

WILEY-VCH

Edited by  
Paul Hudson

# Cyanobacteria Biotechnology

**Volume 12**

Series Editors:  
S. Y. Lee, J. Nielsen,  
G. Stephanopoulos



Advanced Biotechnology



## **Cyanobacteria Biotechnology**



# **Cyanobacteria Biotechnology**

*Edited by*  
*Paul Hudson*

**WILEY-VCH**

**Volume Editor****Prof. Dr. Paul Hudson**

KTH Royal Institute of Technology  
Science for Life Laboratory  
Tomtebodavägen 23A  
171 65 Solna  
Sweden

**Series Editors****Prof. Sang Y. Lee**

KAIST  
373-1;Guseong-Dong  
291 Daehak-ro,Yuseong-gu  
305-701 Daejeon  
South Korea

**Prof. Dr. Jens Nielsen**

Chalmers University  
Department of Chemical and Biological  
Engineering  
Kemivägen 10  
412 96 Göteborg  
Sweden

**Gregory Stephanopoulos**

Massachusetts Institute of Technology  
Department of Chemical Engineering  
Massachusetts Ave 77  
United States

**Cover:** Culture Flasks in microbiological  
laboratory / science photo, fotolia

■ All books published by **WILEY-VCH** are carefully produced. Nevertheless, authors, editors, and publisher do not warrant the information contained in these books, including this book, to be free of errors. Readers are advised to keep in mind that statements, data, illustrations, procedural details or other items may inadvertently be inaccurate.

**Library of Congress Card No.:**

applied for

**British Library Cataloguing-in-Publication Data**

A catalogue record for this book is available from the British Library.

**Bibliographic information published by the Deutsche Nationalbibliothek**

The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <<http://dnb.d-nb.de>>.

© 2021 WILEY-VCH GmbH, Boschstr. 12, 69469 Weinheim, Germany

All rights reserved (including those of translation into other languages). No part of this book may be reproduced in any form – by photoprinting, microfilm, or any other means – nor transmitted or translated into a machine language without written permission from the publishers. Registered names, trademarks, etc. used in this book, even when not specifically marked as such, are not to be considered unprotected by law.

**Print ISBN:** 978-3-527-34714-8

**ePDF ISBN:** 978-3-527-82492-2

**ePub ISBN:** 978-3-527-82491-5

**oBook ISBN:** 978-3-527-82490-8

**Cover Design** Adam-Design,  
Weinheim, Germany

**Typesetting** SPi Global, Chennai, India

**Printing and Binding**

Printed on acid-free paper

10 9 8 7 6 5 4 3 2 1

## Contents

**Foreword: Cyanobacteria Biotechnology** xv

**Acknowledgments** xviii

### Part I Core Cyanobacteria Processes 1

- 1 Inorganic Carbon Assimilation in Cyanobacteria: Mechanisms, Regulation, and Engineering** 3  
*Martin Hagemann, Shanshan Song, and Eva-Maria Brouwer*
- 1.1 Introduction – The Need for a Carbon-Concentrating Mechanism 3
- 1.2 The Carbon-Concentrating Mechanism (CCM) Among Cyanobacteria 4
- 1.2.1 C<sub>i</sub> Uptake Proteins/Mechanisms 5
- 1.2.2 Carboxysome and RubisCO 8
- 1.3 Regulation of C<sub>i</sub> Assimilation 10
- 1.3.1 Regulation of the CCM 10
- 1.3.2 Further Regulation of Carbon Assimilation 13
- 1.3.3 Metabolic Changes and Regulation During C<sub>i</sub> Acclimation 14
- 1.3.4 Redox Regulation of C<sub>i</sub> Assimilation 15
- 1.4 Engineering the Cyanobacterial CCM 16
- 1.5 Photorespiration 17
- 1.5.1 Cyanobacterial Photorespiration 17
- 1.5.2 Attempts to Engineer Photorespiration 19
- 1.6 Concluding Remarks 20
- Acknowledgments 21
- References 21
- 2 Electron Transport in Cyanobacteria and Its Potential in Bioproduction** 33  
*David J. Lea-Smith and Guy T. Hanke*
- 2.1 Introduction 33
- 2.2 Electron Transport in a Bioenergetic Membrane 34
- 2.2.1 Linear Electron Transport 34

2.2.2	Cyclic Electron Transport	37
2.2.3	ATP Production from Linear and Cyclic Electron Transport	37
2.3	Respiratory Electron Transport	38
2.4	Role of Electron Sinks in Photoprotection	41
2.4.1	Terminal Oxidases	41
2.4.2	Hydrogenase and Flavodiiron Complexes	41
2.4.3	Carbon Fixation and Photorespiration	43
2.4.4	Extracellular Electron Export	44
2.5	Regulating Electron Flux into Different Pathways	45
2.5.1	Electron Flux Through the Plastoquinone Pool	45
2.5.2	Electron Flux Through Fdx	46
2.6	Spatial Organization of Electron Transport Complexes	47
2.7	Manipulating Electron Transport for Synthetic Biology Applications	48
2.7.1	Improving Growth of Cyanobacteria	49
2.7.2	Production of Electrical Power in BPVs	49
2.7.3	Hydrogen Production	50
2.7.4	Production of Industrial Compounds	50
2.8	Future Challenges in Cyanobacterial Electron Transport	51
	References	52

### **3 Optimizing the Spectral Fit Between Cyanobacteria and Solar Radiation in the Light of Sustainability Applications** 65

*Klaas J. Hellingwerf, Que Chen, and Filipe Branco dos Santos*

3.1	Introduction	65
3.2	Molecular Basis and Efficiency of Oxygenic Photosynthesis	67
3.3	Fit Between the Spectrum of Solar Radiation and the Action Spectrum of Photosynthesis	72
3.4	Expansion of the PAR Region of Oxygenic Photosynthesis	74
3.5	Modulation and Optimization of the Transparency of Photobioreactors	79
3.6	Full Control of the Light Regime: LEDs Inside the PBR	81
3.7	Conclusions and Prospects	82
	References	83

## **Part II Concepts in Metabolic Engineering** 89

### **4 What We Can Learn from Measuring Metabolic Fluxes in Cyanobacteria** 91

*Xiang Gao, Chao Wu, Michael Cantrell, Melissa Cano, Jianping Yu, and Wei Xiong*

4.1	Central Carbon Metabolism in Cyanobacteria: An Overview and Renewed Pathway Knowledge	91
4.1.1	Glycolytic Routes Interwoven with the Calvin Cycle	91
4.1.2	Tricarboxylic Acid Cycling	94



4.2	Methodologies for Predicting and Quantifying Metabolic Fluxes in Cyanobacteria	95
4.2.1	Flux Balance Analysis and Genome-Scale Reconstruction of Metabolic Network	95
4.2.2	<sup>13</sup> C-Metabolic Flux Analysis	96
4.2.3	Thermodynamic Analysis and Kinetics Analysis	99
4.3	Cyanobacteria Fluxome in Response to Altered Nutrient Modes and Environmental Conditions	101
4.3.1	Autotrophic Fluxome	101
4.3.2	Photomixotrophic Fluxome	104
4.3.3	Heterotrophic Fluxome	105
4.3.4	Photoheterotrophic Fluxome	105
4.3.5	Diurnal Metabolite Oscillations	106
4.3.6	Nutrient States' Impact on Metabolic Flux	107
4.4	Metabolic Fluxes Redirected in Cyanobacteria for Biomanufacturing Purposes	108
4.4.1	Restructuring the TCA Cycle for Ethylene Production	108
4.4.2	Maximizing Flux in the Isoprenoid Pathway	109
4.4.2.1	Measuring Precursor Pool Size to Evaluate Potential Driving Forces for Isoprenoid Production	109
4.4.2.2	Balancing Intermediates for Increased Pathway Activity	110
4.4.2.3	Kinetic Flux Profiling to Detect Bottlenecks in the Pathway	111
4.5	Synopsis and Future Directions	112
	Acknowledgments	112
	References	112
<b>5</b>	<b>Synthetic Biology in Cyanobacteria and Applications for Biotechnology</b>	<b>123</b>
	<i>Elton P. Hudson</i>	
5.1	Introduction	123
5.2	Getting Genes into Cyanobacteria	123
5.2.1	Transformation	123
5.2.2	Expression from Episomal Plasmids	125
5.2.3	Delivery of Genes to the Chromosome	127
5.3	Basic Synthetic Control of Gene Expression in Cyanobacteria	129
5.3.1	Quantifying Transcription and Translation in Cyanobacteria	130
5.3.2	Controlling Transcription with Synthetic Promoters	134
5.3.2.1	Constitutive Promoters	136
5.3.2.2	Regulated Promoters that Are Sensitive to Added Compounds (Inducible)	137
5.3.2.3	CRISPR Interference for Transcriptional Repression	139
5.3.3	Controlling Translation	141
5.3.3.1	Ribosome Binding Sites (Cis-Acting)	141
5.3.3.2	Riboswitches (Cis-Acting)	142
5.3.3.3	Small RNAs (Trans-Acting)	143

5.4	Exotic Signals for Controlling Expression	143
5.4.1	Oxygen	144
5.4.2	Light Color	144
5.4.3	Cell Density or Growth Phase	145
5.4.4	Engineering Regulators for Altered Sensing Properties: State of the Art	147
5.5	Advanced Regulation: The Near Future	148
5.5.1	Logic Gates and Timing Circuits	148
5.5.2	Orthogonal Transcription Systems	151
5.5.3	Synthetic Biology Solutions to Increase Stability	152
5.5.4	Synthetic Biology Solutions for Cell Separation and Product Recovery	154
5.6	Conclusions	157
	Acknowledgments	158
	References	158
<b>6</b>	<b>Sink Engineering in Photosynthetic Microbes</b>	<b>171</b>
	<i>María Santos-Merino, Amit K. Singh, and Daniel C. Ducat</i>	
6.1	Introduction	171
6.2	Source and Sink	172
6.3	Regulation of Sink Energy in Plants	177
6.3.1	Sucrose and Other Signaling Carbohydrates	178
6.3.2	Hexokinases	179
6.3.3	Sucrose Non-fermenting Related Kinases	180
6.3.4	TOR Kinase	181
6.3.5	Engineered Pathways as Sinks in Photosynthetic Microbes	182
6.3.6	Sucrose	183
6.3.7	2,3-Butanediol	187
6.3.8	Ethylene	187
6.3.9	Glycerol	188
6.3.10	Isobutanol	188
6.3.11	Isoprene	189
6.3.12	Limonene	189
6.3.13	P450, an Electron Sink	190
6.4	What Are Key Source/Sink Regulatory Hubs in Photosynthetic Microbes?	191
6.5	Concluding Remarks	194
	Acknowledgment	195
	References	195
<b>7</b>	<b>Design Principles for Engineering Metabolic Pathways in Cyanobacteria</b>	<b>211</b>
	<i>Jason T. Ku and Ethan I. Lan</i>	
7.1	Introduction	211
7.2	Cofactor Optimization	212

- 7.2.1 Recruiting NADPH-Dependent Enzymes Wherever Possible 215
- 7.2.2 Engineering NADH-Specific Enzymes to Utilize NADPH 217
- 7.2.3 Increasing NADH Pool in Cyanobacteria Through Expression of Transhydrogenase 218
- 7.3 Incorporation of Thermodynamic Driving Force into Metabolic Pathway Design 219
  - 7.3.1 ATP Driving Force in Metabolic Pathways 220
  - 7.3.2 Increasing Substrate Pool Supports the Carbon Flux Toward Products 222
  - 7.3.3 Product Removal Unblocks the Limitations of Product Titer 223
- 7.4 Development of Synthetic Pathways for Carbon Conserving Photorespiration and Enhanced Carbon Fixation 225
- 7.5 Summary and Future Perspective on Cyanobacterial Metabolic Engineering 229
  - References 229

## 8 Engineering Cyanobacteria for Efficient Photosynthetic

### Production: Ethanol Case Study 237

*Guodong Luan and Xuefeng Lu*

- 8.1 Introduction 237
- 8.2 Pathway for Ethanol Synthesis in Cyanobacteria 238
  - 8.2.1 Pyruvate Decarboxylase and Type II Alcohol Dehydrogenase 238
  - 8.2.2 Selection of Better Enzymes in the Pdc-AdhII Pathway 240
  - 8.2.3 Systematic Characterization of the Pdc<sub>ZM</sub>-Slr1192 Pathway 241
- 8.3 Selection of Optimal Cyanobacteria “Chassis,” Strain for Ethanol Production 242
  - 8.3.1 *Synechococcus* PCC 6803 and *Synechococcus* PCC 7942 243
  - 8.3.2 *Synechococcus* PCC 7002 245
  - 8.3.3 *Anabaena* PCC 7120 245
  - 8.3.4 Nonconventional Cyanobacteria Species 246
- 8.4 Metabolic Engineering Strategies Toward More Efficient and Stable Ethanol Production 246
  - 8.4.1 Enhancing the Carbon Flux via Overexpression of Calvin Cycle Enzymes 248
  - 8.4.2 Blocking Pathways that Are Competitive to Ethanol 248
  - 8.4.3 Arresting Biomass Formation 249
  - 8.4.4 Engineering Cofactor Supply 249
  - 8.4.5 Engineering Strategies Guided by *In Silico* Simulation 250
  - 8.4.6 Stabilizing Ethanol Synthesis Capacity in Cyanobacterial Cell Factories 251
- 8.5 Exploring the Response in Cyanobacteria to Ethanol 253
  - 8.5.1 Response of Cyanobacterial Cells Toward Exogenous Added Ethanol 254
  - 8.5.2 Response of Cyanobacteria to Endogenous Synthesized Ethanol 255

8.6	Metabolic Engineering Strategies to Facilitate Robust Cultivation Against Biocontaminants	256
8.6.1	Engineering Cyanobacteria Cell Factories to Adapt for Selective Environmental Stresses	256
8.6.2	Engineering Cyanobacteria Cell Factories to Utilize Uncommon Nutrients	258
8.7	Conclusions and Perspectives	258
	References	259
<b>9</b>	<b>Engineering Cyanobacteria as Host Organisms for Production of Terpenes and Terpenoids</b>	<b>267</b>
	<i>João S. Rodrigues and Pia Lindberg</i>	
9.1	Terpenoids and Industrial Applications	267
9.2	Terpenoid Biosynthesis in Cyanobacteria	270
9.2.1	Methylerythritol-4-Phosphate Pathway	270
9.2.2	Formation of Terpene Backbones	272
9.3	Natural Occurrence and Physiological Roles of Terpenes and Terpenoids in Cyanobacteria	274
9.4	Engineering Cyanobacteria for Terpenoid Production	275
9.4.1	Metabolic Engineering	277
9.4.1.1	Terpene Synthases	277
9.4.1.2	Increasing Supply of Terpene Backbones	285
9.4.1.3	Engineering the Native MEP Pathway	286
9.4.1.4	Implementing the MVA Pathway	287
9.4.1.5	Enhancing Precursor Supply	288
9.4.2	Optimizing Growth Conditions for Production	289
9.4.3	Product Capture and Harvesting	291
9.5	Summary and Outlook	292
	Acknowledgments	293
	References	293
<b>10</b>	<b>Cyanobacterial Biopolymers</b>	<b>301</b>
	<i>Moritz Koch and Karl Forchhammer</i>	
10.1	Polyhydroxybutyrate	301
10.1.1	Introduction	301
10.1.2	PHB Metabolism in Cyanobacteria	302
10.1.3	Industrial Applications of PHB	305
10.1.3.1	Physical Properties of PHB and Its Derivatives	305
10.1.3.2	Biodegradability	306
10.1.3.3	Application of PHB as a Plastic	306
10.1.3.4	Reactor Types	306
10.1.3.5	Production Process	307
10.1.3.6	Downstream Processing	308
10.1.4	Metabolic Engineering of PHB Biosynthesis	308
10.1.5	Limitations and Potential of PHB Production in Cyanobacteria	310

10.2	Cyanophycin Granules in Cyanobacteria	311
10.2.1	Biology of Cyanophycin	311
10.2.2	Genes and Enzymes of CGP Metabolism	315
10.2.2.1	Cyanophycin Synthetase	315
10.2.2.2	Cyanophycin Degrading Enzymes	316
10.2.3	Regulation of Cyanophycin Metabolism	317
10.2.4	Cyanophycin Overproduction and Potential Industrial Applications	318
	Acknowledgement	319
	References	319
<b>11</b>	<b>Biosynthesis of Fatty Acid Derivatives by Cyanobacteria: From Basics to Biofuel Production</b>	<b>331</b>
	<i>Akihito Kawahara and Yukako Hihara</i>	
11.1	Introduction	331
11.2	Overview of Fatty Acid Metabolism	332
11.2.1	Fatty Acid Biosynthesis	332
11.2.2	Fatty Acid Degradation and Turnover	335
11.2.3	Accumulation of Storage Lipids	336
11.3	Basic Technologies for Production of Free Fatty Acids	337
11.3.1	Production of Free Fatty Acids in <i>E. coli</i>	337
11.3.2	Production of Free Fatty Acids in Cyanobacteria	338
11.4	Advanced Technologies for Enhancement of Free Fatty Acid Production	339
11.4.1	Enhancement of Fatty Acid Biosynthesis	339
11.4.2	Enhancement of Carbon Fixation Activity	345
11.4.3	Engineering of Carbon Flow: Modification of Key Regulatory Factors	345
11.4.4	Engineering of Carbon Flow: Deletion of Competitive Pathways	346
11.4.5	Mitigation of the Toxicity of FFAs	347
11.4.6	Enhancement of FFA Secretion	348
11.4.7	Induction of Cell Lysis	349
11.4.8	Recovery of Produced FFAs from Medium	350
11.4.9	Identification of Cyanobacterial Strains Suitable for FFA Production	350
11.5	Hydrocarbon Production in Cyanobacteria	351
11.6	Advanced Technologies for Enhancement of Hydrocarbon Production	353
11.6.1	Enhancement of Alk(a/e)ne Biosynthesis	353
11.6.2	Improvement of the Performance of Alkane Biosynthetic Enzymes	354
11.7	Basic Technologies for Production of Fatty Alcohols	355
11.8	Advanced Technologies for Enhancement of Fatty Alcohol Production	355
11.9	Basic Technologies for Production of Fatty Acid Alkyl Esters	356
11.10	Perspectives	357
	References	358

<b>12</b>	<b>Product Export in Cyanobacteria</b>	<b>369</b>
	<i>Cátia F. Gonçalves, Steeve Lima, and Paulo Oliveira</i>	
12.1	Introduction	369
12.2	Secretion Mediated by Membrane-Embedded Systems	373
12.2.1	Proteins	373
12.2.2	Extracellular Polymeric Substances (EPS)	377
12.2.3	Soluble Sugars and Organic Acids	379
12.2.4	Fatty Acids	381
12.2.5	Alcohols	382
12.2.6	Terpenes	384
12.3	MV-Mediated Secretion	386
12.3.1	Structure and Biogenesis of Bacterial MVs	386
12.3.1.1	Cyanobacterial MVs	388
12.3.2	MVs as Novel Biotechnological Tools	389
12.4	Concluding Remarks	391
	Acknowledgments	392
	References	392

### **Part III Frontiers of Cyanobacteria Biotechnology** 407

<b>13</b>	<b>Harnessing Solar-Powered Oxic N<sub>2</sub>-fixing Cyanobacteria for the BioNitrogen Economy</b>	<b>409</b>
	<i>James Young, Liping Gu, William Gibbons, and Ruanbao Zhou</i>	
13.1	Introduction	409
13.2	Physiology and Implications of Oxic Nitrogen Fixation	410
13.2.1	Ecological Range	411
13.2.2	Balancing Photosynthesis and Nitrogen Fixation	412
13.2.3	Energetic Demands and How the Cells Adapt	412
13.2.4	Impacts of Continuous Light vs Dark-Light Cycles	416
13.3	Major Biotechnology Applications for Diazotrophic Cyanobacteria	417
13.3.1	General Economic and Environmental Considerations of Diazotrophic Cyanobacteria	417
13.3.2	Metabolic Engineering of N <sub>2</sub> -Fixing Cyanobacteria for Carbon Compound Production	420
13.3.2.1	Direct Production of Biofuels	420
13.3.2.2	Cyanobacteria as a Fermentable Substrate	420
13.3.3	Metabolic Engineering of Nitrogen Fixing Cyanobacteria for Nitrogen-Rich Compound Production	422
13.3.3.1	Ammonia	422
13.3.3.2	Guanidine	423
13.3.3.3	Cyanophycin	423
13.3.3.4	Amino Acids and Proteins	423
13.3.4	Application of Diazotrophic Cyanobacteria in Agriculture	425

13.4	Conclusions	428
	References	428
<b>14</b>	<b>Traits of Fast-Growing Cyanobacteria</b>	<b>441</b>
	<i>Meghna Srivastava, Elton P. Hudson, and Pramod P. Wangikar</i>	
14.1	Introduction	441
14.2	Why Is Growth Rate Significant?	442
14.3	An Overview of Factors Affecting the Growth Rates of Cyanobacteria	446
14.3.1	Light Intensity and Quality	448
14.3.2	Mixotrophic Growth	451
14.3.3	Circadian Rhythm	451
14.3.4	Additional Factors Relating to Growth Rates in Cyanobacteria	452
14.3.4.1	Cell Morphology	453
14.3.4.2	Genome Size	453
14.3.4.3	Saltwater Tolerance	454
14.3.4.4	Nutrient Supplementation	454
14.3.5	Carbon Storage	455
14.4	Overview of the Fast-Growing Model Cyanobacteria	455
14.4.1	<i>Synechococcus elongatus</i> UTEX 2973	455
14.4.2	<i>Synechococcus elongatus</i> PCC 11801	456
14.4.3	<i>Synechococcus</i> sp. PCC 11901	456
14.4.4	<i>Synechococcus</i> sp. PCC 7002	457
14.5	Relationship Between Light Usage and Growth Rate in Model Strains	458
14.5.1	Case Study: The <i>pmgA</i> Mutant of <i>Synechocystis</i>	458
14.5.2	Case Study: The <i>S. elongatus</i> 7942 and <i>S. elongatus</i> 2973 Strains	460
14.6	Molecular Determinants of Fast Growth of <i>S. elongatus</i> UTEX 2973	460
14.7	Carbon Fluxes in Fast-Growing Strains Determined Using Metabolic Flux Analysis	463
14.8	Engineering Cyanobacteria for Fast Growth	465
14.8.1	Calvin Cycle Enzymes	465
14.8.2	PEP Carboxylase	466
14.8.3	Carbon and Light Uptake Proteins	467
14.9	Conclusion	468
	References	468
<b>15</b>	<b>Cyanobacterial Biofilms in Natural and Synthetic Environments</b>	<b>477</b>
	<i>Christian David, Rohan Karande, and Katja Bühler</i>	
15.1	Motivation	477
15.2	Introduction to Biofilms: Biology and Applications	478
15.3	Cyanobacteria in Natural Biofilms and Microbial Mats	483
15.4	Introduction to (Photo-)biotechnology	484
15.5	Benefits of Microscale Systems for (Photo-)biofilm Cultivation	487

15.6	Oxygen Accumulation and Its Impacts	488
15.7	Resource Management in Biofilms	491
15.8	Applications of Photosynthetic Biofilms	493
15.8.1	Biofilms Enable High Cell Densities	497
15.8.2	Biofilms Enable Continuous Production	498
15.9	Outlook	499
	References	499
<b>16</b>	<b>Growth of Photosynthetic Microorganisms in Different Photobioreactors Operated Outdoors</b>	<b>505</b>
	<i>Eleftherios Touloupakis and Pietro Carozzi</i>	
16.1	Background	505
16.1.1	Photobiological Hydrogen Production	506
16.1.2	Polyhydroxyalkanoate Production by Photosynthetic Microbes	508
16.1.3	Photobioreactors	509
16.2	Case Studies of Outdoor Cultivations of Photosynthetic Microorganisms	513
16.2.1	Outdoor Cultures of Purple Non-Sulfur Bacteria for H <sub>2</sub> and PHB Production	513
16.2.2	Outdoor Cultures of Cyanobacteria	516
16.3	Conclusion	517
	Acknowledgments	519
	References	519
	<b>Index</b>	<b>531</b>



## Foreword: Cyanobacteria Biotechnology

Dear reader,

This book comes on the heels of a first era of development of photosynthetic cyanobacteria as microbial catalysts. We are now poised for the beginning of a second one.

Cyanobacteria have been studied for decades as model organisms for various aspects of photosynthesis, such as water oxidation, light sensing, harvesting and conversion, regulation of the Calvin–Benson–Bassham cycle, the circadian rhythm, and nutrient starvation. Although cyanobacteria have traditionally been cultivated at large scale as supplemental foodstuffs or sources of antioxidants, it was only 20 years ago that reliable genetic engineering was developed for model strains in a demonstration of ethanol biosynthesis by *Synechococcus elongatus*. It is fitting in the foreword of such a book to ask where does the field of cyanobacteria metabolic engineering stand today?

From an industrial perspective, the use of light to power CO<sub>2</sub> fixation and conversion is attractive, as both substrates are abundant and cheap. Furthermore, microbial cells are regenerative catalysts and already a proven and accepted technology for some products. However, microbial conversion of light energy to a desired chemical comes with, to borrow from an old proverb, “many a slip ‘twixt the cup and the lip.” There is an energy loss at each step of the conversion process, including light capture, electron transport, and within the stoichiometry of the native metabolic network. Introduction of additional conversion steps to a target chemical brings more inefficiency. Thus, from the same industrial perspective that prioritizes low costs, it is clear that a potential cyanobacteria process must be made to operate near the theoretical energy conversion limit, or perhaps, the conversion limit must be increased, to ensure adoption. Metabolic engineering and biotechnology aim to elucidate the mechanisms of these inefficiencies, as well as devise and test designs for mitigating them. To date, advances in these areas are not sufficient for cyanobacteria to have widespread industrial use.

However, there is hope. As this book will show, our capabilities in studying and manipulating cyanobacteria is now highly advanced and include facile gene editing, rapid design and implementation of biosynthetic pathways, and powerful techniques for mapping metabolic fluxes and photosynthetic processes. Application of these tools within basic research continuously reveals new features of cyanobacteria

metabolism, which become new targets for optimization. The discovery of novel, fast-growing strains has shown that photosynthesis *can* power CO<sub>2</sub> uptake and cell growth at rates significantly higher than previously thought, and many metabolic engineering strategies described in this book can now be ported to such strains. Bold, novel designs for engineering light harvesting, atmospheric nitrogen fixation, and bioreactors will also lead to improvements in productivity in the medium term. Interestingly, our efforts will likely aid by advances in crop engineering, where the chloroplast metabolism is often a target. As cyanobacteria serve as models for the chloroplast of C3 plants, plant scientists are also studying (and engineering) cyanobacteria.

This book should serve as a guide for engineering cyanobacteria and is thus intended for multiple audiences. Researchers at the beginning of their careers, such as graduate students, should find the book useful to learn what has been achieved to date, and for a given specialization, where the most lucrative research lines lie. Cyanobacteria are understood well enough to make rational modifications for purpose, but mechanisms for regulation of key pathways are not yet known. This book is also useful for non-academics, such as biotechnologists seeking to exploit photosynthesis for industrial use. There are several chapters detailing metabolism of cyanobacteria for certain chemical classes, as well as descriptions of state-of-the-art methodologies for creating new strains, analyzing them, and even scale-up.

This book is divided into three parts: Core Cyanobacteria Processes, Concepts in Metabolic Engineering, and Frontiers of Cyanobacteria Biotechnology. Each chapter is written by experts and makes extensive reference to recent literature and patent filings. The initial chapters describe key metabolic processes unique to cyanobacteria, namely, CO<sub>2</sub> uptake and fixation (Chapter 1 by Hagemann et al.), the photosynthetic electron transport chain (Chapter 2 by Lea-Smith and Hanke), and light harvesting (Chapter 3 by Branco dos Santos and colleagues). In keeping with the theme of this book, these chapters also describe engineering strategies to improve efficiencies of these processes.

Key concepts in metabolic engineering of cyanobacteria are introduced in the subsequent four chapters. Xiong and colleagues (Chapter 4) give an overview of how to measure and interpret metabolic fluxes, and how this technology has revealed new aspects of cyanobacteria metabolism. Hudson (Chapter 5) reviews the state of the art of synthetic biology in cyanobacteria, a rapidly growing sub-discipline, with perspective on what should be considered for an industrial process. In Chapter 6, Ducat and colleagues describe the source-sink balance in cyanobacteria and plants, namely, how do cells sense an imbalance in energy and adapt so as to dissipate it, and can this be exploited for bio-production? In Chapter 7, Ku and Lan compile examples of metabolic engineering in cyanobacteria to derive heuristics for future engineering regarding reaction driving forces, kinetics, and stability. The middle of the book explores in more detail metabolic pathways for the biosynthesis of compounds of industrial interest. The ethanol biosynthesis pathway (Chapter 8, Luan and Lu) is the most studied, has been deployed at pilot scale, and is a model for understanding limitations in large-scale production. Terpenes (Chapter 9, Rodrigues and Lindberg), storage polymers (Chapter 10, Koch and Forchhammer), and fatty acids (Chapter 11, Kawahara and Hihara) are higher value compounds that find use outside of biofuels.

In Chapter 12, Oliveira and colleagues describe our current understanding of how cyanobacteria secrete or pump compounds outside of the cell and give perspective on future engineering of product export.

The third part begins by highlighting N<sub>2</sub>-fixing cyanobacteria (Chapter 13, Zhou and colleagues), strains that are underrepresented in the literature but have potential application for fertilizer, and production of nitrogen-rich chemicals. Chapter 14 by Wangikar and colleagues reports on recently discovered fast-growing cyanobacteria, their unique attributes in energy and carbon metabolism, and how CO<sub>2</sub> fixation and growth rate could be enhanced in other strains. The book is concluded by two chapters on cultivation technology. In Chapter 15, Bühler and colleagues describe cyanobacteria biofilms as a new format for high-density cultivation, but one that comes with unique challenges in terms of mass transfer to and from the biocatalyst. In Chapter 16, Touloupakis and Carlozzi summarize their work on outdoor cultivation of photosynthetic bacteria, where the day/night cycle causes periodicity in cell growth and, in this case, hydrogen production.

The book highlights several “grand challenges,” in cyanobacteria biotechnology that awaits a new generation of scientists and engineers. Can we achieve a high, stable partitioning of carbon away from biomass and into a product of interest? What new metabolic pathways can mitigate the energetic costs of carbon fixation and photorespiration and can these be integrated with the native metabolism? How can we expand the PAR spectrum for cyanobacteria? How can we improve tolerance to “industrial stresses,” such as high light (compounded by eventual cultivation in atmospheric CO<sub>2</sub>), salt, and bio-contaminants? What is the optimal reactor configuration that balances cost with productivity?

One theme is that drawing parallels to heterotrophic bacteria will only take us so far in engineering cyanobacteria; these microbes have unique regulation mechanisms that we must continue to elucidate. To paraphrase a summation by Ducat in Chapter 6, cyanobacteria metabolism may be evolved and optimized to anticipate fluctuating conditions and not for continuous secretion of fixed carbon as biotechnologists would like. This book is meant to inform and inspire scientists to solve such challenges. We all are looking forward to the next era of cyanobacteria engineering.

## Acknowledgments

While editing this book, I relied heavily on my research group at KTH Royal Institute of Technology. I acknowledge them for reviewing drafts and for enlightening discussions about content: Ivana Cengic, Michael Jahn, Lun Yao, Kiyon Shabestary, Markus Janasch, Johannes Asplund-Samuelsson, and Jan Karlsen. I am also grateful to Matilda Klett for advice, support, and inspiration.

## **Part I**

### **Core Cyanobacteria Processes**



## 1

## Inorganic Carbon Assimilation in Cyanobacteria: Mechanisms, Regulation, and Engineering

Martin Hagemann, Shanshan Song, and Eva-Maria Brouwer

University of Rostock, Institute of Biosciences, Department of Plant Physiology, A.-Einstein-Str. 3, D-18059 Rostock, Germany

### 1.1 Introduction – The Need for a Carbon-Concentrating Mechanism

Cyanobacteria are oxygenic phototrophs that occur in almost all photic habitats on the Earth. During their long-lasting evolution, they adapted to a wide range of abiotic conditions. Among them, the availability of the photosynthetic substrate inorganic carbon ( $C_i$ , including  $CO_2$  and dissolved inorganic carbon, mostly bicarbonate –  $HCO_3^-$ ) can fluctuate on different timescales. It is generally accepted that oxygenic photosynthesis evolved in cyanobacteria at least 2.7 billion years ago, a time when  $C_i$  was at much higher levels in the Earth atmosphere (6000 ppm) and the oceans [1–3]. Because of the activity of photosynthetic organisms, particularly after the rise of eukaryotic algae and plants, the cyanobacterial habitats became strongly depleted of  $C_i$ , accompanied by an increase in molecular oxygen ( $O_2$ ). Over the long term, cyanobacteria adapted from a  $C_i$ -rich,  $O_2$ -free atmosphere to a  $C_i$ -poor,  $O_2$ -rich atmosphere. This change had drastic consequences for  $C_i$  assimilation, in particular for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the carboxylating enzyme of the Calvin–Benson–Bassham cycle (CBBC). RubisCO is not a *bona fide* carboxylase; it evolved from an enolase in the methionine salvage pathway [4]. In CBBC, RubisCO catalyzes the activation of the acceptor molecule ribulose 1,5-bisphosphate (RuBP) by changing its enol into the reactive keto conformation; this activation allows  $CO_2$  binding to RuBP and its carboxylation. Then, the non-stable carboxylation product is hydrolytically split into two stable molecules of 3-phosphoglycerate (3PGA). Generally, RubisCO is characterized by a rather low speed and low affinity toward  $CO_2$ . Cyanobacterial RubisCO proteins, belonging to the “type 1” RubisCOs, have a particularly high  $K_m$  for  $CO_2$  of approximately 300  $\mu M$ , which is about 10-fold higher than the concentration of dissolved  $CO_2$  in water [5]. In addition, RuBP activated by RubisCO can also react with  $O_2$  in the oxygenase reaction, which leads to the formation of one-molecule 3PGA and one-molecule 2-phosphoglycolate (2PG). The latter product has inhibitory effects

on the enzymes of CBBC in plants, such as triosephosphate isomerase and sedoheptulose 1,7-bisphosphate phosphatase [6, 7]. Hence, 2PG concentrations need to be kept low; this is maintained by the 2PG salvage pathway called photorespiration (see Section 1.5). In conclusion, the long-term change in the atmospheric composition introduced problems for RubisCO, which were solved by the evolution of the carbon-concentrating mechanism (CCM, see Section 1.2) in cyanobacteria, as well as in many other photosynthetic organisms [8].

$C_i$  availability not only changes over long timescales but can also fluctuate on short timescales. The solubility of  $CO_2$  in water is mainly influenced by pH, temperature, and total ion content. In general, high temperature decreases the solubility of gases, including  $CO_2$ . It can also be dissolved in water, leading to the formation of bicarbonate and carbonate, whereby the occurrence of the different  $C_i$  species depends on pH. For example, in the slightly alkaline water of oceans, less than 1% of  $C_i$  is in the form of  $CO_2$  and most of the dissolved  $C_i$  is present as bicarbonate (87.5%). Carbonate dominates in alkaline lakes, while acidic waters preferentially contain only  $CO_2$ . The pH-dependent interconversion of the  $C_i$  species is rather slow and can be highly accelerated in biological systems by carbonic anhydrases (CA). Such enzymes, which belong to different enzymatic classes, are present in all cyanobacteria and many other photosynthetic organisms [9]. In addition to abiotic factors, the  $C_i$  availability is also strongly influenced by biotic factors, especially in eutrophic waters. In dense phytoplankton population, for example, in cyanobacterial surface scums that appear during the so-called bloom situations, the high photosynthetic activity will strongly deplete  $C_i$  and  $O_2$  will accumulate to high levels [10]. Heterotrophic organisms, for example, bacteria specifically associated with cyanobacteria, may not only provide vitamins and other growth-stimulating molecules but can also locally enrich water with  $CO_2$  released via respiration. Hence, cyanobacteria also have to acclimate to short-term variations in  $C_i$ ; hence, the response to varying  $C_i$  needs to be tightly regulated at different levels.

## 1.2 The Carbon-Concentrating Mechanism (CCM) Among Cyanobacteria

The above-mentioned problems of RubisCO due to the long-term depletion of the atmospheric  $CO_2$  and the enrichment of  $O_2$  initiated diverse adaptation mechanisms. For example, RubisCO proteins of type 1 in C3 plants increased the  $CO_2$  affinity to respond to low atmospheric  $CO_2$  partial pressure [11]. Cyanobacteria used another strategy; they did not invest in the improvement of RubisCO but instead evolved a CCM. CCM is defined as the measures that result in higher  $C_i$  affinity of the intact photosynthetic organism/cell compared to the  $CO_2$  affinity of its RubisCO. Hence, the CCM works to increase the  $CO_2$  partial pressure in the vicinity of RubisCO, which allows the enzyme to efficiently perform the carboxylation reaction and at the same time suppresses the oxygenase reaction to a large extent. The cyanobacterial CCM is a so-called biophysical CCM because it mainly depends on transport/uptake of  $C_i$  by different mechanisms, leading to high intracellular



accumulation of the charged bicarbonate, which in contrast to CO<sub>2</sub> cannot easily diffuse from the cell through biological membranes. RubisCO is confined to the carboxysome, a bacterial micro-compartment with a protein shell containing in addition CA that efficiently converts bicarbonate into CO<sub>2</sub> (Figure 1.1). Hence, RubisCO operates in a highly CO<sub>2</sub> enriched environment inside the carboxysome, which is also believed to exclude O<sub>2</sub>, thereby minimizing the competing oxygenase reaction. When the cyanobacterial CCM evolved among cyanobacteria is still a matter of discussion. One common assumption dates it to the low CO<sub>2</sub>, high O<sub>2</sub> period about 400 million years ago [12], while much earlier dates for its appearance have also been reported [13]. Because of the notion that the CCM is functionally and structurally well conserved in the majority of cyanobacterial clades, including the basal and most distant forms, it is more parsimonious to assume that the CCM evolved much earlier than 400 million years ago in the cyanobacterial radiation [14].

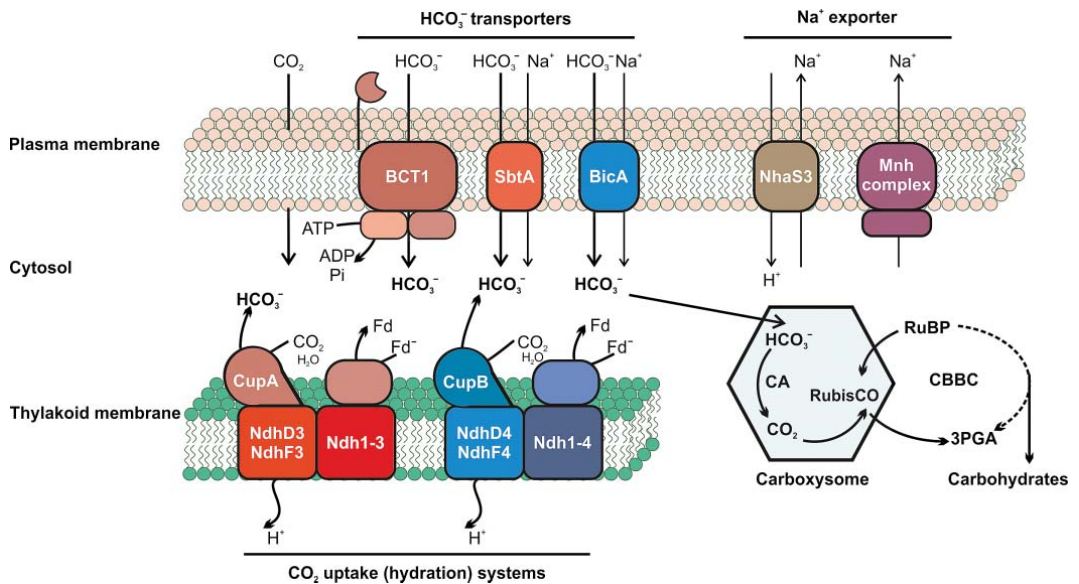
### 1.2.1 C<sub>i</sub> Uptake Proteins/Mechanisms

Over the past 30 years, five different C<sub>i</sub> uptake systems have been identified in different model cyanobacteria (Figure 1.1). Their activity allows a high accumulation of bicarbonate inside the cells, which can exceed the external amount of 100–1000-fold [15].

First, the bicarbonate transporter BCT1 has been characterized [16]. BCT1 belongs to the group of primary active ATP-binding cassette (ABC) transporters, which energize the uptake of substrates via ATP hydrolysis. In cyanobacteria, BCT1 is usually encoded by the *cmp* operon, which comprises the genes for the ATP-binding subunit, the bicarbonate pore protein(s), and a periplasmic bicarbonate-binding protein that determines the high bicarbonate affinity of the system. The *cmp* operon expression is strongly stimulated by the activator protein CmpR under low C<sub>i</sub> conditions (LC, usually ambient air), which is often encoded upstream of the structural *cmp* operon [17].

Second, the sodium-dependent bicarbonate transporter (SbtA) has been identified in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which is a high-affinity bicarbonate transporter presumably executing a bicarbonate/Na<sup>+</sup> symport [18]. The Na<sup>+</sup> dependency of SbtA has been concluded from the high stimulation of the transporter by increasing Na<sup>+</sup> amounts and its dysfunction upon mutation of Na<sup>+</sup> transport protein J (NtpJ), a protein assumed to be involved in sodium export [19]. However, it turned out that NtpJ is not involved in Na<sup>+</sup> export but instead is part of the Ktr-type K<sup>+</sup> transport system [20, 21]. Hence, whether or not Na<sup>+</sup> and bicarbonate transport are indeed directly coupled in SbtA still needs to be verified experimentally on isolated SbtA reconstituted into liposomes. Like BCT1, *sbtA* expression is highly induced under LC conditions.

Third, BicA, another widespread bicarbonate transporter, was identified in *Synechococcus* sp. PCC 7002, which belongs to the SulP family and is also believed to couple bicarbonate uptake to Na<sup>+</sup> symport [22]. In contrast to BCT1 and SbtA, the BicA transporter exhibits a more constitutive, low-affinity, but highly active bicarbonate uptake.



**Figure 1.1** Schematic presentation of cyanobacterial carbon-concentrating mechanism (CCM). The cyanobacterial CCM utilizes three bicarbonate transporters, BCT1, SbtA, and BicA, located in the plasma membrane. The two thylakoid-embedded CO<sub>2</sub> uptake (hydration) systems convert cytoplasmic CO<sub>2</sub> to bicarbonate: Ndh1-3 and Ndh1-4 comprising the cyanobacteria-specific small subunits NdhD3/4, NdhF3/4, and CupA/B. Among them, the expression of transporters BCT1, SbtA, and Ndh1-3 complex (in warm color) are strongly induced under LC conditions, while BicA and Ndh1-4 complexes (in cold color) are constitutively expressed. The Na<sup>+</sup> gradients are kept by at least two Na<sup>+</sup> export systems: NhaS3 (brown) and Mnh complex (purple). The accumulated bicarbonate penetrates into carboxysomes, where bicarbonate is dehydrated back to CO<sub>2</sub> by carbonic anhydrase (CA) in proximity to RubisCO and fixed into carbohydrates through CBBC.

In addition to the three bicarbonate transporters, two CO<sub>2</sub> uptake (hydration) systems have been identified. CO<sub>2</sub> is a gas and no direct transport protein has been characterized to date. Hence, CO<sub>2</sub> passively diffuses inside the cell driven by its partial pressure gradient, whereby the diffusion can be accelerated by specific aquaporin-like channels [23]. Inside the cell, CO<sub>2</sub> is efficiently converted into bicarbonate by two specialized NDH1-like complexes located in the thylakoid membrane [19, 24–26]. The conversion of CO<sub>2</sub> into bicarbonate fulfills two tasks; it maintains the partial pressure gradient for CO<sub>2</sub>, promoting inward diffusion, and reduces the diffusion of CO<sub>2</sub> that is not fixed by RubisCO in the carboxysome from the cell. The latter function has been demonstrated by the observation that cyanobacterial cells release CO<sub>2</sub> during the first minutes after illumination of darkened cells, a time span when RubisCO is still inactive because of missing activation via carbamylation, but carboxysome-located CA is already working [27].

The cyanobacterial NDH1-like complex is involved in different functions. In addition to CO<sub>2</sub> hydration into bicarbonate, it participates in respiration and cyclic electron transport around photosystem 1 [24, 26]. Proteomic and mutant studies revealed that these multiple tasks are fulfilled by structurally different complexes, which are associated with a different set of auxiliary subunits, leading to different complex sizes (see e.g. [25]). Among them, the two small complexes Ndh1-MS and Ndh1-MS' are specifically involved in the CO<sub>2</sub> uptake [26]. In *Synechocystis*, one has been characterized as a constitutive system with low CO<sub>2</sub> affinity named as Ndh1-4 (NDH1-MS'), while the other represents a high-affinity system named as Ndh1-3 (NDH-MS) that is highly induced under LC conditions [19, 25]. Cyanobacteria-specific small NDH1 subunits have been identified, which also play an essential role in the CO<sub>2</sub> to bicarbonate conversion [26, 28, 29]. Among them, the CupA or CupB subunits, which reach into the cytoplasm on the distal membrane part of the NDH-MS complexes, are specifically involved in the CO<sub>2</sub> uptake [19, 30–32].

Despite the impressive progress in functional and structural studies of NDH1 complexes, some questions remained open for a long time [26]. For example, the nature of the electron donor for the cyanobacterial NDH1 has been discussed because the NAD(P)H<sub>2</sub>-oxidizing subunits were never identified in isolated NDH1 complexes nor harbor cyanobacterial genome homologs for these proteins. Recently, the structures of the large NDH1 complex from *Thermosynechococcus elongatus* have been solved, which unequivocally showed that reduced ferredoxin donates electrons to the complex. Hence, the NDH1 complex among cyanobacteria is not a *bona fide* NAD(P)H<sub>2</sub> dehydrogenase complex [33]. Another open question is related to the mechanism, which permits the CO<sub>2</sub> conversion by the specialized NDH1-MS complexes. It is assumed that because of light-driven trans-thylakoid proton transport via the NdhD subunits [32], an alkaline region is created at the cytoplasmic site of the NDH1-MS complexes, which forms the predicted “alkaline pocket” driving the CO<sub>2</sub> conversion [34]. Recently, the CA EcaB was found at the thylakoid membrane, where it seems to be specifically linked to the NDH1-3 and NDH1-4 (NDH1-MS) complexes involved in CO<sub>2</sub> uptake in *Synechocystis* [35]. The authors concluded that EcaB might be involved in the proton extraction by the

NDH1-3 and NDH1-4 complexes to allow for continuous CO<sub>2</sub> to bicarbonate conversion. Moreover, the EcaB protein also seems to regulate the CO<sub>2</sub> uptake activity of these specialized NDH1 complexes [35]. The structure of the NDH-MS complex has recently been solved by cryo-electron microscopy, which permitted to gain a mechanistic understanding of the CO<sub>2</sub>-hydrating reaction performed by CupA [36].

The multiple systems involved in C<sub>i</sub> uptake among cyanobacteria seem to cooperate in the accumulation of bicarbonate inside the cell. This redundancy explains why the mutation of single bicarbonate transporters or CO<sub>2</sub> uptake systems causes usually no severe phenotype, i.e. these mutants can still grow at ambient air conditions (LC). Only under specific conditions do growth phenotypes of single mutants become visible, such as variation of the external pH to make CO<sub>2</sub> or bicarbonate the predominantly available C<sub>i</sub> form [32]. However, the combined inactivation of several transporters, including a *Synechocystis* mutant with all five C<sub>i</sub> uptake systems inactivated [37], resulted in the characteristic high CO<sub>2</sub>-requiring (HCR) phenotype. The HCR phenotype of these mutants directly supports the notion that despite the existence of an intact carboxysome, the accumulation of high cellular bicarbonate concentrations is an essential part of the cyanobacterial CCM. This has also been shown in experiments expressing a highly active recombinant CA in the cytoplasm of *Synechococcus elongatus*. The activity of this human CA collapsed the internal accumulation of bicarbonate, resulting in the HCR phenotype [38].

It should be noted that not all cyanobacteria possess all the five described C<sub>i</sub> uptake systems [39]. For example, it has been shown that *Microcystis* spp. ecotypes are characterized by variable numbers of C<sub>i</sub> transporters, especially the SbtA protein is often absent while BicA is retained [40]. Competition experiments showed that strains having both SbtA and BicA outcompete *Microcystis* spp. with only BicA under LC conditions. Consistently, *sbtA* and *bicA* genotypes of *Microcystis* strains were found predominantly during cyanobacterial bloom development, leading to C<sub>i</sub>-limiting conditions, while *bicA*-only genotypes were successful in seasons with enriched C<sub>i</sub> amounts in lakes, in agreement with its low-affinity, high-flux characteristics [41]. Picoplanktonic cyanobacteria living in the open oceans are characterized by large genome reductions. Consistently, the genomes of *Prochlorococcus* spp. and marine *Synechococcus* spp. have lost several C<sub>i</sub> uptake systems [42]. The molecular basis of C<sub>i</sub> acquisition in these ecological important cyanobacteria, which are believed to perform approximately 20% of annual CO<sub>2</sub> fixation, is not well understood and needs further investigations. Bioinformatic analysis showed that their genomes harbor many yet uncharacterized proteins belonging to protein families of characterized C<sub>i</sub> uptake systems. Hence, these proteins represent excellent candidates for the further study on new C<sub>i</sub> uptake systems among cyanobacteria [39].

### 1.2.2 Carboxysome and RubisCO

The final C<sub>i</sub> fixation by RubisCO is performed in the bacterial micro-compartments called carboxysomes (Figure 1.1). Carboxysomes are polyhedral bodies, resembling phage capsids, which became visible in all cyanobacterial cells via transmission

electron microscopy. Their specific function in CO<sub>2</sub> fixation was demonstrated in attempts to localize RubisCO and the site of <sup>14</sup>CO<sub>2</sub>-incorporation in cyanobacterial cells, both are confined to the carboxysome structures. Despite a similar function, two phylogenetically distinct carboxysomes and RubisCO types are present among cyanobacteria, distinguishing the so-called  $\beta$ -cyanobacteria and the so-called  $\alpha$ -cyanobacteria [43, 44].  $\beta$ -cyanobacteria, to which the majority of the cyanobacterial phylum belongs, harbor RubisCO proteins of the subclass 1B and  $\beta$ -carboxysomes (Ccm-type). In the model cyanobacterium *S. elongatus*, most carboxysomal proteins are encoded in one large *ccm* operon that forms a superlocus also comprising operons encoding RubisCO and some C<sub>i</sub> uptake systems [45]. The  $\alpha$ -cyanobacteria, which form a phylogenetic separate clade of picoplanktonic cyanobacteria dominated by oceanic *Prochlorococcus* spp. and *Synechococcus* spp. [46], contain RubisCO type 1A and  $\alpha$ -carboxysomes (Cso-type), which were most probably acquired via horizontal gene transfer from proteobacteria [47]. Because most cyanobacteria, including all well-investigated model strains, belong to the class of  $\beta$ -cyanobacteria, we will here shortly describe only this type.

The structure and genesis of  $\beta$ -carboxysomes were intensively characterized over the past 20 years. These carboxysomes assemble from the inside out [48]. First, hexadecameric RubisCO molecules and CA assemble together with CcmM to a pro-carboxysome, which is subsequently surrounded by the shell proteins. In the case of *S. elongatus* and *Synechocystis*, a  $\beta$ -type CA named as CcaA has been identified, which is responsible for the rapid conversion of bicarbonate into CO<sub>2</sub> inside the carboxysomes [49, 50]. Structural analyses revealed that CcaA forms well-packed trimer-of-dimers organization. Moreover, it binds with high affinity to CcmM [51]. However, the CA-type inside carboxysomes is variable among cyanobacteria [47, 52]. CcaA homologs are only found in a rather low number of cyanobacterial genomes. This implied the question, which CA is replacing the CcaA in other cyanobacterial strains. In the filamentous strain *Nostoc (Anabaena)* sp. PCC 7120, one isoform of CcmM has been shown to exhibit CA activity [53]. This CA-like domain is well conserved in cyanobacterial CcmM proteins, in addition to RbcS-like domains. The structure showed that this CcmM domain resembles  $\gamma$ -type CAs. It became obvious that CcmM-mediated CA activity seems to be more widespread among cyanobacteria than CcaA. In addition to its CA function, the CcmM protein plays a major role as a scaffold protein for the semicrystalline order of RubisCOs inside carboxysomes. Recently, the structural basis for CcmM and RubisCO interaction has been resolved, which showed unexpectedly that the RbcS-like domain of CcmM did not directly interact with RbcS in RubisCO, but CcmM rather binds to a region between RbcL dimers [54]. The RubisCO-CcmM structures are then anchored by CcmN to the inner carboxysomal shell [45].

The polyhedral protein shell of  $\beta$ -carboxysomes is mainly composed by CcmK proteins that form hexameric structures with a central pore, which is believed to facilitate the flux of bicarbonate and RuBP toward the carboxysome lumen and of 3PGA out of it [55]. CcmKs are the main constituents of the shell. In *Synechocystis*, four different CcmK proteins are present, which can be expressed to different extents under fluctuating C<sub>i</sub> levels. The variable composition of carboxysome shell

by different CcmKs is anticipated to impact the function of the carboxysome because the CcmK homologs mostly differ in sequences around the pore-forming part, which influence their size and charge and possibly provide a range of selectivity toward RuBP and 3PGA as well as possibly bicarbonate, CO<sub>2</sub> and O<sub>2</sub> [45]. However, this assumption needs experimental support, which is hampered because of the difficulty to isolate intact, functional carboxysomes from cyanobacteria. Another hexameric shell protein is CcmP, which is found in lower abundances compared to CcmKs. The CcmP protein can form stacked dimers with a central chamber and gated pores, which are believed to allow a controlled permeation of larger molecules such as RuBP and 3PGA across the carboxysomal shell [45, 56]. Finally, the pentameric shell protein CcmL forms the vertices in the icosahedral carboxysomal shell [57].

As mentioned above, mutation of genes for structural carboxysomal proteins or for CA always resulted in the HCR phenotype, which shows that the compartmentalization of RubisCO and CA inside the carboxysome is an essential part of the cyanobacterial CCM (e.g. [58]). The *Synechocystis*  $\Delta ccmM$  mutant accumulated high amounts of photorespiratory intermediates because RubisCO is exposed to much lower CO<sub>2</sub> and higher O<sub>2</sub> partial pressure than in intact carboxysomes [59].

### 1.3 Regulation of C<sub>i</sub> Assimilation

Environmental fluctuations in the available C<sub>i</sub> must be measured by cyanobacteria in order to coordinate the CCM activity and the downstream utilization of fixed carbon by the primary carbon metabolism. This regulation is achieved at different levels, including transcriptional but also posttranscriptional control. Using model cyanobacteria, the activity of the CCM and the carbon metabolism has been compared mainly under two conditions. Usually, cells grown under CO<sub>2</sub>-supplemented conditions (1–5% CO<sub>2</sub>, defined as high carbon, HC) have been challenged by shifts to ambient air, LC (0.04% CO<sub>2</sub>) conditions. This shift scenario has been selected because many mutants with defined mutations in CCM components are characterized by the HCR phenotype and can be only cultivated and characterized at HC conditions. However, the HC to LC shift protocol usually needs to include a medium change, i.e. HC-grown cells are harvested by centrifugation or filtration to remove the C<sub>i</sub>-enriched medium at time point zero before cells are then suspended in C<sub>i</sub>-poor fresh medium. This treatment disturbs the steady-state light conditions and might be stressful for the cells, i.e. it is probably more physiological to shift from LC into HC conditions, which simply involves a change in the composition of the gas stream through the cultures.

#### 1.3.1 Regulation of the CCM

The activity of the CCM is clearly higher in LC- than in HC-grown cyanobacterial cells, which is directly seen in the enhanced photosynthetic C<sub>i</sub> affinity of LC cells [34]. This change is mainly regulated at the transcriptional level, influencing the differential expression of C<sub>i</sub> uptake systems, while RubisCO and carboxysome