Cyclic Di-GMP: Second Messenger Extraordinaire

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Abstract

28 Cyclic dinucleotides are highly versatile signaling molecules in both prokaryotes and eu-
29 karyotes involved in the control of various important biological processes. The best-studied
30 example is bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP). Known since the late 1980's it is now
31 recognized as near-ubiquitous second messenger in bacteria that coordinates diverse aspects
32 of bacterial growth and behavior including motility, virulence, biofilm formation or cell cycle
33 progression. In this Review, we discuss important new insights into the molecular principles
34 of c-di-GMP synthesis and degradation, and its function in cellular control and give a short
35 overview on the signaling versatility of other CDNs including c-di-AMP, cAMP-GMP and
36 cGAMP.

37

38
Introduction

39 The roles of the prototypical second messengers cAMP and cGMP have been studied for
40 over 50 years, whereas recognition of their larger relatives, the cyclic dinucleotides (CDNs),
41 lagged behind. The first CDN was discovered in 1987, when Moshe Benziman reported on
42 "*an unusual cyclic nucleotide activator*" that was able to stimulate cellulose synthase from
43 *Acinetobacter xylinum* and identified this compound as bis-(3'-5')-cyclic diguanylic acid (c-
44 di-GMP)[1]. More than 20 years later, c-di-AMP was discovered as a factor involved in DNA
45 repair in *Bacillus subtilis*[2]. Moreover, different versions of c-GMP-AMP (cGAMP) were first
46 discovered in bacteria[3] and later in mammalian cells[4] and were shown to have prominent roles
47 in bacterial virulence and the innate immune response. Despite of their chemical similarities,
48 different CDNs seem to have distinct evolutionary origins with enzymes involved in their
49 synthesis and breakdown being structurally unrelated[2,5,6]. The idea that different CDNs
50 evolved in parallel emphasizes the potency and versatility of this macrocyclic ring with two
51 purine moieties as key carrier of cellular information.

52 The discovery of CDNs has provided novel entry points into studying important biologi-
53 cal processes and cell behavior, including how bacteria coordinate their own growth and rep-
54 lication cycle, how they adapt to surfaces by forming multicellular consortia called biofilms,
55 or how pathogenic bacteria control their virulence and persistence. This was possible by first
56 identifying the enzymes involved in CDN synthesis and degradation, followed by the charac-
57 terization of specific effectors and target molecules. The CDN field is now rapidly expanding
58 into different directions exploring signaling aspects at the atomic, molecular and cellular lev-

59 els. In the past years, we have learned that CDNs are widespread and immensely versatile
60 signaling molecules that regulate cellular processes at multiple levels of control and that are
61 well integrated with other global regulatory pathways in bacteria like phosphorylation net-
62 works[7], quorum sensing[8] or with other small signaling molecules like cGMP, cAMP or
63 ppGpp[9-11]. In this Review, we discuss some of the recent advances in CDN biology without
64 the claim to be comprehensive and with a primary focus on aspects of c-di-GMP signaling. C-
65 di-GMP is not only the most widespread CDN in bacteria but so far is also the most intensely
66 studied and best understood member of this family of second messengers. We first summarize
67 properties of the core components of c-di-GMP signaling, enzymes involved in its synthesis
68 and breakdown as well as effector proteins that convert dynamic changes of c-di-GMP con-
69 centrations into specific cellular responses. We then highlight recent progress in understand-
70 ing the most prominent cellular processes regulated by c-di-GMP. While we draw some paral-
71 lels between c-di-GMP and other CDNs, c-di-AMP signaling in bacteria and the role of
72 cGAMP in the mammalian innate immune response will not be discussed in detail. Instead we
73 refer readers to some excellent and comprehensive recent reviews on CDNs and their promi-
74 nent role in bacterial physiology[2,4,12-16].

75

76 Makers and breakers

77 The c-di-GMP monomer shows 2-fold symmetry with two GMP moieties fused by a 5'-
78 3' macrocyclic ring (Fig. 1a). High-resolution structures of c-di-GMP, in solution or bound to
79 protein, indicated that the ligand exists either as elongated monomer or as condensed interca-
80 lated dimer[3,17]. At physiological concentrations c-di-GMP is a monomer in solution[4,18] argu-
81 ing that intercalated dimers form by successive binding of two molecules to specific effector
82 proteins. Cellular levels of c-di-GMP are regulated in response to environmental and internal
83 cues. This is achieved through the activity of two antagonistic enzyme families, diguanylate
84 cyclases (DGCs) and c-di-GMP specific phosphodiesterases (PDEs) (Figure 1a) with equiva-
85 lent enzyme activities being responsible for c-di-AMP metabolism (Box 2). DGCs and PDEs
86 are found in members of all major bacterial phyla, representing two of the largest known fam-
87 ilies of signaling proteins in the bacterial kingdom[2,5,6,12]. The synthesis of c-di-GMP is cata-
88 lyzed by DGCs through the cooperative action of two catalytic GGDEF domains that arrange
89 in an antiparallel fashion with one GTP molecule bound by each protomer. Pioneering struc-
90 tural and mechanistic studies with PleD, a DGC from *Caulobacter crescentus,* proposed
91 modes of substrate binding, catalytic mechanism, enzyme activation and product inhibition

92 for this class of enzymes[5,7,19-21]. A metal-catalyzed mechanism was proposed, whereby two
93 GTP molecules are positioned in an antiparallel manner to enable the formation of two inter-
94 molecular phosphodiester bonds[8,22] (Figure 1b). The requirement for dimerization conveys a
95 simple mechanism to control DGC activity by using an accessory domain that forms ho-
96 modimers in a signal-dependent manner. In the case of PleD or its *Pseudomonas aeruginosa*
97 homolog WspR this is facilitated by an N-terminal receiver domain, which dimerizes upon
98 phosphorylation[5,9-11,19,23] (Figure 1b). An alternative mechanism for the activation of DGCs
99 was proposed recently for DgcZ from *Escherichia coli*, an enzyme with a catalytic GGDEF
100 domain fused to an N-terminal zinc-binding (CZB) domain. DgcZ is a constitutive dimer with
101 its activity being allosterically regulated by the CZB domain[22] (Figure 1c). When zinc is pre-
102 sent, the GGDEF domains of DgcZ, although facing each other, are not positioned in a cata-
103 lytically competent conformation. DgcZ activation in the absence of zinc may occur via repo-
104 sitioning of the GGDEF domains to enable the productive encounter of bound substrates mol-
105 ecules (Figure 1b).
106 The arrangement of the catalytic GGDEF domains was also implicated in feedback inhi-
107 bition. Many of these enzymes are subject to non-competitive product inhibition by binding of
108 c-di-GMP to the allosteric I-site on the surface of the GGDEF domain[5,21]. In PleD or WspR,
109 an intercalated c-di-GMP dimer binds to this primary site and to a secondary binding site
110 thereby immobilizing the GGDEF domains in a non-productive state[19,23] (Figure 1b). Product
111 inhibition of DGCs may establish precise cellular threshold concentrations of c-di-GMP or
112 contribute to the reduction of stochastic perturbations and increased stability of c-di-GMP
113 networks by maintaining c-di-GMP levels in defined concentration windows[21]. While a func-
114 tional connection between the I-site and product inhibition is clearly established, c-di-GMP
115 binding to some GGDEF domains may also serve other purposes like protein-protein interac-
116 tion[24] (see chapter on receptors, below).
117 Structurally and mechanistically distinct c-di-GMP specific PDEs have been described
118 that are based on EAL and HD-GYP domains, respectively. EAL-type PDEs hydrolyze c-di-
119 GMP in the presence of Mg^{2+} or Mn^{2+} to yield the linear pGpG dinucleotide[25]. EAL domain-
120 containing proteins are active as dimers[26,27]. But in contrast to DGCs, where the fusion of two
121 GTP molecules requires a dimer arrangement of the enzyme, the necessity of this quarternary
122 arrangement for PDE catalysis is not intuitive. Recent structural studies implied a regulatory
123 role for EAL dimerization. Based on distinct structural arrangements of EAL dimers, a clam-
124 shell-like opening and closing of the EAL dimer was proposed to regulate PDE activity[27,28].
125 The evolutionary conserved dimerization interface is formed by two helices, $\alpha5$ and $\alpha6$, with

126 α5 directly connecting via the β5-α5 loop (loop 6) to two central Asp residues that coordinate
127 the metal ions in the active site[26,27,29] (Figure 1d). Structural and biophysical studies revealed
128 that the α5-loop6 region undergoes substantial rearrangements during the clam-like move-
129 ments of the EAL dimer, indicating that this part of the protein may play a hinge-joint-like
130 role to couple EAL conformation to catalytic activity via the positioning of metal ions in the
131 active site[27,28]. Consistent with this, accessory domains known to control PDE activity directly
132 communicate with the EAL dimerization region[28]. The observation that substrate binding can
133 induce EAL dimerization and also determines the conformation of the α5-loop6 region pro-
134 posed a bidirectional allosteric communication between EAL domains and regulatory do-
135 mains with the α5-loop6 region serving as central communication platform[27-29]. Interestingly,
136 EAL domain-containing proteins that have adopted roles as c-di-GMP effectors seem to ex-
137 ploit similar c-di-GMP–mediated dimerization and α5-loop6 remodeling to regulate cellular
138 processes[30] (see below).
139 A second, unrelated family of c-di-GMP-specific phosphodiesterase harbors conserved
140 HD-GYP domains[31]. Recently, the first structure of an active HD-GYP PDE was solved im-
141 plicating a novel trinuclear iron-binding site in catalysis[32]. While EAL-based enzymes convert
142 c-di-GMP into the linear product pGpG, HD-GYP hydrolyzes c-di-GMP in a one-step reac-
143 tion to yield two molecules of GMP[32]. Thus, for bacteria that lack HD-GYP-domain proteins
144 it remained unclear how pGpG is further catabolized into GMP. This puzzle was solved re-
145 cently by demonstrating that the oligoribonuclease Orn, a ribonuclease hydrolyzing two- to
146 five-nucleotide-long RNAs, is the primary enzyme capable of removing pGpG[33,34].
147 Despite detailed knowledge on structure and function of DGCs and PDEs, it has re-
148 mained challenging to assign physiological roles to individual enzymes under laboratory con-
149 ditions. Genetic studies often fail to disclose clear phenotypes. Since only few specific input
150 signals have been identified for these enzymes so far, this may be due to the limited physio-
151 logical conditions that are assayed in the laboratory. Evidence for this was provided by a re-
152 cent study of PDEs in *E. coli*. Despite of a total of 13 PDEs being encoded in the genome of
153 this organism, only PdeH is able to reduce c-di-GMP levels and license motility in growing *E.*
154 *coli* cells[35,36] (see below). The observations that most PDEs are readily expressed and that a
155 large fraction of these enzymes can be genetically activated to substitute for PdeH in motility
156 control implied that most of these enzymes simply lack the appropriate stimuli under labora-
157 tory conditions[37]. DGCs and PDEs also engage in downstream signaling through direct inter-
158 actions with their target molecules thereby providing a platform for “spatially localized” con-
159 trol of cellular processes[24,38,39]. Within such supra-molecular complexes, these proteins not

160 only regulate the synthesis and degradation of c-di-GMP, but can also act as “c-di-GMP sen-
161 sors” to control neighboring partner proteins[39].

162

163 C-di-GMP receptors

164 While "makers and breakers" explain how c-di-GMP levels are controlled in time and
165 space, c-di-GMP pathways ultimately hinge on the respective effectors that bind c-di-GMP
166 and on their downstream targets, cellular components that are regulated by specific c-di-GMP
167 effectors. Given the global influence of c-di-GMP on bacterial cell physiology and given the
168 sheer abundance of DGCs and PDEs in some bacteria, it must be assumed that a large number
169 of such effectors and cellular targets exist. Several families of effector proteins and RNAs
170 have been identified and are now well characterized structurally and functionally[40]. This in-
171 cludes mRNA riboswitches[41], transcription regulators[42], proteins containing PilZ domains, a
172 small prototypical c-di-GMP binding unit[36,43,44] and proteins harboring degenerate GGDEF
173 and EAL domains[45]. With one of these examples the field has recently come full circle. The
174 discovery of c-di-GMP goes back to the observation that c-di-GMP activates the membrane-
175 bound BcsAB cellulose synthase complex in *Gluconacetobacter xylinus* thereby boosting the
176 production of this exopolysaccharide matrix component[1]. The availability of the structure of
177 the BcsAB complex now revealed an elegant mechanism, whereby c-di-GMP binding to the
178 C-terminal PilZ domain of BcsA releases autoinhibition of the glycosyltransferase activity to
179 activate the complex[46] (Figure 1e). This example illustrates how c-di-GMP effectors such as
180 PilZ or the newly discovered YajQ protein family[47] can act as versatile adaptors that link c-di-
181 GMP signal input to the activity of enzymes complexes or transcription factors.

182 The discovery that c-di-GMP binds to a range of transcription factors such as members of
183 the response regulator or CRP/FNR families in a way that was not predictable from protein
184 sequence, argued for a more versatile nature of effector-ligand interactions[48-50]. This is sup-
185 ported by the identification and characterization of a range of novel c-di-GMP effectors, an
186 endeavor that was greatly aided by the introduction of innovative high-throughput methods
187 and biochemical techniques (Box 1). One of the most exciting recent discoveries is the emer-
188 gence of ATPases as molecular targets of c-di-GMP. The first example is FleQ, a bacterial
189 enhancer-binding protein from *P. aeruginosa.* While members of this family of transcription
190 factors are normally activated by phosphorylation, FleQ activity is controlled by c-di-GMP[51].
191 Structural studies revealed that the second messenger interacts with the AAA+ ATPase do-
192 main of FleQ at a site distinct from the ATP binding pocket. Binding of c-di-GMP obstructs

FleQ ATPase activity, thereby altering its quaternary structure and transcriptional activity[50]. Similarly, c-di-GMP specifically binds to MshE, an AAA$^+$ ATPase involved in the assembly of mannose sensitive hemagglutinin (MSHA) pili in *Vibrio cholerae*[52,53]. The observation that HxrA, a MshE homolog and type 2 secretion (T2S) ATPase from *P. aeruginosa* also specifically binds c-di-GMP opened up the exciting possibility that this general protein secretion pathway that employs a pilus-like extrusion mechanism might also be controlled directly by c-di-GMP[52]. The idea that c-di-GMP might have taken a more global control over bacterial protein secretion is reinforced by some recent observations indicating that this second messenger also controls Type 6 (T6S)[54] as well as Type 3 secretion (T3S)[55]. While the exact role of c-di-GMP in T6S is yet unclear, its influence on T3S seems to be direct and again mediated via a central ATPase. It was shown that the flagellar export ATPase FliI from a range of distantly related bacteria specifically bind c-di-GMP[55]. Binding of c-di-GMP to FliI and to its homolog HrcN from the virulence related T3SS inhibits ATPase activity arguing that it directly interferes with flagellar export and T3S. The authors of this study proposed that the c-di-GMP binding arrangement might be widely conserved among the rotary export ATPases, making the second messenger central to the function of many of these secretion proteins. It will be interesting to compare the c-di-GMP binding mode of the individual members of this family once structural information is available. Finally, sensor histidine kinases, the central components of phosphorylation pathways in bacteria, have also been identified as c-di-GMP targets. The histidine kinase CckA from *C. crescentus* was shown to bind c-di-GMP via its catalytic and ATPase domain, thereby shifting the kinase/phosphatase balance of this bifunctional enzyme[7] (see below). The discovery that ATPases serve as regulatory hubs for c-di-GMP may reflect on the global role of c-di-GMP in monitoring bacterial cell physiology. ATPases often function as central regulatory switches governing key cellular processes. Apparently, c-di-GMP leverages part of its global influence by seizing control over these essential cellular players.

Physiological roles of c-di-GMP

Development and morphogenesis

Several bacteria make use of c-di-GMP to control morphogenesis and developmental transitions. This includes *Caulobacter crescentus*, an aqueous organism with an inherently asymmetric life cycle. *C. crescentus* produced two specialized progeny cells during each division cycle, a motile swarmer (SW) and a sessile stalked cell (ST). Predivisional cells of *C.*

226 *crescentus* are highly polarized with a stalk and adhesive holdfast exposed at one cell pole
227 and a flagellum, pili and a chemotaxis apparatus assembled at the opposite pole. While the
228 surface attached ST progeny re-initiates chromosome replication (S-phase) and cell division
229 (G2-phase) immediately following division, the newborn SW cell is motile but blocks replica-
230 tion throughout an extended period (G1-phase). Replication and division resume when the
231 SW cell differentiates into a ST cell, a process during which it ejects its flagellum, retracts its
232 pili and replaces them with a holdfast and a stalk[56] (Figure 2). Recent studies identified c-di-
233 GMP as a major driver of *C. crescentus* pole morphogenesis and cell cycle control[7,57,58]. Mu-
234 tants unable to synthesize c-di-GMP lost all polar appendages and showed striking cell mor-
235 phology aberrations[58]. Levels of c-di-GMP oscillate during the *C. crescentus* cell cycle with
236 trough values in the motile SW, a peak during the swarmer-to-stalked cell transition and in-
237 termediate concentrations during division[58,59] (Figure 2). The increase in c-di-GMP concen-
238 tration during the SW-to-ST transition is produced primarily by PleD, a DGC that is activated
239 when cells enter S-phase[58,60]. PleD activity is confined to the ST cell by two antagonistic his-
240 tidine kinases, PleC and DivJ, which position to opposite poles of dividing cells and differen-
241 tially segregate into SW and ST progenies (Figure 2). While PleC acts as phosphatase keeping
242 PleD~P levels low in SW cells, DivJ acts as kinase to impel PleD phosphorylation in ST
243 cells[60]. Counteracting PDEs are thought to keep c-di-GMP levels low in the motile SW cell.
244 One of these, PdeA, localizes to the flagellated pole before division and later partitions into
245 the newborn SW cell where it authorizes motility by keeping c-di-GMP levels low. PdeA is
246 removed by specific proteolysis during the SW-to-ST transition coincident with PleD activa-
247 tion, thereby contributing to the sharp upsurge of c-di-GMP at this stage of the cell cycle[57].
248 But how does c-di-GMP oscillation instigate the exact timing of *C. crescentus* cell cycle
249 events? The TipF-TipN pathway regulating flagellar polarity illustrates such an example. Up-
250 on binding of c-di-GMP, TipF localizes to the pole opposite of the stalk where it connects
251 with its polar receptor, the birth scar protein TipN[61]. TipF then recruits flagellar proteins to
252 this subcellular site to initiate flagellar assembly in the predivisional cell. TipF is stable when
253 bound to c-di-GMP but is rapidly degraded when c-di-GMP levels drop in the SW cell. Re-
254 moval of TipF was proposed to reset the flagellar polarization state and to avoid misposition-
255 ing of the flagellar motor at the incipient stalked cell pole[61] (Figure 2). Recent studies also
256 linked c-di-GMP oscillations to the G1-S cell cycle transition and chromosome replication
257 control. The transcription factor CtrA acts as inhibitor of replication initiation in *C. crescen-*
258 *tus*. CtrA is phosphorylated and active in swarmer cells (G1) where it binds to the origin of
259 replication (Cori) to block replication initiation[56]. During differentiation into ST cells, CtrA is

260 inactivated to license replication start. CtrA activity is controlled by the bifunctional cell cycle
261 histidine kinase CckA, which phosphorylates CtrA through the phosphotransfer protein ChpT.
262 CckA exhibits kinase activity in the SW cell but adopts strong phosphatase activity during the
263 G1-S transition, thereby reversing the phosphate flux through the CckA-ChpT-CtrA cascade
264 and inactivating CtrA. Concurrent with its dephosphorylation, CtrA is degraded by the ClpXP
265 protease[56]. Both dephosphorylation and degradation of CtrA are controlled by the c-di-GMP
266 upshift during G1-S. While degradation is mediated by the ClpXP protease adaptor PopA,
267 which binds to c-di-GMP and delivers CtrA to the protease[45,62,63], CtrA inactivation results
268 from c-di-GMP directly interfering with the CckA kinase-phosphatase switch. Biochemical
269 and structural studies demonstrated that c-di-GMP binds to CA domain of CckA, thereby in-
270 hibiting its default kinase activity and stimulating phosphatase activity (Figure 2)[7]. In addition
271 to adopting a cyclin-like role to drive G1-S, c-di-GMP imposes spatial control on CckA dur-
272 ing division to install asymmetric replication of future daughter cells. In predivisional cells
273 CckA positions to opposite cell poles, adopting kinase and phosphatase activity at the flagel-
274 lated and stalked pole, respectively. This leads to a gradient of CtrA~P in the cell and to
275 asymmetric replication initiation with the Cori at the ST pole being activated before cell divi-
276 sion is completed, while the Cori at the flagellated pole remains inactive[64,65]. The unequal dis-
277 tribution of c-di-GMP was proposed to control differential activity of CckA at opposite poles.
278 While the bulk of dividing cells experiences high levels of c-di-GMP, a microenvironment
279 with low levels of c-di-GMP was proposed to promote CckA kinase activity at the flagellated
280 pole[7] (Figure 2). The authors of this study proposed that CckA sequestration to the flagellated
281 pole could shield the protein from the cellular pool of c-di-GMP. How such a low c-di-GMP
282 microenvironment is organized and which PDEs are involved in this spatial control remains to
283 be shown.

284 Asymmetric distribution of c-di-GMP during cell division was also observed in other
285 bacteria arguing that this might represent a general principle controlling cell behavior and/or
286 reproduction[59]. For example, during the *P. aeruginosa* cell cycle, c-di-GMP levels drop dur-
287 ing a short period after cell division in the daughter cell that inherits the polar flagellum. This
288 pattern is caused by the asymmetric distribution of Pch, a PDE that during division localizes
289 to the chemotaxis machinery at the flagellated cell pole[66]. Akin to the G1 period of the *Cau-*
290 *lobacter* cell cycle, reduction of c-di-GMP at this stage of the *P. aeruginosa* division cycle
291 may promote diversity in the swimming behavior, which in turn could help to adapt to new
292 environments.

293 Streptomycetes undergo a complex life cycle with two distinct filamentous cell forms. Ger-
294 minating spores develop into vegetative hyphae, which grow into the substrate to scavenge
295 nutrients. Upon nutrient depletion aerial hyphae are formed, which eventually differentiate in-
296 to long chains of spores[67]. Recently, c-di-GMP was found to have a key role in the transition
297 from vegetative mycelial growth to the formation of a reproductive aerial mycelium[42]. Dele-
298 tion of genes encoding proteins involved in c-di-GMP metabolism had a notable effect on
299 colony morphology and development [67]. Moreover, increasing internal levels of c-di-GMP
300 blocked development, while depleting c-di-GMP caused premature spore production bypass-
301 ing the formation of aerial hyphae[42]. Premature sporulation is also observed in mutants lack-
302 ing BldD, the master regulator of *Streptomyces* development that represses a global regulon of
303 ~170 sporulation genes[67]. Recently, a direct connection between these two key components of
304 developmental control was identified when BldD was shown to be a c-di-GMP effector pro-
305 tein that represses its target genes in a manner that depends on its binding to c-di-GMP[42]. A
306 drop in cytoplasmic c-di-GMP levels, which causes the BldD dimer to fall apart and dissoci-
307 ate from the DNA, may then trigger BldD inactivation and sporulation. Other examples illus-
308 trating the broad impact of c-di-GMP on development and morphogenesis in bacteria include
309 *Myxococcus xanthus*[68], *Bdellovibrio bacteriovorus*[69] or cyanobacteria[70].
310

311 ***Motile-sessile transition and biofilm formation.***

312 Controlling the motile-sessile transition of bacteria is a universal feature of c-di-GMP.
313 Generally, low levels of c-di-GMP are associated with motility of individual cells, while in-
314 creased c-di-GMP concentrations direct bacteria into surface attached communities and bio-
315 films. But rather than being a simple on/off switch, complex regulatory steps seem to be in-
316 volved in a multi-stage process leading to surface colonization[71]. In line with motility being a
317 primary target of c-di-GMP, building and operating the bacterial flagellar motor is highly reg-
318 ulated. This includes regulation of flagellar gene expression[49,51], motor assembly[55,61] or motor
319 function[36,72]. While controlling flagellar gene expression is likely to be part of a long-term ad-
320 aptation strategy, tuning motor activity might be important for rapid decisions during bacterial
321 surface encounter. For example, in *E. coli* and *Salmonella enterica* increased c-di-GMP levels
322 result in flagellar obstruction by the c-di-GMP effector protein YcgR, which in its c-di-GMP-
323 bound form interacts with the flagellar rotor/stator interface[36,73] (Figure 3a). To block YcgR
324 activity and to authorize swimming these bacteria co-express the PDE PdeH together with
325 their flagellar genes. A similar mechanisms was proposed to tune motility in *Bacillus subtilis*,
326 where PdeH controls motility by preventing flagellar obstruction by the YcgR homolog

327 DgrA[74]. YcgR has high ligand affinity arguing that the flagellar motor may respond to small
328 spikes of c-di-GMP that are required to initiate surface attachment. Consecutive steps of sur-
329 face colonization may involve incremental steps of c-di-GMP increase and the sequential ac-
330 tivation of distinct cellular processes. This could be accomplished by a successive interven-
331 tion of DGCs harboring distinct levels of feedback inhibition[21] and by the activation of c-di-
332 GMP receptors with gradually reduced affinities[75]. For example, in *P. aeruginosa* different
333 DGCs, PDEs and receptor proteins are required at discrete stages of biofilm formation[71].

334 Upon surface contact, bacteria rapidly change their program, expose adhesins, activate
335 surface motility organelles and produce an extracellular matrix to protect the developing mi-
336 crocolonies. This adaptation is coordinated by c-di-GMP at the transcriptional (e.g.[76]), transla-
337 tional (e.g.[77]) and posttranslational level (e.g.[78]). For example, c-di-GMP regulates Type IV
338 pili (T4P), the prototypical surface adherence and motility organelles, in various bacteria in-
339 cluding *M. xanthus*[79], *V. cholera*[53], *P. aeruginosa*[80], *C. crescentus*[58] or *Clostridium difficile*[81]
340 (Figure 4). Likewise, in *E. coli* the production of the two principle biofilm matrix compo-
341 nents, curli fibers and cellulose, is regulated by c-di-GMP[82]. During the motile-sessile switch,
342 c-di-GMP levels increase as a result of σ^S-induced expression of DgcE and other DGCs and
343 the consecutive downregulation of the PDE PdeH, which acts as gatekeeper for motility and is
344 part of the large flagellar regulon[36,83] (formerly: YegE and YhjH[35]). Increased global c-di-
345 GMP levels then set in motion a local control module consisting of DgcM and PdeR, a
346 DGC/PDE pair that directly interacts with and stimulates the transcription factor MlrA, which
347 in turn activates the expression of the central curli regulator CsgD. Interestingly, the role of
348 PdeR and DgcM is not primarily a catalytic one but rather to sense the global increase in c-di-
349 GMP and in response serve as co-activators for MlrA[39]. CsgD then mediates transcription of
350 curli genes and at the same time induces the expression of DgcC, the primary DGC to allo-
351 sterically activate cellulose production via the cellulose synthase complex (Figures 1e, 3b)[46].
352 This is a prime example of how different levels of the c-di-GMP network are interconnected
353 to generate highly flexible and expanding responses, which in this case enable for differential
354 tuning of individual matrix components. An alternative exopolysaccharide, poly-beta-1,6-N-
355 acetyl-glucosamine (PGA) can promote *E. coli* surface adherence and biofilm formation.
356 PGA biogenesis and secretion requires the Pga complex (PgaA-D) and its allosteric activation
357 by c-di-GMP. Both the *pgaABCD* operon and two DGCs, DgcT and DgcZ, are controlled by
358 Csr, a global regulatory system that mediates *E. coli* virulence and biofilm formation[84]. Re-
359 cent findings indicated that c-di-GMP activates the Pga machinery by binding directly to both

360 PgaC and PgaD, the two inner membrane components of the Pga complex to stimulate their
361 glycosyltransferase activity[78] (Figure 3c).
362 While the processes driving biofilm formation are relatively well understood, mecha-
363 nisms underlying biofilm dispersal have remained understudied. Given the prominent role of
364 c-di-GMP in biofilm formation, careful control of the second messenger must also be linked
365 to active biofilm dispersal[85]. Such an escape mechanism was identified in *Pseudomonas fluo-*
366 *rescens*, where the LapA surface protein mediates surface adhesion and stabilization of bio-
367 films[86]. At high c-di-GMP levels, c-di-GMP binds to LapD to help sequester the LapG prote-
368 ase in the periplasm. When c-di-GMP levels drop upon induction of the PDE RapA, LapD is
369 inactivated thereby releasing the protease to cleave the LapA adhesin and to weaken the bio-
370 film (Figure 3d).
371 As biofilms contribute to acute and chronic infections, it is not surprising that the c-di-
372 GMP network is under selective pressure in human patients. Slow growing, autoaggregative
373 *P. aeruginosa* isolates from airways of patients with cystic fibrosis were shown to harbor mu-
374 tations leading to strong activation of some of the major DGCs[87,88]. The observation that such
375 variants effectively persisted in animal models and in the presence of sub-inhibitory concen-
376 tration of antibiotics, despite of reduced growth rates *in vitro*, indicated that they may have an
377 important role in persistence during antimicrobial chemotherapy[87].
378

379 ***Role of c-di-GMP in bacterial virulence***

380 C-di-GMP modulates virulence of animal and plant pathogens[12]. Processes controlled by
381 c-di-GMP include host cell adherence, secretion of virulence factors, cytotoxicity, invasion,
382 resistance to oxidative stress, and modulation of the immune response. Importantly, recent
383 findings have linked c-di-GMP to the most prominent secretion systems for virulence factors
384 including T2SS, T3SS and T6SS[52,54,55]. This opens up the possibility that c-di-GMP interferes
385 with these processes on a more global scale. An emerging example for the importance of c-di-
386 GMP in virulence is *Clostridium difficile*. In contrast to most Gram-positive bacteria *C. dif-*
387 *ficile* encodes a large number of enzymes involved in c-di-GMP turnover [89]. In the course of
388 infections, *C. difficile* undergoes a c-di-GMP-mediated switch from a motile to a surface ad-
389 herent state with cells adhering to the intestinal mucosa via T4P and other adhesins[90]. This
390 transition is mediated by a total of 16 c-di-GMP-responsive riboswitches, 12 of which being
391 OFF switches (Type I) and four being ON switches (type II)[91]. Through these regulatory ele-
392 ments, c-di-GMP controls the expression of flagella, pili, adhesion factors and other virulence

393 factors including toxins TcdA and TcdB, the main virulence factors of *C. difficile*[81,91-94] (Fig-
394 ure 4). For example, a collagen binding protein (CBP) and its specific protease are inversely
395 controlled by Type I and II riboswitches, respectively[91]. Expression of the protease at low c-
396 di-GMP concentrations effectively prevents host cell adherence, while expression of the CBP
397 at high c-di-GMP concentrations promotes attachment to host tissue. Thus, c-di-GMP-
398 mediated riboswitches control *C. difficile* host colonization by coordinating motility, toxin
399 production, surface adhesion and biofilm formation.

400 Another example for the prominent role of c-di-GMP in virulence is the 2011 German
401 outbreak of *E. coli* O104:H4, which caused a unusually high incidence of haemolytic uraemic
402 syndrome (HUS)[95]. The genome of the causative strain showed characteristics of both enter-
403 haemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) and revealed the pres-
404 ence of a highly expressed diguanylate cyclase (*dgcX*), which is prevalent in EAEC O104:H4
405 strains[96]. This indicated that the outbreak strain and EAEC in general produce high levels of
406 c-di-GMP and likely form biofilms in the host. The observation that the *dgcX* gene is inserted
407 at the *attB* locus, the integration site for phage lambda, and is flanked by prophage elements,
408 suggested acquisition by horizontal gene transfer. The analysis of *E. coli* O104:H4 also em-
409 phasized the key importance of adaptation and regulatory flexibility of the c-di-GMP net-
410 work. While strong adherence, together with Shiga-toxin expression, is a key virulence factor
411 of *E. coli* O104:H4, this strain produces curli but is cellulose negative. The authors of this
412 study speculated that the strong pro-inflammatory effect of curli together with the absence of
413 cellulose (which normally counteracts this effect) may facilitate entry into the bloodstream
414 and kidneys where this pathogen can cause life-threatening hemolytic uremic syndrome[96].
415 Given their unique distribution in bacteria and their importance in bacterial virulence it is not
416 surprising that bacterial CDNs did not go unnoticed by the host's immune system. Recent ev-
417 idence points to a prominent role for c-di-GMP and c-di-AMP as PAMPs, pathogen-
418 associated molecular patterns that are specifically recognized by the innate immune system of
419 the host (Box 2).
420

421 Conclusion and outlook

422 Above we have summarized some of the recent findings describing mechanistic and func-
423 tional aspects of c-di-GMP signaling in bacteria. Although c-di-AMP was discovered more
424 recently, the field is picking up rapidly exposing comparable physiological complexity (Box
425 2). It is possible that additional CDNs still await their discovery offering even greater signal-

426 ing diversity by varying either the nucleotide composition or linkage chemistry. But why are
427 CDNs so prevalent in controlling important biological processes in bacteria? One major ad-
428 vantage of second messenger-based networks over other information transfer systems based
429 on protein-protein interaction might be the ease with which they are able to evolve. For ex-
430 ample, recruiting additional cellular processes into an existing c-di-GMP network seems rela-
431 tively straightforward, considering that c-di-GMP often binds on the surface of pre-existing
432 protein domains with only a few amino acids contributing to ligand affinity and specificity
433 (Figure 5a). Simple recruitment of additional effectors together with the rapid expansion of
434 makers and breakers by gene duplication might thus have predisposed CDN-based regulatory
435 networks for the coordination of global metabolic and behavioral transitions in bacteria.

436 CDN based second messengers also offer various advantages in signal transduction. Their
437 rapid cellular diffusion stages an instantaneous and global internal response. At the same time
438 CDNs may act in a highly specific manner either through temporal or spatial control. For ex-
439 ample, the combination of DGCs or PDEs with distinct inhibition constants and substrate af-
440 finities, respectively, together with effector proteins or RNAs of matching c-di-GMP affinities
441 would permit cells to regulate different processes in a highly specific manner (Figure 5b). Al-
442 ternatively, spatial organization with DGCs and/or PDEs interacting directly with their re-
443 spective targets in combination with effective mechanisms isolating individual signaling
444 modules from each other would permit parallel CDN signaling modules with highly specific
445 readouts (Figure 5c). CDNs like c-di-GMP control the expression, activity, stability, localiza-
446 tion or interaction of specific proteins (e.g.[42,61,78,97]). Moreover, c-di-GMP can control the
447 same biological process at different levels including transcription, translation or allosteric
448 control (Figure 5d) (e.g.[52]). Such a multi-layered signaling architecture can impose tight con-
449 trol and continuous evaluation power over strictly unidirectional cellular processes like cell
450 cycle progression or processes with considerable metabolic cost like the motile-sessile switch.
451 It can also provide bacteria with the ability to rapidly sample the environment and to adjust
452 their behavior without the need for *de novo* protein synthesis. Or it could serve to integrate
453 two distinct processes but at the same time uncouple them if necessary by the use of distinct
454 DGC/PDE modules (Figure 5d). An example of such a process is illustrated by the production
455 of *E. coli* curli and cellulose (see above). Finally, it could be used to define activity windows
456 for specific cellular processes, for example by sequential expression control (module 1) and
457 inactivation of a downstream effector (module 2), which is either turned off by c-di-GMP or
458 subject to c-di-GMP mediated degradation.

459 Despite the advances in the CDN field, many important questions remain to be addressed
460 in the future. For example, are there additional CDNs to be included in this emerging signal-
461 ing paradigm? Which cellular activities do specific CDN networks control and how extensive-
462 ly do these compounds interfere with basic cellular processes in bacteria? What is the exact
463 architecture of CDN networks and how do they contribute to the highly dynamic behavior of
464 bacterial cells? And how do CDN-based networks integrate with other signaling networks like
465 quorum sensing, phosphorylation cascades or regulation by ppGpp? It is safe to predict that
466 this field of research will continue to provide exciting novel insights into bacterial signaling,
467 growth and behavior.
468
469

470 ## Box 1: Toolkit for CDN analysis

471 Several novel approaches were developed in the past years to identify and characterize
472 CDN effector proteins on a global scale. This included affinity pull-downs followed by sub-
473 sequent mass spectrometry analysis. Trivalent chemical scaffolds with a CDN binding, a bio-
474 tin sorting, and a crosslinking moiety were used as capture compounds in combination with
475 strepdavidin coated magnetic beads[98,99]. A similar approach used c-di-GMP coated sepharose
476 beads for affinity pull-down[100]. The advantage of these methods is that potential binding pro-
477 teins can directly be isolated from cell extracts without the need for time consuming fractiona-
478 tion or biochemical purification. Moreover, once specific binding proteins have been identi-
479 fied, such pull-down methods can also be employed for diagnostic purposes in combination
480 with specific antibodies[78]. Both methods were successfully applied with different bacteria in-
481 cluding *Pseudomonas, Caulobacter, Listeria, Streptomyces* or *Bdellovibrio*[42,98,101,102]. A more
482 indirect approach made use of the complete ORFeome and subsequent testing of cell lysates
483 with a high-throughput binding assay[52,103]. Differential radial capillary action of ligand assay
484 (DRaCALA) was developed to directly mix proteins with (radio)-labeled nucleotide(s) on a
485 nitrocellulose membrane. Upon washing the filters free ligand will diffuse away, while ligand
486 specifically bound by proteins will be immobilized at contact site[103]. Application of these
487 techniques as well as more conventional approaches like Isothermal Titration Calorimetry
488 (ITC) or Microscale Thermophoresis (MST) have led to the identification of a plethora of
489 novel effector proteins[52,55,100,104].

490 Several tools and biomarkers were established for *in vitro* and *in vivo* analysis of c-di-
491 GMP. This includes sensitive high performance liquid chromatography-coupled tandem mass
492 spectrometry (HPLC-MS/MS) to accurately determine the concentration of second messen-
493 gers in cell extracts[105,106], fluorescence-based reporters fused to c-di-GMP dependent promot-
494 ers or riboswitches[107-110], and a c-di-GMP specific FRET probe that allows direct measure-
495 ments of c-di-GMP concentrations in individual live cells[59,66].

496

497

498 ## Box 2: CDNs beyond c-di-GMP

499 The CDN c-di-AMP was discovered as a ligand bound to the N-terminal domain of the
500 DNA damage-sensing protein DisA of *B. subtilis*[2]. Biochemical studies identified this domain
501 as diadenylate cylcase (DAC), the founding member of a family of enzymes that converts
502 ATP to c-di-AMP. Specific PDEs associated with DHH-DHHA1 or HD domains hydrolyze c-
503 di-AMP into pApA or AMP[111-113]. C-di-AMP is essential in a variety of different bacteria and
504 any dysregulation causes abnormal phenotypes[114,115]. A recent report suggested that in *Lis-*
505 *teria monocytogenes* this effect is due to overshooting levels of (p)ppGpp, a global second
506 messenger linked to carbon metabolism and nutrient starvation[116]. C-di-AMP is associated
507 with a growing list of cellular functions primarily in gram-positive bacteria. These include
508 cell wall homeostasis[115,117-120], DNA integrity[2,121-123], potassium homeostasis[104,124-126] and os-
509 moprotection[127,128], gene expression[129,130], biofilm formation[131,132], sporulation[133], metabo-
510 lism[102], resistance to antibiotics[134], and, similar to c-di-GMP, cell-mediated adaptive immune
511 response (see below).

512 c-GMP-AMP is of special interest because it is produced by bacteria and metazoans[3,135].
513 Bacterial c-GMP-AMP shows 3'-3' linkage and is produced by the dinucleotide synthase
514 DncV originally identified in *V. cholera*[3]. Structural studies revealed that in the first nucleo-
515 tidyl transfer reaction DncV preferably recognizes ATP and GTP as acceptor and donor nu-
516 cleotides, respectively[136]. c-GMP-AMP is required for host colonization by *V. cholera* and for
517 exoelectrogenesis in different delta-proteobacteria[137,138]. Mammalian c-GMP-AMP (2'-3')
518 (cGAMP) has adopted a prominent role in a vertebrate innate immunity pathway responsible
519 for surveillance of cytoplasmic DNA[139]. cGAMP is synthesized by the cGAMP synthase
520 (cGAS), which is activated in response to binding cytoplasmic DNA[140,141]. cGAMP binds to
521 and activates the host receptor STING which in turn recruits TANK-binding kinase 1 (TBK1)
522 to phosphorylate IFN regulatory factor 3 (IRF3), ultimately leading to type I interferon (IFN)
523 production. Evolutionary studies recently revealed that the cGAS-STING function is con-
524 served in anemone, which diverged from the human lineage more than 500 million years ago.
525 Because Anemone cGAS produces a bacteria-like 3'-3' linked CDN that is recognized by
526 Anemone STING, it was proposed that cGAMP (2'-3') is a recent vertebrate innovation and
527 that during evolution the protein components of this pathway remained structurally conserved,
528 while chemical changes in the second messenger were driving functional innovation[142].

529 Recent evidence suggests that c-di-GMP and c-di-AMP, secreted or released outside bac-
530 teria, are also sensed by STING (stimulator of interferon genes) thereby converging with the
531 cGAS-cGAMP cytosolic DNA surveillance pathway[143-145]. Interestingly, bacteria seem to

532 have evolved strategies to dampen IFN production by avoiding STING activation. Group B
533 *Streptococcus* was recently shown to express an ectonucleotidase, CdnP, which hydrolyzes
534 extracellular bacterial c-di-AMP to attenuate the cGAS-STING axis[146].
535
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537
538

Figure legends

Figure 1: Components of the c-di-GMP signaling network. (a) Principles of c-di-GMP signaling. Enzymatic reactions are depicted as grey arrows. GGDEF, EAL and HD-GYP represent conserved catalytic domains of diguanylate cyclases and phosphodiesterases, respectively. **(b)** Schematic of DGC activation. Upper panel: phosphorylation-dependent activation of PleD from *C. crescentus*. Receiver-domains (Rec) are shown in green and GGDEF-domains in orange. Phosphorylation-induced dimerization of Rec-domain stem leads to dimerization and activation of GGDEF-domains. Lower panel: metal-dependent activation of *E. coli* DgcZ. DgcZ is a constitutive dimer. Zinc-depletion from the CZB-domain leads to competent positioning of the GGDEF-domains. **(c)** Structure of the zinc-binding diguanylate cyclase DgcZ from *E. coli* (PDB: 4H54)[22]. GGDEF-domains (orange) and zinc-binding CZB-domain (grey) are highlighted. Zinc metal ions are depicted as red spheres. C-di-GMP (magenta) binding to antipodal inhibitory I-sites (IP & IP') and GTPαS (green) binding to active sites (A & A') are shown. **(d)** Overlay of the EAL domains of the phosphodiesterase PdeL in the tight, substrate-bound (blue; PDB: 4LJ3) and relaxed (apo) conformation (grey, PDB: 4LYK)[27]. Inlet: zoom-in of the active site and conserved loop 6 region. The loop 6 conformations in the relaxed, apo (yellow) and tight, c-di-GMP-bound (orange) dimer are indicated. Yellow and magenta spheres indicate the positions of Mg^{2+}-ions in the relaxed and tight protein conformations, respectively. The conserved double-aspartic acid motif (D262, D263) and anchoring glutamate (E235), which determine the structural arrangement of loop 6 are highlighted. **(e)** Structure of *Rhodobacter sphaeroides* cellulose synthase complex with the BcsA subunit (green), its C-terminal PilZ-domain (magenta) and the BscB subunit (grey) (PDB: 4P02) [46]. The cytoplasmic membrane is outlined in grey. A dimer of c-di-GMP bound to the PilZ domain is marked.

Figure 2: Role of c-di-GMP in *C. crescentus* pole morphogenesis and cell cycle progression. A schematic of the *Caulobacter* cell cycle is shown with flagellated swarmer cells (SW, G1-phase), stalked cells (ST, S-phase) and predivisional cells (division) indicated. Polar organelles (flagellum, pili, stalk and holdfast) of individual cell types are marked. The replication status of the circular chromosome is indicated schematically with SW cells being replication silent while chromosome replication initiates in ST cells. Cell type-specific levels of c-di-GMP are as indicated. The subcellular localization of the DGC PleD, the PDE PdeA, the flagellar placement protein TipF and the sensor histidine kinases PleC, DivJ and CckA are

marked at individual stages of the cell cycle. Individual panels highlight stage-specific processes at the stalked and flagellated poles. Autophosphorylation of histidine kinases (DivJ, CckA) and phosphotransfer to response regulators (PleD, CtrA) are indicated. **a)** Flagellar assembly. TipF binds to c-di-GMP to localize to the flagellated pole, where it recruits flagellar components PflI and FliG to initiate flagellar assembly. **b)** Low c-di-GMP levels at the flagellated pole of dividing cells and in SW cells promote TipF degradation by the ClpXP protease and CckA kinase activity. The CckA kinase activates the CtrA replication initiation inhibitor by phosphorylation via the P-transfer protein ChpT. PdeA and as yet unidentified PDE(s) contribute to the reduction of the c-di-GMP concentration at this cell cycle stage. **c)** Degradation of PdeA and CtrA by the ClpXP protease during the SW-to-ST transition and at the stalked pole of the dividing cell. PleD and as yet unidentified DGC(s) contribute to the upshift of c-di-GMP upon entry into S-phase and in the predivisional cell. Activation of the protease adaptor PopA by c-di-GMP leads to the degradation of CtrA. **d)** Inactivation of CtrA by the CckA phosphatase during the SW-to-ST transition and at the stalked pole of the dividing cell. PleD and as yet unidentified DGC(s) contribute to the upshift of c-di-GMP upon entry into S-phase and in the predivisional cell. Binding of c-di-GMP causes the switch of the CckA histidine kinase from its default kinase to the S-phase-specific phosphatase state.

Figure 3: Role of c-di-GMP in biofilm formation and dispersal. Bacterial surface attachment, biofilm formation and dispersal are indicated schematically in the central panel. **(a)** c-di-GMP-mediated control of flagellar motility in *E. coli*. DGCs (orange), PDE (blue) and the c-di-GMP effector YcgR (purple) are highlighted. YcgR interacts with and curbs the flagellar motor upon binding of c-di-GMP. PdeH adopts a key role to inactivate YcgR by keeping c-di-GMP levels low thereby enabling motor function. **(b)** c-di-GMP-dependent production of amyloid curli fibers and cellulose in *E. coli*. The global (DgcE/PdeH) and local (DgcM/PdeR) modules of DGCs and PDEs controlling *csgD* transcription are indicated. DgcM/PdeR sense the global concentration of c-di-GMP and, in response, activate the transcription factor MlrA. The global transcription factor CsgD then activates the expression of curli components and of DgcC, the main activator of the cellulose synthase complex. **(c)** c-di-GMP-mediated synthesis of poly-beta-1,6-N-acetyl-glucosamine (GlcNAc) in *E. coli*. The Csr global regulatory system co-regulates the *pga* genes encoding components of the GlcNAc synthesis machinery (PgaA-D) and *dgcT* and *dgcZ* encoding two DGCs (orange) responsible for the allosteric activation of PgaCD. The histidine kinase BarA is stimulated by short-chain fatty acids and through the phosphorylation of the response regulator UvrY, activates the expression of two small RNAs,

606 CsrB and CsrC, which in turn antagonize the translation inhibitor CsrA. **(d)** Biofilm escape
607 mechanism in *P. fluorescens* Pf0-1. The LapA surface protein mediates *P. fluorescens* surface
608 adhesion and contributes to the stabilization of biofilms. Under phosphate starvation condi-
609 tions LapA is degraded by the periplasmic protease LapG resulting in biofilm escape. If
610 enough phosphate is available, LapG is sequestered by its partner LapD in its c-di-GMP
611 bound conformation. When phosphate becomes limited the RapA PDE is expressed through
612 the phosphate control system Pst/PhoRB, leading to a drop of c-di-GMP, a conformational
613 change of apo-LapD and the release of the protease.
614
615
616 **Figure 4: Role of c-di-GMP in virulence of *Clostridium difficile*.** *C. difficile* virulence is
617 regulated by c-di-GMP-specific riboswitches. Type-I riboswitches and type-II riboswitches
618 control the expression of factors involved in motility, surface attachment and virulence. Type-
619 I riboswitches (OFF-switches) inhibit translation upon c-di-GMP binding, while type-II-
620 riboswitches (ON-switches) promote translation of target genes when bound to c-di-GMP. In-
621 creasing levels of c-di-GMP stimulate the expression of adhesion factors such as type-4-pili
622 (T4P) and collagen-binding proteins (CBP) and inhibit the expression of flagellar genes and
623 the CBP protease. The gene encoding the sigma factor SigD is co-regulated with flagellar
624 genes. Thus, when the c-di-GMP concentration is low, cells not only express motility and an-
625 ti-adhesion genes, but also express the SigD-dependent Cdiff toxins TcdA and TcdB.
626
627
628 **Figure 5: General concepts of c-di-GMP signaling modules.** Effectors (E) (c-di-GMP bind-
629 ing proteins), diguanylate cyclases (DGC) and phosphodiesterases (PDE) are labeled. c-di-
630 GMP molecules are indicated as black circles or as spatial gradient in **(c)**. **(a)** Evolutionary di-
631 agram of recruiting cellular processes into an existing CDN network. Minor modifications of
632 the surface of a specific protein can mediate specific binding of c-di-GMP, which in turn can
633 modulate the protein's activity, stability or interaction with a partner. **(b)** and **(c)** Network ar-
634 chitecture involved in pathway-specific signaling. C-di-GMP-dependent processes can be
635 specifically regulated by temporal **(b)** or spatial **(c)** separation. Temporal regulation relies on
636 effector proteins with different ligand affinities and on DGCs and/or PDEs with specific inhi-
637 bition and activation constants, respectively. This allows establishing precise cellular thresh-
638 olds of c-di-GMP thereby activating specific downstream effectors and pathways. Spatially
639 separated signaling relies on some form of compartmentalization, for example with a specific

640 DGC/PDEs module interacting with its specific effector. To avoid unwanted crosstalk with
641 other effectors and cellular pathways, spatially confined modules need to be effectively insu-
642 lated. This can occur via the action of the module-specific PDE or by a general cellular PDE
643 that restrains leakage of c-di-GMP. **(d)** C-di-GMP can interfere with the same biological pro-
644 cess at different levels of control. E.g. c-di-GMP can control gene expression (transcrip-
645 tion/translation) or control the activity of one of the resulting proteins as indicated. Expression
646 and allosteric control can be mediated by the same module comprising a DGC and PDE (ar-
647 rows) or can be modulate independently by different DGC/PDEs modules.

648

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Figure 1

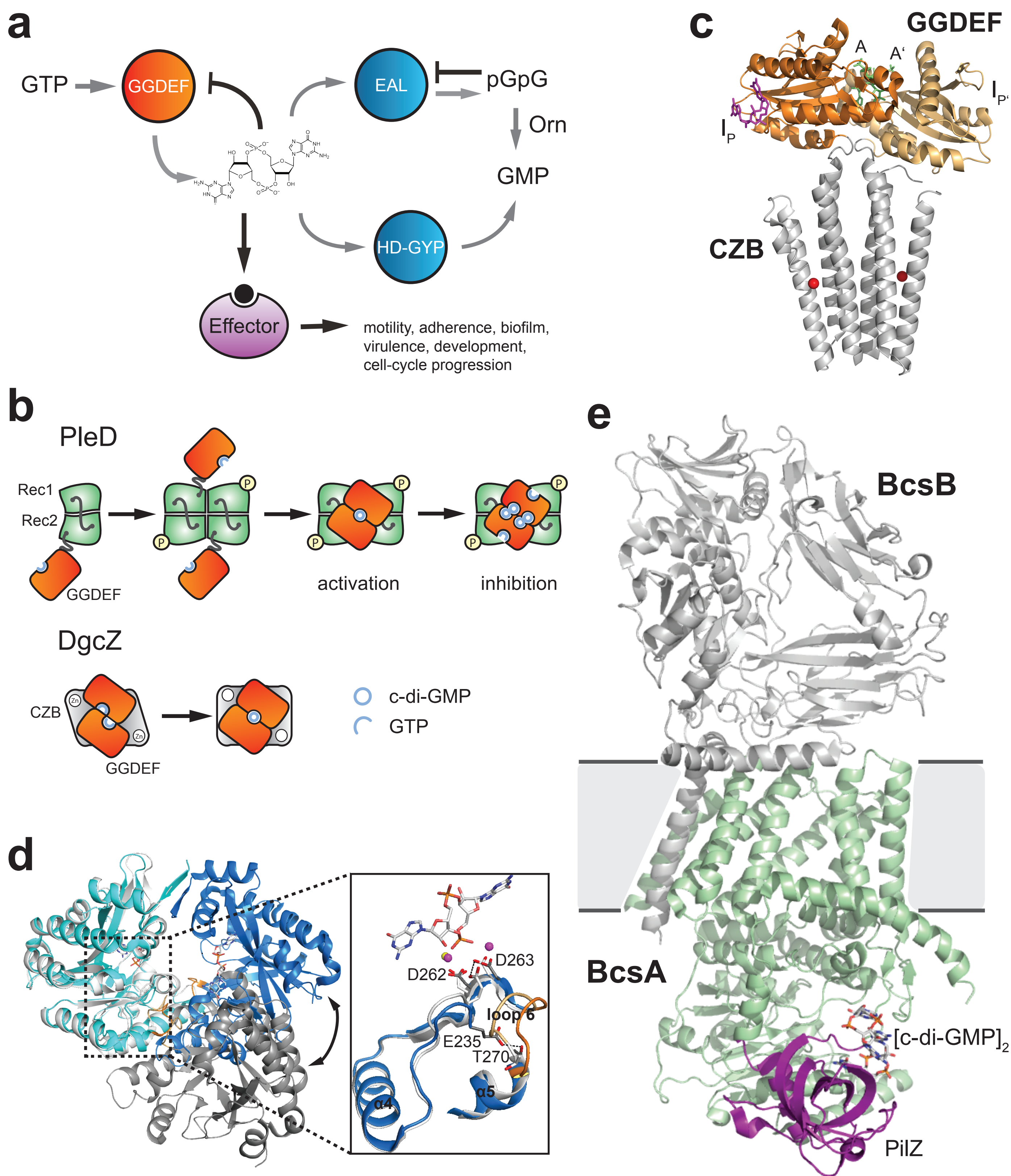

Figure 2

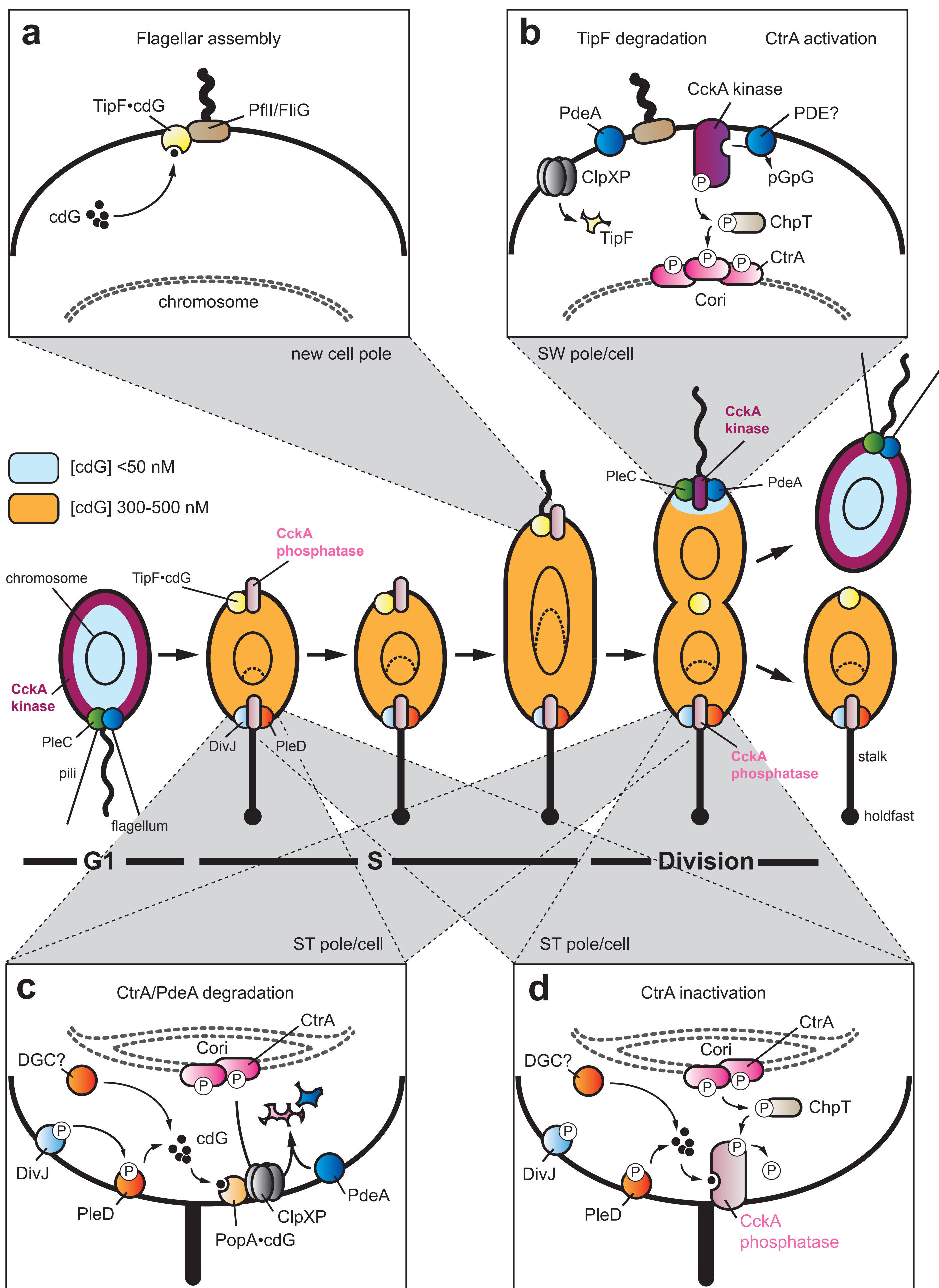

Figure 3

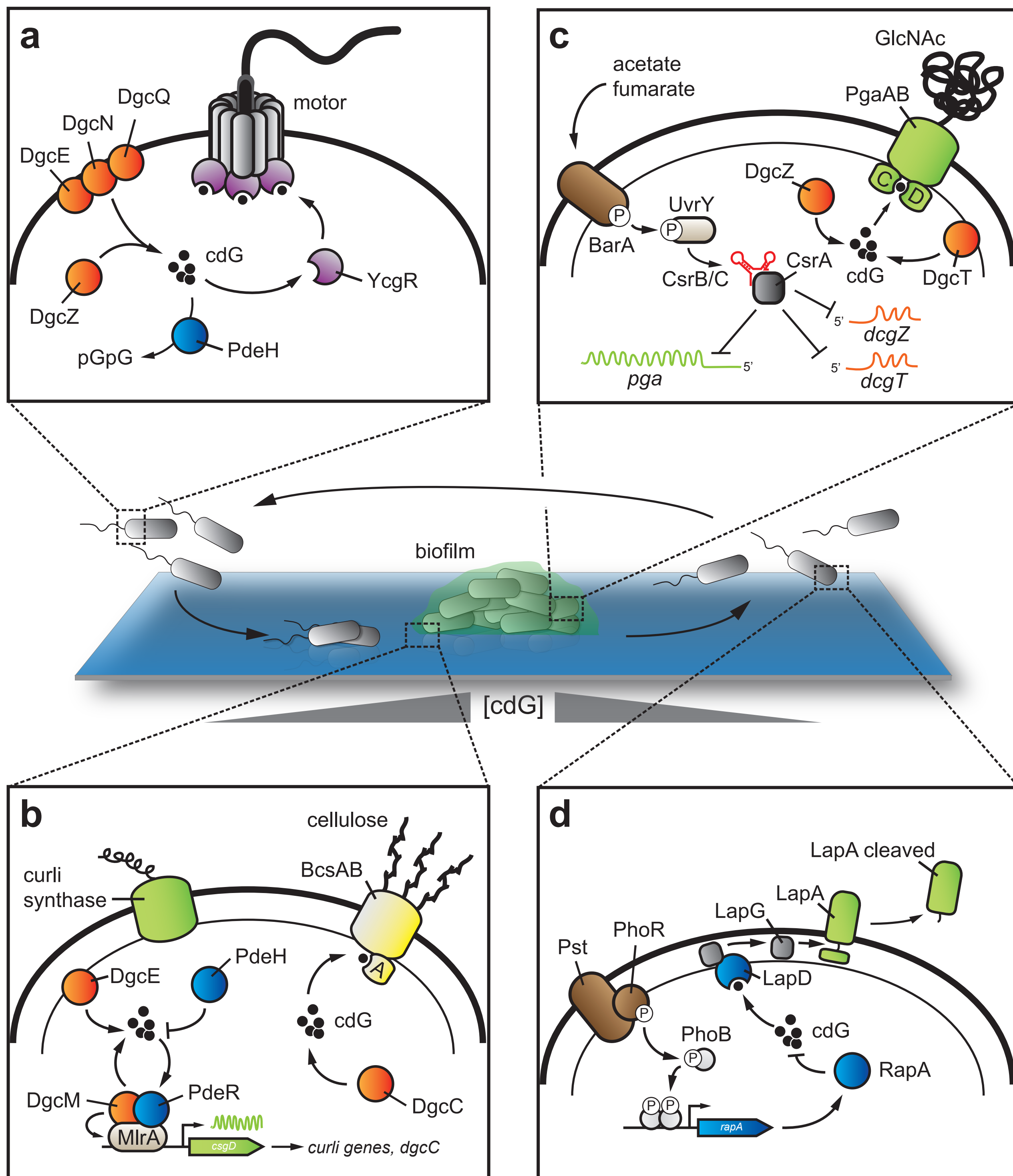

Figure 4

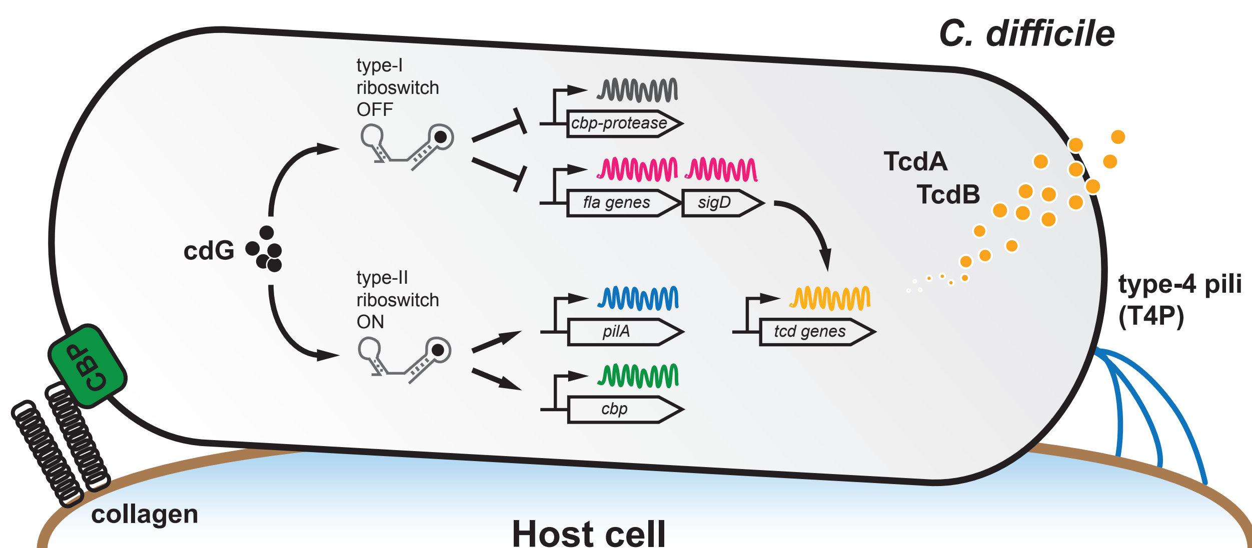

Figure 5