Chapter 38
Conclusions

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Abstract The universal impact of cyclic di-nucleotide second messengers, with the most prominent example of cyclic di-GMP, on microbial physiology and behavior has been demonstrated in a multitude of studies performed in microorganisms from the phylogenetic tree throughout. Here we address some of the still open fundamental questions in this vast research field.

Keywords Biofilm formation · Cyclic di-GMP · Life style · Motility · Turnover enzymes

Given the universal importance of the second messenger cyclic di-GMP in regulation of numerous aspects of bacterial physiology and behaviour, it had a surprisingly slow rise to prominence. Functionality for some of its turnover

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S.-H. Chou et al. (eds.), *Microbial Cyclic Di-Nucleotide Signaling*,
https://doi.org/10.1007/978-3-030-33308-9_38

proteins had already been discovered before the molecule was known, but the mechanism of action could not be concluded. Cyclic di-GMP's claim to fame started in 1987 with the identification of its role as an allosteric activator of the cellulose synthase in the fruit-degrading bacterium *Komagataeibacter xylinus* (formerly known as *Gluconacetobacter xylinus/Acetobacter xylinum*). Even long after this functionality had been unraveled, cyclic di-GMP was considered restricted to this species and its function limited to regulation of cellulose production. The full potential for global regulation of microbial physiology by cyclic di-GMP (and subsequently other cyclic di-nucleotide second messengers) was only realized after its turnover proteins had been initially identified early in the twenty-first century. Experimental (wet-lab) work and bioinformatics converged to unravel physiological functionality and to demonstrate that the enzymes involved in synthesizing and degrading cyclic di-GMP were encoded in numerous copies on most bacterial genomes. Subsequently did bioinformatic analysis also predict the first cyclic di-GMP receptor by phenotype/synteny assessment just to be experimentally confirmed soon afterwards. The rest is history and in the meantime, an ever-increasing number of processes regulated by cyclic di-GMP has been reported. In this book, the current snapshot of the still expanding breadth and depth of cyclic di-GMP second messenger signaling systems and the subsequently discovered cyclic di-AMP and cyclic GAMP (cGAMP) second messenger signaling systems has been captured and their broad impact on various aspects of bacterial physiology and metabolism has been described.

Cyclic di-GMP has been identified in most bacterial branches of the phylogenetic tree. Nevertheless, various physiological and metabolic traits that cyclic di-GMP is regulating are surprisingly similar in diverse species with promotion of biofilm formation, inhibition of different types of motility, cell cycle regulation, and inhibition of acute virulence traits as the most widespread and well-investigated examples. Similarly, cyclic di-AMP directs osmohomeostasis and cell wall remodeling in various bacteria.

Despite all the discoveries made within the field of microbial cyclic di-nucleotide signaling, fundamental questions remain open. We list some of them below.

- Compared to other nucleotide-based second messengers in bacteria, cyclic di-GMP appears to be special because a single bacterial cell typically encodes multiple enzymes to synthesize and degrade cyclic di-GMP as well as multiple effectors that can bind this molecule. What is the evolutionary advantage of this multiplicity and versatility? And, if so, why has cyclic di-GMP been specifically selected?
- In this line, so far, readily accessible bacterial organisms have mainly been investigated for cyclic di-GMP signaling. There is a need to broaden cyclic di-GMP studies to include bacterial (and archaeal) models from larger than the actual spectrum of habitats, especially covering extreme environments.
- Have we indeed discovered all true major functions of cyclic di-GMP turnover proteins? And of the turnover proteins of other cyclic di-nucleotide second messengers? Furthermore, what is the most basic role of this and other nucleotide second messenger signaling systems?
- Most bacteria maintain multiple copies of the enzymes that synthesize and degrade cyclic di-GMP, but these numbers vary vastly between species. By contrast, enzymes for making cAMP or other nucleotide-based second

messengers seem to be less numerous. So, what are the selective forces that maintain multiple enzymes?

- Each diguanylate cycase and phosphodiesterase is usually comprised of many different domains often including several N-terminal signaling domains. What is the spectrum of environmental cues and signals that regulates a specific turnover enzyme? Some cyclic di-GMP metabolizing proteins even contain antagonistic diguanylate cyclase and phosphodiesterase domains in a single protein. How are these antagonistic activities coordinated?
- Many bacteria from different branches of the phylogenetic tree possess multiple cyclic di-GMP turnover proteins. Intriguingly, cyclic di-GMP synthesizing and degrading enzymes from the same species more often than not possess and affect very different functions. What are the mechanisms that ensure specificity and link one enzyme to one particular process and insulate it from other processes? How is temporal and/or spatial regulation further achieved in the different systems? Along the same lines, are there subcellular pools of localized cyclic di-GMP within a bacterial cell?
- Cyclic di-nucleotide signaling is a highly transmissible trait frequently found on plasmids. What are the selective forces that promote dissemination of this signaling system? Also, what particular functionality serves this horizontal gene transfer and how can its advantages been assessed?
- What are the effectors that bind cyclic di-GMP and why are there so many different types of effectors? Despite the multiplicity of effectors, what evolutionary forces maintain the motility to sessility transition that has been observed in all bacteria investigated? How are different levels of cyclic di-GMP monitored by a cell and how are these different levels coordinated to affect cellular physiology and function?
- Cyclic di-GMP promotes the transition from motility to sessility on the single-cell level ultimately resulting in the formation of a multicellular biofilm. The steps from a free-swimming cell to a multicellular community have been investigated in a few model bacteria. Can these developmental programs be generalized to other bacteria? Also, how are adhesins, exopolysaccharides, and motility structures differentially regulated temporally and spatially by cyclic di-nucleotides and their individual turnover components?
- Typically, a bacterial species can form morphologically different biofilms under different growth conditions. What are the factors that regulate microbial physiology and extracellular matrix components under the different growth conditions and how do these components shape a biofilm?
- Cyclic di-GMP signaling networks are closely interconnected with other signaling systems, such as two-component systems and quorum sensing. How are these different systems interconnected in the different organisms?
- Last, but not least, due to the accumulation of knowledge, how will bioinformatics analysis aid the future extrapolation and prediction of microbial behavior?

Many of the questions above also relate to alternative, less explored cyclic di-nucleotide second messengers. In addition, many more questions certainly can be posed, and, eventually, answered.