Yin and Yang of Biofilm Formation and Cyclic di-GMP Signaling of the Gastrointestinal Pathogen Salmonella enterica Serovar Typhimurium

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Abstract
Within the last 60 years, microbiological research has challenged many dogmas such as bacteria being unicellular microorganisms directed by nutrient sources; these investigations produced new dogmas such as cyclic diguanylate monophosphate (cyclic di-GMP) second messenger signaling as a ubiquitous regulator of the fundamental sessility/motility lifestyle switch on the single-cell level. Successive investigations have not yet challenged this view; however, the complexity of cyclic di-GMP as an intracellular bacterial signal, and, less explored, as an extracellular signaling molecule in combination with the conformational flexibility of the molecule, provides endless opportunities for cross-kingdom interactions. Cyclic di-GMP-directed microbial biofilms commonly stimulate the immune system on a lower level, whereas host-sensed cyclic di-GMP broadly stimulates the innate and adaptive immune responses. Furthermore, while the intracellular second messenger cyclic di-GMP signaling promotes bacterial biofilm formation and chronic infections, oppositely, Salmonella Typhimurium cellulose biofilm inside immune cells is not endorsed. These observations only touch on the complexity of the interaction of biofilm microbial cells with its host. In this review, we describe the Yin and Yang interactive concepts of biofilm formation and cyclic di-GMP signaling using S. Typhimurium as an example.

Introduction
Regulation of virulence properties of a microbial organism and its interaction with a potential host is highly dependent on environmental conditions. As has been observed exemplarily by laboratory studies, plate-grown cells of the gastrointestinal pathogen Salmonella enterica serovar Typhimurium are hardly virulent, while liquid-grown S. Typhimurium cells readily invade host cells [1], a regulation occurring already at the transcriptional level [2]. Part of this delicate regulation between acute virulence and commensalism/chronic infection is executed by a small molecule whose local or global concentration responds readily to environmental conditions, namely the ubiquitous second messenger cyclic diguanylate monophosphate (cyclic di-GMP) [3–6]. Cyclic di-GMP is
one, and perhaps, the most important member of a larger family of cyclic di- and oligonucleotide second messengers that primarily include the predominantly Gram-positive cyclic di-AMP and the hybrid molecule 3′,3′-cyclic AMP-GMP [7, 8]. Recently, the discovery of a broad panel of additional cyclic di- and oligonucleotides has been substantialized including compounds previously only predictively chemically synthesized [9–14]. Cyclic oligonucleotide second messengers possess a major role in the regulation of the activity of nucleases in CRISPR/Cas-based innate immune response against bacteriophages [14–16]. On an evolutionary scale, the metazoan viral defense cGAS-STING (cyclic GAMP synthase – stimulator of interferon genes) pathway with the 2′,3′-cyclic GMP-AMP analog as the second messenger has its foundation in microbial components [13, 17].

The spatial and temporal intracellular concentration of the second messenger cyclic di-GMP which occurs in over 75% of all bacterial species is adjusted on the single-cell level by a multitude of turnover proteins and receptors [18] and consequently delicately regulates a wide variety of physiological and metabolic traits that channel into acute versus chronic virulence, and sessility versus motility, as well as concomitantly in the promotion of antimicrobial and detergent tolerance and tolerance against the immune response. In this context, cyclic di-GMP can direct fundamental processes such as carbon source catabolism, respiration, cell division, and cell shape by affecting global molecular processes such as RNA turnover, proteolysis, protein acetylation, secretion, and the catalytic activity of biofilm matrix biosynthesis enzymes. This physiological and behavioral consequences have a wide impact not only in the clinical, industrial, and agricultural setting, but also shape the ecology in oceans and affect geochemical relevant global compounds and cycles, such as the denitrification cycle [19–23]. The conformational flexibility and the ability to form various types of oligomers and few amino acids sufficient to define binding make it challenging to predict the binding sites of cyclic di-GMP [5, 24].

Environmental and intrinsic signals received by cyclic di-GMP turnover proteins determine not only the (acute) virulence properties of microorganisms, but can also provoke the expression of different types of biofilms such as *Pseudomonas aeruginosa* biofilm formation in the urinary tract versus laboratory-grown biofilms [25]. The multitude of signals that direct the turnover activity of the cyclic di-GMP second messenger signaling system (equally as those of other second messengers and phosphotransfer signal transduction systems, chemotaxis systems, and other, which are discussed here in the context of relevant cross talk) on the transcriptional, post-transcriptional, and post-translational level integrates into a specific output response which is equally dependent on the receptor and target proteome status combined with the rest of the proteome [6]. The predominant extracellular matrix components that cover the bacterial cells in a honeycomb-like fashion include amyloid curli fimbriae and the exopolysaccharide cellulose. Curli and (phosphoethanolamine modified) cellulose possess clearly defined features, which point to opposite functionality [27–29]. In this mini-review, we discuss the Yin and Yang functionality of the extracellular matrix components, biofilm formation, biofilm regulators, and cyclic di-GMP signaling in bacterial and bacterial-host interactions taking mainly the gastrointestinal pathogen *S. Typhimurium* as an example (Fig. 1).

**Amyloid Curli Fimbriae and the Exopolysaccharide Cellulose as Opposing Extracellular Matrix Components of *S. Typhimurium* Biofilms**

A highly hydrophobic outer shell encloses cells of the plate-grown rdar (red, dry, and rough) morphotype of *S. Typhimurium*, *Escherichia coli*, and other enterobacteria upon expression of two major extracellular matrix components: amyloid curli fimbriae and the exopolysaccharide cellulose (Fig. 2; [30–32]). These two polymeric extracellular matrix components tightly interact to display a full biofilm phenotype (bacterial wood), but with each of these matrix components actually to possess a distinct and frequently opposite functionality (Fig. 2). The extracellularly polymerized amyloid curli with subunits characterized by 5 parallel pseudo-repetitive beta-strands converts the cell surface toward hydrophobicity with promiscuous adhesive properties toward proteins and surfaces (Fig. 2a; [31, 33–37]). Consequently, biofilm cells expressing curli fimbriae interact tightly with abiotic and biotic surfaces [28, 30]. In contrast, expression of the exopolysaccharide cellulose leads to an overall hydrophilic cell surface as measured by the contact angle of bacterial macrocolonies [31]. Cellulose provides predominantly flexible cell-cell interactions in a static rich culture medium, while under continuous flow in minimal medium cellulose contributes to surface adherence and cell-cell interactions [31, 38–40]. Although the expression of these two matrix components is tightly coupled in plate-grown biofilms through direct and indirect regulation by the bistably expressed transcriptional regulator CsgD, the con-
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Fig. 1. The cyclic di-GMP signaling network of *S. Typhimurium* ATCC14028 and its effect on biofilm formation, motility, and virulence-related phenotypes. The genome of *S. Typhimurium* codes for 22 conserved and evolved cyclic di-GMP turnover proteins. The effect of a gene, as assessed upon deletion, on particular phenotypes (csgD = production of the biofilm activator CsgD; cellulose = biosynthesis of the exopolysaccharide cellulose; m = apparent motility; FlhDC = inhibition of the class I flagellar regulon activator FlhDC; IL-8 = secretion of the proinflammatory cytokine IL-8 by the epithelial cell line HT-29; iv = invasion of the intestinal cell line HT-29; sm = survival in macrophages; co = colonization of the gastrointestinal tract (as assessed by analysis of feces)) in the strain *S. Typhimurium* ATCC14028 is indicated; green = promotion of phenotype; red = suppressive effect on phenotype. In brackets, not consistently observed. The response regulator SsrB in its unphosphorylated form activates expression of the csgD biofilm regulator gene; FlhDC = class I flagellar regulon activator FlhDC; Cyclic di-GMP, cyclic diguanylate monophosphate; CHASE, cyclase/histidine kinase-associated sensing extracellular domain; CSS, redox-sensing domain with C(x)30CSS motif; GAPES, gammaproteobacterial periplasmic sensor domain; HAMP, histidine kinases, adenylate cyclases, methyl accepting proteins, phosphatases signal transduction domain; MASE, membrane-associated sensor; MHYT, methionine, histidine, tyrosine and threonine containing integral membrane sensor domain; PAS-PAC, Per-Arnt-Sim domain – C-terminal to PAS domain.

The Cyclic di-Nucleotide Second Messenger Signaling System

The universally conserved predominantly bacterial secondary messenger cyclic di-GMP was initially identified in the bacterium *Komagataeibacter xylinus* (orig-
Acetobacter ([Gluconacetobacter] xylinum) to activate the biosynthesis of the exopolysaccharide cellulose [42]. Interactive with other nucleotide signaling systems and upon integration of still unknown signaling pathways, cyclic di-GMP acts as a nearly ubiquitous signal currency to exponentially translate and integrate a multitude of environmental and intracellular signals into the opposite sessility/motility lifestyle behavior concomitant with the cell cycle, cell morphology, metabolism, secondary metabolites, and physiology. The local and global elevation of the cyclic di-GMP signal thus results in the transition from a motile planktonic growth of single cells to an often sessile multicellular biofilm.

The turnover of cyclic di-GMP is controlled by ubiquitous GGDEF and EAL or HD-GYP single or hybrid domain proteins encoded by numerous gene copies in variable numbers and ratios grossly correlated with genome size within a phylum [4, 43−45]. Thereby, cyclic di-GMP is synthesized from two molecules of GTP by the diguanylate cyclase activity of GGDEF domains and hydrolyzed to linear pGpG or GMP through the phosphodiesterase activity of EAL or HD-GYP domains [46]. Both the N-terminal signaling domains and the catalytic domains can receive regulatory signals which allosterically regulate the synthesis and hydrolysis of the messenger [47, 48]. For example, in the plant pathogen *Agrobacterium tumefaciens*, the level of cyclic di-GMP is controlled by a

Fig. 2. The extracellular matrix components of the rdar biofilm, the exopolysaccharide cellulose, and amyloid curli fimbriae possess distinct features and furnish *S. Typhimurium* cells with a distinct biological function. (a) While expression of the exopolysaccharide cellulose provides a hydrophilic cell surface, expression of the amyloid curli fimbriae leads to a more hydrophobic surface as exemplified by assessment of surface tension [31]. A larger contact angle Θ indicates a more hydrophobic surface. Expression of the exopolysaccharide cellulose prevents adhesion (b), invasion (c), and secretion of the proinflammatory cytokine IL-8 (d) of *S. Typhimurium* to the gastrointestinal epithelial cell line HT-29, while expression of the amyloid curli fimbriae promotes adhesion, invasion, and secretion [83, 101, 186]. However, this microbial behavior is context dependent [41]. Rdar, red, dry, and rough.
hybrid GGDEF-EAL protein, DcpA, that confers either diguanylate cyclase or phosphodiesterase activities depending on the absence or presence of the pteridine reductase PruA [49]. Similarly, in P. aeruginosa, the GGDEF-EAL protein MucR, confers diguanylate cyclase activity when the bacterium is planktonic while the EAL domain is active conferring a phosphodiesterase function when in a biofilm, with the activity growth responsive to nitric oxide [50]. In S. Typhimurium, the hybrid GGDEF-EAL protein STM3388, the homolog of MucR, subsequently represses and activates production of the biofilm regulator CsgD during the growth phase [51].

In the signaling cascade downstream of synthesis, specific effector proteins directly or indirectly mediate the physiological output and phenotypes. Since the first cyclic di-GMP receptor was identified in 1987, namely the cellulose synthase of Gluconacetobacter xylinus [52, 53], with subsequent identification of C-terminal PilZ as the binding domain, numerous receptors with distinct cyclic di-GMP binding motifs including RNA aptamers have been elucidated [54–56].

Despite the wealth of experimental data that address various aspects of the cyclic di-GMP signaling system and its physiological consequences, one major question remains unanswered: how are biofilm extracellular matrix components differentially regulated to promote the various temporal and spatial restricted types of biofilms [57] and do these different types of biofilms display distinct tolerance profiles? For example, the phosphodiesterase BinA of Vibrio fischeri adjacent of the Spy exopolysaccharide operon downregulates production of a cellulose-like exopolysaccharide [58]. In S. Typhimurium, the evolved phosphodiesterase STM0551 within the type 1 fimbrial gene cluster represses the adjacent fimbrial genes [59].

**Regulation of Motility versus Sessility by Cyclic di-GMP Signaling**

Perhaps the most fundamental feature of cyclic di-GMP is to confer the sessility versus motility lifestyle switch. First demonstrated with model signaling proteins including the diguanylate cyclase AdrA and the phosphodiesterase YhjH, high cyclic di-GMP levels enhance biofilm formation [46, 60], while low cyclic di-GMP levels result in promotion of bacterial motility which can result in a planktonic lifestyle [60–62]. Cyclic di-GMP ubiquitously regulates sessility versus motility in all investigated bacteria with a multitude of physiological and metabolic adjustments, beyond the simple stimulation of biofilm transcription factors and biosynthesis enzymes that synthesize extracellular matrix components and post-translational downregulation of flagellar-based motility [5, 20, 63]. Such a concomitant adjustment occurs, for example, in Vibrio cholerae where high level of cyclic di-GMP promotes DNA repair through the VpsT and VpsR cyclic di-
GMP-dependent biofilm regulators. This regulation positively induces expression of the DNA repair gene 3-methyladenine glycosylase (*tag*) offering higher tolerance to DNA damaging conditions [64].

In *S. Typhimurium* and *E. coli*, the *csgD*-mediated biofilm has been shown to be a major hub of cyclic di-GMP regulation with the orphan response regulator CsgD to promote the transcription of genes encoding biofilm matrix components. These include the *csgBAC* operon of minor and major curli fimbriae subunits and, indirectly, cellulose production by activating the gene for the diguanylate cyclase AdrA [66–68]. CsgD-mediated biofilms contribute not only to the transmission of *S. Typhimurium*, but also to biofilm formation and persistence in the context of the gastrointestinal tract, plants, and other environments (Fig. 3; [68–70]). The bistable expression of CsgD ensures different subpopulations of cells with distinct biofilm formation and virulence properties [40, 69], which provide the multicellular cell population with various immediate physiological possibilities.

With a cyclic di-GMP binding motif absent, *csgD* expression itself is a major target of cyclic di-GMP signaling on the transcriptional and post-transcriptional level (Fig. 1; [71, 72]). In contrast to the ubiquitous intracellular role of cyclic di-GMP, application of cyclic di-GMP extracellularly inhibits biofilm formation in bacteria such as *Staphylococcus aureus* (Fig. 4a; [73]). Indeed, extracellular cyclic di-GMP has been proposed as a treatment option against biofilm diseases; however, the effective mechanisms have been poorly explored.

*S. Typhimurium* and *E. coli* possess an array of fimbriae that can potentially promote biofilm formation
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batch cultures demonstrated a type 1 fimbriae and csgD-mediated biofilm after 24 h that transforms into a solely csgD-mediated biofilm after 48 h [77]. Surprisingly, when grown on a silicone surface mimicking a urinary catheter in the artificial urine medium, csgD expression repressed biofilm formation as a csgD mutant showed higher biofilm formation as the wild type (Fig. 4b; Xiaoda Wang and Ute Römling, unpublished work; [78]). One explanation is possible repression of alternative biofilm matrix components such as type 1 fimbriae by csgD. Another possibility is that adhesive components are surface-selective with deletion of csgD to expose silicone-specific adhesins on the cell surface. In this context, the composition of the biofilm of P. aeruginosa formed on the surface of the silicone catheter under urinary tract growth conditions has been shown to be fundamentally different from medium-grown biofilms [79]. Motility is commonly negatively regulated by cyclic di-GMP in various bacteria [46, 80]. A wide variety of motility modes are repressed by cyclic di-GMP signaling including flagella-mediated swimming and swarming motility and type IV pili surface motility [81]. In S. Typhimurium, post-translational regulation by cyclic di-GMP, which binds to the PilZ domain protein YcgR, leads to a conformational change in the protein [80, 82]. Consequently, cyclic di-GMP loaded YcgR can form a complex with FliG and FliM proteins that are part of the flagella rotor. Although cyclic di-GMP can also inhibit expression of the flagellar regulon cascade in E. coli, overexpression of diguanylate cyclases in S. Typhimurium enhanced cell-associated flagellin most likely in the form of flagella [83]. This scenario is consistent with the idea that flagella have multiple roles as propellor of motility, as surface sensor and adherence factor, even constituting an extracellular matrix component of biofilms (Fig. 4c; [84, 85]).

Acute Virulence Phenotypes Regulated by Cyclic di-GMP Signaling

Acute infections are based on short-term expansion of microbes that mostly involve planktonic (and motile) bacterial cells to employ the repertoire of virulence factors to invade and severely damage the tissue and to cause a substantial immune response. During acute infection by S. Typhimurium, the microorganisms are hypothesized to predominantly form biofilms in the gastrointestinal lumen with a fraction of cells breaching the epithelial cell lining. Nine cyclic di-GMP turnover proteins contribute to cecum colonization in the microbiota-depleted streptomycin-treated mouse model [91]. The contribution of (predicted) phosphodiesterases such as STM3615 (YhjK) and diguanylate cyclases such as STM2672 (YfN) and Salmonella-specific STM4551 points to a complex role of cyclic di-GMP in persistent gut colonization (Fig. 1; [91]), with STM3615 also deficient in the colonization of mesenteric lymph nodes and the spleen (Lamprekostopoulos, Römling, and W.-D. Hardt, unpublished observations). Although the biofilm regulator csgD and curli are expressed in the gastrointestinal tract, the panel of regulatory cyclic di-GMP turnover proteins is distinct compared to regulation of plate-grown biofilms [51, 71, 92]. The putative phosphodiesterase STM3615, though, has an unconventional role in regulation of rdar biofilm formation in the background of deletion of dsbA dsbB genes involved in periplasmic disulfide bond formation, with the involvement of the catalytic activity to be tested [93].

Although S. Typhimurium causes acute gastroenteritis, the disease is self-limiting in most immune-competent individuals due to the massive immune response combined with neutrophil influx. A particularly invasive S. Ty-
phimurium clone, ST313, with enhanced virulence resembling typhoid fever and reduced biofilm formation, has emerged in Africa in HIV and malaria infected individuals and upon malnutrition [94]. The infection process of S. Typhimurium is regulated by cyclic di-GMP signaling and biofilm components at various stages as dissected by experimental studies with cell culture and animal models to show unique and distinct contributions of individual cyclic di-GMP turnover proteins (see below; [69, 83, 91, 95–97]).

Close association with epithelial cells is a characteristic of gastrointestinal pathogens and one of the first contacts of the bacteria with host tissue. S. Typhimurium forms biofilms on intestinal epithelial cells [98, 99]. Surprisingly, the two csgD-activated extracellular matrix components curli and cellulose have opposing roles in cell adherence. Curli fimbiae promote adhesion, while the exopolysaccharide cellulose inhibits adhesion of S. Typhimurium to the gastrointestinal cell line HT-29 (Fig. 2b; [83, 99, 100]). While a similar adherence pattern has been observed in a commensual and urinary tract infection E. coli strain [28, 101], the functionality of cellulose is context dependent; in the probiotic strain E. coli Nissle 1917, cellulose production promotes adhesion [41].

Invasion or uptake of S. Typhimurium into cells of the epithelial cell lining is one of the key steps in the pathogenicity of S. Typhimurium [102]. Saturation of the bacterial cell with cyclic di-GMP by overexpression of the diguanylate cyclase AdrA had a profound negative effect on invasion into the gastrointestinal epithelial cell line HT-29 [83]. Inhibition of virulence properties can be partially or even fully restored upon deletion of the biofilm regulator csgD, identifying csgD as one central hub for the acute virulence versus biofilm switch at the epithelial cell lining in S. Typhimurium [83]. Relieve of invasion occurs further through inhibition of the production of cellulose and capsule extracellular matrix components (Fig. 2c; [31, 65, 83]). Cellulose production can be activated, though, by cyclic di-GMP independently of CsgD [31, 103]. Possible mechanisms of reduction of invasion are prevention to establish adherence and shielding the type III secretion system-1 nanomachine via production of the cellulose exopolysaccharide [83, 104].

Dissecting the effect of individual cyclic di-GMP turnover proteins showed that 10 out of 20 deletions of individual GGDEF/EAL domain genes altered the invasion phenotype with 7 mutants showing a conventional and three mutants showing an unconventional phenotype. The molecular basis of interference with invasion has started to become unraveled for some of these GGDEF/EAL domain proteins (see below).

Breaching the epithelial cell lining by S. Typhimurium causes massive secretion of the pro-inflammatory cytokine IL-8, which subsequently attracts neutrophils to clear the infection [105]. Again, flooding the bacterial cell with cyclic di-GMP abolishes induction of the pro-inflammatory cytokine IL-8 in the epithelial cell line HT-29 (Fig. 2d, 3; [83]). Deletion of the genes for 6 cyclic di-GMP turnover proteins affects IL-8 secretion, while three of those proteins affect both invasion and IL-8 secretion. In contrast, secreted cyclic di-GMP and cyclic di-GMP systemically applied to the host is commonly immunostimulatory, with cyclic di-GMP recognized as a non-cytotoxic adjuvant [17, 83, 106–110].

Macrophages take up Salmonella beyond the gastrointestinal epithelial barrier for transport to inner organs via the blood stream [111–113]. S. Typhimurium uptake and survival in macrophages has been investigated in Salmonella susceptible animal models by mimicking Salmonella enterica serovar Typhi infection in humans long before the invasive S. Typhimurium ST313 clone emerged [69, 114]. S. Typhimurium produces cellulose within the Salmonella-containing vacuole in macrophages, which restricts its proliferation and attenuates acute virulence during systemic infection of Salmonella susceptible mice (Fig. 5a; [39, 96]). Different mechanisms can lead to alteration of cellulose production such as the MtgC virulence factor which interacts with the F1F0 ATP synthase to restrict cellulose biosynthesis. The cellulase BcsZ, which substantially upregulates virulence of S. Typhimurium, suggests that dysregulated biosynthesis of cellulose and not expression of the cellulose synthase BcsA is a determinative virulence modifying factor. Of note, ST313 clone members of S. Typhimurium possess a number of single nucleotide polymorphisms inside open reading frames and in intergenic regions, which have been shown to modulate virulence of this organism. For example, an amino acid substitution in the sensory Cache 1 domain of the diguanylate cyclase STM1987 causes reduced cellulose production but enhanced murine and human macrophage survival [97]. Equally, ST313 representatives harbor mutations in the gene for the alkaline phosphatase superfamily member BcsG [115]. As BcsG stabilizes the cellulose synthase BcsA post-translationally in combination with covalent modifications of the glucose subunits growing glucan chain by the phosphoethanolamine phospholipid headgroup [27, 29], the reduced cellulose biosynthesis is predicted to lead to enhanced proliferation in macrophages. Thus, intracellular proliferation without cellulose production opposes growth restriction upon biosynthesis of the cellulose biofilm matrix.
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Component, which basically oppositely reflects biofilm formation versus planktonic cell proliferation in the extracellular gastrointestinal space. Whether and, if so, why cellulose-producing bacteria are more susceptible to intracellular antimicrobial defense mechanisms needs to be shown. The channeling of glucose into cellulose instead of glycolysis which is required for S. Typhimurium in macrophages might restrict proliferation [116].

In bone-marrow-derived macrophages, at least three different subpopulations of intracellular Salmonella: fast, moderate, and slow growing have been identified [95]. Counterintuitively, the cellulose-producing slow-growing subpopulation was significantly more depleted upon deletion of three cyclic di-GMP-specific phosphodiesterases required for proliferation in macrophages, is also required for luminal colonization [91].

Intracellular and extracellular cellulose production provides an excellent example of the distinct roles of biofilms within host cells versus the luminal space. Within host cells, proliferation of planktonic cells is promoted, while slow-growing cells in a cellulose-producing biofilm status might persist without destruction of the host cell on a longer time scale. Outside host cells in the extracellular gastrointestinal space, the large number of biofilm cells outnumbers the few planktonic cells that breach the epithelial cell lining. Whether other biofilm types are expressed in immune cells cannot be excluded.

Contribution of Type III Secretion System-1

Cyclic di-GMP contributes at many stages to adjust virulence properties of S. Typhimurium; however, which of these processes are affected on the molecular level remains unknown. S. Typhimurium possesses two type III secretion systems (TTSS-1 and TTSS-2) needle-like nanomachines that tip adhere to the epithelial cell to inject effector proteins for host cell manipulation. Invasion of S. Typhimurium into epithelial cells requires genes of the Salmonella pathogenicity island 1 that code for the TTSS-1 [117]. TTSS-1 is regulated by a variety of extra- and intracellular signals [118–120] such as small intestine growth conditions with low oxygen and high salt to promote optimal expression of TTSS-1 proteins. Regulatory pathways for TTSS-1 expression converge at the transcriptional regulator HilA [117]. Polysaccharide components on the surface of bacteria have been demonstrated to interfere with invasion and/or type III secretion system functionality. The length of the O-antigen chain of lipopolysaccharide [121–123] and likewise in S. Typhi, the Vi-capsule [124] counteracts invasion of host cells. Inhibition by extracellular matrix components is, however, not universal as in P. aeruginosa biosynthesis of extracellular biofilm matrix does not seem to correlate with interference with type III secretion functionality [90]. On the other hand, in S. Typhimurium, adhesive curli fimbriae mediate adherence [28, 83], a prerequisite for TTSS-1 functionality and invasion [125].

In many bacteria including S. Typhimurium, TTSS-1/2 systems are subject to regulation by cyclic di-GMP signaling. Cyclic di-GMP can be closely linked as expression of type III secretion system components can be upregulated in bio-

Fig. 5. Contribution of biofilm components and virulence factors to virulence and biofilm formation in S. Typhimurium. (a) Cellulose production and csgD expression of S. Typhimurium establish extracellular and intracellular biofilms [5, 39, 40, 91, 95, 96]. Left: S. Typhimurium produces cellulose inside macrophages. Right: S. Typhimurium produces cellulose in extracellular biofilms. (b) The phosphorylated SsrB response regulator of the SsrA/SsrB 2-component system stimulates expression of the TTSS-2 2-component system inside the Salmonella-containing vacuole of macrophages [131], while the unphosphorylated SsrB response regulator aids promoter activation of the csgDEFG operon encoding csgD, the rdar biofilm activator, and additional genes required for the biogenesis of amyloid curli fimbriae [120, 132]. Cyclic di-GMP, cyclic di guanylate monophosphate.
films [93, 126] and required for the formation of mature biofilms and multicellular behavior [127, 128].

In S. Typhimurium, csgD and cyclic di-GMP signaling interfere with TTSS-1 functionality downstream of the activity of the TTSS-1 central regulator HiiA [6, 83, 91]. Thereby, cyclic di-GMP-mediated csgD expression has been exemplarily shown to inhibit the secretion of the TTSS-1 effector SopE2 [83, 91, 128, 129]. In csgD competent cell, cyclic di-GMP turnover proteins, diguanylate cyclases, and phosphodiesterases regulate secretion of effector proteins by their scaffold rather than by catalytic activity [71]. Those findings are consistent with results from P. aeruginosa where TTSS-mediated cytotoxicity toward the CHO cell line is affected by cyclic di-GMP signaling [90]. The subset of GGDEF/EAL mutants demonstrating alteration in cytotoxicity toward the CHO cell line was only partially overlapping with the subset contributing to virulence in a burn wound mouse model.

### Contribution of Type III Secretion System-2

The TTSS-2 is required for survival and proliferation of S. Typhimurium inside the Salmonella-containing vacuole [130]. The SsrA-SsrB 2-component system is regulated by the transcriptional regulator HilD, which affects the expression of HiiA coordinating the expression of TTSS-1 and TTSS-2. A major transcriptional regulator of the TTSS-2 is the response regulator SsrB phosphorylated by its cognate histidine kinase SsrA [131]. On the other hand, though, in the lumen of the gastrointestinal tract of the nematode Caenorhabditis elegans unphosphorylated SsrB is an anti-virulence factor to be required for the activation of transcription of the biofilm regulator csgD (Fig. 5b; [132]). The association of biofilm formation with TTSS-2 is more tight than previously thought as the TTSS-2-encoded MerR-like transcriptional regulator MlrB represses csgD expression inside macrophages [133]. These findings showed that biofilm formation is tightly counterregulated with virulence in S. Typhimurium by even using the same components. Of note, not only SsrB but also CsgD directs biofilm formation in its unphosphorylated form [72].

### Regulation of the IL-8 Response by Cyclic di-GMP Signaling

In the absence of cellulose production, S. Typhimurium can effectively bind to and invade epithelial cells via curli fimbriae and subsequently trigger production of the pro-inflammatory cytokine via curli-bound flagellin (Fig. 2d; [37, 56, 101]). High levels of cyclic di-GMP, though, did not trigger IL-8 production by HT-29 cells [83]. Stimulation of secretion of the pro-inflammatory cytokine IL-8 is recovered upon deletion of csgD, which relieves the secretion of monomeric flagellin. The non-stimulatory phenotype of S. Typhimurium during high cyclic di-GMP concentrations may possibly be a result of inhibition of the secretion of monomeric flagellin inducing IL-8 in the HT-29 cell line [134].

Similar to invasion, the cyclic di-GMP signaling system regulates flagellin secretion, as monitored by stimulation of the secretion of the pro-inflammatory cytokine IL-8, by a complex network of cyclic di-GMP turnover proteins (Fig. 1, 3; [91]). While the diguanylate cyclase STM1287 conventionally represses IL-8 secretion, the two EAL domain proteins, STM0468 and STM4264, and the GGDEF-EAL proteins, STM1703 and STM2503, stimulate IL-8 secretion equally as the degenerated GGDEF-EAL protein STM3375. Several of the EAL proteins seem to work in the same pathway as double mutants do not additively diminish the phenotype.

### Flagella Regulon-Related Phenotypes Affected by Cyclic di-GMP Signaling

With the flagellar regulon cascade delicately manipulated on different levels, the flagellum is not only a bacterial virulence factor with swimming and swarming motility to promote colonization and tissue invasion [83, 136]. Monomeric flagellin is recognized as a major pathogen-associated molecular pattern (PAMP) and systemic antigen being a major antigen in Crohn’s disease [136, 137]. Differential in vivo affinities for cyclic di-GMP for the flagellar motor break, the cyclic di-GMP receptor YcgR (2 μM), and subsequently the cellulose synthase BcsA (8 μM) involved in the inhibition of bacterial motility and in increase in cellulose-based biofilm matrix production, respectively, ensure coordinated steps toward S. Typhimurium biofilm formation [135, 138]. During bacterial infection, the polymeric flagellar filament can act as a virulence factor with secreted monomeric flagellin as an immunogen triggering innate as well as adaptive host response.

Recognition of flagellin monomers by epithelial cells occurs by pattern recognition receptors. Toll-like receptors (TLRs) are a group of important transmembrane pattern recognition receptors and until now, 15 TLRs have been identified, from which TLR 1–10 are found in humans [139]. TLRs have been found to reside...
on the surface or within cell compartments of not only epithelial and innate immune cells, but also neuronal cells, endothelial cells, and other cell types. After recognition of PAMPs, TLRs trigger a signaling cascade, which leads to the release of pro-inflammatory cytokines in order to subsequently promote an immune response. Recognition of flagellin by TLR5 and in the case of plants by FLS2 [140] subsequently leads to NF-kB activation, chemokine release, T-cell activation, and other inflammatory phenotypes with flagella production to be shut off at the later stage of infection [141, 142]. In bacterial infection of plants, high intracellular cyclic di-GMP concentrations drastically reduce the virulence of Pseudomonas syringae pv. tomato (Pto) DC3000 through inhibition of flagellar motility among other pleiotropic effects resulting from cyclic di-GMP signaling on bacterial behavior [143, 144]. Stimulation of the secretion of monomeric flagellin has been observed in response to host cells [82], and it remains to be shown whether this stimulation involves a cyclic di-GMP signaling pathway. Equally whether, and how, secretion of monomeric flagellin is coupled to the flagella biosynthesis process is unknown [128]. In conclusion, the above described work shows that secretion of monomeric flagellin is dependent on the expression of biofilm regulators and cyclic di-GMP signaling [83, 91].

The two evolved EAL domain only proteins STM1344 and STM1697 do not possess phosphodiesterase activity nor do these proteins bind cyclic di-GMP but inhibit the flagella regulon by inhibiting the activity of the class 1 regulator FlhD:C4 through protein-protein interactions [145–147]. In this way, STM1344 and STM1697, both contribute to regulation of swimming motility and phase variation of flagellar expression [145, 146]. Both proteins promote virulence presumably by their contribution to the delicate regulation of expression of flagellar antigenic filaments. Furthermore, STM1344 promotes resistance to Salmonella-induced oxidative stress and inhibits rapid macrophage killing [148].

On the other hand, the EAL-only protein YhjH is the only motility-dedicated phosphodiesterase [92, 135]. YhjH, despite possessing catalytic activity, is actually more closely related to STM1344 and STM1697 than to any other EAL domain in S. Typhimurium [67, 149]. Three diguanylate cyclases differentially feed into the inhibition of motility addressing the YcgR motor break (STM2672), the BcsA cellulose synthase (STM1987), or both receptors (STM4551) [135].

**Bacterial Cyclic di-GMP Signaling in Immunity**

Overgrowth of the microbial flora is prevented by an outer and inner mucus layer on the surface of the epithelium [150], which provides a mechanical, physicochemical, and biological barrier accumulating bacteriolytic enzymes like lysozyme and antimicrobial peptides secreted from Paneth cells [151, 152]. In addition, nutritional immunity challenges, for example, iron acquisition by microbial produced siderophores [153, 154]. Microbial secreted cyclic di-GMP can contribute not only to stimulate innate immunity, but to overcome nutritional immunity [12, 109]. Upon ingestion, few S. Typhimurium cells penetrate the mucus layer to reach the mucosal cell lining as the first barrier [155]. In mammalian as well as in plant host cells, innate immune receptors located in the cell membrane and intracellular receptors recognize PAMPs and induce an innate immune response known as pattern-triggered immunity (PTI) as a first line of response [156, 157]. Commensal bacteria trigger low-level PTI, evade, or even suppress PTI in order to successfully colonize the host [158]. Pathogen-PAMPs include the following: lipid A part of the lipopolysaccharide present in the outer membrane of Gram-negative bacteria, components of the bacterial cell wall such as peptidoglycan, microbial DNA, and physiological amyloids such as curli [159]. Another PAMP that plays an important role in triggering mucosal innate immune responses, as described above, is flagellin [160].

Based on initial reports [106, 161], cyclic di-GMP was recognized as a PAMP, to trigger protective host innate and adaptive immune responses [110]. The comprehensive stimulation of immunity might contribute cyclic di-GMP to be delivered exogenously in a murine model of bacterial pneumonia. A local or systemic administration of cyclic di-GMP prior to challenge with Klebsiella pneumoniae resulted in significantly increased animal survival and bacterial reduction in the lung and blood [110]. In combination with the initiation of robust innate and adaptive immune responses characterized by enhanced accumulation of neutrophils and alphabeta T cells, as well as activated natural killer cells and macrophages expressing inducible nitric oxide synthase and nitric oxide, the cell recruitment was associated with early elevated expression of chemokines and type I cytokines. These initial fundamental findings established cyclic di-GMP and subsequently analogous cyclic di-nucleotides not only as effective immune-modulators and enhancers, but also as potential anti-biofilm and anticancer agents. The subsequent identification of cyclic di-GMP and other cyclic di-nucleotide receptors in mammals paved the way for the identification of the central cGAS-cGAMP-
STING axis for sensing and responding to cytoplasmic nucleic acids [162–164]. Cyclic di-GMP can also activate the intracellular sensor STING, suggesting that cytosolic bacteria release this immune activator [38, 165]. Upon Salmonella infection and subsequent cyclic di-GMP release, STING activation induces Interferon Regulatory Factor 1 responsible for TH17 subspecification in the mucosal immune system [166]. The association between high cyclic di-GMP concentration in the colon and stabilization of STING by cyclic di-GMP-induced ubiquitination in a mouse model of spontaneous colitis indicates an underestimated degree of interkingdom cross talk by this ubiquitous second messenger [167]. These observations in an animal model also put forward that substantial secretion of cyclic di-GMP and other cyclic di-nucleotides can occur, perhaps not only by gastrointestinal bacteria such as E. coli, but also lung pathogens like M. tuberculosis [168, 169].

The Yin and Yang of Biofilm Formation in the Gastrointestinal Tract

The biofilm regulator csgD is expressed in the gastrointestinal tract and can be required for colonization (Fig. 6; [170, 171]). Consequently, curli (and other physiological microbial amyloids) are produced during acute and chronic infections [172–175]. These amyloid PAMPs are recognized by TLR1/TLR2 in combination with the CD14 adaptor [176–178] and intracellular NOD-like receptors [179]. Thereby, recognition of curli by host immune components leads, on the one hand, to the strengthening of the epithelial barrier function and dampens inflammation in the gut [180, 181] and, on the other hand, upon breaching of the intestinal barrier, to autoantibody formation with delayed onset of autoimmunity, inflammation, and functioning as a seed to promote enhanced aggregation leading to neurogenerative diseases [173, 182–185]. The csgD biofilm activator is impaired or absent in the invasive S. Typhimurium clone ST313, equally as in S. Typhimurium [94]. The deficiency to produce curli may contribute to enhanced translocation as demonstrated for non-ST313 curli mutants [181]. Thus, in the luminar space, production of these physiological amyloids prevents their own, other amyloids and bacterial cell translocation to protect the host from systemic disease and overshooting inflammatory processes.

**Conclusion**

Although biofilms are commonly considered as one physiological state in a bacterium, various modes of biofilm formation exist that might have different consequences on microbial host interactions. Thereby, even concomitantly expressed extracellular matrix components can play opposite roles in microbial physiology. The major regulator of biofilm formation, the ubiquitous second messenger cyclic di-GMP, delicately regulates biofilm formation and pathogen-host interactions. Thereby, extracellularly of bacteria, the role of cyclic di-GMP and other cyclic di-nucleotides in a host environment is in stark contrast to the intracellular role of cyclic di-GMP in bacteria. Cyclic di-GMP is an intracellular second messenger signaling molecule in bacteria that promotes biofilm formation, which transforms cells into a low virulence, (relatively) low immunogenic status that is more similar to persistent commensalism than reflecting an acutely virulent pathogen. However, microbial secreted or systemically and mucosally applied cyclic di-GMP, cyclic di-AMP, and other cyclic di-nucleotides either stimulate or also inhibit immune responses. In this way, bacteria have the possibility to distinctively manipulate the immune system response. To what extent this secretion process occurs in bacteria, whether and how it is regulated, and in the case of secretion of cyclic di-nucle-
otides by *E. coli* and *M. tuberculosis*, its precise molecular mechanisms need to be unraveled. Furthermore, the interaction of biofilms with immune cells such as M cells and dendritic cells at the interface between innate and adaptive immune response with cyclic di-nucleotide-producing bacteria has not been thoroughly explored.

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**References**

1. Kouropitsky Y, Gollop R, Belausov E, Pinto R, Sela Saldinger S. *Salmonella enterica* growth conditions influence lettuce leaf internalization. *Front Microbiol*. 2019;10:639.
2. Hamilton S, Bongaerts RJ, Mulholland F, Cochrane B, Porter J, Lucchini S, et al. The transcriptional programme of *Salmonella enterica* serovar Typhimurium reveals a key role for tryptophan metabolism in biofilms. *BMC Genomics*. 2009 Dec 11 [cited 2021 May 12]; 10(1):599.
3. D’Argenio DA, Miller SI. Cyclic di-GMP as a bacterial second messenger. *Microbiology*. 2004 Aug;150(Pt 8):2497–502.
4. Römling U, Galperin MY, Gomelsky M. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev*. 2013 Mar;77(1):1–52.
5. Römling U, Jonas K, Meleiros O, Grancharova N, Lamprokostopoulou A. Hierarchical control of rdat genotype development of *Salmonella enterica* by cyclic di-GMP. In: The second messenger cyclic di-GMP [Internet]. American Society of Microbiology; 2010. p. 137–55. Available from: https://www.asm.sci.org/content/book/10.1128/9781555816667.ch10.
6. Römling U. Cyclic di-GMP signaling in *Salmonella enterica* serovar Typhimurium. In: Chou S-H, Guiliani N, Lee VT, Römling U, editors. Microbial cyclic di-nucleotide signaling [Internet]. Cham: Springer International Publishing; 2020. p. 395–425.
7. Corrigan RM, Gründling A. Cyclic di-AMP: another second messenger enters the fray. *Nat Rev Microbiol*. 2013 Aug;11(8):513–24.
8. Davies BW, Bogard RW, Young TS, Mekalanos J. Coordinated regulation of accessory genetic elements produces cyclic di-nucleotides for *V. cholerae* virulence. *Cell*. 2012 Apr;149(2):358–70.
9. Aravind L, Anantharaman V, Iyer LM. Evolutionary connections between bacterial and eukaryotic signaling systems: a genomic perspective. *Curr Opin Microbiol*. 2003;6(5):490.
10. Blommers MJ, Haasnoot CA, Walters JA, van der Marel GA, van Boom JH, Hilbers CW. Solution structure of the 3’-5’ cyclic di nucleotide d(pApA). A combined NMR, UV melting, and molecular mechanics study. *Biochemistry*. 1988 Nov;27(22):8361–9.
11. Burroughs AM, Zhang D, Schäffer DE, Iyer LM, Aravind L. Comparative genomic analyses reveal a vast, novel network of nucleotide-centric systems in biological conflicts, immunity and signaling. *Nucleic Acids Res*. 2015 Dec;43(22):10633–54.
12. Gao YG, Robinson H, Guan Y, Liaw YC, van Boom JH, van der Marel GA, et al. Molecular structure of two crystal forms of cyclic triacyl-acyl amide at 10 Å resolution. *J Biol Chem*. 1998 Aug;16(1):69–76.
13. Whiteley AT, Eaglesham JB, de Oliveira Mann CC, Morohouse BR, Lowey B, Nieminen EN, et al. Bacterial cGAS-like enzymes synthesize diverse nucleotide signals. *Nature*. 2019 Mar 14 [cited 2021 May 6];567(7747):194–9. Available from: https://www.nature.com/articles/s41586-019-0941-9.
14. Kazlauskiene M, Kostiuik G, Venclovas V. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiology*. 2010 Aug;150(Pt 8):2497–502.
15. Lau RK, Ye Q, Ouyang Z, Troxell B, Xu H, Moh A, et al. Characterization of the cyclic di-GMP signalling pathway in type III CRISPR-Cas systems. *Science*. 2017 Aug;357(6351):605–9.
16. Lau HK, Ye Q, Birkholz EA, Berg KR, Patel L, Mathews IT, et al. Structure and mechanism of a cyclic di-GMP activated bacterial endonuclease mediating bacteriophage immunity. *Mol Cell*. 2020 Feb;77(4):723–35.e6.
17. Morehouse BR, Govande AA, Millman A, Keszei AFA, Lowey B, Ofrim G, et al. STING cyclic di-nucleotide sensing originated in bacteria. *Nature*. 2020 Oct;586(7829):429–33.
18. Christen M, Kulasekara HD, Christen B, Kulasekara BR, Hoffman LR, Miller SI. Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division. *Science*. 2010 Jun;329(5983):1295–7.
19. Martin-Rodriguez AJ, Reyes-Darias JA, Martin-Mora D, Gonzalez JM, Krell T, Römling U. Reduction of alternative electron acceptors drives biofilm formation in *Shewanella alga*. *NPJ Biofilms Microbiomes*. 2021 Jan;7(1):9.
20. Fernandez NL, Huseb BY, Nhu NTQ, Franklin JL, Dufour YS, Waters CM. *Vibrio cholerae* adapts to sessile and motile lifestyles by c-di-GMP regulation of cell shape. *Proc Natl Acad Sci U S A*. 2020 Nov;117(46):29046–54.
21. He M, Ouyang Z, Troxell B, Xu H, Moh A, Piesman J, et al. Cyclic di-GMP is essential for the survival of the lyme disease spirochete in ticks. *PLoS Pathog*. 2011 Jun;7(6):e1002133.
22. Joshi A, Mahmoud SA, Kim SK, Ogahal JL, Lee VT, Chien P, et al. c-di-GMP inhibits Lon-dependent proteolysis of TiOy in *Vibrio cholerae*. *PLoS Genet*. 2020 Jun;16(6):e1008897.
23. Osborne DO, Soo VW, Konieczny I, Wood TK. Polyphosphate, cyclic AMP, guanosine tetraphosphate, and c-di-GMP reduce in vitro *L. monocytes* activity. *Bioengineered*. 2014;5(4):264–8.
24. Römling U. Cyclic di-GMP, an established secondary messenger still speeding up. *Environ Microbiol*. 2012 Aug;14(8):1817–29.
25. Cole SJ, Lee VT. Cyclic Di-GMP signaling contributes to *Pseudomonas aeruginosa*-mediated catheter-associated urinary tract infection. *J Bacteriol*. 2016 Jan;198(1):91–7.

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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1998 Mar;  4(3):  2352–63.

2006 May;  60(3):  602–16.

2006 Oct;  28(11):  3031–40.

2016 Oct;  21(2):  177.

2012 Jun;  37(37):  34568–72.

2018 Aug;  200(15):  e00005-18.

2018 Aug;  200(15):  e00005-18.

2017 Nov;  325(6101):  279–81.

2018 Aug;  200(15):  e00005-18.
Virulence versus Biofilm of *Salmonella Typhimurium*

65 Gibson DL, White AP, Snyder SD, Martin S, Heiss C, Azadzi P, et al. *Salmonella* produces an O-antigen capsule regulated by AgD3 and important for environmental persistence. *J Bacteriol*. 2006 Nov;188(22):7722–30.

66 Latasa C, Roux A, Toledo-Aranar A, Chigo JM, Gamazo C, Penades JR, et al. BapA, a large secreted protein required for biofilm formation and host colonization of *Salmonella enterica* serovar Enteritidis. *Mol Microbiol*. 2005 Dec;58(5):1322–39.

67 Römling U. Characterization of the rdar morphotype, a multicellular behaviour in Entero-
bacteraeae. *Cell Mol Life Sci*. 2005 Jun; 62(11):1234–46.

68 Tan MS, White AP, Rahman S, Dykes GA. Role of fimbriae, flagella and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 to plant cell wall models. *PLoS One*. 2016;11(6):e0158311.

69 Zakikhany K, Harrington CR, Nimtz M, Hinner JM, Beloin C. Multiple cryptic but functional chaperone-usher systems require the GGDEF-EAL domain to mediate biofilm formation on HEp-2 cells and chicken intestinal epithelium. *Catheter-associated urinary tract infec-
tion. Proc Natl Acad Sci U S A*. 2019 Mar;116(13):6353–40.

70 Wolfe AJ, Visick KL. The message out: cy-
clic-Di-GMP regulates multiple levels of flag-
ella-based motility. *J Bacteriol*. 2008 Jan; 190(2):463–75.

71 Wu CM, Poste G. Virulence and environmental adaptation of *Salmonella enterica* serovar Typhimurium. *PLoS One*. 2013 Sep;8(5):e013212.

72 Yue S, Feng JX, et al. Cyclic di-GMP signalling in the cache 1 signaling domain of diguanyl-
ate cyclase STM1987 leads to increased in vivo fitness of invasive *Salmo-
nella enterica* serovar Typhi. *EMBO J*. 2006 Oct;25(20):4292–429.

73 Ahmed I, Lamprokostopoulo A, Le Guyon S, Streek E, Barthel M, Peters V, et al. Complex c-di-GMP signaling networks mediate transition between virulence properties and biofilm formation in *Salmonella enterica* sero-

74 Vila J, Sáez-López E, Johnson JR, Römling U, Dobrindt U, Cantón R, et al. *Escherichia coli*: an old friend with new tidings. *FEBS Microbiol Rev*. 2016 Jul;40(4):437–63.

75 Kolenda R, Ugorski M, Grzymajlo K. Every-
thing you always wanted to know about *Salmo-
nella* biofilm formation on HEp-2 tissue cul-
tures and its curli fimbriae interact with the an-
microbial peptide LL-37. *PLoS Pathog*. 2010 Jul;6(7):e1000110.

76 Römling U. Unphosphorylated CsgD promotes virulence by repress-
ing cellulose production. *Proc Natl Acad Sci U S A*. 2015 Apr;112(16):5183–8.

77 Dörr C, Kalia Y, Musicha P, Han-
son K, Ververidis K, et al. Cyclic-di-GMP regulation promotes survival of a slow-
replcating subpopulation of intracel-
ular *Salmonella Typhimurium*. *Proc Natl Acad Sci U S A*. 2019 Mar;116(13):6353–40.

78 Römling U. Modulation of biofilm formation in *Salmonella enterica* serovar Typhimurium by the periplasmic DsbA/DsbB oxidoreductase system requires the GQDEE-EAL domain protein STM3615. *PLoS One*. 2014;9(8): e106095.

79 MacKenzie KD, Wang Y, Musicha P, Han-
sen K, Palmer MB, Herman DJ, et al. Parallel evolution leading to impaired biofilm for-
mation in invasive *Salmonella* strains. *J Innate Immun*. 2022;14:275–292
DOI: 10.1159/000519573
103 Solano C, Garcia B, Valle J, Berauscin C, Ghigo JM, Gamazo C, et al. Genetic analysis of Salmonella enteritidis biofilm formation: critical role of cellulose. Mol Microbiol. 2002 Feb;43(3):793–808.

104 Zorrozúa V, García B, Latasa C, Echeverz M, Toledo-Aran A, Valle J, et al. Coordination of cyclic-di-GMP repression of Salmonella motility through YcgR and cellulose. J Bacteriol. 2013 Feb;195(3):417–28.

105 Tsolis RM, Young GM, Solnick JV, Bäumler AJ. From bench to bedside: stealth of enteroinvasive pathogens. Nat Rev Microbiol. 2008 Dec;6(12):883–92.

106 Steinberger O, Lapidot Z, Ben-Israël Z, Amikam D. Elevated expression of the CD4 receptor and cell cycle arrest are induced in Jurkat cells by treatment with the novel cyclic dinucleotide 3',5'-cyclic diguanylic acid. FEBS Lett. 1999 Feb;444(1):125–9.

107 Oggunniyi AD, Paton JC, Kirby AC, McCullers JA, Cook J, Hyodo M, et al. c-di-GMP is an effective immunomodulator and vaccine adjutant against pneumococcal infection. Vaccine. 2008 Aug;26(36):4676–85.

108 Martinez-Gil M, Ramos C. Role of cyclic di-GMP in the bacterial virulence and evasion of the plant immunity. Curr Issues Mol Biol. 2018;25:199–221.

109 Cai T, Cang H, Yang B, He ZG. Cyclic dimeric guanosine monophosphate: activation and inhibition of innate immune response. J Innate Immun. 2019;11(3):242–8.

110 Kaoalis DK, Newstead MW, Zeng X, Hyodo M, Hayakawa Y, Bhan U, et al. Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. Infect Immun. 2007 Oct;75(10):4942–50.

111 Fields PJ, Swanson RV, Haidaris CG, Heftron F. Mutations of Salmonella Typhimurium that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A. 1986 Jul;83(14):5189–93.

112 Geddes K, Cruz F, Heftron F. Analysis of cells targeted by Salmonella type III secretion in vivo. PLoS Pathog. 2007 Dec;3(12): e196.

113 Jiang L, Wang P, Song X, Zhang H, Ma S, Wang J, et al. Salmonella Typhimurium rep programs macrophase metabolism via T3SS effector SopE2 to promote intracellular replication and virulence. Nat Commun. 2021 Feb;12(1):879.

114 Pulford CV, Perez-Sepulveda BM, Canals R, Bevington JA, Bengtsson RJ, Wenner N, et al. Stepwise evolution of Salmonella Typhimurium ST313 causing bloodstream infection in Africa. Nat Microbiol. 2021 Mar; 6(3):327–38.

115 Singletary LA, Karlinsky JE, Libby SJ, Mooney JP, Lokken KL, Tsolis RM, et al. Loss of multicellular behavior in epidemic African non-typhoidal Salmonella enterica serovar Typhimurium ST313 strain D23580. MBio. 2016 Mar;7(2):e02265.

116 Petersen E, Miller SI. The cellular microbiology of Salmonella interactions with macrophages. Cell Microbiol. 2019 Nov;21(11):e13116.

117 Ellermeier JR, Slauch JM. Adaptation to the host environment: regulation of the SPI2 type III secretion system in Salmonella enterica serovar Typhimurium. Curr Opin Microbiol. 2007 Feb;10(1):24–9.

118 Hansen-Wester I, Hensel M. Salmonella pathogenicity islands encoding type III secretion systems. Microbes Infect. 2001 Jun; 3(7):549–59.

119 Lostrohp CP, Lee CA. The Salmonella pathogenicity island-1 type III secretion system. Microbes Infect. 2001;3(14–15):1281–91.

120 Desai SK, Winardhi RS, Periasamy S, Dykas MM, Jie Y, Kenney LJ. The horizontally-acquired response regulator SsrB drives a Salmonella lifestyle switch by relieving biofilm silencing. Elife. 2016 Feb;5:e10747.

121 Augustin DK, Song Y, Baek MS, Saya W, Singh G, Taylor B, et al. Presence or absence of lipopolysaccharide O antigens affects type III secretion by Pseudomonas aeruginosa. J Bacteriol. 2007 Mar;189(6):2203–9.

122 Pérez-Gutiérrez C, Llopamt MP, Skurnik M, Bengoechea JA. Expression of the Yersinia enterocolitica pYV-encoded type III secretion system is modulated by lipopolysaccharide O-antigen status. Infect Immun. 2007 Mar;75(3):1512–6.

123 West NP, Sansonetti P, Mounier J, Exley JM, Sansonetti P, Mounier J. From bench to bedside: stealth of enteroinvasive pathogens. Nat Rev Microbiol. 2008 Dec;6(12):1247–53.

124 Miyake M, Zhao L, Ezaki T, Hirose K, Khan MM, Jie Y, Kenney LJ. The horizontally-acquired type III secretion systems. Microbes Infect. 2001 Jun; 3(7):549–59.

125 House D, Bishop A, Parry C, Dougan G, Achenbaum JA, Cook J, Hyodo M, et al. c-di-GMP is an effective immunomodulator and vaccine adjutant against pneumococcal infection. Vaccine. 2008 Aug;26(36):4676–85.

126 Prouty AM, Gunn JS. Comparative analysis of lipopolysaccharide O antigens affects type III secretion system by Pseudomonas aeruginosa. J Bacteriol. 2001 Mar;189(6):2203–9.

127 Yap MN, Yang CH, Barak JD, Jahn CE, Sassone-Corsi P, Chen NJ, Li G. The horizontal acquisition of the SPI-2 type III secretion system. Microbes Infect. 2001;3(14–15):1281–91.

128 Pérez-Gutiérrez C, Llopamt MP, Skurnik M, Bengoechea JA. Expression of the Yersinia enterocolitica pYV-encoded type III secretion system is modulated by lipopolysaccharide O-antigen status. Infect Immun. 2007 Mar;75(3):1512–6.

129 Collazo CM, Galán JE. The invasion-associated type III secretion system of Salmonella enteric serovar Typhimurium directs the translocation of Sip proteins into the host cell. Mol Microbiol. 1997 May;24(4):747–56.

130 Jennings E, Thurston TLM, Holden DW. Salmonella SPI-2 type III secretion system effectors: molecular mechanisms and physiological consequences. Cell Host Microbe. 2017 Aug;22(2):217–31.

131 Walthers D, Carroll RK, Navarre WW, Libby SJ, Fang FC, Kenney LJ. The response regulator SsrB activates expression of diverse Salmonella pathogenicity island 2 promoters and counters silencing by the nucleoid-associated protein H-NS. Mol Microbiol. 2007 Jul;60(2):477–93.

132 Desai SK, Padmanabhan A, Harshes S, Zaidel-Bar R, Kenney LJ. Salmonella biofilms program innate immunity for persistence in Caenorhabditis elegans. Proc Natl Acad Sci U S A. 2019 Jun;116(25):12462–7.

133 Echarren ML, Figueroa NR, Vitor-Horen L, Pucciarelli MG, Garcia-Del Portillo F, Soncini FC. Balance between bacterial extracellular matrix production and intramacrophase proliferation by a Salmonella-specific SPI-2-encoded transcription factor. Mol Microbiol. 2021 Aug;116(4):1022–32.

134 Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol. 2003 Dec;4(12):1247–53.

135 Le Guyon S, Simm R, Rehn M, Römling U. Dissecting the cyclic di-guanosine monophosphate signalling network regulating motility in Salmonella enterica serovar Typhimurium. Environ Microbiol. 2015; 17(11):4818.

136 Vijay-Kumar M, Gewirtz AT. Role of flagellin in Crohn’s disease: emblematic of the progress and enigmas in understanding inflammatory bowel disease. Inflamm Bowel Dis. 2009 May;15(5):789–95.

137 Hjama IA, Dar PA, Shahnawaz I, Jaume JC, Lee JH. Bacterial flagellin—a potent immunomodulatory agent. Exp Mol Med. 2017 Sep; 49(9):e373.

138 Pultz IS, Christen M, Kulasekara HD, Kennard A, Kulasekara B, Miller SL. The response threshold of Salmonella PilZ domain proteins is determined by their binding affinities for c-di-GMP. Mol Microbiol. 2012 Dec;86(6):1424–40.

139 Kumagai Y, Takeuchi O, Akira S. Pathogen recognition by innate receptors. J Infect Chemother. 2008 Apr;14(2):86–92.

140 Colaianni NR, Parys K, Lee HS, Conway JM, Kim NH, Edlbacher N, et al. The complex immune response to flagellin epitope variation in commensal communities. Cell Host Microbe. 2021 Apr;29(4):635–49.e9.

141 Cummings LA, Wilkerson WD, Bergbaken T, Cookson BT. In vivo, flIC expression by Salmonella enterica serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. Mol Microbiol. 2006 Aug;61(3):795–809.

DOI: 10.1159/000519573
Virulence versus Biofilm of Salmonella Typhimurium

2005 Sep; 150 Furter M, Sellin ME, Hansson GC, Hardt KD, Engl C, Waite CJ, McKenna JF, Bennett MH, Pfeilmeier S, Saur IM, Rathjen JP, Zipfel C, Johansson ME, Sjövall H, Hansson GC. The role of Paneth cells and their antimicrobial plant immune response in the mammalian intestinal epithelium. J Innate Immun. 2019;11(3):249–62.

2009 Jun; 191(12): 3928–37. Antelo GT, Vila AJ, Giedroc DP, Capdevila DA. Molecular evolution of transition metal bioavailability at the host-pathogen interface. Trends Microbiol. 2021 May;29(5): 441–57.

2011 Apr; 193(7): 1610–11. Gehlert SR, Le Gros C, Sadowski RB, et al. The EAL-like protein STM1344 regulates virulence phenotype. Mol Microbiol. 2013 Dec;90(6): 1216–32.

2017 Sep; 199(18): e00179-17. Furler M, Sellin ME, Hansson GC, Hardt WD. Mucus architecture and near-surface swimming affect distinct Salmonella Typhimurium infection patterns along the murine intestinal tract. Cell Rep. 2019 May; 27(9):2665–78.e3.

2012; 498(7454): 380–4. Simm R, Ahmad I, Rhen M, Le Gouyon S, Römling U. Regulation of biofilm formation in Salmonella enterica serovar Typhimurium. Future Microbiol. 2014;9(11): 1261–82. Salmonella enterica serovar Typhimurium. Infect Immun. 2008 Mar;76(3): 1048–58.

2012; 43(6): 670–4. Hurley BP, McCormick BA. Translating tissue culture results into animal models: the case of Salmonella Typhimurium. Trends Microbiol. 2003 Dec;11(12): 562–9.

167 Shmuel-Galia L, Humphries P, Lei X, Ceglia S, Wilson R, Jiang Z, et al. Dysbiosis exacerbates colitis by promoting ubiquitination and accumulation of the innate immune adaptor STING in myeloid cells. Immunity. 2021 Jun;54(6): 1137–53.e8.

2015 Sep;6:8330. White AP, Gibson DL, Grassl GA, Kay WW, Finlay BB, Vallance BA, et al. Aggregation via the red, dry, and rough morphotype is not a virulence adaptation in Salmonella enterica serovar Typhimurium. Infect Immun. 2008 Mar;76(3): 1048–58.

2005 Sep; 5(3):01168-14.disposed bacterial flagellin in the innate immune system. Semin Immunopathol. 2007 Sep;29(3): 275–88.

2003 Mar;147(6): 670–4. Hurley BP, McCormick BA. Translating tissue culture results into animal models: the case of Salmonella Typhimurium. Trends Microbiol. 2003 Dec;11(12): 562–9.

2019 Jul; 670: 4–14. Helicobacter pylori binds to human siderocalin and inhibits its antibacterial activity. Nat Commun. 2015 Sep;6:8330.
Oppong GO, Rapsinski GJ, Tursi SA, Biesecker SG, Klein-Szanto AJ, Goulian M, et al. Biofilm-associated bacterial amyloids dampen inflammation in the gut: oral treatment with curli fibres reduces the severity of hapten-induced colitis in mice. NPJ Biofilms Microbiomes. 2015;1:15019.

Oppong GO, Rapsinski GJ, Newman TN, Nishimori JH, Biesecker SG, Tükel Ç. Epithelial cells augment barrier function via activation of the Toll-like receptor 2/phosphatidylinositol 3-kinase pathway upon recognition of Salmonella enterica serovar Typhimurium curli fibrils in the gut. Infect Immun. 2013 Feb;81(2):478–86.

Tursi SA, Lee EY, Medeiros NJ, Lee MH, Nicastro LK, Buttaro B, et al. Bacterial amyloid curli acts as a carrier for DNA to elicit an autoimmune response via TLR2 and TLR9. PLoS Pathog. 2017 Apr;13(4):e1006315.

Gallo PM, Rapsinski GJ, Wilson RP, Oppong GO, Sriram U, Goulian M, et al. Amyloid-DNA composites of bacterial biofilms stimulate autoimmunity. Immunity. 2015 Jun 16;42(6):1171–84.

Sampson TR, Challis C, Jain N, Moiseyenko A, Ladinsky MS, Shastri GG, et al. A gut bacterial amyloid promotes a-synuclein aggregation and motor impairment in mice. Elife. 2020;9:e53111.

Bian Z, Yan ZQ, Hansson GK, Thörén P, Normark S. Activation of inducible nitric oxide synthase/nitric oxide by curli fibers leads to a fall in blood pressure during systemic Escherichia coli infection in mice. J Infect Dis. 2001 Feb;183(4):612–9.

Wang D, Ding X, Rather PN. Indole can act as an extracellular signal in Escherichia coli. J Bacteriol. 2001 Jul;183(14):4210–6.

Elmanfi S, Zhou J, Sintim HO, Könönen E, Gürsoy M, Gürsoy UK. Regulation of gingival epithelial cytokine response by bacterial cyclic dinucleotides. J Oral Microbiol. 2019;11(1):1538927.

Gao J, Tao J, Liang W, Jiang Z. Cyclic (di)nucleotides: the common language shared by microbe and host. Curr Opin Microbiol. 2016 Apr;30:79–87.

Kim CC, Falkow S. Delineation of upstream signaling events in the salmonella pathogenicity island 2 transcriptional activation pathway. J Bacteriol. 2004 Jul;186(14):4694–704.