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Karolinska Institutet, Stockholm, Sweden

CHARACTERIZATION OF C-DI-GMP SIGNALLING IN SALMONELLA TYPHIMURIUM

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Stockholm 2007

Published by Karolinska Institutet. Printed by [name of printer]

ISBN 978-91-7357-417-4

ABSTRACT

Signal transduction via cyclic nucleotides is a general mechanism utilized by cells from all kingdoms of life. Identification of cyclic diguanosine monophosphate (c-di-GMP) as an allosteric activator of the cellulose synthase in *Gluconacetobacter xylinus* 20 years ago, paved the way for the discovery of a novel general signalling system which is unique to bacteria.
In this thesis, the c-di-GMP signalling network leading to the formation of a biofilm behavior in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), the rdar morphotype, is described. The rdar morphotype is characterized by the expression of the extracellular matrix components cellulose and curli, which are controlled by the transcriptional regulator CsgD. Curli production is directly activated by CsgD, whereas cellulose biosynthesis is indirectly activated by CsgD via the expression of the GGDEF domain protein AdrA. AdrA is one of 20 proteins carrying a GGDEF and/or EAL domain.
The development of a detection method for c-di-GMP based on high pressure liquid chromatography and mass spectrometry (MS) and structural characterisation of c-di-GMP by MS-MS analysis allowed the characterisation of the c-di-GMP pathway in S. Typhimurium. We demonstrate that the GGDEF domain synthesizes c-di-GMP, whereas the EAL domain is responsible for c-di-GMP degradation. A high c-di-GMP concentration positively regulates the biosynthesis of adhesive matrix components and biofilm formation, whereas it inversely regulates motility in *S.* Typhimurium, *Escherichia coli* and *Pseudomonas aeruginosa*. These findings indicate that c-di-GMP is a general regulator of the transition from motility to sessility in Bacteria.
Overexpression of AdrA in the wild type strain *S.* Typhimurium UMR1 resulted in upregulation of cellulose and curli. The effect of c-di-GMP on curli expression was finally mapped to the level of CsgD transcription or mRNA stability. We further demonstrate that at least two subsets of GGDEF domain proteins are involved in CsgD expression. Chromosomally encoded AdrA controls cellulose production, but is not involved in CsgD expression, whereas the GGDEF-EAL domain proteins STM2123 and STM3388 control CsgD expression, but cannot functionally replace AdrA. Since all three proteins display apparent diguanylate cyclase activity, there are separate c-di-GMP pools dedicated to regulation of CsgD expression and cellulose biosynthesis.
Further on, four of 15 EAL domain proteins, STM3611, STM1827, STM1703 and STM 4264, are involved in CsgD expression. All EAL domain proteins displayed apparent phosphodiesterase activity in vivo, which is, however, not directly correlated with the effect on CsgD expression, Therefore, the c-di-GMP pools degraded by the four EAL domain proteins are dedicated to CsgD expression to different extends. Other subsets of EAL domain proteins regulate pellicle formation, biofilm in liquid culture and flagella mediated motility.
Although molecular mechanisms of c-di-GMP synthesis and degradation are unraveling, major questions regarding the targets of c-di-GMP signaling are still open. Bioinformatic studies predicted that the PilZ domain may function as a c-di-GMP binding domain. We experimentally demonstrate that c-di-GMP binds to the PilZ domains of the cellulose synthase from *G. xylinus* and YcgR from *Escherichia coli*. By inactivation and over expression of YcgR in *S.* Typhimurium we showed that this protein regulated motility in a c-di-GMP dependent way when c-di-GMP levels were enhanced.
In summary, this work shows that c-di-GMP is a central regulator of a biofilm behaviour in S. Typhimurium. Although many details about the c-di-GMP metabolism were unravelled in this work, the molecular mechanisms how c-di-GMP exerts its function remain to be discovered.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. **Simm, R**., Morr, M., Kader, A., Nimtz, M., and Romling, U. (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol* **53**: 1123-1134.

II. Kader, A., **Simm, R**., Gerstel, U., Morr, M., and Romling, U. (2006) Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of Salmonella enterica serovar Typhimurium. *Mol Microbiol* **60**: 602-616.

III. **Simm, R**., Lusch, A., Kader, A., Andersson, M., and Romling, U. (2007) Role of EAL-containing proteins in multicellular behavior of Salmonella enterica serovar Typhimurium. *J Bacteriol* **189**: 3613-3623.

IV. Ryjenkov, D.A., **Simm, R**., Romling, U., and Gomelsky, M. (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. *J Biol Chem* **281**: 30310-30314

CONTENTS

LIST OF ABBREVIATIONS

AdrA	AgfD regulated protein A
AgfD	Agregative fimbriae D
Bcs	Bacterial cellulose synthesis
Bdar	Brown dry and rough
cAMP	Cyclic adenosine monophosphate
C. crescentus	*Caulobacter crescentus*
c-di-GMP	Cyclic diguanosine monophosphate
cGMP	Cyclic guanosine monophosphate
CR	Congo red
Csg	Curli subunit gene
E. coli	*Escherichia coli*
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
G. xylinus	*Gluconacetobacter xylinus*
l-di-GMP	Linear diguanosine monophosphate
mRNA	messenger ribonucleic acid
P. aeruginosa	*Pseudomonas aeruginosa*
Pdar	Pink dry and rough
Rdar	Red dry and rough
RP-HPLC	Reversed phase high-performance liquid chromatography
S. aureus	*Staphylococcus aureus*
Saw	Smooth and white
S. epidermidis	*Staphylococcus epidermidis*
Serovar	Serological variant
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
V. cholera	*Vibrio cholera*
VPS	Vibrio polysaccharide
X. campestris	*Xanthomonas campestris*

1 INTRODUCTION

Signal transduction via small molecules is a general mechanism utilized by cells integrating internal and external information to elicit suitable responses. In eukaryotic cells cyclic purine nucleotides regulate diverse functions including photomorphogenesis, cell movement, cell differentiation, and immunity (Saran *et al.*, 2002; Soderling and Beavo, 2000; Walden, 1998). In bacteria cAMP is a common second messenger involved in transcriptional regulation (Botsford and Harman, 1992; Smith *et al.*, 2004), whereas the role of cGMP in bacterial physiology is more uncertain (Camilli and Bassler, 2006).

This thesis describes the recognition of a novel cyclic nucleotide mediated signaling system, which is unique to bacteria. The focal point is the characterization of the cyclic diguanosine monophosphate (c-di-GMP) signaling network in the human pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium).

1.1 SALMONELLA ENTERICA

The genus *Salmonella* comprises gram negative flagellated rods belonging to the family enterobacteriacea. According to the Kauffman and White scheme the genus is divided into seven subspecies consisting of more than 2500 serological variants (serovars) based on the variable lipopolysacharide O antigen and the flagella H antigen (Popoff *et al.*, 2004). Six of seven subspecies (group I, II, IIIa, IIIb, IV and VI) are classified as *Salmonella enterica*, while subspecies group V comprises the species *Salmonella bongori*.

Serovars from all seven subspecies can cause disease in humans, but generally serovars grouped under subspecies I are isolated from patients (Aleksic *et al.*, 1996). Clinically, subspecies I serovars can be classified in two groups. One group constitutes the human specific serovars *Salmonella enterica* serovar Typhi (*S.* Typhi) and *Salmonella enterica* serovar Paratyphi (*S.* Paratyphi) A – C. These serovars cause enteric fever which is responsible for ~200,000 deaths world wide every year (Crump *et al.*, 2004). Serovars of this group spread via the fecal oral route. The second group includes zoonotic serovars with broader host range. Zoonotic serovars normally cause self-limiting gastroenteritis in humans manifested by diarrhea, nausea vomiting, intestinal cramping and fever which affect billions of people world-wide every year. Serovars of the second group are mainly transmitted by contaminated food products. *Salmonella enterica* serovar Enteriditis (*S.* Enteriditis) and *S.* Typhimurium are the most important causes of gastroenteritis.

Although gastroenteritis normally is uncomplicated, the disease can become systemic with fatal outcome in the young, elderly and patients with weakened immunity. Problems to treat systemic *Salmonella* infections with antibiotic therapy have increased significantly, mainly due to the appearance of antibiotic resistance and the emergence of multidrug resistant strains (Cloeckaert and Schwarz, 2001).

Salmonella enterica colonizes the intestinal tract of warm and cold blooded animals, often without causing disease, but with occasional outbreaks of acute infections. One

mechanism of colonization of the gut might be the adherence to epithelial cells. The bacteria are transferred between hosts, either by close contact or via vehicles such as water or food products. Food borne Salmonellosis is often spread via meat and eggs, but has also been reported to occur after spread via vegetables and grains. The colonization of the intestinal systems in cattle and poultry and the food borne spread of *Salmonella* may be facilitated by the ability of the bacteria to adhere to and colonize different surfaces. This adherent phenotype is a multicellular behavior of bacteria referred to as biofilms.

2 BIOFILMS

The term biofilm has been defined in a more or less stringent way over the last three decades. One broad definition states that bacterial biofilms are “matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces.” (Costerton *et al.*, 1995)

Most, if not all, bacteria have the ability to form biofilms and these structures are found in all ecological niches. The complex 3D-architecture of a mature biofilm gives rise to heterogeneous microenvironments within the structure. These microenvironments that differ with respect to osmolarity, oxygen tension, nutritional supply and cell density produce diverse bacterial phenotypes even in single species biofilms (Fux *et al.*, 2005). Because of this, biofilms are compared to eukaryotic systems where different celltypes perform defined and specialized functions (Costerton *et al.*, 1995). The diversity offered by the multicellular behaviour of biofilms may provide the bacterial population with benefits compared to their planktonic counterparts by having subpopulations equipped to survive various conditions (Stoodley *et al.*, 2002). Under natural conditions a biofilm is not only constituted of one species, but is a complex multispecies community. For example the dental plaque consists of several hundred species of bacteria (ten Cate, 2006).

2.1 IMPLICATIONS OF BACTERIAL BIOFILMS

With respect to the human host, biofilms have dual functions. They may be advantageous for health, but can also create important health problems. Biofilms of commensal bacteria attached to the epithelial surface which protect against pathogens have a clear beneficial function (Collado *et al.*, 2007; Soerjadi *et al.*, 1982). However, bacterial biofilms are also associated with pathogenic processes. Indwelling medical devices such as voice prosthesis, catheters, prosthetic heart valves and replacement joints are prone to biofilm formation (Donlan and Costerton, 2002). The biofilms might lead to chronic infections in the host. These persistent infections are very difficult to remove and are highly resistant to antimicrobial therapy. Immunocompromised patients frequently suffer from chronic infections, initiated by colonization with biofilm forming bacteria. These bacteria often originate from the commensal flora e.g. *Staphylococcus epidermidis* (*S. epidermidis*) or the environment e.g. *Pseudomonas aeruginosa* (*P. aeruginosa*). However, biofilm related infections are not only a curse of compromised patients, but are also difficult to eliminate in apparently healthy individuals. Many women suffer from recurrent urinary tract infections. In a mouse model of bladder infection it has been demonstrated that uropathogenic *E. coli* can invade the superficial epithelia of the bladder in a type I fimbriae dependent manner (Anderson *et al.*, 2004). These bacteria colonize the cytoplasm of the epithelial cells as aggregates interconnected by fibrous structures and surrounded by polysaccharide. These multicellular communities may be responsible for chronic and recurrent urinary tract infections (Anderson *et al.*, 2004).

As previously mentioned, *Salmonella enterica* readily form biofilms on a variety of surfaces e.g. epithelial cells, plants and grains, which may facilitate the maintenance and transmission of bacteria leading to food borne outbreaks of Salmonellosis (Barak

et al., 2007; Boddicker *et al.*, 2002; Lapidot *et al.*, 2006). In addition to this, *Salmonella* form biofilms on abiotic surfaces such as stainless steel and glass which may constitute reservoirs that contaminate food and water supplies (Hood and Zottola, 1997). Biofilms have also been demonstrated on gall stones in the carrier stage of *S*. Typhi, probably contributing to the persistence of the infection (Lai *et al.*, 1992).

In the industrial context beneficial biofilms are used in bioremediation processes of human and manufacturing wastes (Singh *et al.*, 2006). However, detrimental effects of biofilms may also result in corrosion of metal surfaces (Coetser and Cloete, 2005) as well as contamination during food processing with short shelf-life and food-borne illnesses as result (Midelet and Carpentier, 2002). In addition to this, biofilms cause big economic problems to the agricultural industry where e.g. mastitis results in impaired animal health, reduced milk production and large financial losses (Melchior *et al.*, 2006).

2.2 BIOFILM FORMATION

It is generally accepted that biofilms are formed via several sequential stages, starting with the attachment of bacteria to a surface. After the initial reversible attachment, bacteria may migrate over the surface. Once irreversibly attached, they divide to form microcolonies, which are the basic structures of biofilms. The bacteria produce extracellular material that firmly attaches the cells to the surface and to each other. Over time the biofilm develops into a complex three dimensional structure where the bacteria are surrounded by the matrix components. Within the biofilm there are regions with lower cell number and matrix density, which are referred to as water channels. The water channels can function in transporting nutrients and waste products deep into the structure (Watnick and Kolter, 2000).

Once formed, the mature biofilm is by no means a static construction. There is a constant remodelling of the biofilm structure e.g. by enzymes degrading and producing the matrix. Cells may be broken off from the biofilm surface or actively dissociate and spread to novel environments for colonization. The dynamic behaviour of the biofilm and the alteration between different phenotypes during biofilm development indicate that a strictly controlled regulatory network is effective. Cells switch between a sticky sessile phenotype favouring attachment and community behaviour and a motile phenotype for dispersal and/or dissociation of cells.

2.3 THE BIOFILM MATRIX

Although the extracellular matrix is a universal attribute of bacterial biofilms, the composition differs depending on the organisms present, their physiological status, the environmental conditions and the surface. Extracellular polysaccharides and proteins are integral structural components of most matrixes, but other components like nucleic acids, glycoproteins, membrane vesicles, enzymes, phospholipids and material from the environment are also associated with the biofilm (Allison, 2003; Schooling and Beveridge, 2006). One of the major functions of the matrix is probably to provide the biofilm with mechanical support maintaining the structural complexity. However, biofilm forming organisms may benefit in many ways from the matrix. Biofilms are

resistant against a wide array of factors that normally eradicate bacterial populations, including biocides, bacteriophages, amoebas, host immune responses and antibiotics (Costerton *et al.,* 1999). Other functions of the biofilm matrix are trapping of nutrients and protection against long term desiccation (White *et al.,* 2006).

2.3.1 Extracellular polysaccharides

Extracellular polysaccharides are important structural determinants of the biofilm architecture. Unrelated bacterial species can utilize polysaccharides of similar structure as matrix components. Cellulose, for example, is a vital constituent of the biofilm matrix of diverse bacteria such as the fruit degrading bacteria *Gluconacteobacter xylinus* (*G. xylinus*), the plant pathogens *Agrobacterium tumefaciens* and *Rhizobium leguminosarum,* and bacteria of the family enterobacteriaceae including the human pathogen *S.* Typhimurium and commensal *E. coli* (Bokranz et al., 2005; Ross et al., 1991; Zogaj et al., 2001; Zogaj et al., 2003).
Another common extracellular polysaccharide is the β-1, 6 linked N-acetylglucosamine (PIA or PNAG) which was originally described in *S. epidermidis* and *Staphylococcus aureus (Mack et al., 1996; Maira-Litran et al., 2002)* . PIA is synthesised by the gene products of the icaADBC-operon. Homologues of these genes are found in various bacteria including the human pathogens *Yersinia pestis* and *Bordetella pertussis; the* human commensal *E. coli,* the plant commensal *Pseudomonas fluorescens,* and the plant pathogen *Ralstonia solanacearum* (Lasa, 2006).

In addition to this, bacteria have the ability to utilize different polysaccharides under different conditions. *S.* Enteriditis has the capacity to express several exopolysaccharides, which may be differentially produced and contribute to the biofilm formation under different conditions. For example, cellulose was shown to be important for biofilm formation on chicken epithelial cells as well as plastic, whereas colanic acid was required for biofilm formation on the epithelial cells, but not on plastic (Ledeboer and Jones, 2005). Gibson et al postulated that the salmonella O-antigen capsule is not involved in multicellular behaviour since mutations in the *yih*-genes encoding the enzymes producing the capsule do not alter aggregative phenotypes. On the other hand, the capsule appears to be important for survival during desiccation stress (Gibson *et al.,* 2006). However, Barak et al showed that the O-antigen capsule, and cellulose are important for colonization on alfalfa sprouts, whereas colanic acid is not (Barak *et al.,* 2007).

2.3.2 Proteinacious components

Proteins as components of the structural matrix can have structural and enzymatic functions. Proteins with structural function can be adhesive fimbriae or monomeric adhesins. Enzymes such cellulase, may modulate the macromolecules within the matrix thereby influencing the structural composition. The extracellular proteins involved in biofilm formation are diverse. Filamentous structures such as curli and type I fimbriae have been shown to be important for biofilm formation (Austin *et al.,* 1998).

In S. Typhimurium, curli is required for biofilm formation under different conditions (Austin *et al.,* 1998; Romling *et al.,* 1998a; Romling *et al.,* 1998b). Curli are involved

in the cell-cell interactions as well as cell-surface attachment. It was shown in *S.* Typhimuiurm and *E. coli* that curli deficient strains form flat, less developed biofilms in a flow cell system compared to curli producing strains (Jonas *et al.*, 2007; Kikuchi *et al.*, 2005). In addition to this, curli have been implicated in attachment and invasion of host cells, binding to host proteins and activation of the immune system (Barnhart and Chapman, 2006).
In *S.* Typhimurium, the biofilm associated protein A (BapA) has been demonstrated to function in biofilm formation (Latasa *et al.*, 2005; Romling *et al.*, 1998a). BapA belongs to a family of large surface proteins which are involved in the initial attachment of bacteria to surfaces and promote cell-cell interactions leading to biofilm maturation (Latasa *et al.*, 2006). BapA was first described in *S. aureus* (Cucarella *et al.*, 2001), but homologues, with similar function are found in diverse bacteria like Enterococcus faecalis, Pseudomonas putida and several coagulase negative Staphylococci (Latasa *et al.*, 2006). In S. Typhimurium, BapA was demonstrated to be required for pellicle formation (Latasa *et al.*, 2005). BapA was on the other hand shown to be dispensable for biofilm formation in a flow cell system (Jonas *et al.*, 2007). However overexpression of BapA cannot functionally overcome curli deficiency in biofilm development (Latasa *et al.*, 2005). The specific function of BapA in biofilm formation is not known, but the protein may function in strengthening cell-cell interactions via homophilic interactions between BapA molecules acting like receptor and ligand on different cells (Latasa *et al.*, 2006). In addition to a function in biofilm formation, it was shown that BapA mutants have a decreased ability to invade the intestinal epithelium and access internal organs in BALB-C mice. The role of BapA in virulence is not evident, but may be due to interactions of BapA with structures on the host cell surface or by decreased ability to form biofilms (Latasa *et al.*, 2005).

Different adhesive protein structures may be important for biofilm formation at different stages of maturation as well as under different environmental conditions. In E. coli for example several adhesive components participate in biofilm formation. Type I fimbriae play a critical role in the initial cell-surface interaction, (Van Houdt and Michiels, 2005), but may be important at later stages as well. Antigen 43 (Ag43) is a self-recognized adhesin which mediates auto aggregation of cells through Ag43-Ag43 interactions, promoting micrcolony development (Kjaergaard *et al.*, 2000). The adhesive curli-fimbriae, structural components of the biofilm matrix in many enterobacteriacea (Zogaj *et al.*, 2003), promote cell-cell and cell-surface interactions aiding the initial attachment to surfaces, microcolony formation and biofilm maturation (Barnhart and Chapman, 2006; Van Houdt and Michiels, 2005) . Conjugative pili are reported to facilitate unspecific cell-cell and cell-surface interactions promoting biofilm development (Ghigo, 2001). Adhesive components can substitute each other. For example, the expression of conjugative pili can eliminated the need for Curli, Ag43 and type I fimbriae in a biofilm flow cell system (Reisner *et al.*, 2003).

3 RDAR-MORPHOTYPE

Biofilm forming strains of *S.* Typhimurium, *E. coli* and many other enterobacteria readily employ a multicellular behaviour characterized by expression of the extracellular polymers cellulose and curli (Bokranz *et al.*, 2005; Romling, 2005; Zogaj *et al.*, 2001; Zogaj *et al.*, 2003). When these bacteria are grown on agar plates supplemented with the diazo dye Congo Red (CR), expression of cellulose and curli results in dark purple dry and wrinkled colony morphology called the red dry and rough phenotype (rdar) (fig. 1). The colour is a consequence of CR-binding to the extracellular matrix components changing the spectrophotometric properties of the dye. The absorbance spectrum of CR differs upon binding of cellulose and curli. Strains producing only cellulose appear pink dry and rough (pdar) on CR-plates whereas production of curli results in brown dry and rough (bdar) colony morphology. If none of the matrix components are expressed the phenotype is smooth, white and glossy and goes under the name smooth and white phenotype (saw). The different CR morphotypes constitute an excellent tool for genetic studies of the regulatory networks leading to synthesis of the extracellular matrix components and biofilm formation in *S.* Typhimurium.

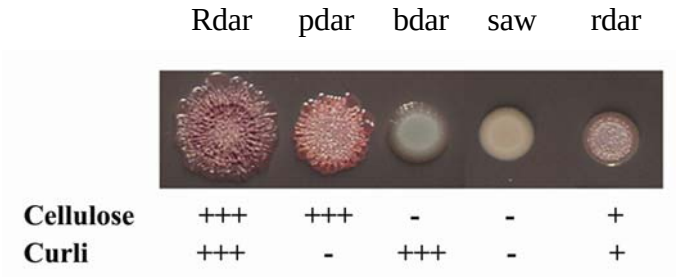

Figure 1. Colony morphology prototypes of *Salmonella enterica* serovar Typhimurium ATCC14028 after incubation at 28°C for 24h. From left to right are examples of semi-constitutive rdar morphotype (MAE52 (UMR1 P*csgD*1)), pdar morphotype, bdar morphotype, saw morphotype as well as a regulated rdar morphotype (UMR1). UMR1 expresses a highly regulated rdar morphotype only at ambient temperatures. The semi-constitutive strain MAE52 differs compared form strain UMR1 by a point mutation in the csgD promoter, which results in temperature independent and increased expression of the rdar morphotype (Romling *et al.*, 1998b).

3.1 REGULATION OF RDAR MORPHOTYPE EXPRESSION

The rdar morphotype is regulated by CsgD (previously called AgfD). CsgD is categorized in the LuxR superfamily of transcriptional regulators carrying an N-terminal receiver domain and a C-terminal DNA-binding helix-turn-helix domain. Homology between the N-terminus of CsgD and members of the FixJ family of response regulators, suggests a role for CsgD as the effector protein in a two component system. Response regulators in two component systems are activated by phosphorylation of an associated sensor kinase. However, no cognate sensor kinase has

been identified for CsgD. CsgD expression is complex and influenced by many environmental conditions such as temperature, osmotic pressure, nutrient starvation and oxygen tension (Gerstel and Romling, 2001, 2003; Romling, 2005).

The information for curli biosynthesis is carried by the curli synthesis genes (*csg*) in the divergently transcribed *csgBAC* and *csgDEFG* operons. CsgA and CsgB are the structural components building up the curli fibers (Hammar *et al.*, 1996). Although *csgC* is part of the *csgBAC* operon, the function is unkown. The *csgDEFG* operon encodes four accessory proteins required for curli synthesis. CsgE and F are periplasmic proteins interacting with CsgG, an outer membrane lipoprotein (Loferer *et al.*, 1997). These proteins are required for proper secretion and assembly of the CsgA and CsgB subunits.
Although, direct binding to the promoter of *csgBAC* has never been demonstrated, CsgD is required for the transcriptional regulation of the *csgBAC* operon. CsgD has not been demonstrated to regulate the expression of the *csgDEFG* operon and consequently does not regulate its own expression (Barnhart and Chapman, 2006).

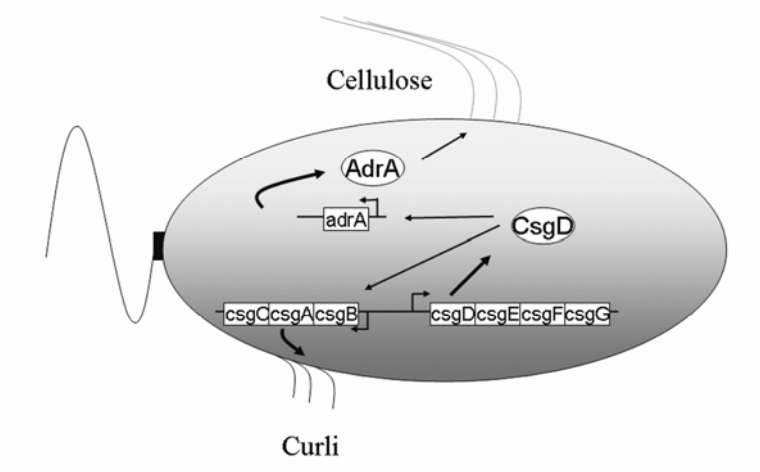

Figure 2. Model of the regulatory network leading to rdar morphotype development. Depicted here is the CsgD mediated regulatory network which results in the rdar morphotype development when *S.* Typhimurium is cultured on Congo-Red agar plates at ambient temperature. CsgD directly activates transcription of the *csgBAC* operon, which results in expression of the structural proteins CsgA and CsgB. In addition to this, CsgD activates the transcription of AdrA, which in turn produces c-di-GMP required to activate cellulose biosynthesis. CsgD is expressed from the *csgDEFG* operon in response to multiple environmental signals. However, unlike many other transcriptional regulators, CsgD does not regulate its own expression.

The bacterial cellulose synthesis genes (*bcs*) are expressed from two divergently transcribed operons (*bcsABZC-bcsEFG*). BcsA and BcsB form the cellulose synthase complex where BcsA contain the catalytic site. BcsZ is a cellulase, degrading cellulose. The function of the other *bcs*-encoded proteins is still unknown. The expression of the

bcs-genes appears to be constitutive when *S*. Typhimurium is cultivated on CR-agar (Zogaj *et al.*, 2001). Cellulose synthesis is indirectly regulated by CsgD via transcriptional regulation of AdrA (AgfD regulated protein A). AdrA is a protein carrying an N-terminal MASE2 domain (Nikolskaya *et al.*, 2003) and a C-terminal GGDEF domain. GGDEF domain proteins are involved in modulating the intracellular concentrations of the nucleotide cyclic diguanosine monophosphate (c-di-GMP) (Romling, 2005; Zogaj *et al.*, 2001).

CsgD has regulatory functions besides the activation of transcription of cellulose and curli. The *Salmonella* capsular polysaccharide and BapA are other surface associated structures positively regulated by CsgD (Gibson *et al.*, 2006; Latasa *et al.*, 2005). In *E. coli*, transcriptome analysis has revealed several additional genes up and down-regulated by CsgD. Among the genes transcriptionally modulated by constitutive CsgD expression is an EAL-domain containing, putative phosphodiesterase (yoaD) (Brombacher *et al.*, 2006). A role for CsgD in the regulation of metabolic processes has also been indicated, but falls outside of the scope for this thesis (Brombacher *et al.*, 2006).

4 CYCLIC DIGUANOSINE MONOPHOSPHATE

C-di-GMP (fig. 3) was first discovered in *G. xylinus*, where it was identified as an allosteric activator of the cellulose synthase (Ross *et al.*, 1987). Today, 20 years later, c-di-GMP is recognized as a ubiquitous second messenger (Romling *et al.*, 2005) unique to bacteria, but with potential applications outside the bacterial kingdom (Amikam *et al.*, 1995; Brouillette *et al.*, 2005; Karaolis *et al.*, 2005a; Karaolis *et al.*, 2005b; Karaolis *et al.*, 2007a; Karaolis *et al.*, 2007b; Steinberger *et al.*, 1999).

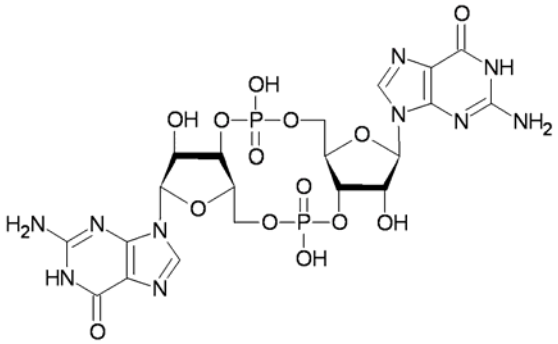

Figure 3. Chemical structure of cyclic diguanosine monophosphate (c-di-GMP)

The realization that c-di-GMP is a general second messenger in bacteria is mainly derived from its implication as a regulator of biofilm related phenotypes in diverse bacteria and the recognition that c-di-GMP metabolising proteins occur in most bacteria. C-di-GMP is positively regulating exopolysaccharide production, biosynthesis of adhesive fimbriae (Haussler, 2004; Kader *et al.*, 2006) and biofilm formation. On the other hand c-di-GMP inhibits motility. Today it is established that c-di-GMP is central to the regulation from motile to sessile bacterial cells (Rahman *et al.*, 2007; Simm *et al.*, 2004).

Several studies have also demonstrated that c-di-GMP is a regulator of virulence factors in different bacteria. For example, c-di-GMP negatively regulates Cholera toxin production in *V. cholera* (Tamayo *et al.*, 2005; Tischler *et al.*, 2002; Tischler and Camilli, 2005). Inactivation of the EAL domain protein STM1344 in *S.* Typhimurium resulted in a mutant with reduced resistance to hydrogen peroxide (Hisert *et al.*, 2005). In addition to this the mutant demonstrated increased speed of killing macrophages (Hisert *et al.*, 2005).

Caulobacter crescentus (*C. crescentus*) is an example where the transition from motility to sessility is an integral part of the cell development, which is regulated by c-di-GMP (Aldridge *et al.*, 2003; Paul *et al.*, 2004). *C. crescentus* undergo a developmental life cycle starting with motile cells carrying polar flagellum and pili. Once approaching a surface, the pili retract and the cells release the flagellum. A stalk is developed at the same pole. The stalked cell adheres to a surface and undergoes unsymmetrical cell division resulting in an attached, stalked cell and a new flagellated,

motile cell. The polar development is regulated by the c-di-GMP synthesising activity of the phosphorylated response regulator PleD which localizes to the flagellated pole driving the stalked cell formation (Paul *et al.*, 2004).

Other examples of phenotypes, suggested to be regulated by c-di-GMP, include resistance to phage infection and heavy metal ions in *E. coli* and photosynthesis in *Synechococcus elongatus* (Brown *et al.*, 1986; Chae and Yoo, 1986; Romling *et al.*, 2005)).

4.1 DIGUANYLATE CYCLASES AND PHOSPHODIESTERASES

The initial characterization of c-di-GMP-signalling in G. xylinus led to the discovery of six homologues proteins involved in the turnover of the molecule (Tal *et al.*, 1998). These proteins were found to have two conserved domains, named GGDEF and EAL based on short conserved amino acid motifs. Considering the domain architecture and amino acid sequences of the G. xylinus proteins responsible for turning over c-di-GMP, the identification of the catalytic regions and assignment of functions to the different domains was not obvious.

Genetic experiments (Ausmees *et al.*, 2001) as well as homology modelling of the GGDEF domain against an adenylate cyclase, to which the GGDEF domain show low sequence similarity, suggested that the GGDEF domain is responsible for the synthesis of c-di-GMP (Pei and Grishin, 2001). In accordance with this prediction, the EAL domain was assumed to be responsible for the degradation of the molecule.

It was only recently that the work of several research groups established by biochemical methods that the synthesis of c-di-GMP is catalysed by GGDEF domains, which function as diguanylate cyclases, (Paul *et al.*, 2004; Ryjenkov *et al.*, 2005). Although GGDEF domains spontaneously dimerize the low catalytic activity of the dimeric GGDEF domain indicated that there is additional regulatory mechanisms involved in controlling c-di-GMP synthesis (Paul *et al.*, 2004). It was shown by Paul et al that the GGDEF domain protein PleD from C. crescentus is activated by phosphorylation which leads to dimerization and enzymatic activity (Paul *et al.*, 2007). The GGDEF motif is an integral part of the catalytic domain and mutation of any amino acid residue in the GGDEF motif was shown to result in inactive diguanylate cyclases. However Thormann et al demonstrated c-di-GMP production upon overexpression of MxdA, a GGDEF domain protein with a NVDEF-motif (Thormann *et al.*, 2006).

C-di-GMP specific phosphodiesterases, where the EAL domain is the active domain, catalyse the hydrolysis of c-di-GMP to the apparently inactive linear diguanosine monophosphate (l-di-GMP) (Christen *et al.*, 2005; Schmidt *et al.*, 2005; Tamayo *et al.*, 2005). The active site of the EAL domain is not identified, but amino acid sequence alignment of experimentally verified active phosphodiesterases revealed conserved motifs. Within the EAL domain, which are degenerate in some EAL-domain proteins that do not function as phosphodiesterases. The EAL-motif is on the other hand not perfectly conserved in the active phosphodiesterases, where the alanine (A) often is

exchanged for other aliphatic amino acids. The glutamate (E) in the EAL motif has been suggested to function in coordinating the metal ion co-factor necessary for proper activity. The EAL domain acts as a monomer and is active in alone.

A second class of c-di-GMP specific phosphodiesterases has recently been demonstrated to hydrolyse c-di-GMP to GMP (Ryan *et al.*, 2006). This class of phosphodiesterases share the conserved HD-GYP sequence motifs, previously predicted to have c-di-GMP hydrolysing activity (Galperin *et al.*, 2001b)

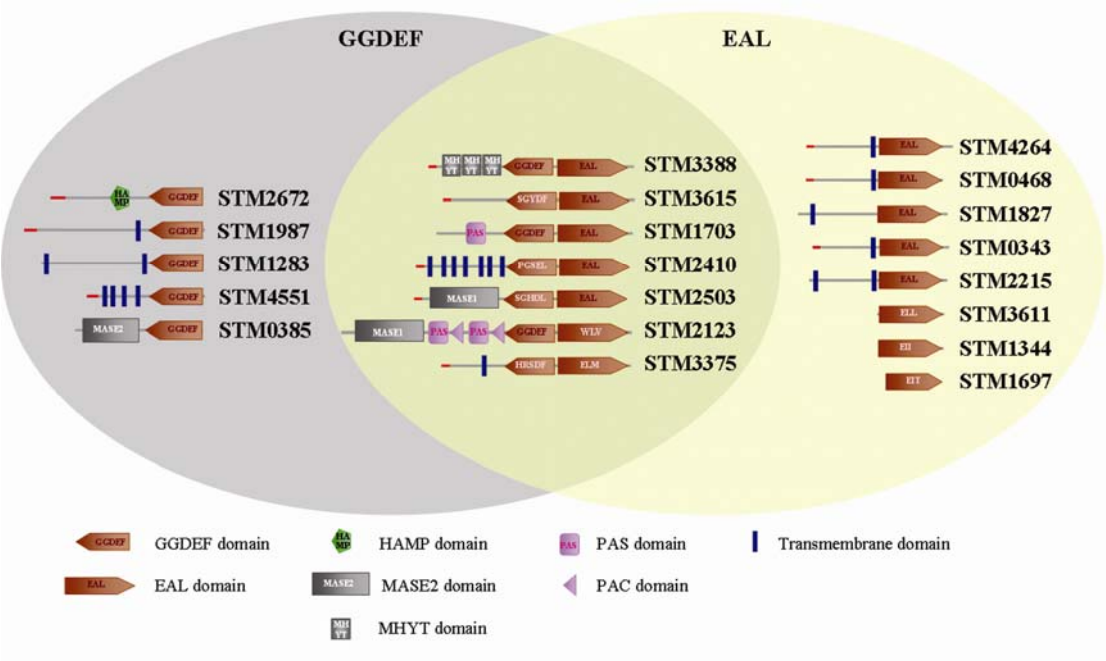

Figure 4. Venn diagram displaying the domain architecture of the GGDEF/EAL domain proteins in *S.* Typhimurium. Five proteins contain the GGDEF domain, eight proteins have the EAL domain, whereas seven proteins comprise both the GGDEF and EAL domains. Some proteins have additional domains in the N-terminal regions. These domains are mainly predicted sensors of environmental stimuli and may be required for proper activity of the protein. The different domains include: the HAMP domain that has a putative role in signal transduction (Hulko *et al.*, 2006), the MASE1 and MASE2 domains implicated in sensing signals at the membrane (Nikolskaya *et al.*, 2003), as well as MHYT, a membrane domain predicted to sense signals like O_2, CO or NO (Galperin *et al.*, 2001a). The PAS domain, often function as an oxygen sensor and the PAC domain is often found associated with the PAS domain where it may be important for the proper PAS-fold. Transmembrane regions may anchor the protein to the membrane

4.1.1 Distribution of c-di-GMP metabolising proteins in bacteria

Although database searches and cross-comparison of genome sequences revealed an abundance of proteins with GGDEF, EAL and HD-GYP domains (Galperin *et al.*,

2001b), the c-di-GMP signalling system seems to be restricted to bacteria since no confirmed copies were found in Archae or Eukarya (Galperin, 2004). The number of GGDEF, EAL and HD-GYP domain proteins encoded in single genomes varies from none in some bacteria reaching ~100 in others. Proteobacteria are especially rich in putative c-di-GMP metabolising proteins, whereas, for example, Firmicutes tend to have just a few of these domains per genome (Galperin, 2005). The distribution of the GGDEF, EAL and HD-GYP domains in different bacteria is biased towards free-living species. Bacteria such as *Helicobacter Pylori* that are well adapted to the human host have no copies of either domain. Environmental water species, on the other hand, like *P. aeruginosa* and *V. cholera*, which frequently adapt to quickly changing and adverse environments, have a total of ~60 proteins each.
The *S.* Typhimurium (LT2) genome encodes 20 proteins with the GGDEF and/or EAL domain, five of these proteins have the GGDEF domain, eight proteins contain the EAL domain whereas seven proteins constitute both domains (fig 4). *S.* Typhimurium does not encode any HD-GYP domain protein.

4.1.2 Function of GGDEF and EAL domains

Systematic approaches to elucidate the role of GGDEF and EAL domain proteins in different species have revealed apparently functional and non-functional domains with respect to the diguanylate cyclase and phosphodiesterase activities (Kader *et al.*, 2006; Kulasakara *et al.*, 2006; Lim *et al.*, 2006; Ryan *et al.*, 2007; Simm *et al.*, 2007).

Proteins with one output GGDEF or EAL domain make up the majority of putative diguanylate cyclases and phosphodiesterases whereas tandem arrangement is found in ~27% the GGDEF and/or EAL domain proteins as annotated in the SMART database (http://smart.embl-heidelberg.de/). Three possibilities may be imagined for the function of sequential domains where (i) both domains (ii) only one domain or (iii) none of the domains have the predicted enzymatic activity.

4.1.2.1 Proteins with functional GGDEF and EAL domains

The phytochrome BphG1, from *Rhodobacter Sphaeroides*, has relatively complex domain architecture with the domain order PAS-GAF-PHY-GGDEF-EAL. The PAS-GAF-PHY segment, commonly found in phytochromes is responsible for the detection of light. Biochemical studies suggested that the full length protein is a light independent, c-di-GMP specific phosphodiesterase. Whereas the truncated protein, lacking the EAL domain, has light induced diguanylate cyclase activity (Tarutina *et al.*, 2006). Although EAL domains function as monomers, the EAL domain of BphG1 spontaneously dimerizes. The diguanylate cyclase activity of GGDEF domains requires dimerization in combination with a conformational change for activity. The authors suggest a regulatory mechanism where dimerization of the EAL domain sterically prevents the conformational change in the GGDEF domain which would result in an enzymatically functional diguanylate cyclase

In *V. parahaemolyticus*, scrG, a PAS-GGDEF-EAL domain protein that is a positive regulator of swarmer cell differentiation was demonstrated to require the EAL domain for proper function (Kim and McCarter, 2007). Although overexpression of ScrG

resulted in decreased c-di-GMP concentration whereas overexpression of the truncated protein $ScrG_{\Delta EAL}$ lacking the EAL domain, resulted in increased c-di-GMP concentration it is premature to assign diguanylate cyclase activity to the GGDEF domain. There are two reasons for this, (i) the GGDEF motif is poorly conserved (HDDDF) indicating an enzymatically inactive domain and (ii) point mutations in selected amino acids, previously known to inactivate diguanylate cyclases, did not alter the effect of the $ScrG_{\Delta EAL}$ on c-di-GMP mediated polysaccharide production compared to the wild type protein (Kim and McCarter, 2007).

Although it has never been demonstrated experimentally it is conceivable that both the GGDEF and EAL domains are active simultaneously in one protein. This scenario would lead to a highly localised regulation of c-di-GMP signalling, which might only be feasible if the protein is in close contact with the effector protein.

4.1.2.2 EAL domain as a phosphodiesterase under allosteric control

A more common finding is GGDEF-EAL domain proteins with only one activity indicating one active and one inactive domain. Recently, as a first function of a degenerate GGDEF domain, CC3396 a GGDEF-EAL domain protein was shown to bind GTP (Christen *et al.*, 2005). By doing so, the activity of the successive EAL-domain was positively regulated. Several other GGDEF-EAL domain proteins have been shown to require the function of the degenerate GGDEF domain for proper activity of the successive EAL domain (Kazmierczak *et al.,* 2006; Kuchma *et al.*, 2007). It has been speculated, that in agreement with CC3396, the phosphodiesterase activity of these proteins is under allosteric control from GTP binding to a degenerated GGDEF domain. A physiological function for GTP stimulated c-di-GMP degradation was proposed by Chirsten et al. suggesting that uncontrolled phosphodiesterase activity could result in depletion of GTP. To avoid drainage of the GTP pool phospho-diesterases allosterically activated by GTP, quickly turn off the activity in response to low GTP levels (Christen *et al.*, 2005).

Bioinformatic analysis of the EAL domain proteins in *S.* Typhimurium identified two proteins (STM1344 and STM3611) with the similar domain architecture where basically the whole protein constitutes the EAL domain. Alignment of the amino acid sequences from these two proteins revealed ~30% similarity (our unpublished data). Alignment of the amino acid sequence of STM3611 against the consensus EAL sequence revealed that most of the conserved motifs predicted to be characteristic for an active phosphodiesterase were conserved (Schmidt *et al.*, 2005), our unpublished data). This is in agreement with experimental data indicating that STM3611 is a functional phosphodiesterase (Simm *et al.*, 2004)(fig. 5). A similar analysis of the amino acid sequence from STM1344 demonstrated a degenerate alignment compared to the consensus sequence that indicated an enzymatically inactive EAL-domain protein (Simm, Römling unpublished data). Analysis of mutants in STM1344 and STM3611on CR plates after incubation at 28°C for 20h demonstrated apparently opposite functions for these proteins. The STM3611 mutant was upregulated in the rdar morphotype compared to the wild type, had increased expression of CsgD as shown by western blotting as well as increased expression of Curli demonstrated by coomassie staining of CsgA after depolymerization and enrichment (fig. 5). The STM1344 mutant

displayed downregulated rdar morphotype, as well as undetectable levels of CsgD and CsgA (fig. 5). In the swimming assay for flagellum mediated motility in semisolid agar the STM3611 mutant displayed ~70% reduction in motility, whereas the STM1344 mutant demonstrated a hyper motile phenotype (fig 5). The results for STM3611 are characteristic for a mutant in a functional phosphodiesterase, while STM1344 demonstrated a regulatory function consistent with diguanylate cyclase activity. Cross complementation of STM1344 with confirmed phosphodiesterases and diguanylate cyclases indicated that STM1344 does not contain either enzymatic activity (Simm, Römling unpublished data). The function of STM1344 is at present not known, but it appears that the protein does not have c-di-GMP metabolising activity.

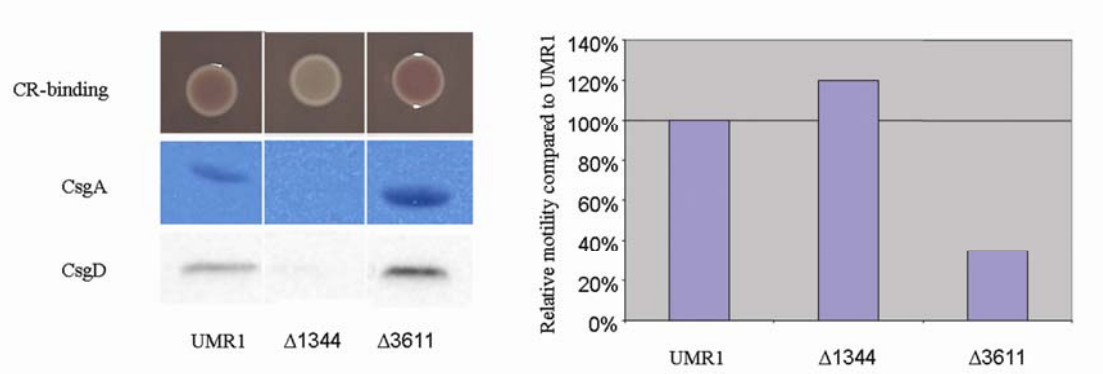

Figure 5. Effects of STM1344 and STM3611 mutations on the rdar morphotype development and flagellum mediated swimming motility. Mutation in the EAL domain protein STM3611 resulted in increased expression of the rdar morphotype, as well as the increased CsgA and CsgD levels compared to the wild type UMR1. In contrast, mutation in the EAL domain protein STM1344, results in downregulation of the rdar morphotype as well as decreased CsgA and CsgD expression (left). The effect on flagellum mediated swimming motility by inactivation of STM3611 is a motility defective mutant displaying approximately ~70% reduction in motility compared to the wild type UMR1. Deletion of the STM1344 gene results in a hypermotile mutant compared to UMR1, with ~20% increased motility (right).

4.1.2.3 Unorthodox function of GGDEF and EAL domains

Searching for regulators of small RNAs in *E. coli*, Suzuki et al identified CsrD, a GGDEF-EAL domain protein with amino acid sequences that deviated from the GGDEF and EAL consensus. The protein is involved in regulation of functions normally mediated by c-di-GMP signalling, like biofilm formation and flagella mediated motility. Experimental evidence from the authors show that CsrD lacks both diguanylate cyclase and phosphodiesterase activity (Suzuki *et al.*, 2006). It was shown that CsrD binds small RNA and targets them for RNAse E degradation.

4.2 SPECIFICITY OF C-DI-GMP SIGNALLING

Multiple GGDEF and EAL domain proteins in single genomes raised questions about the specificity of these proteins. The abundance of GGDEF and EAL domain proteins imply either redundant functions or a highly regulated signalling network. Intuitively, it

is difficult to justify a system of up to 100 proteins with similar activity, involved in the synthesis of a hypothetically freely diffusible signalling molecule. However, several factors indicate strictly controlled synthesis and degradation of c-di-GMP, which non-redundantly regulate different phenotypes in a spatial and temporal fashion as a response to external or internal stimuli. First, many of the c-di-GMP metabolising domains are associated with different sensory domains indicating a regulatory function in response to diverse environmental signals (fig. 5 (Jenal, 2004; Romling, 2005) Second, genetic experiments identifying a role for c-di-GMP metabolising proteins in different phenotypes suggested non-redundant functions (Aldridge and Jenal, 1999; Romling *et al.*, 2000; Slater *et al.*, 2000; Tischler *et al.*, 2002). Third, the regulation of various GGDEF and EAL domain proteins differ under diverse environmental conditions and growth phases. The apparent activity of different GGDEF or EAL domain proteins in vivo differ widely (Kader *et al.*, 2006; Kulasakara *et al.*, 2006; Simm *et al.*, 2005; Simm *et al.*, 2007).

4.3 SIGNALS REGULATING C-DI-GMP METABOLISM

The output domains (GGDEF and EAL) regulating c-di-GMP metabolism are found connected to a multitude of membrane associated and cytoplasmic signal sensing domains (fig. 4). Therefore it is likely that various external and internal signals control the enzymatic activities. It was for example demonstrated that oxygen binds to the PAS domain of PDEA1 from G. xylinus and possibly regulates the phosphodiesterase activity (Tomita *et al.*, 2002). The GAF domain is encoded in eukaryotic and prokaryotic genomes. In eukaryotes, the GAF domain is often associated with cAMP or cGMP dependent phosphodiesterases. The GAF domain binds small molecules, such as cGMP, which results in modulation of the protein activity (Martinez *et al.*, 2002). The function of the GAF domain in bacteria is uncertain, but it is possible that it binds cGMP, cAMP or perhaps c-di-GMP. Regulation by environmental signals may also take place via two-component systems, which consist of one sensor kinase that receives the signal and activates a second protein, which is the response regulator carrying out the effector function. The response regulator is activated via phosphorylation of a receiver domain. Phosphorylation is required for full activity of the diguanylate cyclases Rrp1 in *Borrelia burgdorferi* (Ryjenkov *et al.*, 2005), WspR in *P. aeruginosa* (Hickman *et al.*, 2005) and PleD in *C. crescentus* (Paul *et al.*, 2004).

By solving the crystal structure of the GGDEF domain containing response regulator PleD Chan et al. provided important information regarding the regulation of c-di-GMP synthesis in PleD. Apart from the active site (A-site) of the diguanylate cyclase, which is actually constituted by the GGDEF-motif, they identified an inhibitory site (I-site), which binds two intercalated c-di-GMP molecules, which results in a feedback inhibition mechanism (Chan *et al.*, 2004). The I-site is located in the GGDEF domain and is composed of a conserved RxxD sequence motif which was demonstrated to be important for binding. This motif is found in 57% of GGDEF and 27% of GGDEF/EAL domain proteins and may constitute a general mechanism for regulating the c-di-GMP synthesising activity of these proteins (Chan *et al.*, 2004).

The vast amount of GGDEF, EAL and HD-GYP domains in bacteria and their association with diverse signal input domains has lead to the speculation that environmental signals converge at the c-di-GMP signalling network deciding the life style of bacteria.

4.4 EFFECTOR PROTEINS IN C-DI-GMP SIGNALLING

Although it is established that the synthesis and degradation of c-di-GMP is due to the activity of GGDEF and EAL domains respectively, little is known about the targets of c-di-GMP signalling. Until recently, the only implicated direct target for c-di-GMP was the cellulose synthase of *G. xylinus* (Ross *et al.*, 1987).

A recent in silico study by Amikam and Galperin identified the PilZ domain as a likely c-di-GMP binding domain (Amikam and Galperin, 2006). The PilZ-domain is named after PilZ (PA2960) which is a 118 amino acid protein required for twitching motility in *P. aeruginosa*. Twitching motility in *P. aeruginosa* is reduced under high intracellular concentrations of c-di-GMP indicating that it is a target for c-di-GMP signalling (Huang *et al.*, 2003; Kazmierczak *et al.*, 2006; Simm *et al.*, 2004). A PSI-BLAST search starting with the PilZ-domain of GSU3263, a response regulator of *Geobacter sulfurreducens* resulted in more than 600 domains present in different proteins in diverse bacteria. The distribution of the PilZ domain correlated well with other proteins involved in c-di-GMP turnover, with an absolute correlation between the presence of consensus GGDEF and EAL-domains and PilZ domain proteins in beta, gamma and delta Proteobacteria. Interestingly, the PilZ-domain was found in several proteins regulating c-di-GMP dependent phenotypes like exopolysaccharide production and motility. Consistent with the apparent absence of a c-di-GMP signalling system in Eukaryota and Archae there are no PilZ-domain proteins found in any organisms from these kingdoms. Eubacterial genomes on the other hand may encode several PilZ-domain proteins. The number of PilZ domain proteins encoded in a single genome ranges from none in some bacteria like *S. aureus*, two in *S.* Typhimurium, five in *V. cholera* up to 15 in *Bdelovibrio bacteriovorus* (Amikam and Galperin, 2006). Recently published experimental evidence confirms that the PilZ-domain binds c-di-GMP in vitro (Christen *et al.*, 2007; Merighi *et al.*, 2007; Pratt *et al.*, 2007; Ramelot *et al.*, 2007; Ryjenkov *et al.*, 2006).

5 AIMS OF THE THESIS

The general aim of the thesis was to characterize the c-di-GMP signalling network in S. Typhimurium.

Specific aims were:

I. Develop a method to detect c-di-GMP in nucleotide extracts from bacteria. Analyze the function of the GGDEF and EAL domains in c-di-GMP metabolism. Elucidate the role of c-di-GMP in the physiology of *S.* Typhimurium.

II. Characterize the function of the GGDEF and/or EAL domain proteins in rdar morphotype development. Analyze the role of the GGDEF and/or EAL domain proteins in different c-di-GMP mediated functions.

III. Identify and characterize targets in the c-di-GMP signalling network in order to understand the molecular mechanisms of c-di-GMP signalling.

6 RESULTS AND DISCUSSION

6.1 PAPER I:

GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility.

Characterization of the rdar morphotype in *S.* Typhimurium revealed that the extracellular matrix consists of the major structural polymers cellulose and curli (Romling *et al.*, 2000; Romling, 2005; Zogaj *et al.*, 2001). The biosynthesis of cellulose is dependent on AdrA, a protein composed of an N-terminal MASE2 domain and a C-terminal GGDEF domain (Nikolskaya *et al.*, 2003; Romling *et al.*, 2000; Zogaj *et al.*, 2001). In G. xylinus, proteins with the GGDEF and EAL domains control the intracellular concentrations of c-di-GMP, the allosteric activator of the cellulose synthase (Tal *et al.*, 1998). Although cyclic nucleotide mediated signal transduction is a general regulatory mechanism in both pro- and eukaryotes the c-di-GMP signalling was considered to be restricted to a few plant associated bacterial species. However, the sequencing of bacterial genomes revealed an abundance of GGDEF and EAL domain proteins throughout the bacterial kingdom which led to the prediction that c-di-GMP may function as a general second messenger in bacteria (Galperin *et al.*, 2001b). Although structural modelling and genetic experiments predicted c-di-GMP synthesising activity for the GGDEF domain (Ausmees *et al.*, 2001; Pei and Grishin, 2001), experimental evidence of the catalytic function was missing.

In this study we developed a high pressure liquid chromatography (HPLC)-mass spectrometry (MS) based method to detect and characterize c-di-GMP. This technical development allowed us to demonstrate that overexpression of the GGDEF domain protein AdrA significantly increased the c-di-GMP levels in S. Typhimurium. Two point mutations in the GGDEF motif changing the sequence to AIDE resulted in an inactive protein unable to activate c-di-GMP production. Co-expression of STM3611 (Shih), a protein that consist of solely an EAL domain, resulted in decreased c-di-GMP production, The activity of STM3611 was lost by mutating a glutamate to an alanine in the conserved EAL motif. The results indicate that the diguanylate cyclase activity is confined to the GGDEF domain and that the EAL domain is the catalytic domain for c-di-GMP degradation. However, the experiments were carried out in vivo and an indirect regulatory function, of AdrA and STM3611, in the c-di-GMP turnover could not be excluded. The concept of diguanylate cyclase activity confined to the GGDEF domain and the phosphodiesterase activity residing in the EAL domain was generalized using the GGDEF domain protein YedQ and the EAL domain protein STM1827. These proteins have a different domain architecture compared to AdrA and STM3611 with only the GGDEF and EAL domain in common, respectively.
The protein encoded on the YedQ expressing plasmid was wrongly designated YhcK in our publication and the original study where it was constructed (Ausmees *et al.*, 2001). However, gene sequencing identified the protein as YedQ (Gjermansen *et al.*, 2006). Biochemical characterization of several GGDEF domain proteins from different bacterial species has established that the GGDEF domain harbour the catalytic activity responsible for c-di-GMP synthesis (Paul *et al.*, 2004; Ryjenkov *et al.*, 2005). The crystal structure of the response regulator PleD from C. crescntus revealed that the

GGDEF domain is part of the active site of the GGDEF domain (Chan *et al.*, 2004). Characterization of diguanylate cyclases showed that mutation of any amino acid residue in the GGDEF motif results in enzymatically inactive proteins (Chan *et al.*, 2004; Ryjenkov *et al.*, 2005). The catalytic activity of the GGDEF domain requires dimerization (Chan *et al.*, 2004; Paul *et al.*, 2007; Ryjenkov *et al.*, 2005). The mechanism proposed for the catalytic activity of the diguanylate cyclase, PleD, involves binding of GTP to the GGDEF domain. Following this, phosphorylation induced dimerization of two substrate binding PleD monomers, bringing the GGDEF-GTP complexes in close contact, results in catalyzed condensation of the GTP molecules into c-di-GMP (Chan *et al.*, 2004; Paul *et al.*, 2007).

Although the active site of the EAL domain has not been identified, biochemical characterization of several proteins has revealed that the EAL domain has c-di-GMP specific phosphodiesterase activity (Christen *et al.*, 2005; Schmidt *et al.*, 2005; Tamayo *et al.*, 2005). C-di-GMP hydrolysis is catalyzed by monomeric EAL domain proteins and the major product is a linear di-GMP molecule (l-di-GMP) (Schmidt *et al.*, 2005). A small amount of the l-di-GMP is further hydrolysed to GMP, but cleavage of the second phosphodiester bond appears to be unspecific (Christen *et al.*, 2005; Schmidt *et al.*, 2005).
Summarizing own experimental evidence and data from the literature, Schmidt et al., concluded that c-di-GMP specific phosphodiesterase activity is characteristic for proteins with consensus amino acid motifs within the EAL domain (Schmidt *et al.*, 2005). Proteins with amino acids deviating significantly from the consensus sequence have been shown to lack c-di-GMP specific catalytic activity (Suzuki *et al.*, 2006), Simm, Römling unpublished results). A discrimination of proteins containing putative active and inactive domains with respect to c-di-GMP hydrolyzing activity is possible with reasonable certainty by sequence comparison of the investigated EAL domain with the consensus sequence for functional phosphodiesterases (Schmidt *et al.*, 2005), own unpublished observation).

Considering the biological function of c-di-GMP, we demonstrated that the intracellular c-di-GMP concentration mediated by GGDEF domain proteins such as AdrA correlates with the amount of cellulose biosynthesis in S. Typhimurium. On the other hand, overexpression of the EAL domain protein STM3611 lead to decreased c-di-GMP concentrations and cellulose biosynthesis. Therefore cellulose biosynthesis in S. Typhimurium requires c-di-GMP as an activator.

To broadly investigate the effects of c-di-GMP in S. Typhimurium, we investigated also other biofilm related phenotypes such as adherence behaviour in different media and flagella-mediated motility. In summary, c-di-GMP mediated biofilm behaviour and inhibited flagella-mediated swimming and swarming motility.

To generalize the effects of c-di-GMP on biofilm formation and motility, AdrA and STM3611 were overexpressed in E. coli and P. aeruginosa. Assuming that the ectopic expression of diguanylate cyclase and phosphodiesterase increase and decrease the c-di-GMP concentration respectively under all environmental conditions we demonstrate that c-di-GMP activates biofilm formation, but inhibits flagella mediated motility in S.

Typhimurium, E. coli and P. aeruginosa, where c-di-GMP also inhibited type IV pili mediated twitching motility.

Based on the experiments described above we developed the concept of c-di-GMP as a general secondary messenger regulating the transition from motile to sessile cells. This function is today accepted as the major general effect of c-di-GMP signalling in bacteria (Kazmierczak *et al.*, 2006; Kim and McCarter, 2007; Kuchma *et al.*, 2007; Morgan *et al.*, 2006; Rahman *et al.*, 2007; Romling and Amikam, 2006; Thormann *et al.*, 2006). However, the broad impact of c-di-GMP regulation on bacterial physiology is just starting to be appreciated. In addition to the transition from motile to sessile behaviour and single cell- to community life styles, the effect on virulence phenotypes indicates that c-di-GMP is arising as a second messenger regulating the adaptation from acute to persistent infections (Cotter and Stibitz, 2007; Dow *et al.*, 2006; Tamayo *et al.*, 2007)

6.2 PAPER II:

Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of Salmonella enterica serovar Typhimurium.

In S. Typhimurium there are five proteins containing the GGDEF domain, eight proteins with the EAL domain and seven proteins with both domains (Romling, 2005). The reason for this wealth of proteins predicted to be involved in the turnover of a supposedly freely diffusible second messenger was not understood. However, since deletion of the GGDEF domain protein AdrA results in a strain deficient in cellulose production, but with motility comparable to the wild type S. Typhimurium UMR1 (Romling *et al.*, 2000), it appeared that the different GGDEF domain proteins do not have redundant functions. We hypothesized that overexpression of the GGDEF domain protein AdrA and the EAL domain protein STM3611 resulted in altered cellulose production and flagella mediated motility most likely due to the excessive production and depletion of c-di-GMP (paper I, (Simm *et al.*, 2004)). Consequently, overexpression of AdrA influenced most, if not all, phenotypes affected by c-di-GMP signalling in S. Typhimurium, but normally not regulated by this protein under physiological conditions. In this study we analyze the role of all GGDEF domain proteins in the development of the rdar phenotype in S. Typhimurium.

By altering the concentration of c-di-GMP in the wild type strain S. Typhimurium UMR1, which is proficient in cellulose and curli expression it was shown that the entire rdar phenotype was affected. This finding indicated that not only cellulose synthesis, but also curli production is under c-di-GMP control. The effect of c-di-GMP on curli biosynthesis was confirmed in a cellulose deficient mutant of S. Typhimurium UMR1. Successive studies concentrated on to investigate on which regulatory level c-di-GMP mediated the regulation of curli biosynthesis. Investigating the expression of the structural component of curli fimbriae, CsgA and CsgD on the transcriptional and protein level indicated that the effect of c-di-GMP on curli biosynthesis is mainly mediated via control of the CsgD expression on the level of transcription or mRNA stability.
Transcriptome analysis in V. cholera revealed that increased c-di-GMP concentration results in significantly enhanced expression of the response regulator VpsT (Vibrio

polysaccharide synthesis). VpsT, which is a homologue of CsgD, positively regulates transcription of the *vps* genes required for production of the VPS polysaccharide. Considering the timing of altered vpsT transcription, the authors speculate that it is a direct response to increased c-di-GMP levels (Beyhan *et al.*, 2006).
How the transcriptional activity of CsgD as well as VpsT is modulated by c-di-GMP is not know. Different possibilities can be envisioned where (i) c-di-GMP binds directly to a transcriptional regulator modulating its DNA-related activity which consequently leads to enhanced *vpsT* expression , (ii) c-di-GMP binds to a receptor protein which interacts with a transcriptional regulator or (iii) c-di-GMP interacts directly with the DNA by base-pairing, possibly altering the structure of the DNA. In addition to this it is possible that c-di-GMP controls the stability of the mRNA, either directly by base pairing, or indirectly by modifying the activity of e.g. a ribonuclease.

Although overexpression of AdrA from a plasmid indicated a positive c-di-GMP mediated feedback loop regulating CsgD transcription, AdrA inactivation had no effect on CsgD expression in S. Typhimurium UMR1. In other words, AdrA is not required for CsgD transcription at physiological protein levels. Analysis of the rdar phenotype in mutants of the 11 other GGDEF domain proteins revealed that the GGDEF-EAL domain proteins STM2123 and STM3388 are involved in the positive regulation of CsgD expression. The regulatory contribution of the two proteins is temporally different with STM2123 acting early in the development of the rdar phenotype whereas STM3388 has a role at a later stage. By comparing the CsgD levels in a double mutant of STM2123 and STM3388 with the respective single mutants the effect on CsgD expression was demonstrated to be additive indicating that the two GGDEF-EAL domain proteins act in two different pathways.
Consistent with our observation that STM2123 and STM3388 enhance the intracellular c-di-GMP concentration up to six times, Garcia et al observed that, by overexpressing a subset of the GGDEF domain proteins from S. Typhimurium in an *adrA* mutant, six proteins, including STM2123 and STM3388, could functionally replace *adrA* indicating diguanylate cyclase activity (Garcia *et al.*, 2004). It is worth noting that the GGDEF domain protein STM1283 could only complement the adrA mutant in minimal media, but not in rich media indicating activation by an environmental signal.

Quantification of the c-di-GMP level in strains expressing STM2123 and STM3388 demonstrated six and three times increased concentration of c-di-GMP. This should be compared to the 10,000 time increase in the c-di-GMP levels when AdrA is overexpressed.
The reason for the great differences in apparent diguanylate cyclase activity between the three proteins is not fully understood. However, STM2123 and STM3388 are two of the most complex GGDEF domain proteins encoded by *S.* Typhimurium. In addition to the GGDEF domain, STM2123 has an N-terminal MASE1 domain followed by two sequential PAS-PAC domains as well as a C-terminal EAL domain (fig. 4). Bioinformatic studies predicted a functional GGDEF domain and an EAL domain lacking phosphodiesterase activity (Simm, Römling unpublished data). A similar analysis of the GGDEF and EAL domains of STM3388 predicts that both domains may have the expected enzymatic activity. In addition to the GGDEF and EAL domains, the N-terminus of STM3388 constitutes three sequential MHYT domains. AdrA on the other hand is composed of a GGDEF domain and an N-terminal MASE2 domain. Considering that all three proteins have N-terminal domains predicted to sense environmental signals it is possible that the conditions are suboptimal for the diguanylate cyclase activity of STM3388 and STM2123.

Chan et al predicted that all GGDEF domain proteins with the RxxD motif N-terminal of the GGDEF motif are subject to product inhibition (Chan *et al.*, 2004). Both STM2123 and AdrA have the RxxD motif, but considering the high in vivo production of c-di-GMP, feedback inhibition may not be a regulatory mechanism in AdrA. STM3388 which show the lowest apparent activity does not contain the RxxD motif. In the case of STM3388 it is possible that the low apparent diguanylate cyclase activity is dependent on phosphodiesterase activity in the EAL domain. However, phosphodiesterase activity has not been experimentally demonstrated in STM3388.

In a systematic study assessing the activity of GGDEF/EAL domain proteins in P. aeruginosa, Kulasakara et al detected diguanylate cyclase activity in eight of 33 GGDEF domain containing proteins (Kulasakara *et al.*, 2006). The quantification was done by reversed phase high performance liquid chromatography (RP-HPLC) with a detection limit of 12pmol/mg cells. The wild type level of c-di-GMP in P. aeruginosa has been estimated to be < 1 pmol/mg cells (Meissner *et al.*, 2007; Simm *et al.*, 2004). Therefore it cannot be excluded that other GGDEF domain proteins in P. aeruginosa have diguanylate cyclase activity, inducing subtle changes in c-di-GMP concentration, as presented above for STM2123 and STM3388.

Based on the work in paper I, in this work we further refined the analysis of c-di-GMP concentrations by HPLC and MS analysis. This technical development allowed us to determine c-di-GMP concentration in S. Typhimurium UMR1 wild type cells and consequently the effect of individual GGDEF/EAL domain proteins on the intracellular c-di-GMP concentration. We demonstrated that the c-di-GMP concentration varies over time in S. Typhimurium UMR1, whereby the c-di-GMP concentration of 578 fmol/mg cells after 10 h of plate-growth continuously decreases to 214 fmol/mg cells after 24 h of growth. We further showed that STM2123 and STM3388 do not contribute significantly to the total c-di-GMP pool under our experimental conditions. On the other hand, AdrA is required for the majority of the intracellular c-di-GMP concentration at 16h, but is not significantly involved in c-di-GMP production at 10h and 24h.
We hypothesise that the c-di-GMP mediated regulation on CsgD expression is dependent on a localized pool of c-di-GMP, which is highly regulated by STM2123, STM3388 and maybe other associated proteins, possibly in response to environmental signals sensed by the N-terminal sensory domains of these proteins. The c-di-GMP produced by AdrA on the other hand is most likely confined to the cell membrane, since the MASE2 domain is predicted to be a membrane spanning domain sensing, so far unknown, environmental signals. AdrA may be in close contact with the cellulose synthase and possibly other regulatory proteins localising the c-di-GMP pool dedicated to cellulose production to the membrane compartment. Upon overexpression of AdrA the regulatory mechanisms at the membrane are overcome and c-di-GMP spills over into the CsgD restricted pool, leading to increased CsgD expression.

6.3 PAPER III:

Role of EAL-containing proteins in multicellular behavior of Salmonella enterica serovar Typhimurium.

Following the characterization of the GGDEF domain proteins in paper II the characterization of the proteins involved in c-di-GMP metabolism was expanded to include the role of the eight EAL domain proteins in development of the rdar

morphotype. In addition to this all 15 EAL domain mutants were screened for other c-di-GMP regulated phenotypes such as pellicle formation, biofilm formation in liquid culture and flagella mediated motility.

A subset of four EAL domain proteins (STM1703, STM4264, STM3611 and STM1827) showed a phenotype consistent with the predicted function for a phosphodiesterase in the development of the rdar phenotype. This means that since phosphodiesterases degrade c-di-GMP, a mutation in an EAL domain protein with phosphodiesterase activity display an upregulated rdar phenotype due to increased c-di-GMP concentration. The four proteins differ in their contributions to regulation of the rdar phenotype. Mutants of STM3611 and STM1827 were moderately upregulated with respect to the rdar phenotype. Consistent with upregulation of the rdar morphotype, these mutants had upregulated expression of CsgD and increased c-di-GMP levels. Mutants of STM4262 and STM1703 were highly upregulated with respect to the rdar morphotype. Both mutants displayed upregulated expression of CsgD and increased c-di-GMP levels. However, STM4264 was shown to contribute to the regulation of rdar morphotype expression via degradation of c-di-GMP from two separate pathways/pools. One c-di-GMP pool/pathway mediates CsgD expression, whereas the other c-di-GMP pool/ pathway controls cellulose synthesis independent of CsgD. STM1703 on the other hand exerts its regulation of the rdar phenotype entirely via CsgD at a level downstream of STM4264 in the regulatory network.
In agreement with the above described findings, correlation of CsgD expression and c-di-GMP concentration showed that the c-di-GMP degraded by STM1703 is dedicated to a higher extend to CsgD expression than the c-di-GMP degraded by STM4264. Microarray analysis of E. coli MC4100 and its mutant with the homologue of STM1703 (*yciR*) demonstrated increased expression of the csgBAC operon in the mutant (Weber *et al.*, 2006). This finding is in agreement with our data that STM1703 represses CsgD expression. In addition to this, genetic analysis identified Mad, a GGDEF domain protein, which positively controls CsgD and curli expression. Mad has no homologue in S. Typhimurium. This finding demonstrated that CsgD expression is differentially regulated by c-di-GMP in closely related bacteria.
In V. cholera at least two Phosphodiesterase (MbaA and CdgC) have been shown to negatively regulate the expression of the CsgD homologue Vps T. In addition to this the EAL domain protein VieA is a negative regulator of the response regulator VpsR leading to VPS production (Tischler and Camilli, 2004). VpsR and VpsT have been shown to regulate each others expression (Casper-Lindley and Yildiz, 2004; Yildiz *et al.*, 2004), which raises the possibility that also VieA acts via VpsT which in turn modulates the transcription of *vpsR*.
Summarizing our data and data from the literature, we hypothesize that CsgD and its homologues are major targets for regulation by c-di-GMP signalling.

Investigation of the contribution of EAL domain proteins to other biofilm phenotypes showed that most, but not all EAL domain proteins involved in rdar morphotype expression also played a role in biofilm formation (adherence, cell clumping) and pellicle formation in liquid media. The STM1703 mutant was significantly upregulated in biofilm formation in liquid media after 48h incubation at 28°C in shaken glass tubes, whereas the increase in the STM4264 and STM1827 mutants was moderate. The STM3375 mutant was downregulated in biofilm formation. There was a significant increase in pellicle formation in the STM4264 and STM1703 mutants, while the pellicle formation of the STM1827 mutant was moderately up-regulated.

Investigation of biofilm-related phenotypes showed that the two EAL domain proteins STM3375 and STM3611 positively regulate flagella mediated motility as demonstrated by swimming and swarming assays.
The STM3375 homologue in E. coli (CsrD) was recently shown to lack both phosphodiesterase and diguanylate cyclase activity (Suzuki *et al.*, 2006). The function of the protein is not fully understood, but it may bind small RNAs and expose them for degradation by RNAseE. This finding suggests that STM3375 is not involved in c-di-GMP metabolism, but rather controls motility by an alternative mechanism.
On the other hand, in *Serratia*, PigX, a STM3375 homologue, was demonstrated to control production of the pigment prodigiosin which has antimicrobial effects. The in vivo function of PigX is dependent on the EAL domain.

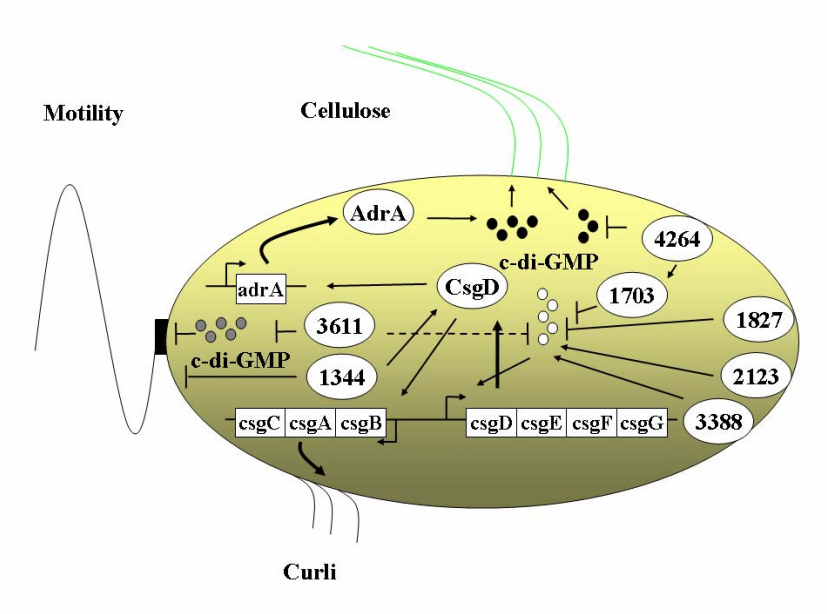

Figure 5. Working model of the regulatory network of GGDEF and EAL domain proteins involved in rdar morphotype development and flagellum mediated motility. CsgD is the central regulator of both curli and cellulose biosynthesis, by activating the expression of the csgBAC operon and AdrA respectively. Spatially separated c-di-GMP pools regulate specific functions. One pool (indicated in black), which is regulated by the GGDEF domain protein AdrA and the EAL domain protein STM4264 is probably allocated at the membrane regulating cellulose synthesis in close proximity to the cellulose synthase. A second c-di-GMP pool (indicated in white) regulates the CsgD expression. This pool is controlled by at least six proteins, the two GGDEF-EAL domain proteins STM2123 and STM3388 functioning as diguanylate cyclases, as well as the three EAL domain proteins STM3611, STM1827 and STM4264 and one GGDEF-EAL domain protein STM1703 functioning as phosphodiesterases. The function of the EAL domain protein STM1344 is not certain at present, but the protein is a positive regulator of CsgD expression.
STM3611 and STM1344 play a role in the regulation of flagella mediated motility. STM3611 is a phosphodiesterase degrading c-di-GMP, which results in increased motility, whereas STM1344 exerts negative control on the motility by an unidentified mechanism.

Ectopic expression of the EAL domain from the acknowledged phosphodiesterase YahA resulted in restoration of the prodigiosin production in a *pigX* mutant. This result indicates that PigX has phosphodiesterase activity thus regulating the prodigiosine production via degradation of c-di-GMP (Fineran *et al.*, 2007).
Sequence alignments between EAL domains with proven phosphodiesterase activity lead Scmidt et al to postulate that it is possible to predict if an EAL domain posses phosphodiesterase activity (Schmidt *et al.*, 2005). Similarly, Suzuki et al have found that CsrD homologues have degenerate amino acid sequence compared to the consensus sequence of functional phosphodiesterases but have instead alternative conserved amino acid motifs (Suzuki *et al.*, 2006). Considering that the amino acid sequence of PigX is not available, it is not possible to compare the PigX sequence to other EAL domain proteins and CsrD. Consequently it is not known if PigX should be predicted to function as a phosphodiesterase or as CsrD.
In the case of STM3375 the sequence alignment demonstrates 93% identity compared to CsrD with a degenerate EAL domain sequence suggesting a protein with a similar function as CsrD.

In the screen for functions of the EAL domain proteins in c-di-GMP mediated phenotypes the unconventional EAL domain protein STM1344 displayed a function, which differs from a conventional phosphodiesterase (fig. 5). The function of this protein is presently not known, but cross complementation of STM1344 with conventional phosphodiesterases and active diguanylate cyclases indicated a function distinct from c-di-GMP metabolism (Simm, Römling unpublished results).

Taken together, the results presented in paper II and paper III show that individual c-di-GMP metabolizing proteins are dedicated to the regulation of specific functions. We hypothesized that the expression and/or activity of different proteins is dependent on environmental conditions, temporal regulation and/or localization. Commitment of individual proteins with c-di-GMP synthesizing activity/GGDEF domain containing proteins or c-di-GMP degrading activity/EAL domain containing proteins to the regulation of distinct functions has been shown in similar systematic studies in *V. cholera*, *P. aeruginosa* and *X. campestris* (Kulasakara *et al.*, 2006; Lim *et al.*, 2006; Ryan *et al.*, 2007).

6.4 PAPER IV:

The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria.
In recent years a significant amount of work has been invested in understanding the mechanisms controlling the synthesis and degradation of c-di-GMP by GGDEF, EAL and HD-GYP domain proteins. C-di-GMP has been implicated as a regulator of diverse functions such as biofilm formation, motility, virulence and photosynthesis. However, major questions of the c-di-GMP signalling network remained unanswered. The only effector protein that was proposed to be a direct target for c-di-GMP signalling is the cellulose synthase of *G. xylinus*. In a publication from 2006, Amikam and Galperin predicted that the PilZ domain may function as a c-di-GMP binding domain. The PilZ domain was found at the C-terminus of bacterial cellulose synthases. The distribution of PilZ domains in different bacterial species correlated with the presence of GGDEF

and EAL domain proteins. The hypothesis that PilZ is a c-di-GMP binding domain was supported by the realization that several PilZ domain containing proteins are involved in c-di-GMP regulated phenotypes such as motility and polysaccharide production (Amikam and Galperin, 2006). S. Typhimurium has two PilZ domain proteins, the cellulose synthase BcsA and YcgR, a protein which was shown to regulate motility in E. coli (Ko and Park, 2000).

In this study we experimentally demonstrate by equilibrium dialysis between purified protein and c-di-GMP that YcgR from E. coli specifically binds c-di-GMP with a binding affinity that corresponds to physiologically relevant c-di-GMP concentrations. In a similar way, by expressing truncated forms of the proteins constituting the PilZ domain alone we show that the individual PilZ domains of YcgR from *E. coli* and BcsA from *G. xylinus* are sufficient for c-di-GMP binding. In addition to this we show that a point mutation changing a conserved arginine residue to glutamine in the PilZ domain results in a protein unable to bind c-di-GMP.
Although our study was the first published experimental evidence of the PilZ domain as a c-di-GMP binding protein, several subsequent publications have generalized the concept. The c-di-GMP binding capacity of the PilZ domain was independently demonstrated by three additional research groups in proteins from *V. cholera, P. aeruginosa and C. crescentus (Christen et al., 2007; Pratt et al., 2007; Ramelot et al., 2007)*.

The binding characteristics of YcgR suggests that c-di-GMP binds as a dimer at a single binding site on the protein. Gel filtration showed that, despite a higher molecular weight, YcgR with bound c-di-GMP elutes at a later time point compared to ligand-free YcgR. This observation indicates that YcgR undergoes a conformational change upon c-di-GMP binding resulting in a more compact protein. Possibly the N-terminal domain of YcgR (PilZN) somehow interacts with the ligand stabilizing the YcgR-c-di-GMP complex.
The structures of two PilZ domain proteins PA4608 and VCA0042 have been solved by NMR (Ramelot *et al.*, 2007) and X-ray crystallography (Protein Data Bank number 1YLN), respectively. Bioinformatic analysis, mutational studies and NMR-analysis upon titration of c-di-GMP assigned the c-di-GMP binding site to the β2 and β3 strand of the β-barrel structure (Christen *et al.*, 2007; Merighi *et al.*, 2007; Pratt *et al.*, 2007; Ramelot *et al.*, 2007; Ryjenkov *et al.*, 2006). NMR studies of the protein PA4608 indicated that the N-terminus of the PilZ domain may be dynamic (Christen *et al.*, 2007; Ramelot *et al.*, 2007). This region contains three conserved arginines which have been demonstrated to be required for c-di-GMP binding in different PilZ domain proteins (Christen *et al.*, 2007; Ryjenkov *et al.*, 2006)). It is hypothesized that upon c-di-GMP binding, the region shifts and wraps around the ligand to stabilize the protein-ligand interaction (Christen *et al.*, 2007). A similar mechanism may explain the migration of ligand-bound YcgR at an apparently smaller size compared to the ligand free protein despite of the higher molecular weight.

The biological effects of inactivation of YcgR and its homologue STM1798 in E. coli TOB1 and *S.* Typhimurium UMR1, were tested on rdar morphotype development, biofilm formation in liquid media, pellicle formation and motility. We detected no changes in the phenotypes compared to the E. coli and S. Typhimurium wild type

strains. We speculated that the inability to show a difference in the phenotypes was due to suboptimal conditions e.g. low c-di-GMP levels or an inactive protein. We inactivated STM3611 in S. Typhimurium UMR1; the homologous mutant in E. coli TOB1 could not be constructed. The STM3611 mutant has reduced flagella-mediated motility and increased c-di-GMP concentration (see paper III). We could demonstrate that inactivation of STM1798 in the STM3611 mutant restored swimming and swarming motility almost to wild type levels. On the other hand, overexpression of STM1798 (or YcgR from *E. coli*) in the double mutant STM3611 STM1798 repressed the motility to the level of the STM3611 single mutant. In contrast to this, overexpression of the YcgR mutant protein R>D incapable of c-di-GMP binding had no effect on motility. None of the biofilm related phenotypes were affected by YcgR inactivation or expression in the STM3611 mutant. In conclusion, STM1798 (YcgR) is an intracellular receptor for c-di-GMP regulating the flagella mediated motility in S. Typhimurium.
Later on, a similar effect of c-di-GMP levels on PilZ domain protein regulated motility was seen in C. crescentus. The proteins DgrA and DgrB, small PilZ domain proteins, bind c-di-GMP and negatively regulate motility when c-di-GMP concentrations are increased (Christen *et al.*, 2007). There was no detectable role in regulation of motility for these proteins under conditions with physiological c-di-GMP concentrations. The authors speculate that DgrA regulate motility by controlling the protein levels of FliL, which is required for flagellar rotation (Christen *et al.*, 2007).

Although c-di-GMP binding was formally not demonstrated for the BcsA of S. Typhimurium, it was shown for the homologue in *G. xylinus*. Considering the high identity in the amino acid sequence between these proteins, the mechanism of activation is predicted to be similar in the two organisms. On the other hand, it was recently demonstrated that alginate biosynthesis in P. aeruginosa is activated upon binding of c-di-GMP to the PilZ domain of Alg44 (Merighi *et al.*, 2007). Since several of the PilZ domain proteins are involved in polysaccharide synthesis, c-di-GMP binding to proteins belonging to the polysaccharide biosynthesis complex may be a general mechanism for allosteric activation of polysaccharide production. In agreement with this, it was recently demonstrated that the PEL polysaccharide in P. aeruginosa is regulated by c-di-GMP binding to PelD (Lee *et al.*, 2007).

In P. aeruginosa it was shown that c-di-GMP binds to seven of the eight identified PilZ domain containing proteins. Few phenotypes were described for PilZ domain proteins in P. aeruginosa. Besides the contribution of Alg44 in alginate biosynthesis, the name giving protein PilZ and PA2989 showed a defect in twitching motility. In addition the *PA2989* mutant showed decreased biofilm formation.

On the other hand, in V. cholera the five identified PilZ domain proteins (PlzA – E) showed a variety of complex phenotypes. Although consensus PilZ domain residues are present, only PlzC and PlzD were demonstrated to bind c-di-GMP by using various membrane or filter binding assays (Pratt *et al.*, 2007). In-frame deletion mutants were gained for *plzB, plzC,* and *plzD.* Investigation of the effect of *plzB, plzC,* and *plzD* mutations that PlzB and partially PlzC are positive regulators of swimming motility, while overexpression while overexpression of PlzD showed a negative regulator of motility independent of c-di-GMP binding. PlzB also positively regulated biofilm

formation in a PilZ dependent way at low c-di-GMP concentrations, whereas PlzB and PlzD function as positive regulators of biofilm formation at high c-di-GMP levels. Interesting is the fact that, in contrast to S. Typhimurium and C. crescentus, PilZ domain proteins are not negative, but positive regulators of motility. (Pratt *et al.*, 2007). In addition, a plzB mutant and a plzC plzD double mutant were affected in colonization of the intestine of infant mice.

Assuming functional specificity for PilZ domain proteins, the number encoded by bacterial genomes is too few to explain the phenotypes modulated by the intracellular concentration of c-di-GMP. As an example, in S. Typhimurium, cellulose production is activated by c-di-GMP binding to the PilZ domain of cellulose synthase, whereas, motility is negatively regulated by binding to the PilZ domain of YcgR. However, we have shown that in addition to controlling cellulose production down stream of the global regulator CsgD, c-di-GMP regulate biofilm formation at a level upstream of CsgD (Kader *et al.*, 2006; Simm *et al.*, 2007). The receptor for c-di-GMP signaling in this system awaits identification.

By solving the crystal structure of PleD from C. crescentus Chan and co-workers discovered an additional c-di-GMP binding site in the GGDEF domain apart from the active site (Chan *et al.*, 2004). Upon ligand binding to the site, the activity of the protein was repressed. This Inhibitory site was shown to include a conserved RxxD motif important for c-di-GMP binding (Chan *et al.*, 2004). In a recent publication, Lee et al demonstrated that c-di-GMP binding to PelD activates the production of the PEL polysaccharide in P. aeruginosa(Lee *et al.*, 2007). PelD does not contain the PilZ domain. Bioinformatic studies demonstrated that the secondary structure of PelD differs significantly from the PilZ domain. An RxxD motif in PelD is located in a similar secondary structure as the motif in PleD, the authors speculate that the binding pocket containing the RxxD motif in PleD may be conserved and responsible for the c-di-GMP binding in PelD (Lee *et al.*, 2007). These results suggest that there may be conserved structures, other than the PilZ domain, that bind c-di-GMP regulating the activity of proteins.

6.5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

In recent years, the perception of c-di-GMP signalling has evolved from c-di-GMP being considered as an allosteric activator of cellulose synthase in a few plant associated bacteria to its recognition as a general second messenger regulating complex physiological processes in diverse bacteria. C-di-GMP has been demonstrated to be a negative regulator of motility and virulence factors as well as being a positive regulator of biofilm formation. It has been postulated that c-di-GMP regulates transition from motility to sessility, conversion form dispersed populations to multicellular communities as well as development from acute to persistent infections. Today we have a fair understanding of the biochemistry behind synthesis and degradation of c-di-GMP, whereby the signals controlling the enzymatic activity of the diguanylate cyclases and phosphodiesterases have started to be recognized. The understanding of effector mechanisms involved in c-di-GMP signalling is increasing with the discovery of the PilZ domain as a c-di-GMP

binding domain. The discovery of the inhibitory RxxD motif in diguanylate cyclases as well as the prediction that a similar motif is required for the regulatory effects of c-di-GMP binding to PelD, indicate that apart from the PilZ domain, there may be other conserved c-di-GMP binding structures in effector proteins.

In *S.* Typhimurium characterization of the c-di-GMP signalling system has revealed a complex regulatory network of GGDEF and EAL domain proteins that control biofilm formation in a hierarchical fashion upstream and down stream of the central transcriptional regulator CsgD. The regulation of CsgD expression takes place on the level of transcription or mRNA stability, whereas the regulation of cellulose synthase down stream of CsgD is allosterically controlled by c-di-GMP binding to the PilZ domain of BcsA. In addition to this, motility is negatively controlled by c-di-GMP, which regulates the transition from motility to sessility. This is mediated via c-di-GMP binding to the PilZ domain of YcgR and the regulation of available c-di-GMP via degradation by STM3611. The number of c-di-GMP mediated phenotypes indicate that additional receptors for c-di-GMP signalling exist, which have not been identified.

Future experiments will aim to strengthen the experimental data on localized c-di-GMP pools regulating different cellular functions. Most likely, compartmentalization of c-di-GMP regulation is achieved through protein complex formation between c-di-GMP metabolizing proteins, c-di-GMP receptors and effector proteins. A reasonable approach would be to visualize the localization of different proteins demonstrated to regulate particular functions by fluorescence microscopy of chromosomally encoded fluorescently labeled proteins. Co-localization of proteins will be a good indicator of protein complex formation. In addition to this a biochemical approach will be undertaken. An appropriate method is immunoprecipitation of affinity tagged chromosomal copies of relevant proteins which will pull down interacting partners. A genome-wide approach like Microarray analysis which compares the wild type strain UMR1 with mutants of relevant GGDEF/EAL domain encoded genes will support the identification of gene products that may be co-regulated with the respective GGDEF/EAL domain protein.

Further studies will be undertaken to biochemically characterize the unorthodox EAL domain protein STM1344. In vivo characterization of the functions regulated by this protein indicates a protein lacking phosphodiesterase activity. Elucidation of the function of STM1344 and comparison of the function of STM1344 with the function of the phosphodiesterase STM3611, a protein with the same domain architecture and high amino acid homology could greatly increase the understanding of the molecular mechanism of c-di-GMP degradation and the functional shift leading to proteins with novel function in the c-di-GMP signalling network of S. Typhimurium.

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7 ACKNOWLEDGEMENTS

I would like to thank the people who have contributed to this thesis one way or the other. First out is **Ute Römling**, my supervisor for giving me the opportunity to work in your lab and for your enthusiasm for science. **Mats Andersson**, my co-supevisor for being easy going and always available when I have needed your advice. You are both very knowledgeable and working with the two of you has been an experience.

Present and former members of the Römling research group: **Kader** for your friendship and more or less serious discussions over the years. **Agaristi** and **Eugenie** for giving me a place to stay. I am forever in dept to you. The original Germans: **Uli** and **Maike**, for taking care of me when I first arrived in Stockholm. I felt welcome in the lab from the first day I arrived. **Xhavit** for teaching me many things during the first couple of months as a research student. **Linda** for making me realize what being a PhD student is really about. **Claudia** for bringing me to play football, taking my mind off other things. **Nina, Katharina, Xiaoda, Devrim, Ylimaz, Michaela, Linnea, Reddy, Tabea, Daniel, Dinesh, Heili** and any other people who I might have forgotten to mention. It has been a pleasure hanging out with you all in the multicultural group that we have constituted. A special thanks to my students **Astrid, Steffie** and **Karl,** I have learnt a lot, supervising you in the lab. People in the Andersson group:**Charlotte** for always having time to talk and for always having something to say. **Katrin P** for all the interesting and fun conversations about science, sports and beyond. **Olivia** for always being happy and nice. And thank you **Jenny** for always being supportive, willing to help and for all the lunches and late trips along the green line.

My collaborators:
Michael Morr, Dmitri Ryjenkov, Mark Gomelsky, Manfred Nimtz

All the other people at MTC whom I should have mentioned, but didn't. My PhD-studies have been…interesting and you have been part of the experience. I am running out of time and space, but I hope the people who deserve a thanx know it.

Säve, Katrin och Jocke med familj för all hjälp det senaste halvåret. Det har betytt enormt mycket för mig och min familj.

Sten och **Kerstin** för att ni alltid ställer upp på alla sätt ni kan.

Min släkt och vänner. Ni vet vilka ni är. Ett specielt tack till min syster **Therese** för att du stått ut med mig som storebror i alla år och för att vi får låna din händige man ibland

Mamma och **pappa** för att ni alltid funnits där och gjort allt för att jag ska kunna uppnå mina drömmar. Ni har stöttat mig i allt jag gjort sedan jag var en liten parvel med halvseriösa drömmar att bli hockey proffs.

Sist men inte minst, min älskade familj, **Maria**, **Isabel** och **Natali**. Ni har utstått en hel del på grund av mina forskarstudier. Tack för att ni finns.

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