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Exploring the Links Between Nucleotide Signaling and Quorum Sensing Pathways in Regulating Bacterial Virulence

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Abstract

The survival of all organisms depends on implementation of appropriate phenotypic responses upon perception of relevant environmental stimuli. Sensory inputs are propagated *via* interconnected biochemical and/or electrical cascades mediated by diverse signaling molecules, including gases, metal cations, lipids, peptides, and nucleotides. These networks often comprise second messenger signaling systems in which a ligand (the primary messenger) binds an extracellular receptor, thereby altering the intracellular concentration of a second messenger molecule which ultimately modulates gene expression through interaction with various effectors. The identification of intersections of these signaling pathways, such as nucleotide second messengers and quorum sensing, provides new insights into the mechanisms by which bacteria use multiple inputs to regulate cellular metabolism and phenotypes. Further investigations of the overlap between bacterial signaling pathways may yield new targets and methods to control bacterial behavior, such as biofilm formation and virulence.

Graphical Abstract

Keywords

Quorum sensing; cyclic di-GMP; cyclic AMP; virulence; biofilm

While bacteria play essential roles in many aspects of human health, as evidenced by the growing body of work on the human microbiome,^{1–2} bacterial infections also can wreak havoc, particularly if the infectious microbes are antibiotic resistant. Currently within the

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United States, nearly 2 million people develop hospital-acquired infections each year, the majority of which are antibiotic resistant and result in nearly 100,000 deaths. In addition, antibiotic resistant infections cost the United States between 21–34 million dollars each year, resulting in a financial strain on the health care system.^{3–5} Development of new and effective antibiotic treatments requires a detailed understanding of the signaling mechanisms that bacteria use to respond to stress, which can lead to a variety of phenotypic changes that result in infections. In addition, gaining insight into the differences in sensing and signaling pathways between infectious and symbiotic bacteria may identify novel pathways that regulate cell decision-making, allowing us to potentially develop new therapeutics to target bacterial infections without harming the commensal populations.

A major challenge in our current understanding of bacterial signaling pathways is the interconnection of numerous signaling molecules. While historically there was a focus on bacterial two component signaling (sensor histidine kinase phosphorylates a response regulator that changes transcription of genes),⁶ it is now apparent that this simple depiction of linear signaling pathways is incomplete. Instead, bacteria have evolved to sense environmental inputs, often using seemingly redundant sensing proteins, and translate these inputs into the production of their own signaling molecules, which include nucleotide-based second messengers and quorum sensing autoinducers.^{7–8} Elucidating the mechanisms by which these bacterial signaling molecules control phenotypic effects and the points within downstream pathways at which the signaling molecules intersect presents a major challenge for the field. However, the results of this work have broad implications for both our fundamental understanding of bacterial physiology and for the rational identification of targets for new compounds that modulate bacterial behavior.

While the links between nucleotide second messengers and quorum sensing have been reviewed previously by the Waters and Sintim groups,^{9–10} in this Perspective, we highlight recent advances in our understanding of the interconnection of signaling pathways within Gram-negative bacteria, focusing particularly on nucleotide-based signaling molecules and quorum sensing autoinducers, and the effects on biofilm formation and other virulence-related bacterial phenotypes.

c-di-GMP and QS

Bacterial adaptation and virulence are regulated by interconnected nucleotide signaling and quorum sensing circuits. Among the most essential signaling molecules in bacteria is the second messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP).¹¹ Initially linked to the allosteric control of cellulose biogenesis in *Gluconacetobacter xylinum* over 30 years ago,¹² c-di-GMP governs an intricate regulatory network orchestrating the transition from the motile planktonic state to sessile biofilm communities.¹³ Cyclic-di-GMP is biosynthesized from two GTP molecules by diguanylate cyclases (DGCs), which contain a conserved GGDEF amino acid motif in the active site. The activity of some DGCs also is subject to allosteric inhibition *via* c-di-GMP binding to a remote site on the enzyme.¹⁴ Hydrolysis of c-di-GMP to the dinucleotide 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) is catalyzed by specific phosphodiesterases (PDEs), which are characterized by conserved EAL or HD-GYP motifs in the active site.¹³ The aforementioned active site residues are

essential for catalytic activity, and genes encoding GGDEF-, EAL-, and HD-GYP-containing enzymes are conserved across bacteria, with many species expressing multiple functional diguanylate cyclases and c-di-GMP-specific phosphodiesterases.¹¹ For example, the *E. coli* K-12 genome encodes 12 DGCs and 13 PDEs. Among these 25 proteins, six contain domains homologous to both the GGDEF- and EAL-containing families, but lack one of the two associated enzymatic activities, while one such dual-domain-containing protein exhibits both DGC and PDE activity. In addition, four proteins in *E. coli* K-12 are homologous to either the GGDEF- and/or EAL-containing families, but are devoid of both DGC and/or PDE activity.¹⁵ These enzymatically inactive proteins nonetheless mediate biofilm production and motility in *E. coli* through interactions with other proteins and small regulatory RNAs (sRNAs). In certain cases, these macromolecular interactions are controlled allosterically by c-di-GMP or GTP binding to the degenerate GGDEF or EAL site.¹¹ Moreover, catalytically competent GGDEF- and EAL-containing enzymes also engage in this complex interaction network to generate spatially-resolved pools of c-di-GMP which vary over the growth curve, facilitating activation of specific c-di-GMP-binding effectors with exquisite spatial and temporal control to mediate distinct aspects of motility and biofilm formation.^{11, 16} These effectors include both proteins structured mRNA elements known as riboswitches which undergo c-di-GMP-specific conformational changes to regulate translation of certain transcripts.¹¹ Families of cyclic-di-GMP-responsive proteins include PilZ, PelD, and FleQ effectors, along with certain enzymatically inactive GGDEF- and EAL-family proteins, as previously discussed herein.¹¹ Signaling cascades mediated by c-di-GMP are responsive to environmental factors such as oxygen availability,¹⁷ light,¹⁸ aminoglycosides,¹⁹ and nutrient deprivation.²⁰ Perception of these diverse inputs relies on various GGDEF- and EAL-family proteins, as many contain a sensory domain which enables integration of the signal *via* modulation of the associated DGC or PDE activity.¹¹

These diverse nucleotide-driven prokaryotic signaling cascades also intersect with bacterial quorum sensing (QS) networks, which involve inter- and intraspecies gene regulation *via* secreted autoinducer molecules in a cell density-dependent fashion.²¹ QS in Gram-negative bacteria relies primarily on diffusible acyl-homoserine lactones (AHLs), while Gram-positive taxa utilize cyclic peptide quorumones. In addition, 4,5-dihydroxy-2,3-pentanedione (autoinducer-2 [AI-2]) regulates quorum interactions both within and across bacterial species.²² The paradigmatic Gram-negative AHL quorum sensing system initially was discovered in *Vibrio fischeri* nearly 40 years ago,²³ and later emerged as a key facet of symbiosis between the bacteria and the *Euprymna scolopes* squid symbiont.²⁴ A single bacterial species may produce multiple AHL molecules with different acyl moieties which typically are sensed in a species-specific fashion.^{25–26} AHLs are biosynthesized by the LuxI-type enzymes, which catalyze lactonization of *S*-adenosylmethionine (SAM) followed by *N*-acylation with various fatty acids.^{27–28} The resulting AHL signal modulates QS gene expression *via* interaction with the LuxR-type transcription factors.²⁹

In contrast to the highly specific nature of AHL-mediated QS, AI-2 regulates bacterial physiology across diverse species, with over 500 bacterial taxa encoding homologs of the LuxS AI-2 synthase.³⁰ Additionally, the periplasmic LuxP- and LsrB-family AI-2 receptors are widely conserved across bacteria,³⁰ further demonstrating the significance of AI-2 as an

interspecies quorumone. AI-2 biosynthesis involves initial cleavage of SAM by LuxS to produce 4,5-dihydroxy-2,3-pentanedione,³¹ which can undergo cyclization and hydration to yield different diastereomeric tetrahydrofurans.³² These distinct forms of AI-2 bind either the LuxP- or LsrB-family receptor to regulate gene expression through a phosphorylation cascade.³⁰

Studies conducted within the past decade have begun to elucidate the interplay between c-di-GMP signaling and QS (Fig. 1). In the pandemic *Vibrio cholerae* biotype E1, distinct QS-mediated pathways regulated by the hydroxidecanone cholerae autoinducer-1 (CAI-1) and AI-2 converge to modulate biofilm formation in a c-di-GMP-dependent fashion. A random genetic reporter screen in *V. cholerae* E1 identified four putative HD-GYP c-di-GMP-specific PDEs that are induced at high cell density by the LuxR-family regulator HapR in response to a phosphorylation cascade initiated by CAI-1 and AI-2 binding to cognate sensor kinases CqsS and LuxP/Q, respectively.³³ Upon induction of PDE expression by HapR, the intracellular c-di-GMP level decreases, which attenuates transcription of the biofilm-associated *vps* gene cluster.^{33–35} In contrast, the pathogenic *V. cholerae* C1 biotype lacks the HapR transcriptional regulator, but similar antagonistic links exist between CAI-1/AI-2 QS and c-di-GMP-induced biofilm formation. In the C1 biotype, transcription of GGDEF protein Vca0930 is positively regulated by the quorum regulatory sRNAs (Qrr), which repress translation of the HapR regulator in HapR⁺ *V. cholerae* strains, further demonstrating the inverse relationship between c-di-GMP and QS.³⁶ In addition, the VieSAB QS circuit also regulates biofilm production in the *hapR*-deficient C1 strain in response to an unknown QS signal. The VieSAB system induces transcription of EAL PDE VieA, resulting in c-di-GMP hydrolysis and concomitant inhibition of biofilm production at high cell density when the unknown autoinducer is abundant.³⁷ In the related *Vibrio parahaemolyticus*, which can cause gastrointestinal illness, the *scrABC* operon modulates biofilm formation and motility by linking QS to the c-di-GMP pool. ScrA, a predicted class V pyridoxal phosphate-dependent aminotransferase, produces an unknown diffusible signal that binds the periplasmic receptor ScrB. The ScrB receptor forms a complex with bifunctional GGDEF/EAL protein ScrC, which functions as a c-di-GMP PDE in the presence of ScrAB to up-regulate swarming and inhibit production of extracellular matrix polymers.³⁸

In the opportunistic pathogen *Pseudomonas aeruginosa*, AHL-mediated QS similarly decreases the c-di-GMP concentration *via* induction of Tyr phosphatase TbpA by AHL-bound LasR. This inhibition of c-di-GMP synthesis likely occurs due to TbpA-catalyzed dephosphorylation and concomitant inactivation of GGDEF-containing DGC TbpB. In accord with these data, depletion of TbpA in *P. aeruginosa* increases the c-di-GMP level, stimulates biofilm formation, and reduces motility.³⁹ Furthermore, reducing the c-di-GMP concentration through overexpression of PDE YhjH induces transcription of both the *rhl* and *pqs* QS regulons in *P. aeruginosa* PAO1, as compared to transcript levels in PAO1 constitutively expressing the DGC WspR to elevate intracellular c-di-GMP.⁴⁰ Importantly, the *rhl* and *pqs* systems are mediated by AHL and quinolone autoinducers, respectively,^{41–43} and up-regulate transcription of several virulence factors, including rhamnolipids, PQS, and pyocyanin.^{44–45} In agreement with the gene expression analyses, production of these *rhl*- and *pqs*-controlled virulence factors increased upon depletion of c-di-GMP, resulting in

elevated pathogenesis against murine macrophages, relative to *P. aeruginosa* with increased c-di-GMP (Fig. 1).⁴⁰ Despite the antagonistic relationship between QS and c-di-GMP signaling in *P. aeruginosa*, a high-throughput screen of microbial extracts identified the cyclopentenone terrein (produced by the fungus *Apsergillus terreus*) as an inhibitor of both c-di-GMP synthesis and QS synthesis/signaling.⁴⁶ Additional experiments using QS reporter strains and LC-MS/MS quantitation revealed that terrein antagonizes the LasR and RhlR receptors in *P. aeruginosa* and inhibits production of OdDHL, BHL, and PQS²⁸ – the three major pseudomonad QS autoinducers.^{43, 47–50}

Furthermore, virulence factor production in *P. aeruginosa* is tuned by the intersecting Las, Rhl, and Pqs QS circuits in response to different starvation conditions.⁵¹ Chemical genetics studies employing QS inhibitors specific for each QS receptor have elucidated crucial functions for the Rhl and Pqs systems in activating pyocyanin, rhamnolipid, and elastase B production under phosphate and iron limitation. Conversely, LasR is dispensable for synthesis of these virulence factors upon nutrient depletion.⁵² While this study did not investigate links between *P. aeruginosa* QS and c-di-GMP, prior work in *Pseudomonas fluorescens* revealed that phosphate depletion induces expression of the c-di-GMP-specific PDE RapA via the PhoBR two-component phosphate-sensing system, resulting in attenuated biofilm formation.⁵³ Intriguingly, *P. aeruginosa* PhoB activates the Rhl and Pqs systems in response to phosphate limitation,^{54–55} suggesting that such conditions impact both QS and c-di-GMP signaling in pseudomonads. In addition, the PhoB transcription factor modulates the synthesis of c-di-GMP and AI-2 in *E. coli* in response to polyphosphate abundance.⁵⁶ These findings suggest interplay between QS and c-di-GMP in mediating the response to phosphate depletion in *P. aeruginosa* and in other opportunistic pathogens, alluding to the potential of disrupting these transduction pathways to attenuate biofilm formation and virulence factor production.

Additional studies have identified antagonistic relationships between c-di-GMP dependent regulation and different QS systems in the opportunistic pathogen *Burkholderia cenocepacia*. In this organism, virulence gene expression is regulated by AHL, as well as *cis*-2-dodecanoic acid (*Burkholderia* diffusible signal factor; BDSF).^{57–58} BDSF is synthesized by RpfF (which bears sequence similarity to enoyl-CoA hydratase) and activates virulence gene expression through activation of receptor RpfR, a multi-domain PAS-GGDEF-EAL protein.⁵⁹ Binding of the BDSF signal to the PAS domain allosterically stimulates activity of the EAL domain to modulate swarming, biofilm formation, and virulence via c-di-GMP degradation (Fig. 1).⁶⁰ BDSF-bound RpfR also induces transcription of *cepI*,³⁷ encoding *N*-octanoyl homoserine lactone (OHL) synthase,⁵⁷ demonstrating that BDSF regulates concentrations of both OHL and c-di-GMP to coordinate gene expression in *B. cenocepacia*. Electrophoretic mobility shift experiments and two-hybrid assays elucidated that RpfR activates *cepI* expression through association with the Fis-family transcriptional regulator GtrR, thereby increasing the affinity of GtrR for *cepI* promoter DNA.⁶¹ Interestingly, the intracellular concentration of c-di-GMP further regulates *cepI* transcription, as c-di-GMP binds to the RpfR EAL domain and reduces the affinity of the GtrR-RpfR complex for target promoter DNA *in vitro*, constituting a complex feedback mechanism through which BDSF down-regulates the c-di-GMP level and stimulates OHL synthesis, while c-di-GMP attenuates OHL production.⁶¹ Subsequent transcriptional

profiling experiments in *B. cenocepacia* expressing RpfR with a mutated EAL domain revealed over 100 differentially expressed transcripts relative to the WT control, demonstrating the global transcriptional impact of converging QS and c-di-GMP signals which act through receptor RpfR.⁶² Expression of QS regulons and other virulence-associated transcripts generally was down-regulated in the EAL mutant and pathogenicity was attenuated in a nematode model, indicating that accumulation of c-di-GMP represses virulence factor production in *B. cenocepacia*.⁶² While the clinical efficacy of disrupting QS and c-di-GMP signaling remains largely untested, the aforementioned studies depict the importance of understanding the antagonistic cross-talk between these fundamental signaling mechanisms to optimize anti-virulence and anti-biofilm therapies.

3',5'-cAMP and QS

QS-mediated regulation also intersects with adenosine 3',5'-cyclic monophosphate (3',5'-cAMP)-dependent signaling (Fig. 2), another important nucleotide second messenger system in numerous bacterial species. In *Escherichia coli*, 3',5'-cAMP primarily regulates carbon catabolism *via* the 3',5'-cAMP receptor protein (CRP), a transcription factor that interacts with RNA polymerase to induce expression of over 100 genes.^{63–64} The activity of adenylate cyclase (CyaA) in *E. coli* is modulated by carbon-source availability; an abundance of glucose indirectly inhibits CyaA-catalyzed 3',5'-cAMP synthesis, whereas glucose limitation activates cyclase activity and increases intracellular 3',5'-cAMP.⁶³ Stimulation of CyaA is mediated by phosphorylated glucose transport protein IIA (IIA^{glc}) which accumulates during glucose starvation, as phosphoryl-IIA^{glc} participates in phosphate transfer to glucose during uptake.⁶³ This cyclic nucleotide also mediates expression of virulence- and biofilm-associated genes in other bacterial taxa,⁶⁵ and regulates cell division in the archaeon *Halobacterium salinarum*.⁶⁶

In contrast to the generally antagonistic regulatory relationship between QS- and c-di-GMP-dependent cascades, 3',5'-cAMP positively regulates AI-2 signaling in both vibrios and pseudomonads. In the halophilic and sepsis-inducing *Vibrio vulnificus*, transcription of the AI-2-binding global transcriptional regulator SmcR (homologous to *Vibrio fischeri* LuxR)⁶⁷ is induced by the 3',5'-cAMP-CRP complex.⁶⁸ Additionally, the related pathogen *Vibrio cholerae* up-regulates production of the CAI-1 quorumone in a CRP-dependent fashion. The resulting increase in CAI-1 synthesis induces expression of *hapR*, encoding a transcriptional activator of haemagglutinin (HA)/protease. Accordingly, deletion of either *crp* or *cqsA* (encoding CAI-1 synthase) decreases production of HA/protease.⁶⁹ In addition, the reduced HapR level in the *crp* and *cqsA* mutants elevates biofilm formation and cholera toxin (CT) production,⁶⁹ in agreement with the repressive role of HapR on genes encoding exopolysaccharides and CT (Fig. 2).^{70–71} Collectively, these findings demonstrate that 3',5'-cAMP-CRP elevates CAI-1 synthesis, resulting in up-regulated HA/protease production and attenuated synthesis of CT and biofilm.⁶⁹ Thus, while inhibition of 3',5'-cAMP and/or CAI-1 signaling is an attractive method to repress HA/protease expression, such a strategy likely would stimulate production of CT and biofilm, highlighting important considerations in developing clinically effective inhibitors of virulence and biofilm formation. These studies reveal that catabolite repression modulates pathogenicity in vibrios *via* regulation of CAI-1 and AI-2 in *V. cholerae* and *V. vulnificus*, respectively. Similarly, the *lasR* promoter

in *Pseudomonas aeruginosa* contains the prototypical CRP-binding consensus sequence, and subsequent β -galactosidase reporter experiments revealed that *P. aeruginosa* Vfr (homologous to *E. coli* CRP) induces *lasR* expression, linking 3',5'-cAMP-dependent regulation to AHL signaling (Fig. 2).⁷² Interestingly, separate research identified an inverse correlation between 3',5'-cAMP and c-di-GMP levels in *P. aeruginosa*,⁷³ further demonstrating the overlapping regulatory features which tune the balance between virulence gene expression and biofilm production.

E. coli CRP also regulates QS via modulation of AI-2 signaling. However, 3',5'-cAMP-CRP inhibits AI-2 production by indirectly repressing *luxS* expression, indicating that antagonistic links exist between 3',5'-cAMP and autoinducer production in certain taxa.⁷⁴ In addition, 3',5'-cAMP-CRP directly activates transcription of the *lsr* operon, thereby increasing AI-2 uptake (Fig. 2);⁷⁴ thus 3',5'-cAMP links the intracellular AI-2 level to carbon catabolism. Intriguingly, enterohemorrhagic *E. coli* O157:H7 deficient for the *luxS* AI-2 synthase exhibit decreased swimming motility and reduced production of the translation inhibitor Shiga toxin 2 (Stx2), demonstrating important functions for AI-2 signaling and/or SAM metabolism (from which AI-2 is derived) in host colonization and virulence factor expression.⁷⁵ Additional research in *E. coli* has identified complex interactions between 3',5'-cAMP, c-di-GMP, AHL-type QS, and AI-2-mediated QS. Although *E. coli* does not produce AHL autoinducers due to the lack of a LuxI homologue, it does express a LuxR-type AHL receptor (SdiA) which can intercept AHLs produced by other taxa.⁷⁶ The SdiA regulon includes the EAL-encoding gene *ydiV*, which is induced through direct binding of SdiA to the promoter, indicating that *E. coli* modulates c-di-GMP signaling in response to extraneous AHLs in multi-species bacterial communities. Furthermore, SdiA and YdiV converge to up-regulate the 3',5'-cAMP concentration in *E. coli* through unknown mechanisms, presumably resulting in global transcriptional changes mediated by 3',5'-cAMP-bound CRP (Fig. 2). The intracellular AI-2 level also is regulated cooperatively by SdiA and YdiV, as the *sdia-ydiV* double mutant exhibits decreased expression of the AI-2 receptor *lsrR* and the *lsrACDBFG* operon involved in AI-2 uptake.⁷⁷ These discoveries reveal the convergence of multiple nucleotide and QS signaling systems in the integration of environmental and ecological factors to tune global gene expression in *E. coli*. Thus, bacterial behavior is coordinated not only by interplay between individual nucleotide and QS systems, but also by regulatory hierarchies linking different nucleotide-driven pathways and distinct QS circuits.

c-di-GMP and 3',5'-cAMP

The intersection between the nucleotide signals c-di-GMP and 3',5'-cAMP (Fig. 3) is typically antagonistic and associated with changes in biofilm formation. To date, the changes in biofilm formation has been demonstrated to typically be caused by altered transcript levels that encode for proteins involved in the various nucleotide metabolic pathways, rather than through direct binding to cNMP metabolic enzymes. For instance, in *Vibrio cholerae*, 3',5'-cAMP binding to CRP inhibits biofilm formation through repression of biofilm-associated gene expression.⁷⁸ The dysregulated genes include those encoding a number of diguanylate cyclases and c-di-GMP phosphodiesterases, as well as two transcriptional regulators (*vpsR* and *vpsT*) that control expression of the *vps* genes that encode for the

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biosynthetic machinery to produce *Vibrio* polysaccharide (VPS), a key component of biofilms (Fig. 3).^{79–80} The DGC-encoding gene *cdgA* was identified as the key target of cAMP-CRP mediated biofilm inhibition, as *cdgA* up-regulates transcription of genes encoding proteins involved in VPS biosynthesis and matrix protein production.⁸¹

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Additionally, 3',5'-cAMP and c-di-GMP cooperate to regulate expression of *gfpA* in *V. cholerae*, which encodes a protein that promotes attachment to aquatic surfaces and host epithelial cells in the intestine.⁸² Depleting c-di-GMP levels leads to increased *gfpA* transcription but requires the presence of the adenylate cyclase (encoded by *cya*) and the 3', 5'-cAMP receptor CRP, demonstrating that *gfpA* transcription is under CRP control (Fig. 3). Although reduced c-di-GMP concentration results in increased 3',5'-cAMP synthesis and *gfpA* expression, levels of *crp* transcript and protein do not change and c-di-GMP does not interfere with binding of 3',5'-cAMP-CRP to the *gfpA* promoter.⁸³ As c-di-GMP levels do not globally impact the CRP regulon, the mechanism of *gfpA* transcriptional regulation by c-di-GMP and 3',5'-cAMP-CRP is unclear. However, these data suggest an unknown mediator protein governs the expression of specific CRP transcriptional targets in a c-di-GMP-dependent fashion and highlight the intersection of c-di-GMP/cAMP signaling at the level of transcriptional control.

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The links in *Vibrio* between biofilm formation, c-di-GMP, and cAMP are mirrored in other bacterial species. Studies in *Pseudomonas aeruginosa* demonstrated similar indirect intersecting mechanisms of biofilm regulation by c-di-GMP and cAMP. Binding of cAMP to Vfr, the *P. aeruginosa* homologue of CRP, results in up-regulated levels of the biofilm-related response regulator phospho-AlgR, which induces expression of PilY1, a protein involved in pilus assemblies and required for adhesion to surfaces (Fig. 3).⁸⁴ Expression of PilY1 then activates the diguanylate cyclase SadC, increasing c-di-GMP levels and further promoting biofilm formation.^{85–87}

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Similar linkages between c-di-GMP and cAMP at the transcriptional levels are found within *Klebsiella pneumoniae*, highlighting the generality of the antagonistic effects. Expression of type 3 fimbriae (encoded by *mrkABCD*), which facilitate biofilm formation on surfaces,^{88–89} is positively regulated by c-di-GMP through the *mrkHII* operon, which encodes a c-di-GMP-binding PilZ protein that induces fimbriae expression,^{90–91} a LuxR-type regulator,⁹⁰ and a c-di-GMP phosphodiesterase,⁹² respectively. Expression of the phosphodiesterase MrkJ results in decreased c-di-GMP levels and repression of *mrkABCD* transcription and type 3 fimbriae expression.⁹² In contrast, decreased cAMP levels (caused by increased glucose concentration) results in increased MrkA production, while addition of exogenous cAMP abrogates this effect.⁹³ Taken together, recent work has suggested that c-di-GMP and cAMP bind cognate effectors to elicit opposing effects on biofilm formation through differential transcriptional regulation of the type 3 fimbriae operon (Fig. 3). While the mechanisms by which these cyclic nucleotide pathways interconnect are still relatively unknown, these types of studies suggest that additional proteins that monitor 3',5'-cAMP and c-di-GMP levels may occur in many Gram-negative bacteria.

(p)ppGpp, QS, and c-di-GMP

The stringent response in bacteria is activated in response to stress conditions, including nutrient deprivation (amino acid starvation, iron limitation, etc.) and heat shock, and is controlled through production of the nucleotide signal guanosine 5'-tri/diphosphate 3'-diphosphate ((p)ppGpp) by Rel/Spo homologues and small alarmone synthetase proteins (SAS).⁹⁴ Increased (p)ppGpp levels regulate transcription of numerous genes, typically upregulating genes related to stress responses and down-regulating genes related to growth.⁹⁵ As (p)ppGpp exhibits far-reaching regulatory roles, recent work demonstrating that the stringent response can modulate biofilm formation and quorum sensing hints at (p)ppGpp potentially having a role as a “master” second messenger (Fig. 4).⁹⁶

Within *P. aeruginosa*, triggering the stringent response through membrane perturbation leads to increased RelA expression, which regulates the stringent response through (p)ppGpp production, resulting in elevated levels of the AHL synthase genes *las* and *rhl* (Fig. 4).⁹⁷ These data demonstrate a direct link between stringent response induction and quorum sensing and suggest that (p)ppGpp levels are above quorum sensing in the signaling hierarchy.⁹⁸ Furthermore, studies in *Pectobacterium atrosepticum*, a Gram-negative soft rot plant pathogen, have demonstrated that inhibiting the stringent response through supplementation with nutrients or deletion of the (p)ppGpp synthase genes (*relA* *spoT*) decreases production of *P. atrosepticum* AHL (3-oxo-hexanoyl-L-homoserine lactone (OHHL)) and plant cell wall degradation enzymes, which are key virulence factors for infection of plant hosts (Fig. 4).⁹⁹ The effects of (p)ppGpp on AHL-dependent virulence factor production are mediated by increasing expression of *rsmB*, an antagonist of RsmA, which represses transcription of plant cell wall degrading enzymes. These studies directly link (p)ppGpp signaling and downstream pathways to modulation of quorum sensing molecules and virulence.

These results have been extended to the effects on biofilm formation in *Vibrio cholerae*, where experiments have demonstrated that (p)ppGpp levels (modulated through deletion of *rel* genes) control biofilm formation. The *rel* deletion strains exhibit decreased transcript levels of the *vps* genes involved in *Vibrio* polysaccharide production, a major component of *Vibrio* biofilms (Fig. 4).¹⁰⁰ While many further studies remain to be done to further link (p)ppGpp signaling to other cellular pathways, these early results highlight intriguing links to quorum sensing and biofilm formation and suggest that the stringent response may be a potential target for modulating multiple, key bacterial pathways involved in infection.

Links between primary nucleotide metabolism, c-di-GMP, ppGpp, and QS

Recent pharmacological and genetic studies in different bacterial taxa have elucidated associations between primary nucleotide metabolism, biofilm formation, and virulence factor production. Inhibition of the essential purine biosynthesis enzyme 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase with the purine analog azathioprine reduces biofilm production in *E. coli* by depleting purine nucleotide pools and consequently decreasing the c-di-GMP concentration.¹⁰¹ Interestingly, *de novo* pyrimidine synthesis and salvage also influence biofilm production, as disruption of any UMP

biosynthesis gene attenuates curli amyloid synthesis in *E. coli*.¹⁰² Furthermore, the polymeric composition of the *E. coli* biofilm is modulated by the availability of pyrimidine bases; an abundance of pyrimidines favors UMP synthesis through nucleotide salvage and up-regulates cellulose production *via* allosteric activation of DgcQ by UTP. Conversely, reliance on *de novo* pyrimidine synthesis decreases cellulose biogenesis due to allosteric inhibition of DgcQ by *N*-carbamoyl L-Asp (an intermediate in *de novo* pyrimidine synthesis).^{102–103} These findings illustrate the therapeutic potential of modulating nucleotide metabolism to intercept c-di-GMP signaling and disrupt biofilm formation.

In addition, a genome-wide transposon screen in *P. aeruginosa* identified novel associations between uracil metabolism and QS-regulated processes, including biofilm formation and virulence gene expression.¹⁰⁴ Specifically, disruption of *de novo* UMP biosynthesis genes (but not other pyrimidine or purine biosynthesis genes) down-regulates biofilm formation, and exogenous uracil reverses the hypo-biofilm phenotype. Moreover, disruption of *de novo* UMP biosynthesis attenuates transcription of genes involved in all three primary pseudomonad QS systems (Rhl, Las, and Pqs), as well as virulence-associated genes linked to chemotaxis, the type II secretion system, phenazine synthesis, and protease production. Thus, disruption of pyrimidine metabolism is a promising anti-virulence strategy, and this concept is further validated by the anti-biofilm and anti-QS activity of the antimetabolite 5-fluorouracil against *P. aeruginosa*.¹⁰⁴

Furthermore, the second messenger guanosine 3',5'-bis(diphosphate) (ppGpp), which induces the stringent response upon nutrient depletion,^{105–106} inhibits *E. coli* adenylosuccinate synthetase *in vitro*.¹⁰⁷ This enzyme catalyzes GTP-dependent $\mathcal{O}^6$ -phosphorylation of IMP and subsequent condensation with L-Asp to produce adenylosuccinate,¹⁰⁸ suggesting that ppGpp directly represses *de novo* purine synthesis to conserve resources during starvation. Interestingly, crystallographic studies of adenylosuccinate synthetase identified guanosine 2',3'-cyclic monophosphate 5'-diphosphate (5'-pp 2',3'-cGMP) as a high-affinity ligand for the GTP binding site (with affinity exceeding that of ppGpp). While the biological relevance of the 5'-pp 2',3'-cGMP-adenylosuccinate synthetase interaction requires further investigation, studies in our group have identified 2',3'-cyclic nucleotide monophosphates (2',3'-cNMPs) as novel modulators of diverse bacterial processes, including biofilm formation,¹⁰⁹ potentially through regulation of primary nucleotide metabolism. Collectively, these discoveries suggest that multiple nucleotide signals converge to modulate the cellular response to nutrient deprivation.

Outlook and Conclusions

In addition to the nucleotides discussed above, recent studies have identified novel nucleotide-based regulators that likely intersect with the signaling pathways described above. Specifically, our group is focused on determining the functions of 2',3'-cyclic nucleotide monophosphates (2',3'-cNMPs) in bacterial signaling. Recent work has elucidated that 2',3'-cNMPs play a role in *E. coli* biofilm formation,¹⁰⁹ and ongoing studies employing (bio)chemical manipulation of 2',3'-cNMP levels have identified functions for 2',3'-cNMPs in regulating virulence-associated processes, potentially by modulating c-di-GMP signaling and primary nucleotide metabolism. Future efforts seek to elucidate the

interactions of 2',3'-cNMPs with other signaling pathways in controlling behavior in *E. coli* and in other Gram-negative bacteria. Furthermore, additional nucleotide second messengers are emerging as important regulators of prokaryotic physiology, such as diadenosine tetraphosphate (Ap4A), a side product of tRNA aminoacylation, which is found throughout all kingdoms of life.¹¹⁰ Recent genetic and phenotypic studies in *P. fluorescens* suggest that Ap4A stimulates GTP synthesis, ultimately increasing the c-di-GMP level and promoting surface adhesion.¹¹¹ These findings warrant further investigation into potential links between Ap4A and additional facets of nucleotide signaling and quorum sensing. Discovering new nucleotide messengers and determining how signaling molecules intersect must be studied in order to combat the rising antibiotic resistance and biofilm formation of bacteria. By understanding how bacteria utilize nucleotide messengers to regulate phenotypes will allow us to find new drug targets for antibiotics and to reduce biofilm formation in clinical settings. Furthermore, to tackle the increasing number of infections and agricultural damage caused by bacteria, work must be done to understand how bacterial signaling intersects with eukaryotic cells to cause infectious diseases.

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References.

1. Sommer F; Backhed F, The gut microbiota--masters of host development and physiology. *Nat Rev Microbiol* 2013, 11 (4), 227–38. DOI: 10.1038/nrmicro2974. [PubMed: 23435359]
2. Kostic AD; Howitt MR; Garrett WS, Exploring host-microbiota interactions in animal models and humans. *Genes & development* 2013, 27 (7), 701–18. DOI: 10.1101/gad.212522.112. [PubMed: 23592793]
3. Boucher HW; Talbot GH; Bradley JS; Edwards JE; Gilbert D; Rice LB; Scheld M; Spellberg B; Bartlett J, Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2009, 48 (1), 1–12. DOI: 10.1086/595011. [PubMed: 19035777]
4. Spellberg B; Blaser M; Guidos RJ; Boucher HW; Bradley JS; Eisenstein BI; Gerding D; Lynfield R; Reller LB; Rex J; Schwartz D; Septimus E; Tenover FC; Gilbert DN, Combating antimicrobial resistance: policy recommendations to save lives. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2011, 52 Suppl 5, S397–428. DOI: 10.1093/cid/cir153. [PubMed: 21474585]
5. Roberts RR; Hota B; Ahmad I; Scott RD 2nd; Foster SD; Abbasi F; Schabowski S; Kampe LM; Ciavarella GG; Supino M; Naples J; Cordell R; Levy SB; Weinstein RA, Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2009, 49 (8), 1175–84. DOI: 10.1086/605630. [PubMed: 19739972]
6. Prüß BM, Involvement of Two-Component Signaling on Bacterial Motility and Biofilm Development. *J. Bacteriol* 2017, 199, e00259–17. [PubMed: 28533218]
7. Pesavento C; Hengge R, Bacterial nucleotide-based second messengers. *Curr. Opin. Microbiol* 2009, 12 (2), 170–176. [PubMed: 19318291]
8. Galloway WRJD; Hodgkinson JT; Bowden SD; Welch M; Spring DR, Quorum Sensing in Gram-Negative Bacteria: Small-Molecule Modulation of AHL and AI-2 Quorum Sensing Pathways. *Chem. Rev* 2011, 111, 28–67. [PubMed: 21182299]

9. Srivastava D; Waters CM, A tangled web: regulatory connections between quorum sensing and cyclic Di-GMP. *J Bacteriol* 2012, 194 (17), 4485–93. DOI: 10.1128/JB.00379-12. [PubMed: 22661686]
10. Kalia D; Merey G; Nakayama S; Zheng Y; Zhou J; Luo Y; Guo M; Roembke BT; Sintim HO, Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp signaling in bacteria and implications in pathogenesis. *Chem Soc Rev* 2013, 42 (1), 305–41. DOI: 10.1039/c2cs35206k. [PubMed: 23023210]
11. Hengge R, Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol* 2009, 7 (4), 263–273. DOI: 10.1038/nrmicro2109. [PubMed: 19287449]
12. Ross P; Weinhouse H; Aloni Y; Michaeli D; Weinbergerohana P; Mayer R; Braun S; Devroom E; Vandermarel G; Vanboom J; Benziman M, Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* 1987, 325 (6101), 279–281. DOI: 10.1038/325279a0. [PubMed: 18990795]
13. Simm R; Morr M; Kader A; Nimtz M; Romling U, GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol. Microbiol* 2004, 53 (4), 1123–1134. DOI: 10.1111/j.1365-2958.2004.04206.x. [PubMed: 15306016]
14. Romling U; Galperin M; Gomelsky M, Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev* 2013, 77 (1), 1–52. DOI: 10.1128/MMBR.00043-12. [PubMed: 23471616]
15. Hengge R; Galperin M; Ghigo J; Gomelsky M; Green J; Hughes K; Jenal U; Landini P, Systematic Nomenclature for GGDEF and EAL Domain-Containing Cyclic Di-GMP Turnover Proteins of *Escherichia coli*. *J. Bacteriol* 2016, 198 (1), 7–11. DOI: 10.1128/JB.00424-15. [PubMed: 26148715]
16. Sarenko O; Klauck G; Wilke F; Pfiffer V; Richter A; Herbst S; Kaever V; Hengge R, More than enzymes that make or break cyclic di-GMP - local signaling in the interactome of GGDEF/EAL domain proteins of *Escherichia coli*. *MBio* 2017, 8 (5). DOI: 10.1128/mBio.01639-17.
17. Chang A; Tuckerman J; Gonzalez G; Mayer R; Weinhouse H; Volman G; Amikam D; Benziman M; Gilles-Gonzalez M, Phosphodiesterase A1, a regulator of cellulose synthesis in *Acetobacter xylinum*, is a heme-based sensor. *Biochemistry* 2001, 40 (12), 3420–3426. DOI: 10.1021/bi0100236. [PubMed: 11297407]
18. Rajagopal S; Key J; Purcell E; Boerema D; Moffat K, Purification and initial characterization of a putative blue light-regulated phosphodiesterase from *Escherichia coli*. *Photochem. Photobiol* 2004, 80 (3), 542–547. DOI: 10.1562/0031-8655(2004)080<0542:PAICOA>2.0.CO;2. [PubMed: 15453820]
19. Hoffman L; D'Argenio D; MacCoss M; Zhang Z; Jones R; Miller S, Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 2005, 436 (7054), 1171–1175. DOI: 10.1038/nature03912. [PubMed: 16121184]
20. Gjermansen M; Ragas P; Sternberg C; Molin S; Tolker-Nielsen T, Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environ. Microbiol* 2005, 7 (6), 894–906. DOI: 10.1111/j.1462-2920.2005.00775.x. [PubMed: 15892708]
21. Fuqua W; Winans S; Greenberg E, Quorum sensing in bacteria - the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol* 1994, 176 (2), 269–275. [PubMed: 8288518]
22. Waters C; Bassler B, Quorum sensing: Cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol* 2005, 21, 319–346. DOI: 10.1146/annurev.cellbio.21.012704.131001. [PubMed: 16212498]
23. Nealson K; Hastings J, BACTERIAL BIOLUMINESCENCE - ITS CONTROL AND ECOLOGICAL SIGNIFICANCE. *Microbiological Reviews* 1979, 43 (4), 496–518. [PubMed: 396467]
24. Visick K; Foster J; Doino J; McFall-Ngai M; Ruby E, *Vibrio fischeri* lux genes play an important role in colonization and development of the host light organ. *J. Bacteriol* 2000, 182 (16), 4578–4586. DOI: 10.1128/JB.182.16.4578-4586.2000. [PubMed: 10913092]

25. Gould T; Schweizer H; Churchill M, Structure of the *Pseudomonas aeruginosa* acyl-homoserinylactone synthase LasI. *Mol. Microbiol* 2004, 53 (4), 1135–1146. DOI: 10.1111/j.1365-2958.2004.04211.x. [PubMed: 15306017]
26. Watson W; Minogue T; Val D; von Bodman S; Churchill M, Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Mol. Cell* 2002, 9 (3), 685–694. DOI: 10.1016/S1097-2765(02)00480-X. [PubMed: 11931774]
27. More M; Finger L; Stryker J; Fuqua C; Eberhard A; Winans S, Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. *Science* 1996, 272 (5268), 1655–1658. DOI: 10.1126/science.272.5268.1655. [PubMed: 8658141]
28. Parsek M; Val D; Hanzelka B; Cronan J; Greenberg E, Acyl homoserine-lactone quorum-sensing signal generation. *Proc. Natl. Acad. Sci. USA* 1999, 96 (8), 4360–4365. DOI: 10.1073/pnas.96.8.4360. [PubMed: 10200267]
29. Stevens A; Dolan K; Greenberg E, SYNERGISTIC BINDING OF THE *VIBRIO-FISCHERI* LUXR TRANSCRIPTIONAL ACTIVATOR DOMAIN AND RNA-POLYMERASE TO THE LUX PROMOTER REGION. *Proc. Natl. Acad. Sci. USA* 1994, 91 (26), 12619–12623. DOI: 10.1073/pnas.91.26.12619. [PubMed: 7809088]
30. Pereira C; Thompson J; Xavier K, AI-2-mediated signalling in bacteria. *FEMS Microbiol. Rev* 2013, 37 (2), 156–181. DOI: 10.1111/j.1574-6976.2012.00345.x. [PubMed: 22712853]
31. Surette MG; Miller MB; Bassler BL, Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proceedings of the National Academy of Sciences of the United States of America* 1999, 96 (4), 1639–44. [PubMed: 9990077]
32. Chen X; Schauder S; Potier N; Van Dorsselaer A; Pelczar I; Bassler B; Hughson F, Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* 2002, 415 (6871), 545–549. DOI: 10.1038/415545a. [PubMed: 11823863]
33. Hammer B; Bassler B, Distinct Sensory Pathways in *Vibrio cholerae* El Tor and Classical Biotypes Modulate Cyclic Dimeric GMP Levels To Control Biofilm Formation. *J. Bacteriol* 2009, 191 (1), 169–177. DOI: 10.1128/JB.01307-08. [PubMed: 18952786]
34. Wang H; Ayala J; Benitez J; Silva A, The LuxR-Type Regulator VpsT Negatively Controls the Transcription of *rpoS*, Encoding the General Stress Response Regulator, in *Vibrio cholerae* Biofilms. *J. Bacteriol* 2014, 196 (5), 1020–1030. DOI: 10.1128/JB.00993-13. [PubMed: 24363348]
35. Waters C; Lu W; Rabinowitz J; Bassler B, Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic Di-GMP levels and repression of *vpsT*. *J. Bacteriol* 2008, 190 (7), 2527–2536. DOI: 10.1128/JB.01756-07. [PubMed: 18223081]
36. Hammer B; Bassler B, Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* 2007, 104 (27), 11145–11149. DOI: 10.1073/pnas.0703860104. [PubMed: 17556542]
37. Martinez-Wilson H; Tamayo R; Tischler A; Lazinski D; Camilli A, The *Vibrio cholerae* hybrid sensor kinase VieS contributes to motility and biofilm regulation by altering the cyclic diguanylate level. *J. Bacteriol* 2008, 190 (19), 6439–6447. DOI: 10.1128/JB.00541-08. [PubMed: 18676667]
38. Trimble M; McCarter L, Bis-(3'-5')-cyclic dimeric GMP-linked quorum sensing controls swarming in *Vibrio parahaemolyticus*. *Proc. Natl. Acad. Sci. USA* 2011, 108 (44), 18079–18084. DOI: 10.1073/pnas.1113790108. [PubMed: 22006340]
39. Ueda A; Wood T, Connecting Quorum Sensing, c-di-GMP, Pel Polysaccharide, and Biofilm Formation in *Pseudomonas aeruginosa* through Tyrosine Phosphatase TpbA (PA3885). *PLoS Path* 2009, 5 (6). DOI: 10.1371/journal.ppat.1000483.
40. Chua S; Liu Y; Li Y; Ting H; Kohli G; Cai Z; Suwanchaikasem P; Goh K; Ng S; Tolker-Nielsen T; Yang L; Givskov M, Reduced Intracellular c-di-GMP Content Increases Expression of Quorum Sensing-Regulated Genes in *Pseudomonas aeruginosa*. *Frontiers in Cellular and Infection Microbiology* 2017, 7 DOI: 10.3389/fcimb.2017.00451.
41. Diggle S; Matthijs S; Wright V; Fletcher M; Chhabra S; Lamont I; Kong X; Hider R; Cornelis P; Camara M; Williams P, The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and

- PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem. Biol* 2007, 14 (1), 87–96. DOI: 10.1016/j.chembiol.2006.11.014. [PubMed: 17254955]
42. OCHSNER U; REISER J, AUTOINDUCER-MEDIATED REGULATION OF RHAMNOLIPID BIOSURFACTANT SYNTHESIS IN PSEUDOMONAS-AERUGINOSA. *Proc. Natl. Acad. Sci. USA* 1995, 92 (14), 6424–6428. DOI: 10.1073/pnas.92.14.6424. [PubMed: 7604006]
 43. PEARSON J; PASSADOR L; IGLEWSKI B; GREENBERG E, A 2ND N-ACYLHOMOSERINE LACTONE SIGNAL PRODUCED BY PSEUDOMONAS-AERUGINOSA. *Proc. Natl. Acad. Sci. USA* 1995, 92 (5), 1490–1494. DOI: 10.1073/pnas.92.5.1490. [PubMed: 7878006]
 44. Pearson J; Pesci E; Iglewski B, Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol* 1997, 179 (18), 5756–5767. DOI: 10.1128/jb.179.18.5756-5767.1997. [PubMed: 9294432]
 45. Xiao G; Deziel E; He J; Lepine F; Lesic B; Castonguay M; Milot S; Tampakaki A; Stachel S; Rahme L, MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. *Mol. Microbiol* 2006, 62 (6), 1689–1699. DOI: 10.1111/j.1365-2958.2006.05462.x. [PubMed: 17083468]
 46. Kim B; Park JS; Choi HY; Yoon SS; Kim WG, Terrein is an inhibitor of quorum sensing and c-di-GMP in *Pseudomonas aeruginosa*: a connection between quorum sensing and c-di-GMP. *Sci Rep* 2018, 8 (1), 8617 DOI: 10.1038/s41598-018-26974-5. [PubMed: 29872101]
 47. Diggle SP; Cornelis P; Williams P; Cámara M, 4-quinolone signalling in *Pseudomonas aeruginosa*: old molecules, new perspectives. *Int. J. Med. Microbiol* 2006, 296 (2–3), 83–91. DOI: 10.1016/j.ijmm.2006.01.038. [PubMed: 16483840]
 48. Gambello MJ; Iglewski BH, Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. *J. Bacteriol* 1991, 173 (9), 3000–9. [PubMed: 1902216]
 49. Passador L; Cook JM; Gambello MJ; Rust L; Iglewski BH, Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 1993, 260 (5111), 1127–30. [PubMed: 8493556]
 50. Pesci EC; Milbank JB; Pearson JP; McKnight S; Kende AS; Greenberg EP; Iglewski BH, Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 1999, 96 (20), 11229–34. [PubMed: 10500159]
 51. Venturi V, Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol. Rev* 2006, 30 (2), 274–91. DOI: 10.1111/j.1574-6976.2005.00012.x. [PubMed: 16472307]
 52. Welsh MA; Blackwell HE, Chemical genetics reveals environment-specific roles for quorum sensing circuits in *Pseudomonas aeruginosa*. *Cell Chem Biol* 2016, 23 (3), 361–9. DOI: 10.1016/j.chembiol.2016.01.006. [PubMed: 26905657]
 53. Monds RD; Newell PD; Gross RH; O'Toole GA, Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0–1 biofilm formation by controlling secretion of the adhesin LapA. *Mol Microbiol* 2007, 63 (3), 656–79. DOI: 10.1111/j.1365-2958.2006.05539.x. [PubMed: 17302799]
 54. Jensen V; Löns D; Zaoui C; Bredenbruch F; Meissner A; Dieterich G; Münch R; Häussler S, RhlR expression in *Pseudomonas aeruginosa* is modulated by the *Pseudomonas* quinolone signal via PhoB-dependent and -independent pathways. *J. Bacteriol* 2006, 188 (24), 8601–6. DOI: 10.1128/JB.01378-06. [PubMed: 17028277]
 55. Zaborin A; Romanowski K; Gerdes S; Holbrook C; Lepine F; Long J; Poroyko V; Diggle SP; Wilke A; Righetti K; Morozova I; Babrowski T; Liu DC; Zaborina O; Alverdy JC, Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proc Natl Acad Sci U S A* 2009, 106 (15), 6327–32. DOI: 10.1073/pnas.0813199106. [PubMed: 19369215]
 56. Grillo-Puertas M; Rintoul MR; Rapisarda VA, PhoB activation in non-limiting phosphate condition by the maintenance of high polyphosphate levels in the stationary phase inhibits biofilm formation in *Escherichia coli*. *Microbiology* 2016, 162 (6), 1000–8. DOI: 10.1099/mic.0.000281. [PubMed: 27023099]
 57. Lewenza S; Conway B; Greenberg EP; Sokol PA, Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. *J. Bacteriol* 1999, 181 (3), 748–56. [PubMed: 9922236]

58. Ryan RP; Dow JM, Communication with a growing family: diffusible signal factor (DSF) signaling in bacteria. *Trends Microbiol* 2011, 19 (3), 145–52. DOI: 10.1016/j.tim.2010.12.003. [PubMed: 21227698]
59. Boon C; Deng Y; Wang LH; He Y; Xu JL; Fan Y; Pan SQ; Zhang LH, A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *ISME J* 2008, 2 (1), 27–36. DOI: 10.1038/ismej.2007.76. [PubMed: 18049456]
60. Deng Y; Schmid N; Wang C; Wang J; Pessi G; Wu D; Lee J; Aguilar C; Ahrens CH; Chang C; Song H; Eberl L; Zhang LH, Cis-2-dodecenoic acid receptor RpfR links quorum-sensing signal perception with regulation of virulence through cyclic dimeric guanosine monophosphate turnover. *Proceedings of the National Academy of Sciences of the United States of America* 2012, 109 (38), 15479–84. DOI: 10.1073/pnas.1205037109. [PubMed: 22949660]
61. Yang C; Cui C; Ye Q; Kan J; Fu S; Song S; Huang Y; He F; Zhang LH; Jia Y; Gao YG; Harwood CS; Deng Y, *Burkholderia cenocepacia* integrates cis-2-dodecenoic acid and cyclic dimeric guanosine monophosphate signals to control virulence. *Proc. Natl. Acad. Sci. USA* 2017, 114 (49), 13006–13011. DOI: 10.1073/pnas.1709048114. [PubMed: 29158389]
62. Schmid N; Suppiger A; Steiner E; Pessi G; Kaever V; Fazli M; Tolker-Nielsen T; Jenal U; Eberl L, High intracellular c-di-GMP levels antagonize quorum sensing and virulence gene expression in *Burkholderia cenocepacia* H111. *Microbiology* 2017, 163 (5), 754–764. DOI: 10.1099/mic.0.000452. [PubMed: 28463102]
63. Gorke B; Stulke J, Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol* 2008, 6 (8), 613–624. DOI: 10.1038/nrmicro1932. [PubMed: 18628769]
64. Zheng D; Constantinidou C; Hobman J; Minchin S, Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. *Nucleic Acids Res* 2004, 32 (19), 5874–5893. DOI: 10.1093/nar/gkh908. [PubMed: 15520470]
65. Kalia D; Merrey G; Nakayama S; Zheng Y; Zhou J; Luo Y; Guo M; Roembke B; Sintim H, Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp signaling in bacteria and implications in pathogenesis. *Chem. Soc. Rev* 2013, 42 (1), 305–341. DOI: 10.1039/c2cs35206k. [PubMed: 23023210]
66. Baumann A; Lange C; Soppa J, Transcriptome changes and cAMP oscillations in an archaeal cell cycle. *BMC Cell Biol* 2007, 8 DOI: 10.1186/1471-2121-8-21.
67. Lee DH; Jeong HS; Jeong HG; Kim KM; Kim H; Choi SH, A Consensus Sequence for Binding of SmcR, a *Vibrio vulnificus* LuxR Homologue, and Genome-wide Identification of the SmcR Regulon 2008 DOI: 10.1074/jbc.M801480200.
68. Kim SP; Kim CM; Shin SH, Cyclic AMP and cyclic AMP-receptor protein modulate the autoinducer-2-mediated quorum sensing system in *Vibrio vulnificus*. *Current microbiology* 2012, 65 (6), 701–10. DOI: 10.1007/s00284-012-0218-0. [PubMed: 22961036]
69. Liang W; Pascual-Montano A; Silva AJ; Benitez JA, The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. *Microbiology* 2007, 153 (Pt 9), 2964–75. DOI: 10.1099/mic.0.2007/006668-0. [PubMed: 17768239]
70. Kovackova G; Skorupski K, Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. *Mol. Microbiol* 2002, 46 (4), 1135–47. [PubMed: 12421317]
71. Yildiz FH; Liu XS; Heydorn A; Schoolnik GK, Molecular analysis of rugosity in a *Vibrio cholerae* O1 El Tor phase variant. *Mol. Microbiol* 2004, 53 (2), 497–515. DOI: 10.1111/j.1365-2958.2004.04154.x. [PubMed: 15228530]
72. Albus AM; Pesci EC; Runyen-Janecky LJ; West SE; Iglewski BH, Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol* 1997, 179 (12), 3928–35. [PubMed: 9190808]
73. Almblad H; Harrison JJ; Rybtke M; Groizeleau J; Givskov M; Parsek MR; Tolker-Nielsen T, The Cyclic AMP-Vfr Signaling Pathway in *Pseudomonas aeruginosa* Is Inhibited by Cyclic Di-GMP. *J Bacteriol* 2015, 197 (13), 2190–200. DOI: 10.1128/JB.00193-15. [PubMed: 25897033]
74. Wang L; Hashimoto Y; Tsao C; Valdes J; Bentley W, Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in *Escherichia coli*. *J.*

- Bacteriol 2005, 187 (6), 2066–2076. DOI: 10.1128/JB.187.6.2066-2076.2005. [PubMed: 15743955]
75. Sperandio V; Torres AG; Girón JA; Kaper JB, Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. *J. Bacteriol* 2001, 183 (17), 5187–97. [PubMed: 11489873]
 76. Yao Y; Martinez-Yamout MA; Dickerson TJ; Brogan AP; Wright PE; Dyson HJ, Structure of the *Escherichia coli* quorum sensing protein SdiA: activation of the folding switch by acyl homoserine lactones. *J. Mol. Biol* 2006, 355 (2), 262–73. DOI: 10.1016/j.jmb.2005.10.041. [PubMed: 16307757]
 77. Zhou X; Meng X; Sun B, An EAL domain protein and cyclic AMP contribute to the interaction between the two quorum sensing systems in *Escherichia coli*. *Cell Res* 2008, 18 (9), 937–948. DOI: 10.1038/cr.2008.67. [PubMed: 18560382]
 78. Fong JC; Yildiz FH, Interplay between cyclic AMP-cyclic AMP receptor protein and cyclic di-GMP signaling in *Vibrio cholerae* biofilm formation. *J Bacteriol* 2008, 190 (20), 6646–59. DOI: 10.1128/JB.00466-08. [PubMed: 18708497]
 79. Yildiz FH; Dolganov NA; Schoolnik GK, VpsR, a Member of the Response Regulators of the Two-Component Regulatory Systems, Is Required for Expression of vps Biosynthesis Genes and EPS(ETr)-Associated Phenotypes in *Vibrio cholerae* O1 El Tor. *J Bacteriol* 2001, 183 (5), 1716–26. DOI: 10.1128/jb.183.5.1716-1726.2001. [PubMed: 11160103]
 80. Casper-Lindley C; Yildiz FH, VpsT Is a Transcriptional Regulator Required for Expression of vps Biosynthesis Genes and the Development of Rugose Colonial Morphology in *Vibrio cholerae* O1 El Tor. In *J Bacteriol*, 2004; Vol. 186, pp 1574–8. DOI: 10.1128/jb.186.5.1574-1578.2004. [PubMed: 14973043]
 81. Kariisa AT; Grube A; Tamayo R, Two nucleotide second messengers regulate the production of the *Vibrio cholerae* colonization factor GbpA. *BMC Microbiol* 2015, 15, 166 DOI: 10.1186/s12866-015-0506-5. [PubMed: 26286031]
 82. Kirn TJ; Jude BA; Taylor RK, A colonization factor links *Vibrio cholerae* environmental survival and human infection. *Nature* 2005, 438 (7069), 863–6. DOI: 10.1038/nature04249. [PubMed: 16341015]
 83. Bhowmick R; Ghosal A; Das B; Koley H; Saha DR; Ganguly S; Nandy RK; Bhadra RK; Chatterjee NS, Intestinal adherence of *Vibrio cholerae* involves a coordinated interaction between colonization factor GbpA and mucin. *Infection and immunity* 2008, 76 (11), 4968–77. DOI: 10.1128/iai.01615-07. [PubMed: 18765724]
 84. Luo Y; Zhao K; Baker AE; Kuchma SL; Coggan KA; Wolfgang MC; Wong GC; O'Toole GA, A hierarchical cascade of second messengers regulates *Pseudomonas aeruginosa* surface behaviors. *MBio* 2015, 6 (1). DOI: 10.1128/mBio.02456-14.
 85. Belete B; Lu H; Wozniak DJ, *Pseudomonas aeruginosa* AlgR Regulates Type IV Pilus Biosynthesis by Activating Transcription of the fimU-pilVWXY1Y2E Operon 2008 DOI: 10.1128/JB.01623-07.
 86. Kanack KJ; Runyen-Janecky LJ; Ferrell EP; Suh S-J; West SEH, Characterization of DNA-binding specificity and analysis of binding sites of the *Pseudomonas aeruginosa* global regulator, Vfr, a homologue of the *Escherichia coli* cAMP receptor protein 2006. DOI: doi:10.1099/mic.0.29008-0.
 87. Fulcher NB; Holliday PM; Klem E; Cann MJ; Wolfgang MC, The *Pseudomonas aeruginosa* Chp chemosensory system regulates intracellular cAMP levels by modulating adenylate cyclase activity. *Mol Microbiol* 2010, 76 (4), 889–904. DOI: 10.1111/j.1365-2958.2010.07135.x. [PubMed: 20345659]
 88. Jagnow J; Clegg S, *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiology* 2003, 149 (Pt 9), 2397–405. DOI: 10.1099/mic.0.26434-0. [PubMed: 12949165]
 89. Di Martino P; Cafferini N; Joly B; Darfeuille-Michaud A, *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. *Res Microbiol* 2003, 154 (1), 9–16. [PubMed: 12576153]
 90. Tan JW; Wilksch JJ; Hocking DM; Wang N; Srikhanta YN; Tauschek M; Lithgow T; Robins-Browne RM; Yang J; Strugnell RA, Positive autoregulation of mrkHI by the cyclic di-GMP-

- dependent MrkH protein in the biofilm regulatory circuit of *Klebsiella pneumoniae*. *J Bacteriol* 2015, 197 (9), 1659–67. DOI: 10.1128/jb.02615-14. [PubMed: 25733612]
91. Wilksch JJ; Yang J; Clements A; Gabbe JL; Short KR; Cao H; Cavaliere R; James CE; Whitchurch CB; Schembri MA; Chuah ML; Liang ZX; Wijburg OL; Jenney AW; Lithgow T; Strugnell RA, MrkH, a novel c-di-GMP-dependent transcriptional activator, controls *Klebsiella pneumoniae* biofilm formation by regulating type 3 fimbriae expression. *PLoS Pathog* 2011, 7 (8), e1002204 DOI: 10.1371/journal.ppat.1002204. [PubMed: 21901098]
 92. Johnson JG; Clegg S, Role of MrkJ, a phosphodiesterase, in type 3 fimbrial expression and biofilm formation in *Klebsiella pneumoniae*. *J Bacteriol* 2010, 192 (15), 3944–50. DOI: 10.1128/jb.00304-10. [PubMed: 20511505]
 93. Yang J; Wilksch JJ; Tan JW; Hocking DM; Webb CT; Lithgow T; Robins-Browne RM; Strugnell RA, Transcriptional activation of the mrkA promoter of the *Klebsiella pneumoniae* type 3 fimbrial operon by the c-di-GMP-dependent MrkH protein. *PLoS One* 2013, 8 (11), e79038 DOI: 10.1371/journal.pone.0079038. [PubMed: 24244411]
 94. Harms A; Maisonneuve E; Gerdes K, Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* 2016, 354, aaf4268. [PubMed: 27980159]
 95. Ababneh QO; Herman JK, RelA inhibits *Bacillus subtilis* motility and chaining. *J Bacteriol* 2015, 197 (1), 128–37. DOI: 10.1128/JB.02063-14. [PubMed: 25331430]
 96. Petchiappan A; Chatterji D, Antibiotic Resistance: Current Perspectives. *Acs Omega* 2017, 2 (10), 7400–7409. DOI: 10.1021/acsomega.7b01368. [PubMed: 30023551]
 97. Bayse C; Cullinane M; Dénervaud V; Burrowes E; Dow JM; Morrissey JP; Tam L; Trevors JT; O’Gara F, Modulation of quorum sensing in *Pseudomonas aeruginosa* through alteration of membrane properties. *Microbiology* 2005, 151 (Pt 8), 2529–42. DOI: 10.1099/mic.0.28185-0. [PubMed: 16079332]
 98. Schafhauser J; Lepine F; McKay G; Ahlgren HG; Khakimova M; Nguyen D, The stringent response modulates 4-hydroxy-2-alkylquinoline biosynthesis and quorum-sensing hierarchy in *Pseudomonas aeruginosa*. *J Bacteriol* 2014, 196 (9), 1641–50. DOI: 10.1128/JB.01086-13. [PubMed: 24509318]
 99. Bowden SD; Eyres A; Chung JC; Monson RE; Thompson A; Salmond GP; Spring DR; Welch M, Virulence in *Pectobacterium atrosepticum* is regulated by a coincidence circuit involving quorum sensing and the stress alarmone, (p)ppGpp. *Mol Microbiol* 2013, 90 (3), 457–71. DOI: 10.1111/mmi.12369. [PubMed: 23957692]
 100. He H; Cooper JN; Mishra A; Raskin DM, Stringent Response Regulation of Biofilm Formation in *Vibrio cholerae* 2012 DOI: 10.1128/JB.00014-12.
 101. Antoniani D; Rossi E; Rinaldo S; Bocci P; Lolicato M; Paiardini A; Raffaelli N; Cutruzzola F; Landini P, The immunosuppressive drug azathioprine inhibits biosynthesis of the bacterial signal molecule cyclic-di-GMP by interfering with intracellular nucleotide pool availability. *Appl. Microbiol. Biotechnol* 2013, 97 (16), 7325–7336. DOI: 10.1007/s00253-013-4875-0. [PubMed: 23584245]
 102. Garavaglia M; Rossi E; Landini P, The pyrimidine nucleotide biosynthetic pathway modulates production of biofilm determinants in *Escherichia coli*. *PLOS ONE* 2012, 7 (2), doi:10.1371/journal.pone.0031252. DOI: 10.1371/journal.pone.0031252.
 103. Rossi E; Motta S; Aliverti A; Cossu F; Gourlay L; Mauri P; Landini P, Cellulose production is coupled to sensing of the pyrimidine biosynthetic pathway via c-di-GMP production by the DgcQ protein of *Escherichia coli*. *Environ Microbiol* 2017, 19 (11), 4551–4563. DOI: 10.1111/1462-2920.13918. [PubMed: 28892259]
 104. Ueda A; Attila C; Whiteley M; Wood T, Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist. *Microb. Biotechnol* 2009, 2 (1), 62–74. DOI: 10.1111/j.1751-7915.2008.00060.x. [PubMed: 21261882]
 105. Wells DH; Gaynor EC, *Helicobacter pylori* Initiates the Stringent Response upon Nutrient and pH Downshift. In *J Bacteriol*, 2006; Vol. 188, pp 3726–9. DOI: 10.1128/jb.188.10.3726-3729.2006. [PubMed: 16672627]

106. Hauryliuk V; Atkinson GC; Murakami KS; Tenson T; Gerdes K, Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol* 2015, 13 (5), 298–309. DOI: 10.1038/nrmicro3448. [PubMed: 25853779]
107. Hou Z; Cashel M; Fromm H; Honzatko R, Effectors of the stringent response target the active site of *Escherichia coli* adenylosuccinate synthetase. *J. Biol. Chem* 1999, 274 (25), 17505–17510. [PubMed: 10364182]
108. Bass MB; Fromm HJ; Rudolph FB, The mechanism of the adenylosuccinate synthetase reaction as studied by positional isotope exchange. *J Biol Chem* 1984, 259 (20), 12330–3. [PubMed: 6490614]
109. Fontaine BM; Martin KS; Garcia-Rodriguez JM; Jung C; Briggs L; Southwell JE; Jia X; Weinert EE, RNase I regulates *Escherichia coli* 2',3'-cyclic nucleotide monophosphate levels and biofilm formation 2018 DOI: 10.1042/BCJ20170906.
110. Despotovic D; Brandis A; Savidor A; Levin Y; Fumagalli L; Tawfik DS, Diadenosine tetraphosphate (Ap₄A) - an *E. coli* alarmone or a damage metabolite? *Febs j* 2017, 284 (14), 2194–2215. DOI: 10.1111/febs.14113. [PubMed: 28516732]
111. Monds RD; Newell PD; Wagner JC; Schwartzman JA; Lu W; Rabinowitz JD; O'Toole GA, Di-Adenosine Tetraphosphate (Ap₄A) Metabolism Impacts Biofilm Formation by *Pseudomonas fluorescens* via Modulation of c-di-GMP-Dependent Pathways 2010 DOI: 10.1128/JB.01571-09.

Figure 1.

Overview of intersection of c-di-GMP and quorum sensing pathways in *P. aeruginosa* (purple), *V. cholerae* (orange), and *B. cenocepacia* (green). Arrows represent effects or signaling molecule/transcript levels that are promoted by the input, while flat ended lines represent effects/concentrations that are inhibited by the input.

Figure 2.

Cross-talk between cAMP and quorum sensing pathways in *E. coli* (dark green), *P. aeruginosa* (purple), and *Vibrio* species (orange). Arrows represent effects or signaling molecule/transcript levels that are promoted by the input, while flat ended lines represent effects/concentrations that are inhibited by the input.

Figure 3.

Intercommunication between c-di-GMP and cAMP pathways in *P. aeruginosa* (purple), *V. cholerae* (orange), and *K. pneumoniae* (magenta). Arrows represent effects or signaling molecule/transcript levels that are promoted by the input, while flat ended lines represent effects/concentrations that are inhibited by the input.

Figure 4.

Regulation of other nucleotide and quorum sensing pathways by (p)ppGpp in *V. cholerae* (orange), *P. aeruginosa* (purple), and *P. atrosepticum* (blue). Arrows represent effects or signaling molecule/transcript levels that are promoted by the input, while flat ended lines represent effects/concentrations that are inhibited by the input.