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Complex regulatory network encompassing the Csr, c-di-GMP and motility systems of *Salmonella* Typhimurium

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Summary

Bacterial survival depends on the ability to switch between sessile and motile life styles in response to changing environmental conditions. In many species, this switch is governed by (3′-5′)-cyclic-diguanosine monophosphate (c-di-GMP) a signalling molecule, which is metabolized by proteins containing GGDEF and/or EAL domains. *Salmonella* Typhimurium contains 20 such proteins. Here, we show that the RNA-binding protein CsrA regulates the expression of eight genes encoding GGDEF, GGDEF-EAL and EAL domain proteins. CsrA bound directly to the mRNA leaders of five of these genes, suggesting that it may regulate these genes post-transcriptionally. The c-di-GMP specific phosphodiesterase STM3611, which reciprocally controls flagella function and production of biofilm matrix components, was regulated by CsrA binding to the mRNA, but was also indirectly regulated by CsrA through the FlhDC/FliA flagella cascade and STM1344. STM1344 is an unconventional (c-di-GMP-inactive) EAL domain protein, recently identified as a negative regulator of flagella gene expression. Here, we demonstrate that CsrA directly downregulates expression of STM1344, which in turn regulates *STM3611* through *fliA* and thus reciprocally controls motility and biofilm factors. Altogether, our data reveal that the concerted and complex regulation of several genes encoding GGDEF/EAL domain proteins allows CsrA to control the motility-sessility switch in *S.* Typhimurium at multiple levels.

Introduction

The survival of bacteria in diverse environments largely depends on their ability to adjust their life style according to the surrounding conditions. An important factor that mediates the choice of an appropriate life style in various bacteria is the signalling molecule (3′-5′)-cyclic-diguanosine monophosphate (c-di-GMP) (recent reviews by Hengge, 2009; Jonas et al., 2009; Romling and Simm, 2009). In general, high intracellular levels of this second messenger promote sedentary biofilm-associated phenotypes, whereas low concentrations of c-di-GMP favour motility. In several bacteria, c-di-GMP has also been associated with the regulation of virulence and other phenotypes (Cotter and Stibitz, 2007; Tamayo et al., 2007).

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c-di-GMP is synthesized by diguanylate cyclases (DGCs), which contain catalytically active GGDEF domains, and degradation of the second messenger is mediated by phosphodiesterases (PDE), which harbour either EAL or HD-GYP domains (Paul et al., 2004; Simm et al., 2004; Christen et al., 2005; Ryjenkov et al., 2005; Schmidt et al., 2005; Ryan et al., 2006). Notably, individual bacterial genomes frequently encode numerous GGDEF and EAL/HD-GYP proteins (Galperin *et al.*, 2001; Galperin, 2004), implying that the c-di-GMP network is a highly complex and tightly regulated intracellular signalling system (Jonas et al., 2009).

The ability to switch between different life styles is crucial for bacteria such as *Salmonella* that alternate between distinct niches in host organisms during infection as well as in the abiotic environment, in which they can persist for weeks in food, soil, water and other habitats. *Salmonella enterica* serovar Typhimurium contains a c-di-GMP system comprised of 5 GGDEF domain proteins, 7 GGDEF-EAL and 8 EAL domain proteins (Romling, 2005). The characterization of the phenotypes of these proteins has been the subject of several previous studies (Garcia et al., 2004; Kader et al., 2006; Simm et al., 2007; Solano et al., 2009). However, little is known about the regulation of the *Salmonella* c-di-GMP system by intra- and extracellular factors.

In the closely related species *Escherichia coli*, the carbon storage regulator CsrA controls the expression of at least seven of 29 genes encoding GGDEF/EAL domain proteins at the post-transcriptional level (Jonas et al., 2008). Members of the CsrA (RsmA) family are homodimeric RNA-binding proteins (Mercante et al., 2009) that are widely distributed among eubacteria and control various phenotypes including biofilm formation, motility, carbon flux, secondary metabolism, quorum sensing as well as interactions with animal and plant hosts (Romeo, 1998; Babitzke and Romeo, 2007; Lapouge et al., 2008; Lucchetti-Miganeh et al., 2008). In γ-proteobacteria CsrA proteins are antagonized by small non-coding RNAs (sRNAs) that bind and sequester multiple copies of CsrA (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008). Transcription of the sRNAs is controlled by a two-component system (BarA-UvrY in *E. coli*, BarA-SirA in *S.* Typhimurium), permitting the integration of environmental signals into the Csr system (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008).

By directly binding to target mRNAs CsrA can either down- or upregulate the expression of target genes (Babitzke and Romeo, 2007). CsrA binding to the mRNAs of the GGDEF proteins YcdT and YdeH in *E. coli* led to a strong downregulation in transcript levels (Jonas et al., 2008). Both proteins encode DGCs, which inhibit motility. YdeH is also involved in the positive regulation of biofilm formation (Boehm et al., 2009). Thus, by regulating the expression of these GGDEF domain proteins CsrA controls motility and biofilm behaviour in a c-di-GMP dependent manner. In addition, CsrA enhances motility and reciprocally inhibits biofilm formation in *E. coli* by binding to and stabilizing the transcript of the flagella master regulator FlhDC (Wei *et al.*, 2001) and by blocking translation of *pgaA* and destabilizing *pgaABCD* mRNA (Wang *et al.*, 2005), which encodes the synthesis and secretion apparatus of the PGA (poly-β-1,6-N-acetylglucosamine) biofilm polysaccharide adhesin (Itoh et al., 2008).

In contrast to *E. coli*, the role of CsrA in the regulation of the sessility-motility switch is less well understood in *Salmonella. S.* Typhimurium has a CsrA orthologue that is identical in its amino acid sequence to CsrA in *E. coli*. However, *S.* Typhimurium contains neither the *pga* operon nor orthologues of *ycdT*, *ydeH* and most of the other GGDEF/EAL genes that are regulated by CsrA in *E. coli*. Although CsrA regulates genes belonging to the flagella synthesis cascade as well as genes required for virulence (Altier et al., 2000a; Lawhon et al., 2003), no direct mRNA targets have been identified in *Salmonella*. Here, we show that CsrA

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controls the expression of eight genes encoding GGDEF/EAL domain proteins in *S.* Typhimurium by both direct and indirect mechanisms. The complex regulation of these genes enables CsrA to act at multiple levels in the signalling hierarchy mediating the switch between a motile and a sessile life style in *S.* Typhimurium.

Results

CsrA regulates steady state levels of transcripts encoding GGDEF, GGDEF-EAL and EAL domain proteins in *S. Typhimurium*

CsrA regulates GGDEF/EAL domain proteins in *E. coli* by affecting their mRNA steady state levels (Jonas et al., 2008). Since *S.* Typhimurium lacks orthologues of most of the CsrA regulated GGDEF/EAL genes in *E. coli*, we hypothesised that CsrA might control the expression of other GGDEF/EAL domain proteins in *Salmonella*. To identify such genes in *S.* Typhimurium UMR1, we analysed the effect of a *csrA* mutation on the mRNA steady state levels of all 20 genes that encode GGDEF, EAL or GGDEF-EAL domain proteins by quantitative Real-Time RT-PCR. This systematic screen led to the identification of eight genes whose mRNA levels were altered in the *csrA* mutant MAE125 compared to the wild type, when grown as liquid cultures at 37 °C to early stationary phase. Among these were two genes encoding GGDEF proteins (STM1987, STM4551), two genes encoding GGDEF-EAL domain proteins (STM1703, STM3375) as well as four genes encoding the EAL domain proteins STM3611, STM1827, STM1344 and STM1697 (Table 1). These genes have been phenotypically characterized in previous studies and most of them have been assigned to functions in the regulation of biofilm formation, motility and virulence (Table 1). Our data show that the *csrA* mutation caused a significant increase (3 to 7 fold) in the mRNA levels of *STM1987*, *STM1703*, *STM3375*, *STM1827* and *STM1344* (Fig. 1). Also the transcript levels of *STM4551* and *STM1697* were elevated in the *csrA* mutant compared to the wild type, albeit less strongly (1.6 to 1.8 fold). *STM3611* (also known as *yhjH*), encoding an active PDE (Simm et al., 2004), was the only gene which showed a drastic decrease in mRNA steady state levels (>10 fold) in the *csrA* mutant. Ectopic *csrA* expression from pBADcsrA (pCsrA) fully complemented the effect of the *csrA* mutation for all genes that were upregulated in the *csrA* mutant. Expression of *STM3611* was partially restored (to approx. 45 % of wt mRNA levels) by pCsrA. Altogether, these data demonstrate that CsrA down- or upregulates the mRNA steady state level of *STM1987*, *STM4551*, *STM1703*, *STM3375*, *STM3611*, *STM1827*, *STM1344* and *STM1697* in *S.* Typhimurium. Although we did not observe significant changes in the mRNA levels for the remaining 12 genes encoding GGDEF, GGDEF-EAL or EAL domain proteins in *Salmonella* (data not shown), we cannot rule out that CsrA affects the expression of these genes under different growth conditions or that potential changes in the half-lives of these mRNAs in the *csrA* mutant are compensated by unknown homeostatic mechanisms.

Potential CsrA binding sites in the 5′ untranslated leader sequences

CsrA is known to regulate gene expression by binding to specific sites in the 5′ untranslated regions (UTRs) of mRNA targets and thereby to affect mRNA steady state levels (Wei et al., 2001; Wang et al., 2005). Previous studies determined an optimal site [5′-(A/U)CA-GGA-G(U/A)-3′] for high affinity CsrA/RsmA binding (Valverde et al., 2004; Dubey et al., 2005; Schubert et al., 2007). However, the key feature that is recognized by CsrA in *E. coli* RNAs (*pgaA*, *cstA*, *glgC*, *hfq*, *ydeH*, *ycdT, sepL*, *grlR*, *csrB* and *csrC*) is the trinucleotide motif GGA, which is preferentially but not necessarily present in the loops of hairpin structures (Liu et al., 1997; Baker et al., 2002; Dubey et al., 2003; Weilbacher et al., 2003; Wang et al., 2005; Baker et al., 2007; Jonas et al., 2008; Bhatt et al., 2009). We determined the 5′ end of the transcripts, which were regulated by CsrA, and analysed the 5′UTRs for the presence of GGA triplets, the most invariant element of the CsrA binding site. The 5′UTRs of *STM1987*,

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STM3375, *STM1703, STM3611*, *STM1344* and *STM1697* contained at least one (*STM1703*) and at most 11 (*STM1987*) GGA motifs (Table 2), raising the possibility that CsrA directly interacts with the transcripts corresponding to these genes. No GGA triplets were present in the 5′UTRs of the transcripts of *STM1827* and *STM4551* (Table 2). Noticeably, *STM1987*, *STM4551*, *STM1703*, *STM3375*, *STM3611*, *STM1827, STM1344* and *STM1697* are not located in operons and, thus, possess their own promoters and 5′UTRs (S. 1). Likewise, most of the other genes encoding GGDEF/EAL proteins in *Salmonella* are stand-alone genes that are not clustered together with other functionally related genes (unpublished observation).

CsrA binds specifically to untranslated mRNA leaders of STM1987, STM3375, STM3611, STM1344 and STM1697

To determine whether CsrA directly interacts with the transcripts of the eight GGDEF/EAL genes found to be regulated by CsrA in *Salmonella*, gel-mobility shift assays were performed with CsrA protein, purified as previously described (Mercante *et al.*, 2006), and *in vitro* synthesized transcripts, comprising the entire untranslated leader and a small part of the coding region, respectively: *STM1987* (374 nt, 3 nt), *STM4551* (109 nt, 3 nt), *STM3375* (322 nt, 3 nt), *STM1703* (95 nt, 3 nt), *STM3611* (39 nt, 10 nt), *STM1827* (42 nt, 38 nt), *STM1344* (133 nt, 11 nt) and *STM1697* (65 nt, 3 nt). CsrA binding to the transcripts of *STM1987*, *STM3375*, *STM3611*, *STM1344* and *STM1697* was detected as distinctly shifted bands in native gels (Fig. 2A). No shifts were detected for *STM1703* and *STM4551* (S. 2A), suggesting that CsrA regulates these genes by an indirect mechanism or requires additional factors that are necessary for an interaction. Consistent with the gel-shift data, no potential binding site for CsrA was detected in the 5′leader of *STM4551* (Table 2). The leader of *STM1703* contained one GGA motif. However, most previously identified direct CsrA targets in *E. coli* contain at least two binding sites (Mercante et al., 2009). Although a shifted band was seen for *STM1827* at high CsrA concentrations, significant amounts of the free transcript remained unbound (S. 2A). In the 5′UTR of *STM1827* no GGA motifs were found. Thus, it is possible that CsrA interaction with the *STM1827* transcript may not be biologically relevant, although we cannot completely exclude this possibility to this date. We also cannot rule out that CsrA interacts with other regions than the 5′UTRs of the transcripts of *STM4551*, *STM1703* and *STM1827*.

More than one shifted complex was detected for *STM1987*, *STM3375*, *STM1344* and *STM1697* (Fig. 2A), suggesting that two or more CsrA proteins were bound to each transcript, although the stoichiometry of binding was not experimentally determined. In contrast, *STM3611* formed only one shifted complex (Fig. 2A). A nonlinear least-squares analysis of these CsrA binding data yielded apparent K_d values of 53 ± 10 nM for *STM1987*, 34 ± 7 nM for *STM3375*, 119 ± 8 nM for *STM3611*, 58 ± 5 nM for *STM1344* and 33 ± 3 nM for *STM1697*. These binding constants are comparable to the affinities previously measured for the interactions between CsrA and the *E. coli* mRNA targets *glgC* (39 nM), *cstA* (40 nM), *pgaA* (22 nM), *hfq* (38 nM) and *sepL* (23 nM) (Baker et al., 2002;Dubey et al., 2003;Wang et al., 2005;Baker et al., 2007;Bhatt et al., 2009). In contrast, CsrA binding to the transcripts of *ycdT*, *ydeH* (K_ds ≈ 2.5 nM) and *grlR* (K_d ≈ 6 nM) was stronger (Jonas et al., 2008;Bhatt et al., 2009).

The specificity of the CsrA-RNA interactions was investigated by performing competition experiments with specific (*STM1987*, *STM3375*, *STM3611, STM1344*, *STM1697* and *RNA10-2BS*) and non-specific (*phoB* 5′ untranslated region from *E. coli* K-12) unlabelled RNA competitors. Unlabelled *STM1987*, *STM3375*, *STM1344* and *STM1697* RNAs, respectively, were able to compete for CsrA binding with the corresponding labelled transcripts while *phoB* RNA did not compete (Fig. 2B, S. 2B). Unlabelled *STM3611* RNA exhibited complex interactions (Fig. 2B). As the concentration of this RNA was increased, slower migrating bands were observed, the major species of which was also seen in the

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absence of CsrA (last lane), strongly suggesting the formation of complexes between the labelled *STM3611* RNA, unlabelled *STM3611* RNA and CsrA. Thus, to determine binding specificity of *STM3611*, unlabelled RNA10-2BS, which contains two optimal CsrA binding sites (Mercante et al., 2009) was used as a specific competitor for *STM3611* RNA. RNA10-2BS was able to compete for CsrA binding without the formation of novel shifted species, while unlabelled *phoB* RNA did not compete with the *STM3611*-CsrA interaction (Fig. 2B). Altogether, these results indicate that CsrA binds specifically and with high affinity to *STM1987*, *STM3375*, *STM1344*, *STM1697* and *STM3611* RNA.

Indirect regulation of STM3611 through the FlhDC/FliA cascade

The mRNA level of *STM3611* was drastically (>10 fold) downregulated in the *csrA* mutant compared to the wild type (Fig. 1). Consistent with this finding, the level of STM3611 protein was strongly decreased in the *csrA* mutant as observed by Western Blot analysis (Fig. 3A). *STM3611* encodes a PDE, which positively controls flagella function and has a negative effect on the production of the *Salmonella* biofilm matrix components curli and cellulose (Simm et al., 2004;Simm et al., 2007). *STM3611* is under the control of the flagella sigma factor FliA (Ko and Park, 2000;Wang et al., 2004;Frye et al., 2006;Claret et al., 2007). *fliA*, in turn is transcribed from a flagella gene class II promoter, which requires the master regulator of flagella synthesis, FlhDC (class I) for its activation (Chevance and Hughes, 2008).

E. coli and *S.* Typhimurium *csrA* mutants lack flagella and are non-motile (Wei et al., 2001; Lawhon et al., 2003). Furthermore, in *E. coli* CsrA was shown to control flagella synthesis by binding to and stabilizing *flhDC* mRNA (Wei et al., 2001). In *S.* Typhimurium, a microarray-based study revealed that genes belonging to the *flhDC* regulon were strongly downregulated in a *csrA* mutant (Lawhon et al., 2003). We found the mRNA levels of *flhDC, fliA* and genes belonging to the FliA regulon to be significantly decreased in the *csrA* mutant MAE125 compared to the wild type (Fig. 3B). Among these genes was also *STM1798* (*ycgR*), encoding a PilZ domain containing c-di-GMP receptor protein (Ryjenkov *et al.*, 2006), which has previously been shown to be co-regulated with *STM3611* (Ko and Park, 2000; Wang et al., 2004; Frye et al., 2006; Claret et al., 2007).

As we considered it likely that the strong effect of CsrA on the mRNA levels of *STM3611* was mediated, at least partly, by the indirect regulation of *STM3611* through FlhDC and FliA, we attempted to complement the *csrA* mutation with plasmid-borne expression of *fliA* and *flhDC*, respectively. Ectopic expression of *fliA* from pBADfliA in the *csrA* mutant led to strongly elevated mRNA levels of both *STM3611* and *STM1798* (> 5 fold compared to wt) (Fig. 3C). We explain this result with the fact that the *csrA* mutant is deficient in the expression of the anti-sigma factor *flgM*, which is co-transcribed with *fliA* and counteracts FliA's activity in the wild type background (Chevance and Hughes, 2008). Ectopic expression of *flhDC* from pBADflhDC in the *csrA* mutant led only to a partial restoration of *STM3611* mRNA levels (approx. 60 % of wt), but to a complete restoration of *STM1798* transcript levels (approx. 105 % of wt) (Fig. 3D).

Furthermore, analysis of the swimming behaviour of the bacteria revealed that plasmid-borne expression of *flhDC* only partly restored the swimming defect of a *csrA* mutant (Fig. 3E). In contrast, expression of plasmid-borne *csrA* from pBADcsrA enabled the *csrA* mutant to swim in motility agar as the wild type. Overexpression of *fliA* did not restore the swimming ability in the *csrA* mutant, which is in agreement with the model that the other FlhDC regulated class II genes, which assemble the hook-basal body of flagella (Chevance and Hughes, 2008), are shut down in the *csrA* mutant.

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Altogether, our data suggest that CsrA controls the flagella cascade by regulating *flhDC*, which results in the indirect upregulation of *STM3611*. Our data also indicate that CsrA does not exclusively regulate the flagella signalling hierarchy and STM3611 through *flhDC*, but also affects additional pathways.

Direct regulation of STM3611 by CsrA

Gel-shift analysis revealed that CsrA specifically interacted with the transcript of *STM3611* (Fig. 2A,B), suggesting that in addition to the indirect regulation through FlhDC/FliA CsrA might also regulate *STM3611* mRNA stability. We noted that the leader of *STM3611* contains two GGA motifs (Table 2), one of which seems to overlap the Shine-Dalgarno (SD) sequence, at which ribosome binding occurs. Previously identified CsrA mRNA targets in *E. coli*, which contain one CsrA binding site at the SD site and another one further upstream, have been demonstrated to be destabilized by CsrA (Mercante et al., 2009). However, our results show that in a *fliA* deficient background a mutation in *csrA* led to a further reduction in *STM3611* mRNA levels (Fig. 4A), suggesting that binding of CsrA might have a stabilizing effect on *STM3611* mRNA levels, independently of *fliA*. In contrast, the mRNA levels or *STM1798*, whose transcript did not interact with CsrA as determined by gel-mobility shift analysis (S. 2A), were the same in the *fliA* mutant and the *csrA fliA* double mutant (Fig. 4A).

The regulation of *flhDC* by CsrA in *E. coli* has so far been the only example, in which binding of CsrA to a transcript resulted in mRNA stabilization (Wei et al., 2001). It is possible that CsrA-mediated activation processes require the formation of higher order RNA structural alterations that involve CsrA binding to additional uncharacterized sites in the transcript. However, to this date no molecular model exists for CsrA-mediated activation. Our data indicate that CsrA upregulates *STM3611* expression by activating its transcription through the FlhDC/FliA cascade, as well as by directly interacting with *STM3611* transcript (Fig. 4B).

Indirect regulation of STM3611 through STM1344

Besides *STM3611*, CsrA was found to strongly alter the mRNA and protein levels of another EAL domain protein, STM1344 (Fig. 1, Fig. 5A). In contrast to STM3611 and other conventional EAL domain proteins, STM1344 lacks activity as a c-di-GMP specific PDE and displays phenotypes, which are more typical for DGCs: the upregulation of biofilm behaviour and the downregulation of motility (Simm et al., 2009). Furthermore, STM1344 exerts its effect on biofilm behaviour through the PDEs STM3611 and STM1703, which are upregulated in the *STM1344* mutant (Simm et al., 2009). In another parallel study STM1344 (also called YdiV) was identified as a negative regulator of flagella gene expression and it was suggested that STM1344 acts on post class I genes (Wozniak et al., 2008). In agreement with these previous studies, we found that a mutation in *STM1344* caused an upregulation in *STM3611* mRNA levels (Fig. 5B). Furthermore, the transcript levels of *STM1798* and *fliA* were increased in the *STM1344* mutant to similar extents, suggesting that STM1344 might regulate *STM3611* expression through *fliA*. A similar upregulation of *STM3611*, *STM1798* and *fliA* was detected in a *csrA STM1344* double mutant (Fig. 5C), which is in agreement with our model that CsrA acts upstream of STM1344.

The suggested sequential regulation of *STM3611* by *fliA* and *STM1344* was confirmed by additional studies, in which the ability of *Salmonella* to produce the biofilm matrix components curli and cellulose was used as a read-out. Curli and cellulose production can be conveniently visualized on Congo Red (CR) agar plates, on which colonies expressing these surface structures appear as red, dry and rough (rdar) and those that lack them as smooth and white (saw) (Romling, 2005). Under biofilm-inducing conditions (28 °C, LB agar plates

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without salt), the phosphodiesterase STM3611 was found to negatively affect rdar morphotype expression (Simm et al., 2007). Specifically, a mutant in *STM3611* shows a more pronounced rdar phenotype on CR plates than the wild type and the protein level of CsgD, the major activator of curli and cellulose synthesis, is upregulated (Simm et al., 2007). Previous results also revealed that a mutation in *STM1344* downregulates *csgD* expression through STM3611, resulting in a reduced production of curli and cellulose (Simm et al., 2009). Our data show that, similar to the *STM3611* deletion strain, a mutant in *fliA* displayed elevated CsgD levels and enhanced rdar morphotype expression compared to the wild type (Fig. 5D). The same phenotype was also observed in a *STM1344 fliA* double mutant, confirming our model that STM1344 acts upstream of FliA and thereby regulates the PDE STM3611, which in turn positively controls motility behaviour and has a negative impact on the production of biofilm matrix components.

In summary, these data show that CsrA regulates the levels of STM3611 by an additional pathway, involving the direct regulation of *STM1344*, which in turn affects *STM3611* by interfering with the flagella cascade upstream of *fliA* (Fig. 5E). Altogether these data indicate that CsrA controls the PDE STM3611 by at least three distinct pathways: the indirect regulation through *flhDC*, the direct regulation of its mRNA level and the indirect regulation through *STM1344* (Fig. 5E). We suggest that this multi-layer control allows CsrA to precisely regulate the activity of STM3611 and, hence, the switch between motility and biofilm behaviour.

Discussion

The transition between a sessile and a motile life-style requires the complex integration of the pathways that regulate either bacterial behaviour. The present study describes the tight interplay between the Csr, c-di-GMP and motility systems in *S.* Typhimurium. In a recent study the regulation of c-di-GMP signalling by CsrA was investigated in *E. coli* (Jonas et al., 2008). However, the interconnection between Csr and c-di-GMP signalling differs substantially between *E. coli* and *Salmonella*. This let suggest that the utilization of conserved regulatory systems is highly adaptable and can vary between closely related species.

CsrA-mediated downregulation of the DGCs STM1987 and STM4551

Previous studies revealed that CsrA promotes motility and inhibits biofilm-associated phenotypes (Romeo, 1998; Wei et al., 2001; Jackson et al., 2002; Wang et al., 2005; Jonas et al., 2008). In agreement, CsrA was found to directly or indirectly downregulate the c-di-GMP producing enzymes STM1987 and STM4551, respectively, in *S.* Typhimurium (Figs. 1 and 2). Under certain growth conditions STM1987 promotes the synthesis of the biofilm polysaccharide cellulose (Garcia et al., 2004; Solano et al., 2009) and thus acts as an activator of biofilm formation. STM4551 has recently been shown to possess DGC activity, but it also appeared to act by a c-di-GMP-independent mechanism on diverse phenotypes (Solano et al., 2009). In *E. coli* CsrA downregulates the two DGCs YdeH and YcdT (Jonas et al., 2008), which are distinct from STM4551 and STM1987. Similar to STM1987, YdeH promotes the synthesis of a biofilm polysaccharide by a c-di-GMP dependent mechanism (Boehm et al., 2009). However, instead of cellulose, which is not produced by *E. coli* K12, YdeH controls production of the polysaccharide PGA (Boehm et al., 2009).

Complex regulation of the PDE STM3611 and the motility cascade

While CsrA inhibits c-di-GMP synthesis by downregulating STM1987 and STM4551, it upregulates the PDE STM3611 and thus promotes the degradation of c-di-GMP (Simm et al., 2004; Simm et al., 2007). STM3611 is suggested to influence the functionality of the

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flagella motor by degrading a local pool of c-di-GMP (Romling and Amikam, 2006; Wolfe and Visick, 2008). Under conditions when STM3611 is inactive, c-di-GMP accumulates and presumably binds to the receptor protein STM1798, containing a PilZ domain (Ryjenkov et al., 2006). The resulting c-di-GMP-STM1798 complex was suggested to negatively affect motor function (Romling and Amikam, 2006; Wolfe and Visick, 2008). In addition, STM3611 inhibits biofilm behaviour by negatively regulating the expression of *csgD* by a c-di-GMP dependent mechanism (Simm et al., 2007). Thus, by regulating STM3611 CsrA can reciprocally act on motility and biofilm behaviours.

Notably, the regulation of STM3611 by CsrA occurred at multiple levels: by two indirect pathways involving FlhDC (Fig. 3) and STM1344 (Fig. 5), respectively, and by a direct, presumably post-transcriptional mechanism (Figs. 2 and 4). This three-tiered regulatory circuitry must permit precise control of the levels of the PDE STM3611. Furthermore, these results illustrate, for the first time, that CsrA controls the motility cascade at multiple levels (*flhDC* - class I, *STM1344* - post-class I, *STM3611* - class III) in the signalling hierarchy. We suggest that this complex control enables CsrA to coordinate flagella synthesis with flagella function. The feed-forward arrangement that directly regulates STM3611 might allow a rapid onset of the flagella motor, under conditions that favour motility. It is likely that CsrA also controls the expression of the orthologue of STM3611 in *E. coli* (YhjH), although this has not been experimentally demonstrated.

Noticeably, the mRNA levels of two other PDEs, STM1827 and STM1703, were downregulated by CsrA (Fig. 1), probably by indirect mechanisms. Both STM1827 and STM1703 have previously been shown to degrade c-di-GMP and thereby to downregulate *csg* expression (Simm et al., 2007).

Regulation of unconventional GGDEF/EAL domain proteins

CsrA was also found to control GGDEF and/or EAL domain proteins that have apparently lost the ability to metabolize c-di-GMP and have instead evolved alternative functions. The EAL-domain protein STM1344, which was directly downregulated by CsrA (Figs. 1, 2 and 5), contains a degenerate sequence motif in its EAL domain and is inactive in the degradation or binding of c-di-GMP (Simm et al., 2009). STM1344 negatively controls the flagella cascade by an unknown mechanism upstream of *fliA* (present study) and downstream of *flhDC* transcription (Wozniak et al., 2008). The indirect regulation of the PDE STM3611 by STM1344 results in the downregulation of motility and the upregulation of biofilm matrix production. STM1344 also controls the PDE STM1703 (Simm et al., 2009), which negatively regulates *csg* expression and, thus, biofilm behaviour (Simm et al., 2007) (Fig. 6). Hence, despite its degenerate EAL domain, STM1344 still maintains a function in the regulation of c-di-GMP metabolism.

Another EAL domain protein, STM1697, shows high sequence similarity to STM1344, and its mRNA level was directly regulated by CsrA (Figs. 1 and 2). Similar to STM1344, STM1697 does not contain the highly conserved sequence motifs (e.g. EXL, DDFGTG), which have previously been suggested to be critical for PDE activity (Schmidt et al., 2005;Rao et al., 2009), suggesting that STM1697 has no PDE activity. A mutant in STM1697 does not show a distinct phenotype in motility or biofilm behaviour (Simm *et al.*, 2007). However, data from another on-going study suggest that the protein might be involved in the regulation of virulence phenotypes (Lamprokostopoulou and Römling, unpublished).

CsrA also regulated the expression of the unorthodox GGDEF-EAL protein STM3375 (Fig. 1, Fig. 2). The *E. coli* STM3375 orthologue CsrD (YhdA) is itself a component of the Csr system (Jonas et al., 2006;Suzuki et al., 2006), which facilitates RNaseE-dependent

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degradation of the sRNAs CsrB and CsrC, the molecular antagonists of CsrA (Suzuki *et al.*, 2006). Thus, although not directly involved in the synthesis or degradation of c-di-GMP, CsrD controls the activity of CsrA, a global regulator of GGDEF and EAL domain proteins and c-di-GMP levels (Jonas et al., 2008). STM3375 and *E. coli* CsrD are highly similar proteins. They contain identical degenerate GGDEF and EAL motif signatures, as well as an identical sequence in the EAL domain that is unique to putative CsrD homologues (R579-TENQLLVQ-S588) and is required for activity of the *E. coli* protein (Suzuki et al., 2006). This suggests that these proteins function similarly. The finding that CsrA controls the expression of *csrD* (Suzuki et al., 2006;Jonas et al., 2008) and *STM3375* indicates that it regulates its own activity through an autoregulatory loop in both of these species.

Signal integration into the Csr/c-di-GMP system

CsrA activity is also controlled by the BarA-SirA (BarA-UvrY in *E. coli*) two-component system (Fig. 6), which activates the transcription of *csrB* and *csrC* (Altier et al., 2000b;Suzuki et al., 2002;Teplitski et al., 2003;Weilbacher et al., 2003). The chemical nature of the signal acting on the BarA sensor and its orthologues in other bacteria has not been identified yet. However, studies in *E. coli* suggest that the metabolic status of the cells and the external pH regulate the activity of the two-component system (Pernestig et al., 2003;Mondragon et al., 2006;Jonas and Melefors, 2009). In *Salmonella*, the presence of bile salts seems to affect BarA-SirA dependent responses (Prouty and Gunn, 2000) and results from another study have shown that short chain fatty acids affect CsrA mediated phenotypes in a pathway involving SirA, but probably not BarA (Lawhon et al., 2002). Transcription of *csrB* and *csrC* somehow also requires upstream activation by CsrA, indicative of an additional feedback loop (Fig. 6) and strongly suggestive of a homeostatic mechanism controlling CsrA activity (Gudapaty et al., 2001;Suzuki et al., 2002;Weilbacher et al., 2003;Jonas and Melefors, 2009). The tight regulation of CsrA activity by BarA-SirA, CsrD and CsrA itself through the Csr sRNAs ensures that downstream targets of CsrA are precisely coordinated in response to multiple input signals.

Central role of CsrA in the mediation of life-style switches

Our data illustrate that the regulation of GGDEF and EAL domain proteins enables CsrA to tightly control the switch between sessility and motility at multiple levels (Fig. 6). In general, CsrA activates motility pathways and inhibits the production of biofilm factors. Notably however, in contrast to a *csrA* mutant in *E. coli*, in which biofilm formation is strongly enhanced (Jackson et al., 2002), a *S.* Typhimurium *csrA* mutant does not show increased adherence or rdar morphotype expression as would be expected (Teplitski et al., 2006)(our unpublished observations). Instead, colonies of the *csrA* mutant have a mucoid and smooth colony appearance (our unpublished observations). Although the molecular basis of this observation remains unknown, it indicates a complex role of CsrA in the regulation of cell surface / extracellular phenotypes in *Salmonella*.

Finally, CsrA plays an important role in the regulation of *Salmonella* virulence genes (Altier et al., 2000a; Lawhon et al., 2003). Likewise, c-di-GMP has been recently found to affect virulence properties in *Salmonella* (Lamprokostopoulou et al., 2009). In fact, several of the CsrA-regulated genes studied herein affect virulence phenotypes, including STM1344, STM4551 and STM1697 (Hisert et al., 2005; Solano et al., 2009). Thus it is likely, that the regulation of GGDEF and/or EAL domain proteins allows CsrA not only to control motility and sessility, but also a range of other bacterial behaviours that are important for *Salmonella* to adapt to changing environments.

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Experimental Procedures

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. Chromosomal mutations were generated using the Datsenko method (Datsenko and Wanner, 2000). For the construction of the *csrA::kan* mutant allele, the *kan* gene was amplified from pKD4 by PCR using the primer pair CsrAKOup/CsrAKOdn (Table 3) and electroporated into arabinose-treated *S.* Typhimurium ATCC 14028 carrying pKD46. For the construction of the *fli*A mutant MAE1456 (UMR1 *fliA::cat*), the *cat* gene was amplified from pKD3 using the primer pair FliAKOfor/FliAKOrev and introduced by electroporation into arabinose-treated *S.* Typhimurium UMR1 carrying pKD46. Transformants were selected for the gain of kanamycin or chloramphenicol resistance, respectively, and the loss of ampicillin resistance and were verified by PCR using the appropriate control primer pairs (S. 3). To generate MAE125 (UMR1 *csrA::kan*) the *csrA::kan* allele was transferred by P22 transduction into *Salmonella* Typhimurium UMR1. For construction of the *fliA csrA* (MAE1476) and the *STM1344 csrA* (MAE1474) double mutants, the mutant alleles from MAE1456 (UMR1 *fliA::cat*) and MAE424 (UMR1 *STM1344::cat*), respectively, were transduced into the MAE125 background. For the construction of the *fliA STM1344* double mutant MAE1481, the *cat* cassette was removed from MAE1456 followed by the transduction of *STM1344::cat* into the resulting strain (MAE1463). To generate MAE1493 (UMR1 Δ*csrA STM3611*-SPA) and MAE1492 (UMR1 Δ*csrA STM1344*-SPA), the *kan* cassette from MAE125 was flipped out using the the FLP recombinase followed by the transduction of the SPA-tagged *STM1344* and *STM3611* constructs from MAE132 (UMR1 STM1344-SPA kan^r) and MAE130 (UMR1 STM*3611*-SPA kan^r), respectively, into the resulting strain. Bacteria were routinely grown in LB medium at 37 °C with shaking at 200 r.p.m. If necessary, arabinose (0.1 %) or antibiotics were added: ampicillin 100 μg ml^{-1}, kanamycin 50 μg ml^{-1} and chloramphenicol 30 μg ml^{-1}.

Plasmid construction

All plasmids used in this study are listed in Table 1. For construction of pIRF-2 the *fliA* gene was amplified from the *S.* Typhimurium UMR1 chromosome by PCR using the primers pBADfliAfor and pBADfliArev (S. 3). The resultant PCR product was cleaved with *XbaI* and *HindIII* and inserted between the corresponding sites of pBAD30, under the control of the arabinose inducible promoter P_{BAD}. Sequencing verified the integrity of the *fliA* gene.

Quantitative real-time RT PCR

RNA was sampled, treated with RNAprotect Bacterial Reagent (Qiagen) and prepared using the RNeasy Mini Kit with on-column DNA digestion (Qiagen) according to the protocol. After determination of the RNA concentrations using the NanoDrop ND-1000 UV-Vis Spectrophotometer, 1 μg RNA was reverse transcribed in 20 μl reactions using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers were designed with the Primer Express Software v3.0 (Applied Biosystems). Twenty ng of template were used for the real-time PCR reaction using Power SYBR Green PCR Master Mix (Applied Biosystems). The cycling reaction was performed with an ABI 7500 Real Time PCR System (Applied Biosystems) using the standard run mode of the instrument. For detection of primer dimerization or other artefacts of amplification, dissociation curves and non-template controls were included in the real-time PCR analysis. Individual gene expression profiles were normalized against the *recA* gene or the *rrnD* gene (16S rRNA), serving as endogenous controls. All results were analysed using the 7500 SDS Software v1.3.1 (Applied Biosystems) and further prepared using Excel (Microsoft). In all experiments, the change in expression was measured relative to a calibrator, e.g. wild type, which was set to 1. The data values presented in all figures represent the mean values calculated from the results from at

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least three independent repetitions of the experiment. The error bars represent the standard deviations. For statistical evaluation p-values were calculated using the student t-test.

5′ Rapid amplification of cDNA ends (5′RACE)

The 5′ ends of the transcripts of *STM1987*, *STM4551*, *STM3375*, *STM1703*, *STM3611*, *STM1827*, *STM1344*, *STM1697* and *STM1798* were determined using the 5′RACE System for Rapid Amplification of cDNA ends (v2.0 Invitrogen) according to the protocol and as previously described (Jonas *et al.*, 2008). The resulting RACE PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Kit (v3.1).

RNA gel mobility shift assays

Quantitative gel mobility shift assays followed a previously published procedure (Yakhnin *et al.*, 2000). CsrA-His_6 protein was purified as described previously (Mercante *et al.*, 2006). DNA templates for generating *STM1697*, *STM1798*, and *phoB* (non-specific competitor) RNA transcripts were produced by annealing primers STM1697-T7 and GC STM1697-T7, STM1798-T7 and GC STM1798-T7, or phoB-T7 and GC phoB-T7 in TES buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl). DNA templates for STM1344, STM1703, STM1827, STM1987, STM3375, and STM4551 were PCR-amplified from UMR1 genomic DNA using primers STM1344-F-T7 and STM1344-R-T7, STM1703-F-T7 and STM1703-R-T7, STM1827-F-T7 and STM1827-R-T7, STM1987-F-T7 and STM1987-R-T7, 3375-F1(P1)-T7 and 3375-R1(ATG)-T7 or STM4551-F-T7 and STM4551-R-T7 (S. 3). RNA was synthesized in vitro using the MEGAshortscript kit (Ambion, Austin, TX) using the annealed DNA primers (*STM1697*, *STM1798* and *phoB*) or DNA templates (*STM1344, STM1703, STM1827, STM1987, STM3375, and STM4551*) as templates, and RNA was gel purified. *STM3611* and RNA10-2BS RNA were synthesized by Integrated DNA Technologies (Coralville, IA). Transcripts were 5′ end-labelled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. Radiolabelled RNA was gel purified and resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), heated to 85°C and chilled on ice. Increasing concentrations of purified CsrA-His_6 recombinant protein were combined with 50 pM radiolabelled RNA in 10 μl of binding reactions [10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 3.25 ng total yeast RNA, 20 mM DTT, 7.5 % glycerol, 4 U SUPERasin (Ambion, Austin, TX)] for 30 min at 37 °C to allow for CsrA–RNA complex formation. Competition assays were performed in the absence or presence of unlabelled RNA specific and non-specific competitors. Binding reactions were separated using 8-12 % native polyacrylamide gels, and radioactive bands were visualized with a Molecular Dynamics phosphorimager. Free and bound RNA species were quantified with ImageQuant Software (Molecular Dynamics), and an apparent equilibrium binding constant (K_d) was calculated for CsrA–RNA complex formation according to a previously described cooperative binding equation (Mercante et al., 2006). The mean values and standard errors from at least two independent experiments were determined for each transcript. Graphpad Prism version 3.02 for Windows (San Diego, CA) software was used for calculations.

Western Blot analysis

For Western Blot analysis 5 μg of cells were harvested, resuspended in sample buffer, and heated to 95 °C for 10 min. The protein content was analysed by staining with a Coomassie blue solution (20 % methanol, 10 % acetic acid, 0.1 % Coomassie brilliant blue G). Equal amounts of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 15 % (for SPA tagged proteins) or 12 % (for CsgD) resolving gels and 4 % stacking gels and were transferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore). The detection of the SPA tags was done using anti-FLAG antibody (1:2000) and anti-mouse immunoglobulin G conjugated with horseradish peroxidase (1:2000, Jackson ImmunoResearch Laboratories Inc.) as the secondary antibody.

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Detection of CsgD was performed as previously described (Romling et al., 2000) by using the polyclonal anti-CsgD peptide antibody (1:5000) as the primary antibody and goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:2000) as the secondary antibody. Chemiluminescence from the Lumi-Light WB substrate (Roche) was recorded using the LAS-1000 system (Fujifilm). The intensity of the bands was quantified using Adobe Photoshop CS3. Each experiment was repeated at least three times and representative results were chosen for display.

Motility assay

To analyse the swimming behaviour of the bacteria, 0.3 % LB agar plates supplemented with 0.1 % arabinose were inoculated with 4 μl of overnight cultures, which were grown in LB with 0.1 % arabinose for 14 -16 h. The plates were incubated at 37 °C and the diameter of the swimming zone was measured over time. Each experiment was performed at least three times, and a representative result was chosen for display.

Congo Red binding assay

Samples of 5 μl of an overnight culture suspended in water (to an optical density at 600 nm [OD_{600}] of 5) were spotted onto LB agar plates lacking NaCl and supplemented with Congo red (40 μg ml^{-1}) and Coomassie brilliant blue (20 μg ml^{-1}). Plates were incubated at 28°C and the development of the colony morphology and dye binding were analyzed over time.

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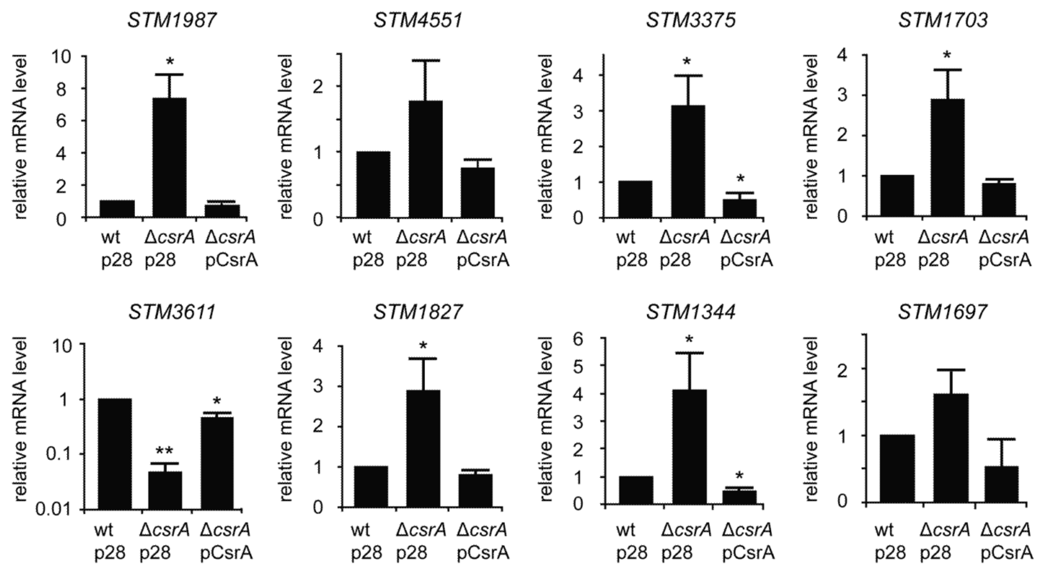

Figure 1.

CsrA regulates the mRNA steady state level of genes encoding GGDEF/EAL domain proteins in *S.* Typhimurium. Relative mRNA levels of *STM1987, STM4551, STM3375, STM1703, STM3611, STM1827, STM134*4 and *STM1697* were measured by quantitative Real-Time RT PCR in the wild type UMR1 (wt) and in the *csrA::kan* mutant MAE125 (Δ*csrA*), carrying the empty vector pBAD28 (p28) or the CsrA vector pBADcsrA (pCsrA), respectively. Note that the data for *STM3611* are displayed with a logarithmic scale. Total RNA was isolated from bacterial cultures grown at 37 °C in LB medium with 0.1 % arabinose to OD_{600} 1.5. The data values represent means with standard deviations (** $P < 0.01$; * $P < 0.05$).

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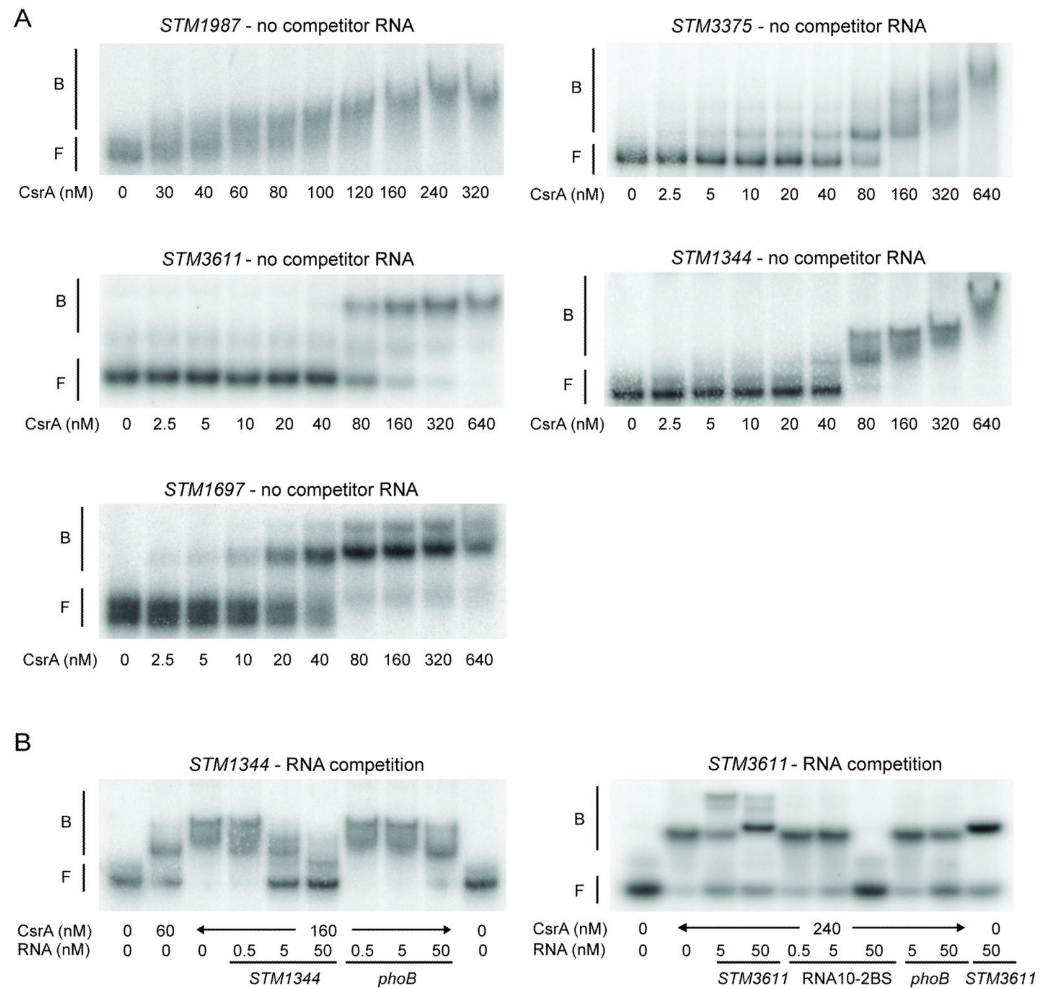

Figure 2.

Direct interaction between CsrA and the transcripts of *STM1987*, *STM3375*, *STM3611*, *STM1344* and *STM1697*. A) Gel mobility shift analyses of CsrA–STM1987, CsrA–STM3375, CsrA–STM3611, CsrA–STM1344 and CsrA–STM1697 interactions in the absence of RNA competitor. The 5′ end-labelled respective transcript was incubated with CsrA at the indicated concentrations. The positions of free (F) and bound (B) RNA are shown. B) Competition reactions for STM1344 and STM3611 using specific (STM1344, STM3611 or RNA10-2BS) or non-specific (*phoB*) unlabelled RNA competitors. The concentration of competitor RNA is shown at the bottom of each lane.

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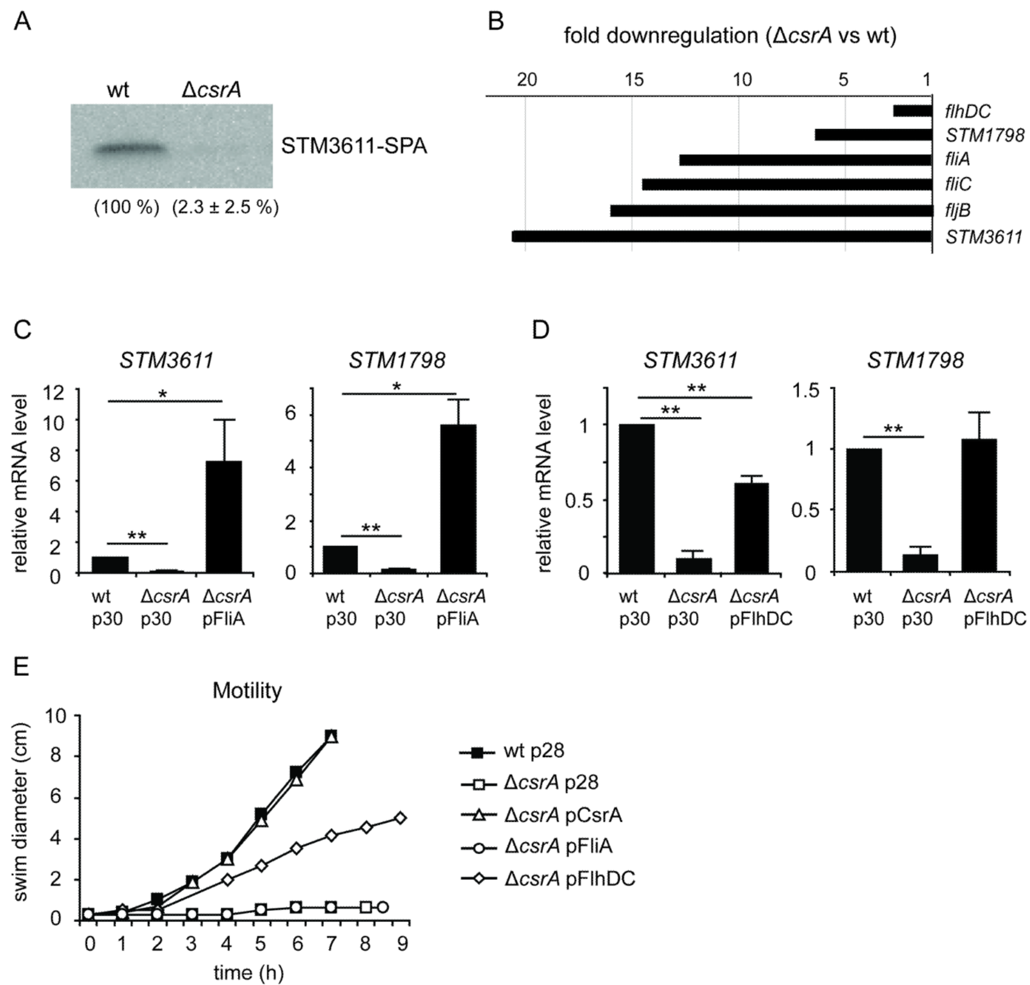

Figure 3.

Indirect regulation of STM3611 by CsrA through the flagella cascade. A) Protein levels of SPA-tagged STM3611 in the wild type (wt) and the *csrA* deficient background (Δ*csrA*). The bacteria were grown at 37 °C to OD_{600} 1.5. B) A mutation in *csrA* results in a strong downregulation of flagella genes. mRNA levels were measured in the *csrA* mutant and the wild type by quantitative Real-Time RT PCR after growing the bacteria at 37 °C to OD_{600} 1.5. C) Complementation of the effect of a *csrA* mutation on *STM3611* and *STM1798* mRNA levels by plasmid-borne expression of *fliA* from pIRF-2 (pFliA). p30 corresponds to the empty vector control pBAD30. The values represent means with standard deviations (** $P < 0.01$; * $P < 0.05$). D) Complementation with the plasmid pAS-0081 (pFlhDC). The values represent means with standard deviations (** $P < 0.01$; * $P < 0.05$). E) Restoration of swimming motility in the *csrA* mutant. Swimming motility of the wild type strain carrying the empty vector pBAD28 (wt p28) or the *csrA* mutant MAE125 (Δ*csrA*) carrying pBAD28, pBADcsrA (pCsrA), pAS-0081 (pFlhDC) or pIRF-2 (pFliA), respectively, was analysed in 0.3 % LB agar with 0.1 % arabinose at 37 °C. Four μl of overnight cultures, which were grown in LB with 0.1 % arabinose, were used for the experiment. The swimming diameter vs. time is displayed.

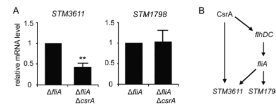

Figure 4.

Regulation of STM3611 by CsrA. A) Effect of a *csrA* mutation on *STM3611* and *STM1798* mRNA levels in a *fliA* deficient background. RNA was isolated from bacterial cultures grown at 37 °C in LB medium with 0.1 % arabinose to OD_{600} 1.5. mRNA levels of *STM3611* and *STM1798* were measured by quantitative Real-Time RT PCR in the *csrA* mutant MAE125 and the *fliA csrA* double mutant MAE1476 (*fliA csrA*). The values represent means with standard deviations (** $P < 0.01$). B) Schematic model depicting the inferred direct and indirect regulation of *STM3611* by CsrA.

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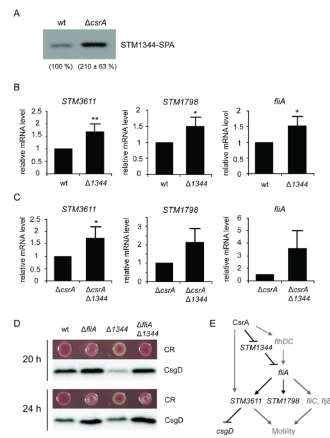

Figure 5.

CsrA-mediated regulation of STM1344 and its downstream effects. A) Protein levels of SPA-tagged STM1344 in the wild type (wt) and the *csrA* deficient background (Δ*csrA*). The bacteria were grown at 37 °C to OD_{600} 1.5. B) Effect of a mutation in *STM1344* (Δ*1344*) on the mRNA levels of *STM3611*, *STM1798* and *fliA* measured by quantitative Real-Time RT PCR, after growth of the bacteria at 37 °C to OD_{600} 1.5. The values represent means with standard deviations (** $P < 0.01$; * $P < 0.05$). C) Effect of a double mutation in *csrA* and *STM1344* (Δ*csrA* Δ*1344*) on the mRNA levels of *STM3611, STM1798* and *fliA*. The values represent means with standard deviations (* $P < 0.05$). D) STM1344 regulates rdar morphotype expression and CsgD levels through *fliA*. Rdar morphotype expression and CsgD protein levels were analysed in the wild type UMR1 and its isogenic mutants in *fliA*, *STM1344* and *fliA STM1344*. The bacteria were grown for 20 h or 24 h, respectively, at 28 °C on Congo Red (CR) LB agar plates without salt. E) Schematic model depicting the regulation of *STM3611* by STM1344 through *fliA*. The model illustrates that CsrA controls the flagella cascade at multiple levels, through FlhDC, STM1344 and STM3611.

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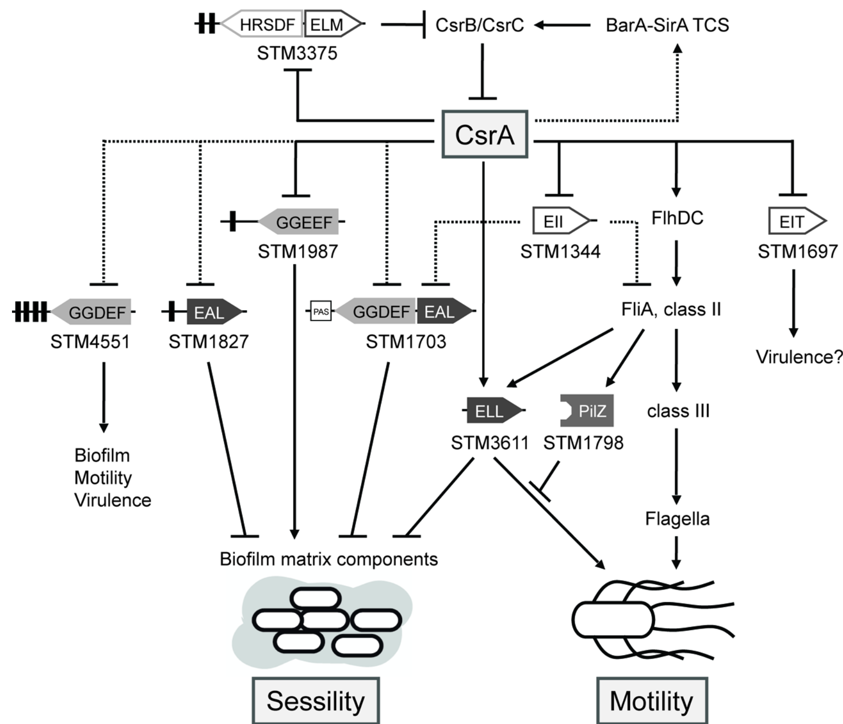

Figure 6.

Schematic model illustrating the interplay between the Csr-, the c-di-GMP and the flagella regulatory system in *Salmonella* Typhimurium. Apparent direct (solid line) and indirect (dashed line) roles of CsrA in the expression of genes encoding GGDEF and EAL domain proteins resulting in the tight control of the sessility-motility switch in *S.* Typhimurium. STM4551 and STM1987 possess DGC activity (Garcia et al., 2004; Solano et al., 2009), whereas STM1703, STM1827 and STM3611 act as PDEs (Simm et al., 2004; Simm et al., 2007). The c-di-GMP metabolizing activities of these proteins control phenotypes in motility, biofilm formation or viruence. In contrast, STM1344, STM3375 and possibly STM1697 contain degenerate EAL/GGDEF domains (unshaded), which cannot synthesize or degrade c-di-GMP, but have apparently evolved alternative functions, e.g. regulatory functions (Simm et al., 2009). Notably, CsrA controls the flagella cascade at multiple levels in the hierarchy: by apparent direct regulation of the flagella master regulator FlhDC and STM1344, which influences the flagella cascade upstream of *fliA*, and by apparent direct and indirect regulation of STM3611. Presumably, this multi-layer control allows CsrA to coordinate flagella synthesis with motor function. By regulating STM3375 (CsrD), which, along with RNase E, destabilizes the CsrB and CsrC sRNAs in *E. coli* (Suzuki et al., 2006), CsrA seems to control its own activity by an autoregulatory loop. CsrB and CsrC are positively controlled by the two-component system (TCS) BarA-SirA (Altier et al., 2000b; Teplitski et al., 2003), which allows the integration of environmental signals into the regulatory network. In *E. coli* (Gudapaty et al., 2001) and in *S.* Typhimurium (our unpublished observations) transcription of *csrB* and *csrC* also requires upstream activation by CsrA, probably through the BarA-UvrY (BarA-SirA) TCS (Suzuki et al., 2002), indicative of an additional feedback loop.

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Table 1

GGDEF/EAL proteins regulated by CsrA.

Protein (synonym)	Regulation by CsrA	Motif	Enzymatic activity	Reported phenotype	Reference
STM1987	negative, direct[1]	GGEEF	DGC	Upregulation of cellulose production	(Garcia et al., 2004; Solano et al., 2009)
STM4551	negative, indirect[1]?	GGDEF	DGC	Functions in motility, rdar morphotype expression, virulence	(Solano et al., 2009)
STM3375 (YhdA, CsrD)	negative, direct	HRSDF, ELM	no DGC, no PDE (in *E. coli*)	Regulation of sRNAs (in *E. coli*), upregulation of motility	(Suzuki et al., 2006; Simm et al., 2007)
STM1703 (YciR)	negative, indirect?	GGDEF, EAL	putative PDE, DGC activity not known	Downregulation of the rdar morphotype	(Garcia et al., 2004; Simm et al., 2007)
STM3611 (YhjH)	positive, direct and indirect	ELL	PDE	Upregulation of motility, downregulation of the rdar morphotype	(Simm et al., 2004; Simm et al., 2007)
STM1827	negative, indirect?	EAL	putative PDE	Downregulation of the rdar morphotype	(Simm et al., 2007)
STM1344 (YdiV)	negative, direct	EII	no PDE	Downregulation of motility, upregulation of the rdar morphotype, role in virulence	(Hisert et al., 2005; Simm et al., 2007; Wozniak et al., 2008; Simm et al., 2009)
STM1697	negative, direct	EIT	probably no PDE	Role in virulence (?)	(Lamprokostopoulou and Römling, unpublished)

[1] direct vs indirect regulation inferred from RNA-binding by CsrA to the respective transcripts as observed by gel-mobility shift assays.

Table 2

5′untranslated leader sequences of CsrA regulated genes.

Gene	5'UTR
STM1987	UUUCAUCUGAUACAGGAGGCAGGUAUGGUCUUUUCUGUCAGUGAGGAAGUUACCGUGAAAGAGGGUGGGCCCCGGAUGAUCGUCACCGGUUAUUCCAGCGGAAUGGUAGAGUGUCGCUGGUAUGAUGGUUUUGGCGUAAAGCGGGAGGCAUUCCAUGAGAAUGAGCUCGUGCCAGGCAAGGAAAGGCGCGUGCGGGACGAAGCCCGAUGACAAAACGCCCGGUUAAUGCCGGGCGUUUUGCUGUGGAGAAAAAGAGUCGUCGCGCUGCGACAUGCUGCUACCUGGUUAUGCCAGGAUUGAGUGGUAUCCCUUGAUGAGGUCUGGCUACCGUAAGCCAUCAGGGGGAGUUGUAUCAAUAACCAGGAGUUACCAGGGUG
STM4551	GUUAACGUAAAAACAACAACACCTCGCUUUAGGCGACCUUUUCUCAUACGUUAUUUUCAUGUUGGCGCCCGAUUCCGUGUAUAUCACCGAUAAAUAACGACAAUUACCCAUG
STM3375	UUCAGGCUGGACCAGUGGACAUCCACCGUCACAUCACCUGCCGGCAGUUGACUCUCUUCGAGAUGUUGCACGGAUGCGAGGGUUUUACCGUCCUGCUGUUCUAAGAUCAACGCCUGCAUAACCUGUCCUCACUUUACAUGGUAGAUUGGAAAAAUAGCUACGAAGACUAUACUCGCUAAUUAAAACGUGAUGCCGAUGCAACGCAAUAAAUUGCCAGAUAGAUCCAUUUUGGUAGUAUGCCUGCUUCAUUGCGCGCCGUGGCGAAUUCCGCCUUCAGAUUCGCUUUUUCAUACUGUUUAUAACCGUCGGAGUUAACUCAAGGAUG
STM1703	AUAGAUAAAACAAUACGUUCAGCCAUAAUUACCCUUAUUUAUAACAUGGCUAACCUUUCUUUUCGGGCACCUGGCGCUUAAAUGGGAUGGUACGCAUG
STM3611	AGUCCGGACAGUCACACUCCCAUUAACAGGACAACUGAGAUG
STM1827	UCGCGGUUGGCUUUUGUCGCCAACGCUUAUUGGGCGGCCCGCAUG
STM1344	AACCGUAGCAUUUACAAACGTACUUUUAUUAAAAACGGGCGUGUUCACGCCCUGUGAAAAGUACAAGAUUGAAUAUUGGUUUAUAAUCAGAGUAAGUUAAAAAAAGGAACCGGUUCACCGGUAAACAAGAAAAAUGAUGACGCUUCAAUUAGUAUCUGAAGUUACGUCUGUUUCCGUCCGUAGUGACUGGAUGGCGAAUAGCGCCCUAACCAUGGGACUGGCGUAAUG
STM1697	UUUUAUUGAUUGAUUUGCGUUAAAAGCGCAUUAUCAGGGAGUUAAGAUCAAGUGGAGUGGUCACAUG

GGA - element of putative CsrA binding boxes; **A**, **G** or **U** – transcriptional start site; **AUG** or **GUG** – initiation codon; underlined – predicted Shine Dalgarno sequence

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Table 3

Strains and plasmids used in this study.

Strain/plasmid	Description or genotype	Reference
Strains		
Salmonella enterica serovar Typhimurium ATCC 14028		
UMR1	ATCC 14028-1s Nal^r	(Romling et al., 1998)
MAE125	UMR1 *csrA::kan*	This study
MAE1491	UMR1 Δ*csrA*	This study
MAE130	UMR1 *STM3611-SPA kan^r*	(Simm et al., 2009)
MAE1493	MAE1491 *STM3611-SPA kan^r*	This study
MAE132	UMR1 *STM1344-SPA kan^r*	(Simm et al., 2009)
MAE1492	MAE1491 *STM1344-SPA kan^r*	This study
MAE1456	UMR1 *fliA::cat*	This study
MAE1463	UMR1 Δ*fliA:*101	This study
MAE1476	MAE125 *fliA::cat*	This study
MAE424	UMR1 *STM1344::cat*	(Simm *et al.*, 2007)
MAE1481	MAE1463 *STM1344::cat*	This study
MAE1474	MAE125 *STM1344::cat*	This study
Plasmids		
pKD46	Temperature-sensitive λ red recombinase expression vector	(Datsenko and Wanner, 2000)
pKD4	Template plasmid (*kan^r*) for mutant construction	(Datsenko and Wanner, 2000)
pKD3	Template plasmid (*cat^r*) for mutant construction	(Datsenko and Wanner, 2000)
pBAD28	Arabinose inducible expression plasmid	(Guzman *et al.*, 1995)
pBAD30	Arabinose inducible expression plasmid	(Guzman *et al.*, 1995)
pBADcsrA	*csrA* under control of the plasmid-borne P_{BAD} promoter	(Jonas *et al.*, 2008)
pIRF-2	*fliA* under control of the plasmid-borne P_{BAD} promoter	This study
pAS-0081	*flhDC* under control of the plasmid-borne P_{BAD} promoter	(Sittka *et al.*, 2008)