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Review Article

REVIEW ARTICLE

# Cyclic diguanylate signaling in Gram-positive bacteria

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**One sentence summary:** The majority of bacterial life exists in a surface-associated state, with motility allowing bacteria to disseminate and colonize new environments. Like in Gram-negative bacteria, c-di-GMP signaling in Gram-positives inversely controls the production of flagella and adherence factors and appears to be a major mechanism by which bacteria sense and respond to surfaces.

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## ABSTRACT

The nucleotide second messenger 3′-5′ cyclic diguanylate monophosphate (c-di-GMP) is a central regulator of the transition between motile and non-motile lifestyles in bacteria, favoring sessility. Most research investigating the functions of c-di-GMP has focused on Gram-negative species, especially pathogens. Recent work in Gram-positive species has revealed that c-di-GMP plays similar roles in Gram-positives, though the precise targets and mechanisms of regulation may differ. The majority of bacterial life exists in a surface-associated state, with motility allowing bacteria to disseminate and colonize new environments. c-di-GMP signaling regulates flagellum biosynthesis and production of adherence factors and appears to be a primary mechanism by which bacteria sense and respond to surfaces. Ultimately, c-di-GMP influences the ability of a bacterium to alter its transcriptional program, physiology and behavior upon surface contact. This review discusses how bacteria are able to sense a surface via flagella and type IV pili, and the role of c-di-GMP in regulating the response to surfaces, with emphasis on studies of Gram-positive bacteria.

**Keywords:** cyclic diguanylate; motility; biofilm; adherence; signaling; Gram-positive

## INTRODUCTION

Bacteria utilize multiple modes of motility to colonize new environments, but typically attach to surfaces to persist on them. In natural environments, most microbes exist within biofilms, surface-associated communities of microbes that collectively secrete a protective extracellular matrix (ECM) and undergo significant metabolic and transcriptional reprogramming compared to genetically identical free-living planktonic bacteria (Costerton *et al.* 1995). For environmental persistence, bacteria can adhere to abiotic or biotic surfaces. Pathogenic and commensal bacteria often interact with host cells and tissues; host cell contact often serves as a stimulus of virulence programs for many bacterial pathogens (Galan and Collmer 1999; Lee *et al.* 1999; Siryaporn *et al.* 2014; Persat *et al.* 2015). For some species, biofilm formation in a host is key to persistence, virulence and/or transmission (Roilides *et al.* 2015). If and when surface-associated growth becomes disadvantageous, bacteria have evolved modes of dissociating from the community and migrating toward a more favorable milieu (Abee *et al.* 2011; Hall-Stoodley, Costerton and Stoodley 2014). Thus, many bacteria are capable of switching between free-living planktonic growth and surface association. The ability to sense and respond to contact with a surface is vital to maintaining this facet of bacterial life, yet the mechanisms underlying this process are poorly understood (Galperin 2005). Highly diverse bacteria employ surface sensing; both flagellated Gram-negative *Escherichia coli* and non-flagellated Gram-positive *Staphylococcus aureus* preferentially adhere to hard surfaces rather than chemically equivalent soft ones (Kolewe, Peyton and Schiffman 2015). Bacteria utilize cell surface structures, namely flagella and type IV pili (TFP), in mediating physical attachment and/or motility, and recent work

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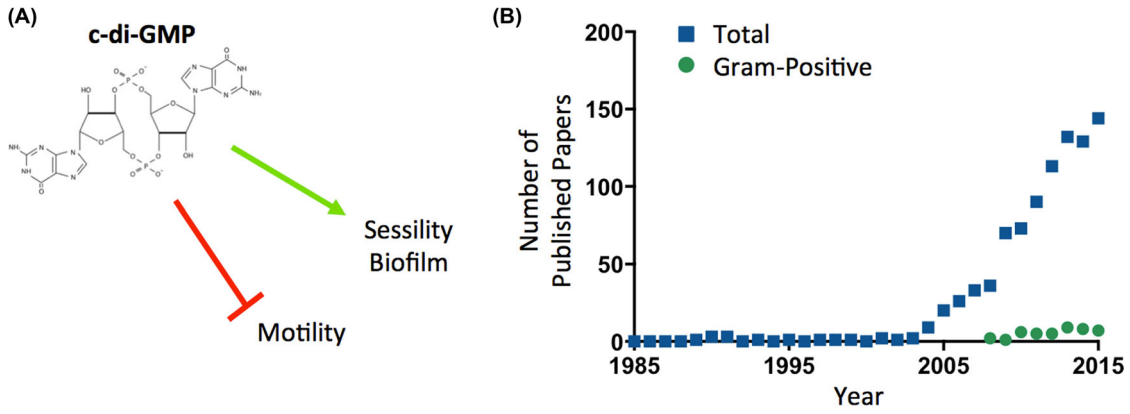


**Figure 1.** Growing interest in c-di-GMP signaling in Gram-positive bacteria. (**A**) The consensus of numerous studies indicates that c-di-GMP negatively regulates properties of free-living bacteria such as flagellum-mediated swimming, while promoting adherent phenotypes such as biofilm development. (**B**) Though c-di-GMP was first described in 1985 (Ross *et al.* 1985, 1987) and interest in c-di-GMP signaling exploded in 2004, c-di-GMP signaling in the context of Gram-positive bacteria went unreported before 2010 (den Hengst *et al.* 2010). Trends in publications including c-di-GMP were determined using the search engine at http://dan.corlan.net/medline-trend.html, with the search terms 'c-di-GMP' to identify the total number of publications. Search results for all years since 2009 were manually sorted to identify the subset of reports on Gram-positive bacteria. The numbers are likely to be underestimates given differences in terminology used for c-di-GMP in earlier studies (e.g. cyclic diguanylic acid, cyclic diguanylate, cyclic di-GMP).

has also implicated these structures in sensing that the bacterium is in contact with a surface (Blair *et al.* 2008; Van Dellen, Houot and Watnick 2008; Clausen *et al.* 2009; Guttenplan, Blair and Kearns 2010; Cairns *et al.* 2013; Lele, Hosu and Berg 2013; Tipping *et al.* 2013; Maier and Wong 2015). How the interaction between a flagellum or pilus and a surface results in the initiation of regulatory programs to promote bacterial adherence is unclear.

The nucleotide second messenger 3′-5′ cyclic diguanylate monophosphate (c-di-GMP, Fig. 1A) has emerged in the last 30 years as a central regulator in the transition between motile and non-motile lifestyles in bacteria (Romling, Galperin and Gomelsky 2013). The vast majority of this research focused on Gram-negative bacteria and has elucidated a paradigm in which, broadly speaking, c-di-GMP inhibits motility (particularly flagellum-based motility) and promotes sessility, including biofilm development (Romling, Gomelsky and Galperin 2005; Hengge 2009; Romling, Galperin and Gomelsky 2013). In contrast, despite the presence of conserved c-di-GMP metabolism genes in Gram-positive genomes, this area of study is relatively underdeveloped (Fig. 1B) (den Hengst *et al.* 2010). Indeed, many aspects of Gram-positive microbiology are poorly characterized. For example, TFP were thought for many years to be specific to certain Gram-negative bacteria, but putative genes for TFP biosynthesis have been identified in multiple Gram-positive genomes, and TFP were recently described in the Gram-positive Clostridia (Varga *et al.* 2006; Imam *et al.* 2011; Bordeleau *et al.* 2015; Purcell *et al.* 2015). Nonetheless, studies in both Gram-negative and Gram-positive bacteria have demonstrated that, while the processes regulated by c-di-GMP are remarkably consistent across diverse bacteria, the mechanisms by which c-di-GMP regulates these pathways vary widely among and within bacterial species. Recent evidence, detailed herein, suggests that c-di-GMP not only inversely regulates motility and sessility, but may also play a role in bacterial surface sensing and adhesion.

In this review, we focus on the roles of c-di-GMP in regulating changes in bacterial physiology, which are often crucial during transitions between motility and surface adherence. We place emphasis on recent studies done in Gram-positive bacteria, which have been relatively understudied in the context of surface-associated behaviors. Gram-positive pathogens are of great medical interest, as many increasingly urgent healthcare threats stem from Gram-positive bacteria such as *Clostridium difficile* and methicillin-resistant *St. aureus* (US Department of Health and Human Services 2013). Up to 60% of nosocomial infections arise from biofilm-related infections, including many caused by staphylococci (O'Toole, Kaplan and Kolter 2000). Nucleotide second messengers beyond c-di-GMP have been implicated in regulating surface behaviors, and we discuss the ways in which the signaling pathways intersect. Quorum sensing also plays a central role in controlling bacterial surface interactions, and the 'crosstalk' between quorum sensing and nucleotide second messenger signaling pathways was recently reviewed by Kalia *et al.* (2013). These signaling pathways function together to coordinate highly orchestrated bacterial behaviors, particularly those involving surfaces.

## LIFESTYLE CHANGES: MOTILE TO SESSILE TRANSITIONS

As noted above, the majority of bacterial life exists in a surface-associated state. Often this form entails development of a biofilm community of microorganisms encased in a matrix of exopolysaccharide (EPS), secreted proteins and/or extracellular DNA (Costerton *et al.* 1995; Flemming and Wingender 2010). If and when environmental conditions become unfavorable, bacteria use various modes of self-driven motility to disperse and identify a suitable new environment (Abee *et al.* 2011; Hall-Stoodley, Costerton and Stoodley 2014). In diverse bacteria, flagella confer the ability to swim through low viscosity liquids and/or to 'swarm' along solid surfaces with the aid of secreted surfactants (Kearns 2010). Propulsion is achieved by the rotation of the rigid, helical flagellar filament(s). Flagellar gene expression and assembly of flagella (diagrammed in Fig. 2A), energetically costly structures for bacteria to produce, are tightly regulated (Chevance and Hughes 2008). Ultimately, flagellar motility, aided by chemotaxis (Sourjik and Wingreen 2012), enables avoidance of undesirable conditions and migration toward favorable environments. Upon interaction with a suitable substratum, the flagellum may participate in attachment, potentially leading to biofilm formation and/or host colonization.

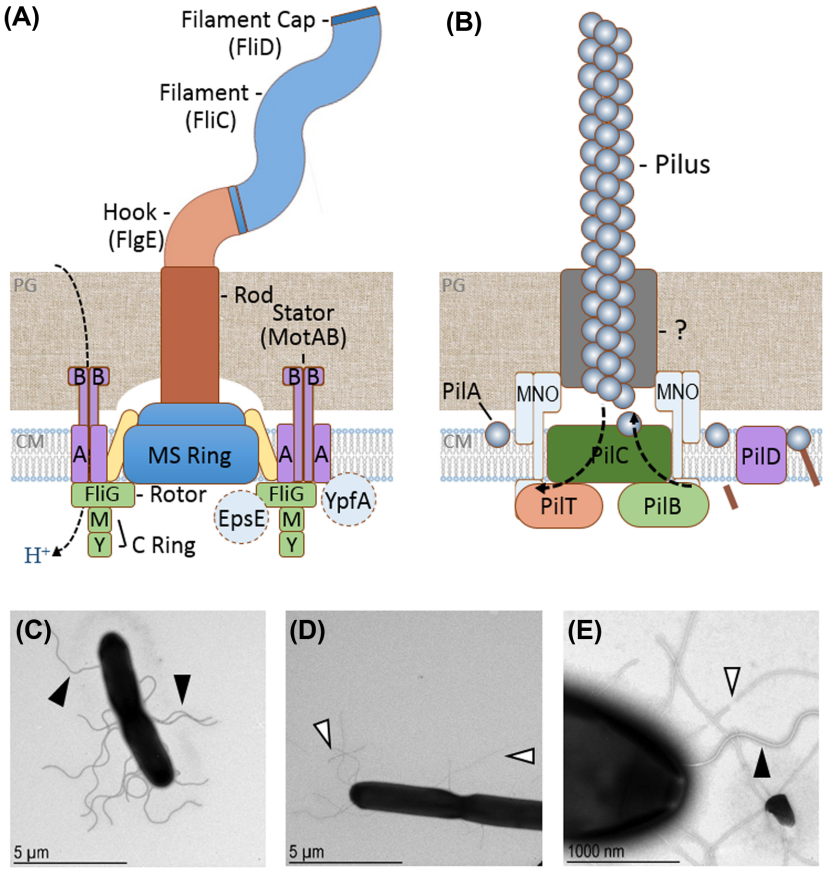


**Figure 2.** Comparison of flagellum and TFP structures. (**A**) Diagram of the flagellum in Gram-positive bacteria, including key structural components of the basal body, hook and filament. Gram-positive bacteria have two basal body rings instead of four, one in the membrane and one in the peptidoglycan layer. The motor that drives flagellar rotation consists of stator proteins MotA and MotB, and rotor FliG, FliM and FliY (FliN). In the absence of ion flow through the motor, the stator and rotor complexes do not engage, and no flagellar rotation occurs. As ions flow though, dependent on adequate membrane potential, the motor and stator engage to generate the torque needed to propel the flagellum. In *B. subtilis*, the EpsE protein that contributes to biosynthesis of EPS production and biofilm formation also serves as a 'clutch', interacting with FliG to impede flagellar rotation. The PilZ domain protein YpfA/DgrA, a post-translational negative regulator of *B. subtilis* motility, interacts with MotA. (**B**) Diagram of the TFP of Gram-positive bacteria. The PilB ATPase powers the assembly of PilA pilin subunits into the base of the growing fiber; the PilT ATPase functions in disassembly of pilin subunits from the base, leading to retraction of the pilus as it shrinks. PilM, PilN and PilO comprise the membrane complex through which the pilus extends. In Gram-positive bacteria, the secretin that spans the outer membrane (PilQ in Gram-negatives) is absent, and the equivalent structure that allows the TFP to cross the peptidoglycan cell wall is unknown. (**C–E**) Comparisons of flagella (black arrowheads) and TFP (white arrowheads) on *C. difficile* by transmission electron microscopy Flagella measure ~20 nm in diameter and can be two to three times as long as the cell (C, E). TFP are thinner and shorter, approximately 5–8 nm in diameter and up to several microns in length (**D**, E).

In pathogens such as *Salmonella enterica*, some pathogenic *E. coli*, *Vibrio cholerae*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *C. difficile* and others, flagella have been implicated in promoting interactions with the host (Yancey, Willis and Berry 1978; Montie *et al.* 1982; Attridge and Rowley 1983; Sato, Okinaga and Saito 1988; Postnova, Gomez-Duarte and Richardson 1996; Josenhans and Suerbaum 2002; Stecher *et al.* 2004; Haiko and Westerlund-Wikstrom 2013; Stevenson, Minton and Kuehne 2015). In some cases, flagella propel the bacterium to the target colonization site. Evidence suggests that *H. pylori* and *V. cholerae* use flagella to transit through stomach and intestinal mucus, respectively, to reach the epithelium below (Freter and O'Brien 1981; Richardson 1991; Eaton *et al.* 1996; Butler and Camilli 2004; Terry *et al.* 2005). In other instances, the flagellum (either the filament itself or the cap protein) serves as an adhesin. Various pathogenic *E. coli* use flagella to attach to epithelial cells, and the *P. aeruginosa* flagellum adheres to mucin, a substratum encountered in the lung (Arora *et al.* 1996, 1998; Scharfman *et al.* 2001; Lillehoj, Kim and Kim 2002; Haiko and Westerlund-Wikstrom 2013).

In contrast to flagella, TFP (Fig. 2B) are thinner, shorter filaments extending from the bacterial surface (Fig. 2C–E). TFP are involved in DNA uptake, surface adhesion, microcolony formation, surface motility, biofilm formation and transformation in many bacteria (Mattick 2002; Giltner, Nguyen and Burrows 2012; Melville and Craig 2013). TFP are well documented to mediate interactions between (and among) the bacteria bearing them and various surfaces (Marceau, Beretti and Nassif 1995; Li *et al.* 2003; Rodgers, Arvidson and Melville 2011). In some instances, flagella or other surface structures promote transient, reversible interactions that are then reinforced by TFP, resulting in more intimate, permanent associations (Conrad 2012). Accordingly, TFP help initiate and maintain biofilm development (O'Toole and Kolter 1998; Moorthy and Watnick 2004; Conrad 2012; Utada *et al.* 2014). TFP also function in host colonization in myriad animal and plant pathogens (Sato, Okinaga and Saito 1988; Virji *et al.* 1991; Nassif *et al.* 1994; Jurcisek *et al.* 2007; Bahar, Goffer and Burdman 2009; Wang, Haitjema and Fuqua 2014).

TFP can mediate motility through the iterative extension, attachment and retraction of the pilus fiber along a solid surface, comparable to a grappling hook (Mattick 2002). Pilus extension and retraction is powered by ATPases with opposing activities. The PilB extension ATPase (named PilF in *Neisseriae*) is required for polymerization of pilin subunits into the base of the pilus fiber, lengthening the pilus (Nunn, Bergman and Lory 1990). Conversely, the PilT ATPase is essential for reversing this via depolymerization of pilin subunits from the fiber, leading to shortening of the pilus and retraction (Wolfgang *et al*. 2000). The TFP-based motility has been described as 'twitching' motility in *Neisseriae*, *Pseudomonas* and a handful of other genera, and TFP-dependent surface motility has more recently been reported in *Myxococcus xanthus* and *C. difficile* (Mattick 2002; Burrows 2012; Dunger *et al*. 2014; Purcell *et al*. 2015). *Clostridium perfringens* demonstrates 'gliding' motility on surfaces that is TFP dependent (Varga *et al*. 2006).

Upon interacting with a surface, bacteria rapidly and dramatically alter their transcriptional program, physiology and behavior; they stop swimming and activate pathways for swarmer-cell differentiation or biofilm development (O'Toole, Kaplan and Kolter 2000; Abel *et al*. 2013; Luo *et al*. 2015). Yet once a bacterium reaches a new surface to potentially colonize, how does it recognize this to initiate its adaptive response? Remarkably, although there is a breadth of knowledge about the events that occur once bacteria have established surface colonization, we know relatively little about the mechanosensing process by which bacteria determine that surface contact has occurred. Intuitively, surface-responsive mechanosensors described to date are structures that extend from the bacterial cell, including flagella and TFP.

## Flagella as surface sensors

Several lines of evidence support a role for flagella in surface-sensing functions beyond motility. First, flagellum-mediated swarming occurs strictly on a surface and involves a switch from a free-swimming form to a coordinately migrating group. In addition, swarming is associated with altered cell morphology and hyperflagellation. It stands to reason that the morphological and behavioral changes result as a consequence of surface contact. In support of this, there is typically a lag between when bacteria contact a surface and when they initiate swarming motility. Several studies have shown that mutations alleviating this lag map to genes encoding regulatory proteins, suggesting a response involving transcriptional and post-transcriptional changes. In *Vibrio parahaemolyticus*, for example, the LonS protease regulates genes encoding the lateral flagella (*laf*) that mediate swarming; mutation of *lonS* resulted in constitutive *laf* gene expression, elongated cell morphology and enhanced swarming (Stewart, Enos-Berlage and McCarter 1997). The LonA protease inhibits motility and promotes biofilm and virulence in *V. cholerae* as well (Rogers *et al*. 2016). Mutation of a *lon* ortholog in *Proteus mirabilis* similarly conferred increased swarming, likely due to elevated transcription of flagellar genes and increased stability of the master regulator FlhD (Claret and Hughes 2000; Clemmer and Rather 2008). Recent studies in *Bacillus subtilis* have implicated the ortholog LonA in inhibition of the swarmer state in liquid medium through proteolysis of the master activator of flagellar synthesis, SwrA (Mukherjee *et al*. 2015). On surfaces, LonA degradation of SwrA is relieved, allowing flagellar biosynthesis and swarming. As differentiation to a swarmer cell is linked to increased virulence and antimicrobial resistance in several species (Mobley and Belas 1995; Wang *et al*. 2004; Overhage *et al*. 2008; Lai, Tremblay and Deziel 2009; Butler, Wang and Harshey 2010; Gode-Potratz *et al*. 2011), it is important to identify the mechanisms behind these surface-induced behaviors.

Flagella are also involved in surface sensing to promote the transcriptional and morphological shifts that occur upon surface contact in biofilm development. Cairns and colleagues inhibited flagellar rotation in *B. subtilis* by three distinct mechanisms and found that all three increased the activity of DegU, a transcriptional regulator known to inhibit flagellar gene expression and activate the expression of multiple genes involved in biofilm development, including the EPS synthetase gene *pgsB* (Ohsawa, Tsukahara and Ogura 2009; Verhamme, Murray and Stanley-Wall 2009; Hsueh *et al*. 2011; Cairns *et al*. 2013). First, a mutation in the stator protein MotB (*motB* D24A) allows normal flagellum biosynthesis but precludes its rotation due to the absence of ion flux through the flagellar motor (Cairns *et al*. 2013). Similarly, a D94A mutation in EpsE, an EPS synthase that also engages with the flagellar motor protein FliG to serve as a clutch for the flagellar motor, abolishes EPS biosynthesis but permits the EpsE-FliG interaction; overexpression of EpsE(D94A) inhibits flagellar function (Fig. 2A) (Guttenplan, Blair and Kearns 2010; Cairns *et al*. 2013). Whether flagellar rotation is impaired by the *motB* mutation or blocked by mutant EpsE, the result is an upregulation of DegU-regulated phenotypes, including *pgsB* transcription and EPS production (Cairns *et al*. 2013). Further, the addition of anti-flagellum antibodies to the growth medium 'tangles' the flagella and physically impedes rotation and stimulates *degU* transcription (Cairns *et al*. 2013). These data suggest that preventing flagellar rotation through various mechanisms, and likely through physical impediment with a surface, results in the initiation of regulatory pathways favoring biofilm formation. It is likely that the responses to a surface leading to either a swarmer phenotype or biofilm development overlap and additional environmental cues determine which avenue the bacterium pursues.

How surface contact by the flagellum results in altered gene transcription and adaptive behaviors is unclear. The prevailing model involves the detection of increased torque on the flagellar motor. As a rotating flagellum interacts with a surface (or surroundings of increased viscosity), rotation is impeded resulting in a higher load on the flagellum. Flagellar rotation is powered by ion flow through the stators, so impaired rotation slows or blocks ion flow (Manson *et al*. 1977; Zhou, Lloyd and Blair 1998). The consequent drop in ion flow upon surface contact has been proposed to stimulate pathways promoting surface attachment phenotypes. Studies using genetic approaches and/or reagents that dissipate membrane potential support the idea that changes in membrane potential occur upon contact of a bacterium, via the flagellum, with a surface (Goulbourne and Greenberg 1981, 1983; Zhou *et al*. 1998; Van Dellen, Houot and Watnick 2008). For example, a genetic screen for genes involved in establishing a *V. cholerae* monolayer, an early step in biofilm development, identified genes involved in generating membrane potential or responding to changes therein (Van Dellen, Houot and Watnick 2008). The same study provided evidence that chemical dissipation of the membrane potential blocked the transition from transient to more permanent surface attachment.

Harshey and Partridge (2015) have proposed that conformational changes occur at the interface between flagellar stators and rotors, which only engage when ions flow and torque is generated. In *Vibrio alginolyticus*, chemically inhibiting $Na^+$ ion flow with phenamil leads to low load on the flagellum, preventing stator-rotor engagement and stalling the flagellum (Fukuoka

*et al.* 2009). Conversely, in *E. coli*, high loads lead to fully engaged stators, resulting in either running or stalled motors (Lele, Hosu and Berg 2013; Tipping *et al.* 2013). It is possible that the flagellar motor senses changes in torque, and either increasing or decreasing the load could result in conformational changes that are sensed by the bacterium.

Nonetheless, the question remains: How do changes in ion motive force and membrane potential (or structural changes) result in the transcriptional or post-transcriptional changes that drive attachment? That membrane potential is capable of influencing gene expression has been documented, but to our knowledge it has not been shown to regulate the expression of motility or adherence genes (Patton, Yang and Bayles 2006; Alteri *et al.* 2011).

### TFP as surface sensors

The nature of the TFP as a surface-attachment fiber positions it as a sensor that can transmit information about surface contact to the bacterial cell. PilB-mediated extension of the TFP filament, which can be several microns in length, facilitates bacterial adherence to a surface, often via non-specific interactions (Craig, Pique and Tainer 2004; Giltner, Nguyen and Burrows 2012). Retraction of the anchored pilus by PilT can generate substantial force (exceeding 100 pN) (Maier *et al.* 2002; Biais *et al.* 2008; Clausen *et al.* 2009), bringing the bacterium in closer proximity with the surface. An attached TFP can thus withstand considerable applied force. This is made possible by the addition of pilin subunits to extend the length of the pilus in response to applied force (Maier, Koomey and Sheetz 2004; Clausen *et al.* 2009). In addition, the pilin subunits themselves can deform in response to applied force, allowing the pilus to stretch significantly (Biais *et al.* 2010). The mechanisms by which TFP mediate surface interactions were recently reviewed in detail (Maier and Wong 2015).

Recent evidence suggests that TFP function is modulated in response to surface interactions. In *N. gonorrhoeae*, TFP retraction can occur at high or low speeds, both of which are reduced by the force applied to the pilus fiber (Clausen *et al.* 2009). Interestingly, the switch between high- and low-speed retraction is regulated by oxygen availability and proton motive force (Kurre and Maier 2012; Kurre *et al.* 2013). The two-speed mode of retraction is conserved in *M. xanthus*, and likely other bacterial species (Clausen *et al.* 2009); however, the role of oxygen concentration and proton motive force in these systems has not been investigated. It is tempting to speculate that changes in proton motive force and concomitant changes in membrane potential as a consequence of flagellum-mediated attachment could influence the speed of TFP retraction and bacterial twitching motility.

## CYCLIC DIGUANYLATE: A KEY SIGNAL GUIDING SURFACE ASSOCIATION

The cyclic dinucleotide 3′,5′-cyclic diguanylic acid (cyclic diguanylate, c-di-GMP) is now a well-established regulator of the switch between a free-living, often motile lifestyle and a sessile, surface-associated form in bacteria, favoring the latter (Romling, Galperin and Gomelsky 2013). This intracellular signaling molecule impairs flagellum-based motility in numerous species by inhibiting flagellar gene expression, flagellum biosynthesis and/or flagellum function (Wolfe and Visick 2008). c-di-GMP further promotes sessility by positively regulating the production of ECM components, including protein adhesins and extracellular polysaccharides (Hengge 2009; Romling, Galperin and Gomelsky 2013). Therefore, c-di-GMP is a key factor influencing if and how a bacterium responds to a surface.

The intracellular level of c-di-GMP is controlled by enzymes with opposing activities: GGDEF-domain containing diguanylate cyclases (DGC, synthases) and EAL- or HD-GYP-domain containing phosphodiesterases (PDE, hydrolases) (Galperin, Nikolskaya and Koonin 2001). In turn, these enzymes are controlled at the transcriptional and post-transcriptional levels. Most bacterial species encode multiple, often numerous GGDEF, EAL and HD-GYP domains, resulting in complex c-di-GMP signaling systems (Romling, Gomelsky and Galperin 2005). In bacteria with complex c-di-GMP metabolic pathways, it is likely that only a subset of the DGC and PDE genes are expressed under a given condition, presumably in response to specific extracellular cues. Accordingly, numerous transcriptional profiling and mutagenesis studies have identified genes encoding GGDEF, EAL and HD-GYP domains as targets of regulation in response to growth conditions or gene mutations (Lee *et al.* 1998; Fong and Yildiz 2008; Waters *et al.* 2008; Dineen, McBride and Sonenshein 2010; Bernier *et al.* 2011; Saujet *et al.* 2011; Antunes *et al.* 2012; Chen *et al.* 2012; Martin *et al.* 2013). Redundancy among proteins with the same enzymatic function is also possible (McKee *et al.* 2014). In addition, GGDEF, EAL and HD-GYP domains are frequently found in tandem, in a single polypeptide, with additional sensory or regulatory domains (e.g. PAS, REC, HAMP, GAF) with potential to post-translationally control the activity of the associated enzymatic domain (Chou and Galperin 2016). For example, the *Caulobacter crescentus* DGC PleD consists of two tandem phosphoreceiver (REC) domains and a C-terminal GGDEF domain; phosphorylation of the first REC domains enhances c-di-GMP synthesis by the GGDEF domain by promoting formation of the active PleD dimer (Paul *et al.* 2004). The cumulative effects of active DGC(s) and/or PDE(s) in a cell determine the phenotypic output. Some DGC and PDE broadly affect c-di-GMP-regulated processes in a species, likely because they affect global intracellular c-di-GMP levels (Tischler, Lee and Camilli 2002; Tischler and Camilli 2004, 2005; Kulasakara *et al.* 2006; Lim *et al.* 2007; Pratt, McDonough and Camilli 2009). Other DGC and PDE have more targeted roles, impacting only a subset of processes (Kulasakara *et al.* 2006; Beyhan, Odell and Yildiz 2008). These observations have led to a model in which some DGC and PDE are restricted spatially within the cell, causing localized fluxes in c-di-GMP (Christen *et al.* 2010; Abel *et al.* 2013).

Changes in c-di-GMP are sensed by specific intracellular receptors, and numerous protein and RNA-based receptors have been identified to date. We refer the reader to a recent, comprehensive review of protein receptors of c-di-GMP, which highlights the diversity of protein structures and the conservation of 'signature motifs' that mediate interaction with the c-di-GMP ligand (Chou and Galperin 2016). Of note among these receptors is the widespread PilZ domain, which, like GGDEF, EAL and HD-GYP domains, is often present in proteins with additional modules and binds c-di-GMP with RxxxR and DxSxxG motifs (each motif interacting with one of the guanine bases) (Amikam and Galperin 2006; Benach *et al.* 2007; Ramelot *et al.* 2007). Some PilZ-domain containing proteins post-translationally inhibits the rotation of the flagellum in response to c-di-GMP binding (discussed in more detail below) (Ryjenkov *et al.* 2006; Boehm *et al.* 2010; Fang and Gomelsky 2010; Paul *et al.* 2010; Chen *et al.* 2012), while others positively regulate biofilm-associated ECM production (Weinhouse *et al.* 1997; Ryjenkov *et al.* 2006; Merighi *et al.* 2007; Pratt *et al.* 2007). Other c-di-GMP binding proteins, such as VpsT, FleQ/FlrA, MrkH, XcCLP, BldD and others, are transcription factors that directly

control gene expression in response to c-di-GMP (Hickman and Harwood 2008; Chin *et al*. 2010; den Hengst *et al*. 2010; Krasteva *et al*. 2010; Tao *et al*. 2010; Wilksch *et al*. 2011; Srivastava *et al*. 2013; Chambers *et al*. 2014). Recently, it was demonstrated that c-di-GMP binds and controls the function of certain ATPases, including the *Pseudomonas* flagellar rotary ATPase FliI, type 3 secretion export ATPase HrcN, type VI secretion ATPase ClpB2 and the *V. cholerae* MSHA TFP secretion ATPase MshE (Jones *et al*. 2015; Roelofs *et al*. 2015; Trampari *et al*. 2015). c-di-GMP binding by FliI, which is conserved in several bacterial species (Trampari *et al*. 2015), may regulate flagellar rotation, but this has not been demonstrated experimentally. However, binding of c-di-GMP to MshE was shown to be critical for secretion of the MshA pilin subunits, surface attachment and biofilm development (Roelofs *et al*. 2015). As interaction of MSHA with a surface is associated with decreased flagellar motility (Utada *et al*. 2014), the direct sensing of c-di-GMP by MshE influences both TFP and flagellar function.

In Gram-positive bacteria, two protein receptors for c-di-GMP have been identified: the PilZ-domain protein that regulates flagellar motility in *B. subtilis* and the transcription factor BldD in *Streptomyces coelicolor* discussed in more detail below (den Hengst *et al*. 2010; Chen *et al*. 2012; Gao *et al*. 2013). It is likely that other protein receptors remain to be identified. Two distinct types of c-di-GMP specific riboswitch (classes I and II) have also been described (Sudarsan *et al*. 2008; Lee *et al*. 2010). These riboswitches, especially the class I c-di-GMP riboswitch, are predicted in the genomes of both Gram-negative and positive genomes. The Firmicutes in particular appear to encode numerous class I c-di-GMP riboswitches, suggesting that this mechanism of regulation plays an important role in c-di-GMP signaling in these organisms. While the biochemical interactions between these riboswitches and the c-di-GMP ligand have been well studied, few studies have evaluated the physiological outcomes of these interactions (Karunker *et al*. 2013; Soutourina *et al*. 2013; Bordeleau *et al*. 2015; Peltier *et al*. 2015; Kariisa, Weeks and Tamayo 2016).

Studies of c-di-GMP signaling in Gram-positive species to date largely support a conserved role for c-di-GMP in controlling the switch from motile to sessile lifestyles, but elucidate some distinctions in mechanisms of regulation. In the next section, we review the findings published to date on c-di-GMP signaling in Gram-positive species. For each organism, we discuss the composition of the c-di-GMP metabolic network, the known or suspected c-di-GMP receptors, and the motile-sessile transitions regulated by c-di-GMP. The specific contributions of c-di-GMP to controlling surface interactions are highlighted.

## *Streptomyces coelicolor*

The genus *Streptomyces* is of biomedical interest because *Streptomyces* species synthesize antimicrobial compounds and are the source of the majority of known antibiotics (Barka *et al*. 2016). The *Actinobacteria* phylum, including *S. coelicolor* and *S. venezuelae*, exhibits extreme morphological transitions between spore formation and surface-associated mycelium production. Non-motile *Streptomyces* species disseminate to new environments as spores. Given the appropriate conditions, the bacteria germinate into vegetative cells that reproduce without separating, forming filamentous vegetative hyphae called mycelia. If triggered by extracellular stress such as nutrient limitation, they differentiate into reproductive aerial hyphae that form and release spores (Ensign 1978; de Jong *et al*. 2009; Barka *et al*. 2016). Mutants incapable of forming aerial hyphae are referred to as 'bald' to differentiate them from wild-type colonies, which look fluffy after hyphae development (Merrick 1976). Bald *Streptomyces* mutants are typically incapable of secreting antibiotic compounds, which usually occurs during aerial hyphae development (Barka *et al*. 2016). *Streptomyces* does not undergo motile-sessile transitions or form morphologically and metabolically distinct biofilms, but they are surface responsive; aerial hyphae only form on solid media (Ensign 1978). A subset of *Streptomyces* species including *Streptomyces griseus* and *Streptomyces venezuelae* can sporulate in liquid culture, but the majority do not form spores without differentiation into aerial hyphae (Daza *et al*. 1989; Glazebrook *et al*. 1990; Yague *et al*. 2012). *S. coelicolor* grown in agitated liquid culture enters stationary phase rather than differentiating and producing spores, while cells grown in static liquid culture only differentiate after creating a surface by secreting proteins that form a rigid film at the air–liquid interface (van Keulen *et al*. 2003; Manteca *et al*. 2008).

Aerial hyphae formation in *S. coelicolor* was the first process identified as regulated by c-di-GMP in a Gram-positive bacterium (den Hengst *et al*. 2010). *S. coelicolor* encodes five GGDEF proteins, one EAL domain protein, four GGDEF-EAL fusions and one HD-GYP domain protein (Table 1) (Chou and Galperin 2016; Tschowri 2016). Increasing intracellular c-di-GMP, by either overexpressing an endogenous DGC gene (*cdgA* or *cdgB*) or deleting a PDE gene (*rmdA* or *rmdB*), inhibits both hyphae formation and sporulation (den Hengst *et al*. 2010; Tran *et al*. 2011; Hull *et al*. 2012). Elevated c-di-GMP also inhibits antibiotic production (den Hengst *et al*. 2010; Tran *et al*. 2011). Conversely, overexpression of an exogenous PDE to lower c-di-GMP levels accelerates spore formation, resulting in 'pseudo-bald' cells that sporulate in the absence of aerial hyphae development (Tschowri *et al*. 2014). These data indicate that c-di-GMP regulates developmental progression toward spore formation and release. However, there is redundancy between the *rmdA* and *rmdB* PDEs, as deletion of both genes is necessary to cause a discernible increase in cytoplasmic c-di-GMP level (Hull *et al*. 2012).

*S. coelicolor* encodes no PilZ domains or c-di-GMP riboswitches, although two of its predicted EAL domains do not have conserved active sites and may be receptor proteins rather than PDEs (Hull *et al*. 2012; Chou and Galperin 2016). *Streptomyces* species and some other Actinobacteria encode a novel c-di-GMP receptor protein, the transcriptional regulator BldD (Tschowri *et al*. 2014; Chou and Galperin 2016). BldD is a master regulator controlling the transition between spore and mycelium production (den Hengst *et al*. 2010). To date, BldD is the only c-di-GMP receptor protein specific to Gram-positive bacteria to be structurally characterized, and it does not share c-di-GMP binding motifs with PilZ domains. Rather, BldD employs a previously unknown c-di-GMP binding mechanism. BldD functions as a dimer with no direct contact between monomers, which only interact when binding c-di-GMP (den Hengst *et al*. 2010; Tschowri *et al*. 2014). A tetramer of c-di-GMP, rather than the monomers or dimers bound by PilZ proteins, anchors the dimer interface, coupling BldD dimerization and function to c-di-GMP availability (Tschowri *et al*. 2014). Deletion of *bldD* accelerates sporulation in pseudo-bald cells, phenocopying PDE overexpression and supporting the role of BldD as a c-di-GMP receptor (Tschowri *et al*. 2014). Among the targets of BldD regulation are the DGC genes *cdgA* and *cdgB*, providing a potential regulatory loop (Tran *et al*. 2011). By controlling the timing of differentiation in cells growing on solid medium, c-di-GMP influences a lifestyle shift between surface association and dispersion even in a non-motile bacterium, as *Streptomyces* hyphae development and spore release is the only way for the organism to spread to new environments.

**Table 1.** The *S. coelicolor* c-di-GMP signaling network.

| Gene | Domain[a] | Activity *in vitro* | Deletion phenotype | Overexpression phenotype |
|---|---|---|---|---|
| *cdgB* | GGDEF | DGC | Bald<br>Blocked sporulation<br>Impaired antibiotic production | Bald<br>Blocked sporulation<br>Impaired antibiotic production |
| SCO5345 | GGDEF | | n.d. | n.d. |
| SCP1.113 | GGDEF | | n.d. | n.d. |
| SCO4931 | GGDEF | | n.d. | n.d. |
| SCO1398 | GGDEF | | n.d. | n.d. |
| *cdgA* | GGDEF-EAL | | n.d. | Bald<br>Blocked sporulation<br>Impaired antibiotic production |
| *rmdA* | GGDEF-EAL | PDE | Delayed sporulation | n.d. |
| *rmdB* | GGDEF-EAL | PDE | Delayed sporulation | n.d. |
| *rmdA/rmdB* | | | Bald<br>Blocked sporulation<br>Elevated c-di-GMP | n.d. |
| SCO5511 | GGDEF-EAL* | | | n.d. |
| SCO1397 | EAL* | | | n.d. |
| SCO5218 | HD-GYP | | | n.d. |
| *bldD* | BldD | Binds c-di-GMP | Pseudo-bald[b]<br>Accelerated sporulation<br>Disregulated *cdgA*, *cdgB*, SCO5511 | n.d. |

[a]Asterisks indicate sequences divergent from the consensus.
[b]Colonies appear bald but sporulation occurs, bypassing the need for aerial hyphae development.
n.d., not determined.

## *Bacillus subtilis*

*Bacillus subtilis* is a ubiquitous saprophytic microorganism in soil and vegetation, where it can adopt a biofilm lifestyle to cope with the harsh, often nutrient limiting environment. *B. subtilis* can also undergo sporulation in response to nutrient limitation at high cell density, although no specific nutritional signals have been identified (Sonenshein 2000). Sporulation occurs in response to a regulatory phosphorelay that culminates in the phosphorylation and activation of the master transcriptional regulator, Spo0A (Sonenshein 2000). These metabolically dormant spores allow prolonged survival and are produced within biofilms along with flagellated, motile cells and EPS-producing cells (Vlamakis *et al.* 2008). Because it is easily cultured and genetically tractable, *B. subtilis* is considered a model organism for the study of Gram-positive bacteria and of bacterial biofilm and spore formation.

The biochemical and biological activities of the predicted *B. subtilis* c-di-GMP synthesis, hydrolysis and binding enzymes have been systematically characterized (Table 2). *B. subtilis* encodes four GGDEF domain proteins, two EAL domain proteins, one GGDEF-EAL fusion protein and one PilZ domain protein (Chen *et al.* 2012; Gao *et al.* 2013). These c-di-GMP signaling proteins are conserved among *Bacillus* species, including the pathogens *Bacillus cereus* and *Bacillus anthracis* (Chou and Galperin 2016). In *B. subtilis*, growth in standard rich medium results in c-di-GMP levels below the limit of detection (Gao *et al.* 2013). Overproduction of YhcK/DgcK or YtrP/DcgP, which contain GGDEF domains, significantly increases intracellular c-di-GMP (Gao *et al.* 2013). The GGDEF-EAL fusion protein YkoW/DgcW also appears to function primarily as a DGC, as its overproduction elevates cytoplasmic c-di-GMP levels (Gao *et al.* 2013). These three DGCs are also capable of synthesizing c-di-GMP *in vitro* (Gao *et al.* 2013). However, the biological roles of these cyclases are unknown, as deletion of the individual genes or deletion of all three functional cyclase genes does not result in any observable phenotypic changes in biofilm formation or motility under standard growth conditions (Chen *et al.* 2012; Gao *et al.* 2013).

The YuxH/PdeH EAL domain protein is a functional PDE *in vitro* (Gao *et al.* 2013). Deletion of *yuxH/pdeH* alone increases cytoplasmic c-di-GMP levels and results in impaired flagellar swarming motility (Chen *et al.* 2012; Gao *et al.* 2013). The EAL domain of the YkuI protein has a conserved active site and the purified domain binds c-di-GMP, but neither the domain nor the full-length protein exhibits catalytic activity *in vitro* (Minasov *et al.* 2009). Chen *et al.* (2012) observed slightly impaired motility in a Δ*ykuI* strain, but the motility of a Δ*yuxH/pdeH*, Δ*ykuI*, Δ*ykoW/dgcW* triple mutant was indistinguishable from that of the Δ*yuxH/pdeH* single mutant, suggesting that YuxH/PdeH is the only significant c-di-GMP PDE in the conditions studied.

*B. subtilis* encodes two GGDEF domain containing proteins, YdaK and YybT, that have degenerate active site sequences lacking conserved residues in the cyclase active site (Gao *et al.* 2013). YdaK appears to be catalytically inactive but can bind c-di-GMP, suggesting that YdaK functions as a receptor rather than a DGC (Gao *et al.* 2013). Interestingly, the YybT GGDEF domain is coupled to a PDE domain that degrades cyclic diadenylate monophosphate (c-di-AMP). The YybT GGDEF domain cannot synthesize c-di-GMP but exhibits weak ATPase activity *in vitro* (Rae *et al.* 2010).

The single PilZ domain protein, YpfA/DgrA, is a confirmed c-di-GMP receptor controlling flagellar motility in a post-translational manner. YpfA/DgrA is necessary for c-di-GMP-dependent inhibition of flagellar motility (Chen *et al.* 2012; Gao *et al.* 2013). In a Δ*yuxH/pdeH* strain with elevated c-di-GMP and reduced swarming motility, deletion of *ypfA/dgrA* restores wild-type motility, while overproduction of YpfA/DgrA exacerbates the motility defect (Gao *et al.* 2013). Overproduction of YkuI or YdaK in the Δ*yuxH/pdeH* mutant background does not impact motility, so if these proteins are bona fide c-di-GMP receptors, they likely control pathways other than motility (Gao *et al.* 2013).

**Table 2.** The *B. subtilis* c-di-GMP signaling network.

| Gene[a] | Domain[b] | Activity | Deletion phenotype | Overexpression phenotype |
|---|---|---|---|---|
| *yhcK/dgcK* | GGDEF | DGC | None | Elevated c-di-GMP<br>Impaired motility |
| *ytrP/dgcP* | GGDEF | DGC | None | Elevated c-di-GMP<br>Impaired motility |
| *ydaK* | GGDEF* | None | None | None[d] |
| *yybT* | GGDEF* | Weak ATPase | None | n.d. |
| *yuxH/pdeH* | EAL | PDE | Elevated c-di-GMP<br>Impaired motility | n.d. |
| *ykuI* | EAL | None | Slightly impaired motility | None[d] |
| *ykoW/dgcW* | GGDEF-EAL | DGC | none | Elevated c-di-GMP (entire protein)<br>Impaired motility (GGDEF alone) |
| *ypfA/dgrA* | PilZ | Binds c-di-GMP | Enhanced motility<br>Increased biofilm/pellicle formation[c]<br>Restored motility in DGC overexpression strains | Impaired motility[d] |

[a]Names are according to (Chen *et al.* 2012; Gao *et al.* 2013).
[b]Asterisks indicate sequences divergent from the consensus.
[c]Phenotype observed by Chen *et al.*, but not Gao *et al.*
[d]Tested in Δ*yuxH/pdeH* mutant with elevated c-di-GMP.
n.d., not determined

YpfA/DgrA interacts with the flagellar motor protein MotA, suggesting that it regulates motility through protein complex formation (Chen *et al.* 2012). YpfA/DgrA also affects the *B. subtilis* response to air–liquid interfaces. *B. subtilis* forms architecturally complex biofilm colonies on agar surfaces and thick, wrinkled pellicles that persist for roughly 3 days in standing liquid culture (Branda *et al.* 2001). Deletion of individual DGC or PDE genes has no effect on these processes, but deletion of the *ypfA/dgrA* receptor gene increases the production of ECM, accelerates the development of 3D wrinkles in biofilm colonies and delays disassembly of pellicles, resulting in more robust multicellular communities at environmental interfaces (Chen *et al.* 2012). Mutation of *spo0A*, the transcriptional regulator that controls sporulation, disrupts the transcription of EPS biosynthesis genes and abolishes biofilm architecture and pellicle formation (Branda *et al.* 2001). Spo0A also controls the transcription of *yuxH/pdeH*, further linking c-di-GMP-dependent biofilm and pellicle formation to stationary phase onset and sporulation (Branda *et al.* 2001; Chen *et al.* 2012).

*B. subtilis* encodes a very small c-di-GMP regulatory network, with only four metabolic enzymes and one confirmed receptor protein identified to date. None of the *B. subtilis* DGC or PDE genes are essential, and the successful construction of a 'c-di-GMP null' strain deficient in *ydaK*, *yhcK/dgcK*, *ykoW/dgcW*, *ytrP/dgcP* and *yuxH/pdeH* confirms that c-di-GMP is not essential for viability or growth under the conditions tested (Gao *et al.* 2013). Nor is c-di-GMP accumulation toxic to *B. subtilis* as a *yuxH/pdeH* mutant strain with no ability to hydrolyze the second messenger is also viable (Chen *et al.* 2012).

## *Clostridium difficile*

The spore-forming Gram-positive intestinal pathogen *Clostridium difficile* is the most common cause of hospital-acquired infections in the United States, causing antibiotic-associated diarrhea and potentially fatal colitis (Erik *et al.* 2008; Miller 2010; Drekonja *et al.* 2011; Lessa, Winston and McDonald 2015). *C. difficile*-associated disease is driven by protein cytotoxins, TcdA and TcdB, that cause apoptosis and necrosis, disrupting the integrity of the intestinal barrier and eliciting massive inflammation (Voth and Ballard 2005; Denève *et al.* 2008; Kuipers and Surawicz 2008; Vlamakis *et al.* 2008; McCollum and Rodriguez 2012; Gao *et al.* 2013). *C. difficile* is an obligate anaerobe, but persists in the oxygenated environment as metabolically dormant endospores, which are highly resilient to environmental stresses, including oxygen, heat and ethanol (Gerding *et al.* 2008). *C. difficile* spores germinate in the digestive system of susceptible hosts and establish highly persistent infections, with a 10%–20% recurrence rate after initial treatment (Drekonja *et al.* 2011; McCollum and Rodriguez 2012). Vegetative *C. difficile* adheres to mammalian cells *in vitro* and expresses several surface and cell wall proteins that interact with the mammalian ECM and potentially contribute to host cell interaction (Eveillard *et al.* 1993; Karjalainen *et al.* 1994; Waligora *et al.* 2001; Calabi *et al.* 2002; Cerquetti *et al.* 2002; Hennequin *et al.* 2003; Janoir *et al.* 2007; Barketi-Klai *et al.* 2011). There is evidence that persistence involves interaction with the intestinal mucosa and/or epithelial surface, and biofilms have been suggested to contribute to persistence (Borriello 1979; Borriello *et al.* 1988; Gomez-Trevino *et al.* 1996; Goulding *et al.* 2009; Lawley *et al.* 2009; Semenyuk *et al.* 2015). Flagella may or may not play a role in adhesion to host tissues; assays performed with different *C. difficile* strains, mutations and animal models have resulted in conflicting conclusions, leaving the role of flagella in *C. difficile* colonization and virulence unclear (Tasteyre *et al.* 2001; Dingle, Mulvey and Armstrong 2011; Aubry *et al.* 2012; Baban *et al.* 2013; Stevenson, Minton and Kuehne 2015).

In contrast to the small c-di-GMP metabolic networks encoded by *S. coelicolor* and *B. subtilis*, *C. difficile* encodes 37 predicted c-di-GMP cyclases and PDEs, devoting nearly 1% of its genome to c-di-GMP metabolism, and c-di-GMP is present in the cytoplasm of multiple wild-type *C. difficile* strains (Sebaihia *et al.* 2006; Purcell *et al.* 2012, 2015). The metabolic activity of 28 of these enzymes has been confirmed by heterologous expression in *V. cholerae* and/or in 'c-di-GMP null' *B. subtilis* (Bordeleau *et al.* 2011; Gao *et al.* 2014). The others may function under specific conditions in *C. difficile* and/or serve as receptors. *C. difficile* encodes only one PilZ domain and no other predicted receptor domains, yet it encodes 12 predicted class I and four predicted class II c-di-GMP riboswitches (Soutourina *et al.* 2013; Chou and Galperin 2016).

While no mutations of DGC or PDE genes or associated phenotypes have been reported, multiple traits are affected by inducible overproduction of the endogenous DccA synthase,

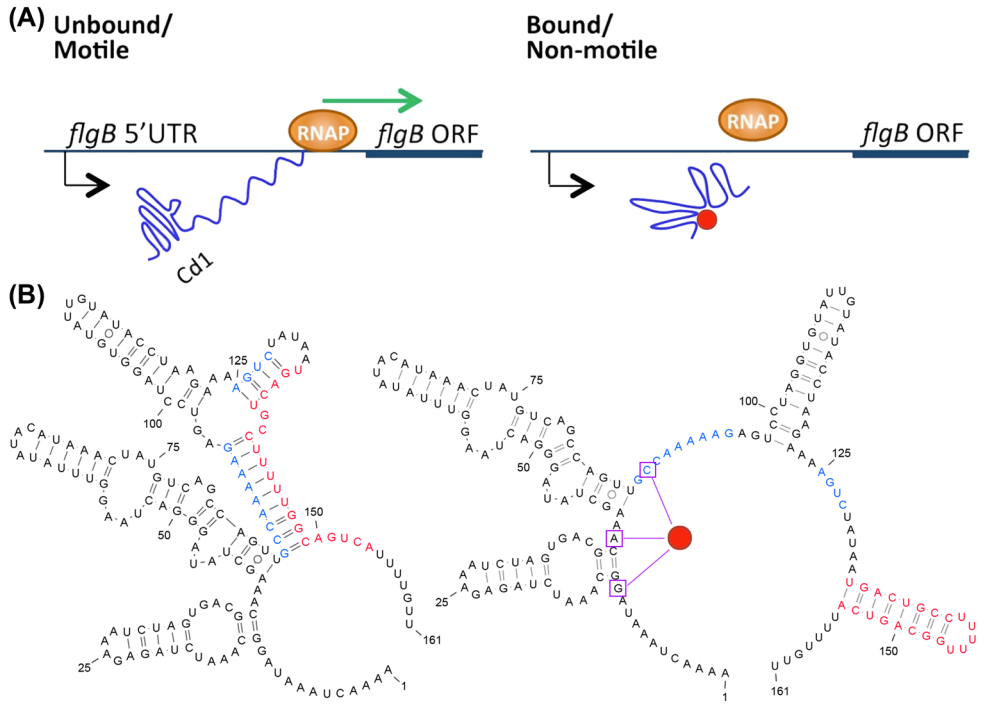


**Figure 3.** Control of flagellar gene expression by the Cd1 riboswitch in *C. difficile*. (**A**) Cd1 is a class I c-di-GMP riboswitch upstream of *flgB*, the first gene in a large (~23 kb) flagellar operon in *C. difficile*. In the proposed model, in the absence of c-di-GMP, the Cd1 riboswitch structure allows synthesis of the full-length flagellar gene transcript, promoting biosynthesis of flagella and motility. In the presence of c-di-GMP (represented by the red circle), Cd1 folds into an alternate structure that includes a Rho-independent transcription terminator. This leads to destabilization of the RNAP-DNA complex, premature transcription termination, and reduced flagellar gene expression and motility. (**B**) The predicted structures of Cd1 with and without c-di-GMP (red circle) were determined based on alignment with the *in vitro*-characterized riboswitch Vc2 and using Mfold. The conserved stemloops are labeled (P2 and P3) (Sudarsan *et al.* 2008; Lee *et al.* 2010). Unlike Vc2, Cd1 is not predicted to form a P1 stem in the presence of c-di-GMP (Smith *et al.* 2009). The predicted contact residues for c-di-GMP, based on the sequence alignment with Vc2, are boxed. The predicted Rho-independent transcription terminator sequence is indicated with red text. The anti-terminator sequence complementary to a portion of the terminator (green text) is proposed to preclude formation of the transcription terminator, allowing transcription read-through.

which can elevate cytoplasmic c-di-GMP levels across three orders of magnitude (Purcell *et al.* 2012). Initial studies of DccA induction revealed that c-di-GMP represses *C. difficile* flagellar motility through semi-solid medium and promotes autoaggregation into fibril-linked clumps that sediment out of liquid culture (Purcell *et al.* 2012). Regulation of swimming motility occurs through a type I c-di-GMP riboswitch in the 5′ untranslated region (UTR) of the *flgB* flagellar biosynthesis operon, which undergoes premature transcription termination in response to c-di-GMP (Fig. 3) (Purcell *et al.* 2012; Soutourina *et al.* 2013). The *flgB* operon includes *fliA/sigD*, which encodes the flagellar alternative sigma factor SigD, and thus elevated c-di-GMP inhibits the transcription of multiple flagellar operons and impairs motility (Purcell *et al.* 2012; El Meouche *et al.* 2013). SigD also regulates the transcription of *tcdR*, which encodes a positive transcriptional regulator of the toxins TcdA and TcdB (El Meouche *et al.* 2013; McKee *et al.* 2013). The supernatants of *C. difficile* cultures with elevated c-di-GMP contain less secreted toxin and have significantly reduced cytopathicity toward human cells (McKee *et al.* 2013). Thus, c-di-GMP controls a major virulence trait of *C. difficile* indirectly through the flagellar c-di-GMP riboswitch.

TFP, which have not been extensively studied in Gram-positive bacteria, contribute to c-di-GMP-induced autoaggregation of *C. difficile* (Bordeleau *et al.* 2015). c-di-GMP positively regulates TFP biosynthesis via a type II c-di-GMP riboswitch in the 5′ UTR of the *pilA1* pilin gene, which forms a terminator stem loop in the absence of c-di-GMP (i.e. in contrast to the flagellar riboswitch) (Soutourina *et al.* 2013; Bordeleau *et al.* 2015). Biofilm formation on abiotic surfaces is similarly stimulated by riboswitch-dependent c-di-GMP regulation of TFP biosynthesis (Soutourina *et al.* 2013; Purcell *et al.* 2015). In addition, TFP were also shown to mediate motility across agar surfaces, a process augmented by elevated c-di-GMP (Purcell *et al.* 2015).

TFP are nearly absent from wild-type *C. difficile* grown in liquid culture but can be induced by increased c-di-GMP (Bordeleau *et al.* 2015; Purcell *et al.* 2015). A colorimetric reporter based on a fusion of the *pilA1* c-di-GMP riboswitch to a beta-glucuronidase (*gusA*) gene has been generated and used to evaluate c-di-GMP levels in *C. difficile* in different growth conditions (Mani and Dupuy 2001; Emerson *et al.* 2009; Purcell *et al.* 2015). TFP-based motility of the historical, virulent *C. difficile* 630 strain is relatively surface insensitive (Purcell *et al.* 2015). In contrast, *C. difficile* R20291, a strain associated with recent disease epidemics, accumulates higher levels of c-di-GMP and upregulates transcription of TFP genes during growth in biofilms or on agar surfaces compared to growth in liquid culture, suggesting that it has a greater capacity to sense and respond to surfaces (Purcell *et al.* 2015). R20291 also initiates transcription at the *pilA1* promoter at a higher rate than 630 in the absence of c-di-GMP manipulation, which may allow R20291 to form more robust biofilms and exhibit greater surface motility than 630 (Purcell *et al.* 2015). R20291 c-di-GMP accumulation and TFP-dependent motility on agar surfaces are highly dependent on surface hardness, increasing during biofilm formation on plastic and with the agar percentage between 0.9% and 1.8% agar (Purcell *et al.* 2015). These results provide strong evidence that c-di-GMP plays a role in surface sensing as well as surface adhesion. Furthermore, given that c-di-GMP inversely regulates

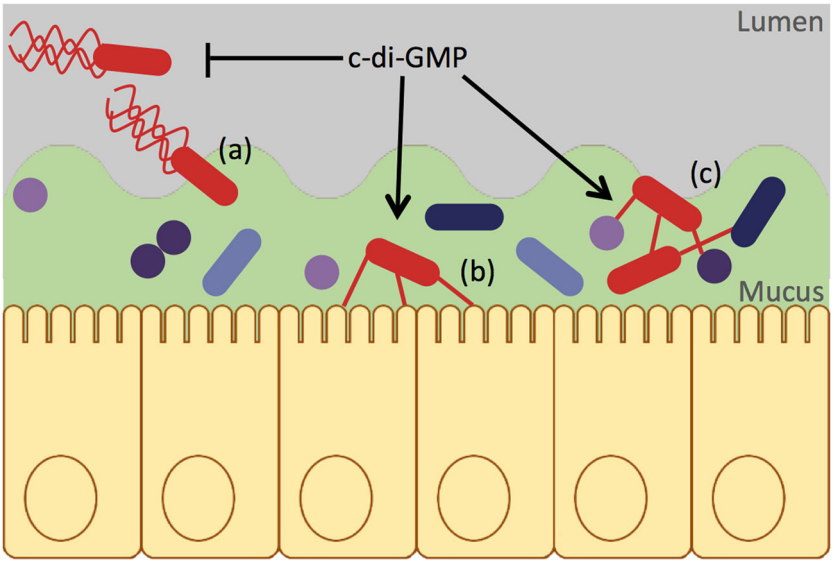


**Figure 4.** Model of surface sensing via TFP in *C. difficile*. Intracellular c-di-GMP inhibits flagellar biosynthesis and stimulates TFP formation in *C. difficile*, both through riboswitch-dependent mechanisms controlling expression of the respective genes. Based on these data, we propose that flagella and TFP are produced under distinct spatiotemporal conditions in the large intestine, though there may be conditions under which both are produced. Peritrichous flagella may allow *C. difficile* (depicted as red bacilli) to swim through the viscous intestinal lumen and mucosa (**a**). TFP promote *C. difficile* biofilm formation, motility across solid surfaces and aggregation *in vitro* and may allow *C. difficile* to adhere to the intestinal epithelium (**b**) and/or other intestinal bacteria (**c**). Not shown, c-di-GMP also promotes the production of other putative adhesins, by directly promoting the transcription of the respective genes and/or inhibiting the production of the ZmpI protease that cleaves these adhesins (Cafardi *et al.* 2013; Soutourina *et al.* 2013; Hensbergen *et al.* 2015; Peltier *et al.* 2015). Decreases in c-di-GMP may thus restore flagellum biosynthesis and downregulate the putative adhesins, allowing *C. difficile* to detach and disseminate.

flagella and TFP, the two structures may function during distinct spatiotemporal conditions. For example, it is possible that flagella play a role in *C. difficile* motility through the intestinal lumen and mucus, while TFP mediate attachment to pre-existing microbial communities in the mucosa or to the host epithelium (Fig. 4).

Disruption of TFP genes abolishes c-di-GMP-induced surface motility but only reduces biofilm formation by *C. difficile* 630, suggesting that c-di-GMP regulates multiple surface interaction mechanisms (Purcell *et al.* 2015). Another type I c-di-GMP riboswitch represses the transcription of *zmpI*, which encodes a secreted zinc metalloprotease (Cafardi *et al.* 2013; Soutourina *et al.* 2013; Peltier *et al.* 2015). *C. difficile* ZmpI degrades the human ECM components fibronectin and fibrogenin, to which several *C. difficile* surface proteins adhere (Waligora *et al.* 2001; Calabi *et al.* 2002; Hennequin *et al.* 2003; Janoir *et al.* 2007; Barketi-Klai *et al.* 2011; Cafardi *et al.* 2013). In addition, ZmpI cleaves two *C. difficile* surface proteins with predicted roles in binding collagen, another mammalian ECM component (Hensbergen *et al.* 2015). Both of these predicted adhesins are under the positive transcriptional control of type II c-di-GMP riboswitches, suggesting that high levels of c-di-GMP promote adhesion to host cells by ECM-binding proteins as well as TFP, and that low levels of c-di-GMP allow the production of flagella and an adhesin-cleaving protease to promote motility away from surfaces (Soutourina *et al.* 2013; Hensbergen *et al.* 2015; Purcell *et al.* 2015).

## *Listeria monocytogenes*

*Listeria monocytogenes*, a causative agent of food-borne illness (listeriosis), is responsible for far-ranging outbreaks of disease, in part because its biofilms can survive on contaminated food during shipping and can persist in processing plants for years to cause repeated outbreaks (Møretrø and Langsrud 2004; McCollum *et al.* 2013). The composition and regulation of listerial biofilm formation is poorly understood, though recently c-di-GMP was implicated in this process (Chen *et al.* 2014).

*L. monocytogenes* encodes a relatively minimal set of c-di-GMP metabolic enzymes: three proteins with a GGDEF domain, three with EAL domain PDEs and one with a degenerate GGDEF domain (Table 3) (Chen *et al.* 2014). The DGC enzymatic activities of the *L. monocytogenes* GGDEF-domain proteins DgcA, DgcB and DcgC were evaluated in a heterologous organism, *E. coli*. Overexpression of the corresponding genes increases synthesis of curli fimbrae and inhibits swimming motility, consistent with increased c-di-GMP in *E. coli* (Christen *et al.* 2006; Chen *et al.* 2014). The EAL-domain proteins PdeB, PdeC and PdeD were similarly shown to be functional PDEs. The purified proteins hydrolyze c-di-GMP *in vitro*, and all three enhance motility in the *E. coli* system (Chen *et al.* 2014). There are no predicted PilZ domains or c-di-GMP riboswitches predicted in the *L. monocytogenes* genome (Chou and Galperin 2016). However, PssE, which contains a degenerate GGDEF domain, lacks enzymatic activity but binds c-di-GMP *in vitro* with micromolar affinity and was hypothesized to serve as a c-di-GMP receptor (Chen *et al.* 2014).

As with *B. subtilis*, no phenotypic changes in flagellar motility or biofilm formation are observed in strains with mutations in individual or pairs of the PDE genes (Chen *et al.* 2014). The triple Δ*pdeB/C/D* mutant displays severely inhibited motility through soft agar, suggesting overlapping functions between the three PDEs. Notably, abolishing all c-di-GMP hydrolysis in the Δ*pdeB/C/D* mutant does not appear to be detrimental to viability or growth (Chen *et al.* 2014). It appears that unchecked c-di-GMP accumulation is not toxic in either *B. subtilis* or *L. monocytogenes* (Chen *et al.* 2012, 2014). Elevated c-di-GMP levels in the Δ*pdeB/C/D* mutant strain, or in a strain overexpressing a heterologous DGC gene, result in increased synthesis of EPS (Chen *et al.* 2014). More subtly, simultaneous deletion of *pdeB* and *pdeC* augments EPS biosynthesis compared to the wild type (Chen *et al.* 2014). This c-di-GMP-induced EPS production requires the

**Table 3.** The *L. monocytogenes* c-di-GMP signaling network.

| Gene | Domain[a] | Activity[b,c] | Deletion phenotype |
|---|---|---|---|
| *dgcA* | GGDEF | DGC[b] | Reduced EPS synthesis[d] |
| *dgcB* | GGDEF | DGC[b] | Reduced EPS synthesis[d] |
| *dgcC* | GGDEF | DGC[b] | None |
| *dgcA/B/C* | | | None |
| *pssE* | GGDEF* | Binds c-di-GMP[c] | Reduced aggregation[d]<br>Reduced EPS synthesis[d] |
| *pdeB* | EAL | PDE[b,c] | None |
| *pdcC* | EAL | PDE[b,c] | None |
| *pdeD* | EAL | PDE[b,c] | None |
| *pdeB/C/D* | | | Aggregation<br>Impaired motility<br>Increased disinfectant/desiccation tolerance<br>Impaired virulence<br>Increased EPS synthesis |

[a]Asterisks indicate sequences divergent from the consensus.
[b]Assayed by heterologous expression in *E. coli* and *in vitro* assays.
[c]Assayed using purified proteins *in vitro*.
[d]Phenotype in *pdeB/C/D* triple mutant background (elevated c-di-GMP).

activity of PssE, the degenerate GGDEF domain protein, supporting a role for PssE as a c-di-GMP receptor (Chen *et al.* 2014; Koseoglu *et al.* 2015). Interestingly, PssE is encoded in a locus with genes involved in EPS biosynthesis (Fig. 5), genetically linking its function as a c-di-GMP sensor directly to controlling EPS production (Chen *et al.* 2014; Koseoglu *et al.* 2015). Deletion of *dgcA*, *dgcB* or *dgcC* (or combinations thereof) results in no detectible phenotypic changes in a wild-type background (Chen *et al.* 2014). However, individual deletion of *dgcA* or *dgcB*, but not *dgcC*, reduces EPS biosynthesis in the Δ*pdeB/C/D* background (Koseoglu *et al.* 2015). It is clear that *L. monocytogenes* c-di-GMP homeostasis is tightly regulated and the bacterium is capable of modulating the expression levels and/or activities of other c-di-GMP metabolic enzymes in order to prevent individual gene deletions from perturbing cellular c-di-GMP levels.

The contribution of this EPS to 3D biofilm formation of *L. monocytogenes* is unclear. However, increased EPS production increases bacterial aggregation and tolerance to disinfectants and desiccation. Furthermore, c-di-GMP-induced EPS impedes bacterial motility and inhibits host cell invasion and spread between organs in the mouse model (Chen *et al.* 2014). In the absence of EPS synthesis, elevated c-di-GMP has no effect on aggregation, motility or systemic spread through a host but does cause an EPS-independent defect in intracellular invasion (Chen *et al.* 2014). In a medium that promotes biofilm formation, the Δ*pdeB/C/D* mutant shows somewhat increased biofilm formation even in mutant strains incapable of synthesizing Pss-encoded EPS, suggesting that c-di-GMP regulates listerial sessility through an additional mechanism (Chen *et al.* 2014).

## *Staphylococcus aureus* and *Streptococcus mutans*

Several Gram-positive pathogens that do not metabolize c-di-GMP have either retained or acquired the ability to respond to c-di-GMP in biofilms, which are often multispecies. *Staphylococcus aureus* is an opportunistic human pathogen and frequent cause of nosocomial infections, which typically arise from contact with bacterial biofilms on medical devices (O'Toole, Kaplan and Kolter 2000; McCarthy *et al.* 2015). *Streptococcus mutans* is part of the human oral flora, and the biofilms it forms on teeth are a majority contributor to dental caries (Loesche 1986). Both genomes encode a single protein with a degenerate GGDEF domain but no predicted PDEs, known receptor proteins or riboswitches (Karaolis *et al.* 2005; Holland *et al.* 2008; Yan *et al.* 2010; Chou and Galperin 2016). Thus, there are no intact c-di-GMP signaling networks in these organisms.

The *St. aureus* degenerate GGDEF-containing *gdpS* gene encodes a protein with no DGC activity *in vitro* (Holland *et al.* 2008). Deletion of *gdpS* reduces biofilm formation in some *Staphylococcus* species and enhances it in others, leaving its broader role unclear (Holland *et al.* 2008; Fischer *et al.* 2014). In *St. epidermidis*, deletion of *gdpS* inhibits biofilm development; this can be complemented by the expression of a truncated *gdpS* gene lacking the GGDEF domain, confirming that GdpS regulation of biofilm formation is independent of c-di-GMP biosynthesis (Holland *et al.* 2008). Similarly, inactivation of the *St. aureus gdpS* gene alters the expression of several virulence genes, and *gdpS* with the GGDEF domain mutated or deleted can restore transcription to wild-type levels (Shang *et al.* 2009). It appears that GdpS contains a vestigial GGDEF domain whose role in regulating biofilm and virulence has been conserved in organisms that do not metabolize c-di-GMP.

*Streptococcus* species have no conserved DGC or PDE genes but do have *gcp* (SMU_2140c), also referred to as *pde1* in *Streptococcus pneumoniae* and *pdeA* in *Strep. mutans*. These orthologs have similarity to *B. subtilis* YybT, which has an enzymatically inactive GGDEF domain and a DHH domain with c-di-AMP-specific PDE activity (Rae; Yan *et al.* 2010; Bai *et al.* 2013; Peng *et al.* 2015). Yan *et al.* (2010) reported that insertional inactivation of *gcp* inhibits biofilm formation in *Strep. mutans*, while Peng *et al.* (2015) reported that deletion of the equivalent *pdeA* increases cytoplasmic c-di-AMP and enhances biofilm formation in *Strep. mutans*; both of these phenotypes are partially complemented by expressing the gene *in trans*. These divergent findings may result from the difference between insertional inactivation and gene deletion, but it appears that the role of *gcp/pdeA* in regulating streptococcal biofilm formation is c-di-GMP independent.

Both *Staphylococcus* and *Streptococcus* species exhibit reduced biofilm formation when treated with extracellular c-di-GMP, and *St. aureus* virulence in a mouse model is reduced when the mice are administered c-di-GMP after infection (Brouillette *et al.* 2005; Karaolis *et al.* 2005; Holland *et al.* 2008; Yan *et al.* 2010). This may be due to an increased host innate immune response

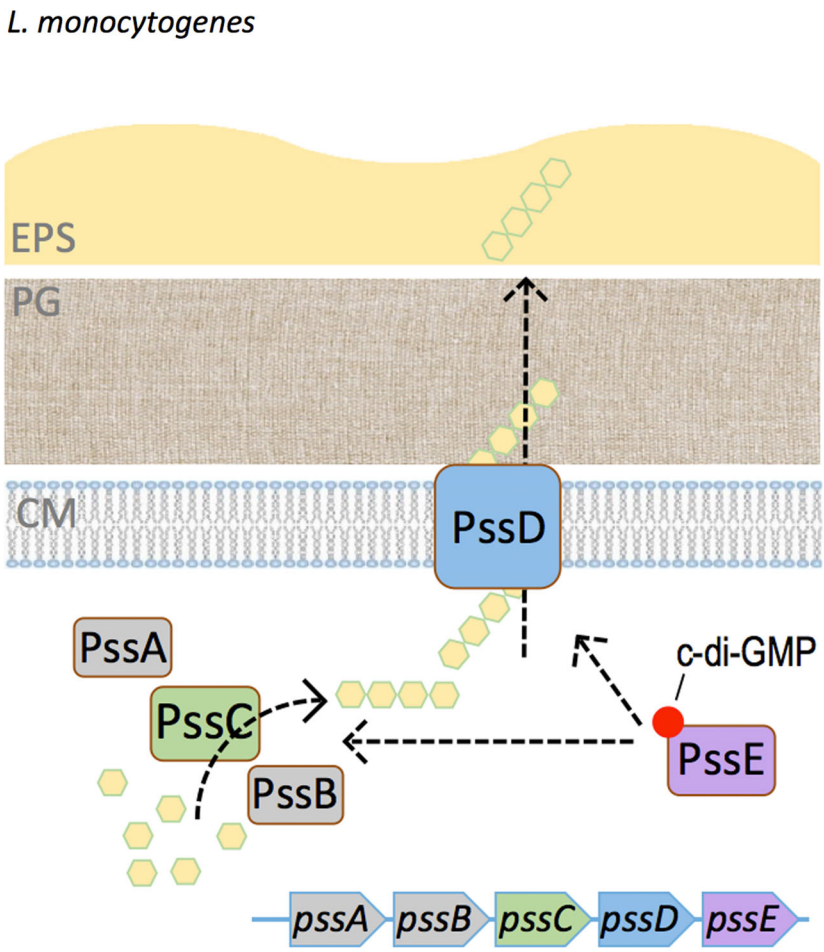


**Figure 5.** c-di-GMP promotes EPS production in *L. monocytogenes*. *Listeria monocytogenes* encodes three DGCs and three PDEs that control intracellular c-di-GMP levels (not shown). C-di-GMP positively regulates EPS production through PssE, an enzymatically inactive GGDEF domain protein that functions as a c-di-GMP receptor. The *pssE* gene is the fifth in a putative operon containing four genes predicted to be involved in EPS biosynthesis. PssC is a putative type 2 glycosyltransferase predicted to be involved in incorporation of the relevant sugars into the EPS. PssD has homology to BscB, the B subunit of the bacterial cellulose synthase. BscB does not catalyze cellulose synthesis, but are involved in export of the polymer. Thus, PssD may be involved in translocation of the as yet unknown EPS across the membrane. The remaining genes in the *pssA-E* locus, *pssA* and *pssB*, are predicted to encode a transmembrane protein and deacetylase, respectively. Their contributions to *L. monocytogenes* EPS biosynthesis have not been determined.

stimulated by c-di-GMP. While mammals do not produce c-di-GMP, the production of cytoplasmic receptors with high affinity for c-di-GMP, such as STING and DDX41, allows detection of microbially derived c-di-GMP (Burdette *et al.* 2011; Sauer *et al.* 2011; Parvatiyar *et al.* 2012). Consequently, c-di-GMP stimulates type I interferon production and the recruitment of neutrophils and other immune cells to sites of infection, which could inhibit bacterial colonization and biofilm formation (Karaolis *et al.* 2007; Yan *et al.* 2009). Alternatively (or in addition), it is also possible that c-di-GMP crosstalk from other bacterial species within established biofilms is a signal of an unfavorable environment. c-di-GMP promotes biofilm formation in multiple bacterial species and is likely to be present at high levels in the cytoplasm of many individual cells within a multispecies biofilm (Purcell *et al.* 2015). Cells in biofilms can disperse or lyse when environmental conditions become unfavorable, and genomic DNA from lysed cells can serve as an inhibitor of subsequent biofilm formation (Berne, Kysela and Brun 2010). It is possible that c-di-GMP released by lysed cells similarly inhibits biofilm formation and/or promotes dispersal in species that do not otherwise employ c-di-GMP signaling such as *St. aureus* and *Streptococcus* spp.

## C-DI-GMP AND THE BACTERIAL RESPONSE TO A SURFACE

c-di-GMP is poised to control the bacterial response to surface contact, whether by preventing contact (e.g. by inhibiting the production of flagella) or regulating the physiological response to contact (e.g. by impacting flagellar function and/or EPS biosynthesis). Overall, there is relatively little known about the extracellular conditions governing intracellular c-di-GMP levels and downstream processes. There is growing evidence that c-di-GMP levels change during interactions with a surface, both in Gram-positive and Gram-negative bacteria, suggesting that surface contact itself is a stimulus for c-di-GMP synthesis.

Examination of the localization of WspA and WspR, two proteins in a chemosensory-like system regulating biofilm production in *P. aeruginosa*, suggests that c-di-GMP increases during surface association. WspR, a DGC composed of an N-terminal phosphoreceiver domain fused to a C-terminal GGDEF domain, was found to cluster in the cytoplasm in a phosphorylation-dependent manner (Hickman, Tifrea and Harwood 2005; Guvener and Harwood 2007). In liquid medium, the WspR clusters only appeared in a *wspF* mutant background that renders

WspR-mediated c-di-GMP synthesis constitutively active. In a wild-type background, however, WspR clusters did not appear in broth-grown bacteria, but did appear in cells grown on an agar surface. Clusters were observed regardless of the nutrient source, as numerous media and supplements were tested, suggesting that surface contact primed WspR clustering. A later study showed that clustering of phosphorylated WspR was associated with increased DGC activity, suggesting that while phosphorylation stimulates WspR DGC activity, it is clustering of the protein that potentiates its enzymatic function (Huangyutitham, Guvener and Harwood 2013). These findings support a model in which enzymatically active WspR becomes localized during surface growth, restricting c-di-GMP production within the cell under these conditions.

Indeed, *P. aeruginosa* grown on an agar surface has an intracellular concentration of c-di-GMP approximately three to five times higher than bacteria grown in the same liquid media (Kuchma, Griffin and O'Toole 2012), consistent with surface contact serving as a stimulus to increased c-di-GMP to switch from a motile to a sessile lifestyle. The surface-exposed PilY1 protein, which is involved in TFP biogenesis and twitching motility, plays a key role in this signaling pathway. The opposing activities of the SadC DGC and BifA PDE modulate c-di-GMP levels to control biofilm formation and swarming motility in *P. aeruginosa* (Kuchma *et al.* 2007; Merritt *et al.* 2007). A *bifA* mutant displays a swarming defect that is suppressed by mutation of *pilY1*, implicating PilY1 as a negative regulator of flagellar swarming (Kuchma *et al.* 2010). Epistasis analysis showed that PilY1 acts specifically upstream of SadC (as opposed to other DGC in *P. aeruginosa*) to inhibit swarming, presumably by promoting production or DGC activity of SadC (Kuchma *et al.* 2010). Recent work reveals a role for PilY1-mediated surface sensing in inducing virulence of *P. aeruginosa* (Siryaporn *et al.* 2014; Persat *et al.* 2015), implicating surface sensing beyond influencing motile/sessile transitions. How the PilY1 protein transmits information about surface contact is unclear, but may involve structural changes to a mechanosensitive von Willebrand Factor A domain within the PilY1 protein (Siryaporn *et al.* 2014). Altogether, studies of the Wsp system and PilY1 in *P. aeruginosa* indicate that c-di-GMP levels increase during growth on surfaces, and provide mechanisms by which this occurs and lead to altered bacterial behavior.

Additional evidence that c-di-GMP participates in the response to surface contact comes from the analysis of *C. difficile* motility on agar surfaces. As detailed above, a type II c-di-GMP riboswitch positively regulates transcription of the *C. difficile* pilin subunit gene *pilA1* in response to c-di-GMP binding (Bordeleau *et al.* 2015). Using a biosensor consisting of a reporter gene under the control of this riboswitch, it was shown that *C. difficile* strain R20291 increases its cytoplasmic c-di-GMP content in response to prolonged contact with surfaces (Purcell *et al.* 2015). These results are supported by direct measurements of intracellular c-di-GMP by chromatographic and mass spectrometric methods. Both c-di-GMP accumulation and TFP-dependent motility depend on surface hardness and are more pronounced at 1.5% and 1.8% agar than at 0.9% agar (Purcell *et al.* 2015). One per cent agar is a good model for healthy gastrointestinal tissue, as both exhibit surface elasticities of roughly 10 kPa (Parker *et al.* 1990; Cha, Kim and Kim 2014). Interestingly, inflammation during *C. difficile* infection 'stiffens' the intestinal epithelium and increases its elastic modulus to 25 kPa, which is better modeled by higher agar percentages (Parker *et al.* 1990; Razzaq and Sukumar 2006; Cha, Kim and Kim 2014). *C. difficile* adheres to intestinal epithelia at sites of inflammatory damage in hamster and mouse models of disease, suggesting that intestinal inflammation caused by *C. difficile* toxins may prime the epithelial surface for *C. difficile* adhesion and spread (Goulding *et al.* 2009; Lawley *et al.* 2009).

## INTERACTIONS BETWEEN NUCLEOTIDE SIGNALING NETWORKS

c-di-GMP controls motility behaviors and surface-dependent developmental strategies such as ECM secretion and biofilm formation, energetically costly processes that are subject to regulation by multiple signaling networks. There is considerable regulatory overlap among c-di-GMP and other nucleotide second messenger signaling pathways, as recently highlighted (Kalia *et al.* 2013; Hengge *et al.* 2016). Specifically, the nucleotide signaling molecules (p)ppGpp ('alarmones' guanosine pentaphosphate and tetraphosphate), c-di-AMP and guanosine triphosphate (GTP) regulate many of the same processes as c-di-GMP, especially those related to surface behaviors and adaptation to stress. Indeed, biofilm formation often increases bacterial resilience to stress (O'Toole, Kaplan and Kolter 2000). The role of c-di-GMP in regulating biofilm formation and development is detailed above. The 'alarmones' guanosine pentaphosphate and tetraphosphate (together referred to as (p)ppGpp), which participate in the response to environmental stress and/or nutrient availability are also known regulators of biofilm formation in Gram-negative and Gram-positive bacteria (Taylor *et al.* 2002; Lemos, Brown and Burne 2004; Boehm *et al.* 2009; Chavez de Paz *et al.* 2012; He *et al.* 2012; Wexselblatt *et al.* 2012; Sugisaki *et al.* 2013; de la Fuente-Núñez *et al.* 2014; Gupta, Kasetty and Chatterji 2015). Like c-di-GMP, the more recently identified c-di-AMP also influences biofilm formation (Du *et al.* 2014; Peng *et al.* 2015); to date, this has only been described in bacteria that do not utilize c-di-GMP, so it is unclear if these are overlapping or mutually exclusive mechanisms. Ultimately, the use of multiple signaling pathways allows bacteria to respond to diverse extracellular stimuli with convergent cellular responses that are fundamental to bacterial survival.

Many Gram-positive bacteria display extreme resilience to extracellular stresses, such as nutrient limitation, desiccation, and disinfectants and antibiotics. Multiple nucleotide signaling pathways influence these stress responses, including c-di-GMP as described above (An *et al.* 2013; Kalia *et al.* 2013; Corrigan *et al.* 2015). In many Gram-positive bacteria, cellular levels of GTP also regulate the stress response and entry into stationary phase via the nutrient-regulated transcriptional regulator CodY (Sonenshein 2005). c-di-GMP regulation of EPS synthesis in *L. monocytogenes* affects resilience to external stress, which is also regulated by c-di-AMP and (p)ppGpp (Liu *et al.* 2006; Kaplan Zeevi *et al.* 2013; Chen *et al.* 2014). c-di-AMP and (p)ppGpp also impact the *B. subtilis* response to external stresses such as antibiotics that target the cell wall, although c-di-GMP regulation of the stress response has not been studied in this organism (Luo and Helmann 2012; Kriel *et al.* 2014). *S. coelicolor* is considered a source of rather than a target for antibiotics, but these secondary metabolites are produced in order to compete with surrounding bacteria for nutrients and their synthesis is coordinately regulated with the nutritional stress response (Lian *et al.* 2008). Nutrient stress signaled by (p)ppGpp regulates antibiotic production in *S. coelicolor*, which is intimately tied to the developmental progression regulated by c-di-GMP (Chakraburtty *et al.* 1996; Tschowri 2016).

Another stress response largely specific to Gram-positive bacteria is endospore formation, which occurs almost exclusively in *Streptomyces, Bacillus* and *Clostridium* species. c-di-GMP regulates the kinetics of *Streptomyces* sporulation and release, which is also regulated by (p)ppGpp (Tschowri 2016). In *B. subtilis*, (p)ppGpp affects spore formation by regulating the transcription of *spo0A*, whose gene product regulates both spore formation and transcription of the c-di-GMP PDE *yuxH/pdeH*, suggesting that c-di-GMP metabolism and sporulation are linked in this species (Eymann, Mittenhuber and Hecker 2001; Chen *et al*. 2012). Sporulation in *Bacillus* species is also subject to regulation by c-di-AMP, and GTP (via CodY) regulates sporulation in *B. subtilis* and *C. difficile*, providing further levels of overlap in the second messenger control (Sonenshein 2005; Witte *et al*. 2008; Oppenheimer-Shaanan *et al*. 2011; Zheng *et al*. 2015). The specific hierarchy of nucleotide signaling mechanisms and their impact on sporulation has not been reported.

As in Gram-negatives, c-di-GMP controls transcriptional regulation of flagellar genes or impairs flagellar motor rotation in Gram-positive species. (p)ppGpp inhibits the transcription of flagellar genes in Gram-negative motility by directly inhibiting RNA polymerase and by activating the transcriptional regulator protein DksA (Lemke, Durfee and Gourse 2009; Pal *et al*. 2012; Liu, Bittner and Wang 2015). (p)ppGpp does not appear to interact with RNAP in Gram-positive bacteria, which have no DksA homolog (Krasny and Gourse 2004; Dalebroux *et al*. 2010). Instead, elevated (p)ppGpp levels in *B. subtilis* result in increased SigD activity, which leads to greater levels of flagellar biosynthesis and swimming motility (Ababneh and Herman 2015). It is unclear how broadly applicable this mechanism is among other species.

Gram-positive pathogens often secrete protein toxins or other virulence factors, which are subject to multiple layers of regulation. To date, GTP and c-di-GMP have been identified as nucleotide signals influencing *C. difficile* cytotoxin secretion (Sonenshein 2005; Dineen *et al*. 2007; Dineen, McBride and Sonenshein 2010; El Meouche *et al*. 2013; McKee *et al*. 2013). c-di-GMP, (p)ppGpp, c-di-AMP and GTP have all been implicated in *L. monocytogenes* virulence factor production (Taylor *et al*. 2002; Lobel *et al*. 2012; Witte *et al*. 2013; Chen *et al*. 2014; Lobel *et al*. 2015). This multifactorial regulation allows overlapping regulatory networks to integrate multiple signals that influence virulence.

A considerable divergence between nucleotide signaling networks in Gram-negative and Gram-positive bacteria is the presence of cyclic mononucleotides in the former. Some Gram-negative bacteria, especially Enterobacteria, utilize cyclic adenylate (cAMP) to control carbon utilization depending on available nutrients (carbon catabolite repression). Several studies have demonstrated crosstalk between c-di-GMP and cAMP signaling pathways in Gram-negatives (Coggan and Wolfgang 2012; Almblad *et al*. 2015; Kariisa, Grube and Tamayo 2015; Luo *et al*. 2015). However, cAMP does not appear to be used by Gram-positive species, which employ a different mechanism of carbon metabolism regulation involving CodY and CcpA (Sonenshein 2007). Cyclic guanylate (cGMP) regulates environmental stress response in some cyanobacteria and proteobacteria species and was recently reported to regulate c-di-GMP synthesis in *Xanthomonas campestris,* but cGMP-dependent signaling has not been reported in any Gram-positive bacteria to date (Cadoret *et al*. 2005; Marden *et al*. 2011; An *et al*. 2013; Gomelsky and Galperin 2013). There is unlikely to be any regulatory overlap between c-di-GMP and the recently discovered hybrid cyclic nucleotide cyclic AMP-GMP (cGAMP) as there are no Gram-positive homologs to the cGAMP cyclase DncV (Davies *et al*. 2012; Kellenberger *et al*. 2015).

## CONCLUSIONS AND FUTURE DIRECTIONS

Microbial interactions with surfaces are mostly studied at a whole cell level because evaluating the presence of bacterial cells on or near a surface is straightforward compared to monitoring intracellular fluctuations in proton motive force or second messenger concentration. However, these transient internal perturbations are what enable cells to sense a surface and alter their physiology or behavior in order to attach to or translocate across it. Surface and extracellular structures such as flagella and TFP have been recognized as potential surface sensors. This is likely an incomplete list, and roles in surface sensing may be identified in other cell envelope components. For example, other pilus and fimbrial structures and mechanosensitive channels may facilitate responses to surfaces (Naismith and Booth 2012). c-di-GMP has long been recognized as a key switch regulating microbial transitions between motility and surface-associated sessility. c-di-GMP is a 'second messenger', conveying intracellular signals in response to extracellular primary signals that bacterial cells cannot directly internalize (Romling, Galperin and Gomelsky 2013). Recent evidence that c-di-GMP levels in both Gram-negative and Gram-positive bacteria fluctuate in response to contact with solid surfaces reveals that physical as well as chemical stimuli from the environment influence intracellular regulation. Changes in cytoplasmic c-di-GMP levels regulate the synthesis and function of extracellular structures in diverse bacteria; these processes allow bacterial cells to establish and maintain contact with surfaces.

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