate, fumarate, TMAO and DMSO. Preference for the electron acceptor is hierarchical in the order of their redox potential.
- Bacteria which use nitrate as respiratory terminal acceptor generally reduce nitrate to ammonia via nitrite by the action of dissimilatory nitrate and nitrite reductases. *E. coli* has three nitrate reductases and two major nitrite reductases.
- NarG nitrate reductase, encoded by *NarGHJI* operon, is the major inducible respiratory nitrate reductase and is cytoplasmic in nature.
- *NapFDAGHBC* operon encodes a periplasmic cytochrome c linked nitrate reductase NapA that mediates anaerobic respiration at low concentrations of nitrate.
- *NarZYWV* operon codes for an assimilatory nitrate reductase NarZ.
- *E. coli* has two major nitrite reductases that reduce nitrite to ammonia viz. NirBD and NrfA.
- NirBD, encoded by *NirBDC* operon, is a cytoplasmic nitrite reductase that helps in rapid detoxification of nitrite generated by membrane-bound nitrate reductase, NarG.
- NrfA, encoded by *NrfABCDEFG* operon, is a membrane-bound periplasmic respiratory nitrite reductase complex that is coupled to formate oxidation via quinoles and thus helps in generation of pmf.
- NO_3^- and NO_2^- are transported across the cell membrane via three membrane transporters NarK, NarU and NirC.
- Nitrate reduction enzyme activation is a two-component system that has two sensor kinases NarX and NarQ and two response regulators NarL and NarP; these sensor kinases cross react with both response regulators.
- The cognate partner for NarX sensor kinase is NarL, while that for NarQ sensor kinase is NarP.
- NarQ recognizes both nitrate and nitrite, whereas NarX recognizes nitrate only.

- On receiving signal (presence of nitrate), NarX can phosphorylate both NarL and NarP. On removal of signal (presence of nitrite), it can dephosphorylate NarL only by its phosphatase activity.
- NarQ can phosphorylate and dephosphorylate both NarL and NarP.
- Nitrate leads to accumulation of NarL-P that activates the gene for respiratory nitrate reductase NarG.
- Nitrite lowers the amounts of NarL-P and increases the amounts of NarP-P which in turn activates nrfA.
- In presence of nitrate, NarL suppresses genes of alternate respiration pathways.
- Formate oxidation is important for supplying electrons to nitrate or any other electron acceptor during anaerobic respiration. There are three formate dehydrogenases in *E. coli* coded by three different operons, *fdnGHI*, *fdoGHI* and *fdhF*.
- In the absence of nitrate, *E. coli* can use fumarate, DMSO or TMAO as terminal electron acceptors under anaerobic conditions.
- Fumarate reductase (encoded by *frdABCD* operon) reduces fumarate to succinate, and its expression is under the control of FNR and NarL factors.
- The primary DMSO reductase (encoded by *dmsABC*) has a broad specificity that can support anaerobic respiration with either DMSO or TMAO, and its expression is mediated by FNR and NarL.
- The primary TMAO reductase is encoded by *torCAD*. It is not repressed by nitrate and is strongly induced by TMAO. Its expression is dependent upon a sensor histidine kinase-response regulator pair, TorSR.2.

Questions

1. *E. coli* is a facultative anaerobe. What is the basis for choice of alternate terminal acceptor?
2. Differentiate between assimilatory and dissimilatory nitrate reduction.
3. What are the key differences between NarG, NapF and NarZ operons with respect to inducibility and localization?
4. What are the different components of *narG* operon?
5. What is the physiological significance of *NapF* operon?
6. Why *E. coli* is known to be a poor nitrate utilizer?
7. Differentiate between denitrifying and ammonifying nitrite reductases.
8. Where are NirBD and NrfA nitrite reductases located? How does their expression vary in response to nitrate concentration?
9. What is the probable advantage of the differential expression of NirBD and NrfA nitrite reductases?
10. Write a note on the mechanism of signal transduction by the nitrate and nitrite sensing two-component system.
11. How do the two sensor kinases NarX and NarQ differ with respect to interaction with their response regulators?
12. NarXL and NarPQ operon regulation is contrasting. Comment.

13. Explain cross-regulation network of signal transduction for nitrate/nitrite signaling.
14. Why is formate oxidation important during anaerobic respiration?
15. Differentiate between the three formate dehydrogenases, fdh-N, fdh-H and fdh-O, with respect to their role and localization.
16. What is fumarate respiration?
17. Write a note on DMSO and TMAO respiration.
18. State True or False with explanations:
    a. Under anaerobic conditions, the main difference in the electron transport system is that the terminal electron acceptor is other than oxygen.
    b. Major difference between assimilatory and dissimilatory nitrate reduction is with respect to final product.
    c. NarG and NapF differ in their affinity for NO_3^-.
    d. *E. coli* utilizes NO_3^- as source of nitrogen.
    e. NarG and NapF operons are also inducible by NO_2^- in addition to anaerobic conditions.
    f. NirBD and NrfA nitrite reductases function via formate reduction.

References

Bonnefoy, V., & Demoss, J. A. (1994). Nitrate reductases in *Escherichia coli*. *Antonie van Leeuwenhoek, 66*(1–3), 47–56.

Clarke, T. A., Mills, P. C., Poock, S. R., Butt, J. N., Cheesman, M. R., Cole, J. A., Hinton, J. C. D., Hemmings, A. M., Kemp, G., Söderberg, C. A. G., Spiro, S., Van Wonderen, J., Richardson, D. J. (2008). *Escherichia coli* cytochrome c nitrite reductase NrfA. *Methods in Enzymology 437*, 63–77.

Dow, J. M., Grahl, S., Ward, R., Evans, R., Byron, O., Norman, D. G., Palmer, T., & Sargent, F. (2014). Characterization of a periplasmic nitrate reductase in complex with its biosynthetic chaperone. *The FEBS journal*, *281*(1), 246–260.

Jia, W., & Cole, J. A. (2005). Nitrate and nitrite transport in *Escherichia coli*. *Biochemical Society Transactions, 33*(1), 159–161.

Jones, H. M., & Gunsalus, R. P. (1987). Regulation of *Escherichia coli* fumarate reductase (frdABCD) operon expression by respiratory electron acceptors and the fnr gene product. *Journal of Bacteriology, 169*(7), 3340–3349.

Jones, S. A., Gibson, T., Maltby, R. C., Chowdhury, F. Z., Stewart, V., Cohen, P. S., et al. (2011). Anaerobic respiration of *Escherichia coli* in the mouse intestine. *Infection and Immunity, 79*(10), 4218–4226.

Jormakka, M., Törnroth, S., Byrne, B., & Iwata, S. (2002). Molecular basis of proton motive force generation: structure of formate dehydrogenase-N. *Science, 295*(5561), 1863–1868.

Lee, A. I., Delgado, A., & Gunsalus, R. P. (1999). Signal-dependent phosphorylation of the membrane-bound NarX two-component sensor-transmitter protein of *Escherichia coli*: Nitrate elicits a superior anion ligand response compared to nitrite. *Journal of Bacteriology, 181*(17), 5309–5316.

Lundberg, J. O., Weitzberg, E., Cole, J. A., & Benjamin, N. (2004). Nitrate, bacteria and human health. *Nature Reviews Microbiology, 2*(7), 593–602.

McCrindle, S. L., Kappler, U., & McEwan, A. G. (2005). Microbial dimethylsulfoxide and trimethylamine-N-oxide respiration. *Advances in Microbial Physiology, 50,* 147–201e.

Noriega, C. E., Lin, H. Y., Chen, L. L., Williams, S. B., & Stewart, V. (2010). Asymmetric cross-regulation between the nitrate-responsive NarX–NarL and NarQ–NarP two-component regulatory systems from Escherichia coli K-12. *Molecular Microbiology, 75*(2), 394–412.

Overton, T. W., Griffiths, L., Patel, M. D., Hobman, J. L., Penn, C. W., Cole, J. A., et al. (2006). Microarray analysis of gene regulation by oxygen, nitrate, nitrite, FNR, NarL and NarP during anaerobic growth *of Escherichia coli*: New insights into microbial physiology. *Biochemical Society Transactions, 34*(1), 104–107.

Stewart, V., Lu, Y., & Darwin, A. J. (2002). Periplasmic nitrate reductase (NapABC enzyme) supports anaerobic respiration by *Escherichia coli* K-12. *Journal of Bacteriology, 184*(5), 1314–1323.

Wang, H., & Gunsalus, R. P. (2003). Coordinate regulation of the *Escherichia coli* formate dehydrogenase fdnGHI and fdhF genes in response to nitrate, nitrite, and formate: roles for NarL and NarP. *Journal of Bacteriology, 185*(17), 5076–5085.

Chapter 23
Response to Phosphate Starvation and Osmotic Homeostasis

Rani Gupta and Namita Gupta

1 Response to Phosphate Starvation

E. coli assimilates phosphates in the form of either inorganic phosphate, organic phosphate or phosphonates. Of these, inorganic phosphates (Pi) are the preferred source. When Pi is in excess, it is taken up by the low-affinity transporter (Pit). However, when the bacterium encounters phosphate deficiency, the gene for high-affinity Pi-specific transporter (Pst) and gene for utilization of alternate phosphate sources (organic phosphates and phosphonates) are de-repressed. These genes are coregulated and are part of Pho regulon which is activated nearly 100-folds during phosphate starvation (Wanner, 1993).

2 Components of Pho Regulon

The Pho regulon of *E. coli* comprises more than 38 genes arranged in eight separate operons. These include all the different genes required for uptake of phosphorous (Pi) or alternate forms for their assimilation into high energy compound, ATP. Finally, phosphorous is incorporated into various biomolecules (Santos-Beneit, 2015). The key genes which are induced on phosphorous deficiency include:

- Periplasmic alkaline phosphatase (phoA) that can use organic phosphate esters to generate phosphate.
- An outer membrane porin anion channel (phoE).
- High-affinity phosphorous transport system (Pst) and a repressor PhoU.
- Genes for phosphonate uptake and breakdown (Phn).
- Proteins for uptake of specific phosphorylated forms such as UgpB (periplasmic protein for uptake of glyceraldehyde 3-phosphate) and UgpG (phosphodiesterase for hydrolysis of glycerophosphoryldiesters to monoesters).

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_23

3 Two-Component PhoBR Regulates Pho Regulon in *E. Coli*

The Pho regulon is regulated by a two-component regulatory system comprising a sensor kinase (PhoR) along with its cognate response regulator (PhoB) which is a DNA-binding transcriptional regulator of genes having phosphate box (PHO box) as signature sequence. The two-component system regulates phosphorus assimilation under Pi stress and are named differently in different organisms, for example, PhoBR in *E. coli*; PhoPR in *Bacillus subtilis*; and PhoSR in *Corynebacterium glutamicum,* etc. Similar to all two-component systems, PhoB phosphorylation is crucial to transcriptional regulation of Pho regulon. Thus, the signals which regulate Pho regulon in turn also determine the amount of phosphorylated PhoB. This PhoR-PhoB regulation is Pi-regulated. However, there is Pi-independent regulation as well where there is an alternate sensor CreC (formerly known as PhoM), while another form of regulation requires acetyl phosphate (Wanner & Wilmes-Riesenberg, 1992). PhoBR two-component system of bacterial signalling is unique as it functions in assimilation with ABC transporter which senses phosphorus concentration.

Phosphate uptake

As described earlier, there are two uptake systems, low-affinity transporter (Pit) and high-affinity transporter (Pst) which function under high- and low-phosphate availability, respectively. The key differences between these two systems are presented in Table 1.

During phosphate starvation, both transportable and non-transportable phosphorylated compounds can serve as source of phosphorous. Hence, the Pho regulon codes for several transporter proteins which transport specific phosphorylated forms. The transportable compounds have specific transport proteins which facilitate uptake, like for glycerol 3-phoshate, Ugp transporter of Pho regulon are transcribed namely, UgpB, a periplasmic glycerol 3-phosphate binding protein. They are directly assimilated into phospholipids; however, if diasters are present, they are first hydrolyzed to

Table 1 Differences between Pit and Pst transporters

Pit system	Pst system
Low-affinity transporter	High-affinity inner membrane transporter
Single component system	Multi-component system comprising PstA, PstB, PstC and PstS • PstA—Integral membrane protein • PstB—Permease • PstC—Integral membrane protein • PstS—Periplasmic Pi binding protein
Proton gradient driven	ATP driven
Constitutively expressed	Expressed during phosphate starvation
	Part of Pho regulon, encoded by an operon which also codes for a negative regulator PhoU

monoesters by UgpG phosphodiesterase. Non-transportable organophosphates are hydrolyzed in periplasm by alkaline phosphatases and depending upon the concentration they are taken up by low-affinity or high-affinity transporters. Phosphonates (Pn) have C-P bonds instead of C-O-P linkage. *E. coli* has nearly fourteen genes for uptake and degradation of phosphonates, all of which are part of Pho regulon.

Signal transduction pathway during phosphorus starvation

Pho regulon is activated under Pi-starved conditions; since sensor kinase PhoR does not have periplasmic domain, it is possible that its PAS domain senses at cytoplasmic side. PhoR, under high Pi conditions, functions as phosphatase and dephosphorylates PhoB. For its phosphatase activity, it requires one additional protein PhoU. For signal transduction, PhoBR two-component system requires five additional proteins, and of these, four are part of Pst transporter and one is metal-binding protein PhoU. Inactivation of PhoU abolishes as Pho regulon repression but does not affects transport. PhoU is a regulator, and it functions along with other Pst components and also with phosphate sensor kinase.

Pst system (Transport phosphate during phosphate starvation)

Pst system is a typical ABC transporter having different components as periplasmic substrate binding protein (PstS), two transmembrane integral proteins (PstA and PstC) and a nucleotide binding protein (PstB), the permease. Wide variety of mutations in Pst system established the role of different component proteins in the transport and regulation of Pho regulon. Few of these are as follows:

- PstS and PstB mutation abolishes both transport and Pho repression
- PstA mutation abolishes transport but does not affect repression

This mutation analysis shows that transport and repression are independent per se.

PhoR histidine kinase Type I domain organization

PhoR domain organization is homologous to EnvZ histidine kinase. It has a cytoplasmic N-terminal domain and its C-terminus is connected to two transmembrane α-helices having a short 6–7 amino acid periplasmic domain. PhoB is a response regulator and in *E. coli* it usually exists as a homodimer. It gets phosphorylated at its N-terminal aspartate residue and oligomerization of its active dimers on DNA is achieved in a cooperative manner.

Signal transduction

During excess Pi availability: During Pi availability, dimerization of PhoR is prevented. PhoR is in monomeric form, and the Pho regulon is repressed. The series of events which is suggested for Pi-mediated repression are:

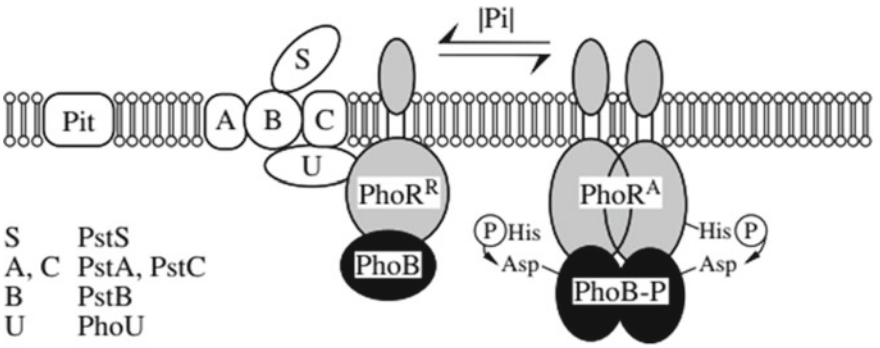


Fig. 1 Signal transduction of Pho regulon *Source* Vershinina and Znamenskaya (2002). With kind permission from Springer Nature

1. PstS, which is a high-affinity Pi transport, is saturated during Pi-sufficient conditions.
2. Pi bound PstS complexes with integral membrane complex, PstABC.
3. PstS-PstABC in turn bind to PhoU to form a repressor complex PstS-PstABC-PhoU.
4. This repressor complex maintains PhoR as monomer and in turn PhoR dephosphorylates PhoB and results in repression of Pho regulon.

During Pi starvation: Under Pi starvation, PstS is not saturated, and thus, the repressor complex does not form, and consequently, PhoR is released to form dimer which autophosphorylates to sense Pi starvation. This in turn phosphorylates PhoB, the response regulator, which up-regulates Pho regulon (Fig. 1; Vershinina & Znamenskaya, 2002; Vuppada et al., 2018).

4 Cross-Regulation: Pi-Independent Regulation of Pho Regulon

Sensor CreC/PhoM—Cross-regulation of PhoBR is mediated via activation of PhoB response regulator independent of its cognate sensor kinase PhoR. It was observed in PhoR mutant where two Pi-independent controls induced by glucose and acetyl phosphate involving CreC sensor kinase. Both controls are independent of each other. This cross-regulation is important in understanding coordinated control of Pi availability under different environmental conditions (Hsieh & Wanner, 2010).

Box 1: Pho Regulon and Virulence (Lamarche et al., 2008)

Pho regulon is not only important for phosphate homeostasis but also offers a complex network important for stress response and virulence as well.

Pst inactivation—Increased sensitivity if *E. coli* ExPEC strain to bactericidal serum and also reduction of capsular antigen.

Pst deletion—Reduced virulence of avian pathogenic *E. coli.*

PhoU mutant—Reduced colonization in murine urinary tract in a competitive infection assay with wild-type strain (Buckles et al. 2006).

Pst mutation—Reduced virulence of *Edwardsiella tarda*; proteome analysis showed the absence of virulence proteins.

PhoBR mutation—Decreased virulence; genes such as siderophores are differentially expressed in *vibrio cholera*, affect sporulation in *Clostridium perfringens* and affect endotoxin production in *C. perfringens.*

PhoR as drug target in *Mycobacterium tuberculosis*

PhoR-PhoB two-component system controls the expression of 78 virulent genes in *Mycobacterium tuberculosis* and its disruption attenuates growth as it severely affects complex lipid synthesis. Hence, PhoR sensor kinase is projected as a prospective drug target as sensor histidine kinase is absent in humans (Suwanto & Giri-Rachman, 2012).

5 Osmohomeostasis

Osmohomeostasis is the metabolic regulation of osmotic pressure in the cell in order to maintain constant water content of cellular fluids. Bacteria have the ability to survive under a variety of physiological stresses. They respond very fast to environmental stimuli and alter their metabolic functions for survival through a complex coordinated metabolic regulation. Osmolarity is one of the stimuli which is sensed through two-component system. It activates osmoregulon for transcription of various osmogenes responsible for osmoprotection.

6 Osmotic Stress and Turgor

The bacterial cell cytoplasm maintains a positive turgor pressure in the cell membrane so that transport of nutrients is possible by water movement from outside to inside. However, when cells encounter high osmotic potential (hypertonic) or lower osmotic potential than the ideal one, there is a water movement from outside to inside and vice versa. This might lead to either loss of water (hypertonic environment) or excess

intake of water in the cell (hypotonic environment). This is a hindrance to maintain cell shape and integrity. In order to maintain cell shape, the bacterium must maintain minimum outward pressure called turgor (0.6 M Pa). When the cell is subjected to high external osmolarity, it switches on osmohomeostatic mechanism which counterbalances external pressure to prevent cell collapse. The solutes which are imported to counterbalance osmotic stress are called osmoprotectants.

Detailed studies on osmoadaptation show that it is a two-step process wherein at the first instance there is K^+ influx along with glutamate accumulation. Subsequently, osmoprotectants such as trehalose, proline, glycine, betaine and glycine betaine (N-methylated amino acid derivates) are either synthesized within the cell or transported in from the environment. Accumulation of these protectants activates K^+ efflux as higher concentrations of K^+ are toxic to cell, and consequently, the cell resumes normal growth. Osmoprotectants are not harmful to cells probably because they are generally excluded from water pockets of proteins while ion accumulation interferes with proteins and can affect their conformation and resulting in denaturation (Crowe et al., 1990). Eukaryotes also accumulate similar osmoprotectants as prokaryotes. A good osmoprotectant is one which can counteract osmotic dehydration.

Further, in gram-negative bacteria, the cell permeability is also altered during high osmotic stress via shift in ratio of porins wherein smaller pore porins (OmpC) are expressed more than larger pores ones (OmpF) to restrict the movement of solutes inside the cell.

Thus, osmoadaptation involves three phases (Sleator & Hill, 2002):

1. Primary response by K^+ uptake
2. Secondary response by accumulation of osmoprotectants
3. Porin regulation in gram-negative bacteria.

7 Primary Response to Osmohomeostasis: ATP-Driven K^+ Uptake

E. coli has three different active transport systems for K^+ uptake:

- Kdp
- TrkA
- TrkD (Kup).

Trk systems are constitutively expressed low-affinity transport systems that maintain cell turgor in normal conditions. During high osmotic conditions, the cell is subjected to extreme K^+ limitation, and a high-affinity (Km = 2 μM) K^+ uptake system, Kdp-ATPase, is induced which is a response of a two-component signal system (Freeman et al., 2013).

E. coli Kdp-ATPase is encoded by the *KdpFABC* operon and is made up of four protein subunits:

- KdpF—important for stability

- KdpA—binds and transports K^+
- KdpB—is a P-type ATPase and contains the highly conserved phosphorylation site
- KdpC—helps in assembly of Kdp-ATPase complex.

Kdp-ATPase is synthesized in response to a two-component signaling system comprising of a sensor kinase KdpD and its cognate response regulator, KdpE. Phosphorylated KdpE interacts with KdpABC operon to up-regulate high-affinity K^+ transporters (Sleator & Hill, 2002; Freeman et al., 2013). The sensor kinase comprises a cytoplasmic N-terminal domain (NTD), a transmembrane domain (TM) followed by an arginine rich C-terminal domain (CTD). NTD has an ATP binding domain while CTD has an active histidine residue for phosphorylation. NTD regulates response to osmotic stress and K^+ limitation as ΔNTD KdpD protein becomes de-regulated. It also regulates phosphatase activity for dephosphorylation of KdpE.

Organization of Kdp operon in bacteria suggests that the structural genes of this operon are always coded by single operon. However, the sensor genes KdpD and KdpE may or may not be in the same arrangement as KdpFABC. The *E. coli* Kdp operon consists of KdpFABC in one operon while KdpDE are present as another operon downstream of KdpFABC. These operons remain repressed under high K^+ concentrations when low-affinity K^+ constitutive transporters are functional. KdpFABC operon is induced under low K^+ concentration (<2 mM K^+) and high osmotic concentration (Fig. 2).

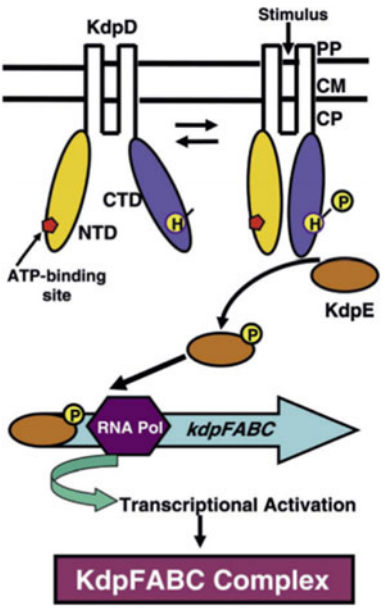


Fig. 2 KdpD-KdpE phosphorelay in *E. coli* *Source* Ballal et al. (2007). With kind permission from Springer Nature

The three structural proteins of Kdp-ATPase, i.e., KdpA, KdpB and KdpC, are localized in inner membrane and form a part of ATP-dependent ABC transport. Among the three, KdpA is periplasmic and is believed to bind to K^+ while KdpB is a transmembrane protein facilitating movement of K^+ across the membrane. It has transmembrane hydrophobic domain along with ATP-binding domain facing cytosol. Function of KdpC is not known (Ballal et al., 2007).

Kdp potassium (K^+) transport

Thus, on exposure to high osmolarity, there would be rapid uptake of K^+ by high-affinity transporters, and to maintain internal osmolarity, there has to be counter ion to balance it. The major counter ion in *E. coli* is glutamate. Along with this, other components glutamine, gamma glutamyl peptides, glutathione and gamma-glutamyl glutamine also contribute to balance K^+ influx.

8 Secondary Response: Osmoprotectant Accumulation

The primary response to osmolarity is K^+ influx with a purpose of osmohomeostasis. However, too much K^+ influx may be deleterious to cells as it may inhibit key enzymes (Finlay & Falkow, 1997). Hence, after reaching the threshold concentration, the cells show K^+ efflux along with increase in concentrations of compatible solutes either by uptake or by synthesis. These solutes, also known as osmoprotectants, protect the cell integration by maintaining turgor. They accumulate in cells and counterbalance the osmotic difference between the cell's surroundings and the cytosol. High accumulation of osmoprotectants does not disturb metabolic functions of the cell. Some of the compatible solutes are glycerol, trehalose, amino acids and their derivatives such as proline, taurine, β-alanine, glycine, betaine and proline betaine. Eukaryotes also accumulate similar osmoprotectants as prokaryotes. Some of the osmoprotectants have multiple roles as shown in Table 2.

Trehalose accumulation: Trehalose is a non-reducing disaccharide of glucose, and its synthesis is upregulated in several yeasts and bacteria by several environmental stimuli including changes in osmolarity. The trehalose accumulation is a result of activation of ostBA trehalose operon coding for ostA trehalose-6-phosphate synthetase and ostB trehalose-6-phosphatase. The ostA and ostB genes are activated by high

Table 2 Osmoprotectants and their roles

Osmoprotectant	Function	Host
Betaine	Cold tolerance	Listeria monocytogenes
Trehalose	Thermotolerance Ethanol tolerance	Yeast
Trehalose	Desiccation tolerance	*Streptomyces, Saccharomyces,* Resurrection plants, Nematodes

osmotic concentration, and also, synthetase is activated by K-glutamate (accumulated component as primary response to osmotic stress). The ostBA operon is also activated during stationary phase and under starvation by RpoS sigma factor.

$$\text{Glucose-6-phospahte + UDP glucose} \xrightarrow{\text{OstA}} \text{Trehalose-6-phospahte} \xrightarrow{\text{OstB}} \text{Trehalose}$$

Noteworthy is that exogenous trehalose does not provide osmoprotection. This is clear from the fact that catabolism of trehalose-6-phosphate, an inducer of trehalose transport protein, is depleted by phosphatase activity induced during osmotic stress. Further, activation of periplasmic trehalose by osmotic stress is also responsible for uptake of trehalose from outer environment after its breakdown to glucose. Glucose is taken up by PEP-PTS system as glucose-6-phosphate and is again accumulated as trehalose (Fig. 3).

Osmoprotectant in halobacteria—Ecotine (1, 4, 5, 6 tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is a cyclic amino acid produced by *Brevibacterium linens*. Ecotine also protects *E. coli* under osmotic stress when provided in the culture medium (Roberts, 2005).

Glycine-betaine accumulation: Glycine-betaine is another osmoprotectant which accumulates intracellularly during osmotic stress. However, in *E. coli* glycine-betaine de novo synthesis does not take place and it accumulates only when choline and

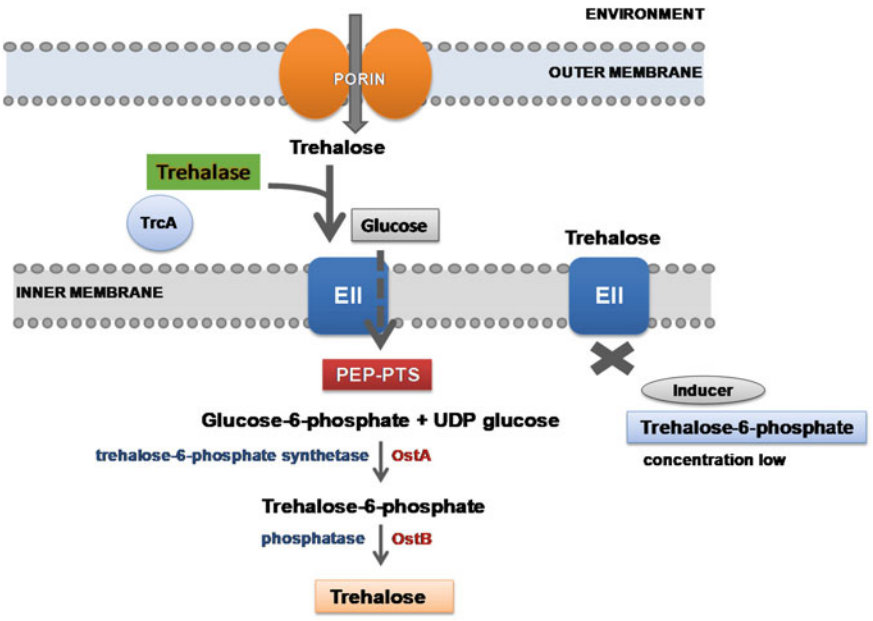


Fig. 3 Pathway of trehalose accumulation

glycine-betaine aldehyde is present in the growth medium. *E. coli* can efficiently transport these precursors and convert them to glycine-betaine by membrane-bound oxygen-dependent choline dehydrogenase coded by *bet* operon in *E. coli* along with NAD(P)-dependent betaine aldehyde dehydrogenase. This can happen only in the presence of oxygen in the presence of choline containing medium. Thus, choline is an osmoprotectant in aerobic conditions while betaine aldehyde and glycine-betaine can function under both aerobic and anaerobic conditions.

Bet operon comprises BetT (choline transporter), BetI (regulator), BetB (glycine-betaine dehydrogenase) and BetA (choline dehydrogenase). BetA is a membrane-bound enzyme which can oxidize both choline and glycine-betaine aldehyde in the presence of oxygen. BetB is soluble protein and has NAD(P)-dependent glycine-betaine aldehyde dehydrogenase activity with marked substrate specificity. Thus, along with BetT transporter, choline from external environment is taken up and is efficiently converted in the cell. However, since some *E. coli* strains and *S. typhimurium* lack this system, it suggests that glycine-betaine pathway may not be essential for osmoprotection.

Regulation of Bet genes—Bet operon comprises four genes:

- BetT—choline transporter
- BetI—regulator
- BetB—glycine-betaine dehydrogenase
- BetA—choline dehydrogenase.

Bet genes cluster is induced by high osmolarity and also by choline in the environment. Aerobic conditions further increase expression of these genes. BetI is regulator which shows homologies with repressor protein. Figure 4 describes the organization of Bet operon, and choline and glycine-betaine aldehyde transport in *E. coli*.

Glycine-betaine and trehalose are known as osmoprotectants. The former is also known to increase cold tolerance, while the latter increases desiccation tolerance in *Saccharomyces cerevisiae*. Genetic engineering studies have shown that transgenic plants with BetA gene from *E. coli* and TPSI gene (trehalose-6-phosphate synthase) from yeast conferred similar protection. Metabolic engineering of glycine-betaine pathway from *E. coli* to related bacteria such as *S. typhimurium*, *Azospirillum brasilense* and *Synechococcus* group of cyanobacteria showed osmotolerance in the presence of choline. In cyanobacteria, glycine-betaine accumulation also protected photosynthetic apparatus against salt stress and also protected against low-temperature stress.

Proline accumulation: Intracellular proline accumulation was demonstrated first in *Salmonella oranienburg* by Christain (1955) followed by its demonstration in

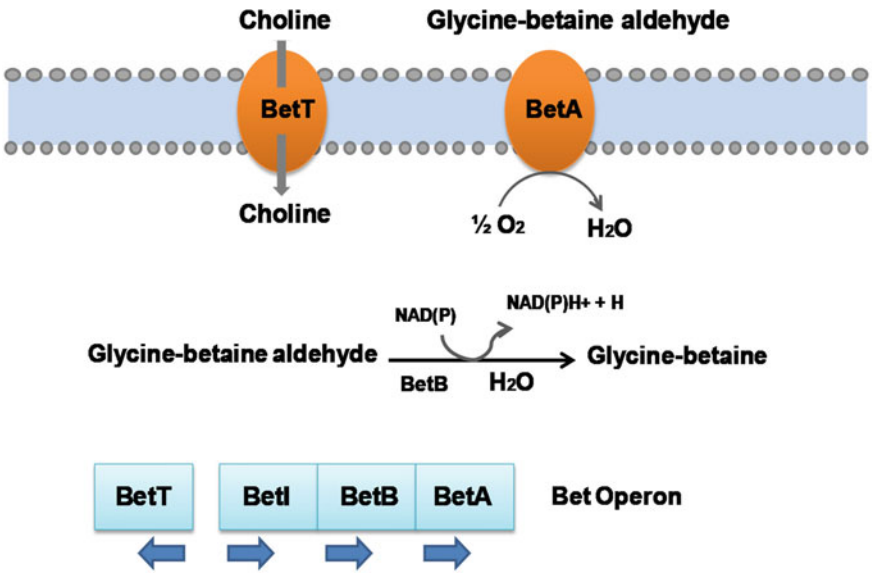


Fig. 4 Regulation of *Bet* genes

aerobic bacteria, *E. coli* and *S. typhimurium* by Measures (1975). Proline accumulation was a result of efficient uptake rather than its overexpression as proline biosynthesis is subject to feed lack inhibition. Also, *E. coli* mutants which lack feedback inhibition accumulated much higher proline than wild type and also exhibit better osmotolerance.

There are three proline uptake systems known in enteric bacteria, PutP, ProP and ProU.

- **PutP transport**: It is a high-affinity transport which is responsible for proline as a nutrient and plays no role during osmotic stress as it is inhibited in those conditions.
- **ProP transport**: It is a low-affinity transport that is overexpressed during high osmolarity. In addition to proline, it can allow several other protectants though with low affinity, viz. taurine (eukaryotes), ectoine (some halophilic and halotolerant bacteria) and glycine-bataine and its analogs.
- **ProU system**: Studies have indicated that in the absence of PutP and ProP transporters, there is a third proline transporter, ProU which functions under conditions of high osmolarity (Csonka, 1982). Later, it was found that it is an efficient transporter for glycine-betaine in *E. coli* and *S. typhimurium.*

ProP and ProU function as transporters during osmotic stress (Sleator & Hill, 2002).

ProU is a multi-component ABC transporter. ProU locus codes for three proteins, ProV, ProW and ProX. ProV, a hydrophilic protein associated with cytoplasmic

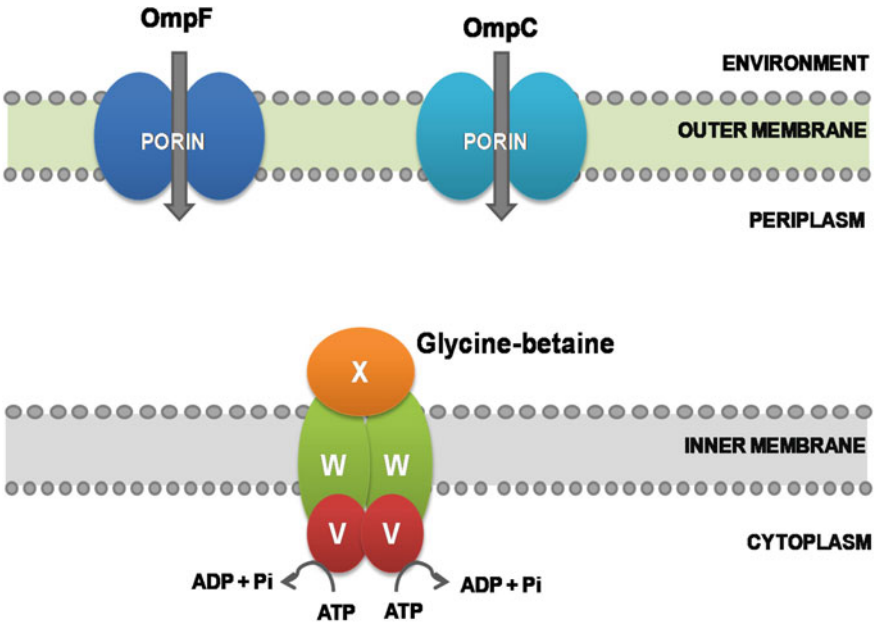


Fig. 5 Pathway of proline accumulation

membrane, is a nucleotide binding protein. It is an energy-coupling component associated with ATP hydrolysis. ProW is a transmembrane hydrophobic protein integrated in inner membrane. Both the proteins exist as homodimers. ProX is a periplasmic glycine-betaine binding protein (Fig. 5).

The glycine-betaine uptake is a typical ABC transporter mechanism and through porins the substrate reaches periplasm. ProU can also permit other osmoprotectants just like ProP. Strains defective in both ProP and ProU transporters are no more protected against osmotic stress in the presence of excess proline in medium showing that there is no other alternative mode of uptake of osmoprotectants. Sudden lowering of osmolarity is encountered after uptake of osmoprotectant; a rapid decrease in intracellular concentration of osmoprotectant has been observed as a result of rapid efflux which is independent of ProP and ProU system.

Kdp versus ProU

Kdp	ProU
K^+ increase occurs transiently immediately on loss of turgor, and it stops when turgor is maintained	Expression is maintained as long as osmotic stimulus persists

The presence of K^+ is prerequisite for ProU expression

ProU expression is a not a two-component response and is not dependent on EnvZ and ompR system which mediate osmoregulation of porins. Uptake of glycine-betaine triggers K^+ efflux and thus lowers the amount of secondary messenger K-glutamate, thereby decreasing Pro U expression. This has been observed in in vitro experimentation of increased ProU expression in the presence of K-glutamate concentration. However, in vitro effect on ProU expression was much lower than in vivo level suggesting multiple regulatory controls of ProU expression in vivo.

Higgins et al. (1988) suggested that high osmolarity causes changes in DNA supercoiling of ProU promoter which may also stimulate transcription. However, both the mechanism's activation by K-glutamate and its effects on DNA structure may function in synergism to activate ProU transcription.

In addition to these, a mechanism was proposed that involves silencer sequence downstream of ProU promoter. Recently, it has been reported that histone like H-NS protein binding to these sites act as a repressor of ProU transcription in vitro and repression is abolished in the presence of K-glutamate (Sleator & Hill, 2002).

Porin regulation in gram-negative bacteria

The outer membrane of *E. coli* has two major porins, OmpC and OmpF (37,000 MW), coded by *ompC* and *ompF* loci. OmpC is the smaller porin channel, while OmpF is the larger porin channel and are expressed at constant levels all the time.

However, during change in osmolarity, the relative ratio of OmpC and OmpF also changes. The relative ratio of OmpC and OmpF is low in low osmolarity and high in high osmolarity. Thus, in a lake environment, where there is low osmolarity, OmpF is preferentially synthesized, whereas in intestinal tract to avoid uptake of toxic compounds and bile salts, smaller porin OmpC is of advantage. The change in porin composition does not affect intracellular osmotic pressure, thus does not account for homeostatic response to osmolarity, but it is simple response to osmolarity. Porins make aqueous pores in the outer membrane facilitating movement of hydrophilic molecules across the outer membrane.

Porin synthesis: a two-component system

EnvZ, a histidine kinase/phosphatase, is the sensor kinase. It is a transmembrane protein which undergoes autophosphorylation under high osmolarity. This, in turn, phosphorylates the response regulator OmpR which is a DNA binding protein. The relative regulation of OmpC and OmpF operon is due to the fact that OmpC is activated by phosphorylated OmpR, but it has a low-affinity binding site. This in turn suggests that in order to activate OmpC operon a high concentration of building up of phosphorylated form of OmpR is a must. On the other hand, OmpF has both low-affinity and high-affinity binding sites which function in both conditions when OmpR-P is abundant and when OmpR-P is limited. First, high-affinity binding site activates OmpF (at this time OmpC is poorly expressed) while low-affinity site turns off expression of OmpF and activates OmpC. Thus, when osmolarity is high, EnvZ is autophosphorylated and in turn OmpR is phosphorylated and OmpR-P is abundantly

made available. This in turn activates OmpC and represses OmpF resulting in more OmpC protein than OmpF and the ratio of OmpC: OmpF is increased. On the contrary, when bacterium is changed from high to low osmolarity, phosphatase activity of EnvZ dephosphorylates both EnvZ and OmpPR reversing the effect by increasing OmpF to OmpC.

There is an additional mechanism for regulation of OmpC to OmpF ratio by antisense RNA regulation. This involves a gene *micF* adjacent to OmpC which is transcribed in opposite direction to OmpC. However, transcript *micF* (90 nucleotides) is complementary to transcript OmpF 5'RNA. Under high osmolarity, OmpR-P which activates OmpC transduces *micF* region also. This transcript hybridizes with OmpF transcript and blocks translation bringing additional control to down-regulate OmpF under high osmotic conditions. The observation was further supported from the report that when *micF* was introduced on a high copy plasmid there was inhibition of ompF (Fig. 6; Delihas, 1995; Ramani et al., 1994).

However, deletion mutants revealed that *micF* mutants did not affect OmpF level during high osmolarity when OmpR-P is sufficient, while at low osmolarity (6% sucrose), the mutant failed to reduce OmpF in comparison to wild type where OmpF

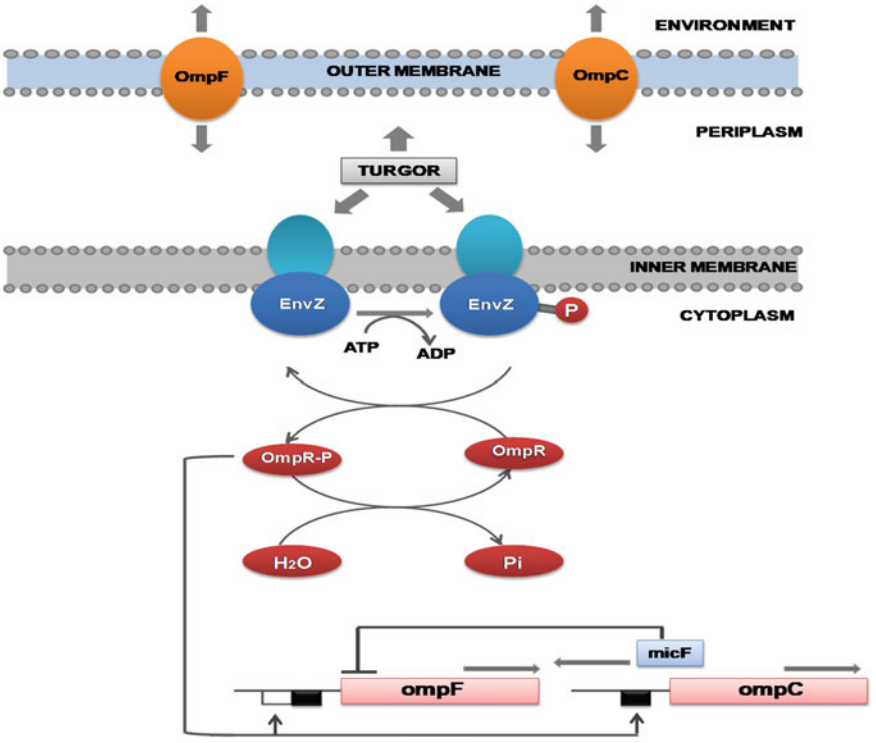


Fig. 6 Regulation of Porin synthesis

was lower. This suggests that *micF* poses additional control at low and medium osmolarity when OmpR-P is supposed to be less.

Additional information in support of model

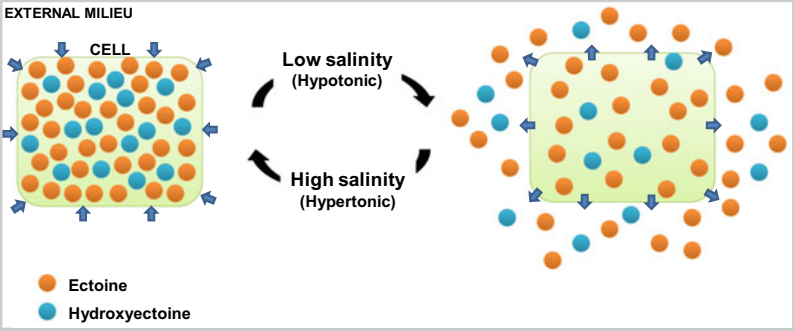
1. In vitro, EnvZ accepts phosphate group from ATP and in turn of phosphorylates OmpR-P.
2. High osmolarity led to high concentration OmpR-P.
3. Mutant EnvZ deletion was found constitutive for expressing osmoresponse that is OmpC high and OmpF reduced. At same time, mutant had constitutively OmpR-P in phosphorylated form in *E. coli* suggesting that EnvZ rather responds to low osmolarity by activating phosphatase activity.

Mechanosensitive channels (MS channels) function in dilute solutions and protect against cell lysis (See details in Chap. 6: Solute transport).

Box 2: Osmoprotectants and Application in Biotechnology

1. **Trehalose and Glycine-betaine pathway engineering for introducing stress tolerance in microbes and plants (Strøm,** 1998)—Glycine-betaine and trehalose are known as osmoprotectant, while the former is also known to increase cold tolerance as well and latter desiccation tolerance in *Saccharomyces cerevisiae*. The genetic engineering studies showed that transgenic plants with BetA gene from *E. coli* and TPSI (trehalose-6-phosphate synthase) from yeast conferred similar protection. Metabolic engineering of glycine-betaine pathway from *E. coli* to related bacteria, *S. typhimurium*, *Azospirillum brasilen* and *Synechococcus* group of cyanobacteria showed recipient to become osmotolerant when supplied with choline. In cyanobacteria, glycine-betaine accumulation also protected photosynthetic apparatus against salt stress and also protected against low-temperature stress.
   Metabolic engineering of plants for stress tolerance is very important as plants are after subjected to several stresses, viz. drought, salinity and cold. Further, several important crops such as rice, potatoes, tomatoes lack glycine-betaine accumulation system. Plants have precursors for synthesis of both glycine-betaine and trehalose. Thus, bet genes were proposed as possible way of introducing glycine-betaine accumulation (Le Rudulier et al., 1984; Paiva & Panek, 1996). Similarly, several successful attempts have been made.
2. **Milking *Halomonas elongata* for osmoprotectants**—*H. elongata* is a gram-negative halophilic bacterium, and it accumulates large amount of compatible solutes ecotines and hydroxyl ectoines on exposure to osmotic stress in the presence of high salinity. Ectoine is a compatible solute which helps organisms survive extreme osmotic stress by acting as an osmolyte.

Sauer and Galinski (1998) developed a novel bioprocess to overproduce these solutes. High-density cell biomass was exposed to salinity when it accumulated these solutes followed by osmotic down shock by shifting to low salinity. In such alternate cycling, the bacterium first accumulated these solutes during osmotic shock and secreted these on lowering osmotic stress and repeating these cycles with the repeated shock. By standardizing time of accumulation, the solutes were secreted in high amounts, and this cycling was termed bacterial milking.



Summary

- *E. coli* assimilates phosphates in the form of either inorganic phosphate, organic phosphate or phosphonates.
- The Pho regulon of *E. coli* comprises more than 38 genes involved in uptake and assimilation of inorganic phosphate (Pi).
- The Pho regulon is regulated by PhoBR two-component system, wherein PhoB is the response regulator and PhoA is the sensor kinase.
- Two phosphate uptake systems are present: low-affinity transporter (Pit) and high-affinity transporter (Pst) which function under high and low phosphate availability, respectively.
- PhoB is the response regulator and functions by dimerization or oligomerization on the DNA.
- Under high Pi conditions, PhoR functions as phosphatase and dephosphorylates PhoB with the help of PhoU.
- PhoU is a metal-binding regulator protein and assists both Pst and PhoR in their functions.
- PhoR domain organization is homologous to EnvZ (HK class I) kinase with dimer interface.
- During Pi availability, dimerization of PhoR is prevented. Thus, PhoR is in monomeric form and the Pho regulon is repressed.

- Under Pi starvation, PhoR is released to form dimer which autophosphorylates and in turn phosphorylates PhoB which up-regulates Pho regulon.
- Cross-regulation of PoBR is mediated via activation of PhoB response regulator by CreC sensor kinase (induced by glucose) or by acetyl phosphate induction.
- Various component genes of the Pho regulon, Pst, PhoBR and PhoU, are also involved in bacterial virulence.
- Osmoregulation is a complex process that plays an important role in regulating the cellular response to different solute concentrations in the growth medium of the organisms.
- Osomolarity is one of the environmental stimuli which is sensed though two-component system to activate osmoregulon for transcription of various osmogenes responsible for osmoprotection.
- The sensory kinase in this two-component system is EnvZ, and the response regulator is OmpR. These two components interact to bring about the differential regulation of two genes, *ompF* and *ompC,* which are responsible for the production of outer membrane porins OmpF and OmpC which control the permeability of polar solutes across the outer membrane.
- The *ompF* and *ompC* are expressed at all times at constant levels; however, the individual porins OmpF and OmpC are present in various relative abundances based on medium osmolarity.
- High osmolarity results in more OmpR molecules that are phosphorylated, and a high level of OmpR-P activates the *ompC* gene and represses *ompF* gene by binding to the low-affinity sites present on their promoters.
- At low osmolarity, the level of OmpR-P is decreased and it only binds to high-affinity site present on the promoter of *ompF* gene and activates it while it is unable to activate *ompC* gene.
- Other response regulators of porin proteins include integration of host factor at the transcriptional level and also through *micF*, which encodes an antisense RNA for translation of ompF.
- The K^+ homeostasis is regulated by different combinations of nonspecific channels and specialized transport systems including constitutively expressed systems, such as Trk and specialized, inducible higher K^+ affinity, i.e., the KdpD/KdpE system.
- At high osmolarity, the cell switches on osmohomeostatic mechanism which counterbalances external pressure to prevent cell collapse by import of osmoprotectants such as trehalose, proline, glycine, betaine and glycine betaine (N-methylated amino acid derivates).
- Glycine-betaine and trehalose are known as osmoprotectants. The former is also known to increase cold tolerance and the latter desiccation tolerance. Proline accumulation also exhibits better osmotolerance.
- During changes in osmolarity, the relative ratio of porins OmpC and OmpF changes which is regulated by EnvZ-ompR two-component system.

Questions

1. Differentiate between low-affinity and high-affinity phosphate transporters.
2. Explain two-component transduction during phosphate starvation in *E. coli.*
3. What is osmohomeostasis?
4. Name two-component system important for signal transduction during osmotic stress in *E. coli.*
5. Explain primary response to osmotic stress in bacteria. Why this response is a transient response?
6. What are osmoprotectants? Give example.
7. Trehalose accumulation protects the cell against osmotic stress, however, supplying trehalose in the medium does not render protection to the cell. Why?
8. Glycine-betaine accumulation is not a major osmoprotectant in *E. coli* unless choline or glycine-betaine aldehyde is provided in the medium. Why?
9. Explain proline accumulation as a way to counterbalance osmotic stress in *E. coli.*
10. Explain molecular basis of differential regulation of porins in *E. coli* during osmotic stress and other environmental stresses.

References

Ballal, A., Basu, B., & Apte, S. K. (2007). The Kdp-ATPase system and its regulation. *Journal of Biosciences, 32*(3), 559–568.

Buckles, E. L., Wang, X., Lockatell, C. V., Johnson, D. E., & Donnenberg, M. S. (2006). PhoU enhances the ability of extraintestinal pathogenic Escherichia coli strain CFT073 to colonize the murine urinary tract. Microbiology, 152(1), 153–160.

Christian, J. H. B. (1955). The influence of nutrition on the water relations of Salmonella oranienburg. *Australian Journal of Biological Sciences, 8*(1), 75–82.

Crowe, J. H., Carpenter, J. F., Crowe, L. M., & Anchordoguy, T. J. (1990). Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. *Cryobiology, 27*(3), 219–231.

Csonka, L. N. (1982). A third L-proline permease in Salmonella typhimurium which functions in media of elevated osmotic strength. *Journal of Bacteriology, 151*(3), 1433–1443.

Delihas, N. (1995). Regulation of gene expression by trans-encoded antisense RNAs. *Molecular Microbiology, 15*(3), 411–414.

Finlay, B. B., & Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiology and Molecular Biology Reviews, 61*(2), 136–169.

Freeman, Z. N., Dorus, S., & Waterfield, N. R. (2013). The KdpD/KdpE two-component system: integrating K + homeostasis and virulence. *PLoS Pathogens, 9*(3), e1003201.

Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G., & Bremer, E. (1988). A physiological role for DNA supercoiling in the osmotic regulation of gene expression in S. typhimurium and E. coli. *Cell*, *52*(4), 569–584.

Hsieh, Y. J., & Wanner, B. L. (2010). Global regulation by the seven-component Pi signaling system. *Current Opinion in Microbiology, 13*(2), 198–203.

Lamarche, M. G., Wanner, B. L., Crépin, S., & Harel, J. (2008). The phosphate regulon and bacterial virulence: A regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiology Reviews, 32*(3), 461–473.

Le Rudulier, D., Strom, A. R., Dandekar, A. M., Smith, L. T., & Valentine, R. C. (1984). Molecular biology of osmoregulation. *Science, 224*(4653), 1064–1068.

Measures, J. C. (1975). Role of amino acids in osmoregulation of non-halophilic bacteria. *Nature, 257,* 398–400.

Paiva, C. L., & Panek, A. D. (1996). Biotechnological applications of the disaccharide trehalose. *Biotechnology Annual Review, 2,* 293–314.

Ramani, N., Hedeshian, M., & Freundlich, M. (1994). micF antisense RNA has a major role in osmoregulation of OmpF in Escherichia coli. *Journal of Bacteriology, 176*(16), 5005–5010.

Roberts, M. F. (2005). Organic compatible solutes of halotolerant and halophilic microorganisms. *Saline Systems, 1*(1), 5.

Santos-Beneit, F. (2015). The Pho regulon: A huge regulatory network in bacteria. *Frontiers in Microbiology, 6,* 402.

Sauer, T., & Galinski, E. A. (1998). Bacterial milking: A novel bioprocess for production of compatible solutes. *Biotechnology and Bioengineering, 57*(3), 306–313.

Sleator, R. D., & Hill, C. (2002). Bacterial osmoadaptation: The role of osmolytes in bacterial stress and virulence. *FEMS Microbiology Reviews, 26*(1), 49–71.

Strøm, A. R. (1998). Osmoregulation in the model organism *Escherichia coli*: Genes governing the synthesis of glycine betaine and trehalose and their use in metabolic engineering of stress tolerance. *Journal of Biosciences, 23*(4), 437–445.

Suwanto, A. S., & Giri-Rachman, E. A. (2012). Molecular cloning of PhoR sensor domain from *Mycobacterium tuberculosis* for structure-based discovery of novel anti-tubercular. *Journal of Life Sciences, 6*(3), 268–275.

Vershinina, O. A., & Znamenskaya, L. V. (2002). The Pho regulons of bacteria. *Microbiology, 71*(5), 497–511.

Vuppada, R. K., Hansen, C. R., Strickland, K. A., Kelly, K. M., & McCleary, W. R. (2018). Phosphate signaling through alternate conformations of the PstSCAB phosphate transporter. *BMC Microbiology, 18*(1), 8.

Wanner, B. L. (1993). Gene regulation by phosphate in enteric bacteria. *Journal of Cellular Biochemistry, 51*(1), 47–54.

Wanner, B. L., & Wilmes-Riesenberg, M. R. (1992). Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in control of the phosphate regulon in Escherichia coli. *Journal of Bacteriology, 174*(7), 2124–2130.

Part X
Cell to Cell Signalling

Chapter 24
Quorum Sensing, Bioluminescence and Chemotaxis

Rani Gupta and Namita Gupta

1 What is Quorum Sensing and Why do Bacteria Talk?

The word 'quorum' means 'sufficient numbers', and with reference to microbial culture, it refers to cell density. Thus, quorum sensing is a cell density-dependent phenomenon that enables bacterial cell to cell communication allowing them to recognize and react to the density of their surrounding cell population. Quorum sensing involves a particular threshold concentration of extracellular chemical signal molecules (known as pheromones and autoinducers) which accumulate as cell density is built up. The molecules are constitutively produced by each cell in low concentration and are released into the surrounding environment, either actively or passively. As the population grows, their concentration automatically increases in the extracellular surrounding (Fig. 1).

These extracellular signaling molecules (known as pheromones or autoinducers) are identified by neighboring cells via specific receptors. When the inducer binds to the receptor, it activates transcription of certain genes, including those for its own synthesis, and hence, it is termed as autoinducer. As the population grows, the concentration of the inducer passes a threshold, causing more inducer to be synthesized. This forms a positive feedback loop, and the receptor becomes fully activated. Activation of the receptor induces the up-regulation of other specific genes, causing all of the cells to begin transcription at approximately the same time. This process enables populations of bacteria to synchronously regulate gene expression and therefore alter their behavior. The low basal expression of the inducer molecule is not enough by itself to activate gene expression, and the rapid diffusion of the molecules out of the cell ensures that it will not accumulate without input from other cells.

Why do bacteria talk to each other?

Quorum sensing enables bacteria to coordinate their behavior according to the density of their local population and with respect to availability of nutrients, defense against other microorganisms. It is used by pathogenic bacteria to coordinate their virulence

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_24

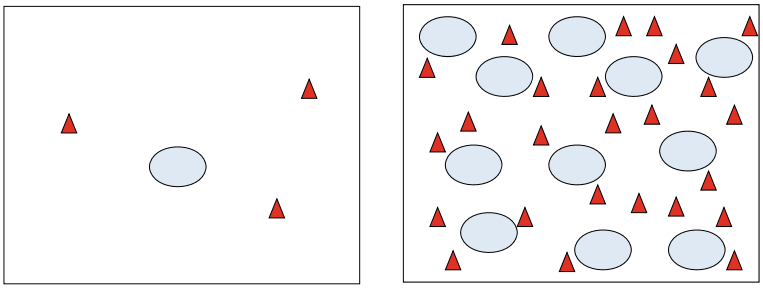


Fig. 1 Diagrammatic representation of quorum sensing phenomenon

in order to escape the immune response of the host and to establish a successful infection. Quorum sensing is found among both gram-negative and gram-positive bacteria and is used by bacteria to coordinate various biological activities including bioluminescence, sporulation, competence, antibiotic production, biofilm formation, virulence factor secretion, fruiting body formation in myxobacteria, etc.

2 Discovery of Quorum Sensing

The phenomenon of quorum sensing was discovered in the late 1960s by J. Woodland Hastings while studying bioluminescence in the marine bacteria *Vibrio fischeri* that lives as a mutualistic symbiont in the photophore of the Hawaiian bobtail squid. It was observed that the cultures produced light only when large numbers of bacteria were present. Luminescence was initiated by the accumulation of an activator molecule or an 'autoinducer'.

Initially, quorum sensing was thought to be a communication channel between bacteria of the same kind. However, the work of Bonnie Bassler, Professor of Microbiology from Princeton, has established that quorum sensing is a universal phenomenon used by bacteria to communicate with other bacterial species in its vicinity. Bassler's group reported that *Vibrio fischeri* could grow when in symbiotic association with fish and squids and not in free-living state. Further, cell extract of other bacteria could also induce bioluminescence in this species putting forth that bacteria could sense signals not only to their own kind, but to other bacteria as well. Thus, bacteria have a universal chemical language, a 'Bacterial Esperanto' that they use to talk between species. This bacterial cross talk has implications in mixed species populations such as biofilms.

3 Signaling Molecules: Autoinducers

Signaling molecules in quorum sensing are known as autoinducers as they up-regulate their own synthesis. Some of the properties of these molecules are as follows:

1. These are synthesized intracellularly and secreted outside.
2. These are generally low molecular weight molecules.
3. As the number of cells in a population increases, the extracellular concentration of autoinducer increases, and then, these binds to their respective cognate receptors on the cell membranes.
4. They trigger signal transduction cascades that result in population-wide changes in gene expression.

Chemical nature of autoinducers—There are two types of autoinducers:

- Acylated homoserine lactones (AHSLs)
- Oligopeptide autoinducers/autoinducing polypeptides (AIPs).

Some bacteria also use autoinducers other than these two, for example, autoinducer 2 (AI-2) or furanosyl borate diester

Acylated Homoserine Lactones (AHLs)

AHLs are produced by different species of gram-negative bacteria. They vary in the length and composition of the acyl side chain, which often varies from 4 to 18 carbons attached by an amide bond to a common homoserine lactone moiety. AHLs are synthesized by AHL synthase. Variety of AHLs and their microbial sources are presented in Fig. 2.

AHLs act by combining with and activating transcription factors that regulate the expression of specific genes. Receptors for AHLs include a number of transcriptional regulators called R proteins, which function as DNA-binding transcription factors or sensor kinases. Some of the well-known phenomena mediated by AHLs are listed below:

- Bioluminescence in *Vibrio fischeri*
- Production and release of virulence factors and biofilm formations in *Pseudomonas aeruginosa*
- Antibiotic production by *Erwinia carotovora* and *Pseudomonas aeruginosa.*

Oligopeptide Autoinducers/Autoinducing Polypeptides (AIPs)

Oligopeptides known as autoinducing polypeptides (AIPs) are produced by gram-positive bacteria (Fig. 3). They usually result from post-translational modification of a larger precursor molecule. They require specialized transport mechanisms (often ABC transporters). Unlike some AHLs, most oligopeptides do not act as transcription factors themselves. Instead, they work by stimulating two-component phosphorelay system that regulates gene transcription. When the peptide inducer molecules accumulate as a result of cell density and reach the threshold concentration, they

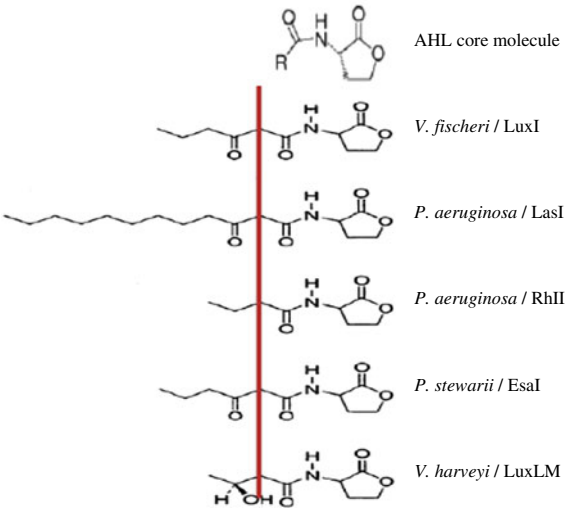


Fig. 2 Variety of AHLs and their microbial sources

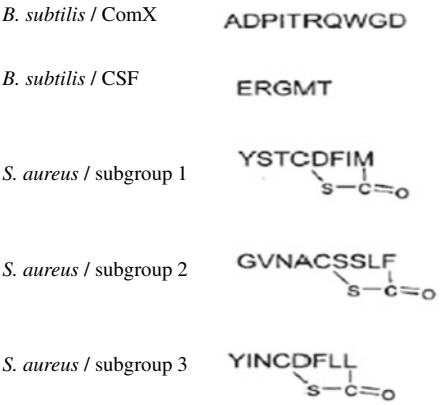


Fig. 3 Autoinducing polypeptides (AIPs) produced by gram-positive bacteria

interact with a sensor kinase at the cell membrane leading to its autophosphorylation and then phosphorylate regulator protein. The regulator protein functions as transcription factor and alters gene expression. For example, competence stimulating peptide (CSP) is a 17-amino acid peptide autoinducer required for competency and subsequent genetic transformation in *Staphylococcus.*

AIPs are involved in expression of different physiological processes in some bacteria such as:

1. Mating in *Enterococcus faecalis*

2. Stimulation of sporulation by *Bacillus subtilis*
3. Production of many toxins and other virulence factors by *Staphylococcus aureus.*

Autoinducer 2

Some bacteria use molecules other than AHLs and AIPs as the signaling molecules. For example, furanosyl borate diester (AI-2) is involved in bioluminescence system in *Vibrio harveyi* (Fig. 4). AI-2 is now known to be produced in non-luminescent bacteria as well (both gram negative and gram positive).

AI-2 is bound in periplasm to protein Lux P; its receptor is membrane-bound histidine kinase (Lux Q).

All the three types of autoinducers are observed in the best-studied examples of bioluminescence by *Vibrio* species, *V. fischeri* and *V. harveyi.*

α-hydroxyketones (AHKs)

A recently discovered class of AIs, namely α-hydroxyketones (AHKs), is predominantly produced by aquatic γ-proteobacteria, including *Legionella* and *Vibrio* spp, that cause Legionnaires' disease and cholera, respectively (Tiaden et al., 2010). 'Legionella quorum sensing' (*lqs*) or 'cholera quorum sensing' (*cqs*) genes encode enzymes that produce and sense the AHK molecules 'Legionella autoinducer-1' (LAI-1; 3-hydroxypentadecane-4-one) or cholera autoinducer-1 (CAI-1; 3-hydroxytridecane-4-one). AHK signaling regulates the virulence of *L. pneumophila* and *V. cholerae*, pathogen-host cell interactions, formation of biofilms or extracellular filaments, expression of a genomic 'fitness island' and competence.

4 Quorum Sensing in Gram-Negative Bacteria: Bioluminescence

Bioluminescence refers to the process of visible light emission in living organisms mediated by enzyme catalysis. Bioluminescence is well described for two species of *Vibrio*, *V. fischeri* and *V. harveyi*. It was known for long that certain luminescent bacteria grow only when their population exceeds the density of 10^7 cells/ml. This

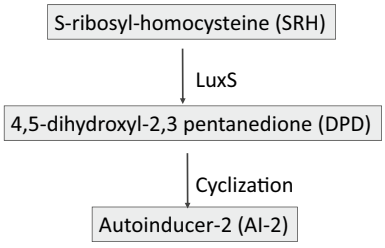


Fig. 4 AI-2 synthesis in *Vibrio harveyi*

is now attributed to the phenomenon of quorum sensing. Light emission from a single bacterium would be both biologically irrelevant and energetically wasteful. Overall emission from a bacterial population is however sufficient for eukaryotes like squids that have evolved organs dedicated to bioluminescence. Squid utilizes this symbiont derived bioluminescence in certain nocturnal behaviors including hunting and counter illumination and hence in communication, defense and/or attraction. In return, the bacteria obtain a well-established niche of stable source of nutrients in the light organ.

The phenomenon of bioluminescence has been observed in many different organisms including bacteria, fungi, fish, insects, algae and squid. However, the biochemical and genetic mechanisms underlying bioluminescence are particularly well understood in members of *Vibrionaceae.*

In all *Vibrio* species, the autoinducers are largely N-acetyl-L-homoserine lactones where the acyl chains may vary. *Vibrio harveyi* have additional signal molecules, unrelated to AHL family, known as the autoinducer-2 (AI-2) that are furanosyl borate diesters.

Biochemistry of bioluminescence

The enzyme that catalyzes the bioluminescent reaction is called luciferase, and the substrates are often referred to as luciferins. Luciferase is heterodimer of two subunits: α & β subunit. It catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde resulting in the emission of a blue-green light at 490 nm (Fig. 5).

Quorum sensing in *Vibrio fischeri*

V. fischeri utilizes two major kinds of systems for bioluminescence which operate through signal transduction. The first and important one is the LuxI/LuxR system which acts by direct activation of *lux* operon at high cell densities. The other systems viz. Ain system (AinS-AinR) and LuxS-LuxPQ system are two de-repressing systems that have less impact on bioluminescence and utilize histidine kinases for signal transduction (Miyashiro & Ruby, 2012).

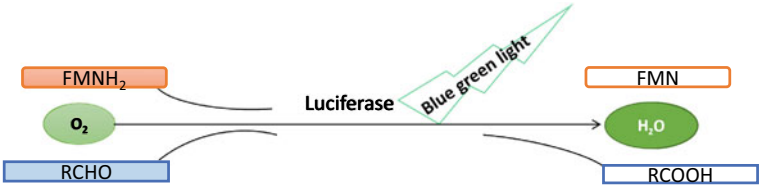


Fig. 5 Biochemical reaction catalyzed by enzyme luciferase utilizes flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde to generate bioluminescence

Table 1 Genes involved in *lux* operon viz *luxCDABE* genes and their respective functions

Gene	Function
IuxAB	Encodes α and β subunit of luciferase
luxR	Positive transcriptional regulator in presence of AI
luxI	LuxI synthase that catalyzes the synthesis of autoinducer N-3-oxohexanoyl-homoserine lactone (HSL) using S-adenosylmethionine (SAM) and acyl carrier protein (ACP)
luxCDE	Fatty acid reductase genes The IuxCDE genes code for polypeptides that are required for the conversion of fatty acids into the long-chain aldehyde required for the luminescent reaction LuxD (transferase) diverts fatty acyl groups from the fatty acid biosynthesis pathway LuxE (synthetase) activates the acyl groups with AMP; these are then reduced to long-chain aldehyde by LuxC (reductase)

Lux system: Lux I/Lux R system

V. fischeri has two chromosomes, and *luxCDABEG* genes are located on the second chromosome that encode for all the structural components necessary for light production (Table 1). The quorum sensing phenomenon of bioluminescence in this species is more or less similar to that in many non-luminescent bacteria.

Lux I synthase, encoded by *lux I*, synthesizes the autoinducer AI-1 or HSL (beta-Ketocaproyl-homoserine lactone). The autoinducer HSL being nonpolar in nature moves freely back and forth the cell membrane and accumulates in extracellular medium. At high cell density, the autoinducer reaches a threshold and activates the transcription factor LuxR which has two distinct domains: N-terminal autoinducer-binding domain and a separate C-terminus DNA-binding domain. In absence of HSL, the regulator LuxR exists in a confirmation which is unable to bind the DNA. When HSL reaches a critical concentration (~10 nM), it binds to the N-terminus of LuxR and allows conformation change exposing the C-terminal DNA-binding domain. This activates LuxR which then binds at the promoter site, and transcription of lux operon (luciferase) occurs.

At high cell density, the autoinducer reaches a threshold and activates the transcription factor LuxR coded by *lux R* gene, which is present to the left of lux operon. Binding of autoinducer to Lux R facilitates its binding to lux promoter and recruits sigma 70 RNA polymerase for transcription of lux operon leading to the synthesis of luciferase enzyme. The autoinducer also up-regulates its own synthesis by a positive feedback loop (Fig. 6).

Ain system (AinS-AinR) and LuxS-LuxPQ system

V. fischeri regulates luminescence with two other quorum sensing systems based on autoinducer molecules: N-octanoyl-L-homoserine (C8-HSL) synthesized by AinS and furanosyl borate diester (AI-2) synthesized by LuxS. C8-HSL interacts with its cognate receptor kinase AinR, while AI-2 binds to the periplasmic protein LuxP forming LuxP-AI-2 complex sensed by the hybrid sensor kinase LuxQ. These sensor kinases interact with the AIs via their periplasmic domain, while its cytoplasmic

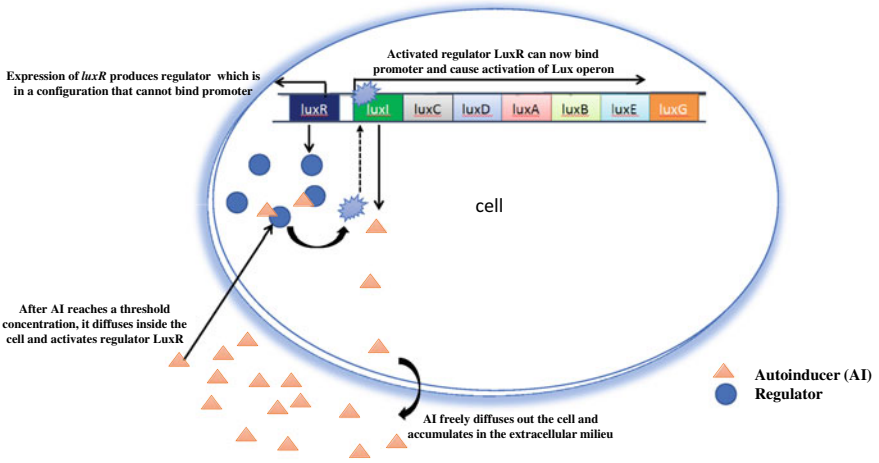


Fig. 6 LuxI/LuxR complex in *V. fischeri*: The nonpolar autoinducer (triangles) moves freely back and forth the cell membrane and accumulates in extracellular medium, and at a critical concentration, it binds and activates the positive regulator LuxR (circles) which in turn binds the promoter site activating transcription of lux operon

domain is the regulator domain with histidine kinase site. Even though Lux I/Lux R system functions at post-translational level to regulate luminescence, these systems impact at the transcriptional level conferring additional checkpoint in regulation of bioluminescence. Both these systems share phosphotransferase protein LuxU which relays the phosphate group from the sensor proteins to LuxO. At high cell densities, these systems operate to cause stable expression of LitR, a transcriptional factor that causes expression of positive regulator LuxR. LitR is a regulator that binds the DNA upstream of the *luxR* gene independent of the *lux* operon and enhances expression of *luxR* without affecting other *lux* genes. In *V. fischeri*, AinS-AinR exerts a greater impact on bioluminescence in comparison with the parallel LuxS-LuxPQ system. The functioning of these systems at low and high cell densities is explained below:

Low cell density

At low cell densities, the corresponding AI is not present at concentrations required to activate cognate receptor kinase. AinR and LuxQ, the sensory proteins, function as kinases and autophosphorylate themselves. This phosphoryl group ultimately transfers in sequence to the shared histidine phophotransferase protein LuxU and then to LuxO. The phosphorylated LuxO cooperates with sigma 54 to activate transcription of the small regulatory RNA, Qrr1. Qrr-1 subsequently silences LitR expression in the presence of the chaperonic protein Hfq.

High cell density

As growth occurs and the cell density increases, the AIs accumulate in the cell culture and bind to their cognate sensor proteins. This converts the sensor proteins into a

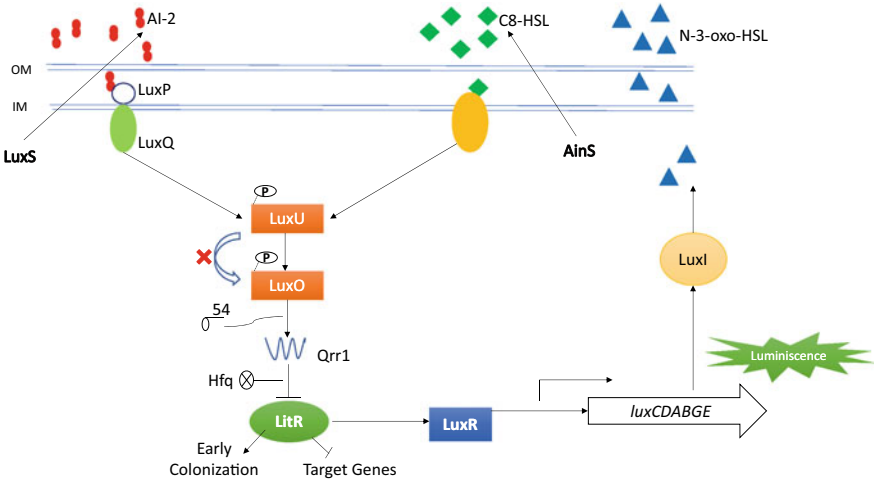


Fig. 7 Quorum sensing in *Vibrio fischeri*

phosphatase which drains the phosphate group in the reverse direction leaving LuxO in the dephosphorylated state. This renders LuxO inactive, and stable expression of LitR occurs. LitR in turn positively regulates the transcription of LuxR, causing a modulation in the expression of downstream luminescence genes. Therefore, QS facilitates deactivation of active kinases at high cell densities to inhibit silencing of LitR mRNA to and in turn allowing stable expression of *lux* operon's positive regulator LuxR (Fig. 7).

Quorum sensing in *Vibrio harveyi*

In *V. harveyi,* three parallel systems function in response to three different autoinducers. These systems operate in same way as the Ain system (AinS-AinR) and LuxS-LuxPQ system described earlier in *V. fischeri* (Liu et al., 2013). The mechanism by which the systems operate is the same as the *lux* operon is switched on by de-repression; however the AIs and sensor proteins are different. Three different AIs and cognate sensor receptors are identified in *V.harveyi* (Table 2).

Autoinducers of all the three systems are sensed by two-component sensor kinases. In these systems as well, LuxU is the shared phosphotransferse protein which relays the phosphate group to LuxO at low cell densities. In turn, LuxO together with sigma54 activates the transcription of small regulatory RNAs that combine with

Table 2 Three different AI's and cognate sensor receptors in *V. harveyi*

Autoinducer	Synthesised by sensor
N-3-hydroxybutyryl-L-homoserine lactone, AI-1	LuxM: LuxN
AI-2	LuxS: LuxQ
3-hydroxytridecan-4-one, CAI-1	CqsA: CqsS

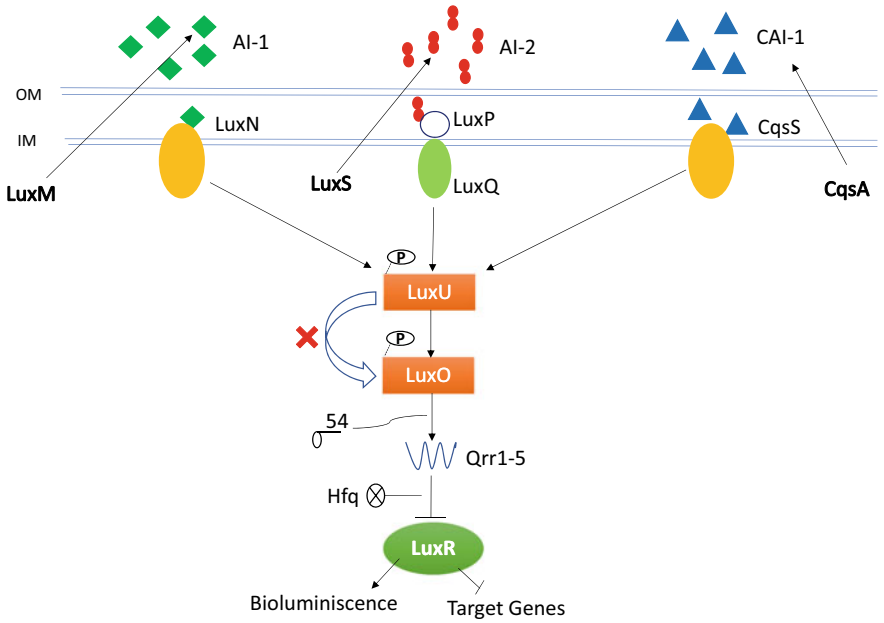


Fig. 8 Quorum sensing in *Vibrio harveyi* by LuxM, LuxS and CqsA

another chaperone protein Hfq to form the repressor complex. This complex binds to $LuxR_{vh}$ (LuxR *V. harveyi*) mRNA, destabilizing it and ultimately leading to its degradation. At high cell densities, the autoinducer accumulates and activates the phosphatase activity of sensory receptors leading to dephosphorylation and thus inactivation of LuxO. This causes stable expression $LuxR_{vh}$ mRNA and therefore switching on *lux* operon (Fig. 8).

ArcB-ArcA system in regulation of bioluminescence

The ArcB-ArcA system described earlier in the chapter directly regulates the expression of *lux* operon in *V. fischeri.* ArcB is the histidine kinase that senses the redox state of the cell's quinone pool. Under poor environment conditions such as low availability of oxygen, it makes much sense for the cells to survive rather than utilizing the available oxygen for bioluminescence. This is where the ArcB-ArcA comes into picture. Upon sensing reduced quinones, ArcB phosphorylates the response regulator ArcA which in turn pleiotropically regulates number of genes. One of these genes is also the *lux* operon. Phosphorylated ArcA binds at the *luxR-luxI* intergenic region inhibiting expression of *lux* operon. ArcB-ArcA system therefore independently regulates bioluminescence depending on the availability of oxygen. This implies that even at high cell densities, when the concentration of AI is high, bioluminescence will not occur if the oxygen concentration is low.

Non-luminescent bacteria also produce an autoinducer similar to AI-2 and not AI-I. Further, the extract of some bacteria such as *Vibrio cholerae* also results in

luminescence in *V. harveyi*. This suggests that the bacterium uses system I for its own type and system-2 to detect presence of other bacteria in its neighborhood.

5 Quorum Quenching

The phenomenon of quorum sensing and its importance in various bacterial processes including generation of virulence factors and pathogenicity is widely known. It directly follows that if quorum sensing can be inhibited it will interfere with pathogenesis and can be a tool for combating diseases. The technique of disrupting quorum sensing is known as quorum quenching. It interferes with the signaling mechanisms and shuts down expression of genes regulated by quorum sensing such as virulence genes in pathogenic bacteria. This technique has a potential to overcome drug toxicities, complicated superinfection and antibiotic resistance.

Quorum quenching was first discovered with the identification of the enzyme AHL-lactonase from *Bacillus* species which has the ability to attenuate virulence in *Erwinia carotovora*. This enzyme cleaves the lactone ring from the acyl moiety of AHLs and renders the AHLs inactive in signal transduction, thereby disrupting the ability of the pathogen to sense its cell density and triggering on the virulent gene expression. Quorum quenching mechanism is now identified in many prokaryotic and eukaryotic organisms. Various enzymes and chemicals can be used for disrupting the quorum signal.

Strategies for quorum quenching

Quorum sensing can be inhibited by the following three strategies:

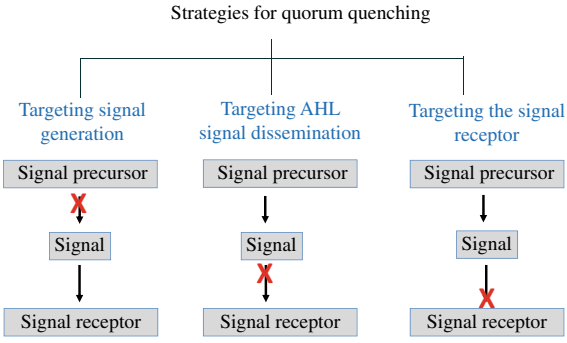


Fig. 9 Three different strategies of quorum quenching

Targeting signal generation—Signal generation can be inhibited by using analogues of precursor of signal molecule. The precursors for AHL signal molecules are acyl—ACP and SAM. Thus, analogues of acyl-ACP and SAM (such as S- adenosylhomocysteine, S-adenosylcysteine, sinefungin and butyryl-SAM) can be used to reduce synthesis of quorum sensing signals.

Targeting AHL signal dissemination—Quorum sensing molecules can be degraded to inhibit signal dissemination. The molecules can be degraded by various means such as:

- Increasing pH (>7) as at higher pH, AHL molecules undergo lactonolysis, and their biological activity is lost
- At higher temperature, AHL undergoes lactonolysis.
- Some plants infected by pathogenic bacteria *E. carotovora* increase the pH at the site of infection, resulting in lactonolysis of AHL molecules.
- Some bacteria produce lactonolysing enzymes (lactonases) such as AiiA produced by *Bacillus* species, AttM and AiiB produced by *Agrobacterium tumefaciens* and QIcA produced by *Rhizobiales* species.

Targeting the signal receptor—Quorum sensing antagonists are analogues of inducer molecules and thus directly bind the signal receptor. One example of such antagonist molecule is 4-nitro-pyridine-N-oxide (4-NPO) from garlic extract (Fig. 9).

6 Chemotaxis

Chemotaxis (*chemo* + *taxis*) is the ability of bacteria to direct their movement toward or away from certain chemicals in their surroundings. Positive chemotaxis occurs if the movement is toward a chemical (chemical attractant) such as a nutrient. However, negative chemotaxis occurs if the movement is in the opposite direction away from a chemical (chemical repellant) such as antibiotics or noxious chemicals. The chemical attractants and repellants are known as chemo-effectors.

The phenomenon of chemotaxis was discovered by Pfeffer and Englemann in 1880, and Julius Adler in the 1960s reported that bacteria use specific receptors to recognize the chemicals. Since then, many studies have been undertaken to understand correlation between flagellar rotation and gene products involved in chemotaxis as well as the mode of signal transduction. Later in 1990s, it was shown that in bacteria such as *Escherichia coli* and *Salmonella enterica* serovar *typhimurium,* chemotaxis-signaling cascade is transduced via protein-protein interactions (Parkinson, 2003).

7 Bacterial Movement

E. coli normally shows random walk composed of runs and tumbles (Fig. 10). Run is represented by smooth swimming in a straight line, while an abrupt turning motion is called a tumble. Bacteria such as *E. coli* typically have 4–10 flagella per cell which move by a rotary motor embedded in the cell envelope. The energy for the motor comes from proton motive force. The rotation of the flagellar motor is reversible and is capable of rotating in two ways:

- Counter-clockwise rotation—When all the flagellar motors in a single cell rotate in counter-clockwise direction, the flagella sweep around the cell in a common axis, forming a single concerted rotating bundle that pushes the cell forward and results in a smooth swimming motion in a straight line.
- Clockwise rotation—When the flagellar motors reverse and rotate in clockwise direction, the flagellar bundle gets dispersed apart and transforms from normal to semi-coiled and then to curly. Each flagellum points in a different direction and acts independently to push and pull the cell in a chaotic tumbling motion. Tumbling episodes enable the cell to try new, randomly determined swimming directions.

Thus, the overall movement of a bacterium is the result of alternating tumble and run phases. They swim straight along a path for a few seconds or less and

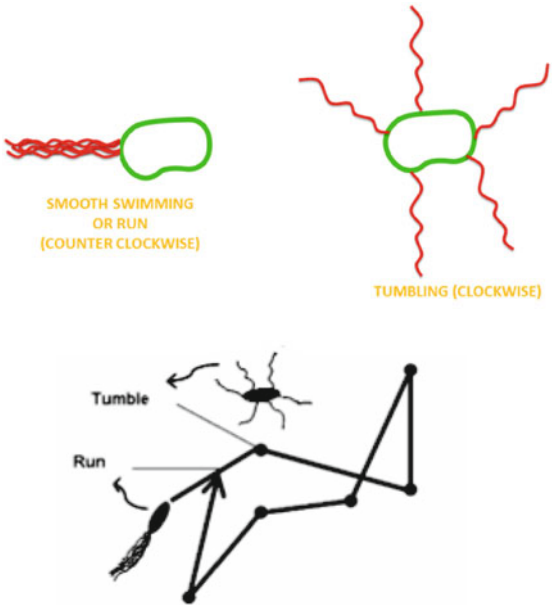


Fig. 10 Swimming behavior of E. coli in the absence of chemo-effectors

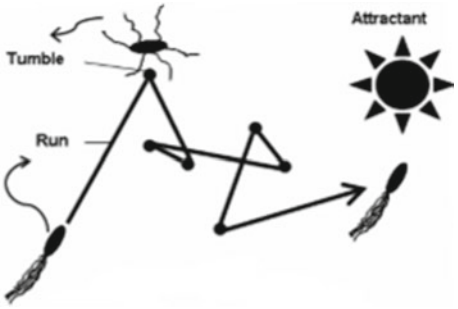


Fig. 11 Swimming behavior of E. coli in the presence of chemoattractant

then tumble. The tumbling movement reorients the bacterium, and they swim in a randomly different direction.

In an attractant or repellent gradient (stimulated conditions), movement in a favorable direction is caused by a bias in the random walk. An increasing chemoattractant concentration or a decreasing chemorepellent concentration decreases the probability of clockwise rotation and thus the probability of tumbles. It directly follows that these responses extend runs that take the cells in favorable directions resulting in net movement toward preferred environments (Fig. 11).

How bacteria senses various attractants and repellants?

E. coli senses chemo-effector gradients in temporal fashion by comparing current concentrations to those encountered over the past few seconds of travel called short-term memory. Typically, the cells compare the concentration over the past 1 s with that observed over the previous 3 s.

Adaptation, i.e., restoration of the pre-stimulus represents the cells' memory of previous environmental conditions and directly affects cellular behavior. After a short period (seconds to minutes) of exposure to a particular concentration of a chemo-effector, bacteria adapt (become desensitized) to it. They do not respond to that concentration of the chemo-effector anymore but may respond to a higher concentration. Thus, adaptation is the means by which bacteria remember the previous concentration of a chemo-effector.

On adapting to a chemo-effector, the bacterium resumes its normal motion (under non-stimulated conditions) consisting of frequent tumbling and runs. This enables the bacteria to stay in that area. On sensing an increased concentration of the chemo-effector, the bacterium changes its motion by suppressing tumbling (in case of an attractant) and increasing tumbling (in case of a repellant). Consequently, the bacterium will swim toward and remain in the area of the higher concentration of chemoattractant or lower concentration of chemo-repellant.

8 Proteins Required for Chemotaxis

Chemotaxis in *E. coli* is a result of signal transmission between the chemoreceptors, located mainly at the poles of the cell, and the flagellar-motor complexes which are randomly distributed around the cell (Bren & Eisenbach, 2000)

Chemoreceptors: The chemoreceptor proteins are called methyl receptor-transducer proteins (MCPs). These are homodimers that span inside to outside surface of membrane with N-terminal sensory domain toward periplasm which receives signals transmit to the C-terminal domain in the cytoplasm. The cytoplasmic domain contains four to six glutamate residues that can methylate and demethylate as a response to availability of ligands. The reversible methylation of glutamates is unique to bacterial chemoreceptors; this family of receptors has therefore been termed methyl-accepting chemotaxis proteins (MCPs). *E. coli* has five such chemoreceptors: Tar (aspartate and maltose) and Tsr (serine) are major receptors, while Trg (ribose, glucose and galactose), Tap (dipeptides) and Aer (aerotactic responses by monitoring redox changes) are minor receptors (Fig. 12). Some ligands generally do not directly bind to the MCPs. Instead, they first bind to periplasmic-binding proteins (known as primary chemotaxis receptors) and then ligand-bound periplasmic-binding proteins interact with the MCPs, which are called secondary chemoreceptors in such cases (Grebe & Stock, 1998). The information from chemoreceptors is transmitted to flagellar motor through several cytoplasmic chemotaxis proteins called Che proteins.

Che proteins: There are essentially six cytoplasmic proteins viz., CheA, CheB, CheR, CheW, CheY and CheZ that process the sensory signals to the flagellar motor (Table 3). Chemotaxis is a two-component signal transduction system with CheA as the histidine kinase and CheY and CheB as the response regulators. CheW is a linker protein that physically bridges CheA to the MCPs. CheY interacts with the flagellar

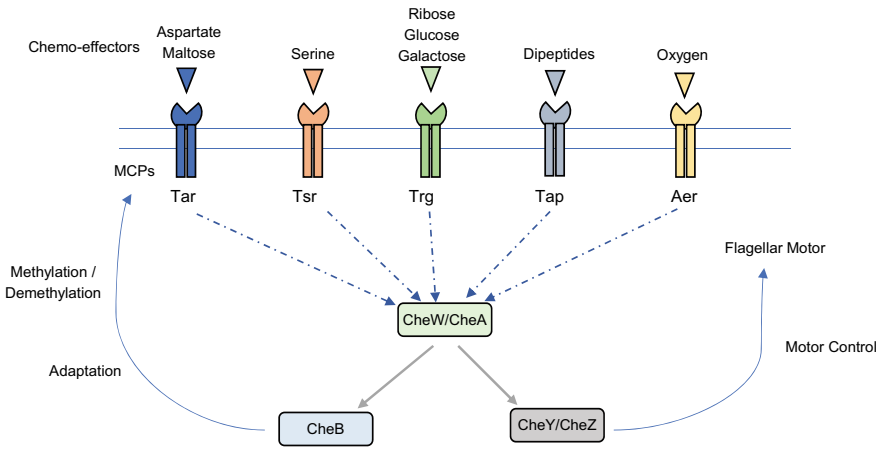


Fig. 12 Chemotaxis signaling components in *E. coli*

Table 3 Signal transduction components and their functions

Signal transduction component	Functions
CheA	CheY and CheB kinase
CheZ	CheY phosphatase acts as switch regulator of CheY
CheW	Linker protein that physically bridges CheA to the MCPs
CheY	Switch regulator of FliM places cell flagellar motor in CW state
CheR	Methylation of MCP receptors
CheB	Demethylation of MCP receptors

motor switching apparatus, and CheZ facilitates the activity of CheY. The remaining two proteins CheR and CheB regulate MCP methylation state.

9 Chemotactic Response

The chemotaxis-specific receptors (MCPs) are stable homodimers which form a ternary structure with a linker protein (CheW) and histidine kinase, CheA. This complex, composed of three MCP dimers: three molecules of CheW and three monomers of CheA, is stabilized by linking CheA monomers at their dimerization site.

It is generally assumed that receptor-bound attractants and repellents inhibit and activate the kinase, respectively. Thus, when an attractant is bound to its respective chemoreceptor, the kinase activity of CheA is inhibited while of arrival of repellent autokinase activity of CheA results in phosphorylation of CheA. Phospho-CheA (CheA-P) transfers phosphoryl groups to specific aspartate residues in the response regulators CheY and CheB. The active forms of these response regulators are short-lived because they quickly lose their phosphoryl group through spontaneous self-hydrolysis.

CheY is a small 14-kDa single-domain molecule which is multifunctional. It acts as a messenger for transmitting the signal to the flagellar-motor supramolecular complex. The phosphorylated CheY has reduced affinity to CheA and increased affinity toward FliM. Hence, as soon as CheY is phosphorylated, it gets released in the cytoplasm and interacts via FliM with the flagellar-motor complex. This effects a change in the direction of rotation by placing cell flagellar motor in clockwise rotation state resulting in increased tumbling frequency. If CheY-P concentration is very low, there is negligible clockwise motion, and the cell will run and vice versa. This signal is terminated upon dephosphorylation of CheY by CheZ, a phosphatase leading to counter-clockwise movement. The activation of the phosphatase depends on unbound CheY-P concentration. Thus, CheZ dephosphorylates free CheY-P which causes dissociation of CheY-P from the flagellar-motor complex by shifting the equilibrium between bound and free CheY-P. Thus, CheY can interact at its C-terminal with three proteins, viz. CheA, CheZ and FliM, however, only one protein at a

time due to an overlapping-binding surface. CheA prefers non-phosphorylated CheY, while FliM and CheZ have higher affinity toward CheY-P.

Adaptation in bacterial chemotaxis, i.e., the temporal comparison of attractant or repellent concentrations is controlled by a feedback mechanism that modulates the methylation level of the MCP receptors by two enzymes, CheB and CheR having methylesterase and methyltransferase activities, respectively.

CheR, a methyltransferase, is constitutively produced and catalyzes S-adenosylmethionine-dependent methylation of specific glutamate residues (four to six residues for each MCP) on the cytoplasmic portion of the receptors during adaptation to positive stimuli. This progressively reduces the attractant-binding affinity to the MCPs resulting in phosphorylation of CheA and setting on the CheY phosphorylation and thus tumbling frequency.

CheB has methylesterase activity which demethylates the receptors during adaptation to negative stimuli. CheB is a response regulator protein which has phosphorylating aspartate residue at its N-terminal which gets phosphorylated by CheA-P. Its effector domain at the C-terminus possesses amidase and esterase activities which are stimulated upon phosphorylation at N-terminal. The interaction of CheB with the receptor demethylates glutamate residue. This in turn results in inhibition of CheA autophosphorylation, thereby leading to transmission of a counter-clockwise signal.

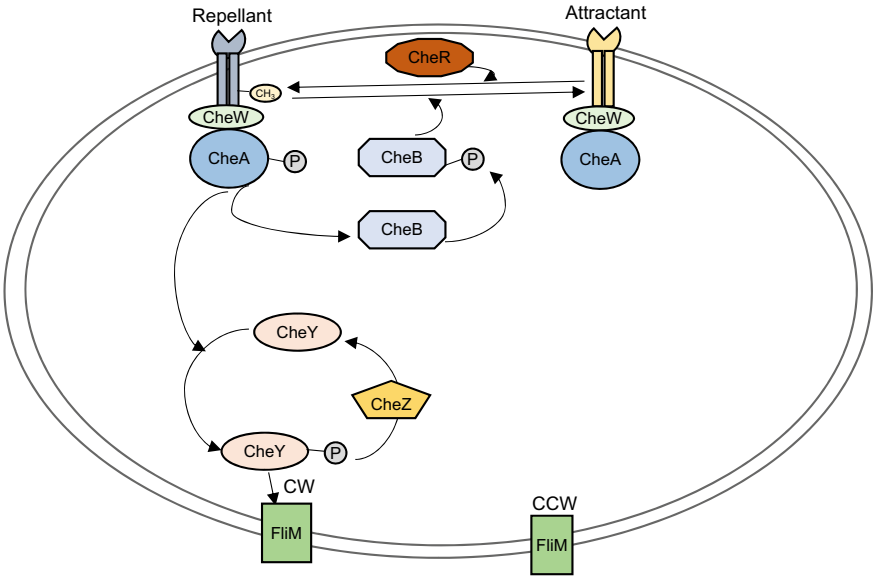


Fig. 13 Chemotaxis switch in *E. coli*

Interestingly, CheB and CheY both are phosphorylated by CheA and thus compete with each other for binding to CheA (Fig. 13).

Thus, the relative rates of methylation and demethylation reactions determine the steady-state level of receptor methylation, and this level regulates the kinase activity of CheA. This regulation occurs only after the initial chemotactic response (Lukat & Stock, 1993; Krembel et al., 2015).

Summary

- Quorum sensing is a cell density-dependent phenomenon that enables bacterial cell to cell communication allowing them to recognize and react to the density of their surrounding cell population.
- Quorum sensing involves a particular threshold concentration of extracellular chemical signal molecules which are constitutively produced by each cell, and their concentration automatically increases in the extracellular surrounding.
- Quorum sensing is found among both gram-negative and gram-positive bacteria and is used by bacteria to coordinate various biological activities including bioluminescence, sporulation, competence, antibiotic production, biofilm formation, virulence factor secretion, fruiting body formation in myxobacteria, etc.
- The phenomenon of quorum sensing was discovered in the late 1960s by J. Woodland Hastings in the marine bacteria *Vibrio fischeri.*
- There are two types of autoinducers: acylated homoserine lactones (AHSLs) and oligopeptide autoinducers/autoinducing polypeptides (AIPs).
- AHLs are produced by different species of gram-negative bacteria.
- AIPs are produced by gram-positive bacteria.
- Furanosyl borate diester (AI-2) is involved in bioluminescence system in *Vibrio harveyi.*
- AI-2 is now known to be produced in non-luminescent bacteria as well (both gram negative and gram positive).
- AIs, namely α-hydroxyketones (AHKs), are predominantly produced by aquatic γ-proteobacteria, including *Legionella* and *Vibrio* spp causing Legionnaires' disease and cholera, respectively.
- *V. fischeri* utilizes two major kinds of systems for bioluminescence via quorum sensing, i.e., LuxI/LuxR system which directly activates *lux* operon and Ain (AinS-AinR) and LuxS-LuxPQ de-repressing systems which utilize histidine kinases for signal transduction.
- In *V. harveyi,* three parallel systems function that correlate separately to three different autoinducers.
- The mechanism by which the systems operate is the same in sense that *lux* operon is switched on by de-repression except that the AIs and sensor proteins are different.
- Quorum quenching was first discovered with the identification of the enzyme AHL-lactonase from *Bacillus* species which has the ability to attenuate virulence in *Erwinia carotovora.*
- Strategies for quorum quenching include targeting signal generation; targeting AHL signal dissemination or targeting the signal receptor.

- Chemotaxis (*chemo* + *taxis*) allows bacteria to move toward or against the presence of certain chemicals in their surroundings and was discovered by Pfeffer and Englemann in 1880.
- The chemical attractants and repellants are known as chemo-effectors.
- Bacteria utilize chemotaxis in locating food and in formation of surface biofilms.
- In the absence of a chemo-effector, *E. coli* executes a random walk composed of runs and tumbles. Smooth swimming in a straight line is called a run, and an abrupt turning motion is called a tumble.
- The flagellar motor rotates in two ways: counter-clockwise and clockwise.
- The chemotactic response in *E. coli* is accomplished by signal transduction between the receptor complex, located mainly at the poles of the cell, and the flagellar-motor complex.
- Chemotaxis is a result of close interaction between methyl-accepting chemotaxis proteins (MCPs) and Che proteins.
- Chemoreceptors bound to ligands in the environment transmit signal to a cytoplasmic 'two-component' signal transduction system, CheYA consisting of a kinase (CheA) and a response regulator (CheY).
- CheW is a linker protein that physically bridges CheA to the MCPs.
- CheY interacts with the flagellar motor FliM and CheZ, a phosphatase. CheY-P has higher affinity with FliM, so most of the phosphorylated form binds; however, free form gets dephosphorylated by CheZ.
- CheR and CheB regulate MCP methylation state.
- Phospho-CheA transfers phosphoryl groups to specific aspartate residues in the response regulators CheY and CheB. The active forms of these response regulators are short-lived because they quickly lose their phosphoryl group through spontaneous self-hydrolysis. CheY-P interacts with FliM and sets flagella move in clockwise direction and thus tumble motion.
- Free CheY-P gets degraded by CheZ, very soon equilibrium of bound CheY-P shifts, and the signal gets destroyed.

Questions

1. What is quorum sensing in bacteria?
2. What are the quorum sensing molecules in gram-negative and gram-positive bacteria?
3. Bioluminescence in *V. harveyi* is a cell density-based de-repression phenomenon. Explain.
4. Bioluminescence in *V. fischeri* is regulated at post-translational level by quorum sensing. Explain.
5. What is quorum quenching?
6. Who discovered chemotaxis and when?
7. How runs and tumbles can be distinguished with respect to flagellar motor rotation and chemo-effector?
8. Name three major components of chemotaxis response in *E. coli.*

9. Name different component proteins involved in signal transduction during chemotaxis in *E. coli*.
10. Explain how bacterium senses chemo-effector gradient by methylation and demethylation?

References

Bren, A., & Eisenbach, M. (2000). How signals are heard during bacterial chemotaxis: Protein-protein interactions in sensory signal propagation. *Journal of Bacteriology, 182*(24), 6865–6873.

Grebe, T. W., & Stock, J. (1998). Bacterial chemotaxis: the five sensors of a bacterium. *Current Biology, 8*(5), R154–R157.

Krembel, A. K., Neumann, S., & Sourjik, V. (2015). Universal response-adaptation relation in bacterial chemotaxis. *Journal of Bacteriology, 197*(2), 307–313.

Liu, H., Srinivas, S., He, H., Gong, G., Dai, C., Feng, Y., Chen, X., & Wang, S. (2013). Quorum sensing in *Vibrio* and its relevance to bacterial virulence. *Journal of Bacteriology and Parasitology*, *4*(172), 3.

Lukat, G. S., & Stock, J. B. (1993). Response regulation in bacterial chemotaxis. *Journal of Cellular Biochemistry, 51*(1), 41–46.

Miyashiro, T., & Ruby, E. G. (2012). Shedding light on bioluminescence regulation in *Vibrio fischeri*. *Molecular Microbiology, 84*(5), 795–806.

Parkinson, J. S. (2003). Bacterial chemotaxis: A new player in response regulator dephosphorylation. *Journal of Bacteriology, 185*(5), 1492–1494.

Tiaden, A., Spirig, T., & Hilbi, H. (2010). Bacterial gene regulation by α-hydroxyketone signaling. *Trends in Microbiology, 18*(7), 288–297.

Chapter 25
Competence and Sporulation in *Bacillus subtilis*

Rani Gupta and Namita Gupta

1 Competence: An Introduction

The ability of bacterial cells to take exogenous DNA fragments from environment is known as *competence*. There are two types of competence—natural competence and nutritional competence. Natural competence is genetically regulated ability of bacteria to uptake DNA (transformation). In nutritional competence, bacteria take up exogenous DNA and utilize it as a source of nutrients. *E. coli* naturally shows nutritional competence but not genetic competence. However, genetic competence can be artificially induced in *E. coli* by Ca^{2+} treatment and is a very important tool in molecular biology.

Uptake of DNA by the cell and its integration into the genome helps bacteria to adapt to the changing environmental conditions such as resistance to antibiotics and evasion of vaccines. Besides transformation, there are other mechanisms of DNA transfer like transduction and conjugation, but only transformation requires proteins encoded by the core genome. The evolutionary significance and consequences of competence as a part of natural bacterial transformation are categorized as an advantage of genetic diversity, source of food and homologous recombination for lateral gene transfer and repair of damaged DNA. Most of the proteins involved in transformation are expressed at specific points during growth of bacterium. Therefore, competence is a short-lived phenomenon to internalize DNA and subsequently transform it.

2 Competence Development in Different Bacteria

Natural genetic competence is present in bacteria such as *Actinobacter*, *Azotobacter*, *Bacillus*, *Streptococcus*, *Haemophilus*, *Neisseria* and *Thermus*. Among all bacteria that show competence, *Bacillus subtilis* is the most studied and hence is considered as a model organism for understanding molecular mechanism of competence and

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_25

DNA integration into its genome. Genetic competence is also known in other species of *Bacillus* such as *B. licheniformis* and *B. cereus,* but it has been studied most extensively in *B. subtilis*, where it is highly regulated via quorum sensing in a density-dependent manner employing a network of complex sensing and regulation cascade. Competence is intimately associated with sporulation pathways (Schultz et al., 2009).

3 Genetic Basis of Competence in *B. Subtilis*

B. subtilis cells synthesize specific DNA uptake machinery for incorporation of DNA present in extracellular medium. The competent cells can uptake any foreign DNA without any sequence specificity. Several proteins required for foreign DNA uptake are expressed by different competence operons. ComG operon encodes seven membrane associated proteins to form a pilin-like structure for binding of DNA to competent cells. The structure is assembled correctly with the help of another protein ComC. Then, a transmembrane channel is formed by a protein comEC encoded by another operon ComE, thus enabling entry of DNA inside the cell. Later, the internalized DNA is stabilized by the action of a DNA translocase protein encoded by ComF operon, DNA-recombination protein RecA and other recombination proteins (Hamoen et al., 2003). The expression of genes encoded by late competence operons for DNA-binding, uptake and recombination is under stringent control which is governed by the competence transcription factor ComK (Fig. 1).

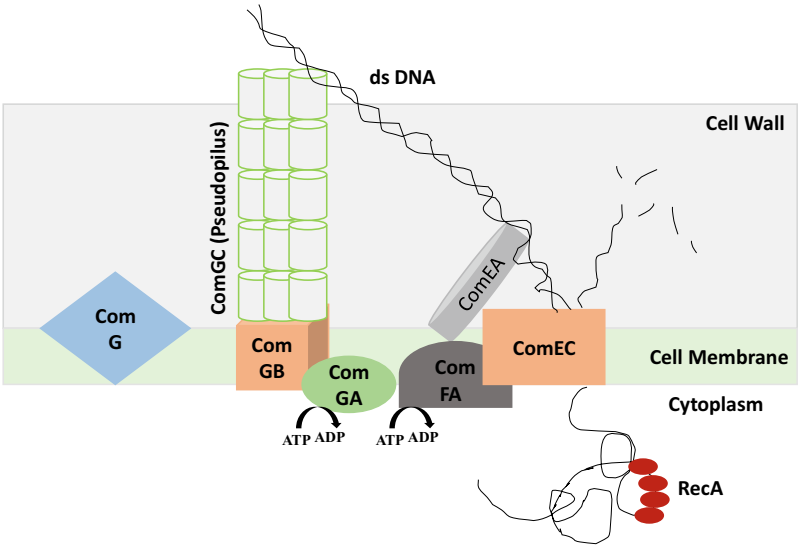


Fig. 1 DNA uptake mechanism of *B. subtilis*

4 ComK: The Master Competence Regulator

Several transcriptional factors work at both transcriptional and translational level for precise competence on-off switching. Of all, the transcription factor ComK is vital for the expression of DNA uptake machinery and recombination genes. ComK undergoes autoinduction by binding to its own promoter ComK, thus initiating self-transcription. The transcription of ComK activated genes starts by binding of ComK to specific sequences known as K-boxes located upstream of ComK-dependent genes. Each K-box is demarcated by presence of two AT-boxes with the consensus sequence AAAA-$(N)_5$-TTTT, separated by a spacer of two, three or four helical turns between the start positions of the repeating AT-box. *Bacillus subtilis* contains 1062 putative ComK-binding sites (K-boxes) in its genome. ComK is functional as a tetramer consisting of two dimers each binding to an AT-box which is accompanied by DNA bending. ComK stabilizes the binding of RNA polymerase, probably by facilitating interactions with upstream DNA, through bending of the promoter area (Hamoen et al., 2002; Susanna et al., 2007).

Repressors of ComK during exponential phase and in complex medium

The positively autoregulated ComK can result in its premature expression during exponential phase. However, in spite of constitutively expressed, the cells enter into competent state only during the stationary phase. This is because the early expression of ComK is prevented both at transcriptional and translational levels simultaneously. The transcription factors mainly involved in negative control of ComK expression during exponential growth are AbrB, CodY and Rok (Hamoen et al., 2003).

During log phase, AbrB is constitutively expressed. It is a transcriptional repressor which acts as transition state regulator resulting in repression of several stationary phase genes involved in various processes such as sporulation, degradative enzyme and antibiotic production and amino acid utilization. It also inhibits the premature expression of ComK during log phase by occupying the transcription initiation site at ComK promoter. Later, during the transition from exponential to stationary phase, the concentration of AbrB protein declines (Fig. 2). During stationary phase, phosphorylated Spo0A and expression of stationary phase genes is up-regulated including ComK. Spo0A down-regulates ABr gene expression.

Expression of ComK is also dependent on the medium composition. This regulation is mediated by another transcription factor CodY which is responsible for repression of several *B. subtilis* early stationary phase genes in a nitrogen-rich medium. CodY is a GTP-sensing transcriptional repressor which binds to intracellular GTP and senses the nutritional status of the medium. When the cells are not under nutritional stress, the availability of amino acids leads to high intracellular levels of GTP which are sensed by CodY, and repression of several genes occurs including ComK. CodY also interrupts the binding of ComK to its own promoter by binding to the RNA polymerase-binding site of ComK promoter. It also explains the reason behind highest expression of ComK under nutritional stress and in minimal medium with glucose as sole carbon source as the repressing effects of CodY and AbrB are relieved.

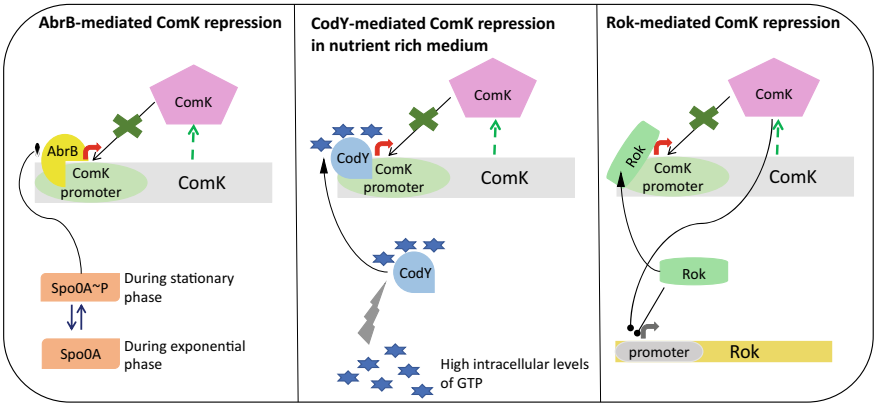


Fig. 2 Transcriptional repression of ComK during exponential phase. Green color dashed arrow represents product of a particular gene. Normal line with arrow depicts positive regulation, and normal line with circle depicts negative regulation

Repressor of ComK *(*Rok) is the third transcriptional repressor of ComK. It has been reported that over-expression of *Rok* gene is directly correlated to inhibited transcription of ComK. Rok not only binds and represses ComK promoter but also its own promoter as well. In addition, high ComK concentration in the medium also represses Rok expression by binding to its promoter. This ComK-dependent repression of Rok expression results into a positive feedback loop for ComK expression (Hamoen et al., 2003) (Fig. 2).

The presence of free ComK protein in the cell during exponential growth is also controlled post-translationally by regulated proteolysis of ComK using three proteins MecA, ClpC and ClpP. It starts with the capture of ComK protein by MecA. This sequestration is enhanced by binding of ClpC to MecA, which alone cannot bind ComK, thus forming a stable ternary MecA/ComK/ClpC complex which is targeted by the protease ClpP. It has also been reported that ClpP interacts with ClpC. Hence, MecA results in proteolytic degradation of ComK by ClpCP. This leads to rapid degradation of ComK and inhibition of its autotranscription (Turgay et al., 1998) (Fig. 3).

Autostimulation of ComK gene expression at the onset of stationary phase

The onset of ComK expression occurs only at a particular window of growth when the cells enter the stationary phase and maximum concentration of ComK is achieved after 2 h of stationary growth. The activation of ComK is under stringent control as its expression drives cells toward competence, thus inhibiting vegetative cell growth, halting DNA replication and ceasing cell division. The autostimulatory transcription of ComK takes place at its own promoter consisting of K-Boxes where ComK binds as dimer at two different sites separated by 31 bp spacer. Binding of another transcription factor DegU in between ComK-dimer-binding sites acts as a priming step

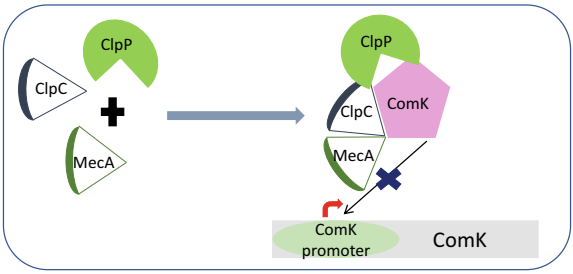


Fig. 3 Post-translational repression of ComK during exponential phase

for efficient binding of ComK. DegU is a response regulator of the DegS/DegU two-component signal transduction system which in its unphosphorylated form binds to ComK promoter. Thus, ComK-DegU complex facilitates initial activation of ComK transcription when ComK protein is present in very low levels. Once ComK concentration exceeds to a certain level, priming by unphosphorylated DegU is not required for functioning of ComK. At the later stages of stationary growth, DegU is phosphorylated by sensor kinase DegS resulting in the production of exo-proteases and other degradative enzymes.

5 Molecular Mechanism of Competence: Induction by Quorum Sensing

The transition of the cell from log phase to stationary phase brings about a significant change in the expression of several genes. This transition is regulated by cell density-dependent quorum sensing by certain signaling molecules known as autoinducers or pheromones. Competence in *B. subtilis* is developed due to the accumulation of two oligopeptide pheromones in the medium. The first pheromone that activates competence regulation pathway is ComX. ComX is synthesized as a 55 amino acid long precursor peptide which is processed at the C-terminal into a mature 10 amino acid oligopeptide. The mature ComX oligopeptide is modified with an isoprenyl group at the tryptophan residue by another protein ComQ located upstream of comX. The modification is essential for the extracellular secretion of ComX (Schneider et al., 2002). Elevated levels of ComX in the medium are detected extracellularly by a membrane spanning sensor kinase ComP which in turn phosphorylates ComA response regulator. Phosphorylation of ComA stimulates its binding to the promoter of *srfA* operon which has a huge size of 30 kb. This induces srfA operon transcription which encodes two proteins one being a lipopeptide antibiotic surfactin and another is a 46-amino acid small peptide ComS. Expression of surfactin, a potent biosurfactant, eliminates active competitors to *B. subtilis* cell in its surroundings, and its lytic action makes the genetic material released from the lysed microbes available

for uptake. ComS, on the other hand, has been reported to be crucial for competence development as it triggers the liberation of ComK from the ternary proteolytic complex MecA/ComK/ClpC. ComS is up-regulated at the onset of stationary phase, and it binds to MecA stimulating the proteolysis of both proteins by the ClpCP complex. This signifies that ComS protects ComK from degradation, thus enabling autotranscription of ComK to occur. ComK levels increase rapidly in the cell due to the positive feedback regulation of ComK expression by ComK (Hamoen et al., 2003; Maamar and Dubnau, 2005).

The second pheromone competence and sporulating factor (CSF) is essential for competence as well as sporulation. CSF is a cationic pentapeptide whose amino acid composition corresponds to the C-terminal 5 amino acids of the 40 amino acid long secreted peptide PhrC. The synthesis of PhrC is regulated by sigma factor H whose promoter has a sigma-H binding site. Expression of sigma-H increases at the end of exponential growth. PhrC is synthesized as a precursor which undergoes processing to form mature pentapeptide CSF which is transported outside the cell through simple diffusion. However, the sensing of CSF occurs intracellularly with the help of an oligopeptide permease Spo0K. At low concentrations of CSF (1–10 nM), competence is induced by inhibition of RapC phosphatase. *rapC* (rap stands for response regulator aspartate phosphatase) gene is located upstream of *phrC* (phr stands for phophatase regulator) gene. Several Rap-Phr system, which comprises an operon consisting of a phosphatase enzyme Rap located upstream of its inhibitory peptide Phr, is critical for regulation of diverse functions in *B. subtilis.*

Here, under normal conditions, RapC dephosphorylates ComA/P two-component system which in turn negatively regulates the s*rfA* operon expression. Thus, repression of RapC at a certain CSF concentration also increases *srfA*/comS expression ultimately increasing the autostimulation of ComK. In addition, ComK also stimulates the transcription of late competence operon, encoding the genes for DNA binding, uptake and integration machinery. Hence, the cells are driven to enter the competent state (Hamoen et al., 2003) (Fig. 4).

Only 10–15% of the total bacterial population enters competence even under optimal conditions, whereas 50–70% of the cells make the switch toward sporulation. Various proteins involved in DNA uptake and regulation of competence are presented in Table 1.

6 Sporulation in *Bacillus Subtilis*

Sporulation is a developmental program which is triggered under extreme nutritional stress as it is the last tactics of cells to survive. The process of spore formation has been best studied in *B. subtilis*. Unlike competence, sporulation is an energy-intensive and irreversible event occurring only in a sub-set of population (Tan and Ramamurthi, 2014). It starts with the formation of a polar septum which divides the cell in two asymmetric compartments, the smaller forespore and the large mother cell. The mother cell engulfs the forespore making it double-membrane bound. Finally, the

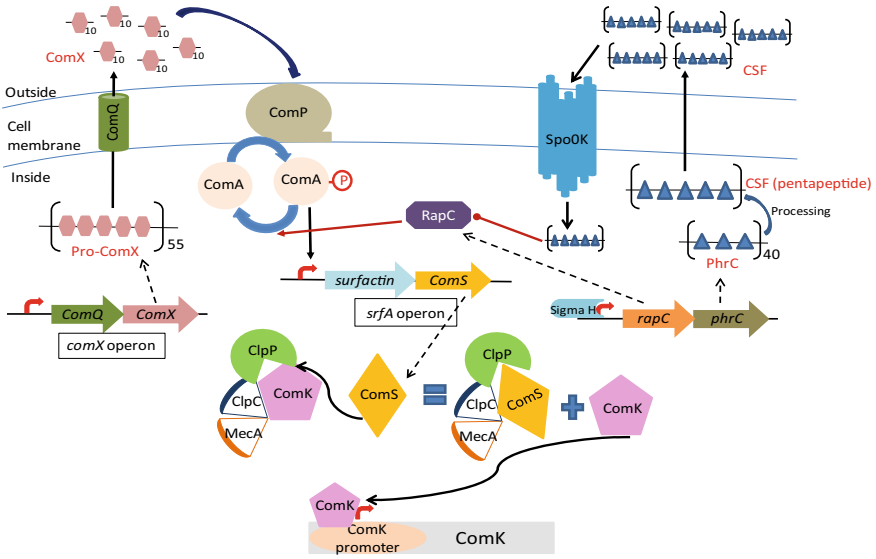


Fig. 4 Competence regulation via quorum sensing during stationary phase. Dashed lined arrows indicate products of the genes coding them; solid lined arrows indicate positive regulation, and solid line with circle indicates negative regulation

mother cell undergoes lysis to release forespore which gets permanently converted into a hard spore, resistant to a variety of environmental hazards such as heat, radiation and toxic chemicals and remains dormant until it senses favorable conditions conducive for growth and undergoes germination. However, sporulation does not initiate automatically upon nutrient deprived conditions but has complex regulation involving multiple factors, and the process of sporulation has been divided into six main stages (0-V) (Fig. 5).

Stage 0: The decision to enter into sporulation

Various survival strategies are also implemented by cells before committing to sporulation. These strategies include activation of flagellar motility to search for nutrients in the environment by chemotaxis, competence for uptake of exogenous DNA, secretion of killing factors to lyse other cells for nutrients and secondary metabolites, a process known as cannibalism (González-Pastor, 2011). Two factors Skf and Sdp are selectively expressed by cells producing biofilms at the onset of cannibalism, and they can kill neighboring isogenic population (López et al., 2009).

The entry into sporulation pathway is controlled by a response regulator protein Spo0A which is phosphorylated via a complex phosphorelay system. As competence is mainly controlled by ComA-ComP two-component system, likewise, sporulation is also controlled by a phosphorelay sensing system **Kin-Spo0F-Spo0B-Spo0A**. It comprises a minimum of five histidine kinases KinA-KinE which senses multiple aspects of the environmental stress and gets autophosphorylated. The activated sensor

Table 1 Proteins involved in DNA uptake and development/inhibition of competence

Proteins involved in DNA uptake	
ComEA	DNA-binding receptor protein
ComG	Modifies cell wall to increase porosity and locally shield or remove negative charges
ComEC	Transportation of DNA inside the cell
ComFA	Transportation of DNA inside the cell
Proteins involved in competence development/inhibition	
ComK	Main regulator of competence
DegU	Helps in priming of ComK transcription when the concentration of ComK is below threshold
DegS	Sensor kinase of DegU
MecA	Negative regulator of competence; holds ComK in a complex
ClpC	Member of HSP 100 chaperone like family; holds ComK in a complex with MecA
ClpP	Conserved protease; holds ComK in a complex with MecA
AbrB	Transition state regulator; acts as both negative and positive regulator of competence
CodY	A GTP-binding transcription factor and a negative regulator of competence
Rok	Repressor of ComK, a negative regulator of competence
ComX	A pheromone which activates ComA/P two-component system
ComP	A protein kinase which activates ComA
ComA	A response regulator protein which activates srfA operon
PhrC	A pheromone which gets converted to competence and sporulation factor (CSF) essential for both competence and sporulation
ComS	A small peptide that triggers liberation of ComK from MecA/ClpC/ClpP complex
Sigma-H	Transcription factor for synthesis of PhrC
RapC	A phosphatase places upstream in a phrC operon, dephosphorylates ComA/P two-component system

kinases phosphorylate the response regulator Spo0F (Spo0F~P) which is an intermediate phosphoacceptor. Spo0F~P transfers the phosphate group to a phosphotransferase Spo0B which rapidly transfers the phosphate to the main response regulator protein Spo0A. Phosphorylated Spo0A (Spo0A~P) governs the expression of several genes involved in the development of sporulation event. In the absence of stress levels, the histidine kinases KinA–E, dephosphorylate Spo0F and stops the event (Hamoen et al., 2003) (Fig. 6).

In addition, the concentration of CSF in the extracellular medium is also important as it regulates the transition from competence to sporulation. As the cells entering stationary phase increase in density, the concentration of CSF in the extracellular medium also increases significantly. At relatively higher concentration (<20 nM),

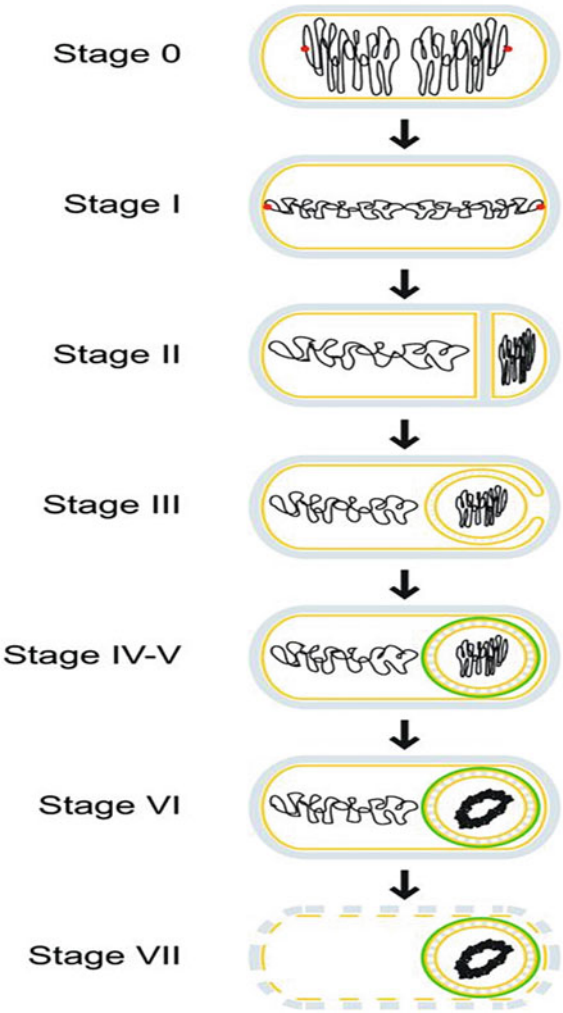


Fig. 5 Different stages involved in endospore development in *Bacillus subtilis* Source: Tan and Ramamurthi (2014). (Public Domain)

CSF leads to inhibition of competence development and drives cell toward sporulation. CSF when present in abundance inhibits phosphorylation of sensory kinase ComP, and thus, concentration of phosphorylated ComA response protein declines in the medium. Consequently, activation of ComS is also hindered, thus inhibiting competence. High CSF concentration also inhibits the action of RapB enzyme. RapB is a constitutive phosphatase responsible for dephosphorylation of sporulation response regulator protein Spo0F. Spo0F is an intermediate phosphoacceptor

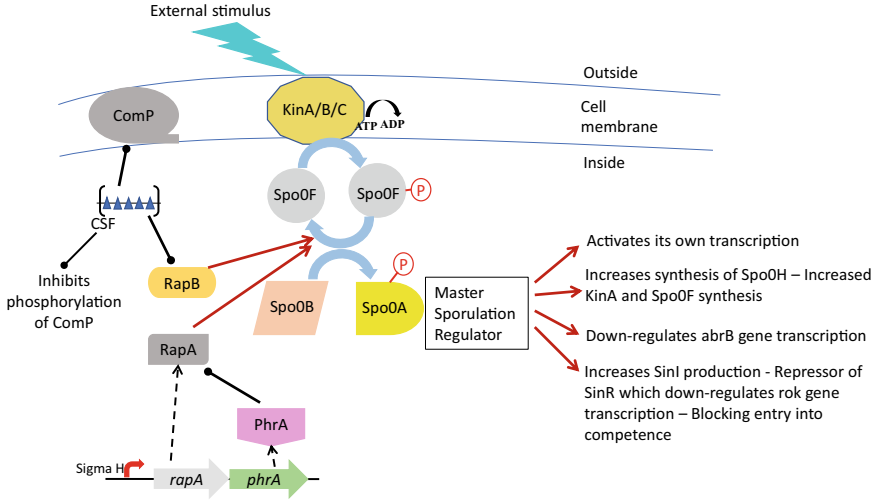


Fig. 6 Regulation mechanism of sporulation via phosphorelay system and inhibition of RapA and RapB phosphatases by CSF and PhrA at the onset of stationary phase. Red arrows indicate positive regulation; dashed arrows indicate products of the gene coding them; lines with circle indicate negative regulation

of the phosphorelay system which phosphorylates Spo0A, the central player in activation of sporulation event. CSF-mediated inhibition of Spo0F dephosphorylation by RapB results in increased Spo0A levels inside the cell, thus progressing the cell toward sporulation. It can be concluded that CSF functions within a physiological concentration range between 1 and 100 nM to decide the fate of cells growing under nutritional stress to acquire competence which is only transient or compelling cells permanently toward sporulation for survival (Schultz et al., 2009).

Another system which regulates the transition of competent cells into sporulating cells is the RapA-PhrA system. It functions in a similar manner as *phrC-rapC* operon. RapA inhibits phosphorylation of Spo0F during early stationary phase when ComX concentration is high in the medium. During late stationary phase, when ComX concentration declines, RapA action is blocked by its own peptide pheromone PhrA, and repression of Spo0F phosphorylation by RapA is relieved which favors establishment of sporulation inside the cell (Schultz et al., 2009).

Spo0A: the master regulator of sporulation

Spo0A is the called the master regulator as its concentration inside the cells is critical to trigger a series of events involved in progression toward sporulation. During the exponential phase, Spo0A level is very low and present majorly in its dephosphorylated form. When cells face unfavorable growth conditions, Spo0A is phosphorylated by the phosphorelay system. Spo0A upon phosphorylation activates its own transcription as well as induces an increase in the synthesis of Spo0H (sigma factor H) which leads to increased transcription of phosphorelay components KinA

and Spo0F. Spo0A~P also binds to certain DNA-binding regions known as 0A box present in the *abrB* gene promoter with a higher affinity. Accumulation of Spo0A~P to a certain threshold results in down-regulation of abrB transcription as described earlier in competence also. This decisive step prepares the cells to enter into competence or sporulation. With further increase in the levels of Spo0A~P, cells committed to enter sporulation block transition to competence completely with the help of another regulatory circuit involving two proteins SinI-SinR. SinI is the repressor of the transcriptional regulator SinR which has been reported to down-regulate Rok transcription and thus directly involved in the transcription of ComK. It also represses *spo0A* promoter. High levels of Spo0A~P stimulate an increase in SinI production. SinI forms a hetero-multimer with SinR and thus inactivates it. This inactivation results in de-repression of Rok, thus blocking entry into competence (Schultz et al., 2009).

Stage I: Formation of axial filament for proper chromosome segregation and distribution

The mother cell and the forespore each should carry one copy of chromosome. Thus, at the starting point, before separation, the cell harbors two chromosomes in the form of an axial filament which is a condensed serpentine like structure extended from pole to pole. The pole to pole extension of DNA is required for proper segregation of chromosomes which is mediated by a DNA-binding protein RacA. This protein binds to GC rich inverted repeats near the origin of replication and forms a highly stable nucleoprotein complex in association with another protein DivIVA for facilitating localization of the chromosomes toward the opposite poles of the cell (Ben-Yehuda et al., 2003). Another protein SpoIIIE is also involved in segregation which acts as a DNA translocase enzyme. After segregation, three other proteins come into picture which ensures that both mother cell and forespore receive one chromosome necessary for proper sporulation. The first protein SirA which is under the control of the master regulator SpoA~P interacts with DnaA protein and inhibits its binding to the origin of replication to prevent another round of replication as the cell enters sporulation (Rahn-Lee et al., 2011). SpoA~P also has a binding site near the origin of replication whose binding to DNA is suggested to further inhibit DNA replication, while cell sporulates. The third protein Sda is indirectly involved in sporulation mechanism as it inhibits the entry of cell into sporulation by binding to KinA, while the cell is in the mode of active replication (Veening et al., 2009).

Stage II: Formation of asymmetric septum (σF activation in fore cell)

The formation of polar septum in place of central septum is the main morphological feature of sporulating cells. The process of asymmetric septation starts with localization of the cell division protein FstZ to the poles, mediated by the help pf SpoIIE (stage II sporulation protein E) protein, which divides the cell into two asymmetric compartments, the forespore and mother cell (Carniol et al., 2005). Other than *spo* genes, the first compartment-specific transcription factor produced by sporulating cell is sigma factor F (σF; SpoIIA) which is specific for forespore and is responsible for transcription of genes in the forespore that is destined to become the spore. It is

inhibited by an anti-sigma factor SpoIIAB which holds the σF. The activation of σF is dependent on SpoIIE which is activated by high levels of SpoA~P and dephosphorylates another protein SpoIIAA. SpoIIAA is an anti-anti-sigma factor which counteracts the inhibition by SpoIIAB and liberates σF (Duncan et al., 1996). Once free, σF binds to the RNA polymerase and promotes transcription of genes whose products are needed for the next stage of sporulation.

It is to be noted that both forespore and mother cell produce σF but its activation occurs only in forespore due to the preferential localization of SpoIIE in forespore and also due to low levels of anti-sigma factor SpoIIAB in the forespore due to genetic asymmetry. Genetic asymmetry occurs due to asymmetric division of axial filament which results in only 30% of the genetic machinery transfer to the forespore, while the 70% still remains in the mother cell. Later, DNA translocase protein SpoIIIE pumps the rest 70% chromosome to the forespore. Therefore, before translocation, limited genetic machinery results in compartment-specific protein expression (Frandsen et al., 1999).

Activation of sigma factor E (σE) in the mother cell

Sigma E is the second sporulation specific sigma factor which is under SpoA~P control and is present in both mother cell and forespore after symmetric division. Initially, it is present as pro-σE which gets activated only in the mother cell. The mature σE is produced by the action of SpoIIGA (Jonas et al., 1988). SpoIIGA is an aspartic protease which itself gets activated by SpoIIR. SpoIIR is produced by the forespore under the control of σF and secreted in the intermembrane space of the septum. SpoIIGA is specifically activated in the mother cell by SpoIIR as SpoIIR has to be acylated to activate SpoIIGA which takes place only in the mother cell due to its de novo ability of fatty acid synthesis (Pedrido et al., 2013). Thus, SpoIIGA activates σE only in the mother cell, and σE promotes transcription of genes whose products are required for engulfment of forespore by mother cell.

Stage III: Engulfment

After activation of σE, the mother cell prepares itself to engulf the forespore which initiates by curving of the polar septum around the forespore. Then, three *spo* proteins SpoIID, SpoIIM and SpoIIP constitutively form a peptidoglycan degradation machinery (autolysin) which degrades the peptidoglycan layer surrounding the forespore and the forespore is swallowed by the mother cell (Abanes-De Mello et al., 2002). However, the peptidoglycan layer is not degraded completely, and the reminiscent portion later acts as a template for synthesis of new peptidoglycan layer, thus making the forespore a double-membrane-bound structure inside the cytosol of mother cell. During the process of engulfment, there is movement of engulfing membranes, and then, there is pinching off event to release forespore carried out by a protein FisB which accumulates at the site of membrane fission and results in membrane remodeling (Doan et al., 2013). Thus, a double-membrane-bound free floating forespore is released inside the mother cell after engulfment.

Activation of sigma factor (σG) in the forespore and sigma factor K (σK) in mother cell

The activation of the third sporulation specific sigma factor G occurs only after engulfment is complete. σG is under the transcriptional control of σF ensuring that it is specifically expressed in the forespore. Once the forespore is engulfed, it cuts off from the mother cell and loses its metabolic activity to a great extent (Camp and Losick, 2009; Doan et al., 2009). For proper nourishment of forespore, a feeding tube channel like structure is formed between mother cell and forespore made up of three *spo* proteins SpoIIAA-SpoIIAH and SpoIIQ which are under the control of σE (Meisner et al., 2008). Activation of σG in forespore is required for transcription of other genes needed further during the late events in the sporulation process.

Another sporulation sigma factor specifically activated in mother cell is sigma K which is also produced in an inactive form as pro-σK. It becomes active after cleavage by an intermembrane protease enzyme SpoIVFB (Lu et al., 1995). Initially, SpoIVFB is also present in an inactive form in association with two other proteins, SpoIVFA and BofA, which becomes active by the action of another enzyme SpoIVB whose activity is regulated by σG.

Stage IV-V: Formation of cortex and coat outside the forespore

The cortex is a thick peptidoglycan layer that is placed between the two membranes surrounding the forespore protoplast and is different from the thin germ cell wall layer immediately surrounding the inner membrane of the forespore. The peptidoglycan layer of the cortex has low level of transpeptidation between glycan chains and is modified by the presence of muramic lactam. This modification is mediated by Mur proteins which are up-regulated by σK (Vasudevan et al., 2007). Cortex is important for providing heat and desiccation resistance and maintaining dormancy of the spore.

The coat layer is made up of proteins deposited on the outer membrane. SpoVM is considered to be the first coat protein produced exclusively by the mother cell which is associated with another protein SpoIVA to form the base layer of the coat (Levin et al., 1993; Ramamurthi et al., 2006). Apart from this, there are around seventy different proteins which assemble to form the complete coat layer. The coat layer protects the spore from lysozyme and other infectious agents like *Tetrahymena.*

Development of spore resistance

The spore is resistant to a variety of environmental stress due to a number of reasons. The first reason is a thick cortex that protects the spore from high temperature and desiccation due to its ability to maintain a partially dehydrated state. Another factor for resistance against heat and desiccation is dipicolinic acid which is synthesized in the mother cell and gets deposited into the core of the forespore. Spore DNA is also bound to small acid soluble proteins (SASP) which protect the spore against UV radiation, hydrogen peroxide, etc. Finally, the mother cell undergoes programmed cell lysis to release the fully mature spore which is surrounded by cortex and coat layers. Signa factors and proteins involved in sporulation are summarized in Table 2.

Table 2 Sigma factors and sporulation proteins involved in spore development

Sigma factors (σ) involved in spore formation	
σF	First forespore specific transcription factor; produced early during sporulation stages and control expression of forespore specific genes
σE	First mother cell specific transcription factor; required for engulfment od forespore by mother cell
σG	Forespore specific transcription factor; produced during late stages of sporulation; activates SpoIVB
σK	Mother cell specific transcription factor; up-regulates Mur proteins required for synthesis of cortex layer outside forspore
Spo proteins involved in spore formation	
Spo0F	A response regulator which acts as an intermediate phosphoacceptor during initiation of sporulation
Spo0B	A phosphotransferase which transfers phosphate group to Spo0A
Spo0A	Master regulator of sporulation; induces formation of axial filaments; also activates SpoIIE, SpoIIGA and multiple other genes
SpoIIE	Involved in the process of formation of asymmetric septum; activates σF
SpoIIAB	An anti-sigma factor which binds to σF and inhibits its activity
SpoIIAA	An anti-anti-sigma factor binds to SpoIIAB and counteracts its inhibition on σF
SpoIIGA	An aspartic protease which cleaves pro-σE to release active form of σE
SpoIIR	Activates SpoIIGA specifically in the mother cell
SpoIIIE	DNA translocase enzyme for transfer of 70% of the DNA from mother cell to forespore
Spo0IID, SpoIIM, SpoIIP	Forms a peptidoglycan machinery to degrade the peptidoglycan layer surrounding forespore to prepare for its engulfment by mother cell
SpoIIAA-SpoIIAH SpoIIQ	Forms a feeding tube like structure between mother cell and forespore for providing proper nourishment to the forespore
SpoIVFB	Activates σK
SpoIVFA	Inhibitor of SpoIVFB
SpoIVB	Activates SpoIVFB
SpoVM-SpoIVA	Coat proteins which form the base layer of coat surrounding the forespore
Other proteins involved in spore formation	
KinA-E	Histidine kinases which sense multiple aspects of environment to initiate process of sporulation
SinR	Repressor of Rok gene which is a repressor of ComK
SinI	Repressor of SinR

(continued)

Table 2 (continued)

RacA	A DNA-binding protein required for proper segregation of chromosomes
DivIVA	Involved in proper segregation of chromosomes along with RacA
SirA	Binds to DnaA protein to inhibit replication during sporulation
Sda	Inhibits entry of cells into sporulation by binding to kina during active replication
FisB	Accumulates at the site of membrane fission during engulfment and involved in membrane remodeling and pinching off event to detach forespore from mother cell

Summary

- Competence is a natural phenomenon shown by bacterial cells to uptake exogenous DNA from the environment.
- It is of two types based on the requirement of the cell: natural and nutritional competence.
- Nutritional competence allows the bacterial cells to use it as a nutrient source, whereas natural competence is useful for genetic engineering and gene regulation.
- Competence is transiently induced by external stimuli and a master competence regulator. ComK drives expression of genes involved in DNA uptake and competence development.
- Expression of ComK is growth specific, and it occurs only during the transition from exponential to stationary phase and is regulated both transcriptionally and post-translationally.
- There are three transcription factors that control ComK expression during exponential phase viz. AbrB which occupies the ComK promoter initiation site; CodY which is a GTP-sensing transcriptional repressor of ComK and Rok which also binds to ComK promoter and inhibits its expression.
- The translational complex MecA/ClpC/ClpP sequesters ComK and results in its proteolytic degradation during exponential phase.
- At the onset of stationary phase, ComK is activated by the action of DegS/DegU two-component system.
- Competence is mainly developed due to quorum sensing which involves ComA/P two-component system.
- Sporulation is another developmental program which is triggered under extreme nutritional stress and is best studied in *B. subtilis.*
- CSF controls competence and sporulation as function of its concentration. Low concentrations of CSF (1–10 nM) induce competence by the inhibition of RapC phosphatase as RapC dephosphorylates ComA/P two-component system, whereas high CSF switches sporulation by inhibiting constitutive phosphatases.
- Various strategies implemented by cells before committing to sporulation include chemotaxis, competence and cannibalism.

- The switch to sporulation pathway is controlled by a master response regulator protein Spo0A which is phosphorylated via a complex phosphorelay system involving Kin A/B/C, Spo0F, Spo0B and Spo0A.
- Spo0A levels block entry into competence with the help of a regulatory circuit involving two proteins SinI-SinR.
- The formation of spore in *B. subtilis* is divided into six main stages viz the decision to enter into sporulation (stage 0); formation of axial filament for proper chromosome segregation and distribution (stage I); formation of asymmetric septum (stage II); engulfment of forespore by mother cell (stage III); activation of sigma factor G in forespore and sigma factor K in mother cell (stage IV); formation of cortex and coat outside the forespore (stage V–VI).
- The sigma factors produced are mainly involved in development of sporulation.

Questions

1. What is competence? Differentiate between natural and nutritional competence.
2. Which is the master regulator of competence?
3. How ComK is regulated at transcriptional and post-translational levels?
4. What do you understand by priming of ComK?
5. Explain the molecular mechanism involved in competence development.
6. Competence is transient and only 10–15% population of cells enter into competence. Explain.
7. Explain cannibalism as a means to avoid entry into sporulation.
8. What is sporulation and what are the different stages of spore formation?
9. How the transcription factor Spo0A helps cells to switch to sporulation?
10. Describe the function of sigma factors in sporulation development.

References

Abanes-De Mello, A., Sun, Y. L., Aung, S., & Pogliano, K. (2002). A cytoskeleton-like role for the bacterial cell wall during engulfment of the *Bacillus subtilis* forespore. *Genes & Development, 16*(24), 3253–3264.

Ben-Yehuda, S., Rudner, D. Z., & Losick, R. (2003). RacA, a bacterial protein that anchors chromosomes to the cell poles. *Science, 299*(5606), 532–536.

Camp, A. H., & Losick, R. (2009). A feeding tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis. Genes & Development, 23*(8), 1014–1024.

Carniol, K., Ben-Yehuda, S., King, N., & Losick, R. (2005). Genetic dissection of the sporulation protein SpoIIE and its role in asymmetric division in *Bacillus subtilis. Journal of Bacteriology, 187*(10), 3511–3520.

Doan, T., Coleman, J., Marquis, K. A., Meeske, A. J., Burton, B. M., Karatekin, E., et al. (2013). FisB mediates membrane fission during sporulation in *Bacillus subtilis. Genes & Development, 27*(3), 322–334.

Doan, T., Morlot, C., Meisner, J., Serrano, M., Henriques, A. O., Moran, C. P., Jr., & Rudner, D. Z. (2009). Novel secretion apparatus maintains spore integrity and developmental gene expression in *Bacillus subtilis*. *PLoS Genetics*, *5*(7).

Duncan, L., Alper, S., & Losick, R. (1996). SpoIIAA governs the release of the cell-type specific transcription factor σF from its anti-sigma factor SpoIIAB. *Journal of Molecular Biology, 260*(2), 147–164.

Frandsen, N., Barák, I., Karmazyn-Campelli, C., & Stragier, P. (1999). Transient gene asymmetry during sporulation and establishment of cell specificity in *Bacillus subtilis*. *Genes & Development, 13*(4), 394–399.

González-Pastor, J. E. (2011). Cannibalism: A social behavior in sporulating Bacillus subtilis. *FEMS Microbiology Reviews, 35*(3), 415–424.

Hamoen, L. W., Smits, W. K., Jong, A. D., Holsappel, S., & Kuipers, O. P. (2002). Improving the predictive value of the competence transcription factor (ComK) binding site in *Bacillus subtilis* using a genomic approach. *Nucleic Acids Research, 30*(24), 5517–5528.

Hamoen, L. W., Venema, G., & Kuipers, O. P. (2003). Controlling competence in *Bacillus subtilis*: shared use of regulators. *Microbiology, 149*(1), 9–17.

Jonas, R. M., Weaver, E. A., Kenney, T. J., Moran, C. P., & Haldenwang, W. G. (1988). The *Bacillus subtilis* spoIIG operon encodes both sigma E and a gene necessary for sigma E activation. *Journal of Bacteriology, 170*(2), 507–511.

Levin, P. A., Fan, N., Ricca, E., Driks, A., Losick, R., & Cutting, S. (1993). An unusually small gene required for sporulation by *Bacillus subtilis*. *Molecular Microbiology, 9*(4), 761–771.

López, D., Vlamakis, H., Losick, R., & Kolter, R. (2009). Cannibalism enhances biofilm development in *Bacillus subtilis*. *Molecular Microbiology, 74*(3), 609–618.

Lu, S., Cutting, S., & Kroos, L. (1995). Sporulation protein SpoIVFB from *Bacillus subtilis* enhances processing of the sigma factor precursor Pro-sigma K in the absence of other sporulation gene products. *Journal of Bacteriology, 177*(4), 1082–1085.

Maamar, H., & Dubnau, D. (2005). Bistability in the Bacillus subtilis K-state (competence) system requires a positive feedback loop. *Molecular Microbiology, 56*(3), 615–624.

Meisner, J., Wang, X., Serrano, M., Henriques, A. O., & Moran, C. P. (2008). A channel connecting the mother cell and forespore during bacterial endospore formation. *Proceedings of the National Academy of Sciences, 105*(39), 15100–15105.

Pedrido, M. E., de Oña, P., Ramirez, W., Leñini, C., Goñi, A., & Grau, R. (2013). Spo0A links de novo fatty acid synthesis to sporulation and biofilm development in *Bacillus subtilis*. *Molecular Microbiology, 87*(2), 348–367.

Rahn-Lee, L., Merrikh, H., Grossman, A. D., & Losick, R. (2011). The sporulation protein SirA inhibits the binding of DnaA to the origin of replication by contacting a patch of clustered amino acids. *Journal of Bacteriology, 193*(6), 1302–1307.

Ramamurthi, K. S., Clapham, K. R., & Losick, R. (2006). Peptide anchoring spore coat assembly to the outer forespore membrane in *Bacillus subtilis*. *Molecular Microbiology, 62*(6), 1547–1557.

Schneider, K. B., Palmer, T. M., & Grossman, A. D. (2002). Characterization of comQ and comX, two genes required for production of ComX pheromone in *Bacillus subtilis*. *Journal of Bacteriology, 184*(2), 410–419.

Schultz, D., Wolynes, P. G., Jacob, E. B., & Onuchic, J. N. (2009). Deciding fate in adverse times: Sporulation and competence in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences, 106*(50), 21027–21034.

Susanna, K. A., Mironczuk, A. M., Smits, W. K., Hamoen, L. W., & Kuipers, O. P. (2007). A single, specific thymine mutation in the ComK-binding site severely decreases binding and transcription activation by the competence transcription factor ComK of *Bacillus subtilis*. *Journal of Bacteriology, 189*(13), 4718–4728.

Tan, I. S., & Ramamurthi, K. S. (2014). Spore formation in *Bacillus subtilis*. *Environmental Microbiology Reports, 6*(3), 212–225.

Turgay, K., Hahn, J., Burghoorn, J., & Dubnau, D. (1998). Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *The EMBO Journal, 17*(22), 6730–6738.

Vasudevan, P., Weaver, A., Reichert, E. D., Linnstaedt, S. D., & Popham, D. L. (2007). Spore cortex formation in *Bacillus subtilis* is regulated by accumulation of peptidoglycan precursors under the control of sigma K. *Molecular Microbiology, 65*(6), 1582–1594.

Veening, J. W., Murray, H., & Errington, J. (2009). A mechanism for cell cycle regulation of sporulation initiation in *Bacillus subtilis*. *Genes & Development, 23*(16), 1959–1970.

Correction to: Fundamentals of Bacterial Physiology and Metabolism

Correction to:
R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3

The original version of this book was published without the citation of authors and co-authors within the chapters. This has now been corrected, and the book has been updated with the changes.

The updated version of the book can be found at
https://doi.org/10.1007/978-981-16-0723-3

R. Gupta and N. Gupta, *Fundamentals of Bacterial Physiology and Metabolism*,
https://doi.org/10.1007/978-981-16-0723-3_26