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

Running title: task distribution of GGDEF domain proteins

Keywords: CsgD/cellulose biosynthesis/curli fimbriae/rdar morphotype/GGDEF/EAL

Abdul Kader[1], Roger Simm[1], Ulrich Gerstel[1], Michael Morr[2] and Ute Römling[1,*]

[1]*Microbiology and Tumor Biology Center (MTC), Karolinska Institutet, Box 280, SE-171 77 Stockholm, Sweden and* [2]*Division of Cell Biology and Immunology, Gesellschaft für Biotechnologische Forschung (GBF), 38124 Braunschweig, Germany*

*For correspondence. Email Ute.Romling@mtc.ki.se; Tel. (+46) 8 524 87319; Fax (+46) 8 330477.

Summary

GGDEF and EAL domain proteins are involved in the turnover of the novel secondary messenger cyclic-di(3'→5')-guanylic acid (c-di-GMP) in many bacteria. In this work the role of the 12 GGDEF domain proteins encoded by the *S.* Typhimurium chromosome in rdar morphtype development was investigated. Previously, it was shown that the GGDEF domain protein AdrA activated the biosynthesis of cellulose by production of c-di-GMP. Enhancement of the c-di-GMP levels by overexpression of the GGDEF domain protein AdrA did lead to the activation of curli fimbriae biosynthesis through the elevated expression of CsgD and CsgA. Although knock-out of the chromosomal copy of *adrA* influenced CsgA expression, CsgD expression was not altered, although more than half of the total cellular c-di-GMP was produced by AdrA at 16 h of growth. On the other hand, chromosomally encoded GGDEF-EAL domain proteins STM2123 and STM3388 were required to additively activate CsgD expression on a transcriptional and post-transcriptional level. Enhanced c-di-GMP levels did overcome temperature regulation of rdar morphotype expression by activation of curli fimbriae as well as cellulose biosynthesis through CsgD expression. Thus in the regulatory cascade leading to rdar morphotype expression c-di-GMP activates several subsequent steps in the network.

Introduction

Although first recognized as an allosteric activator of the cellulose synthase in the fruit-degrading bacterium *Gluconacetobacter xylinus* (Ross, 1987; Tal *et al.*, 1998) the cyclic dinucleotide cyclic-di(3'→5')-guanylic acid (c-di-GMP) emerged recently as a novel secondary messenger in Bacteria. Few examples showed that c-di-GMP turnover is mediated by proteins containing GGDEF and EAL domains (Paul *et al.*, 2004; Schmidt, 2005; Simm, 2004; Tischler and Camilli, 2004). Thereby, product analysis indicated that GGDEF domains perform dinucleotide cyclase activity and EAL domains phosphodiesterase activity (Simm, 2004; Tischler and Camilli, 2004), whereby proteins with both domains display either of the two activities (Tal *et al.*, 1998).

Distinct phenotypes have been detected for GGDEF/EAL domain proteins in many Bacteria (D'Argenio and Miller, 2004; Jenal, 2004; Römling, 2005b). GGDEF/EAL domain proteins modulate the multicellular behaviour commonly termed as biofilm formation through differential expression or functional modification of cell surface components such as exopolysaccharides, fimbriae and flagella (Aldridge *et al.*, 2003; D'Argenio *et al.*, 2002; Ko and Park, 2000; Römling *et al.*, 2000) and the expression of extracellular signalling molecules (Gronewold and Kaiser, 2001; Huber *et al.*, 2001).

GGDEF and EAL protein domains belong to two of the largest superfamilies of bacterial proteins, whereby an individual bacterial genome can encode between 0 to 99 GGDEF/EAL domain proteins (Galperin *et al.*, 2001b; Galperin, 2004). Such a redundancy of paralogous proteins in one cell is a challenge for functional studies, which plan to elucidate the physiological role of signalling pathways via the second messenger molecule c-di-GMP. One might imagine that temporal and spatial expression of GGDEF domain proteins control the specificity of the c-di-GMP response. In addition, differential activation by different

environmental stimuli and product inhibition might restrict c-di-GMP concentrations in the cell.

The *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) genome encodes 5 proteins with GGDEF and 7 with EAL domain, while 7 proteins harbour both domains. Functional studies have shown that overexpression of the GGDEF domain protein AdrA lead to elevated intracellular c-di-GMP concentration, while overpexpression of the EAL domain protein YhjH reduced the c-di-GMP concentration (Simm, 2004). These fluctuating c-di-GMP concentrations regulate cellulose biosynthesis, adherence to abiotic surfaces and swimming and swarming motility (Simm, 2004).

Recently, overexpression of five of 8 tested GGDEF domain proteins including AdrA, complemented a *S.* Typhimurium *adrA* mutant with respect to cellulose biosynthesis as monitored by the Calcofluor binding of colonies on LB agar plates at room temperature (Garcia *et al.*, 2004). An overlapping, yet distinct set of five GGDEF domain proteins, but not AdrA, complemented an STM1987 mutant, which showed deficiency of cellulose production in a carbon-source rich, nutrient-poor medium at 37°C. Those studies suggested a) functional redundancy for 6 of 8 GGDEF domain proteins and b) differential activation of AdrA and STM1987 under different environmental condition. Knock-out of the GGDEF/EAL domain protein STM1703 (*yciR*) lead to enhanced binding of Calcofluor, while overexpression of this gene product abolished Calcofluor binding, suggesting that this protein which contains a GGDEF and EAL domain functions as a phosphodiesterase Besides *adrA*, STM1703 and STM1987 knock-out mutants of the other 5 GGDEF domain proteins which were investigated did not show a phenotype (Garcia *et al.*, 2004).

S. Typhimurium produces the exopolysaccharide cellulose and proteinaceous curli fimbriae (previously called thin aggregative fimbriae *agf*) as extracellular matrix components of the multicellular rdar morphotype (Römling *et al.*, 1998a; Zogaj *et al.*, 2001). Both matrix

components are positively regulated by CsgD (previously called AgfD), a transcriptional regulator with an N-terminal receiver domain of the FixJ family and a C-terminal GerE-like helix-turn-helix motif. While CsgD is suggested to activate biosynthesis of curli fimbriae directly by binding to the promoter of the *csgBA* operon encoding the structural genes for curli fimbriae (Brombacher *et al.*, 2003; Hammar *et al.*, 1995), activation of cellulose biosynthesis by CsgD is indirect (Römling *et al.*, 2000). In this case, CsgD is required for the transcriptional activation of the GGDEF domain protein AdrA whereby AdrA in turn activates cellulose biosynthesis independently of CsgD (Zogaj *et al.*, 2001) through production of the secondary messenger molecule c-di-GMP (Simm, 2004).

The rdar morphotype is highly regulated by environmental conditions such as temperature in the majority of *S.* Typhimurium and *Salmonella enterica* serovar Enteritidis strains due to transcriptional regulation of *csgD* (Römling *et al.*, 1998a; Römling, 2003). Occasionally, semi-constitutive expression of the rdar morphotype is found, whereby promoter-up mutations confer temperature-independent CsgD and rdar morphotype expression (Römling *et al.*, 1998b).

In this work we extend the regulatory network of c-di-GMP physiology in *S.* Typhimurium. We show that elevated c-di-GMP concentrations mediated by AdrA overexpression did not only activate cellulose biosynthesis, but were also required for enhanced expression of curli fimbriae. C-di-GMP concentrations regulate curli fimbriae by enhancing CsgA expression and CsgD expression on the transcriptional and post-transcriptional level. In addition, elevated c-di-GMP concentrations overcome the temperature regulation of the regulated rdar morphotype by expression of CsgD at 37˚C.

In *S.* Typhimurium UMR1, which expresses the regulated rdar morphotype, there is task distribution involving temporally co-expressed GGDEF-EAL domain proteins. Under physiological expression levels, the GGDEF-EAL domain proteins STM2123 and STM3388

co-operate to activate the expression of CsgD on a transcriptional and posttranscriptional level. AdrA was required to activate cellulose biosynthesis and enhanced the expression of CsgA at 28ºC, but, surprisingly, participates only slightly, if at all in CsgD activation, although the majority of the c-di-GMP pool at 16 h is produced by AdrA. Although the same regulatory pattern by GGDEF-EAL domain proteins regulating CsgD and cellulose expression is seen in *S.* Typhimurium MAE52, the promoter-up mutation of *csgD* overcomes rdar morphotype reduction and elevated AdrA expression creates a positive feedback loop for CsgD expression. Thus c-di-GMP acts on various levels of the regulatory network leading to rdar morphotype expression whereby the impact of GGDEF/EAL domain proteins on cellular physiology might be partially determined by the expression level of the respective protein.

Results

c-di-GMP is required for expression of curli fimbriae

Recently, we showed that elevated c-di-GMP levels mediated by the GGDEF domain containing protein AdrA activated cellulose biosynthesis in *S.* Typhimurium, while reduced c-di-GMP levels achieved by expression of the EAL domain protein YhjH abolish cellulose biosynthesis (Simm, 2004). When AdrA was overexpressed in *S.* Typhimurium UMR1 using plasmid pWJB30 phenotypic analysis of colony morphology showed that not only cellulose biosynthesis, but expression of the whole rdar morphotype was enhanced when the cells were grown on agar plates at 28ºC (Fig. 1A). On the other hand, overexpression of YhjH in *S.* Typhimurium UMR1 using plasmid pRGS1 lead to an almost white morphotype. Those phenotypes of *S.* Typhimurium UMR1 indicated that not only cellulose biosynthesis was affected by changes in c-di-GMP concentration, but also the biosynthesis of curli fimbriae, the other extracellular matrix component co-expressed with cellulose (Römling *et al.*, 2000; Zogaj *et al.*, 2001). Modulation of c-di-GMP concentrations by AdrA and YhjH overexpression in strain MAE222 (expressing only curli fimbriae, but not cellulose) lead to up- and down-regulation of the bdar (brown, dry and rough) morphotype, indicative for expression of curli fimbriae (Fig. 1A). To simulate the effect of AdrA from no expression to overexpression strain AdrA1f (*adrA101*::MudJ) was used. Protein gels and Western blot analysis showed upregulated expression of formic-acid resistant CsgA, the subunit of curli fimbriae, when AdrA was overexpressed and downregulation of CsgA expression, when YhjH was overexpressed in comparison with the vector control in the wild type strain *S.* Typhimurium UMR1, the *adrA* mutant strain AdrA1f as well as the *bcsA* mutant strain MAE22 after 16 and 24 hrs (Fig. 1B and data not shown). Those results indicated that curli biosynthesis required the presence of c-di-GMP and was elevated when c-di-GMP levels were increased.

CsgD expression is regulated by c-di-GMP on a transcriptional and post-transcriptional level

To establish whether c-di-GMP acts post-transcriptionally on curli biosynthesis as it has been shown for cellulose biosynthesis (Ross, 1987; Simm, 2004; Zogaj *et al.*, 2001) or further upstream in the regulatory pathway leading to curli biosynthesis, AdrA and YhjH were overexpressed in *S.* Typhimurium UMR1 and expression of components leading to the biosynthesis of curli was investigated. p*csgBA* (Table 1), a *lacZ* transcriptional fusion to the *csgBA* promoter was constructed to measure the transcriptional activity of the *csgBA* operon which encodes the structural subunits required for the biosynthesis of curli fimbriae. To include major regulatory sequences (Arnqvist *et al.*, 1994), the cloned promoter fragment included 186 bp upstream of the transcriptional start site, the 86 bp leader sequences and 78 nucleotides of the *csgB* ORF (Römling *et al.*, 1998a). Enhanced transcriptional activity of the *csgBA* promoter was observed when AdrA was overexpressed and diminished transcriptional activity when YhjH was overexpressed as compared to the vector control after 16 and 24 h of growth at 28ºC (Fig. 1C). Those results suggested that regulation of curli biosynthesis by c-di-GMP occurred upstream of *csgBA* promoter activity. Therefore, in the next step the level of the CsgD protein, the proposed transcriptional activator of the *csgBA* promoter was investigated. The protein expression studies showed significant upregulation of CsgD when AdrA was overexpressed and drastically reduced levels of CsgD when YhjH was overexpressed (Fig. 1D). Analysis of the steady state levels of *csgD* transcript by Northern blotting during overexpression of AdrA and YhjH revealed the same trend (Fig. 1E). Those results showed that c-di-GMP is required for activation of expression of CsgD on a transcriptional level.

To investigate whether CsgD expression is also regulated on a post-transcriptional level by c-di-GMP CsgD was expressed by the arabinose-inducible *araBAD* promoter in pBAD30

(pUMR15), while YhjH was expressed in the same cell by the IPTG-inducible *lac* promoter pRGS3. A decrease in CsgD levels down to 42±3.5% was observed upon increased YhjH expression after 16 h of plate-growth at 28˚C (Fig. 1F). Since CsgD has been cloned with its native Shine-Dalgarno sequence in pBAD30 (Römling *et al.*, 2000), post-transcriptional events from increased ribosome binding to diminished protein degradation can be responsible for elevated CsgD levels.

The other known target of activation by CsgD is the *adrA* promoter (Römling *et al.*, 2000). Thus, the transcriptional activity of the *adrA* promoter was investigated when AdrA and YhjH were overexpressed. Strain AdrA1f is a *S.* Typhimurium UMR1 derivative and contains a MudJ transcriptional fusion to *adrA* (Römling *et al.*, 2000). In *S.* Typhimurium AdrA1f with the vector control *adrA* expression followed CsgD expression and was activated at 28˚C and reduced at 37˚C, which is in concordance with previously reported results (Römling *et al.*, 2000). When YhjH was overexpressed *adrA* transcription was diminished (Fig. 2A) which is in accordance with diminished CsgD expression. However, *adrA* transcription was also diminished when AdrA was overexpressed suggesting that elevated CsgD expression far above wild type levels inhibited the transcription of *adrA*. These results are consistent with observations that a CsgD homologue in *Vibrio haemolyticus* was reported to act as a repressor of capsular polysaccharide production (Guvener and McCarter, 2003). To exclude the observations of artefacts due to a c-di-GMP concentration far above physiological levels, the dependence of *bcsA* transcription on changing c-di-GMP levels was estimated. No significant change in *bcsA* transcription was observed when AdrA and YhjH were overexpressed (Fig. 2B) confirming previous results that *bcsA* transcription is not influenced by knock-out of *csgD* and consequently *adrA* (Zogaj *et al.*, 2001).

Chromosomally encoded adrA *is not required for the expression of CsgD and curli fimbriae in* S. *Typhimurium UMR1*

Overexpression of AdrA from plasmid pWJB30 enhanced the expression of CsgD (Fig. 1D). Thus the effect of an *adrA* knockout on CsgD expression was investigated. The CsgD levels of the wild type strain *S.* Typhimurium UMR1 and its *adrA* knock-out AdrA1f were compared after 16 and 24 h of growth (Fig. 3 and data not shown). Since knock-out of *adrA* in *S.* Typhimurium UMR1 abolished cellulose biosynthesis, *S.* Typhimurium UMR1 derivatives, the *bcsA* knock-out MAE222 and *bcsA adrA* knock-out MAE287, which do not express the extracellular matrix component cellulose were used as internal controls (Fig. 3). CsgD expression of the *S.* Typhimurium UMR1, AdrA1f and other mutants lacking cellulose biosynthesis was not substantially different. The same result was received with other *adrA* mutants (listed in Table 1) in the UMR1 background (data not shown). Thus, *adrA* is not required for CsgD expression when physiological protein concentrations were considered. Consequently, the regulatory network where CsgD activates transcription of *adrA,* whose gene product subsequently produces c-di-GMP is still valid (Simm, 2004). A positive feedback loop where the c-di-GMP concentrations produced by AdrA stimulate CsgD expression does not seem to exist under physiological conditions in *S.* Typhimurium UMR1. Those data are basically in agreement with the phenotypical analysis of the *adrA* mutants of UMR1 which have a visible bdar morphotype (data not shown).

Regulation of CsgD expression by chromosomally expressed GGDEF-EAL domain proteins

Since chromosomally encoded AdrA did not influence CsgD expression, any of the other GGDEF domain proteins was suspected to be responsible for the production of the c-di-GMP required for CsgD expression. To this end, all remaining 11 GGDEF domain proteins of the *S.* Typhimurium UMR1 chromosome were knocked out (Table 1, data not shown).

Morphological analysis of cells grown on CR agar plates revealed that knock-out of STM2123 and STM3388 lead to a reduction of rdar morphotype expression, which was visible from 16 h until the end of the observation period at 48 h (Fig. 4 and data not shown). Importantly, although morphotype expression was reduced the two knock-out mutants showed a colour similar to wild type (a ras (red and smooth) morphotype) indicating that both matrix components cellulose and curli fimbriae were downregulated similarly. Reduced cellulose expression was independently confirmed by reduced Calcofluor binding of the mutant colonies, which was visible already after 10 h of growth in the STM2123 mutant MAE272 (data not shown).

Expression of CsgD was analyzed in *S.* Typimurium UMR1 and knock-out mutants MAE272 (STM2123::Cm) and MAE121 (STM3388::Km) at 10, 16 and 24 h of growth on agar plates at 28˚C. In UMR1, CsgD expression steadily increased during the growth phase (Fig. 5A) Compared with UMR1 expression of CsgD was reduced in the STM2123 knock-out MAE272 at all time points, about 37±7% at 10 h, 37±3% at 16 h and 14±8% at 24h. In contrast, when MAE121 (STM3388::Km) was compared with UMR1, a higher CsgD expression was observed (156±17%) at 10 h, while downregulation of CsgD was 37±3% after growth for 16 h and 25±11% after 24 h (Fig. 5B). Concluded from the phenotypes and molecular data, STM2123 started to act early in rdar morphotype development, while the contribution of STM3388 occurred later. Consistently, Northern blot analysis of transcriptional activity revealed downregulation of the *csgD* transcript in the STM2123 and STM3388 mutants after 16 h of growth (data not shown).

The arrangement of the open reading frames of STM2123 and STM3388 on the *S.* Typhimurium chromosome did not indicate the knock-out of these genes to have polar effects, since the downstream open reading frame is transcribed in opposite direction (data not shown). Complementation of the STM2123 and STM3388 knock-out mutants with the

respective genes on the pBAD30 plasmid lead to enhanced expression of the rdar morphotype and CsgD (data not show). Thereby, cloned STM2123 expressed in MAE272 lead to vigorous production of the rdar morphotype on CR agar plates and elevated CsgD level, whereas the complementation of the respective mutant strain MAE121 by STM3388 was more moderate.

Additive effect of the knock-out of GGDEF domain proteins on CsgD expression

To investigate whether STM2123 and STM3388 work in the same regulatory pathway, the STM2123 STM3388 double mutant MAE275 was constructed. The double mutant showed an even more pronounced reduction of the rdar morphotype being almost white after 16 hrs (Fig. 4). Western blot analysis showed a 60±16% reduction of CsgD expression after 10 h, 54±7% after 16 h and 43±4% reduction after 24 h as compared to UMR1 (Fig. 5B). Since the two mutations had an additive effect on the CsgD concentration when compared to the respective single mutants they are independently affecting CsgD expression.

Double and triple mutants of *adrA* in combination with STM2123 and/or STM3388 were also constructed. The mutants in the *adrA* background did not shown significant changes in CsgD expression as compared to the mutants in the wild type background throughout the growth phase (Fig. 5B).

Effect of AdrA, STM2123 and STM3388 on curli biosynthesis

CsgD expression activates the cellulose and curli biosynthesis. To unambiguously nail down the effects of the GGDEF/EAL domain proteins AdrA, STM2123 and STM3388 downstream of CsgD expression, the levels of the curli subunit CsgA were measured in the single, double and triple mutants of AdrA, STM2123 and STM3388 (Fig. 5C). Downregulation of the curli subunit CsgA was noted in all single knock-out mutants, although to a different degree. STM2123 and STM3388 temporarily downregulated CsgA expression, while STM2123 acted

early in the growth phase and STM3388 at 24 h. The STM2123 STM3388 double mutant showed almost no CsgA expression. Although AdrA did not influence CsgD expression, it played a role in curli biosynthesis downstream of CsgD expression. These results indicate that when *S.* Typhimurium is grown at 28ºC on LB without salt agar plates a) several GGDEF domain proteins are active at the same time and b) have distributed tasks whereby STM2123 and STM3388 are required for elevated CsgD levels, while AdrA is dedicated to cellulose biosynthesis and curli biosynthesis downstream of CsgD expression. Consequently, AdrA might be dedicated to produce c-di-GMP for membrane events.

c-di-GMP production by STM2123 and STM3388

STM2123 and STM3388 are large complex GGDEF-EAL domain proteins (996 and 699 amino acids long) with distinct N-terminal sensor domains (Römling, 2005a). Since chromosomally expressed STM2123 and STM3388 upregulated expression of CsgD like AdrA overexpression from a plasmid, it was concluded that the two proteins displayed diguanylate cyclase activity. The c-di-GMP concentration produced by plasmid-encoded STM2123 (pRGS20) and STM3388 (pRGS19) in *S.* Typhimurium UMR1 was measured after 20 h of plate-growth at 37˚C. STM2123 showed approximately 5-fold higher c-di-GMP concentrations (1.5 pmol/mg cells) than the vector control (0.34 pmol/mg cells), while the c-di-GMP level increased less than 3-fold when STM3388 was expressed (0.93 pmol/mg cells). Thus expression of STM2123 and STM3388 lead to enhanced c-di-GMP concentrations in vivo supporting their function as diguanylate cyclases.

As a next step, the c-di-GMP concentration in *S.* Typhimurium UMR1 and its STM2123 STM3388 double mutant, MAE275, were measured throughout the growth phase at 10, 16 and 24 h of plate-growth at 28˚C (Fig. 6). The c-di-GMP concentration in *S.* Typhimurium UMR1 was low, in the concentration range of 0.5 pmol per mg cells (wet weight) (Fig. 6). At

10 h the c-di-GMP concentration was 578 fmol/mg cells and at 16 h 630 fmol/mg cells. The c-di-GMP concentration decreased over time being as low as 214 fmol/mg cells after 24 h of growth. In MAE275 c-di-GMP concentrations were almost identical as in *S.* Typhimurium UMR1 throughout the growth phase. Thus, expression of STM2123, STM3388 or both proteins did not significantly alter the c-di-GMP concentrations throughout the growth phase. As a comparison the c-di-GMP concentrations were also measured in the *adrA* STM2123 STM3388 triple mutant MAE276. The c-di-GMP concentrations in MAE276 compared with *S.* Typhimurium MAE275 were almost identical at 10 h and slightly lower after 24 h of growth. At 16 h however almost 60% of the c-di-GMP was produced by AdrA. But this significant reduction in the c-di-GMP pool did not lower the CsgD level. On the contrary rather an enhanced CsgD expression was observed (Fig. 5B). Thus the absolute c-di-GMP concentration does not correlate with a certain biological effect, in this case CsgD expression, but is dependent on the presence or absence of specific GGDEF/EAL domain proteins.

Overcoming of the c-di-GMP signalling network by csgD *promoter-up mutations*

The results described above were performed using *S.* Typhimurium UMR1, which shows a highly regulated rdar morphotype only expressed at 28ºC (Römling *et al.*, 1998a). Strain *S.* Typhimurium MAE52 has a promoter-up mutation (P*csgD1*), which leads to an approximately 3-fold higher *csgD* expression at 28ºC and expression at 37˚C (Römling *et al.*, 1998b). To investigate the effect of GGDEF-EAL domain proteins on cellulose biosynthesis and CsgD expression the STM3388 knock-out was transduced to *S.* Typhimurium MAE52. Evaluation of the colony morphology on CR agar plates showed only a slight decrease in rdar morphotype expression, if any change (data not shown). This observation was confirmed by the Calcofluor binding assay (data not shown). Western blot analysis was performed to investigate the CsgD levels in the STM3388 mutant (Fig. 7). The STM3388 knock-out mutant

showed 21.3±3% reduced CsgD levels. Thus, there is a similar regulatory effect of STM3388 on the CsgD expression levels in the MAE52 background as in the UMR1 background. However, the elevated transcriptional activity of *csgD* overcomes visible reduction in morphotype caused by the STM3388 mutant.

The effect of an *adrA* knock-out on CsgD expression was also investigated in strain *S.* Typhimurium MAE52. Western blot analysis consistently showed a significant reduction of CsgD expression in *adrA* knock-out strains AdrA1a (13.5±9%) and AdrA6a (21±9%) as compared to the wild type *S.* Typhimurium MAE52 and the *bcsA* mutant MAE150, which was used as an internal control assessing the effect of loss of the extracellular matrix component cellulose (Fig. 7). Thus, when AdrA expression was conferred a positive feedback loop on CsgD exists in the *S.* Typhimurium MAE52 strain background. A positive feedback is most probably achieved by higher c-di-GMP concentrations produced by elevated amounts of AdrA protein, which leads to elevation of c-di-GMP concentrations in the whole cytoplasm.

Overcome of temperature regulation of CsgD by elevation of c-di-GMP concentrations

S. Typhimurium UMR1 expresses the rdar morphotype only at 28ºC, but not at 37ºC (Römling *et al.*, 1998a). However, recently we had established that cellulose is produced at 37ºC once the second messenger and potential allosteric activator c-di-GMP is provided by overexpression of AdrA (Simm, 2004). It was of interest to see whether c-di-GMP concentrations also affect CsgD protein concentration and the expression of curli fimbriae at 37ºC. Thus, AdrA was overexpressed in *S.* Typhimurium UMR1 and its *bcsA* knock-out mutant MAE222. Western blot analysis detected CsgD as well as CsgA, the subunit of curli fimbriae, in *S.* Typhimurium UMR1, when AdrA was overexpressed in contrast to the vector control (Fig. 8 and data not shown). Thus, elevated c-di-GMP concentrations can overcome the temperature regulation of CsgD expression and rdar morphotype development.

Discussion

GGDEF and EAL domain proteins are abundant in bacterial genomes, up to date more than 1200 and 800 copies are listed in public databases. Most bacterial genomes encode more than one GGDEF and EAL domain protein (Galperin, 2004; Römling, 2005b). 12 GGDEF and 14 EAL domain proteins are encoded by the *S.* Typhimurium genome (7 have GGDEF as well as an EAL domain; (Römling, 2005b)). This redundancy of paralogous proteins raises the question about the functional and regulatory specificity of the proteins. From current data, it is assumed that GGDEF and EAL domains are involved in c-di-GMP turnover, functioning as diguanylate cyclase and phosphodiesterase, respectively (Paul *et al.*, 2004; Ryjenkov *et al.*, 2005; Simm, 2004; Tischler and Camilli, 2004). Recently we proposed regulation of the c-di-GMP pool(s) by the activity of GGDEF and EAL domain proteins to occur on several levels, ranging from transcriptional control of the respective genes over stimulation of enzymatic activity by signal transduction. Spatial location of proteins and product inhibition of the enzymatic activity are other regulatory mechanisms (Chan *et al.*, 2004; Römling, 2005b).

In this study we investigated the function of the 12 GGDEF domain proteins of *S.* Typhimurium in rdar morphotype development. In contrast to the findings of Garcia et al (Garcia *et al.*, 2004) we could show that at least three GGDEF/EAL domain proteins, AdrA, STM2123 and STM3388, were expressed during growth of *S.* Typhimurium on agar plates where they contribute to rdar morphotype development. Overexpression studies in this work and a previous study (Simm et al., 2004) showed that all three GGDEF/EAL domain proteins functioned as diguanylate cyclases, but only AdrA contributed significantly to the enhanced concentration of c-di-GMP in the cell. Also the intracellular c-di-GMP concentrations achieved by induction of the GGDEF/EAL domain proteins with 0.1% arabinose were significantly different. While overexpression of AdrA lead to pmol amounts of c-di-GMP (Simm, 2004), which corresponds to concentrations approximately 10.000 times over the

basal cellular concentration, STM2123 and STM3388 lead to c-di-GMP concentrations approximately 5 and 3 times over the basal level, respectively. The different activities could depend on the concentration or activation of the respective protein. Alternatively, the domain structure might to some extend determine the amount of c-di-GMP produced. While AdrA contains only a GGDEF output domain, STM2123 and STM3388 harbour an EAL domain C-terminal of GGDEF (Fig. 9). In the case of STM2123 the EAL domain contains several deviations in conserved residues predicted to be involved in building up the catalytic site or in metal binding suggesting that the domain is not enzymatically functional (Römling, 2005a). STM3388 however seems to encode a functional EAL domain with consensus residues conserved, suggesting that GGDEF and EAL might both be functional at the same time. On the other hand, knock-out of GGDEF domain proteins with a non-conserved GGDEF-motif, but an EAL domain which contains the consensus residues (Römling, 2005b) slightly upregulated the rdar morphotype, suggesting that they function as phosphodiesterases (A. Kader and U. Römling, unpublished data).

The complex GGDEF-EAL domain proteins STM2123 and STM3388 visibly contributed to the development of the typical rdar colony morphology, while loss of AdrA lead to a bdar morphotype (Fig. 4; (Römling *et al.*, 2000)). Thus a distribution of tasks was observed among the three GGDEF/EAL domain proteins which function as diguanylate cyclases, STM2123, STM3388 and AdrA. The GGDEF domain protein AdrA directed the activation of cellulose biosynthesis and, partially, curli biosynthesis, but did not affect the expression of the transcriptional regulator CsgD. On the other hand, STM2123 and STM3388 activated rdar morphotype expression by activation of expression of the transcriptional regulator CsgD on a transcriptional and post-transcriptional level. Figure 9 summarizes the current model of regulation of rdar morphotype development by GGDEF/EAL domain proteins and c-di-GMP.

How is the task distribution managed? Definitely there is a temporal component, since CsgD has to be expressed in order to subsequently activate *adrA*. Already after 10h of growth, a reduction of Calcofluor binding, curli biosynthesis and CsgD expression was observed in the STM2123 mutant, but not the STM3388 mutant indicating that STM2123 expression arises early in the growth phase.

Overexpression studies did blur the differential functionality of STM2123, STM3388 and AdrA, since all three proteins functioned as diguanylate cyclases and thus conferred overexpression of the rdar morphotype and CsgD. In the same line, it was recently shown that an *adrA* mutant could be complemented by STM2123 and ST3388 for cellulose biosynthesis on agar plates (Garcia *et al.*, 2004). This suggests that overexpression of GGDEF domain proteins allow elevated concentration of the allosteric activator c-di-GMP in the whole cytoplasm. Consequently, saturation of different c-di-GMP pools and activation of several c-di-GMP dependent pathways occured. Those pathways are originally functionally and spatially separated and directed by different GGDEF domain proteins.

Since in the chromosomal context STM2123, STM3388 and *adrA* are expressed and active at the same time, distribution of tasks is envisaged to occur due to stringent regulation of the copy numbers of the protein in combination with a differential spatial localisation of GGDEF domain proteins. However, if this scenario should work also the targets of GGDEF domain proteins are required to be spatially localized. This has been shown in the developmental cycle in *Caulobacter crescentus*, whereby activated PleD is localized at the pole of the cell associated with the membrane where stalk formation and flagella ejection occur (Aldridge *et al.*, 2003; Jenal, 2004). Alternatively, the output domain structure (GGDEF or GGDEF-EAL) may play a role in determination of task distribution. Nevertheless, it was surprising to see that functional co-expression of several GGDEF domain proteins targeting different functions occurred during rdar morphotype expression. Up to date, co-expression of more than one

GGDEF domain protein was shown to affect one phenotype, namely cellulose biosynthesis (Tal *et al*., 1998). Otherwise, co-expression of GGDEF domain proteins has been observed on the transcriptional level (Johnson *et al.*, 2005). Our results show that all the three GGDEF domain proteins are activated by their specific external and membrane stimuli at the same time (Fig. 9).

STM2123 and STM3388 are two of the most complex GGDEF domain proteins encoded by the *S.* Typhimurium chromosome (Römling, 2005a). Both proteins contain also an EAL domain C-terminal of the GGDEF domain as well as different N-terminal sensor domains, MASE1 and MHTY, respectively (Galperin *et al.*, 2001a; Nikolskaya *et al.*, 2003). It is not known what is sensed by the MASE1 and MHTY domain and whether the level of activation is different for STM2123 and STM3388, however, the biological responses were similar. Knockout of STM2123 and STM3388 both reduced CsgD expression by 37% after 16 h of growth. CsgD reduction was 54% in the STM2123 STM3388 double mutant at 16 h of growth, however only a 6%, not significant reduction of the c-di-GMP concentration was observed. In general, deletion of STM2123 and STM3388 did not seem to alter the total c-di-GMP pool significantly, consistent with the observation that the diguanylate cyclase activity of STM2123 seems to be significantly lower than the diguanylate cyclase activity displayed by AdrA.

This work adds one more biological activity of *S.* Typhimurium, curli biosynthesis, to the growing list of phenotypes regulated by c-di-GMP. Before, it has been shown that cellulose biosynthesis, swimming and swarming motility, resistance to H_2O_2 and killing of macrophages are modulated by intracellular c-di-GMP concentrations (Hisert *et al.*, 2005; Römling *et al.*, 2000; Simm, 2004).

Regulation of curli biosynthesis by c-di-GMP is complex. It occurred at the level of CsgD expression and downstream of CsgD expression on a transcriptional and (presumably) post-

transcriptional level. Whether one of the known regulators of *csgD* transcription (Gerstel *et al.*, 2003) is affected by c-di-GMP remains to be shown. It is also not clear whether CsgD is a direct target of c-di-GMP action. However, the expression of CsgD requires only a fraction of the total cellular concentration of c-di-GMP. Although more than half of the c-di-GMP at 16 h was produced by AdrA, the expression of CsgD was not lowered in *adrA* knock-out mutants (Fig. 5B). This finding indicated that the c-di-GMP produced by AdrA belongs to a different pool than the c-di-GMP produced by STM2123 and STM3388 and that the two pools do not communicate with each other. Also in *Gluconacetobacter xylinus*, the total cellular c-di-GMP concentrations did not correlate with the amount of cellulose biosynthesis. Even when c-di-GMP production was severely compromised (4% of total activity), cellulose biosynthesis was still as high as 36% (Tal *et al.*, 1998).

Although in *S.* Typhimurium the c-di-GMP concentrations of the wild type *S.* Typhimurium UMR1 and the triple mutant MAE276 differed by approximately 10% after 10 h of plate growth, the majority of c-di-GMP, 524 fmol/mg, was not produced by any of the three GGDEF domain proteins. Consequently, (an)other GGDEF domain protein(s) is responsible for the c-di-GMP production at 10 h of growth. However, the(se) protein(s) do not play an obvious role in rdar morphotype development, since none of the other GGDEF domain proteins showed a phenotypic difference with respect to rdar morphotype development.

The activation of CsgD expression and curli biosynthesis by c-di-GMP was independent of temperature and occurred at 28˚C and 37˚C. Thus c-di-GMP overcomes the temperature regulation of rdar morphotype expression. Consequently, in the wild type c-di-GMP levels are expected to be lower at 37˚C than at 28˚C. This temperature gradient in c-di-GMP concentration could be achieved by decreased diguanylate cyclase activity or enhanced phosphodiesterase activity. Temperature independent expression of a formerly temperature restricted biofilm phenotype by overexpression of a GGDEF domain protein has also been

reported for *Yersinia pestis* (Kirillina *et al.*, 2004). These findings, if further generalized, establish c-di-GMP as a key regulator of temperature dependent expression of multicellular behaviour.

Acknowledgements

A.K. did the gene knock-outs and Western blot analysis, R.S. developed the methodology for quantification of c-di-GMP, measured the samples and cloned the GGDEF domain proteins, U.G. did the transcriptional analyses and U.R. planned the study, evaluated results, draw the conclusions and wrote the manuscript. We thank Werner Bokranz for the construction of pWJB5 and Fredrik Åslund for construction of the STM3388 and STM3615 knock-outs. We appreciate the help of Manfred Nimtz, who verified the identity of CsgA isolated from protein gels by MALDI-TOF analysis. Mats Andersson provided generous access to the HPLC.

Ulrich Gerstel received a fellowship from the foundation Wenner-Grenska Samfundet. This work was supported by the Karolinska Institutet (‘Elitforskartjänst’ to UR) and Vetenskapsrådet (621-2004-3979).

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Cells were grown on Luria-Bertani (LB) medium without salt agar plates supplemented with Congo Red (40 μg ml^{-1}) and Coomassie brilliant blue G-250 (20 μg ml^{-1}) or 50 μg ml^{-1} Calcoflour (fluorescent brightener 28), when required. Bacterial morphotypes were investigated at 28°C and 37°C at different time points starting from 10 to 48 hours. L (+)-arabinose (up to 0.1%) or IPTG (up to 1mM) were used to express the genes cloned downstream of the *araC* and *lac* promoter in pBAD30 and pLAFR3, respectively. Antibiotics used: ampicillin (Amp; 100μg/ml), chloramphenicol (Cm; 20μg/ml), kanamycin (30μg/ml), nalidixic acid (Nal; 50μg/ml) and tetracycline (Tc; 20μg/ml).

General molecular biology methods

Common molecular biological methods such as isolation of plasmid and chromosomal DNA, PCR, plasmid transformation as well as restriction digestion were carried out using standard protocols (Ausubel *et al;* 1994). PCR products were cleaned using the Quiaquick PCR purification kit (Quiagen) and DNA fragments were recovered from agarose gels using Quiaquick minielute gel purification kit (Quiagen). Primer sequences are provided in Table S1.

Construction of mutants

Knock out of genes was performed by one step gene inactivation following the protocol as developed by Datsenko and Wanner (Datsenko and Wanner; 2000) whereby the entire ORF of the respective gene besides the 40 flanking nucleotides was replaced with either a kanamycin

or chloramphenicol resistance marker. Approximately 300ng of processed PCR product was electroporated in *S.* Typhimurium UMR1 containing pKD46. Recovered colonies were purified at least twice on LB medium containing appropriate antibiotics. All constructed knock-out alleles were transduced to *S.* Typhimurium UMR1 wild type background to verify the phenotype.

An in frame deletion of STM0385 (*adrA*) was constructed by deleting 1027 of 1113 bps of the ORF of AdrA using pWJB5. To construct pWJB5, two approximately 1 kb DNA fragments which overlap the start and stop codon of *adrA* constructed with primers PSIF2/ADRA.A.RAUS-2 and ADRA.R.RAUS/PROC were cloned into pMAK700 (Hamilton *et al.,* 1989) using *Hind*III and *Bam*HI restriction sites. A *Pst*I restriction site had been introduced into the primers to ligate the two fragments. The deletion on the *S.* Typhimurium chromosome was constructed following the protocol as described (Römling, 2001).

Phage transduction was carried out with phage P22 HT105/1 *int*-201 (Schmieger, 1972). Preparation of phage lysates, purification of transductants, checking of pseudolysogenicity and lytic phages were performed as described (Maloy, 1996). Transductants were colony purified twice on LB agar plate containing EGTA and appropriate antibiotics. Auxotrophy was tested routinely.

All constructed mutants were verified by PCR with control primers located up- and down-stream of the targeted open reading frame (ORF). All primers are listed in Table S1.

Plasmid construction

The plasmid p*csgBA* contains the *csgBA* promoter spanning from bps + 164 to –186 relative to the transcriptional start site of *csgBA* fused to *lacZ*. To construct the *csgBA–lacZ* transcriptional fusion p*csgBA*, pUGE1 (Gerstel and Römling, 2001; Gerstel *et al.*, 2003) was cut with *Nco*I and *Bam*HI and the resulting 350 bp fragment was subsequently cloned into the

low copy promoter probe vector pQF50 (Farinha and Kropinski, 1990). STM2123 and STM3388 were cloned into pBAD30 with a C-terminal His-tag. All primers are listed in Table S1.

Plasmids were passed through the restriction deficient strain *S.* Typhimurium LB5010 before electroporation into ATCC14028 derivatives.

Protein Techniques

For Western blot analysis, five milligrams of cells were harvested after growth on LB agar plates without salt, re-suspended in sample buffer and heated at 95°C for 5 to 10 minutes. After the protein content was analyzed by Coomassie blue staining (20% methanol, 10% acetic acid, 0.1% coomassie brilliant blue G), equal amounts of protein were separated by a SDS protein gel (12% separation gel with a 4% stacking gel). The proteins were transferred to a PVDF membrane (Immobilon P; Milipore).

Detection of CsgD was carried out as described by Römling *et al.,* (1998b; 2000) using a polyclonal anti-CsgD peptide antibody (1:5000) and goat anti- rabbit (1:2000) immunoglobulin G conjugated with horse raddish peroxidase (Jackson ImmunoResearch Laboratories Inc.) as primary and secondary antibody. Signals were recorded using the LAS-1000 System (FUJIFILM) and quantified using ImageQuant software (version 5.2). Each experiment was performed at least three times independently.

Analysis of CsgA expression was carried out as described using equal number of cells (Römling *et al.*, 1998a; Römling, 2003). Depolymerization of intact fimbriae was achieved by treating the cell pellet with 99% formic acid for 10 minutes on ice. Depolymerized CsgA subunits were detected either after staining the gel with coomassie brilliant blue over night (identity of bands verified by MALDI-TOF analysis) or by Western blot analysis using an

anti-*E. coli* CsgA anti-serum (1:4000). Experiments were performed on at least three independent occasions.

Transcript analysis

Total RNA was prepared from 10 mg cells grown on LB agar without salt for 16 and 24 h at 28ºC using the hot phenol method exactly as described earlier (Römling *et al.*, 1998a). 10 µg of RNA was loaded on a 1.2% MOPS-formaldehyde gel, run for 4h at 4V cm^{-1} and transferred overnight to a nylon membrane (Amersham Hybond) by capillary blotting using 20xSSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate). As a control for loading equal amounts of RNA, the gel was photographed before RNA transfer. Single-stranded probes complementary to the RNA template on the blot were constructed by asymmetric PCR with primer SP2 (TTTCTCTTTCTGGAGAATGGG) on symmetric PCR templates spanning the region of *csgD* (primers SP2 and SP24 [dTAACTCTGCTGCTACAATCC]) and labelled with the RadPrime Labelling System (Life Technologies) using 30 µCi of [α-^{32}P]dCTP (3,000 Ci/nmol; Amersham). Hybridization (using 6 ng of probe per ml) and washing of blots were carried out according to standard procedures. Signals were analysed with a radioisotope imaging system (PhosphorImager 445SI: Molecular Dynamics) whereby integration of all bands detected by the *csgD* probe was carried out. All experiments were repeated twice.

ß-Galactosidase assays

Promoter activity was assayed at appropriate growth conditions by ß-galactosidase measurements as described (Miller, 1972). ß-galactosidase activity was calculated using the formula: units=$1000\{[OD_{420}- (1.75\times OD_{550})]/t\times V\times OD_{660})\}$ with t = reaction time in min; V=volume of cell suspension in assay in ml; OD_{420} and OD_{550} of reaction solution; OD_{660} of

original cell suspension. All ß-galactosidase measurements were done in duplicates from at least two independent cultures.

Isolation and quantification of c-di-GMP

c-di-GMP was synthesized and purified as described (Simm *et al*., 2004). Nucleotide extracts were prepared by the heat/ethanol method as previously described (Simm *et al*., 2004). Nucleotide extracts equivalent to 10 mg cells (wet weight) were subjected to HPLC-separation using a reversed phase column (Hypersil ODS 5µ; Hypersil-Keystone). Runs were carried out in 0.1 M tri-ethyl-ammonium acteate (TEAA) pH 6.0 at 1ml min^{-1} using a multistep gradient of acetonitrile. Relevant fractions were collected, lyophilised and resuspended in 10 µl water. MALDI-TOF was used to pinpoint all fractions containing c-di-GMP. Thereby the samples were applied to the target as described (Simm, 2004). Quantification by MALDI-TOF was carried out after pooling all fractions, which contained c-di-GMP. A standard curve was established using fractions spiked with a known amount of c-diGMP and c-diAMP as internal control. The isotope area of c-di-GMP and c-di-AMP was calculated and the ratio determined. C-di-GMP measurements were done in triplicates from two independent experiments.

References

Aldridge, P, Paul, R, Goymer, P, Rainey, P, Jenal, U (2003) Role of the GGDEF regulator PleD in polar development of *Caulobacter crescentus*. *Mol Microbiol* 47: 1695-1708

Arnqvist, A, Olsen, A, Normark, S (1994) Sigma S-dependent growth-phase induction of the *csgBA* promoter in *Escherichia coli* can be achieved in vivo by sigma 70 in the absence of the nucleoid-associated protein H-NS. *Mol Microbiol* 13: 1021-1032

Brombacher, E, Dorel, C, Zehnder, AJ, Landini, P (2003) The curli biosynthesis regulator CsgD co-ordinates the expression of both positive and negative determinants for biofilm formation in *Escherichia coli*. *Microbiology* 149: 2847-2857

Bullas, LR, Ryu, JI (1983) *Salmonella typhimurium* LT2 strains which are r- m+ for all three chromosomally located systems of DNA restriction and modification. *J Bacteriol* 156: 471-474

Chan, C, Paul, R, Samoray, D, Amiot, NC, Giese, B, Jenal, U, Schirmer, T (2004) Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci U S A* 101: 17084-17089

D'Argenio, DA, Calfee, MW, Rainey, PB, Pesci, EC (2002) Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *J Bacteriol* 184: 6481-6489

D'Argenio, DA, Miller, SI (2004) Cyclic di-GMP as a bacterial second messenger. *Microbiology* 150: 2497-2502

Farinha, MA, Kropinski, AM (1990) Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J Bacteriol* 172: 3496-3499

Galperin, MY, Gaidenko, TA, Mulkidjanian, AY, Nakano, M, Price, CW (2001a) MHYT, a new integral membrane sensor domain. *FEMS Microbiol Lett* 205: 17-23

Galperin, MY, Nikolskaya, AN, Koonin, EV (2001b) Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* 203: 11-21

Galperin, MY (2004) Bacterial signal transduction network in a genomic perspective. *Environ Microbiol* 6: 552-567

Garcia, B, Latasa, C, Solano, C, Portillo, FG, Gamazo, C, Lasa, I (2004) Role of the GGDEF protein family in *Salmonella* cellulose biosynthesis and biofilm formation. *Mol Microbiol* 54: 264-277

Gerstel, U, Römling, U (2001) Oxygen tension and nutrient starvation are major signals that regulate *agfD* promoter activity and expression of the multicellular morphotype in *Salmonella typhimurium*. *Environ Microbiol* 3: 638-648

Gerstel, U, Park, C, Römling, U (2003) Complex regulation of *csgD* promoter activity by global regulatory proteins. *Mol Microbiol* 49: 639-654

Gronewold, TM, Kaiser, D (2001) The *act* operon controls the level and time of C-signal production for *Myxococcus xanthus* development. *Mol Microbiol* 40: 744-756

Guvener, ZT, McCarter, LL (2003) Multiple regulators control capsular polysaccharide production in *Vibrio parahaemolyticus*. *J Bacteriol* 185: 5431-5441

Guzman, LM, Belin, D, Carson, MJ, Beckwith, J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177: 4121-4130

Hamilton, CM, Aldea, M, Washburn, BK, Babitzke, P, Kushner, SR (1989) New method for generating deletions and gene replacements in *Escherichia coli*. *J Bacteriol* 171: 4617-4622

Hammar, M, Arnqvist, A, Bian, Z, Olsen, A, Normark, S (1995) Expression of two *csg* operons is required for production of fibronectin- and congo red-binding curli polymers in *Escherichia coli* K-12. *Mol Microbiol* 18: 661-670

Hisert, KB, MacCoss, M, Shiloh, MU, Darwin, KH, Singh, S, Jones, RA, Ehrt, S, Zhang, Z, Gaffney, BL, Gandotra, S, Holden, DW, Murray, D, Nathan, C (2005) A glutamate-alanine-leucine (EAL) domain protein of *Salmonella* controls bacterial survival in mice, antioxidant defence and killing of macrophages: role of cyclic diGMP. *Mol Microbiol* 56: 1234-1245

Huber, B, Riedel, K, Hentzer, M, Heydorn, A, Gotschlich, A, Givskov, M, Molin, S, Eberl, L (2001) The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology* 147: 2517-2528

Jenal, U (2004) Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? *Curr Opin Microbiol* 7: 185-191

Johnson, MR, Montero, CI, Conners, SB, Shockley, KR, Bridger, SL, Kelly, RM (2005) Population density-dependent regulation of exopolysaccharide formation in the hyperthermophilic bacterium *Thermotoga maritima*. *Mol Microbiol* 55: 664-674

Kirillina, O, Fetherston, JD, Bobrov, AG, Abney, J, Perry, RD (2004) HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. *Mol Microbiol* 54: 75-88

Ko, M, Park, C (2000) Two novel flagellar components and H-NS are involved in the motor function of *Escherichia coli*. *J Mol Biol* 303: 371-382

Maloy, S, Stewart, V.J, Taylor, R. K. (1996) *Genetic analysis of pathogenic bacteria.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, J (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Nikolskaya, AN, Mulkidjanian, AY, Beech, IB, Galperin, MY (2003) MASE1 and MASE2: two novel integral membrane sensory domains. *J Mol Microbiol Biotechnol* 5: 11-16

Paul, R, Weiser, S, Amiot, NC, Chan, C, Schirmer, T, Giese, B, Jenal, U (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev* 18: 715-727

Ross, P, Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G.A., van Boom, J.H., Benziman, M. (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* 325: 279-281

Ryjenkov, DA, Tarutina, M, Moskvin, OV, Gomelsky, M (2005) Cyclic Diguanylate Is a Ubiquitous Signaling Molecule in Bacteria: Insights into Biochemistry of the GGDEF Protein Domain. *J Bacteriol* 187: 1792-1798

Römling, U, Bian, Z, Hammar, M, Sierralta, WD, Normark, S (1998a) Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. *J Bacteriol* 180: 722-731

Römling, U, Sierralta, WD, Eriksson, K, Normark, S (1998b) Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. *Mol Microbiol* 28: 249-264

Römling, U, Rohde, M, Olsen, A, Normark, S, Reinköster, J (2000) AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways. *Mol Microbiol* 36: 10-23

Römling, U (2001) Genetic and phenotypic analysis of multicellular behavior in *Salmonella typhimurium*. *Methods Enzymol* 336: 48-59

Römling, U (2005a) Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. *CLMS* 62: 1-13

Römling, U, Bokranz, W., Rabsch, W., Zogaj, X., Nimtz, M., Tschäpe, H. (2003) Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. *Int J Med Microbiol* 293: 273-285

Römling, U, Gomelsky, M., Galperin, M.Y. (2005b) c-di-GMP: The dawning of a novel bacterial signalling system. *Mol Microbiol* 67: 629-639

Schmidt, AJ, Ryjenkov, D.A., Gomelsky, M. (2005) Ubiquitous protein domain EAL encodes cyclic diguanylate-specific phosphodiesterase: Enzymatically active and inactive EAL domains. *J Bacteriol* 187: 4774-4781

Schmieger, H (1972) Phage P22-mutants with increased or decreased transduction abilities. *Mol Gen Genet* 119: 75-88

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

Staskawicz, B, Dahlbeck, D, Keen, N, Napoli, C (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* 169: 5789-5794

Tal, R, Wong, HC, Calhoon, R, Gelfand, D, Fear, AL, Volman, G, Mayer, R, Ross, P, Amikam, D, Weinhouse, H, Cohen, A, Sapir, S, Ohana, P, Benziman, M (1998) Three cdg operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. *J Bacteriol* 180: 4416-4425

Tischler, AD, Camilli, A (2004) Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. Mol Microbiol 53: 857-869

Zogaj, X, Nimtz, M, Rohde, M, Bokranz, W, Römling, U (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39: 1452-1463

Figure legends

Fig. 1. Effect of AdrA and YhjH overexpression on regulatory and structural components of the network of rdar morphotye expression in *S.* Typhimurium UMR1.

A. Effect of AdrA and YhjH overexpression on rdar morphotype expression. AdrA overexpression (plasmid pWJB30) enhanced the rdar and bdar morphotype of *S.* Typhimurium UMR1 and MAE222 (UMR1 *bcsA101*::MudJ), respectively, in comparison to the same strains with the vector control pBAD30. YhjH overexpression (plasmid pRGS1) lead to a saw (smooth and white) morphotype, indicative for a lack of expression of curli and cellulose. Cells were grown on CR agar plates at 28ºC for 36 hrs. VC=vector control.

B. Effect of AdrA and YhjH overexpression on expression of formic acid-resistant CsgA as an indicator of curli fimbriae in mutants AdrA1f (UMR1 *adrA101*::MudJ) and MAE222 (UMR1 *bcsA101*::MudJ) as visualized on a Coomassie-stained protein gel. AdrA overexpression lead to enhanced expression of CsgA after 24 hrs of growth as compared to the vector control (VC), while overexpression of YhjH lead to nearly abolished CsgA expression even after 24 hrs growth at 28ºC on LB without salt agar plates. C represents the positive control MAE52.

C. Effect of AdrA and YhjH overexpression on the transcriptional activity of *csgBA* in *S.* Typhimurium UMR1. *CsgBA* transcriptional activity was determined using a transcriptional fusion to β-galactosidase. AdrA overexpression lead to enhanced transcriptional activity of *csgBA* after 16 and 24 hrs of growth on LB without salt agar plates, while overexpression of YhjH lead to low level activity of the *csgBA* promoter as compared to the vector control (VC). The data shown are the averages of two independent experiments done in duplicates with standard deviations.

D. Effect of AdrA and YhjH overexpression on CsgD expression in *S.* Typhimurium UMR1. Western blot analysis showed that CsgD expression was enhanced by AdrA overexpression and reduced, when YhjH was overexpressed as compared to vector control (VC). Cells were grown at 28°C for 16 h and 24 h on LB without salt agar plates.

E. Effect of AdrA and YhjH overexpression on *csgD* transcription in *S.* Typhimurium UMR1. Steady state levels of *csgD* transcript were enhanced when AdrA was overexpressed and reduced, when YhjH was overexpressed compared to vector control (VC) as shown by Northern blot analysis. Arrow marks the *csgD* 2.3 kbp full length mRNA Cells were grown as in D.

F. Effect of YhjH overexpression on CsgD expression from plasmid pBAD30. In the *csgD bcsA* double mutant MAE265 CsgD steady state levels were reduced when YhjH was overexpressed (plasmid pRGS3) as compared to the vector control (VC). Cells were grown at 28 °C for 16 h . Expression of *csgD* and *yhjH* was induced with 0.1% arabinose and 1 mM IPTG from the *araC* and *lac* promotor.

Fig. 2. Effect of AdrA and YhjH overexpression on *adrA* and *bcsA* transcription in *S.* Typhimurium UMR1 background. Effect of AdrA and YhjH overexpression on transcription of *adrA* (strain AdrA1f (UMR1 *adrA101*::MudJ) with plasmids pWJB30 or pRGS1, respectively). Transcriptional activity of *adrA* (measured as ß-galactosidase activity) was elevated at 28ºC and downregulated at 37ºC in accordance with CsgD expression as reported previously (Römling *et al.*, 2000). AdrA and YhjH overexpression decreased *adrA* expression at 28ºC, while overexpression had no effect on the transcriptional activity at 37ºC. Cells were grown at 28°C for 24 h or at 37ºC for 17h on LB without salt agar plates. VC=vector control. Values with standard deviations are displayed which are based on experiments done three times independently using duplicate samples. B. AdrA and YhjH overexpression did not

change transcription of *bcsA* encoding the catalytic subunit for cellulose synthase. Strain MAE165 (MAE32 *bcsA*::MudJ Δ*csgD101*) with plasmids pWJB30 or pRGS3, respectively, was used. When c-di-GMP levels were modulated, the transcriptional activity of *bcsA* (measured as ß-galactosidase activity) was not changed at 28ºC and 37ºC as compared to vector control (VC). Cells were grown as in A. Values with standard deviations are displayed which are based on experiments done three times independently using duplicate samples.

H. **Fig. 3.** Chromosomally encoded AdrA has no effect on CsgD expression in *S.* Typhimurium UMR1. Immunoblot of CsgD protein in *S.* Typhimurium UMR1 (lane 1), MAE260 (UMR1 Δ*adrA103*, lane 2), MAE222 (UMR1 Δ*bcsA*, lane 3) and MAE287 (UMR1 Δ*adrA103 bcsA*::MudJ, lane 4). Cells were grown at 28°C for 16 h on LB without salt agar plates.

Fig. 4. Phenotypic characterization of GGDEF domain protein mutants of *S.* Typhimurium UMR1. Compared to *S.* Typhimurium UMR1, MAE121 (UMR1 ΔSTM3388) and MAE272 (UMR1 ΔSTM2123) show reduced rdar morphotype development, while in the double mutant MAE275 (UMR1 ΔSTM3388 ΔSTM2123) the reduction of rdar morphotype expression was additive. As demonstrated before, MAE260 (UMR1 ΔSTM0385) showed the bdar morphotype, which was further reduced in the triple mutant MAE276 (Δ *adrA*::103 ΔSTM3388::Km ΔSTM2123::Cm). 1, *S.* Typhimurium UMR1; 2, MAE260 (Δ *adrA103*); 3, MAE121 (ΔSTM3388::Km); 4, MAE272 (ΔSTM2123::Cm); 5, MAE267 (Δ *adrA103* ΔSTM3388::Km); 6, MAE274 (Δ *adrA103* ΔSTM2123::Cm); 7, MAE275 (ΔSTM3388::Km ΔSTM2123::Cm); 8. MAE276 (Δ *adrA*::103 ΔSTM3388::Km ΔSTM2123::Cm). Cells were grown at 28°C for 16 hrs on LB without salt agar plates.

Fig. 5. CsgD and CsgA expression in *S.* Typhimurium UMR1 and selected GGDEF domain protein mutants. A. CsgD (immunoblot) and CsgA (Coomassie-stained protein gel) expression at 10, 16 and 24 h of growth in *S.* Typhimurium UMR1. B. CsgD expression at 10, 16 and 24 h of growth in *S.* Typhimurium UMR1 and GGDEF domain protein mutants. Compared to *S.* Typhimurium UMR1, significant reduction of CsgD levels were observed in MAE121 (UMR1 ΔSTM3388), MAE272 (UMR1 ΔSTM2123), the double mutant MAE275 (UMR1 ΔSTM3388 ΔSTM2123) and the triple mutant MAE276 (UMR1 Δ*adrA103* ΔSTM3388 ΔSTM2123) at 16 and 24 h of growth. In MAE260 (Δ*adrA103*) no change in CsgD expression was observed. MAE51 (MAE52 Δ*csgD*), negative control. Data at 10 h were collected from two different plates. C. CsgA expression at 10, 16 and 24 h of growth on Coomassie-stained protein gels. Compared to *S.* Typhimurium UMR1, significant reduction of CsgA levels were observed in MAE260 (Δ*adrA103*), all double and the triple mutant at all time points. MAE272 (UMR1 ΔSTM2123) and MAE121 (UMR1 ΔSTM3388) showed temporal reduction of CsgA.

Fig. 6. c-di-GMP concentrations in *S.* Typhimurium UMR1, MAE275 and MAE276 throughout the growth phase on LB without salt agar plates. In *S.* Typhimurium UMR1 and the STM2123 STM3388 double mutant MAE275 the c-di-GMP levels decreased from 10 h to 24 h (square and diamond symbols). A slightly lower c-di-GMP concentration was observed in MAE275 compared to *S.* Typhimurium UMR1. The *adrA* STM2123 STM3388 triple mutant MAE276 showed a significant decrease (over 50%) of c-di-GMP concentration at 16 h of growth when compared to *S.* Typhimurium UMR1 and MAE275. Values with standard deviations are displayed, which are based on experiments done two times independently in triplicates.

Fig. 7. Immunoblot of CsgD expression in *S.* Typhimurium MAE52 and selected GGDEF domain protein mutants. Feedback regulation of CsgD was observed upon expression of AdrA in AdrA1a (MAE52 *adrA101*::MudJ) and AdrA6a (MAE52 *adrA102*::MudJ compared to *S.* Typhimurium MAE52. A STM3388 knock-out conferred 21.3±3% CsgD reduction in *S.* Typhimurium MAE52. Cells were grown at 28°C for 16 hrs on LB without salt agar plates.

Fig. 8. Temperature regulation of CsgD expression in *S.* Typhimurium UMR1 background is overcome by AdrA overexpression. Immunoblot of CsgD expression of AdrA1f (UMR1 *adrA101*::MudJ) with vector control pBAD30 (left) or AdrA overexpressed in pWJB30 (right).

Fig. 9. Model illustrating the role of different GGDEF domain proteins in *S.* Typhimurium UMR1. STM2123 and STM3388 additively influence the CsgD expression level, while AdrA primarily activates cellulose biosynthesis through the creation of different c-di-GMP pools. Feedback regulation by c-di-GMP produced by AdrA on CsgD expression is seen in strain *S.* Typhimurium MAE52, but not in *S.* Typhimurium UMR1. Domains: HAMP, (histidine kinases, adenylyl cyclases, methyl binding proteins, phosphatases); HTH, helix-turn-helix DNA binding motif of GerE family; MASE1, membrane-associated sensor; MASE2, membrane-associated sensor; MHYT, integral membrane sensory domain with conserved MHYT amino acid pattern; PAS/PAC, Per (periodic clock protein), Arnt (Ah receptor nuclear translocator protein), Sim (single-minded protein). GGDEF and EAL domains with non-conserved residues are assigned the same symbol as GGDEF and EAL domains with consensus sequences, however, the amino acid deviations are indicated (for example, SGHDL instead of GGDEF in STM2503; WLV instead of EAL in STM2123)

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference
Bacterial strains		
S. Typhimurium ATCC 14028		
UMR1	ATCC 14028-1s Nal^r	(Römling *et al.*, 1998a)
MAE32	UMR1 P*csgD2*	(Römling *et al*., 1998b)
MAE51	MAE32 Δ*csgD101*	(Römling *et al*., 2000)
MAE52	UMR1 P*csgD1*	(Römling *et al.*, 1998b)
MAE120	UMR1 STM3615::Km	This study
MAE121	UMR1 STM3388::Km	This study
MAE150	MAE52 *bcsA101*::MudJ	(Zogaj *et al.*, 2001)
MAE165	MAE32 *bcsA101*::MudJ Δ*csgD101*	(Zogaj *et al.*, 2001)
MAE202	MAE52 STM3388::Km	This study
MAE222	UMR1 *bcsA101*::MudJ	(Zogaj *et al*., 2001)
MAE258	UMR1 STM1283::Cm	This study
MAE259	UMR1 STM1987::Cm	This study
MAE260	UMR1 Δ*adrA103*	This study
MAE262	UMR1 STM2672::Cm	This study
MAE263	UMR1 STM4551::Cm	This study
MAE266	UMR1 Δ *adrA103* STM3615::Km	This study
MAE267	UMR1 Δ*adrA103* STM3388::Km	This study
MAE265	UMR1 Δ*csgD*, *bcsA101*::MudJ	This study
MAE270	UMR1 Δ*adrA103* ΔSTM3388	This study

MAE272	UMR1 STM2123::Cm	This study
MAE274	UMR1 Δ*adrA103*, STM2123::Cm	This study
MAE275	UMR1 STM3388::Km STM2123::Cm	This study
MAE276	UMR1 Δ*adrA103* STM3388::Km, STM2123::Cm	This study
MAE279	UMR1 STM2410::Cm	This study
MAE280	UMR1 STM2503::Cm	This study
MAE281	UMR1 STM3375::Cm	This study
MAE282	UMR1 STM1703::Cm	This study
MAE287	UMR1 Δ*adrA103 bcsA101*::MudJ	This study
AdrA1f	UMR1 *adrA101*::MudJ	(Römling *et al.*, 2000)
AdrA1a	MAE52 *adrA101*::MudJ	(Römling *et al.*, 2000)
AdrA6a	MAE52 *adrA102*::MudJ	(Römling *et al.*, 2000)
S. Typhimurium LT2		
LB5010	metA22 metE551 ilv-452 leu-3121 trpC2 xyl – 404 galE856 hsdL6 hsdSA29 hsdsb121 rpsL120 H1-b H2-e,n,x fla-66 nml(-) Fel-2(-)	(Bullas and Ryu, 1983)
Escherichia. coli K-12		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*lacZ*Δ*M15*)	Laboratory collection
Plasmids		
pBAD30	Arabinose-regulated expression vector, Amp^r	(Guzman *et al.*, 1995)
pLAFR3	Broad host range vector, IPTG inducible *lac*	(Staskawicz *et al.*, 1987)

	promoter Tc^r	
pMAK700	Cm^r, Temperature sensitive replicon derived from pSU101	(Hamilton *et al.*, 1989)
pQF50	promoterless *lacZ* gene, RO1600/MB1 origin, Amp^r	(Farinha and Kropinski, 1990)
p*csgBA*	pQF50 containing fragment –186/ + 164 of P*csgBA*	This study
pRGS1	pBAD30:: *yhjH*	(Simm, 2004)
pRGS3	pLAFR3:: *yhjH*	(Simm, 2004)
pRGS19	PBAD30::STM3388	This study
pRGS20	pBAD30::STM2123	This study
pUGE1	pQF50 containing fragment +348/-684 of P*csgD*	(Gerstel and Römling, 2001))
pUGE2	pQF50 containing fragment +348/-685 of P*csgD1*	(Gerstel and Römling, 2001)
pWJB5	pMAK700 Δa*drA103*	This study
pWJB9	pLAFR3:: *adrA*,	(Simm, 2004)
pWJB30	pBAD30::*adrA*	(Zogaj *et al.*, 2001)