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REGULATION OF BACTERIAL ADAPTIVE BEHAVIOR BY THE SECOND MESSENGER CYCLIC DI-GMP AND HOST COMPONENTS

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REGULATION OF BACTERIAL ADAPTIVE BEHAVIOR BY THE SECOND MESSENGER CYCLIC DI-GMP AND HOST COMPONENTS
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

To optimize survival and growth, bacteria have evolved adaptive behaviors that respond to relevant environmental signals. A switch from the motile to the sessile lifestyle is probably the most ancient behavioral transition of microorganisms. Gram-negative bacteria such as *Salmonella* species and *Pseudomonas aeruginosa* have a set of extracellular appendages involved in motility and biofilm formation, but also in interaction with the host. These appendages can be regulated by the bacterial second messenger cyclic di-GMP, which allows a millisecond fast response.

The bacterial second messenger c-di-GMP regulates the transition between sessility and motility and between acute and chronic infection. In this work, the signaling pathway involved in motility in *Salmonella enterica* serovar Typhimurium has been investigated in detail. The phosphodiesterase YhjH specifically downregulates motility by interfering with the flagellar functionality. Three diguanylate cyclases inhibit motility in the *yhjH* background and interact specifically with one of two c-di-GMP receptors affecting motility (Paper 1). Also non-canonical EAL domain proteins such as STM1697 unconventionally inhibit motility by post-transcriptionally interfering with the major flagellar regulator $FlhD_4C_2$ (Paper 2), which downregulates flagellin expression as one final outcome (Paper 2 and Paper 3). STM1697 has also an unconventional phenotype compared to EAL phosphodiesterases with respect to biofilm formation and invasion of the colon adenocarcinoma cell line HT-29 (Paper 2, Paper 4) and affects virulence mediated through the $FlhD_4C_2$ interaction (Paper 2). In general, c-di-GMP metabolizing proteins regulate virulence properties of *S.* Typhimurium such as invasion and production of the pro-inflammatory cytokine interleukin 8 by HT-29, but also secretion of the effector protein SipA from the invasion related type three secretion system and colonization of gut and organs in the streptomycin treated mouse (Paper 4). Surprisingly, c-di-GMP signaling inhibits virulence properties through biofilm components such as the major biofilm regulator CsgD and the cellulose synthase BcsA (Paper 4). These studies show that the c-di-GMP signaling network is involved in virulence in *S.* Typhimurium. In the last study, the human surfactant protein C an innate immune component of the lung, did not have an effect on bacterial growth, but affected biofilm formation and swarming motility of *P. aeruginosa* PAO1 (Paper 5).

In conclusion, this thesis sheds light on how the c-di-GMP signaling network and the surfactant protein C regulate the adaptive behavior of *S.* Typhimurium and *P. aeruginosa*, respectively.

LIST OF SCIENTIFIC PAPERS

I. **Le Guyon** S, Simm R, Rhen M, Römling U. Dissecting the c-di-GMP signaling network regulating motility in *Salmonella enterica* serovar Typhimurium. Environ Microbiol. 2014. doi: 10.1111/1462-2920.12580.

II. Ahmad I, Wigren E, **Le Guyon S**, Vekkeli S, Blanka A, El Mouali Y, Anwar N, Chuah ML, Lünsdorf H, Frank R, Rhen M, Liang ZX, Lindqvist Y, Römling U. The EAL-like protein STM1697 regulates virulence phenotypes, motility and biofilm formation in *Salmonella typhimurium*. Mol Microbiol. 2014. 90:1216-32.

III. **Le Guyon S**, Rhen M, Römling U. Rapid preparation of bacterial flagella from *Salmonella enterica* serovar Typhimurium. Bioprotocol.org. Under revision.

IV. Ahmad I, Lamprokostopoulou A, **Le Guyon S**, Streck E, Barthel M, Peters V, Hardt WD, Römling U. Complex c-di-GMP signaling networks mediate transition between virulence properties and biofilm formation in *Salmonella enterica* serovar Typhimurium. PLoS One. 2011. 6:e28351.

V. **Le Guyon S**, Agerberth B, Bergman P, Johansson J, Rising A, Römling U. Role of the surfactant protein C role in growth inhibition, biofilm formation, motility of *Pseudomonas aeruginosa* PAO1. Manuscript.

Publications by the author, which are not included in the thesis:

I. Monteiro C, Papenfort K, Hentrich K, Ahmad I, **Le Guyon S**, Reimann R, Grantcharova N, Römling U. Hfq and Hfq-dependent small RNAs are major contributors to multicellular development in *Salmonella enterica* serovar Typhimurium. RNA Biol. 2012. 9:489-502.

II. Simm R, Ahmad I, Rhen M, **Guyon SL**, Römling U. Regulation of biofilm formation by *Salmonella enterica* serovar Typhimurium. Future Microbiol. 2014. 9:1261-82.

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LIST OF ABBREVIATIONS

AdrA	AgfD regulated protein A
ATM	Adherence test medium
Bcs	Bacterial cellulose synthesis
cAMP	Cyclic adenosine monophosphate
c-di-GMP	3´-5´-cyclic dimeric guanosine monophosphate
CFU	Colony forming unit
Csg	Curli subunit gene
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
IL-8	Interleukin 8
I-site	Inhibitory site
iT3SS	Invasion associated type three secretion system
LB	Lysogenic broth
LPS	Lipopolysaccharide
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
Rdar	Red, dry and rough
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP	Surfactant protein
SPI	*Salmonella* pathogenicity island
SCV	Small colony variant
TLR	Toll like receptor
UDP glucose	Uridine diphosphate glucose

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1 INTRODUCTION

Bacteria express appendages on their cell surface for survival and virulence. These structures are important for motility, biofilm formation and the early stage of infection among other features. During interaction with higher organisms, these appendages can be recognized by the host and induce a defense response. To escape this attack, bacteria may adjust the expression or modify the structure of their surface appendages.

1.1 GRAM-NEGATIVE SURFACE APPENDAGES: STRUCTURE AND FUNCTIONS

Gram-negative or diderm bacteria exhibit different proteinaceous appendages on their cell surfaces that can be classified into at least six major classes based on their biosynthetic pathway[1]. Major proteinaceous surface appendages common to several bacteria are the flagellum, type three secretion injectisome, amyloid fibers such as curli and type IV pili. Non-proteinaceous appendages include lipopolysaccharide (LPS) and exopolysaccharide appendages such as cellulose.

1.1.1 **Flagellum**

The flagellum is an important surface structure involved in motility, biofilm formation and virulence[2]. The number and localization of the flagellum can vary significantly from one organism to another, for example *Pseudomonas aeruginosa* is monotrichous with one polar flagellum, while *Salmonella enterica* serovar Typhimurium is peritrichous with approximately 6 to 10 flagella arranged around the cell [3, 4]. Assembly of this nanostructure is an ATP-driven process, but the rotation is directed by proton motive force[5]. The structure of this appendage can be divided in three parts: a long extracellular filament used as a propeller, a basal body, which spans across the membrane and contains the motor and a hook that connects the basal body with the filament[6] (**Fig. 1**).

Assembly of the flagellum in bacteria such as *Salmonella* species and *Escherichia coli* is hierarchically organized at the transcriptional level with three classes of promoters or transcriptional checkpoints[7]. In *S.* Typhimurium, the class one promoter genes encode the flagellar master regulator $FlhD_4C_2$. The class two promoter genes are transcribed upon activation by the heterodimer $FlhD_4C_2$ and the

RNA polymerase loaded with sigma factor 70. This class of genes encodes the components of the basal body and sigma factor 28 (FliA). The last transcriptional checkpoint is controlled by the anti-sigma factor FlgM that inhibits FliA. Once the hook basal body is completed, a substrate-specificity switch will lead to FlgM export and relieve inhibition of FliA to subsequently activate the transcription of the class three promoter genes[6]. This class of genes includes hook-associated proteins, motor components, filament subunits FliC and FljB and chemotaxis system proteins. In Pseudomonads, the sigma factor 54 acts together with the flagellar transcriptional activator FleQ to activate class two genes[7].

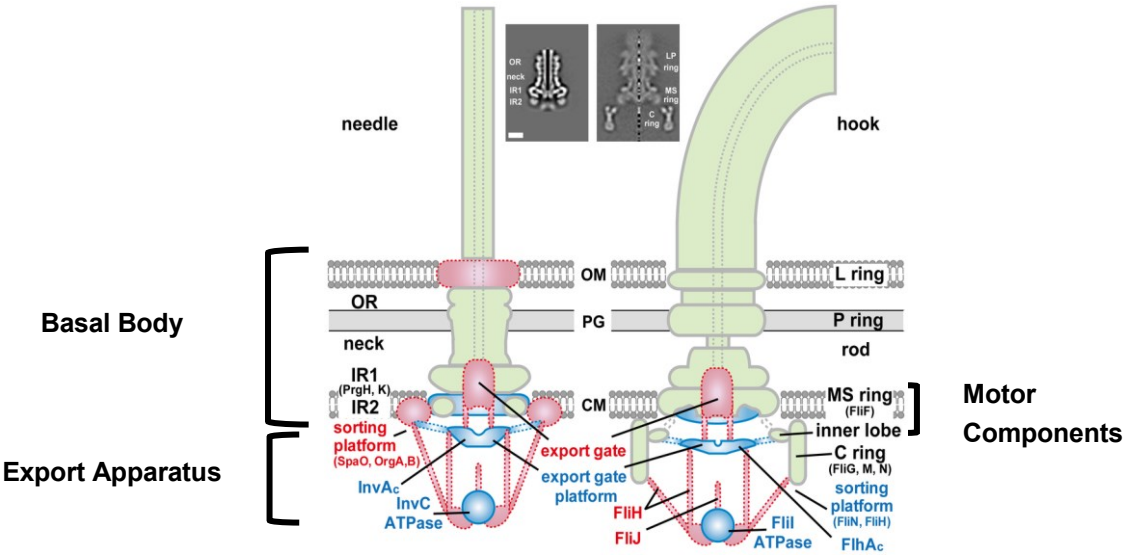

Figure 1: Flagellum and injectisome representation.

The flagellum and injectisome appendages are genetically, structurally and functionally similar. They are close in terms of evolution, their structure can be divided into three parts and the assembly of both appendages is conducted by the type three secretion system. Flagellum and the type three secretion injectisome have membrane-embedded export gates and a cytoplasmic complex containing an ATPase[8]. Both systems possess a molecular ruler to control the length of the flagellar hook or the injectisome needle[9, 10]. In the middle, cryo-electronmicroscopy image of membrane complex. OR: outer membrane ring, IR: inner membrane ring, OM: outer membrane, PG: peptidoglycan layer, CM: cytoplasmic membrane. Adapted from Kawamoto *et al.*, 2013 [11].

1.1.2 **Type three secretion injectisome**

An intact injectisome was first observed in *S*. Typhimurium by electron microscopy and is studied now extensively by cryo-electronmicroscopy[11, 12](**Fig. 1**). This membrane spanning nanostructure is a major virulence factor of the human pathogen *S.* Typhimurium, the plant pathogen *P. syringae* and other bacteria. The main function of the injectisome is to manipulate the host cell by injection of effector proteins whereby the functions depend on bacterial lifestyles and infection modes[13]. This nanostructure can be divided into three parts: a basal body comparable to a channel, an export apparatus with a sorting platform, a needle or a pilus responsible for the translocation of effector proteins into the host cells[14-16].

In *S.* Typhimurium, the invasion associated type three secretion system (iT3SS) is mainly constituted of two pathogenicity islands; *Salmonella* pathogenic island 1 and 2 (SPI-1 and SPI-2)[17, 18]. The iT3SS-1 encoded on SPI-1 is necessary during the early stage of infection while the iT3SS-2 encoded on SPI-2 is required after invasion for bacterial replication and survival in the *Salmonella* containing vacuole in the host cell[19, 20].

The invasion process of *S.* Typhimurium can be divided into four steps: (i) surface scanning and interaction with the mucosal surfaces, (ii) reversible binding mediated by the type 1 fimbriae, (iii) irreversible docking onto the host cell via the injectisome and (iv) manipulation of the host cell to allow invasion[21]. Effector proteins cooperatively mediate entry into the cell, for example SipA (*Salmonella* invasion protein A) encoded on SPI-1 directly binds actin and catalyzes its polymerization. Other iT3SS proteins such as SopE, SopE2 or SopB stimulate Rho family GTPases in the cell[22-24]. In complement, bacteria adjust their motility for surface scanning and use the metabolic products of the gut microbiota upon inflammation as energy sources to ensure successful invasion[21, 25]. As a major route of disease, S*almonella* crosses the epithelial barrier of the gut by invasion of mucosal epithelial cells and induces inflammation after 4-8 hours[24, 26].

1.1.3 **Curli**

Curli are amyloid fibers, 6-12 nm wide, resistant to protease and detergent attack. They are assembled by the type VIII secretion pathway and constitute an important component of the extracellular biofilm matrix[27]. In *Salmonella* species and *E. coli*, curli fibers are commonly coproduced with cellulose resulting in a biofilm, which

displays a distinct morphotype on Congo red agar plates. Biogenesis of curli requires two distinct curli subunit gene (*csg*) operons, *csgBAC* and *csgDEFG*[28]. CsgA is the major subunit protein of the fiber, while CsgB constitutes a minor subunit[29] (**Fig. 2** (**A**)). Export and assembly of CsgA is dependent on CsgG, a pore forming outer membrane lipoprotein and two periplasmic proteins CsgE and CsgF[30, 31]. CsgD is a transcriptional regulator of the FixJ/LuxR family and is required for positive regulation of the curli production[32]. In *P. aeruginosa*, the *fap* operon encodes another class of amyloid fibers involved in biofilm formation[33].

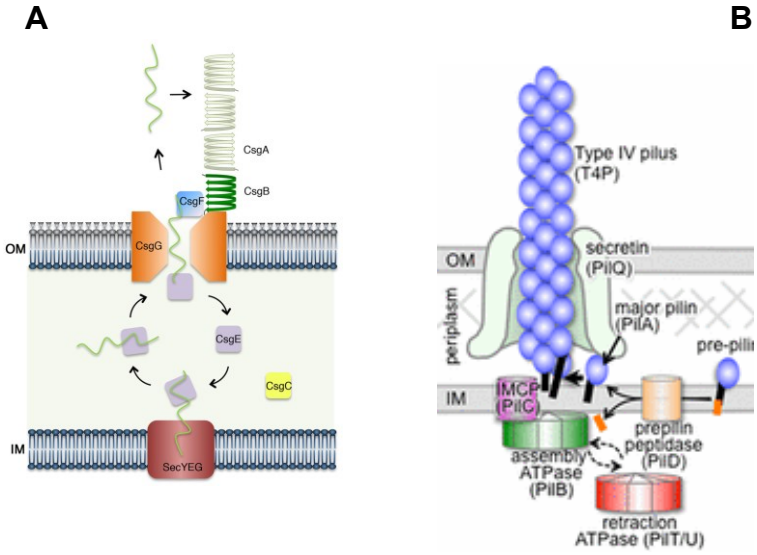

Figure 2: Schematic representation of the curli and type IV pili

(**A**) For curli biogenesis, the soluble major and minor subunits CsgA and CsgB are secreted across the outer membrane through the protein channel CsgG to form amyloid fibers. CsgA and CsgB contain a sec signal sequence that mediates their translocation across the inner membrane, which is cleaved of before passage through the porin forming protein CsgG. (**B**) The secretion system of the type IV pili is homologous of the type II secretion system. IM: inner membrane, OM: outer membrane, IMCP: inner membrane core protein. (**A**) Adapted from Goyal *et al.*, 2014 and (**B**) Melville & Craig, 2013[34, 35].

1.1.4 **Type IV pili**

Type IV pili are cell surface polymers present in Gram-positive and Gram-negative bacteria (**Fig. 2** (**B**)). They are flexible filaments of 1-4 µm length. A major function of type IV pili is twitching motility, associated with biofilm formation and bacterial virulence[36]. The pilus is constituted of the major pilin subunit called PilA and some minor pilins required for assembly or functionality[35, 37]. The secretion of PilA monomers to the cell surface requires the outer membrane secretin PilQ, while

two ATPases, PilB and PilC, are implicated in extension and retraction by assembling and disassembling PilA monomers[38].

1.1.5 **Cellulose and lipopolysaccharide**

Cellulose is the most abundant biopolymer on earth produced by plants, fungi, animals and prokaryotes. In many bacteria including *E. coli* and *S.* Typhimurium, cellulose is one of the major components of the extracellular matrix of biofilm. This exopolysaccharide is composed of straight β-1-4 glucan chains associated by hydrogen bonds. In the model organism *Gluconacetobacter xylinus*, the cellulose macromolecules are crystalline fibrils located on the surface of the cell[39]. Cellulose production is associated with multicellular behavior and its expression can be monitored on Congo agar plate as a pink, dry and rough colony morphology[40-42]. In Enterobacteriaceae, synthesis of cellulose requires the two divergently transcribed bacterial cellulose synthesis (*bcs*) operons *bcsABZC* and *bcsEFG*[28, 43]. The membrane standing complex BcsAB contains the catalytic activity for cellulose synthesis[44] (**Fig. 3** (**A**)). BcsA is a membrane protein with a large cytoplasmic beta-glycosyltransferase 2 domain, which binds UDP-glucose and is responsible for the production of the linear glucan chain; and possess at the C-terminal end a PilZ c-di-GMP binding domain. The glucan chain is thought to be extruded through the outer membrane by the porin BcsC[44, 45].

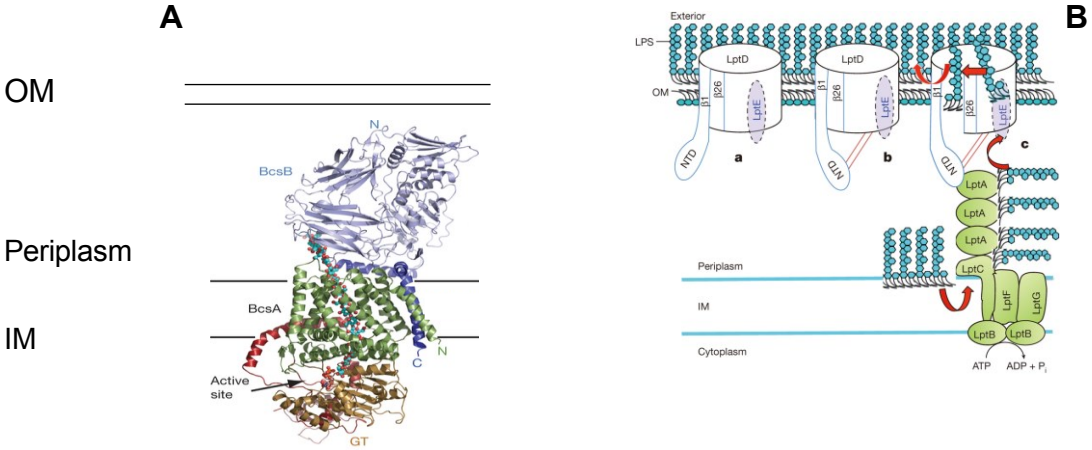

Figure 3: Bacterial cellulose and LPS.

(**A**) BcsA-BcsB is a large inner membrane complex with a large periplasmic and cytosolic part. The catalytic activity of this complex is located in BcsA as indicated by the arrow. The glucose and UDP are represented in blue and red respectively. (**B**) Export of LPS to the outer surface is made by a transmembrane transport complex constituted of seven proteins.

IM: inner membrane. OM: outer membrane. (**A**) Adapted from Morgan *et al.*, 2013 and (**B**) Dong *et al.*, 2013[44, 46].

The lipopolysaccharide (LPS) is an essential component of the outer membrane of Gram-negative bacteria and is constituted of lipid A, core and variable O antigen (**Fig. 3** (**B**)). In *S.* Typhimurium and *P. aeruginosa,* it constitutes an important virulence factor[47].

1.1.6 **Surface appendages as pathogen associated molecular pattern**

Surface appendages are a primary target for the innate and adaptive immune response[48, 49]. Pathogen associated molecular patterns (PAMPs) are conserved microbial molecules not present in the eukaryotic host and thus represent an ideal target to be recognized by the innate immune response. Surface appendages are recognized by pattern recognition receptors (PRRs) in the host. Some of the most studied PRRs belong to the toll like receptor (TLR) family. PAMPs, more correctly designated as MAMPs (microbial- associated molecular pattern), include, for example LPS and curli recognized by TLR-4 and TLR-2, TLR-1 and cluster of differentiation 14 (CD-14) respectively[50, 51].

Bacterial flagellin is a well-characterized PAMP recognized by PRR in mammalian cells and the plant host. In mammals, flagellin is a ligand for cell bound and cytosolic receptors, which include TLR-5 and Nod-like receptors NAIP5 and IPAF[52-54]. Binding of flagellin to these receptors triggers downstream events such as pro-inflammatory response and apoptosis[54].

The flagellin monomer is constituted of four domains: D0, D1, D2 and D3. The conserved domain D1 is recognized by TLR-5 and exposed in monomeric flagellin[55]. The flagellar filament consists of 11 protofilaments that are assembled in a helix like manner (**Fig. 4** (**C**)).

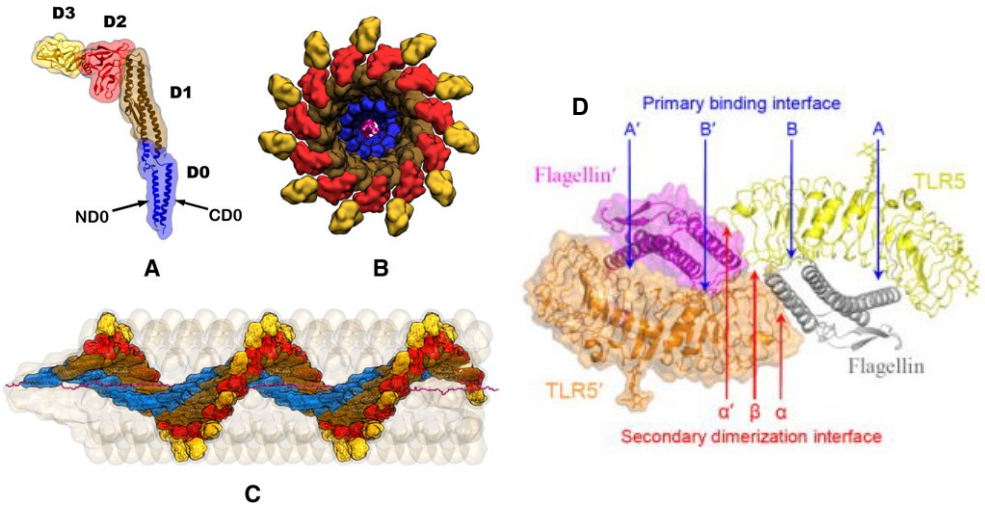

Figure 4: Schematic representation of flagellin domain structure and binding to TLR-5. (**A**) Four domains D0, D1, D2 and D3 compose the flagellin monomer. (**B**) Top view of the 11 proto-filaments. (**C**) Longitudinal view of one protofilament with helical structure. (D) Flagellin and TLR-5 receptor complex. Adapted from Tanner *et al.*, 2011 and Yoon *et al.*, 2012[56, 57].

TLR-5 binding to flagellin can be investigated *in vitro* with the human colon adenocarcinoma cell line HT-29. HT-29 does not express TLR-4, which recognizes LPS. Downstream events of TLR-5 recognition of flagellin such as production of the pro-inflammatory cytokine interleukin 8 (IL-8) or NF-kB activation can be used as biological readout to evaluate the TLR-5-flagellin interaction[58].

1.1.7 **Phase variation and bistable expression of surface appendages**

To avoid recognition by the host or for energy saving, bacteria can regulate the expression of their surface appendages. Two main mechanisms are phase variation and bistable expression. Phase variation is mainly characterized as an ON-OFF system. It occurs in a stochastic manner, but with higher frequency than random mutations. Phase variation is common in Gram-negative bacteria and associated with evasion of the host immunity. Major molecular mechanisms of phase variation are site-specific recombination, gene conversion, epigenetic modification, nested DNA inversion and slipped strand mispairing. For example, the *Salmonella* flagellar switch from flagellin FliC to FljB can be described as an antigenic switch in a phase variation manner (ON-OFF) caused by site-specific inversion of the promoter region of the *fljBA* operon[59, 60] (**Fig. 5**).

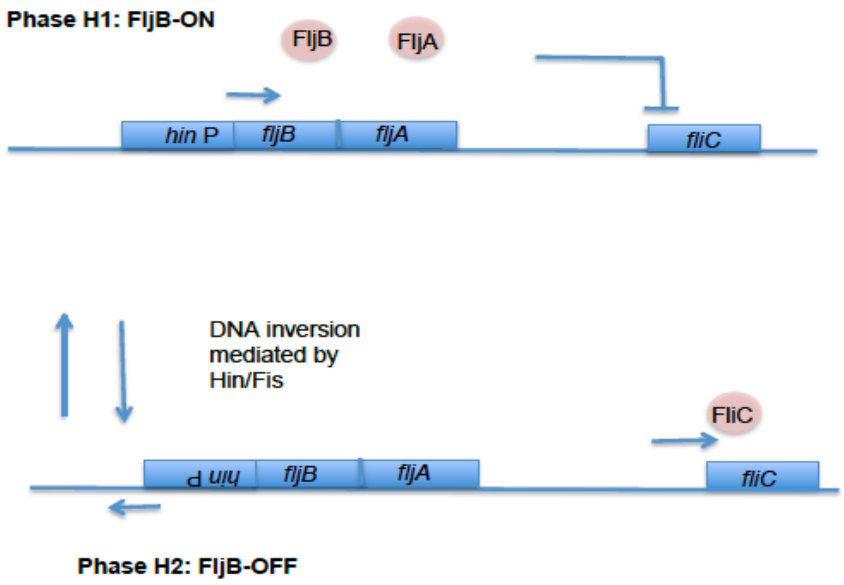

Figure 5: Simplified schema of flagellar phase variation.

During the phase H1, FljB and FljA are expressed. FljA is a transcriptional and post-translational repressor of the phase H2. In the phase 2, the DNA invertase complex Hin/Fis inverts the promoter region of *fljBA* and *fljB* and *fljA* are not transcribed. *fliC* is transcribed as *fljA* transcription is repressed. Adapted from Bonifield and Hughes, 2003 and Hughes et *al*., 1992[4].

Bistability describes coexistence of two distinct populations with an all or nothing expression pattern in the same isogenic cell population[61]. For example, the expression of flagella or the biofilm activator CsgD are bistably expressed in *Salmonella*[62]. Even during the motile phase, not all the cells express flagella. Regulation of this bistability occurs at the post-translational level by interference with the class 1 transcriptional regulator $FlhD_4C_2$. $FlhD_4C_2$ functionality is repressed by the non-canonical EAL domain proteins YdiV and STM1697[63, 64]. Bistable expression is not only restricted to the flagellum system, the injectisome is also subject to bistable expression[65]. One other biological reason for bistability can be found in energy saving for the population. In LB batch culture, it has been observed that bacteria expressing the effector proteins from the SPI-1 locus have a growth disadvantage (retardation) compared to the part of the population not expressing effector proteins[66]. This observation was also made at the single cell level and, with growth retardation correlated with an increase in antibiotic resistance[67].

1.2 BIOFILMS

The multicellular lifestyle, commonly called biofilm formation, is one of the most widespread and ancient adaptive behaviors of microorganisms[68]. Biofilms are defined “as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces”[69]. A biofilm consortium can be composed of different microorganism such as bacteria, fungi or other unicellular organisms such as algae and amoeba. The extracellular matrix is variable depending on the microorganisms’ composition, exerted forces, growth condition, surface, temperature, nutrient availability and other parameters[70]. The extracellular matrix consists of exopolysaccharides, protein, nucleic acids such as eDNA and lipids. Proteinaceous appendages such as flagella, pili and fimbriae contribute to adherence and biofilm matrix stabilization[71]. The biofilm developmental cycle can be dissected in distinct steps; initial and reversible attachment, irreversible binding, formation of microcolonies, development of a biofilm structure and maturation, detachment and dispersion. Surface appendages are essential during almost all steps of biofilm formation[72].

Prior and during the initial attachment, surface structures such as the flagella or pili are important for sensing the surface and to give a ”landing permission”. For example, in *P. aeruginosa*, type IV pili play a complementary role to the Pel polysaccharide biosynthesis locus to slingshot the bacteria on the surface[73].

Irreversible attachment is mediated by formation of extracellular matrix components, for example, exopolysaccharide. The period to switch from reversible to irreversible attachment is highly variable depending on the microorganism[74]. The transition between microcolonies and mature biofilm is mainly driven by metabolic changes and extensive production of extracellular matrix[72]. Detachment of cells from mature biofilm involves specific environmental stimuli, signal transduction pathways and effectors[75, 76].

The composition of the biofilm is highly variable depending on environmental conditions, the microorganisms and the surface colonized. The microorganisms may only constitute 10% of the biofilm, while the extracellular matrix contributes 90% of the mass of the biofilm. In the intestine, the microflora forms a biofilm-like community that promotes physiological functions such as digestion, provides a protection barrier from pathogen invasion of the mucosal epithelium and strengthens the host defense[77]. On the other hand, biofilm formation in enteric pathogens can promote colonization and confer protection from host

immune defenses such as antimicrobial peptides. *S.* Typhimurium is one pathogen that takes advantage of the disruption of the resident intestinal microbiota after antibiotic treatment. This enteric pathogen adheres to the intestinal epithelium with its surface appendages like fimbriae and curli and forms a pathogenic biofilm on biotic surfaces such as gallstones within 14 days[78]. *Salmonella* biofilm formation can be analyzed in the laboratory as pellicle formation at the air-liquid interface in lysogenic broth (LB), a ring in adherence test medium (ATM) or the rdar (red, dry and rough) morphotype on Congo red agar plates[43, 79, 80].

The master regulator of biofilm formation in *S.* Typhimurium is the response regulator CsgD, which constitutes a major hub in the regulation of rdar and pellicle biofilm[81]. The expression of *csgD* in the stationary phase is dependent on RpoS, an alternative sigma factor, and other global regulators[82]. The RNA chaperone Hfq and Hfq dependent sRNAs are involved in *csgD* regulation at the transcriptional and post-transcriptional levels. The carbon storage regulator CsrA, a global RNA-binding protein, also plays an important role in modulation of the biofilm phenotype in *S.* Typhimurium[83].

1.3 THE CYCLIC DI-GMP SIGNALING NETWORK

First discovered in *Gluconacetobacter xylinus* in 1987 as an activator of the cellulose synthase, cyclic-di-GMP (C-di-GMP) has been extensively investigated[84]. In 2004, three research groups independently “rediscovered” c-di-GMP and defined its role as second messenger of bacteria[85-87]. In addition, the biofilm motility switch paradigm was postulated[85] . More recently, c-di-GMP network were dissected in many bacteria and eukaryotic c-di-GMP receptors in mammalians were identified[85]. Since then, the interest for this molecule has been growing, which cumulatively resulted in more than 650 scientific articles about c-di-GMP to date.

1.3.1 **C-di-GMP signaling network architecture**

1.3.1.1 Articulations of the network: Enzymes responsible for c-di-GMP turnover

Usually, a first messenger signal is exponentially amplified by enzymatic production of a second messenger. The intracellular level of the second messenger c-di-GMP is regulated by diguanylate cyclase (c-di-GMP synthesis) and phosphodiesterase (c-di-GMP degradation) activity. Two molecules of guanosine triphosphate (GTP) serve as substrate for c-di-GMP formation with the intermediate product pppGpG. The diguanylate cyclase domain contains a GG(D/E)EF

(Gly-Gly-Asp/Glu-Glu-Phe) motif involved in the enzymatic activity; the condensation of the two GTP. The c-di-GMP synthesis with phosphodiester bond formation requires a GGDEF domain homodimer in antiparallel conformation and the presence of divalent manganese (Mn^{2+}) or magnesium (Mg^{2+}) ions (**Fig. 6**). The third amino acid of the motif, which can be a D (aspartate) or E (glutamate), is involved in catalysis, a mutation of D/E to A (Alanine) abolishes the enzymatic activity[88]. Approximately 60 % of the GG(D/E)EF proteins, like PleD of *Caulobacter crescentus*, contain a product binding site characterized by an RXXD motif (X corresponds to any amino acid) or "I-site" (I for inhibitory) located five amino acids upstream of the GGDEF motif. Upon c-di-GMP binding to the "I-site", allosteric repression or product inhibition occurs which restricts the activity of the diguanylate cyclases[89, 90].

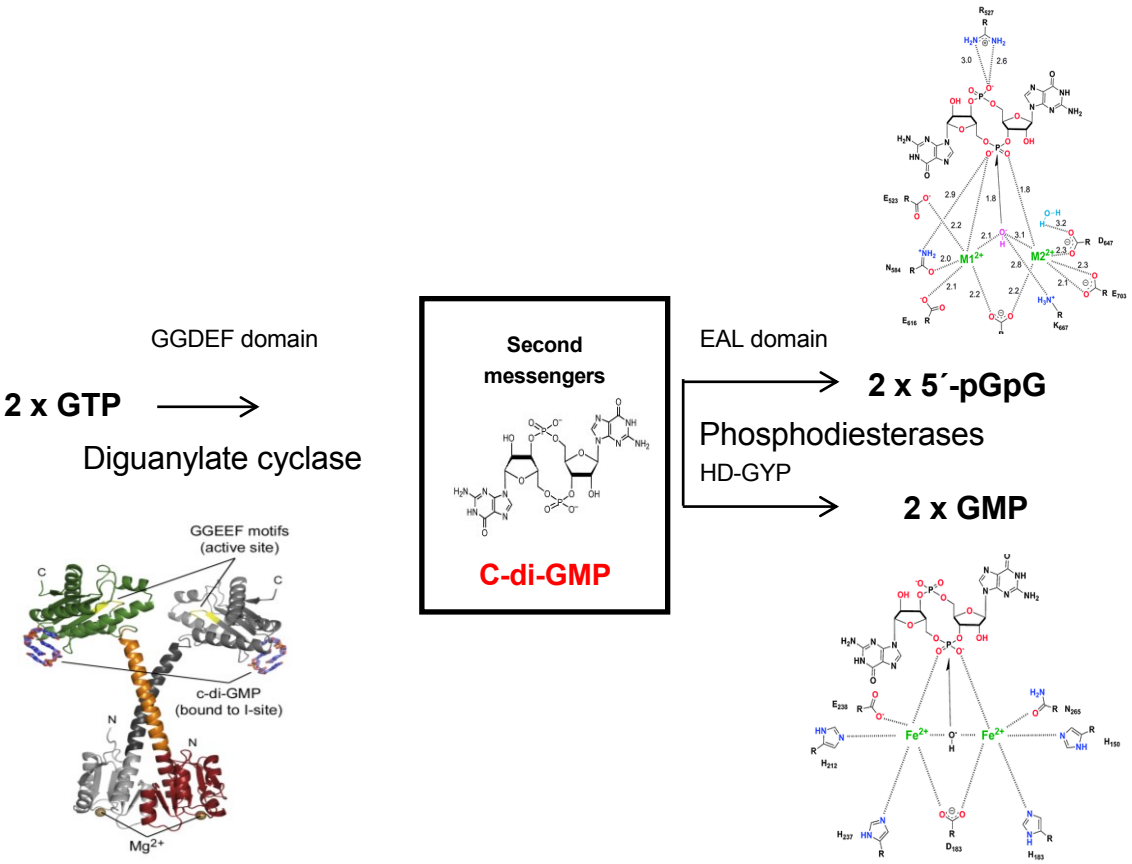

Figure 6: C-di-GMP metabolism and enzymatic components involved.

Synthesis and degradation of c-di-GMP involves diguanylate cyclases and phosphodiesterases. The diguanylate cyclase activity, which performs synthesis of c-di-GMP, contains a GGDEF domain and is represented by WspR from *P. aeruginosa*. The phosphodiesterases performing the degradation of c-di-GMP contain an EAL or HD-GYP domain**.** Adapted from De *et al.*, 2008, Kalia *et al.*, 2013 and Wigren *et al.*,2014[91-93].

Degradation of c-di-GMP is mediated by phosphodiesterases with EAL (Glu-Ala-Leu) or HD-GYP (His-Asp-Gly-Tyr-Pro) domain. Hydrolysis of the c-di-GMP phosphodiester bond leads to the linear di-nucleotide (5´-pGpG) for the EAL and guanosine monophosphate (GMP) for the HD-GYP domain. In the case of the EAL domain, degradation of c-di-GMP requires Mn^{2+} or Mg^{2+} and is inhibited by Ca^{2+} ions. Although a phosphodiesterase activity has been observed for an EAL domain monomer, EAL domains perform optimal catalytic activity as dimers or oligomers[94]. The majority of the EAL domains contain a conserved motif DFG(T/A)GYSS comprising loop 6 implied in maintenance of the secondary structure of the EAL domain and the substrate binding activity[95]. Structural analysis of the phosphodiesterase RocR from *P. aeruginosa* revealed seven amino acids required for Mg^{2+} positioning (**Fig. 6**). The phosphodiester bond breakage is the result of the nucleophilic attack of the resulting OH^- after proton acceptation from a water molecule by the general catalytic base glutamate E in the EGVF motif[96].

The EAL domain proteins can be classified into three classes. The first class contains conserved consensus signature motifs and a conserved loop 6. Class 1 EAL domains are bona fide phosphodiesterases. Member of class 2 EAL domains do not necessarily behave as phosphodiesterases. They have semi-conserved consensus sequences and a degenerated loop 6 such as the phosphodiesterase STM3611 (YhjH) in *S.* Typhimurium. The class 3 EAL domains also contain a degenerated loop 6 and lack residues required for catalysis and c-di-GMP binding activity. Class 3 domain proteins are, for example, STM1697 and YdiV, in *S.* Typhimurium[94].

The other hydrolases that degrade c-di-GMP are the HD-GYP domain proteins. The difference in the degradation product between EAL domains and HD-GYP domains is due to the high efficiency to break down the intermediary product 5´-pGpG by the HD-GYP domain. The first HD-GYP identified was RpfG in *Xanthomonas campestris*[97]. Lately, the crystal structure of the active HD-GYP protein *Pm*-GH revealed the presence of a trinuclear Fe center and an optimal conformation of the catalytic site that allows complete hydrolysis of c-di-GMP into two molecules of GMP[98].

GGDEF-EAL as tandem domains in one protein occurs in 1/3 of the GGDEF domain and 2/3 of the EAL domain proteins. In the tandem proteins, the individual domains can have diguanylate cyclase and phosphodiesterase activity, one of the

enzymatic activities or can be both inactive. GGDEF-HD-GYP tandems exist to a lesser extent[99].

1.3.1.2 Distribution of c-di-GMP metabolizing proteins in the bacterial kingdom

The c-di-GMP metabolizing proteins are present in all the branches of the bacterial kingdom[99]. Within the same phylum or even the same genus, the number and identity of the members of the c-di-GMP signaling network can be different, but is roughly linear to the genome size[100]. Usually, a change to an invasive lifestyle is accompanied by a reduction of the c-di-GMP signaling network components. For example, *Shigella* species have a reduced c-di-GMP network compared to *E. coli* (U. Römling-personal communication). The *S.* Typhimurium signaling network contains 20 proteins, 5 GGDEF domain proteins, 8 EAL domain proteins and 7 GGDEF/EAL domain proteins (**Fig. 7**).

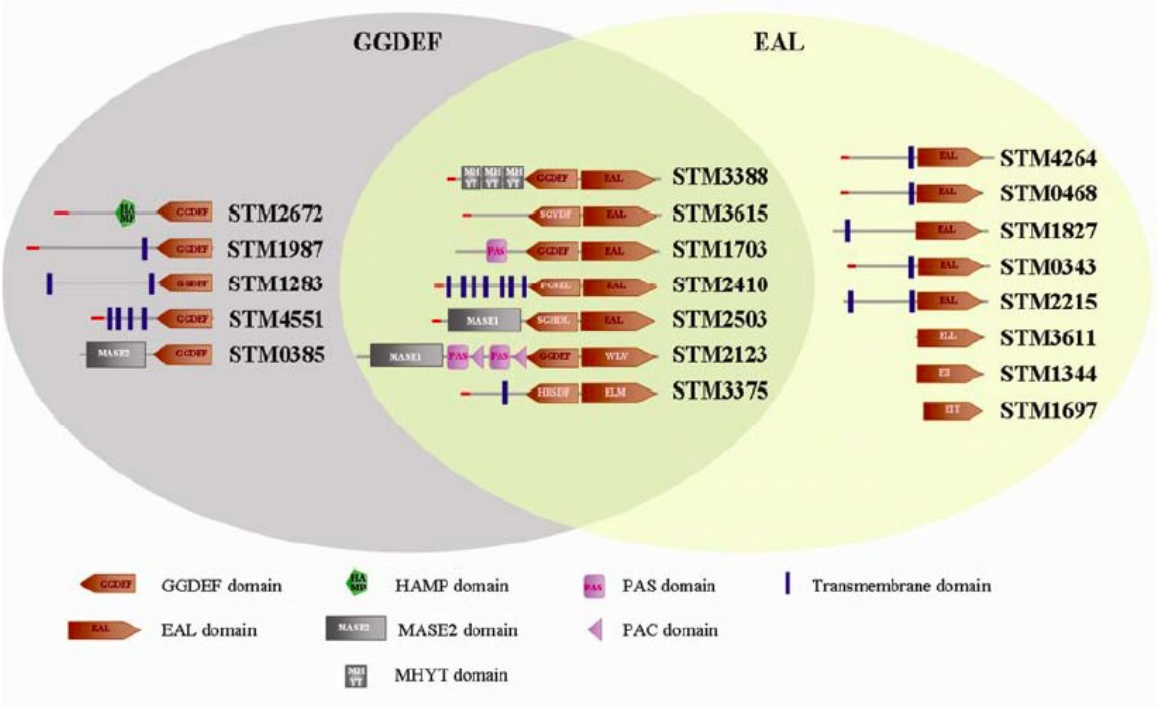

Figure 7: Venn diagram representing the GGGDEF/EAL domain proteins in *S.* Typhimurium. The c-di-GMP signaling network in one bacterial strain usually consists of numerous proteins containing a GGDEF and/or EAL domain. In *S.* Typhimurium as well as in other bacteria, the majority of the c-di-GMP turnover proteins are associated with a N-terminal signaling or sensory domain such as PAS, HAMP, MHYT and PAS-PAC. Sensory domains are more frequently present in GGDEF and GGDEF-EAL domain than in EAL domain proteins[100]. Adapted from Simm, 2007[101].

1.3.1.3 C-di-GMP receptors and effectors as conveying system

The c-di-GMP receptors and effectors transmit the secondary messenger signal to a physiological output (**Fig. 7**). The identification of novel c-di-GMP receptors is essential to understand how c-di-GMP modulates biological functions. C-di-GMP receptors have been identified by bioinformatic prediction, biochemical analysis and structural biology[90, 99, 102, 103].

Regulation of intracellular levels of c-di-GMP by the turnover proteins modulates diverse bacterial behavior and physiology such as biofilm formation, motility, virulence and the cell cycle through the c-di-GMP receptors[99, 103]. Several classes of c-di-GMP receptors have been identified, among them the PilZ domain proteins, degenerated GGDEF and EAL domain proteins, transcription factors and different riboswitches. The c-di-GMP monomer or higher oligomeric structures are usually recognized by short signature motif(s) in the receptors[104]. The c-di-GMP molecule is binding to different motifs due to its high conformational flexibility[104].

A main c-di-GMP receptor family is the PilZ domain protein family. Discovered in 2006, this domain was predicted by bioinformatics analysis and c-di-GMP binding was confirmed by experimentation[105, 106]. The N-terminus of most PilZ domains contains two highly conserved motifs, a (Q/E)RRXXXR and a DZSXXG motif involved in c-di-GMP binding (X corresponds to any amino acid residue and Z to any hydrophobic residue)[97]. Binding of c-di-GMP to PilZ domain proteins can occur with different stoichiometry, affinity and conformation and usually leads to a significant conformational change[103]. Frequently, the same organism contains numerous PilZ domain proteins. *S.* Typhimurium contains two PilZ domain proteins, the flagellar functionality regulator YcgR and the cellulose synthase BcsA. C-di-GMP binds with the same stoichiometry to these two PilZ domain proteins, but with highly different affinities, 43 fold higher for YcgR than BcsA[107]. Another c-di-GMP receptor family, is part of the larger cAMP receptor protein family. The first identified protein to bind c-di-GMP was the transcriptional regulator Clp in *Xanthomonas campestris*. Mutation in the c-di-NMP (N stand for nucleotide) binding site of this protein abolished the ability of Clp to bind c-di-GMP and to target promoters regulating virulence factor genes[108].

The degenerated GGDEF domains harboring the “I-site” can still act as receptors, and upon c-di-GMP binding, be involved in regulation of cellular processes. For example, the membrane protein PelD of *P. aeruginosa* regulates exopolysaccharide synthesis in response to c-di-GMP binding to the RXXD

domain[109]. The response regulator PopA regulates in a similar way cell cycle progression in *C. crescentus*. In *S.* Typhimurium, the GIL domain protein BcsE is required for maximal cellulose production and binds c-di-GMP by the RXGD motif[110]. Also degenerated EAL domain proteins have been identified as potential c-di-GMP receptors. The inactive GGDEF-EAL domain protein LapD from *Pseudomonas fluorescens* regulates cell association of the adhesin LapA by scavenging the periplasmic protease LapG upon c-di-GMP binding[111]. In *P. aeruginosa,* the flagellar transcriptional regulator FleQ controls exopolysaccharide production by repressing the *pel* promoter in the absence of c-di-GMP, while relieving repression in the presence of c-di-GMP[112].

Last but not least, c-di-GMP has been identified as a small molecule binding to the 5´ UTR (untranslated region) of the mRNA to alter gene expression, a so-called riboswitch. Thereby, c-di-GMP regulates diverse cellular functions such as flagella based motility, virulence gene expression and pilus formation[113]. So far, two different classes of riboswitches have been identified that bind c-di-GMP[114, 115].

1.3.1.4 Stimuli and sensory domains

C-di-GMP metabolizing proteins are usually linked to a signaling, input or sensory domain, which is sensing the first messenger. Sensory domains frequently associated with c-di-GMP turnover proteins are PAS, GAF-PHY, hemerythrin and the REC domain. These domains are mostly located at the N-terminal part of the c-di-GMP metabolizing protein[99]. These domains sense, for example, O_2, NO, light, quorum sensing or are regulated by phosphotransfer[116].

1.3.1.5 Non-canonical EAL domain proteins

The non-canonical EAL domain proteins belong to the class 3 EAL domain proteins. These proteins are highly homologous to EAL domains, but lack the determinative consensus amino acids signature and consequently do not have the enzymatic activity or even do not bind c-di-GMP[94]. These proteins are usually involved in protein-protein interactions and are often still part of the c-di-GMP signaling network. One of the well-studied non-canonical EAL proteins is YdiV (STM1344) of *S.* Typhimurium and *E. coli*. The main function of YdiV is to bind the major activator of the flagellar regulon $FlhD_4C_2$ and repress its binding to the promoter DNA. YdiV also acts as a putative adaptor to target $FlhD_4C_2$ for ClpXP-dependent proteolysis[117]. Another example of a non-canonical EAL domain protein is STM1697,

present only in *S.* Typhimurium, which regulates biofilm formation, motility and invasion, also by interference with $FlhD_4C_2$ functionality[64].

1.3.2 **Regulation of cellular function by c-di-GMP**

C-di-GMP controls a wide range of physiological functions spanning from photosynthesis to virulence in a wide variety of bacteria from almost every branch of the phylogenetic tree. The c-di-GMP signaling pathway (**Fig. 8**) occurs in environmental as well as pathogenic bacteria.

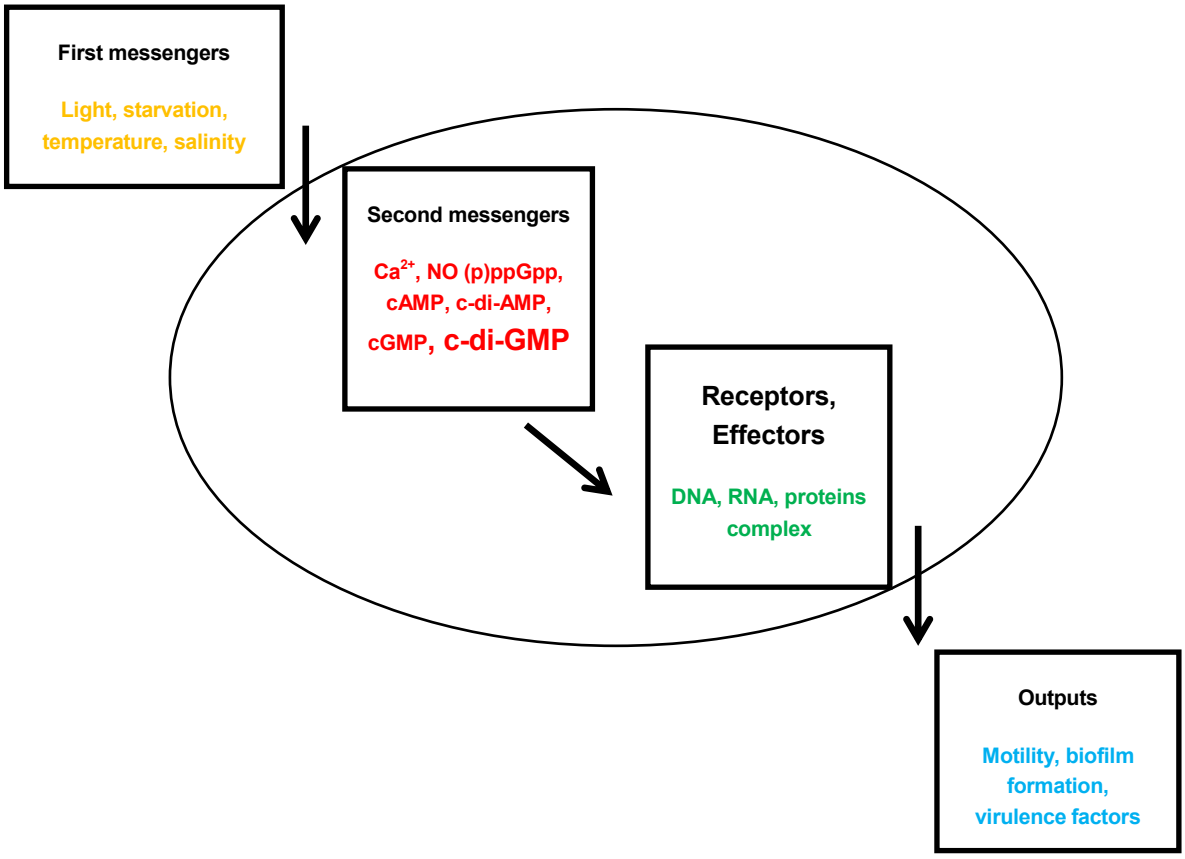

Figure 8: First messengers, second messengers, effectors, outputs.

Second messengers such as c-di-GMP in bacteria are regulated in response to environmental and intracellular stimuli (first messengers) and bind receptors and effectors in the cell. These receptors and effectors will induce specific outputs. Adapted from Agostini *et al.*, 2014[118].

1.3.2.1 Regulation of biofilm formation by c-di-GMP

C-di-GMP is regulating directly or indirectly each step of the biofilm cycle[99]. During initial attachment, c-di-GMP regulates, among other physiological events, the

formation of extracellular appendages. The GGDEF-EAL domain protein LapD from *P. fluorescens* mediates bacterial attachment through the maintenance of the adhesin LapA on the cell surface[119]. Biogenesis of the type IV pili required for surface scanning before microcolony formation is stimulated by c-di-GMP in *P. aeruginosa* and other bacteria[73]. C-di-GMP modulates the production of the extracellular matrix that converts the microcolonies to mature biofilm structures[120, 121]. For example, c-di-GMP stimulates the production of the matrix component, the exopolysaccharide poly-β-1, 6-*N*-acetylglucosamine. Synthesis of this extracellular matrix component occurs through c-di-GMP binding to the two inner membrane proteins PgaC and PgaD[122]. Another important component of biofilm regulated by c-di-GMP is the eDNA that promotes construction of an intricate network of furrows[123, 124]. Dispersal of the biofilm is regulated by the concomitant action of phosphodiesterases and non-toxic level of exogenous nitric oxide in *P. aeruginosa* and other bacteria[125].

In *S.* Typhimurium, CsgD is the major hub for positive regulation of biofilm formation and production of extracellular matrix components. This transcriptional factor of the LuxR family activates curli fimbriae expression and the transcription of *adrA* (AgfD regulated protein A) encoding, a diguanylate cyclase involved in cellulose biosynthesis. The expression of CsgD is regulated by at least six GGDEF/EAL domain proteins. CsgD is expressed in a bimodal way in the biofilm, but high concentration of c-di-GMP abolishes in the cell population the variability[62, 126, 127]. In ATM medium, the GGDEF domain protein STM1987 is involved in cellulose production[128]. To activate cellulose biogenesis, c-di-GMP binds to the C-terminal PilZ domain of the cellulose synthase BcsA. This binding activates the BcsA-BcsB complex by breaking a salt bridge that releases the complex from an auto inhibited state and allows access to substrate and substrate coordination at the active site (**Fig. 3**)[129].

1.3.2.2 Regulation of motility by c-di-GMP

Depending on the bacterial species, the c-di-GMP may be involved in regulation of the flagellum at the transcriptional, post-transcriptional, post-translational and functional level[7]. In *S.* Typhimurium, c-di-GMP regulates the flagellum, motor speed and rotation at the functional level. C-di-GMP binds to the PilZ domain protein YcgR. YcgR interacts with the motor component FliG and induces a bias in flagella rotation (**Fig. 1**)[130, 131]. The other PilZ domain protein, BcsA, is also involved in motility

regulation. High concentration of c-di-GMP will promote the cellulose synthase that provokes an accumulation of cellulose around the cell and hampers flagella rotation[132]. At the transcriptional level, the RNA binding protein CsrA positively regulates $FlhD_4C_2$ by increasing its binding activity and preventing the repression of the heterodimer by the two non-canonical EAL domain proteins YdiV and STM1697[64, 133].

In addition to regulating the transition between motility and biofilm formation, c-di-GMP regulates the cell polarity and morphology of *C. crescentus* and couples this events with DNA replication and cell division[134].

1.3.2.3 C-di-GMP regulation of virulence

Different virulence phenotypes can be affected by c-di-GMP in human, animal and plant pathogens. For example, c-di-GMP signaling regulates adherence to and invasion of the host cells, intracellular infection and modulation of the immune response[99, 135]. In *S.* Typhimurium, high concentrations of c-di-GMP affect secretion of monomeric flagellin, bacterial invasion of an epithelial cell line and secretion of IL-8[136]. During infection by *V. cholerae*, the bile present in the lumen of mice and humans causes an increase in c-di-GMP concentration, while after entry into the mucosal layer, pH augmentation might decrease the concentration of c-di-GMP[137]. Furthermore, low levels of c-di-GMP through the action of the phosphodiesterase VieA subsequently activate cholera toxin production[86]. In *P. aeruginosa*, the clinical phenotype “small colony variant” (SCV) is associated with a high level of c-di-GMP. The YfiBNR operon can regulate the SCV phenotype. Mutant of *yfiR* encoding a negative regulator of the diguanylate cyclase YfiN exhibited a strong resistance to phagocytosis by macrophages[138].

Recently, one activator of the type 1 interferon production, STING (STimulator of INterferon Gene) has been identified as one of the specific cytosolic receptors of c-di-GMP[139]. Several articles have highlighted the immunostimulatory and immunomodulatory properties of c-di-GMP and its possible utilization as an adjuvant for the development of mucosal vaccines. The second messenger has been shown to have a variety of immunomodulatory effects, among others to stimulate dendritic cells and to produce several key cytokines[140]. *In vivo*, pretreatment of mice with c-di-GMP induces a protective effect against mucosal bacterial challenges[140-142]. The adjuvant properties of c-di-GMP have already been used in the development of a live attenuated vaccine for influenza H5N1 virus[143]. Furthermore, c-di-GMP has

been shown to inhibit the proliferation of colonic cancer cells *in vitro* and to improve vaccination against metastatic breast cancer[144, 145].

1.4 HOST COMPONENTS REGULATING BACTERIAL BEHAVIOR

1.4.1 Interactions between host components and biofilm bacteria

Microbial biofilm in the host is a bacterial virulence and defense mechanism leading to chronic infection. Formation of biofilm confers protection to bacterial communities against the hostile environment by limiting penetration of leukocytes into the biofilms, decrease of the macrophage phagocytic capacity and increased tolerance to antibiotics[146]. However, certain components of the innate immune defense such as the antimicrobial peptide LL-37 expressed by neutrophils and epithelial cells are active against biofilm formation in a wide range of bacteria, for example, LL-37 inhibits adherence of bacteria, dissolves mature biofilms and downregulates cell-to-cell communication in *P. aeruginosa*, *E. coli* and *Staphylococcus epidermidis* biofilms[147-149]. A prototype of a biofilm infection in humans is infection of the lung of cystic fibrosis patients by *P. aeruginosa*[150].

The lung is a zone of gas exchange between the environment and host. This organ is composed of respiratory bronchioles, alveolar ducts and alveolar sacs. In the lung, protective immune responses spans from cilial movement to alveolar macrophages and defensins[150]. The surfactant proteins (SPs) are present at the air/surface interface. Surfactants prevent the lung to collapse at expiration. The functions of the surfactant proteins include protection of the host from microbial contamination challenge by different mechanism as listed in **Table 1**.

Name	Property	Family	Interaction with bacterial surface appendages
SP-A	Hydrophilic	Collectin	Flagellin[151], adhesin[152] LPS[153]
SP-B	Hydrophobic	Saposin	LPS[154]
SP-C	Hydrophobic	-	LPS[155]
SP-D	Hydrophilic	Collectin	Flagellin[156], adhesin[157], LPS[158]

Table 1: Surfactant proteins present in the human lung.

Collectins are collagenous carbohydrate-binding proteins. This family is directly involved in innate immunity by their ability to bind pathogens as diverse as viruses, bacteria and fungi. They counteract pathogen invasion in the lung by promoting aggregation of pathogens, stimulation of phagocytosis and modulation of the inflammatory response. The saposins have well conserved structural elements and SP-B is expected to have a similar structure as the antimicrobial peptide NK-lysin. Adhesins, which interacts with the surfactant protein A include Eap in *Staphylococcus aureus* and FimH of uropathogenic *E. coli.* Adapted from Almlén, 2010[159].

1.4.2 **Role of surfactant protein C**

The surfactant protein C (SP-C) is a hydrophobic membrane standing polypeptide. In the lung, the type two alveolar cells express the pro-SP-C gene located on the chromosome 8[160]. A deficiency in SP-C can conduct to an acute respiratory failure and interstitial lung disease, but can be treated with artificial surfactant replacement[161]. SP-C can interact with the bacterial components like the LPS, nevertheless its role in lung immunity remains unclear[162, 163].

2 AIMS OF THE THESIS

The overall aim of this thesis was to understand how a secondary signaling molecule and a component of the host innate immune system regulate bacterial behavior.

The specific aims of this thesis were:

(i) To dissect the c-di-GMP signaling network regulating motility in *S.* Typhimurium

- Determination of the c-di-GMP synthesizing proteins contributing to motility inhibition (Paper 1).
- Determination of c-di-GMP receptors corresponding to c-di-GMP synthesizing proteins (Paper 1).
- Determination of the molecular mechanism by which the non-canonical protein STM1697 regulates motility, virulence properties and biofilm formation (Paper 2).

(ii) To dissect how c-di-GMP signaling regulates virulence properties of *S.* Typhimurium

- Characterization of the c-di-GMP signaling network involved in invasion of *S.* Typhimurium into the host cell line HT-29 (Paper 2 and 4).
- Evaluation of the c-di-GMP signaling network relevant in invasion, which regulates secretion of the effector protein SipA (Papers 2 and 4).
- Identification of the c-di-GMP signaling network, which regulates the secretion of IL-8 (Paper 4).

(iii) To identify surfactant protein C (SP-C) regulated bacterial virulence related phenotypes in *P. aeruginosa*

- Determination of the minimum inhibitory concentration of SP-C on bacterial growth (Paper 5).
- Analyze the effect of SP-C on biofilm formation and motility (Paper 5).

3 MATERIAL AND METHODS

The general principles of major techniques used in this thesis are described. A more broad description of the protocols that were used can be found in the *Materials & Methods* section of the respective papers.

3.1 BACTERIAL STRAINS

3.1.1 *Salmonella enterica* serovar Typhimurium

S. Typhimurium is a Gram-negative bacterium belonging to the family of Enterobacteriaceae. This bacterium is one of the most important food-borne pathogens and follows an oral-fecal route of transmission in humans. From 12 to 36 hours after consumption of contaminated food, symptoms of Salmonellosis such as abdominal cramps, fever, and diarrhea are appearing. In healthy humans, recovery from symptoms occurs without medication, but total intestinal recovery can take several months[164]. *S.* Typhimurium causes a systemic infection in *Salmonella* susceptible mice similar to *S.* Typhi in humans, which constitutes a genetically traceable model to study typhoid fever. Another relevant model is infection of streptomycin treated mice, which mimics human gastroenteritis[26]. *S.* Typhimurium can be conveniently grown and genetically manipulated. Two of the most common genetic engineering tools in S. Typhimurium are λ red recombination and phage transduction[165, 166]. The λ red recombination allows gene replacement with an antibiotic cassette by efficient homologous recombination requiring only 40 base pairs of homology. Phage transduction is based on the ability of the prophage P22 HT105/1 *int-201* to pack and transfer genomic DNA from one bacterial strain to another. A selectable marker allows selection of mutated gene alleles. The bacterial strain used in these studies as the wild type strain is *S.* Typhimurium UMR1, ATCC14028-1s-Nal^r, a single colony nalidixic acid resistant isolate, expressing the rdar morphotype at 28°C[40].

3.1.2 *Pseudomonas aeruginosa* PAO1

P. aeruginosa is an ubiquitous Gram-negative bacterium belonging to the Gammaproteobacteria. *P. aeruginosa* is one of the most frequent nosocomial pathogens mainly due to its ability to form biofilms[167]. *P. aeruginosa* PAO1 is one genetic reference strains isolated from a wound infection in Melbourne, Australia, in

1954 and the background strain used in this study. This is a reference strain for genetic and phenotypic analysis of *P. aeruginosa*[168].

3.2 BACTERIAL VIABILITY ASSAY

In clinical routine, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of an antimicrobial drug is regulated by established protocols. Clinical breakpoints are published by the European Committee on Antimicrobial Susceptibility Testing[169]. To evaluate the bactericidal or bacteriostatic activity of a compound such as the surfactant protein C, bacteria were inocculated in liquid medium containing different concentrations of the antimicrobial agent. The test conditions used were similar to conditions used for evaluation of cationic antimicrobial peptides and followed EUCAST recommendations[170].

3.3 PHENOTYPIC ANALYSIS

Major bacterial behavior relevant to virulence, motility and biofilm formation was investigated in this work. Assays were used in the context of gene deletion and complementation in *S.* Typhimurium and to assess the effect of SP-C on physiology of *P. aeruginosa*.

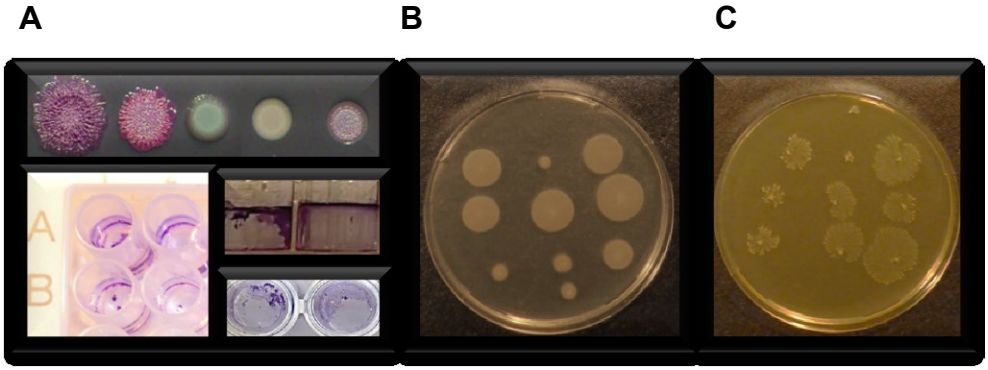

Figure 9: Example of phenotypic assays used in this thesis.

(**A**) Different biofilm models used. Colony morphology on Congo red agar plates, biofilm formed on polypropylene or polystyrene support[171]. Flagella based (**B**) swimming and (**C**) swarming motility.

3.3.1 **Biofilm assays**

3.3.1.1 Rdar morphotype biofilm

Bacterial spotting on Congo red plate is a convenient tool to characterize genotype-biofilm phenotype relation[42]. Observation of colony morphology and color of the spot after 48 h of incubation at 28°C can give information at the level of biofilm

formation and composition of the extracellular matrix. The reference strain *S.* Typhimurium UMR1 displays a wild type red, dry and rough colony phenotype[53]. The color development is dependent on the composition of the extracellular biofilm matrix where cellulose is producing a pink colony and curli are producing a brown colony[34] (**Fig. 9**). This analysis can be complemented by western blot analysis assessing expression of the major biofilm regulator CsgD. The level of expression of CsgD is directly correlated with rdar morphotype development[42, 171].

3.3.1.2 Biofilm formation on abiotic surfaces

Adherence and biofilm formation to an abiotic surface under steady state conditions allows monitoring of biofilm development over time. This frequently used assay is performed in 96 well microtiter dishes for genetic screening or to assess the effect of various growth media on biofilm formation. Staining of bacterial cells with crystal violet allows visualization of microbial adherence and the pattern distribution of biofilm formation at different locations in the well. Dissolution of the dye allows quantification of adherence by measuring the optical density at 590 nm[172].

3.3.2 **Motility assays**

Two distinct flagella-mediated motility behaviors can be observed in *S.* Typhimurium and *P. aeruginosa* PAO1, swimming and swarming motility.

3.3.2.1 Swimming assay

Macroscopic observation of swimming motility occurs in an agar of low concentration (0.2-0.3%). Any medium can be used to assess swimming motility with a low concentration agar.

3.3.2.2 Swarming assay

Macroscopic observation of swarming motility requires a specific medium and higher agar concentration (0.4-0.8%). For *S.* Typhimurium, cells are inoculated in the agar plate. The concentration of the agar > 0.4% will promote surface motility. For *P. aeruginosa* PAO1, bacteria are inoculated as a spot in the middle of the plate and radially spread from the inoculum site[173].

These two analyses can be complemented with evaluation of flagellin expression.

3.3.3 **Virulence associated phenotypes**

Bacterial crossing of the epithelial lining is a key virulence event in the gastrointestinal disease caused by *S.* Typhimurium. Invasion of epithelial cells requires different effector proteins belonging to the iT3SS-1. To characterize how gene products associated with biofilm phenotypes affect virulence and to identify of the c-di-GMP signaling network components, which contribute to virulence regulation, we used several *in vitro* assays.

We utilized the human colon adenocarcinoma HT-29 cell line to evaluate the ability of different *S.* Typhimurium mutants to invade eukaryotic cells. Prior to invasion, the bacteria were grown in hyperosmotic medium and standing culture (microaerophilic conditions) to mimic the gastro-intestinal tract conditions. The infection was performed by co-incubation of cells with bacteria for 1 h. Bacteria were adjusted to 10^7 colony forming units.ml^{-1} (CFU) and 90 % confluent epithelial cells were used. The length of infection has been chosen in order to observe the early stage of invasion.

3.3.3.1 Invasion assay

One hour post-infection, the cells are treated with gentamycin to kill non-invading bacteria, gently washed and resuspended with trypsin to allow determination of intracellular bacteria by CFU counts. The purpose of this experiment was to analyze the relation between specific c-di-GMP genotypes and invasion phenotypes.

3.3.3.2 Interleukin 8 quantification

The pro-inflammatory response induced by TLR-5 activation in the HT-29 cell line is evaluated one hour post-infection by measuring the secretion of IL-8 into the supernatant. Measurement of IL-8 was performed by enzyme linked immune sorbent assay (ELISA).

3.3.3.3 SipA expression

The expression of the effector protein SipA was evaluated by western blot analysis. As SipA is one of the first proteins secreted by iT3SS-1, we choose it as a representative protein. The supernatant of the bacterial culture was analyzed after repeated centrifugation of the bacterial culture and precipitation of the secreted proteins with trichloroacetic acid (TCA). Western blot analysis was performed to

quantify the expression of β-lactamase of a chromosomally encoded SipA-β-lactamase fusion[174].

3.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

C-di-GMP detection and quantification was performed by HPLC. The assay allows quantitative evaluation of c-di-GMP metabolizing proteins under invasion conditions. For the sample preparation, mid-logarithmic bacterial culture was inactivated by formaldehyde and lysed by heating to 95°C[175, 176]. The nucleotides were extracted with ethanol. The bacterial extract was dissolved in HPLC grade water and passed through a Supelcosil LC-18-T column.

4 RESULTS

4.1 PAPER 1: DISSECTING THE C-DI-GMP SIGNALING NETWORK REGULATING MOTILITY IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM.

Aim: to identify and correlate the components of the c-di-GMP signaling network involved in bacterial motility regulation.

Besides biofilm formation, motility is the major target of regulation by c-di-GMP signaling[7]. In *S.* Typhimurium and other bacteria, high concentration of c-di-GMP inhibits motility, while low concentration promotes movement[99, 126]. In *S.* Typhimurium and *E. coli*, various studies have demonstrated that the phosphodiesterase YhjH positively affects swimming and swarming motility[85, 126, 177]. Deletion of this phosphodiesterase elevates the concentration of c-di-GMP and, in coordination with the PilZ domain protein YcgR, downregulates motility[130, 131, 178]. Also cellulose production inhibits motility by mechanically inhibiting flagella rotation[132]. The transcriptional regulator CsgD plays a central role in adhesion and biofilm regulation, but its role in motility has not been addressed in *S.* Typhimurium[179].

In this work, we confirmed that promotion of motility by YhjH is due to regulation by c-di-GMP, while *yhjH* does not affect the expression level of flagellin proteins FliC and FljB. By observation of the motility behavior on swimming and swarming agar plates at 28°C and 37°C, we identified STM2672, STM4551 and STM1987 as key diguanylate cyclases working in concert with YhjH in motility regulation (**Fig. 10**). Subsequent deletion of all these diguanylate cyclases in the *yhjH* mutant restored motility to the wild type level. In addition, deletion of STM2123 restored motility in the triple mutant background Δ*yhjH*Δ*1987*Δ*4551*. These results indicate that the genetic background can be a determinative factor to define the role of diguanylate cyclases. Subsequently, we investigated the partnership between each of the key diguanylate cyclases and motility associated PilZ domain proteins YcgR or BcsA. We could clearly identify an association between STM2672 and YcgR, STM1987 and BcsA, whereas STM4551 is associated with both receptors.

As previously shown for swimming motility, deletion of the two PilZ domain proteins in the Δ*yhjH* background gradually restored swarming to the wild type level. Elevated concentration of c-di-GMP in the triple mutant Δ*yhjH*Δ*ycgR*Δ*bcsA* decreased swarming suggesting that a third receptor could be involved. Unexpectedly, CsgD does not affect flagella-mediated motility. Neither the

csgD mutant nor CsgD overexpression had an effect on motility compared to the wild type. This finding is surprising, since in *E. coli*, CsgD expression inhibits motility[180]. Also CsgD, directly or indirectly transcriptionally regulates components of the flagellar regulon of *S.* Typhimurium[181]. Surprisingly, complementation of the Δ*yhjH* mutant with a plasmid containing *yhjH* provoked a phase variation from FliC to FljB expression suggesting the EAL domain protein to be involved in this phenomenon.

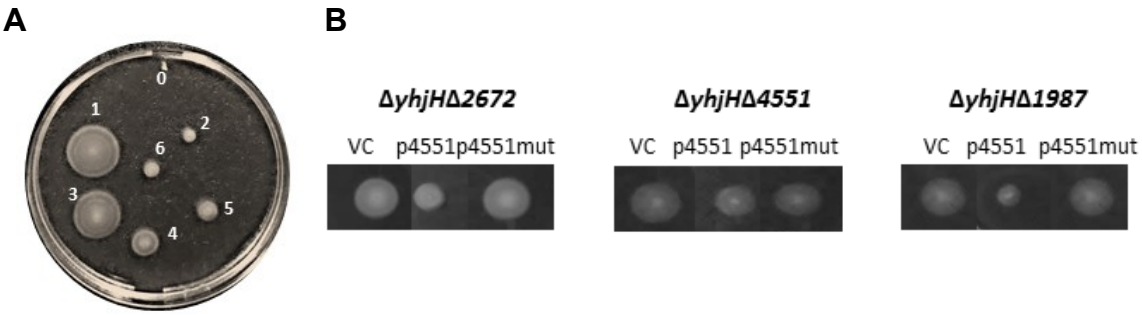

Figure 10: Identification of key diguanylate cyclases working in concert with Δ*yhjH*. (**A**) Swimming motility after 4 hours of incubation at 37°C. 0: negative control, 1: wild type, 2:Δ*yhjH*, 3: Δ*yhjH*Δ2672, 4: Δ*yhjH*Δ4551, 5: Δ*yhjH*Δ1987, 6: Δ*yhjH*Δ1283. (**B**) Complementation of double mutants with the diguanylate cyclase STM4551 and STM4551 containing a mutation in the catalytic site.

4.2 PAPER 2: THE EAL-LIKE PROTEIN STM1697 REGULATES VIRULENCE PHENOTYPES, MOTILITY AND BIOFILM FORMATION IN *SALMONELLA* TYPHIMURIUM.

Aim: to establish the molecular mechanism of the non-canonical EAL domain protein STM1697 to affect bacterial phenotypes.

A previous study (Paper 4) has identified roles for GGDEF/EAL domain proteins in virulence-associated phenotypes, invasion and IL-8 production. Screening for regulation of the invasion phenotype revealed that the non-canonical EAL domain protein STM1697 behaved oppositely to established phosphodiesterases. STM1697 belongs to the third class of EAL domain proteins that do not possess conserved signature motifs and is characterized as non-canonical EAL domain protein[182].

To analyse the molecular mechanism behind this unconventional phenotype, we first assessed bacterial phenotypes affected by the c-di-GMP signaling network such as rdar biofilm morphotype, expression of the biofilm regulator CsgD, secretion of SipA and motility. Usually, EAL domain proteins established as phosphodiesterases downregulate the rdar morphotype and CsgD expression[171].

In bacterial-host interaction, phosphodiesterases upregulate the invasion phenotype and secretion of SipA (Paper 4). However, STM1697 regulates all those different phenotypes in the opposite way.

Binding study and enzymatic analysis of purified STM1697 showed that this non-canonical EAL domain has lost its ability to bind and degrade c-di-GMP, which was in accordance with bioinformatic analysis. The differential scanning calorimetric profil of the positive control YkuI displayed an increase in unfolding temperature in the presence of c-di-GMP. We did not observe a change in unfolding temperature for STM1697 revealing that STM1697 does not bind c-di-GMP.

STM1697 affected biofilm, motility and virulence phenotypes in a similar way as the non-canonical EAL domain protein YdiV[183]. Consequently, we investigated the role of STM1697 in interference with the flagella regulon. STM1697 affected transcription of the class 2 gene *fliA* and class three genes *fliC* and *yhjH*. Transcription of the class 1 *flhD* was not affected by STM1697, suggesting that STM1697 interacts post-transcriptionally with $FlhD_4C_2$. Indeed, size exclusion chromatography with purified components showed that $FlhD_2$ interacts with STM1697 and a subsequent electrophoretic mobility shift assay showed that this interaction inhibits the ability of $FlhD_4C_2$ to bind to a target promoter. Preliminary mutant analysis indicated that STM1697 has a distinct interface to bind to $FlhD_2$ compared to STM1344. Of note, STM1697 additively works with STM1344 in motility and virulence regulation.

We also hypothesized, that STM1697 inhibits invasion of the gastrointestinal cell line HT-29 through inhibition of motility. In line with this hypothesis, deletion of motility components, the flagellin proteins FliC and FljB, the motor component MotA and chemotaxis components CheY, CheZ and CheA affected invasion. A decrease in invasion in those mutants suggested that not only flagellin components but also motor components and chemotaxis components affect invasion. STM1697 has an indirect impact on rdar morphotype biofilm formation through inhibition of $FlhD_4C_2$ via inhibition of the class 3 promoter gene *yhjH*. Interestingly, STM1697 is not functional in the laboratory strain *S.* Typhimurium LT2.

4.3 PAPER 3: RAPID PREPARATION OF BACTERIAL FLAGELLA FROM *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM.

Aim: to simplify pre-existent method for bacterial flagellin analysis of secreted flagellin and make it accessible through the openbiology website.

A semi-quantitative analysis of the flagellin secretion polymerized form complements the phenotypic motility assay. Based on a pre-existing protocol, we developed a protocol upon request of the website bioprotocol.org. We used this approach to quantify the amount of polymerized flagellin and also observed a flagellin switch between the phase 2 to phase 1, potentially mediated by the EAL domain protein (Paper 1 and Paper 2). During the elaboration of this protocol we paid a special focus on the following features: sensitivity: detection of clearly different signals for FliC and FljB, feasibility: reproducibility, reduction of the time procedure and chemicals used and last criterium specifity: the flagellin are the major proteins secreted in the supernatant[184]. In our case, we could clearly identify a signal at 51.6 kDa for FliC and 52.5 kDa for FljB (**Fig. 11**). Semi quantitative analysis can be performed to determine if the amount of flagellin is different from the wild type control with software like Image J (Paper 1). This procedure can also be adapted to isolate flagellin from other bacterial species.

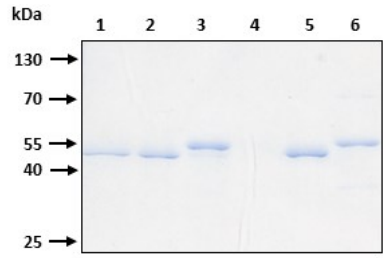

Figure 11: Observation of monomeric flagellin on SDS-PAGE protein gel.

Observation of flagellin expression after Coomassie blue staining. FljB: 52.5 kDa, FliC: 51.6 kDa. 1: wild type, 2: Δ*yhjH*, 3: complementation of Δ*yhjH* with pBAD30 containing *yhjH*, 4: negative control, Δ*fliC* Δ*fljB* 5: Δ*fljB*, 6: Δ*fliC*.

4.4 PAPER 4: COMPLEX C-DI-GMP SIGNALING NETWORKS MEDIATE TRANSITION BETWEEN VIRULENCE PROPERTIES AND BIOFILM FORMATION IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

Aim: to examine how c-di-GMP signaling modulates virulence phenotypes in S. Typhimurium.

Previous experiments showed that c-di-GMP affects at least two virulence properties in *S.* Typhimurium, invasion of HT-29 cells and IL-8 induction in HT-29 cells upon incubation with bacteria[136]. In Lamprokostopoulou *et al.*, 2010, we used overexpression of a diguanylate cyclase which produced five hundred times higher intracellular level of c-di-GMP compared to the wild type concentration[136]. Here, we analysed the effect of individual GGDEF, GGDEF-EAL and EAL domain proteins on virulence phenotypes invasion, secretion of SipA, IL-8 production as well as *in vivo* colonization and systemic spread of the infection in the streptomycin treated mouse model.

For each phenotype investigated, we could observe an opposite effect upon deletion of a distinct panel of GGDEF and EAL domain proteins of variable severity depending on the mutant. For invasion and IL-8, deletion of the GGDEF domain proteins lead to an increase in the invasion phenotypes and IL-8 production, in contrast, deletion of EAL domain proteins decreased the level of invasion as the key virulence phenotype. Complementation of GGDEF and EAL domain mutants with the GGDEF domain protein STM4551 or the EAL domain protein YhjH, restored the phenotype in invasion or IL-8 production to the wild type level respectively. Complementation with a catalytically inactive mutant exhibited a similar phenotype as the deletion mutant. These results confirm a role of c-di-GMP signaling in virulence phenotypes at a physiological relevant concentration.

To preliminary analyse the pathways leading to virulence suppression by c-di-GMP, we determined that the cellulose synthase BcsA and the biofilm regulator CsgD are required for inhibition of invasion and IL-8 production upon high c-di-GMP level. An exception was the mutant *yhjH*, whereby deletion of *csgD* did not recover the wild type invasion level. Another interesting point worth to mention is, that some c-di-GMP signaling network proteins presented an unconventional invasion phenotype like the non-canonical EAL domain protein STM1697 and the GGDEF domain protein STM1283, for instance. Secretion of SipA was also regulated by GGDEF and EAL proteins in agreement with the phenotype observed

in invasion (**Fig. 12**), but the complementation experiments suggested that the catalytic activity is not required for secretion of effector proteins.

To assess the virulence modulation by c-di-GMP *in vivo*, we did caecum colonization and systemic organ colonization experiments in the streptomycin-treated mouse model. Several c-di-GMP metabolizing proteins exhibit a pronounced phenotype in invasion and gut colonization, but no correlation between *in vivo* and *in vitro* experiments can be drawn.

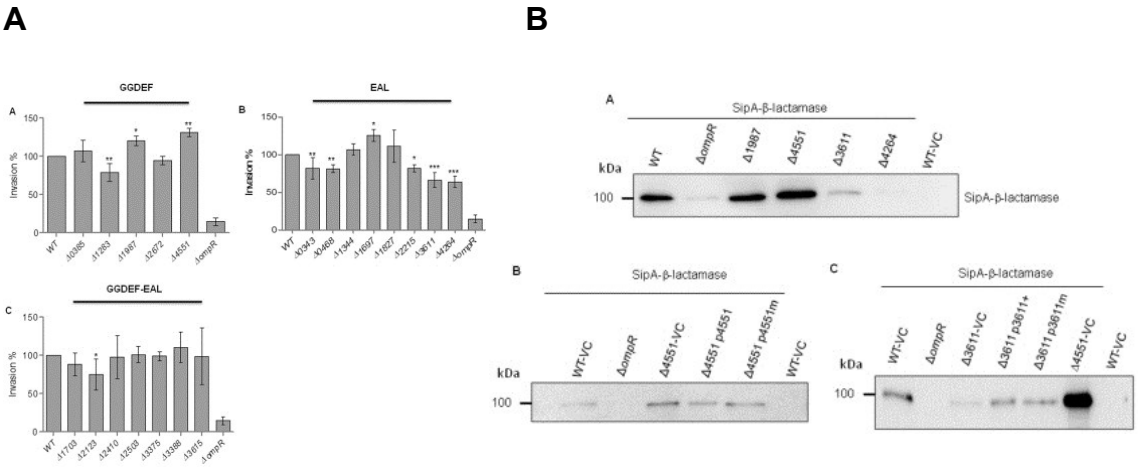

Figure 12: Screening of c-di-GMP metabolizing proteins for a phenotype in invasion and secretion of the SipA effector protein. (**A**) Screening of the c-di-GMP metabolizing proteins for invasion of the HT-29 epithelial cell line. The GGDEF domain proteins STM4551 and STM1987, and the EAL domain proteins YhjH and STM4264 present the most pronounced phenotype observed in that assay. (**B**) The invasion results could be correlated with the amount of secretion of the effector protein SipA.

4.5 PAPER 5: ROLE OF THE SURFACTANT PROTEIN C ROLE IN GROWTH INHIBITION, BIOFILM FORMATION AND MOTILITY OF *PSEUDOMONAS AERUGINOSA* PAO1

Aim: to identify a possible effect of the surfactant protein C on P. aeruginosa PAO1 adaptive behavior and possible interaction with surface appendages in order to improve synthetic surfactant engineering.

The main function of the surfactant protein C is to maintain stability in the lung and take part of the innate immunity of this organ, but little is known about its function. First, we determined the antimicrobial activity of different surfactant protein C variants (synthetic, truncated-SP-C1 and mature version of the surfactant). Then, we

analyzed their role in different bacterial adaptive behaviors such as biofilm formation and motility.

We did not detect any microbial activity for the different versions of the surfactant protein C tested, but we could observe a 30% decrease in biofilm formation in the presence of SP-C1 compared to the control. Motility experiments indicated that swimming activity of *P. aeruginosa* PAO1 is not affected by pre-incubation with SP-C1, while swarming activity of the bacteria is decreased by 25% compared to the control. Swarming activity in *P. aeruginosa* requires the flagellum and type IV pili[185]. Preliminary analysis by microscopy suggested that surfactant protein C induces bacterial clumping in liquid culture. The biofilm assay on polystyrene support confirms the bacterial clumping with a preponderant staining at the bottom of the well.

5 DISCUSSION

In this thesis, the regulation of motility by c-di-GMP signaling in *S.* Typhimurium was analyzed in detail. In general, besides biofilm formation, motility is a major target of c-di-GMP signaling in bacteria[7, 186]. In *S*. Typhimurium, motility is mainly regulated at the post-translational level, while in other bacteria, motility can also be regulated at the transcriptional level by c-di-GMP[130, 131, 178]. For example, in *P. aeruginosa*, the flagellar regulon activator FleQ is a c-di-GMP responsive transcription factor[7]. Of note, in *S.* Typhimurium, the non-canonical EAL proteins regulate the flagellar regulon on the level of the major transcriptional regulator $FlhD_4C_2$. STM1697 and YdiV can interact with $FlhD_4C_2$ and prevent its binding to the target promoter[187] . The interaction with the $FlhD_4C_2$ interface is different for YdiV and STM1697, which can explain their additive effect on motility and virulence phenotypes (Paper 2). The post-translational regulation of $FlhD_4C_2$ is complex, as DnaK and the flagellar regulon component FliT regulate $FlhD_4C_2$ functionality[117, 188].

The phosphodiesterase YhjH is specifically involved in motility in *S.* Typhimurium and *E. coli*[85, 178]. Additional EAL domain proteins involved in motility in S. Typhimurium are the EAL domain protein STM0343 and the non-canonical STM0551[189, 190]. The effect of *yhjH* is probably local as deletion of *yhjH* only marginally increased the level of c-di-GMP in the cell. In any case, deletion of *yhjH* causes YcgR to interact with FliG and via FliM to induce a bias in the rotation of the flagellar filament[131, 183]. Our study showed three different diguanylate cyclases STM2672, STM4551, STM1987 to be involved in motility regulation in *S*. Typhimurium[177, 178]. This set of diguanylate cyclases is overlapping but distinct from the diguanylate cyclases down-regulating motility in *E. coli*[178]. At the receptor level, deletion of the two PilZ domain proteins YcgR and BcsA gradually restored the motility in *S.* Typhimurium[107, 132]. In the triple mutant Δ*yhjH*Δ*ycgR*Δ*bcsA*, overexpression of the diguanylate cyclase AdrA decreased swarming motility. This decrease indicates the potential presence of a third c-di-GMP receptor. Complementary to the motility phenotype, we investigated polymerized flagellin present in the different c-di-GMP mutants. In line with the literature, *yhjH* does not alter expression of polymerized flagellin (Paper 1). The switch in flagellin expression with upregulation of FliC, while FljB is downregulated upon the deletion of STM0343 indicates that STM0343 expresses phosphodiesterase activity *in vivo* as the phenotype of the switch between FliC and

FljB is consistent with our results that the EAL domain protein causes an expression switch between FliC and FljB. In addition, a STM0343 mutant displays reduced motility. In *P. fluorescens*, GacS and SadB, c-di-GMP associated proteins regulate secretion of FliC by affecting FleQ and consequently transcription of *fliC*[191]. In summary, our results indicate that regulation of flagellin expression by c-di-GMP turnover proteins occurs on more levels than previously anticipated.

In this work (Paper 2 and 4), we could identify a role of c-di-GMP signaling in virulence of *S.* Typhimurium. Previous work by the Lasa group, using a *S. enteritidis* strain with deletion of all GGDEF domain proteins indicated that c-di-GMP signaling is not required for virulence regulation[192]. In this study, we have been using systematic screening of deletion mutants in the c-di-GMP turnover proteins to observe a particular phenotype for c-di-GMP signaling in invasion of epithelial cells, induction of the IL-8 response, gut colonization and systemic infection (Paper 4). Our findings are in line with studies by others, who also found a role of c-di-GMP signaling in systemic infection and invasion of Caco-2 cells[193, 194]. Screening of c-di-GMP turnover proteins has also been conducted in other bacteria such as *P. aeruginosa* and *X. campestris* [97, 195]. Our work, seen in the context of the literature, also indicates that invasion of epithelial cells and systemic infection are generally affected by c-di-GMP signaling in bacteria[99, 115, 196]. Also, the invasion related type three secretion system is a general target for c-di-GMP signaling as in the pathogen *P. syringae* and *D. dadantii*, phosphodiesterases are modulating the expression of the injectisome pilus[197, 198].

C-di-GMP regulates bacterial adaptive behavior from motility to virulence factor expression. Furthermore, host components are modulating bacterial behavior. As previously shown, the LL-37 inhibits biofilm formation by preventing the polymerization of the major curli subunit CsgA in *E. coli*[149]. In *P. aeruginosa* and *S. epidermidis*, LL-37 also inhibits biofilm formation. Recently the small peptide 1018, an inhibitor of the second messenger (p)ppGpp, has been shown to efficiently inhibit biofilm formation in Gram-positive and Gram-negative bacteria[199].

As we have shown that the surfactant component SP-C interferes with biofilm formation and swarming motility, we hypothesize that the type IV pili are a target of interaction with SP-C since they are involved in both processes. The interaction between the SP-C and the bacteria leads to clumping, which can serve as a defense mechanism by the host to avoid epithelial damage and increased expectoration of the bacteria. This aggregation of bacteria by an innate immune

component would work in similar way as nanonets formed by the human α-defensin 6, which are build up after *S.* Typhimurium invasion in the gut[200].

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The major conclusions of this thesis are:

- The c-di-GMP signaling network of *S.* Typhimurium regulates motility through one phosphodiesterase and three diguanylate cyclases.
- C-di-GMP metabolizing proteins tightly regulate bacterial motility through specific interactions with two c-di-GMP receptors.
- STM1697 affects biofilm formation, motility and invasion of the HT-29 cell line through its interaction with $FlhD_4C_2$.
- The panels of c-di-GMP metabolizing proteins, which modulate bacterial virulence and host response were defined.
- The major biofilm components, the transcriptional regulator CsgD and the cellulose synthase BcsA are involved in repression of virulence *in vitro*.
- Surfactant protein C inhibits biofilm formation and swarming motility of *P. aeruginosa* and might promote aggregation of the bacterial cells *in vivo*.

The most immediate work to follow up this thesis would be:

C-di-GMP regulation of bacterial adaptive behavior

- Determination of the mechanisms of the specificity of di-guanylate cyclases towards cognate receptors involved in motility inhibition.
- Identification of new c-di-GMP receptors and associated cellular functions.
- To elucidate the molecular mechanism by which the c-di-GMP signaling affects the invasion phenotype.
- To elucidate the molecular mechanism by which the c- di-GMP signaling affects the IL-8 secretion phenotype.
- Determination of the mechanism how GGDEF and EAL domain proteins regulate secretion of the type three secretion effector protein SipA independently of c-di-GMP.
- To characterize the role of other surface appendages involved in bacterial behavior such as virulence.

Bacterial biofilm and host components

- Define the molecular mechanism behind the switch of biofilm modes by the surfactant protein C.
- Investigate whether surfactant protein C has a role in adherence and invasion of host cells by *P. aeruginosa*.

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