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Cyclic-di-GMP regulation of virulence in bacterial pathogens

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Abstract

Signaling pathways allow bacteria to adapt to changing environments. For pathogenic bacteria, signaling pathways allow for timely expression of virulence factors and the repression of anti-virulence factors within the mammalian host. As the bacteria exit the mammalian host, signaling pathways enable expression of factors promoting survival in the environment and/or non-mammalian hosts. One such signaling pathway uses the dinucleotide cyclic-di-GMP (c-di-GMP), and many bacterial genomes encode numerous proteins that are responsible for synthesizing and degrading c-di-GMP. Once made, c-di-GMP binds to individual protein and RNA receptors to allosterically alter the macromolecule function to drive phenotypic changes. Each bacterial genome encodes unique sets of genes for c-di-GMP signaling and virulence factors so the regulation by c-di-GMP is organism specific. Recent works have pointed to evidence that c-di-GMP regulates virulence in different bacterial pathogens of mammalian hosts. In this review, we discuss the criteria for determining the contribution of signaling nucleotides to pathogenesis using a well-characterized signaling nucleotide, cyclic AMP (cAMP), in *Pseudomonas aeruginosa*. Using these criteria, we review the roles of c-di-GMP in mediating virulence and highlight common themes that exist among eight diverse pathogens that cause different diseases through different routes of infection and transmission.

Introduction

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a widely utilized signaling pathway that regulates bacterial adaptation to different environments.[1] C-di-GMP is synthesized from two GTP by diguanylate cyclases (DGCs) that contain a GGDEF domain and degraded into pGpG by phosphodiesterases (PDE-As) that contain EAL or HD-GYP domain. Signaling by c-di-GMP is completed when pGpG is degraded further to GMP by oligoribonuclease and related RNA degradation enzymes.[2-4] Thus, c-di-GMP represents one of the shortest signaling ribonucleotides. In addition, a number of genes can encode proteins with both GGDEF and EAL domains (GGDEF-EAL) indicating that levels of c-di-GMP in the cell are regulated by a complicated number of synthetic and degradative enzymes. Once made, c-di-GMP binds a diverse set of protein receptors and RNA riboswitches.[1] C-di-GMP binding to molecular receptors allosterically alter their function and downstream cellular phenotypes.[5] Genes encoding c-di-GMP signaling components are found in a large number of bacterial pathogens. This review seeks to cover the contribution of c-di-GMP signaling to bacterial pathogenesis for a number of different pathogens to

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identify common themes. For this review, virulence and pathogenesis is considered as the ability of the bacteria to replicate and cause disease symptoms in the mammalian host. Due to space limitation, for each pathogen, we have referenced reviews that cover their virulence factors and their c-di-GMP signaling pathway throughout the text. In order to understand how c-di-GMP contributes to pathogenesis, we adapted the principles of molecular Koch's postulates[6] for signaling nucleotides. For signaling nucleotides to contribute to pathogenesis they should meet the following criteria: 1. the nucleotide should be made during infection, 2. the receptor of the signaling nucleotide should be required for infection, and 3. the process regulated by the signaling nucleotide should be required for infection. Each of these criteria can be assessed experimentally using different approaches. To understand how we assessed c-di-GMP regulation of pathogens using these criteria, first we will briefly describe the role of the well-characterized signaling nucleotide system of cAMP in the virulence of *Pseudomonas aeruginosa*.[7]

In *P. aeruginosa*, cAMP signaling pathway controls the expression of hundreds of genes including type three secretion system (T3SS) that is essential for virulence in a murine model of acute pneumonia.[8] *P. aeruginosa* encodes two adenylate cyclases, *cyaA* and *cyaB*,[8] and one phosphodiesterase, *cpdA*.[9] Once generated, cAMP is bound by the transcription factor Vfr to drive differential expression of more than a hundred genes of which 80% are differentially expressed in the absence of the two cyclases, indicating that cAMP signals through Vfr.[8] Of these genes, Vfr-cAMP binds directly upstream of *exsA*,[10] which encodes the transcription factor for T3SS.[11] To assess the contribution of cAMP to *P. aeruginosa* pathogenesis, strains with altered signaling components were tested in the acute pneumonia model. In this model, *P. aeruginosa* strains that were unable to either synthesize cAMP (Δ*cyaA* Δ*cyaB*), lacked the receptor (Δ*vfr*) or lacked the regulated genes (Δ*exsA* and Δ*pscC*, which cannot perform T3SS) had a similar 2-log defect in the colony forming units (CFU) within the lung and a 3-log CFU reduction of bacteria disseminated to the liver and spleen.[12] Since all steps of the signaling pathway have a similar virulence defect, based on the criteria adapted from molecular Koch's postulates, cAMP signaling is required for acute pneumonia by *P. aeruginosa*. For this review, we applied a similar assessment for c-di-GMP in each of the different pathogens in which c-di-GMP signaling has been studied during mammalian infections. In many pathogens, there can be over 40 genes encoding GGDEF domains and over 30 genes encoding PDE-A domains. This functional redundancy complicates the ability to analyze the contribution of c-di-GMP signaling from the standpoint of controlling the levels of c-di-GMP in the bacterial cell. Nonetheless, a number of different strategies have been used to test the contribution of the c-di-GMP signaling on bacterial pathogenesis.

This review has been divided into three sections to separate the pathogens based on their route of infection/transmission cycle: 1. Healthcare-associated infection pathogens, 2. Gastrointestinal pathogens, and 3. Vector-borne pathogens. These three classes were used in order to consider broadly how pathogens behave inside and outside of mammalian hosts. However, this classification should not be considered as a strict definition as some bacterial pathogens infect through multiple routes of infection.

Healthcare-associated infection pathogens

Healthcare-associated infections (HAI) include central line-associated bloodstream infections, ventilator-associated pneumonias, catheter-associated urinary tract infections (CAUTI) and surgical site infections. One strategy used by HAI pathogens in establishing infections is to form a biofilm on the surfaces of medical devices and subsequently disseminate from the contaminated device into other host tissues to cause systemic infections. HAI pathogens, including *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*, have the potential to utilize c-di-GMP since their genomes encode numerous proteins with GGDEF and EAL/HD-GYP domains. For clinical isolates of *E. coli* and *P. aeruginosa*, the amount of c-di-GMP has been measured in the laboratory setting, and these levels are elevated when specific DGCs are overexpressed and decreased when specific PDE-As are overexpressed.[13] Together, these in vitro studies suggest that all three organisms extensively utilize c-di-GMP as a signaling molecule to regulate bacterial biofilm. Since HAI pathogens cause a number of different infections, the effect of c-di-GMP is discussed for each pathogen, including c-di-GMP receptors and phenotypes that contribute to different infections.

Pseudomonas aeruginosa

P. aeruginosa is an opportunistic pathogen that causes a diverse set of acute and chronic infections. Key virulence factors for acute infections include type II secretion system (T2SS), type III secretion system (T3SS), type IV pili as well as other virulence factors (see review[14]). Chronic infections caused by *P. aeruginosa* include the life-long colonization of the lungs of cystic fibrosis (CF) patients[15] and infections associated with the use of medical devices.[16] A key *P. aeruginosa* virulence factor for CF patients is alginate,[15] a viscous polysaccharide that further occludes the airways, leading to morbidity and mortality. In contrast, infections associated with the use of medical devices often are linked to *P. aeruginosa* forming a biofilm on the device surface. Biofilm formation in *P. aeruginosa* is multifactorial and involves a number of extracellular matrix components (see review[17]).*P. aeruginosa* extensively utilize c-di-GMP to regulate alginate production, biofilm formation, and motility (see reviews[18, 19]). *P. aeruginosa* encodes 16-17 GGDEF domains, 5 EAL domains and 16 GGDEF-EAL domains, and numerous studies have implicated these genes in the regulation of downstream processes.[20] For brevity, we are focusing on the c-di-GMP receptors that mediate the response to c-di-GMP. Biosynthesis of alginate is regulated by c-di-GMP binding to the Alg44 receptor, which has a PilZ domain.[21] C-di-GMP signaling is required for alginate production as Alg44 mutants that are unable to bind c-di-GMP fail to produce alginate.[21] C-di-GMP also regulates biofilm formation through many receptors. *P. aeruginosa* biofilm formation is transcriptionally regulated by c-di-GMP binding to FleQ receptor which activates transcription of the *pel* operon, *psl* operon and the *cdrA* gene,[22-25] while simultaneously repressing flagella genes.[26] The Pel and Psl exopolysaccharide and the CdrA surface proteins contribute to the *P. aeruginosa* extracellular matrix and support biofilm formation. C-di-GMP binds to PelD receptor to activate Pel polysaccharide biosynthesis.[27] C-di-GMP is important for biofilm formation as mutations in PelD that are defective for c-di-GMP binding fail to form biofilm.[27-29] In addition, several other c-di-GMP receptors have been identified that increase biofilm production, repress flagella

motility and repress type IV pili motility. To enhance biofilm formation, c-di-GMP interacts with HapZ, which alters signaling by the SagS histidine kinase;[30] WarA, which regulates LPS biosynthesis;[31] and BlrR, which is a transcription factor.[32] To repress flagella motility, c-di-GMP binds MapZ, which alters CheR1 methyltransferase activity and chemotaxis[33] and FlgZ, which interacts with the MotCD stator proteins.[34] C-di-GMP represses type IV pili by binding FimX, a degenerate GGDEF-EAL that serves as a receptor protein.[35] Together, these studies on *P. aeruginosa* in the laboratory setting show that c-di-GMP binds to a number of receptors to directly activate alginate biosynthesis and biofilm formation while repressing motility mediated by flagella and type IV pili.To address the contribution of c-di-GMP to chronic *P. aeruginosa* infections, several studies have analyzed *P. aeruginosa* strains from sputum samples of CF patients. Isolates from CF sputum are heterogeneous and a subset of these isolates exhibit small colony variant (SCV) phenotype. Using genomic sequencing, several studies have shown that *P. aeruginosa* strains can accumulate mutations during life-long colonization of lungs of CF patients.[36] Sequencing of longitudinal isolates from the lungs of one CF patient identified mutations in the *wspF* gene, which leads to increased c-di-GMP synthesis by the diguanylate cyclase WspR[37], indicating that an increase in c-di-GMP signaling can occur in vivo.[36] In addition to c-di-GMP regulating SCV, c-di-GMP binding to the Alg44 receptor is also needed to activate alginate production during mucoid conversion in the CF lung.[21] Since *P. aeruginosa* in the CF lung can develop SCV phenotype and undergo mucoid conversion, c-di-GMP is implicated in CF infections. Nonetheless, direct evidence that c-di-GMP contributes to CF infection is lacking due to the current limitation in the animal models for CF.[38, 39] Future studies using animal models that better reflect airway infections in CF is needed.

Another type of study determining the contribution of c-di-GMP signaling to chronic infection utilized animal models of medical device infections that typically involve biofilm formation. The experimental designs of these studies have two challenges in genetic redundancy. First is the large number of genes encoding DGCs and PDE-As and the redundancy in the generation and degradation of c-di-GMP. Second is that multiple c-di-GMP regulated pathways can up-regulate biofilm production, leading to complications in determining which of the c-di-GMP-regulated processes is driving biofilm formation and infection. A number of studies have assessed c-di-GMP signaling using implanted catheters in mammalian models of infections by employing strains that were genetically altered to either overproduce or reduce the cellular levels of c-di-GMP. In one study, an *yfiR* mutant was identified with SCV phenotype. Further studies revealed that YfiR is a negative regulator of the YfiN DGC.[40] Thus, mutants in *yfiR* have elevated c-di-GMP levels and are phenotypically SCVs with slower growth rates.[40] When infecting mice using a subcutaneous catheter model, the *yfiR* mutant had a 1-log reduction in CFUs in the subcutaneous catheter and in the surrounding tissue as compared with the parental PAO1 strain at 4-weeks post-infection.[40] At 8-weeks post-infection, the difference between wild type and *yfiR* mutant was decreased to statistically not significant. When co-infected in a competition experiment, the *yfiR* mutant was 8-fold outcompeted by PAO1 at 4 weeks. By 8 weeks, the difference was diminished as the number of PAO1 CFU was reduced to numbers similiar to the *yfiR* mutant. In another study, PAO1 with a vector control or a vector expressing the *yhjH* PDE-A from *E. coli* were grown as a biofilm on silicone catheters which were then implanted in the

peritoneum of mice.[41] Outside the animal, expression of *yhjH* in PAO1 reduced c-di-GMP levels in the cell, which led to a reduction in biofilm formation and an increase in dispersal from pre-formed biofilm. When the silicone catheter with preformed biofilm was implanted in the peritoneum, implants with strains expressing *yhjH* heterologously had a 1-log reduction in CFU associated with the catheter and concomitantly increased CFU in the intraperitoneal cavity by 1.5-log.[41] These results indicate that lower levels of c-di-GMP led *P. aeruginosa* to disseminate from the biofilm on catheter surface. When determining whether expression of *yhjH* had an effect on bacterial colonization of the implanted catheter over time, the authors found that the two different experimental groups showed different levels of clearance of the bacteria from the catheter, indicating that in vivo experiments are more variable than studies in laboratory conditions.[41] A third model of catheter infections used a mice model of catheter-associated urinary tract infection (CAUTI).[42] One part of the study utilized overexpression of two DGCs (*wspR* and *PA1107*) and two PDE-As (*PA2133* and *rocR*). Expression of the DGCs increased the number of CFU in the bladder and the number of CFU in kidney. In contrast, overexpression of the PDE-As reduced the number of CFU the bladder and the number of CFU in the kidney.[42] These results suggest that elevated c-di-GMP levels enhance biofilm formation on the urinary catheter and increased the likelihood of the bacteria to disseminate to the kidneys. To determine which of the DGC/PDE-A is important for CAUTI, a library of in-frame deletion mutants of PA14 was used[43] to assess the contribution of c-di-GMP during CAUTI. From the analysis of 24 mutants, only two genes encoding GGDEF-EAL domains, *morA* and *PA14_07500*, had 1-log reduction in CFU within the bladder indicating a defect in colonization. The subtle effects of in-frame deletion mutants suggest that the signaling redundancy of c-di-GMP may mask any single DGC/PDE-A mutation. Together, these results indicate c-di-GMP signaling enhances the attachment of bacteria to catheters placed in different anatomical locations. However, this enhancement in colonization is about 1-log and can vary for different groups of infected animals.

Two other chronic infection models used to study chronic *P. aeruginosa* pathogenesis are chinchilla model of otitis media and an agar bead model of lung infection in rats. For the chinchilla model of otitis media, *P. aeruginosa* is applied to the middle ear of the animal through a transbullar injection.[44] Infection with *P. aeruginosa* strain MJK8, a strain with a 22 amino acid deletion mutation in the *wspA* gene that results in elevated levels of c-di-GMP[37] was able to persist for a longer amount of time in the chinchilla bulla and caused less morbidity as compared to the parental PAO1 strain.[44] Additional experiments with the MJK8 Δ*psl* and MJK8 Δ*pel* strains did not show a significant difference in the infection from the MJK8 strain.[44] There are two possible explanations for these results. One explanation is that these polysaccharides can act in a redundant manner to mediate their effects in the host. A second explanation is that a different c-di-GMP regulated process is acting in the host to promote biofilm and reduce acute virulence. Together, this study shows that elevated c-di-GMP levels reduce acute virulence and enhance persistence in the otitis media model of infection. Another model of chronic infection was the instillation of agar beads embedded with *P. aeruginosa* into the lungs of rats.[45] A signature-tagged mutagenesis (STM) study revealed a number of genes required for chronic colonization including genes that are involved in type IV pili and alginate biosynthesis among many other functions.[45] Of

the 148 ORFs that showed defects in this infection model, only one encoded a gene, PA4929, that is involved in c-di-GMP signaling.[45] The lack of overlap between genes involved in c-di-GMP and the STM mutants may indicate functional redundancy of DGCs, PDE-As and receptor proteins.The contribution of c-di-GMP signaling to *P. aeruginosa* infections can be assessed using the criteria of molecular Koch's postulates adapted for signaling nucleotides and assessed for each type of infection. For *P. aeruginosa* lung infection in CF patients, there is indirect evidence that strongly indicates the involvement of c-di-GMP in SCV formation and alginate production. However, the ability to directly assess the contribution of the c-di-GMP regulated process for infection requires future development of an appropriate CF animal model. For other infection models, the evidence for c-di-GMP contribution to infection is less clear. In general, there is a positive correlation between c-di-GMP signaling and virulence. Typically, increased c-di-GMP levels are correlated with an enhanced ability of *P. aeruginosa* to persist as biofilms on foreign objects or in the bulla of chinchilla, while decreased c-di-GMP levels are correlated with the reduction of *P. aeruginosa* on foreign objects. One possible reason for the enhancement of biofilm formation in vivo is the production of the PSL polysaccharide which can protect the bacteria from host immune recognition.[46] While these observations generally support the idea that c-di-GMP contributes to these chronic infections, future studies are required to determine the levels of c-di-GMP in the bacteria during infection, to elucidate the processes and pathways regulated by c-di-GMP and to assess contribution of c-di-GMP regulated pathways to chronic infections.

Escherichia coli

E. coli causes numerous types of infection including gastroenteritis, urinary tract infections, meningitis and many others (see reviews [47, 48]). *E. coli* utilizes a number of virulence factors to cause infections including enterotoxins, T3SS effectors and adhesins. The ability of *E. coli* to cause diverse infections is in part due to the plasticity of the genome, which consists of 2,200 core genes and thousands of accessory genes for each distinct pathotype.[49, 50] In a sequencing study of 61 pathogenic and commensal *E. coli* strains 2 GGDEF domains, 4 EAL domains, and 2 GGDEF-EAL domains were found in the core genome.[51]This is a small subset of the total number of genes encoding GGDEF/EAL domains in the pangenome. From the same sequencing study, c-di-GMP receptors including BcsA,[52] BscE,[53] YcgR[54, 55] are found in most strains. Exceptions include some enterohemorrhagic *E. coli* (EHEC) strains that have insertion sequence in *ycgR*, several extraintestinal pathogenic *E. coli* (ExPEC) strains that have frameshift mutations in *bcsA*, and some enteroaggregative *E. coli* (EAEC) and Shiga toxin-producing *E. coli* (STEC) strains that have various mutations leading to a truncated BcsE protein.[51] C-di-GMP signaling in *E. coli* has been extensively studied in K-12 strain that is non-pathogenic (see review[56]). High levels of c-di-GMP activate the production of matrix components, including cellulose, poly-*N*-acetyl glucosamine (PNAG) and curli, and repress flagella motility.[56] C-di-GMP binds the PilZ domain of BcsA to activate cellulose biosynthesis.[57, 58] C-di-GMP binds the complex consisting of PgaC and PgaD to activate productin of PNAG.[59] C-di-GMP bound YcgR inhibits motility by interacting with the flagella machinery.[54, 55] C-di-GMP also activates CsgD to increase expression of curli[60, 61] by a yet to described mechanism.

Since *E. coli* causes numerous distinct infections, the contribution of c-di-GMP has been tested through studies using different bacterial strains on mammalian cells or animal models of infection. For UPEC, a mutation in *yfiR* leads to constitutive activity of the YfiN DGC,[62] in a manner similar to *P. aeruginosa*. Using a murine model of urinary tract infection (UTI), a uropathogenic *E. coli* (UPEC) clinical isolate CFT073 with the Δ*yfiR* mutation was outcompeted by the parental strain by 1-log in the bladder and kidneys, whereas the Δ*yfiN* mutant performed as well the parental strain, indicating that the YfiN DGC is not required for UTI.[62] Interestingly, the virulence defect of the Δ*yfiR* strain is eliminated when *yfiN* is also deleted. The virulence defect associated with elevated c-di-GMP is due to increased cellulose and curli, as a Δ*yfiR* Δ*csgD* Δ*bcsA* triple mutant competes equally with the parental strain.[62] These results indicate that c-di-GMP enhances the production of cellulose and curli, which act as anti-virulence factors for UTI. One other study investigated the contribution of c-di-GMP for adhesion to bladder epithelial cells using CFT073 strain with deletion of individual DGC, PDE, and DGC-PDE genes.[63] The results indicated mutants lacking a subset of genes encoding GGDEF/EAL domains have increased adherence, while mutants in a different subset of genes encoding GGDEF/EAL domains have decreased adherence. Future studies to determine the c-di-GMP levels in the mutants and determining their ability to cause UTI in a mammalian model would reveal which of these genes are active in a mammalian host.

In addition, studies of non-UPEC strains have investigated the contribution of c-di-GMP signaling to pathogenesis. For ETEC, attachment of bacteria to Caco-2 intestinal epithileal cells led to an increased expression of GGDEF domain proteins and *bcs* operon, indicating binding to host cells triggers c-di-GMP synthesis.[64] Additional studies are required to determine if c-di-GMP levels are increased in response to attachment and whether the *bcs* operon contributes to attachment and infection. For EHEC strain O157:H7, expression of the BlrP PDE-A from *K. pneumoniae* was able to reduce cellular c-di-GMP levels, expression of the locus of enterocyte effacement (LEE) T3SS, and attachment to HT-29 human colon epithelial cells.[65] Future studies can determine which c-di-GMP receptor is responsible for regulating the LEE locus and attachment to host cells. For EAEC, genomic sequencing revealed the presence of two genes encoding GGDEF domain (*dgx* and *yneF*) not found in non-pathogenic K-12 strains.[66] Interestingly, all of the clinical strains have lower levels of cellulose at 37 °C.[66] Future studies can test if these additional genes contribute to c-di-GMP signaling and virulence.

Assessing the contribution of c-di-GMP to *E. coli* infection using the criteria of adapted molecular Koch's postulate is complicated by the limited number of studies using the same model to test more than one aspect of the requirement for c-di-GMP signaling during infection. Of these studies on diverse pathogenic *E. coli*, the studies of the UPEC strain CFT073 is the most well characterized. Increased c-di-GMP signaling reduced the competitive index by 1-log in a murine UTI model. This defect was reversed when the c-di-GMP regulated processes, curli and cellulose, are genetically deleted. Together with the ability of the DGC mutant to compete as well as the parental strain, these results indicate that c-di-GMP, cellulose and curli are anti-virulence factors for UTI caused by CFT073. Future studies to determine the concentration of c-di-GMP during infection and which of the

known c-di-GMP regulated processes are responsible for other infections caused by *E. coli* would elucidate the function of the signaling pathway for this important pathogen.

Klebsiella pneumoniae

K. pneumoniae is one of the leading HAI pathogens that form biofilms on the surfaces of medical devices and healthcare facilities.[16] As an opportunistic pathogen, *Klebsiella* utilizes a number of different virulence factors to establish infections. Numerous genetic experiments in different infection models have revealed a number of different virulence genes including capsule, LPS, fimbriae and surface adhesins (see review[67]). *K. pneumoniae* extensively utilizes c-di-GMP signaling, as genome sequencing of 3 strains revealed that there are 12-15 genes encoding proteins with GGDEF domain, 10-15 genes encoding proteins with EAL domain, and 5-6 genes encoding genes encoding proteins with GGDEF-EAL domains.[68] Of the many virulence factors in *K. pneumoniae*, mannose-resistant *Klebsiella*-like (Mrk) hemagglutinin is the only one known to be regulated by c-di-GMP. In a signature-tagged mutagenesis screen for mutants defective for liver abscess from an oral route of infection, a transposon insertion in the *mrkC* gene was avirulent and does not disseminate from the GI tract.[69] *mrkC* is a part of the *mrkABCDF* operon which encodes the Mrk,[70, 71] that forms the type 3 fimbriae that allows attachment to abiotic surfaces and biofilm formation.[72]

In a separate screen for mutants defective in biofilm formation under laboratory conditions, mutants in *mrkABCDF* operon and the adjacent *mrkHIJ* operon were identified.[73] The *mrkHIJ* operon encodes for a PilZ-domain protein (MrkH), a LuxR family transcription factor (MrkI), and a PDE-A (MrkJ).[73, 74] Mutations that ablate in individual genes within the *mrk* operons, except *mrkJ*, have reduced export of the major pili MrkA, hemagglutinin activity and biofilm formation. Additional analysis revealed that MrkH binds c-di-GMP via the PilZ domain and is sufficient to promote expression from the *mrkA* promoter.[73, 7475] In contrast, the *mrkJ* mutant had enhanced levels of MrkA protein and biofilm formation.[73, 76] Since *mrkJ* encodes a PDE-A, the *mrkJ* deletion mutant have elevated levels of c-di-GMP, that binds MrkH to activate type 3 fimbriae expression and biofilm formation. In addition, another PDE-A, YjcC, also reduces c-di-GMP levels, *mrkA* expression, and biofilm formation in a manner similar to MrkJ.[77] Mutants lacking *yjcC* have a 1-log increase in LD_{50} for an intraperitoneal infection, indicating that elevated levels of c-di-GMP lead to a defect in a systemic infection.[77] Since *mrkC* is not required for intraperitoneal infection of mice, elevated levels of c-di-GMP may regulate other processes that are detrimental to systemic infection of mammalian hosts.[69] *K. pneumoniae* encodes the cellulose biosynthesis operon, and cellulose may act as an anti-virulence factor.[78] These studies show that an opportunistic pathogen such as *K. pneumoniae* likely utilizes c-di-GMP regulated processes in a subset of infections in which the Mrk type 3 fimbriae promotes infections while other factors may act as anti-virulence factors.

Currently, two independent studies of *K. pneumoniae* have satisfied one of the criteria of the molecular Koch's postulates adapted for c-di-GMP signaling in pathogenesis. In the study of dissemination to the liver from a GI infection, strains with mutations in *mrkC*, which is a c-di-GMP regulated type 3 fimbriae, are defective for dissemination from the intestines. In a

different model of intraperitoneal infection, a *yjcC* mutant which has elevated c-di-GMP exhibited an increased LD_{50}. Future work is required to determine whether c-di-GMP is made during infection and which c-di-GMP receptor and c-di-GMP-regulated processes are required for various types of infections caused by *K. pneumoniae*.

Summary of HAI pathogens—The contribution of c-di-GMP signaling to these three HAI pathogens is specific to each pathogen and each type of infection. For *P. aerguinosa*, c-di-GMP signaling is required for alginate production and SCV phenotype providing evidence that c-di-GMP is utilized during life-long lung infections of CF patients. For other chronic infections, c-di-GMP signaling also contributes to *P. aeruginosa* infections although additional studies are required to elucidate the receptors and regulated processes involved in each infection. In contrast, c-di-GMP signaling attenuates *E. coli* virulence during UTIs. This defect can be reversed when the curli and cellulose are genetically removed indicating that c-di-GMP activation of curli and cellulose interferes with the infection process. The effect of c-di-GMP regulated process on infection is likely due to the specific molecule that is produced. Many of the c-di-GMP regulated processes include production of extracellular proteins and polysaccharides. Some of these polymers such as cellulose and curli in *E. coli* may be recognized by the mammalian immune system, thus providing a basis for the apparent anti-virulence property of c-di-GMP signaling. In contrast, c-di-GMP regulated extracellular proteins and polysaccharides of *P. aeruginosa* are different in protein sequence and sugar composition. These differences may distinguish which pathogens utilize c-di-GMP signaling to promote infections in mammalian hosts.

Gastrointestinal pathogens

Vibrio cholerae

V. cholerae, the causative agent of cholera, is responsible for seven documented worldwide pandemics.[79] *V. cholerae* infections start with the ingestion of contaminated water and food. Upon ingestion, the bacteria moves though the gastrointestinal tract and the mucosal layer (see review[80]). Biofilm formation by *V. cholerae* enhances survival at low pH[81] and allows the bacteria to enter the small intestines where cholera toxin and the toxin-coregulated pili (Tcp) are expressed. Cholera toxin binds GM1 glangiosides on intestinal epithelial cells[82], moves to the endoplasmic reticulum by retrograde transport and enters the cytoplasm through retrotranslocation from the endoplasmic reticulum.[83] In the cytoplasm, cholera toxin ADP-ribosylates Gsα subunit of adenylate cyclase thereby activating the production of cAMP.[84, 85] cAMP activates protein kinase A to phosphorylate the cystic fibrosis transmembrane conductance regulator (CFTR)[86] chloride channel, leading to export of chloride and carbonate ions and water loss. Intoxication of the intestinal epithelial cells leads to rapid fluid accumulation in the intestinal lumen leading to rice water diarrhea. In addition to cholera toxin, *V. cholerae* use Tcp to attach and colonize the small intestine. As *V. cholerae* exits the host, expression of cholera toxin and Tcp is repressed while genes involved in biofilm production are up-regulated.[81, 87]

V. cholerae extensively utilizes c-di-GMP signaling to regulate biofilm formation (please see review[88]). The *V. cholerae* genome contains 31 genes encoding GGDEF domains, 12 EAL

domains, 9 HD-GYP domains and 10 GGDEF-EAL domains.[88] In addition to enzymes that synthesize and degrade c-di-GMP, *V. cholerae* encodes numerous c-di-GMP binding proteins, including PilZ domain containing proteins such as PlzC, PlzD and PlzE[89, 90] and transcription factors – VpsT,[91] VpsR,[92] and FlrA,[93] and MshE, which is the export ATPase of the mannose sensitive hemagglutinin (MSHA).[94, 95] Elevated levels of c-di-GMP trigger a transcriptional response that includes increased expression of the *Vibrio* polysaccharide (*vps*) and *msh* operons and the repression of flagellar genes.[96] Vps polysaccharide and MSHA both promote biofilm formation.[97] In addition to promoting interactions between bacteria, MSHA also allows *V. cholerae* to bind to chitin, which is the primary component of exoskeleton of crustaceans in marine estuaries and allows the bacteria to have a reservoir outside the host.[98, 99] Furthermore, *V. cholerae* in the biofilm state is more tolerant to hypotonic stress in fresh water environments[100] as well as low pH[81] and surfactant[101] stresses that mimic the mammalian upper GI tract. Together, c-di-GMP drives biofilm formation that promotes bacterial survival outside the mammalian host and the mammalian upper GI tract.

A key question is whether or not c-di-GMP promotes *V. cholerae* virulence inside the mammalian host. Several lines of evidence indicate that c-di-GMP levels are decreased in the small intestines. A study for down-regulated genes in the infant mouse model revealed that the entire *msh* operon and the DGC VC2224 were down-regulated in the intestines.[102] Studies using recombination-based in vivo expression technology (RIVET) to detect expression of genes by *V. cholerae* during infection showed that *ctx* and *tcpA* were highly expressed early in infection, whereas *msh* operon and DGCs (VC0130, VC0956, VC1593 and VC2697) were expressed late in infection.[103] These studies indicate a coordinated down-regulation of DGC and DGC-regulated *msh* operon upon entry into the host. One reason for reduction of c-di-GMP and down-regulation of c-di-GMP-regulated genes was revealed by studies using *V. cholerae* that constitutively express MSHA.[102] In an infant mouse model, the *V. cholerae* with the *msh* operon placed under the constitutive *lac* promoter had a 1,000-fold decrease in competition with the parental strain only when the infected mice were breast fed by wild-type mothers. This defect was not observed in pups that were fed by $IgA^{-/-}$ mothers because the milks lacks IgA which binds MSHA and opsonizes bacteria expressing MSHA. These results show that IgA is responsible for host immune clearance of MSHA expressing *V. cholerae*.[102] Together these studies indicate that *V. cholerae* suppresses c-di-GMP-regulated MSHA to evade host IgA and allow for productive infection in the intestinal tract. As the bacteria exits the host, DGCs are expressed, leading to elevated c-di-GMP levels that can drive biofilm formation as the bacteria returns to the environment.[103] Another proposed signal for this increased c-di-GMP production is the temperature change from the homeostatic mammalian host to the lower ambient environment.[104] The ability of *V. cholerae* to cause epidemics is attributed to hypervirulence of the bacteria exiting infected hosts.[105] This is likely through the ability of bacterial biofilm to better survive the acidic environment as they re-enter the GI tract of the next host. Overall, *V. cholerae* uses c-di-GMP in the infection cycle, but within the mammalian host, DGCs are repressed and c-di-GMP-regulated MSHA acts as an anti-virulence factor (Figure 1).

C-di-GMP signaling in *V. cholerae* pathogenesis as assessed by the adapted molecular Koch's postulates for signaling nucleotides indicates that it is an anti-virulence signaling pathway. Genes encoding DGCs are down-regulated in the host upper GI tract and up-regulated later in the infection. C-di-GMP regulated MSHA is recognized by host IgA and serves as an anti-virulence factor. Together, these studies indicate that c-di-GMP is an anti-virulence factor in the mammalian host. However, this does not mean that c-di-GMP signaling does not contribute to *V. cholerae* transmission and epidemics. C-di-GMP activation of MSHA, and presumably the Vps polysaccharide, promotes biofilm formation that allows the bacteria to survive hypotonic stress in the environment and the acidic pH of the stomachs of subsequent human hosts. The reciprocal regulation of c-di-GMP as the bacteria enters and exits the mammalian host allows the bacteria to survive both host immune stress and environmental stress. Future work to determine the concentration of c-di-GMP in *V. cholerae* throughout the GI tract during infection will increase our understanding of the contribution of c-di-GMP signaling to *V. cholerae* pathogenesis.

Salmonella enterica serovar Typhimurium

S. eneterica serovar Typhirium is a facultative anaerobic bacterium that causes gastroenteritis (see reviews).[106-108] During infection of the gastrointestinal tract, *S.* Typhimurium utilizes two T3SS systems to interact with the host: T3SS-1 mediates entry of *S.* Typhimurium into epithelial cells and T3SS-2 allows the bacteria to survive in the phagosome of macrophages. Currently, neither T3SSs have been shown to be regulated by c-di-GMP.

S. Typhimurium regulates c-di-GMP levels using five GGDEF domain containing proteins, seven EAL domain containing proteins, and seven proteins with both GGDEF and EAL domains.[109, 110] C-di-GMP signaling increases production of biofilm matrix components including exopolysaccharide cellulose and curli fimbriae and represses flagella motility.[111-114] C-di-GMP binds two PilZ domain protein receptors to independently regulate flagella and cellulose. YcgR, binds c-di-GMP and inhibits flagellar rotation.[54, 55, 115, 116] BcsA binds c-di-GMP leading to an allosteric shift that no longer occludes the active site of BscB, the cellulose synthase, thereby allowing UDP-glucose to be polymerized into cellulose.[58] In addition to these PilZ domain receptors, c-di-GMP activates transcription of *csgD* through a yet to be identified receptor.[117, 118] CsgD is a Lux-family transcriptional regulator that positively regulates expression of the diguanylate cyclase *adrA* and the *cgsABC* operon that encodes the curli fimbraie.[56, 111] In turn, AdrA generates c-di-GMP to drive cellulose production. In summary, c-di-GMP enhances biofilm formation by activating cellulose and curli while simultaneously down-regulating flagella motility.

The contribution of c-di-GMP signaling on pathogenesis has been tested in several animal models of infection. In one study, overexpression of AdrA from an ectopic plasmid decreased cell surface attachment on gastrointestinal epithelial HT-29 cells and decreased virulence in a mouse ligated ileal loop infection.[119] The defect caused by elevated levels of c-di-GMP is reversed in part by deletion of the *bcsA* and *csgD* genes.[119] In another study based on intravenous mouse infection, a *cgdR* PDE-A mutant with elevated c-di-GMP levels was attenuated.[120] Another approach to test the effect of c-di-GMP regulated processes on

pathogenesis, bacterial aggregation was used as a surrogate phenotype for c-di-GMP levels.[121] After separating aggregate from planktonic cells, the aggregates had higher levels of c-di-GMP, CgsD and curli fimbriae, whereas planktonic cells had lower levels of c-di-GMP, CsgD and curli. In an oral route of murine infection, the planktonic cells outcompeted the aggregate cells by over 2-logs.[121] The planktonic cells maintained a 2-log competitive advantage over the homogenized aggregates indicating that there are additional changes to the aggregated cells beyond their ability to form biofilm.[121] In another study of intraperitoneal infection, the contribution of c-di-GMP regulated processes was assessed using a mutant defective in extracellular matrix components.[122] The mutant lacking *csgA*, *bcsE*, *wcaM* and *yihO* had an increase in virulence as compared to wild-type in an intraperitoneal mouse infection.[122] Recently, an interesting study was conducted on the *pga* operon which encodes the c-di-GMP-regulated biosynthetic proteins for PNAG exopolysaccharide, that was evolutionarily lost in *S.* Typhimurium.[123] *S.* Typhimurium expressing *pga* operon had reduced survival in macrophages and reduced virulence in an ileal loop competition experiment and intragastric infections. Interestingly, this study also showed that a mutant lacking *bcsA* is more virulent than the isogenic parental strain.[123]

The adapted molecular Koch's postulates for signaling nucleotides can be used to assess the contribution of c-di-GMP signaling to *S.* Typhimurium infection. Results from various studies indicate that increase in cellular levels of c-di-GMP attenuates *S.* Typhimurium in ligated ileal loop and intravenous infections. These defects were likely caused by cellulose and curli since removal of genes responsible for cellulose and curli synthesis reversed the observed attenuation. In addition, introduction of PNAG in *S.* Typhimurium, a polysaccharide that is synthesized by some *E. coli* strains, is also detrimental to virulence in the mammalian host (Figure 1). Together, these results suggest that c-di-GMP signaling and c-di-GMP-regulated processes are detrimental to *S.* Typhimurium infections. Future work is needed to assess the c-di-GMP levels in the bacterial cells during infection to reveal if *S.* Typhimurium turns off c-di-GMP signaling in the mammalian host.

Clostridium difficile

Antibiotic use in clinical settings is a primary risk factor for *C. difficile* infection (CDI).[124] CDI is a leading cause of health-care associated infections that present with a wide spectrum of diseases ranging from mild self-limiting diarrhea to pseudomembrane colitis and toxic megacolon, which often results in death (see review[125]). Ingested *C. difficile* spores germinate into their metabolically active vegetative form that express numerous genes including the TcdA/TcdB toxins, which are essential for virulence, flagella, type IV pili, adhesins, and extracellular degradative enzymes (see reviews[126, 127]). *C. difficile* TcdA and TcdB toxins are large glycosylating toxins that inactivate the Rho and Rac family of GTPases [127]. The roles of flagella and type IV pili in *C. difficile* pathogenesis are still under investigation. *C. difficile* flagella is recognized by the TLR5 receptor to elicit host proinflammatory responses.[128] Lastly, *C. difficile* metalloprotease ZmpI cleaves host fibronectin and fibrinogen[129, 130] and clostridial surface proteins,[130, 131] but the function of these proteins during infections has yet to be determined. In summary, *C. difficile* uses a number of virulence factors to cause infection in mammalian hosts.

C. difficile is unique among the pathogens discussed because its c-di-GMP signaling is primarily mediated through the action of two classes of riboswitches (see review[132]), consisting of twelve Cdi1[133] and four Cdi2.[134] The two classes of riboswitches respond differently to c-di-GMP. Cdi1 riboswitches binding to c-di-GMP lead to premature termination,[133] while Cdi2 riboswitches binding to c-di-GMP allow for transcription of the full-length transcripts.[135] The Cdi1_3 riboswitch is located upstream of the *flgB* operon [133] that encodes several early stage flagella proteins, including the SigD sigma factor. High levels of c-di-GMP repress expression of SigD.[136, 137] SigD positively regulates the transcription of *tcdR*, an alternative sigma factor that directs transcription of *tcdA* and *tcdB*.[138, 139] The consequence of elevated c-di-GMP levels and the repression of *sigD* by Cdi1_3 is the lack of expression of *tcdR* and *tcdA*/*tcdB*, leading to decrease cytoxicity in vitro.[137] The riboswitch Cdi1_12 prematurely terminates the mRNA for zinc metalloprotease ZmpI (CD2830) in the presence of high c-di-GMP levels.[135] Zmp1 cleave two adhesins called CD2831 and CD3246,[131] and the 5'UTR of these two genes contain Cdi2 class riboswitches, Cdi2_3 and Cdi2_1, respectively.[135] The ZmpI metalloprotease and its two substrates are reciprocally regulated by c-di-GMP. At high levels of c-di-GMP, expression of ZmpI is repressed while its substrates are expressed. For the Cdi2_4 riboswitch, high c-di-GMP levels allow for full-length transcription of type IV pilli that promotes bacterial motility and aggregation in vitro.[140] Aside from riboswitches, PilB1, the type IV pili ATPase, is a protein receptor for c-di-GMP.[141] Together, high levels of c-di-GMP activate expression of adhesins and type IV pili while repressing flagella, toxins and metalloprotease.

Currently, the contribution of c-di-GMP to *C. difficile* infection cannot be fully assessed by the modified molecular Koch's postulates for signaling nucleotides. While c-di-GMP regulates gene expression of virulence factors in *C. difficile*, very little is known about whether c-di-GMP regulates pathogenesis during infection. Further studies are needed that explore whether c-di-GMP is required for virulence. Outstanding questions include whether DGCs and PDE-As are differentially expressed during infection, whether c-di-GMP levels are altered during infection, and whether c-di-GMP riboswitch are required for infection. Answering these questions will allow for a more complete understanding of how c-di-GMP regulates pathogenesis of *C. difficile*.

Summary of GI pathogens—Studies of c-di-GMP in two GI pathogens, *V. cholerae* and *S.* Typhimurium, clearly show that the expression of c-di-GMP regulated extracellular proteins, such as MSHA and curli, and polysaccharides, such as cellulose, are detrimental to the ability of the bacteria to cause infections in mammalian hosts. Curli and cellulose are also found in *E. coli* and appear to be anti-virulent during UTI. Expression of *E. coli* PNAG in *S.* Typhimurium also attenuated infection. These results indicate that c-di-GMP positively regulates expression of surface molecules that attenuates the bacterial pathogen during infections. Nonetheless, c-di-GMP regulated processes serve the bacteria outside the mammalian host and, in the case of *V. cholerae*, may aid the infection cycle outside the mammalian GI tract. Future studies to determine how well mammalian host recognize these c-di-GMP regulated surface molecules in a manner similar to MSHA will reveal why c-di-GMP attenuates mammalian infections by these GI pathogens.

Vector-borne pathogens

Two notable vector-borne bacterial pathogens, *Yersinia pestis* and *Borrelia burgdorferi*, use c-di-GMP signaling in their infection life cycle. In both cases, the bacteria require expression of different factors to survive and infect mammalian hosts and insect vectors (Figure 2).

Yersinia pestis

Y. pestis, the causative agent of bubonic plague, spreads from one mammalian host to another through the flea vector. Upon entry into the mammalian host, *Y. pestis* utilizes the type III secretion system and Pla protease to avoid immune clearance and establish systemic infection.[142] Fleas feeding on the infected mammalian host ingest blood meal containing *Y. pestis*. *Y. pestis* utilizes the hemin storage system (*hms*) to form a biofilm on the proventriculus of the flea that effectively blocks the digestive tract, leading to starvation of the flea.[143] The starving fleas continuously bite additional hosts in an attempt to feed, leading to transmission of *Y. pestis* to additional mammalian hosts.[143]

The *hms* genes in *Y. pestis* are regulated by c-di-GMP. C-di-GMP is synthesized from two DGCs, HmsT and y3730 (HmsD),[144-146] and degraded by the HmsP PDE-A.[147, 148] When c-di-GMP levels are high, *Y. pestis* utilize the *hms* genes to produce an exopolysaccharide that mediates biofilm formation.[149] The Hms exopolysaccharide is recognized by antibodies that recognize the intercellular adhesin (ica) of *Staphylococcus epidermidis*, which is a PNAG polysaccharide.[145] Although the c-di-GMP receptor responsible for Hms exopolysaccharide has not yet been experimentally determined, HmsR and HmsS are homologous to the PgaC and PgaD in *E. coli*[150] that serve as the heteromeric c-di-GMP receptor for PNAG synthesis.[59] Together, these studies reveal that the c-di-GMP pathway in *Y. pestis* regulates Hms exopolysaccharide and biofilm formation.

Several studies have characterized the roles of c-di-GMP and the Hms exopolysaccharide using various *Y. pestis* mutants in mice infections. A mutant lacking both DGCs, *hmsT* and *hmsD*, is able to infect mice intranasally and subcutaneously with LD_{50} similar to the parental strain, indicating that c-di-GMP is dispensable for mammalian infections.[145] A *Y. pestis hmsP* mutant lacking the HmsP PDE, that has constitutively high c-di-GMP levels, can infect via the intranasal route of infection, albeit with about a 2.3-fold increase in LD_{50} as compared to the parental strain[145]. In contrast, the same *hmsP* mutant had a 1,000-fold increase in LD_{50} in a subcutaneous infection.[145] The defect in subcutaneous infection for the *hmsP* mutant was suppressed by an additional mutation in *hmsR* that prevents the synthesis of the Hms exopolysaccharide.[145] Together, these results indicate that the Hms exopolysaccharide and biofilm formation prevent systemic infection of mammalian hosts.

When *Y. pestis* is ingested by fleas, transcription of the *hmsT* gene is induced.[146] The lower temperature in the flea also contributes to accumulation of the HmsT protein, which can lead to an increase in c-di-GMP level and the production of the Hms exopolysaccharide. The *Y. pestis hmsT hmsD* double mutant lacking DGCs was unable to block the proventriculus of the flea allowing the fleas to clear the mutant when inoculated at a lower dose.[146] This matches previous studies in which mutants in *hmsR* are also unable to block the fleas and

the number of bacteria in the fleas is reduced. Together, these studies show that *Y. pestis* increases c-di-GMP in the flea to form a biofilm and block the flea. The blocked flea will bite other mammalian hosts and infect them with *Y. pestis*. Once in the mammalian host, *Y. pestis* must reduce levels of c-di-GMP to allow dissemination and systemic infection.

Evaluation of the current literature of c-di-GMP signaling in *Y. pestis* pathogenesis indicates that c-di-GMP signaling is an anti-virulence factor in the mammalian host. Mutants that increase c-di-GMP levels are defective in establishing murine infections, whereas mutants lacking c-di-GMP have similar LD_{50} as the parental strain indicating that c-di-GMP signaling is dispensable for mammalian infections. The virulence defect of mutants with elevated c-di-GMP can be reversed by a secondary mutation in *hmsR* indicating that reduction in virulence caused by elevated c-di-GMP signaling is due to the production of the Hms exopolysaccharide that is cross-reactive to PNAG antibodies. Similar to *E. coli*, *V. cholerae* and *S.* Typhimurium, increase in c-di-GMP signaling and c-di-GMP regulated processes attenuates *Y. pestis* pathogenesis during infection of mammalian hosts. However, the infection cycle of *Y. pestis* is biphasic with the flea serving as an insect vector critical for the spread of *Y. pestis* to new mammalian hosts. In the flea, c-di-GMP signaling and c-di-GMP-regulated Hms exopolysaccharide allow the bacteria to block the proventriculus of the flea. This blockage serves two important functions: first, *Y. pestis* that cannot block the flea is cleared from the insect host; second, blocked fleas cannot feed and continue to bite mammalian host in an attempt to feed. As a consequence, the bacteria are spread to new mammalian hosts. Similar to c-di-GMP regulation of *V. cholerae* infections, c-di-GMP signaling pathway acts as an anti-virulence factor in the mammalian host, but serves an important function in the infection cycle within the insect vector.

Borrelia burgdorferi

B. burgdorferi is the causative agent of Lyme disease.[151] After transmission from the tick vector to a mammalian host, *B. burgdorferi* can replicate at the bite site, disseminate and form foci of infection in various tissues.[152] Since the genome of *B. burgdorferi* lacks genes encoding recognizable toxins, secretion systems and virulence factors found in other pathogens, Lyme disease is thought to be caused by the mammalian host immune response to the spirochete.[151]

B. burgdorferi utilizes c-di-GMP signaling (see review[153]). C-di-GMP is produced by the DGC Rrp1, which must be phosphorylated by Hk1 to be active.[154] C-di-GMP is degraded by two phosphodiesterases: PdeA (BB0363)[155] and PdeB (BB0374).[156] *B. burgdorferi* encodes one PilZ domain protein, PlzA, that contain the consensus RXXXR DXSXXG motifs.[157, 158] In vitro, c-di-GMP regulation of *B. burgdorferi* occurs by altering the expression of several genes encoding proteins, including Bdr, OspE, and OspF as well as other OspE-related proteins.[159, 160] The function of c-di-GMP regulation of *B. burgdorferi* in the mammalian host and insect vector was investigated using individual mutants *rrp1*, *hk1*, *pdeA*, *pdeB* and *plzA*. Mice infected subcutaneously with a needle injection of *hk1*,[161] *rrp1*,[159, 162] or *plzA*[158] mutants were all capable of establishing an infection similar to the parental strain. However, when mice were infected using the tick vector, the *hk1*, *rrp1* and *plzA* single mutant strains were defective in infection of mice.[158, 160, 162] The defect of these

mutants is due to their inability to persist in the tick larvae and nymphs.[158, 159, 161] Even ticks microinjected with *B. burgdorferi hk1*, *rrp1* or *plzA* mutant cleared the bacteria,[162] indicating that c-di-GMP signaling pathway is critical for *B. burgdorferi* survival in the tick vector.

The role of c-di-GMP signaling was also assessed using mutants lacking the two PDEs, *pdeA* or *pdeB*. The *pdeA* mutant was avirulent in mice through the intraperitoneal and subcutaneous route, by tail vein injection, or through tick bite.[155] The *pdeA* mutant was able to survive in the tick, indicating the defect in the mice is due to elevated levels of c-di-GMP, not just an inability of the mutant to persist in the tick vector.[155] In contrast, *pdeB* mutant was able to infect mice subcutaneously, but unable to infect via tick bite.[156] The *pdeB* mutant had a similar defect in survival in ticks, indicating that *pdeA* and *pdeB* have different functions in *B. burgdorferi*. Future studies determining the levels of c-di-GMP in the parental, *pdeA,* and *pdeB* mutant *B. burgdorferi* strains will aid in interpretation of the function of phosphodiesterases in pathogenesis. *B. burgdorferi* with elevated levels of c-di-GMP fails to spread from the infection site,[155] indicating that the bacteria must reduce c-di-GMP levels upon entry into the mammalian host.

The contribution of c-di-GMP signaling to *B. burgdorferi* infections assessed by the adapted molecular Koch's postulates suggests that c-di-GMP signaling is an anti-virulence factor in the mammalian host. Similar to *Y. pestis*, *B. burgdorferi* utilizes the c-di-GMP signaling pathway to survive within the insect host, but the pathway must be turned off for successful infection of mammalian hosts. Future studies are needed to identify the c-di-GMP regulated processes that promote survival in the tick and attenuate infection of *B. burgdorferi* in the mammalian host. Identification of these c-di-GMP regulated processes will complete our understanding of c-di-GMP regulation of *B. burgdorferi* pathogenesis.

Summary of vector-borne pathogens—Studies from these two c-di-GMP utilizing vector-borne pathogens reveal that the bacteria utilize c-di-GMP levels in the insect vector in order to survive and potentially induce the vector to feed more often. Once back in the mammalian host, the c-di-GMP pathway must be turned off in order to allow the bacteria to disseminate from the subcutaneous site to cause systemic infection.

Conclusion

C-di-GMP regulation of bacterial virulence is specific to each pathogen, strain, and type of infection. Differences in the composition and structure of extracellular matrix components could underlie the variation in immune response in the mammalian host. For HAI pathogens, there are some infections, such as *P. aeruginosa* infection in CF patients and on medical devices, that are enhanced by high c-di-GMP levels and c-di-GMP-regulated production of alginate, Psl and Pel exopolysaccharides. In contrast, most infections of mammalian host by GI and vector-borne pathogens are attenuated by high levels of c-di-GMP and the c-di-GMP-mediated activation of extracellular matrix components, including polysaccharides (cellulose and PNAG) and surface protein polymers (MSHA and curli). Bacteria expressing these extracellular matrix components in the mammalian host can be recognized by the immune system leading to immune clearance. To enhance infection, a subset of pathogens

reduces c-di-GMP levels during infection in the mammalian host. Outside the mammalian host, these pathogens benefit from c-di-GMP activation of extracellular matrix components that enhance biofilm formation. For GI pathogens that exit into the environment, bacteria in the biofilm state can better survive hypotonic stress and re-enter into the acidic environment of the mammalian stomach. For vector borne pathogens, biofilms allow the bacteria to survive in the insect vector. Future studies determining the levels of c-di-GMP in the bacteria during infection of mammalian host, the contribution of c-di-GMP receptors and regulated processes will further reveal how c-di-GMP signaling pathway regulates bacterial pathogenesis.

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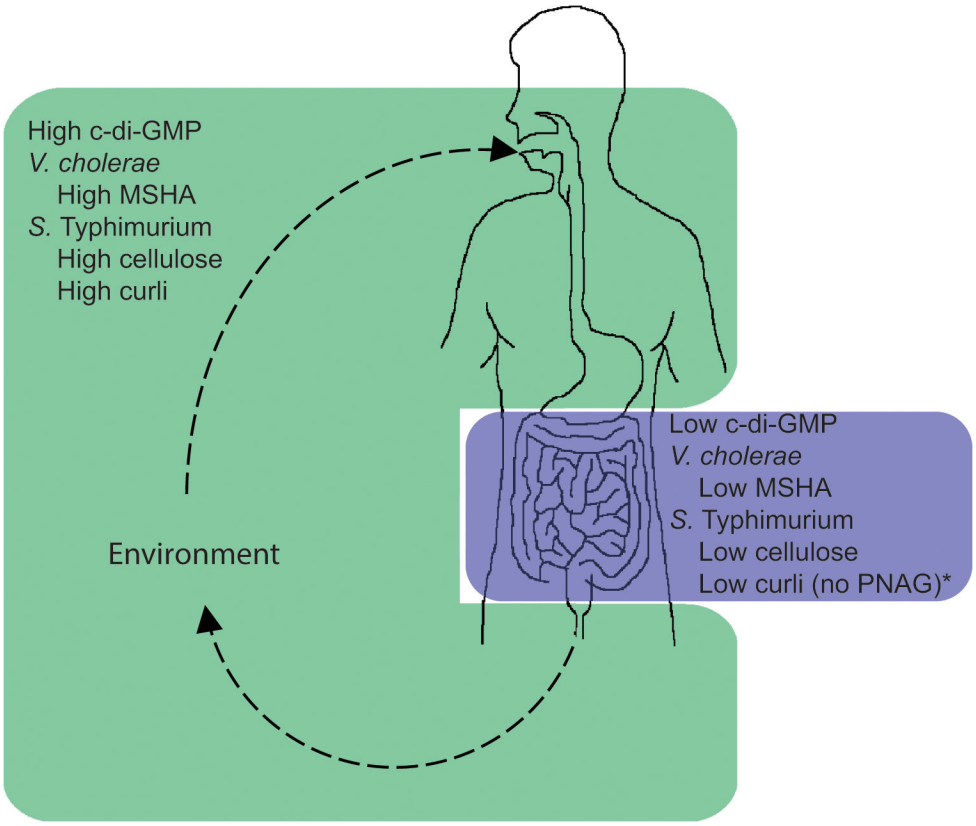

Figure 1.

C-di-GMP signaling in gastrointestinal pathogens. Repression of c-di-GMP-regulated extracellular matrix components in the mammalian host prevents host recognition and immune clearance. Activation of c-di-GMP-regulated extracellular matrix components outside the mammalian host allows bacteri to survive environmental stresses and reentry through the low pH of the stomach. Once inside the mammalian intestine, bacteria reduces c-di-GMP levels in order to establish a successful infection. * *S.* Typhimurium does not encode the *pga* genes encoding for the biosynthetic genes for producing the PNAG polysaccharide. Heterologous expression of *pga* genes attenuates virulence of *S.* Typhimurium.

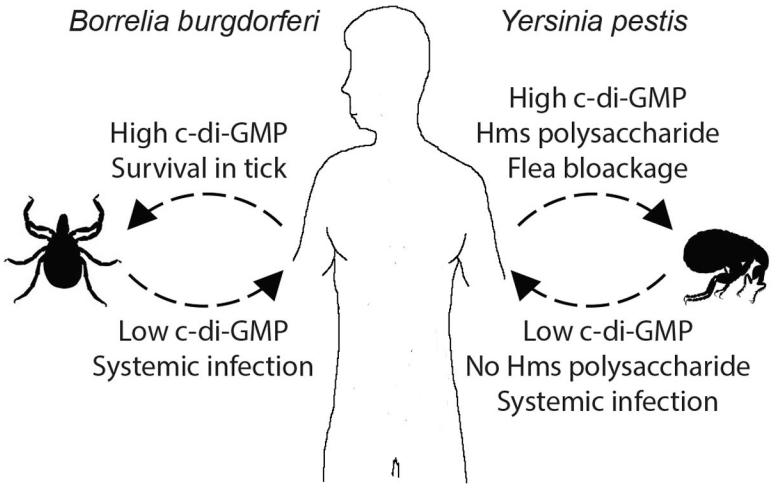

Figure 2.

C-di-GMP signaling in vector-borne pathogens. Repression of c-di-GMP-regulated extracellular matrix components in the mammalian host prevents host immune recognition and clearance, thus allowing disseminating systemic infection. Activation of c-di-GMP-regulated extracellular matrix components in the insect vector allows bacteria to survive the insect and infect additional mammalian hosts.