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The RNA binding protein CsrA controls c-di-GMP metabolism by directly regulating the expression of GGDEF proteins

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Summary

The carbon storage regulator CsrA is an RNA binding protein that controls carbon metabolism, biofilm formation and motility in various eubacteria. Nevertheless, in *Escherichia coli* only five target mRNAs have been shown to be directly regulated by CsrA at the post-transcriptional level. Here we identified two new direct targets for CsrA, *ycdT* and *ydeH*, both of which encode proteins with GGDEF domains. A *csrA* mutation caused mRNA levels of *ycdT* and *ydeH* to increase more than 10-fold. RNA mobility shift assays confirmed the direct and specific binding of CsrA to the mRNA leaders of *ydeH* and *ycdT*. Overexpression of *ycdT* and *ydeH* resulted in a more than 20-fold increase in the cellular concentration of the second messenger c-di-GMP, implying that both proteins possess diguanylate cyclase activity. Phenotypic characterization revealed that both proteins are involved in the regulation of motility in a c-di-GMP dependent manner. CsrA was also found to regulate the expression of five additional GGDEF/EAL proteins and a *csrA* mutation led to modestly increased cellular levels of c-di-GMP. All together, these data demonstrate a global role for CsrA in the regulation of c-di-GMP metabolism by regulating the expression of GGDEF proteins at the post-transcriptional level.

Keywords

CsrA; post-transcriptional regulation; GGDEF; c-di-GMP; motility

Introduction

Successful adaptation of bacteria to different niches depends on their ability to adjust their life style according to the requirements of the environment. Bacteria have evolved numerous mechanisms to sense external signals, to translate them into complex cellular responses and, thereby, to mediate responses to physiological demands. The *Escherichia coli* carbon storage system, with the RNA binding protein CsrA as the central player, exemplifies such an adaptive regulatory cascade [see reviews by (Babitzke and Romeo, 2007; Lucchetti-Miganeh *et al.*, 2008)]. The Csr regulatory system is widely distributed among eubacteria

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(White *et al.*, 1996) and has been found to control a variety of virulence-linked physiological traits (Lucchetti-Miganeh *et al.*, 2008).

CsrA was originally identified as a regulator of glycogen biosynthesis (Romeo *et al.*, 1993), acting as an RNA binding protein on the expression of its target mRNAs (Liu and Romeo, 1997). Beside controlling glycogen synthesis, CsrA and its homologs in various bacteria have widespread regulatory functions, including roles in biofilm formation (Jackson *et al.*, 2002; Wang *et al.*, 2005), motility (Wei *et al.*, 2001; Yakhnin *et al.*, 2007), carbon metabolism (Baker *et al.*, 2002; Sabnis *et al.*, 1995), secondary metabolism (Heeb and Haas, 2001; Heeb *et al.*, 2005; Kay *et al.*, 2005), quorum sensing (Heurlier *et al.*, 2004; Lenz *et al.*, 2005) and numerous functions in the interactions with animal and plant hosts (Altier *et al.*, 2000; Barnard *et al.*, 2004; Heeb and Haas, 2001). CsrA is a homodimer containing two identical RNA-binding surfaces located on opposite sides of the protein, whose structure and function recently has been elucidated in considerable detail (Mercante *et al.*, 2006; Schubert *et al.*, 2007). Despite its global role in bacterial adaptation, only a few direct mRNA targets have been identified, including five in *E. coli*. By binding to mRNA leaders and preventing translation, followed by destabilizing of the transcript, CsrA has been shown to downregulate expression of the *glgCAP* operon (Baker *et al.*, 2002), encoding the glycogen synthesis apparatus, the *cstA* gene (Dubey *et al.*, 2003), involved in carbon starvation and the *pga* operon, encoding the biofilm polysaccharide poly-β-1,6-N-acetyl-D-glucosamine (PGA) (Wang *et al.*, 2005). Regulation of the RNA chaperone gene *hfq* is also mediated by CsrA binding and translation inhibition, although this does not result in *hfq* mRNA destabilization (Baker *et al.*, 2007). CsrA also upregulates the expression of certain target genes. The mRNA of *flhDC*, which is required for flagellum biosynthesis, is stabilized by CsrA binding to the *flhDC* leader (Wei *et al.*, 2001). However, the detailed biochemical mechanism for this activation has not been elucidated.

Regulation of CsrA activity is mediated in part by the action of the two small non-coding RNAs (sRNAs) CsrB and CsrC (Romeo, 1998; Weilbacher *et al.*, 2003). During the past years sRNAs have been recognized as important players in gene regulation, in most cases by base pairing with target mRNAs (Majdalani *et al.*, 2005; Romby *et al.*, 2006; Storz *et al.*, 2005). However, CsrB and CsrC RNAs antagonize the activity of CsrA by binding to and therefore sequestering this protein (Liu *et al.*, 1997). Transcription of the Csr sRNAs is controlled by the two-component system (TCS) BarA-UvrY (Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003), thus permitting the integration of environmental signals into the Csr signalling network. Expression of *csrB* and *csrC* also requires CsrA. This regulation may be mediated indirectly through the BarA-UvrY system (Gudapaty *et al.*, 2001; Suzuki *et al.*, 2002). This auto-regulatory mechanism has been described as a homeostatic system, which leads to tight regulation of CsrA activity. Recently, a new regulatory factor, CsrD (YhdA) has been shown to influence the Csr system (Jonas *et al.*, 2006; Suzuki *et al.*, 2006). By targeting CsrB and CsrC for degradation by RNase E, CsrD acts positively on CsrA activity (Suzuki *et al.*, 2006). The apparent membrane protein CsrD contains degenerate GGDEF and EAL domains. Such domains have been shown to be associated with the turnover of the second messenger cyclic di-GMP (c-di-GMP) (Ryjenkov *et al.*, 2005; Schmidt *et al.*, 2005; Simm *et al.*, 2004), which can mediate the switch between a motile and sessile life style in diverse bacteria [see reviews by (D'Argenio and Miller, 2004; Jenal, 2004; Romling, 2005; Romling and Amikam, 2006)]. In contrast to other GGDEF/EAL proteins, CsrD was demonstrated to lack both diguanylate cyclase (DGC) and phosphodiesterase (PDE) activities, indicating that CsrD is neither involved in the production nor in the degradation of the second messenger (Suzuki *et al.*, 2006). In contrast, CsrD was found to be an RNA binding protein, although its detailed mechanism of action in CsrB/C decay has not been resolved.

Despite the detailed knowledge about the molecular mechanisms of the Csr signalling system, limited information is available concerning the integration of the Csr cascade into other global networks. In order to identify novel direct targets for CsrA that might help us to better understand the global impact of the Csr network, we conducted a genome wide search for genes, whose transcript levels rapidly change upon pulse overproduction of CsrA. Our search revealed that CsrA is a regulator for several GGDEF/EAL proteins, in particular of the two GGDEF proteins YcdT and YdeH. Both proteins produce c-di-GMP *in vivo* and control flagella mediated swimming motility.

Results

Identification of novel mRNA targets for CsrA by microarray

To screen for novel direct CsrA targets we decided to adopt a microarray based approach, which has previously been used to identify direct sRNA targets (Papenfort *et al.*, 2006; Tjaden *et al.*, 2006; Vogel and Wagner, 2007). Our strategy involved the pulse overexpression of *csrA*, followed by the immediate analysis of changes in whole genome expression patterns. The approach is based on the assumption that CsrA not only blocks the translation of many of its mRNA targets, but also secondarily destabilizes them. Hence, pulse overexpression of CsrA from an inducible vector is expected to lead to a rapid decrease in the transcript level of the directly regulated targets. Changes in the transcript levels of indirect CsrA targets are assumed to occur first at later time points after *csrA* induction. Such differential changes in the transcript level can be monitored over time by microarray analysis. To verify that our approach was working, we first monitored *csrA* expression as well as the expression of the known direct target *pgaA* and the known indirect target *csrB* in response to *csrA* overexpression by quantitative real-time reverse transcriptase PCR (RT-PCR). Our data show that addition of arabinose (at 0 minutes) to *E. coli* KJ157 (KSB837 *csrA::kan*), carrying the arabinose inducible vector pBADcsrA, resulted in a strong upregulation of *csrA* expression within 2 minutes (Fig. 1A). Consistent with our prediction mRNA levels of the direct target *pgaA* dramatically decreased within less than 10 minutes (Fig. 1B). The expression of the sRNA CsrB, known to be indirectly and positively controlled by CsrA (Gudapaty *et al.*, 2001), began to increase after a delay of ~12 minutes (Fig. 1C). These data suggest that our approach was successful in discriminating between direct and indirect targets for CsrA.

In the next step we screened for novel direct CsrA targets by using an Affymetrix whole genome *E. coli* array. Since *pgaA* mRNA was downregulated within less than 12 minutes, we compared the transcriptional profiles 4 and 12 minutes after arabinose addition with the profile before arabinose induction (0 minutes). To eliminate genes downregulated in a CsrA-independent manner, we normalised the observed signal ratios against the signal ratios resulting from induction of the vector control pBAD28.

Four of the genes showing the strongest repression (> 7 fold) 12 minutes after pBADcsrA expression belonged to the *pga* operon (Fig. 1D). The mRNA levels of *ycdT*, followed the same kinetics upon CsrA overexpression as the *pga* mRNAs, suggesting that *ycdT* may be regulated by CsrA in a similar manner. Database search revealed that *ycdT* is located directly adjacent to the *pga* operon but on the reverse strand. *ycdT* encodes a transmembrane protein with a C-terminal GGDEF domain (Fig. 1E, F). Among the most downregulated genes in response to CsrA overexpression we found another GGDEF protein encoding ORF, *ydeH* (Fig. 1D, G). YdeH is predicted to encode a cytoplasmic protein, not containing any known domains involved in signalling (Fig. 1G).

The two GGDEF proteins YcdT and YdeH are regulated by CsrA

To confirm the effect of CsrA overexpression on *ycdT* and *ydeH* transcripts, we determined the kinetics of CsrA-dependent downregulation by RT-PCR. In accordance with our array data, *ycdT* and *ydeH* mRNA levels decreased strongly upon arabinose addition in late exponential phase (OD_{600} 1.5) (Fig. 2A). The mRNA level of *ycdT* was halved within 4 minutes and reached a minimum of 3 % between 12 and 24 minutes. *ydeH* mRNA decreased to 50 % within five minutes and continued to decrease to approximately 22 % after 24 minutes. Similar results were observed when arabinose induction was performed earlier during growth at OD_{600} 0.5 (Fig. 2B). In contrast, addition of arabinose to a strain carrying the empty vector pBAD28 did not affect the levels of *ycdT* and *ydeH* transcripts (Fig. 2C).

To test the effect of a *csrA* mutation on *ycdT* and *ydeH* expression, we measured the mRNA levels of *ycdT* and *ydeH* by RT-PCR along the entire growth curve in the wild type and isogenic *csrA* mutant strains. In the wild type strain, expression of *ycdT* slightly decreased within the first 8 h of growth (Fig. 2D), whereas *ydeH* mRNAs remained at constant levels (Fig. 2E). Between 8 and 24 h the expression of both genes strongly increased. In the *csrA* mutant, *ycdT* and *ydeH* mRNA levels were significantly elevated. *ycdT* expression was more strongly upregulated (up to 30-fold) during exponential growth compared to later time points (Fig. 2D), whereas the transcript levels of *ydeH* were approximately 10 fold higher throughout the growth (Fig. 2E). Monitoring *csrA* transcript levels over time in the wild type strain shows that *csrA* expression rapidly decreased between 8 and 24 h (Fig. 2F), demonstrating that *csrA* is inversely regulated with *ycdT* and *ydeH*. However, the fact that CsrA activity is in large part under the control of CsrB and CsrC makes it difficult to correlate *csrA* mRNA levels with its activity. Nevertheless, these data confirm that CsrA is a negative regulator of *ycdT* and *ydeH* expression.

Effects of other components of the BarA-UvrY-Csr cascasde on ydeH and ycdT expression

CsrA is antagonized by the CsrB and CsrC sRNAs. These sRNAs are transcriptionally activated by the BarA-UvrY two-component system and negatively controlled by CsrD at the level of RNA stability (Suzuki *et al.*, 2002; Suzuki *et al.*, 2006; Weilbacher *et al.*, 2003). By using genetic mutants we tested the contribution of these components on *ycdT* and *ydeH* expression. Disruption of *csrB* and *csrC* resulted in a slight decrease in *ycdT* and *ydeH* mRNA levels (approx. 70 %) compared to the wild type (Fig. 3). Similar weak effects were observed in *uvrY* and *barA* mutants. These modest effects are consistent with the earlier finding that levels of CsrA protein normally exceeds the binding capacity of these small RNAs (Gudapaty *et al.*, 2001). A more pronounced effect on *ycdT* and *ydeH* mRNAs was observed in a *csrD* mutant, in which the cellular levels of CsrB and CsrC are increased. Compared to the wild type the mRNA levels of *ycdT* and *ydeH* were approximately 3-fold increased (Fig. 3). All together, these results indicate that the entire Csr regulatory network is involved in the regulation of the expression of *ycdT* and *ydeH*.

CsrA directly interacts with the ycdT and ydeH transcripts

Previous studies have suggested that CsrA binds to the consensus sequence, ACA-GGAUG, with the GGA motif representing the most highly conserved nucleotides (Baker *et al.*, 2002; Dubey *et al.*, 2005). To make predictions about the binding of CsrA to the *ycdT* and *ydeH* mRNAs, we analysed the 5′ leader sequences of both transcripts for the existence of potential CsrA binding motifs. Since no information about the *ycdT* promoter was available in the data base, we determined the transcriptional initiation site of *ycdT* by Rapid Amplification of 5′ cDNA Ends (5′ RACE). A single band was observed for a PCR reaction, amplifying the 5′ non-translated region of the *ycdT* transript (Fig. 4A). Sequencing of the RACE PCR product identified the nucleotide A, 35 bp upstream of AUG, as the transcription start site. The -10 and -35 regions of *ycdT* [TATTAA (-10) and TTGACA

(-35)], separated by a 19 bp spacing region, exhibited 4 and 6 bp of identity with respect to the consensus sequences for these promoter elements [TATAAT (-10) and TTGACA (-35)] (Hawley and McClure, 1983). We identified two potential CsrA binding sites with degenerate motifs in the 5′ non-translated region of the *ycdT* mRNA, one of them close to the transcription start and the other one overlapping the AUG translation initiation start codon (Fig. 4A). The *ydeH* transcript starts 29 nucleotides upstream of the initiation codon AUG (Yamamoto and Ishihama, 2006). Also in the 5′ non-translated region of the *ydeH* mRNA two potential CsrA binding sites binding sites were found, one of which overlaps the Shine-Dalgarno sequence and the other one is close to the 5′ end of the transcript (Fig. 4B).

To experimentally determine whether CsrA directly binds to the *ycdT* and *ydeH* transcripts, quantitative RNA gel mobility shift assays were performed with a *ycdT* transcript, consisting of a 36 nt leader and the first 20 nt of the coding region, and a *ydeH* transcript containing the 29 nt untranslated leader and the first 25 nt of the coding sequence. CsrA bound strongly to both *ycdT* and *ydeH* transcripts (Fig. 4C, D). For the *ycdT* transcript, two distinct complexes were observed at 2.5 nM CsrA, and essentially all of the starting RNA was shifted at 80 nM CsrA (Fig. 4C). For the *ydeH* transcript, two distinct shifted complexes were formed at 5 nM CsrA. However, complete binding was not seen until 320 nM CsrA, and at this concentration essentially all of the RNA was present in the upper complex (Fig. 4D). These gel shift patterns suggested that two CsrA proteins were bound to each transcript at higher CsrA concentrations, although the stoichiometry of binding was not experimentally determined. A nonlinear least-squares analysis of these data yielded an apparent equilibrium binding constant (K_d) of 2.6 ± 0.3 nM for *ycdT* and 2.3 ± 0.1 nM for *ydeH*.

The specificity of CsrA interaction with *ycdT* and *ydeH* transcripts was investigated by performing competition experiments with specific (*ycdT* or *ydeH* transcripts) and non-specific (*Bacillus subtilis trp* leader) unlabelled RNA competitors. Both *ycdT* and *ydeH* RNAs were able to compete for binding to CsrA while *B. subtilis trp* RNA did not effectively compete with the CsrA-*ycdT* or CsrA-*ydeH* interaction (Fig. 4E, F). These results establish that CsrA binds specifically to both *ycdT* and *ydeH* RNA.

In most cases CsrA downregulates its direct mRNA targets by binding to the leader, preventing translation and destabilizing the transcript (Baker *et al.*, 2002; Dubey *et al.*, 2003; Wang *et al.*, 2005). However, in the case of *hfq* the binding of CsrA to the leader does not lead to mRNA destabilization, but to altered transcription (Baker *et al.*, 2007). To test whether CsrA influences *ycdT* and *ydeH* mRNA levels by modulating promoter activity, we constructed plasmid-borne transcriptional *ycdT*- and *ydeH-lacZ* fusions, containing the upstream intergenic region and only 2 or 3 nucleotides of each transcript (*ycdT* -547 to +2; *ydeH* -222 to +3). Measuring of β-galactosidase activity of these reporter fusions revealed that both promoters were highly active in the wild type but were not altered by a *csrA* mutation (Fig. 4G). In contrast, *lacZ* expression from a control plasmid carrying the *csrB* promoter, which has earlier been reported to be regulated in a CsrA-dependent manner (Gudapaty *et al.*, 2001), was clearly decreased in the *csrA* mutant (Fig. 4G). This demonstrates that CsrA does not change transcription of *ycdT* and *ydeH*, but rather modulates the stability of the messages.

YcdT and YdeH regulate motility

Proteins with GGDEF and EAL domains have been demonstrated to be involved in the regulation of bacterial physiology, including motility, biofilm formation, cell morphology and virulence [see reviews by (Cotter and Stibitz, 2007; D'Argenio and Miller, 2004; Jenal, 2004; Romling *et al.*, 2005; Romling and Amikam, 2006)]. To characterise the phenotype of *ycdT* and *ydeH* in motility we analysed the swimming behaviour of strains, in which *ycdT* and *ydeH* were expressed from pBADycdT and pBADydeH, respectively, as well as

respective knock-out mutants. Overexpression of both pBADycdT and pBADydeH led to a strong repression of swimming behaviour (Fig. 5A). The same effect was observed in a *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) background, although *S.* Typhimurium do not contain orthologs of these proteins (Fig. 5B). Mutations in *ydeH* and *ycdT* led to slightly increased swimming ability compared to the wild type. A *ydeH ycdT* double mutant was, however, not more motile than the wild type (Fig. 5C).

Earlier studies have demonstrated that site-directed mutations in the GGDEF signature sequence of other proteins disrupt the function of this domain (Garcia *et al.*, 2004; Paul *et al.*, 2004; Simm *et al.*, 2004). To test whether the repressing effect of *ycdT* and *ydeH* overexpression on motility was due to the activity of both proteins as diguanylate cyclases, we engineered mutants, in which the two first glycine residues of the respective GGEEF motifs were replaced by two alanine residues (GGEEF → AAEEF). The swimming behaviour of *E. coli* or *Salmonella* expressing the plasmids encoding these mutant YdeH and YcdT variants (pBADycdT-mut, pBADydeH-mut) was indistinguishable from the bacteria expressing the empty vector (Fig. 5A, B). This strongly suggests that the effect of YcdT and YdeH on motility is mediated by the second messenger c-di-GMP.

Previous results have demonstrated that a *csrA* mutant is strongly impaired in motility and that CsrA upregulates *flhDC* expression by binding to and stabilizing this mRNA (Wei *et al.*, 2001). To test whether deletions in *ycdT* or *ydeH* can compensate for the swimming defect of the *csrA* mutant we constructed *csrA ycdT* and *csrA ydeH* double mutants as well as a *csrA ycdT ydeH* triple mutant. All three strains were not more motile than the *csrA* mutant (Fig. 5D), suggesting complex regulation of motility by pathways within the Csr network.

Beside their impact on motility, many proteins with GGDEF domains have been shown to regulate biofilm formation. In accordance, results of a parallel ongoing study show that YdeH significantly affects biofilm formation (C. Goller and T. Romeo, unpublished). Furthermore, the effect on biofilm formation seems to be mediated through increased synthesis of the biofilm polysaccharide PGA. YcdT has earlier been characterised regarding its phenotype in biofilm formation (Wang *et al.*, 2005). However, neither biofilm formation nor *pgaA-lacZ* expression was influenced by YcdT under the given conditions (Wang *et al.*, 2005).

In Enterobacteriaceae several GGDEF proteins have been shown to control biofilm formation by regulating the expression of curli fibres (Romling, 2005). Here, we analysed the expression of curli by analysing the colony morphology on Congo Red (CR) agar plates as well as the ability to form pellicles and to adhere to glass culture tubes at the air-liquid interface. However, we were not able to detect distinct *ycdT* or *ydeH* dependent phenotypes with respect to CR binding, pellicle formation or glass adherence at the air-liquid interface, suggesting that neither YcdT nor YdeH influence curli production under the conditions tested (data not shown).

AFM analysis of YcdT and YdeH mediated phenotypes

To further investigate the phenotypes mediated by YcdT and YdeH, we employed Atomic Force Microscopy (AFM), a technique recently shown to be a suitable tool for the study of bacterial morphology (Jonas *et al.*, 2007). We allowed the bacteria to grow and to adhere to the substratum mica, which was submerged in the growth medium. For immobilization, the samples were air-dried at room temperature prior to AFM analysis. Images of the wild type strain carrying pBAD28 showed rod-shaped bacteria expressing flagella and pili-like structures (Fig. 6A). Overexpression of pBADydeH resulted in a clear reduction in the abundance of flagella (Fig. 6A, B), suggesting a role for YdeH in the regulation of flagellum

biosynthesis. We also noted that YdeH overexpression completely abolished the appearance of the pili-like structures (Fig. 6A, C), indicating that flagella and pili synthesis might be co-regulated by YdeH. In contrast to *ydeH*, overexpression of *ycdT* did not affect the occurrence of flagella or pili or another distinct phenotype (Fig. 6), indicating that YcdT might have functions in the cell different from YdeH, which cannot be visualized by AFM under the conditions we have tested.

YcdT and YdeH influence c-di-GMP levels in vivo

Both YcdT and YdeH contain GGDEF domains with consensus motifs, which are predicted to be dedicated to the synthesis of c-di-GMP (Ausmees *et al.*, 2001; Paul *et al.*, 2004; Schmidt *et al.*, 2005; Simm *et al.*, 2004). So far, proteins with a highly conserved active site motifs have been shown to possess DGC activity (Ausmees *et al.*, 2001; Kirillina *et al.*, 2004; Paul *et al.*, 2004; Simm *et al.*, 2004; Weber *et al.*, 2006). In contrast, CsrD with a degenerate motif (HRSDF) failed to produce c-di-GMP (Suzuki *et al.*, 2006). The amino acid sequences of the GGDEF domain of YcdT and YdeH perfectly match the conserved GG(D/E)EF motif as well as additional more extended conserved amino acid signatures of other enzymatically active proteins (Fig. 7A). Together with the finding that site-directed mutations of the GGDEF domains of YcdT and YdeH disrupted the effect on motility (Fig. 5), this strongly suggests that both proteins synthesize c-di-GMP. To prove this experimentally, we measured the c-di-GMP concentrations produced by *E. coli* MG1655 containing plasmid-encoded *ydeH* (pBADydeH), *ycdT* (pBADycdT) or the empty vector using HPLC and MALDI-TOF. Expression of *ydeH* resulted in clearly increased c-di-GMP levels (2215.3 fmol mg^{-1} cells) compared to the low levels, close to the limit of detection, in the control strain, carrying pBAD28 (94.6 fmol mg^{-1} cells). Even higher levels were detected when *ycdT* was overexpressed (7213.0 fmol mg^{-1} cells) (Fig. 7B). These data provide strong evidence that both of these proteins function as diguanylate cyclases (DGC) *in vivo*.

The strong effect of CsrA on *ycdT* and *ydeH* transcript levels led us to analyse the overall effect of CsrA on the cellular c-di-GMP pool, by measuring the levels of the second messenger in the wild type strain MG1655 and its *csrA* mutant. We were able to consistently detect slightly elevated c-di-GMP levels in the *csrA* mutant (120.8 fmol mg^{-1} cells) compared to the wild type (74.5 fmol mg^{-1} cells) (Fig. 7C). This finding demonstrates a net effect of CsrA in the regulation of c-di-GMP turnover and is consistent with the previously documented negative effect of CsrA on biofilm formation (Jackson *et al.*, 2002).

Several genes encoding GGDEF and EAL proteins have previously been shown to cross-complement phenotypes (Garcia *et al.*, 2004; Simm *et al.*, 2004), even between different species (Simm *et al.*, 2005). In *Salmonella*, a mutation in the GGDEF gene *adrA* results in deficiency in cellulose synthesis due to decreased c-di-GMP levels. Overexpression of enzymatically active GGDEF proteins in such a *Salmonella* mutant leads to the restoration of cellulose production, which can be visualized on agar plates containing the dyes calcofluor (CF) or Congo red (CR). Thus, the ability to produce cellulose can be used as an indicator for DGC activity. We utilized this effect to study the enzymatic activities of YcdT and YdeH in *S.* Typhimurium. Strain MAE103, mutated in *adrA* and carrying pBADycdT, pBADydeH or the controls pBAD28 or pBADcsrA, was allowed to grow on CF and CR agar plates at 28 °C. Overexpression of pBADydeH resulted in a strongly fluorescent colony appearance of *Salmonella* on the CF plates (Fig. 7D) and as pink and rough colonies on the CR plates (data not shown), suggesting that cellulose was produced due to the elevated production of c-di-GMP. However, no dye binding could be observed for the strain carrying pBADycdT, demonstrating that *ycdT* fails to cross-complement an *adrA* mutation in *Salmonella* under the given conditions. In agreement with these data, only subtle changes (< 2-fold) in c-di-GMP levels were observed by HPLC and MALDI-TOF, when *ycdT* was

overexpressed in *Salmonella*, grown at 28 °C on LB agar without salt, whereas plasmid encoded expression of *ydeH* in the same background strain resulted in strongly elevated c-di-GMP levels (> 200 fold) (Fig. 7E). Thus, in contrast to YdeH, which apparently possesses high DGC activity in plate grown *Salmonella* at 28 °C, YcdT appears to produce c-di-GMP at very low concentrations under the given conditions. A previous study has already demonstrated that in *Salmonella* most, but not all GGDEF proteins with conserved sequence signatures could restore cellulose production in an *adrA* mutant (Garcia *et al.*, 2004) and that the cross-complementation ability strongly depended on the experimental conditions.

Global role of CsrA in the regulation of other GGDEF/EAL proteins

To test whether CsrA controls the expression of additional GGDEF/EAL proteins we analysed our array data for the expression patterns of all genes, annotated to contain a GGDEF and/or EAL domain, 4 and 12 minutes after CsrA pulse overproduction. For 4 of the 29 selected genes the signals were too low for reliable detection on the microarray (*yeaI*, *yaiC*, *yhjH* and *ycgG*). Most of the other GGDEF/EAL genes were relatively weakly expressed, but strongly enough for detection on the array. Interestingly, beside *ycdT* and *ydeH* several other genes showed changes in their transcript levels upon CsrA overproduction with an additive effect between 4 and 12 minutes: *yddV* (GGDEF), *yliF* (GGDEF), *dos* (GGDEF-EAL), *yhjK* (GGDEF-EAL), *csrD* (GGDEF-EAL), *yliE* (EAL) and *yjcC* (EAL) (Fig. 8A, E). CsrA dependent repression of these genes was, however, not as strong (1.5 to 2.5 fold) as repression of *ycdT* and *ydeH*. RT-PCR analysis of the kinetics of CsrA-dependent expression confirmed that mRNA levels of *yliE*, *yliF*, *yddV*, *dos* and *csrD* were indeed downregulated upon induction of pBADcsrA (Fig 8B), but remained constant or increased upon induction of the vector control (Fig. 8C). In the *csrA* mutant strain, expression of these genes was moderately increased (between 2 and 6 fold) compared to the wild type (Fig. 8D). Repression of *yhjK* and *yjcC* by CsrA overproduction could not be confirmed (data not shown). Noticeably, *yddV* and *dos* (Mendez-Ortiz *et al.*, 2006) as well as *yliE* and *yliF* are organized as polycistronic units in operons (Fig. 8F, G). The expression patterns of *yliE* and *yliF* as well as *yddV* and *dos* followed almost identical kinetics (Fig 8B, C), indicating that these genes are co-regulated at the mRNA level by CsrA. The observation that CsrD is negatively regulated by CsrA agrees with the earlier finding that expression of a chromosomal *csrD-lacZ* translational fusion was modestly repressed (2-fold) by CsrA (Suzuki *et al.*, 2006). These data confirm that CsrD is part of an additional autoregulatory loop within the Csr system. In summary, our data demonstrate that beside *ycdT* and *ydeH*, genes for several other GGDEF and GGDEF-EAL proteins as well as one EAL protein are negatively regulated by CsrA. This finding suggests a global role for CsrA in the regulation of c-di-GMP metabolism.

Discussion

Post-transcriptional regulation of GGDEF/EAL proteins by CsrA

The present study was initiated with a genome wide search for novel targets for the post-transcriptional regulator CsrA to better understand its role in bacterial adaptation and the cross-talk between the Csr system and other regulatory systems. Our search led to the finding that CsrA controls the expression of several GGDEF/EAL proteins, in particular the GGDEF proteins YcdT and YdeH, by physically binding to and changing their mRNA levels. To our knowledge this is the first example of GGDEF/EAL proteins being regulated at the mRNA level by a global post-transcriptional regulator. This supports the idea that c-di-GMP signalling is a multilayer process, including transcriptional, translational and post-translational levels. The array data also indicated that CsrA controls other mRNAs, some of which with unknown functions, but these effects need to be confirmed and were not the focus of this study.

With binding constants (K_d) of approximately 2.5 nM, CsrA binding to the *ycdT* and *ydeH* transcripts was remarkably strong. The affinities to the other known mRNA targets *pgaA*, *glgC*, *cstA* and *hfq* were approximately 10-fold lower (22 nM, 39 nM, 40 nM and 38 nM, respectively) (Baker *et al.*, 2002; Baker *et al.*, 2007; Dubey *et al.*, 2003; Wang *et al.*, 2005). Noteworthy, for each of the *ycdT* and *ydeH* transcripts only two GGA boxes were found in the 5′ leader sequences and binding of two CsrA proteins per transcript was observed at higher concentrations. For comparison, *pgaA*, *glgC* and *cstA* contain four to six potential CsrA binding sites. Moreover, in the case of *ycdT*, the sequence signature of both sites showed relative poor similarity to the consensus sequence. Thus, in addition to the primary sequence conservations, other determinants seem to largely influence the affinity of CsrA to its targets.

Our array revealed that beside YcdT and YdeH, two additional GGDEF proteins (YddV, YliF), two GGDEF-EAL proteins (Dos, CsrD) and one EAL protein (YliE) were regulated by CsrA. Together with the finding that increased overall levels of cellular c-di-GMP were measured in a *csrA* mutant, this implicates a global role for CsrA in the regulation of c-di-GMP metabolism. It is plausible that under other experimental conditions CsrA might control the expression of additional GGDEF/EAL proteins. For most of the GGDEF/EAL genes, relatively weak signals were detected on the microarray, suggesting that these genes require specific conditions for enhanced expression, different from the standard conditions used in our experiment. Since CsrA homologs are present in many different Gram negative bacteria (White *et al.*, 1996) the role of CsrA in the regulation of GGDEF/EAL proteins might be a conserved feature. With the exception of CsrD, no other of the CsrA regulated GGDEF/EAL genes have homologous genes in *S*. Typhimurium. Therefore, CsrA might act on other GGDEF/EAL proteins in other bacteria. Furthermore, we cannot exclude that our microarray approach, which requires the destabilization of the CsrA mRNA targets upon its binding, failed to identify other important CsrA targets, in which translation is regulated without a corresponding alteration in mRNA stability, similar to the previous findings for *hfq* (Baker *et al.*, 2007).

Interplay between Csr and c-di-GMP signalling

While c-di-GMP-mediated phenotypes and the molecular mechanisms governing c-di-GMP synthesis and turnover have received much attention, the role of the c-di-GMP network in signal transduction, including its linkage to external signals of specific adaptive responses and its interconnection with other global networks is relatively unexplored. Nevertheless, in *E. coli* genes encoding GGDEF/EAL domains were recently reported to be over-represented in the σ^S (RpoS) regulon, suggesting a role for c-di-GMP during the general stress response (Weber *et al.*, 2006). In *Vibrio cholerae* quorum sensing signalling was recently demonstrated to be connected to c-di-GMP signalling through the action of the major quorum sensing regulator HapR (Waters *et al.*, 2008). Furthermore, some GGDEF/EAL proteins, exemplified by the response regulator PleD (Aldridge and Jenal, 1999), contain phospho-receiver domains or other signalling domains, facilitating cross-talk and the integration into other signal cascades (Paul *et al.*, 2008).

Our study revealed a direct link between the global Csr network and c-di-GMP signalling, placing both pathways in a broad cellular context. CsrA activity is controlled by the sRNAs CsrB and CsrC, whose expression levels are regulated by the BarA-UvrY two-component system and the probable inner membrane protein CsrD (Fig. 9). CsrA has previously been shown to control motility and biofilm formation by directly targeting the *flhDC* and *pgaA* mRNAs, respectively. Here, we show that in addition to the regulation of biosynthesis and global regulators CsrA regulates bacterial physiology in a c-di-GMP-dependent pathway by directly controlling the expression of *ycdT* and *ydeH*, which cause c-di-GMP accumulation and thereby favour the sessile life style. The combination of c-di-GMP dependent and c-di-

GMP independent regulatory pathways allows CsrA to regulate biofilm related processes at various levels and thus to trigger the switch between a motile and a sessile life style. The CsrA and c-di-GMP specific adaptive responses are controlled by environmental signals, integrated at multiple sites within the signalling cascade. Although the nature of the signal sensed by BarA is not known, it is proposed to reflect the energy/growth status of the cell (Pernestig *et al.*, 2003). In addition, BarA-UvrY signalling was recently demonstrated to be pH dependent (Mondragon *et al.*, 2006). The prediction that CsrD and YcdT are membrane bound suggests that their activity is controlled from the outside. In addition, transcription of *ydeH* was previously demonstrated to depend on the CpxAR two-component system, responding to cell envelope stress and external copper (Yamamoto and Ishihama, 2005,2006). A future challenge will be the identification of the nature of different input signals, controlling Csr and c-di-GMP signalling.

The roles of YcdT and YdeH in bacterial physiology

Numerous studies have shown that c-di-GMP controls bacterial behaviour [reviews by (D'Argenio and Miller, 2004; Jenal, 2004; Romling, 2005; Romling and Amikam, 2006)]. High levels of this second messenger favour sessility whereas low levels of c-di-GMP promote a motile life style. In accordance, YdeH and YcdT were found to repress swimming behaviour. YdeH seems to act at the level of flagellum synthesis while YcdT seems to modulate flagella function, raising the possibility that individual GGDEF proteins are dedicated to specific functions in the cell. We also observed that in the strain overexpressing YdeH the occurrence of pili was abolished. A recent study proposed a link between c-di-GMP signalling and type 1 pili and flagella expression in the Crohn-disease-associated adherent-invasive *E. coli* strain LF82 (Claret *et al.*, 2007). While similar pathways might exist in *E. coli* K12, to this date we have no evidence for this hypothesis.

Our data show that overexpression of *ydeH* led to highly elevated c-di-GMP levels and to pronounced cellulose production in *Salmonella*. Consitent with these data, results from another parallel study suggest a significant role for YdeH in biofilm formation by regulating PGA synthesis (C. Goller and T. Romeo, unpublished). Although overexpression *ycdT* resulted in a strong accumulation of cellular c-di-GMP, we did not observe a distinct biofilm related phenotype neither in *E. coli* nor in *S.* Typhimurium. In addition, in an earlier study biofilm formation and *pgaA-lacZ* expression were not affected in the *ycdT* mutant XWMGΔT (Wang *et al.*, 2005). Nevertheless, we suspect that not only YdeH, but also YcdT might have an impact on biofilm formation under other experimental growth conditions. The *ycdT* gene and the *pga* operon are divergently organized (Fig. 1E). A comprehensive bioinformatics study has recently demonstrated that chromosomal proximity indicates gene coregulation in prokaryotes independent of relative gene orientation and that adjacent bidirectionally transcribed genes with conserved gene orientation are strongly coregulated (Korbel *et al.*, 2004). Furthermore, the *ycdT* homolog in *Yersinia pestis*, called HmsT, has been reported to be required for biofilm formation (Kirillina *et al.*, 2004). Likewise, there is evidence that *E. coli* and *Y. pestis* produce the PGA polysaccharide as biofilm matrix component (Bobrov *et al.*, 2008; Itoh *et al.*, 2005). Synthesis of this polysaccharide was in a recent study shown to be positively regulated by HmsT, which was suggested to control c-di-GMP levels in close proximity to the glycosyltransferase HmsR, responsible for the production of the polysaccharide (Bobrov *et al.*, 2008). Thus, regulation of PGA synthesis in *Yersinia* seems to occur in a c-di-GMP dependent fashion, similar to the production of the biofilm polysaccharide cellulose in *Salmonella*. Moreover, another recent study showed that the PEL biofilm polysaccharide synthesis in *Pseudomonas aeruginosa* is regulated by c-di-GMP. Here, the PelD protein serves as c-di-GMP receptor, activating the production of the PEL polysaccharide by a yet to be defined mechanism (Lee *et al.*, 2007).

These data suggest that there are related c-di-GMP dependent processes for controlling synthesis of the PGA exopolysaccharide in *E. coli*.

Noticeably, the *pel* genes in *Pseudomonas*, necessary for PEL synthesis, have been suggested to be regulated by the GacS-GacA-Rsm cascade, which is homologous to the BarA-UvrY-Csr pathway in *E. coli* (Goodman et al., 2004), further suggesting that the role of the Csr regulatory network in the regulation of biofilm components may be a conserved feature among γ-proteobacteria.

Experimental procedures

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. Chromosomal *ydeH::cat* and *csrB::cat* mutations were constructed using the Datsenko method (Datsenko & Wanner, 2000). The *cat* gene was amplified from pKD3 by PCR using primers ydeHKOFor2 and ydeHKORev2 or csrBKOFor and csrBKORev (Table 2), respectively, and introduced by electroporation into arabinose-treated BW25141 carrying pKD46. Transformants were selected on chloramphenicol plates, and their insertion sites were confirmed by PCR using the primer pairs ydeHKOtestFor/ydeHKOtestRev and csrBKOtestFor/csrBKOtestRev (Table 2). Mutations were transferred among strains by P1 transduction. For construction of the *csrB csrC* double mutant KJ230, the *csrC::tet* allele from strain TWMG1655 was moved into the *csrB* mutant KJ227, from which the chloramphenicol cassette had been flipped out using the FLP recombinase. Strain KJ311 was generated by removing the chloramphenicol cassette from KJ295 by using the FLP recombinase and subsequent infection with a P1 lysate containing the *ycdT::cat* mutation from XWMGΔT. For construction of strain KJ157, *csrA::kan*^{r} was moved from TRMG into KSB837. To generate the *csrD* mutant KJ205 the *yhdA::cat*^{r} cassette from KJ27 was transduced into MG1655. To generate KJ331, KJ330 and KJ369, *csrA::kan*^{r} was transduced from TRMG into KJ295, XWMGΔT or KJ311, respectively. In most of the experiments, bacteria were grown in LB medium at 37 °C with shaking at 200 r.p.m. If necessary, 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 pBADcsrA, pBADydeH and pBADycdT, the genes for *csrA*, *ydeH* and *ycdT*, were amplified from the MG1655 chromosome by PCR using the primer pairs CsrAForBAD/CsrARevBAD, pBADydeHFor/pBADydeHRev or pBADycdTFor2/pBADycdTRev2, respectively (Table 2). The PCR products of *csrA* and *ydeH* were cleaved with the enzymes *HindIII* and *XbaI*, while the product of *ycdT* was cut with *SacI* and *XbaI*. After cleavage of the pBAD28 vector at the corresponding sites followed by dephosphorylation (Shrimp Alkaline Phosphatase, Roche Diagnostics), the cleaved PCR fragments were inserted using the Rapid DNA Ligation Kit (Roche Diagnostics). For construction of pPYCDT and pPYDEH the upstream intergenic regions of the *ycdT* gene and the *ydeH* gene, including 2 or 3 nucleotides of the respective transcripts (*ycdT* -547 to +2; *ydeH* -222 to +3) were amplified using the primer pairs PycdTFor-EcoRI/ PycdTRev-BamHI or PydeHFor-EcoRI/ PydeHRev-BamHI (Table 2), respectively, and subsequently digested by *BamHI* and *EcoRI*. After removing the P*csrB* insert from vector pCBZ1 (Gudapaty *et al.*, 2001) by *BamHI* and *EcoRI* cleavage, the empty linearised vector was dephosphorylated and ligated with the respective *ycdT* or *ydeH* fragments to create pPYCDT and pPYDEH. Sequencing verified the integrity of all plasmid constructs.

Site-directed mutagenesis

To engineer the mutated *ycdT* and *ydeH* alleles, plasmids pBADycdT and pBADydeH were subjected to site-directed mutagenesis using the high-performance liquid chromatography-purified primer pairs YcdT-Mut-For/YcdT-Mut-Rev and YdeH-Mut-For/YdeH-Mut-Rev (Table 2) and the QuikChange II site-directed mutagenesis kit (Stratagene) to create plasmids pBADycdT-mut and pBADydeH-mut. Mutations introduced into *ycdT* and *ydeH* led to the replacement of the two glycines at positions 359 and 360 (*ycdT*) or 206 and 207 (*ydeH*) in the GGEEF motif by alanine (YcdT G359A, G360A; YdeH G206A, G207A). The mutations were confirmed by sequencing.

RNA extraction

Bacterial cultures were mixed with 2 vol of RNAprotect Bacterial Reagent (Qiagen) and incubated for 5 minutes at room temperature. Total cellular RNA was subsequently prepared by using the RNeasy Mini Kit with on-column DNA digestion (Qiagen). RNA concentrations were determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The quality of the RNA used for the microarray was assessed using the Agilent Bioanalyzer.

Microarray analysis

Microarray analysis was performed at the Bioinformatics and Expression Analysis Core Facility (BEA) at the Karolinska Institute (http://www.bea.ki.se) using the GeneChip *E. coli* Genome 2.0 Array (Affymetrix, P/N 900551, Santa Clara, CA). This array includes approximately 10,000 probe sets for all 20,366 genes present in four strains of *E. coli*. Affymetrix analysis was conducted according to the Affymetrix manual (www.affymetrix.com).The absolute signals from the samples, taken at 0 minutes (before arabinose induction), were compared with the signals from the 4 and 12 minutes samples. The signal ratios resulting from pBADcsrA overexpression were then normalised with the ratios resulting from overexpression of the empty vector pBAD28. Genes, whose expression levels were too low for reliable detection, or whose expression levels were decreased in response to induction of the empty vector pBAD28, were excluded from the analysis.

Quantitative Real-Time RT-PCR

500 ng of total RNA were used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers were designed using the Primer Express Software v3.0 (Applied Biosystems). All RT primers used in this study are listed in Table 2. 0.1 ng template was used for the Real-Time PCR reaction using the Power SYBR Green PCR Master Mix (Applied Biosystems). Analysis 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 artifacts of amplification, a dissociation curve was run immediately after completion of the Real-Time PCR. Individual gene expression profiles were normalised against the *rrnD* gene, serving as an endogenous control. All results were analysed using the 7500 SDS Software v1.3.1 (Applied Biosystems) and further prepared using Excel (Microsoft). The data values presented in all figures represent the mean expression level of quadruplicates from one Real-Time PCR assay, relative to a calibrator value (time point 0 minutes or wild type). The error bars represent the standard error of the mean expression level calculated by the SDS software using the confidence value 95 %. Each experiment was repeated independently and representative data were chosen for presentation

5′ RACE

5′-ends of the *ycdT* transcripts were determined using the 5′ RACE System for Rapid Amplification of cDNA Ends (v2.0, Invitrogen). Three μg of total RNA were reverse transcribed using the primer YcdTGSP1 and the superscript II RT. cDNAs were purified, C-tailed with a terminal deoxynucleotidyl transferase and used as template in a PCR with an anchor primer (AAP), specific for the C-tail, and the gene specific primer YcdTGSP2HindIII (GSP2), complementary to a region upstream of the binding site of GSP1. To increase specificity, a nested PCR was carried out using the nested anchor primer UAP and the gene specific nested primer YcdTGSP3HindIII (GSP3). The PCR products were visualized on a 2 % agarose gel in TBE buffer and subsequently 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). *E. coli* CsrA-His_6 protein was purified as described previously (Mercante *et al.*, 2006). DNA templates for generating *ycdT* and *ydeH* RNA transcripts were produced by annealing primers ycdT-T7 ((-36) − (+20)) and GC ycdT-T7 ((-36) − (+20)) and ydeH-T7 ((-29) − (+25)) and GC ydeH-T7 ((-29) − (+25)) in TES buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl). RNA was synthesized *in vitro* using the MEGAshortscript kit (Ambion) using the annealed DNA primers (for *ydeH* and *ycdT*) or linearized plasmid pPB77 (Babitzke *et al.*, 1994) as templates. After gel purification, transcripts were 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. Radiolabeled 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 80 pM radiolabeled RNA in 10 μl 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 minutes at 37 °C to allow for CsrA-RNA complex formation. Competition assays were performed in the absence or presence of unlabeled RNA specific and non-specific competitor. Binding reactions were separated using 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 two independent experiments were determined for each transcript. Graphpad Prism version 3.02 for Windows (San Diego, CA) software was used for calculations.

β-galactosidase assay

β-galactosidase activity was measured in 10 min reactions using the Miller protocol (1972). Twenty μl of bacterial culture, grown to an OD_{600} of 1.5, were used for each reaction. Each measurement was carried out independently at least two times.

Quantification of c-di-GMP

Nucleotide extracts were prepared essentially as previously described (Simm *et al.*, 2004). For c-di-GMP extraction from liquid cultures, bacteria were grown in LB medium to OD_{600} 1.5 at 37 °C, treated with formaldehyde (0.19 % final concentration) and pelleted by centrifugation. The pellet was resuspended in ice-cold water, heated to 95 °C for 10 minutes, before nucleotides were extracted by ethanol treatment. For c-di-GMP extraction from plate grown bacteria, approximately 100 mg of cells were harvested and immediately suspended in ice-cold 0.19 % formaldehyde, before being boiled for 10 minutes and treated with ethanol. Nucleotide extracts of 10 mg or 50 mg of cells (wet weight) were fractioned by

high-performance liquid chromatography (HPLC) using a reversed-phase column (Hypersil ODS 5μ; Hypersil-Keystone). Runs were carried out with a multistep gradient using 0.1 M triethyl ammonium acetate (pH 6.0) at 1 ml min^{-1} with increasing concentrations of acetonitrile. Relevant fractions were collected, lyophilized, and resuspended in 10 μl water. Fractions containing c-di-GMP were pinpointed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis and pooled. Synthetic c-di-AMP was added to the pooled fractions at a suitable concentration to be used as an internal standard. A standard curve was established using fractions spiked with known amounts of c-di-GMP, using a fixed amount of synthetic c-di-AMP as internal control. The isotope areas of c-di-GMP and c-di-AMP were calculated, and the ratio was determined. Each c-di-GMP measurement was carried out independently at least two times.

AFM microscopy

Sample preparation and AFM imaging were performed as earlier described (Jonas *et al.*, 2007). Bacteria were allowed to grow for 24 h at 28°C on mica slides Grade V-4 (SPI® Supplies, USA) submerged in Petri dishes containing 3 ml LB medium without NaCl. After incubation the mica slides were dipped 3 to 4 times into double distilled water, air-dried at room temperature in a dust-free environment for several hours and mounted onto glass microscope slides. Bacteria were imaged with the BioScope SZ (Veeco Instruments, Woodbury, NJ) operated in the contact mode using V-shaped silicon nitride nanoprobe cantilevers MLCT-AUHW (Veeco) with a spring constant of 0.05 N/m. Images were captured using NanoScope v6.13 (Veeco) and further analysed with the scanning probe software WSxM (Nanotec Electronica, Spain) (Horcas et al., 2007). To quantify flagella expression, the number of flagella and the number of bacteria was counted at five different locations on the microscope slide for each strain. The ratio of flagella per ten bacteria was calculated and the mean and the standard deviation determined. To quantify pili expression, the number of bacteria expressing pili per total number of bacteria was calculated at five different locations for each strain, from which the mean value and the standard deviation were calculated.

Phenotypic assays

To analyze the swimming behaviour of the bacteria, 0.3 % motility agar plates, if necessary supplemented with 0.1 % arabinose, were inoculated with 4 μl of overnight culture and incubated at 37 °C. The diameter of the swimming zone was measured over time. For analysis of colony morphology, bacteria from an overnight culture were streaked onto LB agar plates with or without NaCl supplemented with Congo Red (40 μg ml^{-1}) and Coomassie brilliant blue (20 μg ml^{-1}) or calcofluor (fluorescence brightener 28; 50 μg ml^{-1}). Plates were incubated at 28 °C or 37 °C for 20 h or 24 h. The colony morphology and dye binding was analysed over time. Glass adherence was measured by culturing the bacteria in standing glass culture tubes containing LB medium with or without salt at 28 °C or 37 °C for 24 h. After analysing the formation of pellicles visually, the culture liquid was discarded by decanting and the bacteria, adherent to the glass tubes, were stained with crystal violet solution. The tubes were subsequently rinsed with water, allowed to air-dry in the upside-down position and adherence of the bacteria to the glass was analysed visually.

Bioinformatic analysis

The protein domain structures were analysed using Pfam (http://www.sanger.ac.uk) and UniProt (http://beta.uniprot.org/) and aligned using clustalw (http://www.ebi.ac.uk). The genomic context of the genes was analysed using EcoCyc (http://www.ecocyc.org).

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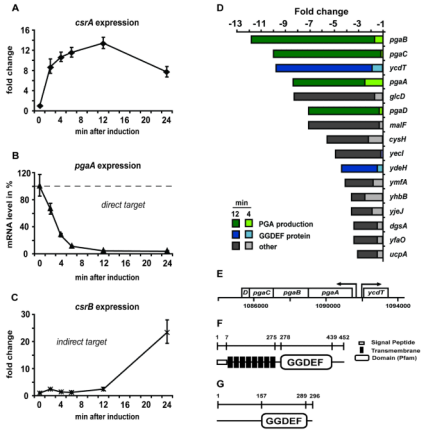

Figure 1.

Identification of *ycdT* and *ydeH* as novel targets for CsrA. Plasmid encoded *csrA* (pBADcsrA) was expressed in KSB837 (*csrA::kan*) upon induction with 0.1 % arabinose at OD_{600} 1.5. (A) Increased *csrA* expression in response to arabinose addition (0 min) was measured by Real-time RT-PCR over time. CsrA is assumed to bind to its direct mRNA targets, to inhibit translation and thereby to destabilize the mRNAs. (B) mRNA levels of the known CsrA target *pgaA* rapidly decreased within a few minutes after pBADcsrA induction. (C) The indirect CsrA target *csrB* was changed in expression first after 12 minutes. (D) A genome-wide screen for genes, whose transcript levels decrease within 4 and 12 minutes (> 3 fold compared to 0 min) in response to CsrA overexpression, identified *ycdT* and *ydeH* as novel CsrA targets. (E) The *ycdT* gene is located adjacent to the *pga* operon and is divergently transcribed. (F) YcdT harbours 8 transmembrane regions and is predicted to contain a GGDEF motif. (G) YdeH contains a GGDEF motif and is predicted to be cytoplasmic.

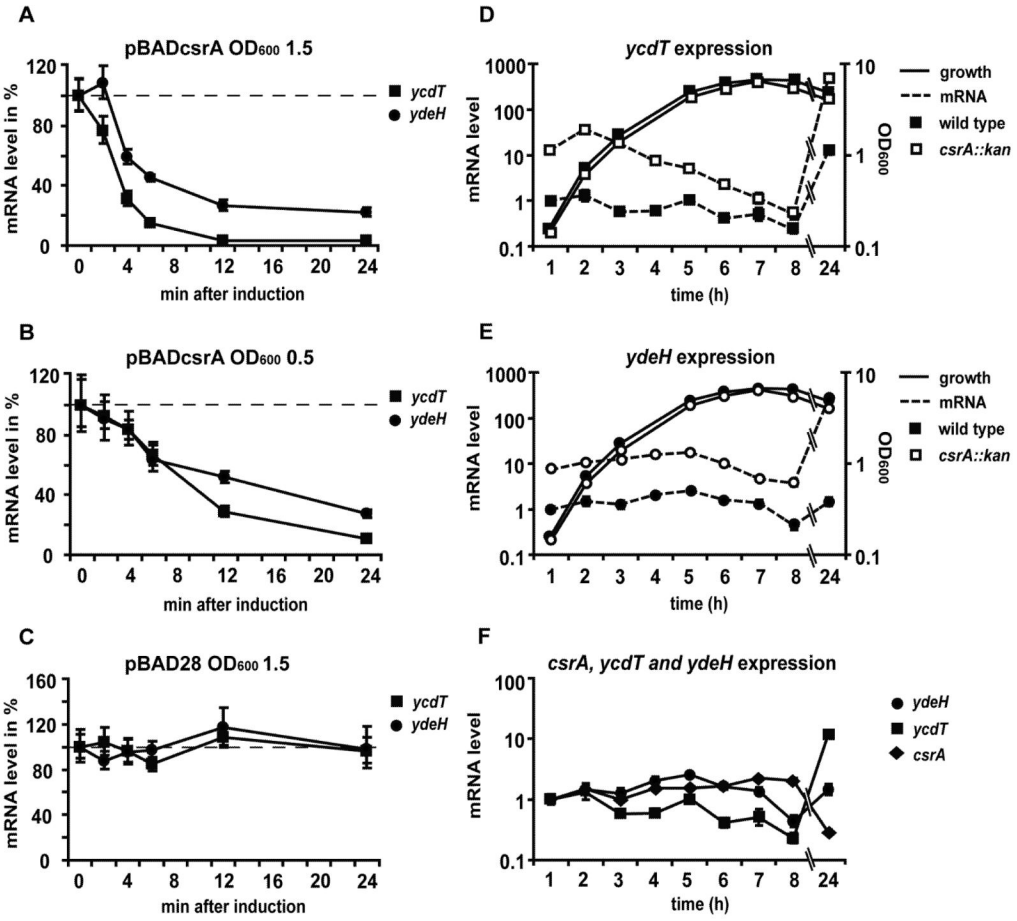

Figure 2.

CsrA dependent regulation of *ycdT* and *ydeH* expression measured by Real-Time RT-PCR. (A) Induction of pBADcsrA with 0.1 % arabinose (at 0 min) leads to a rapid decrease in *ycdT* (squares) and *ydeH* (circles) transcript levels during late (OD_{600} 1.5) exponential growth. (B) Similar results were observed during early exponential growth (OD_{600} 0.5). (C) Induction of pBAD28 had no effect on *ycdT* and *ydeH* mRNA levels. (D) In the *csrA::kan* mutant TRMG1655 (open symbols), *ycdT* mRNA levels (dashed line) were strongly increased compared to the wild type MG1655 (filled symbols) over the entire growth cycle. (E) Likewise, *ydeH* expression (dashed line) was significantly higher in the *csrA* mutant. (F) Analysis of *ycdT*, *ydeH* and *csrA* (diamonds) expression over time in the wild type indicates that *ycdT* and *ydeH* are inversely regulated with respect to *csrA*.

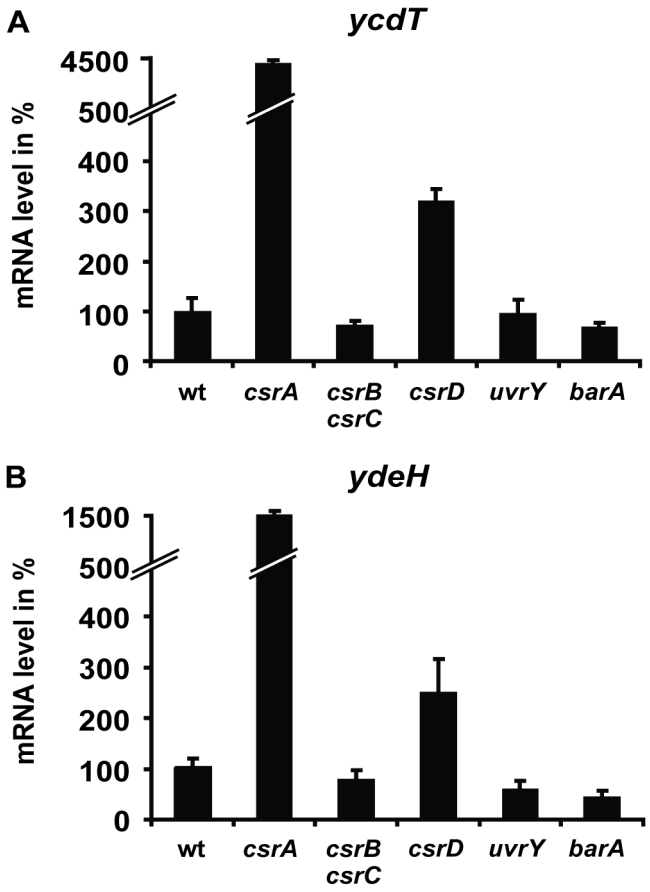

Figure 3.

Effects of other components of the BarA-UvrY-Csr network on *ycdT* and *ydeH* expression. mRNA levels of *ycdT* (A) and *ydeH* (B) were measured in MG1655 (wt), TRMG (*csrA*), KJ230 (*csrB csrC*), KJ205 (*csrD*), AKP200 (*uvrY*) and AKP199 (*barA*) by Real-Time RT-PCR when the bacterial cultures had reached an OD_{600} of 1.5.

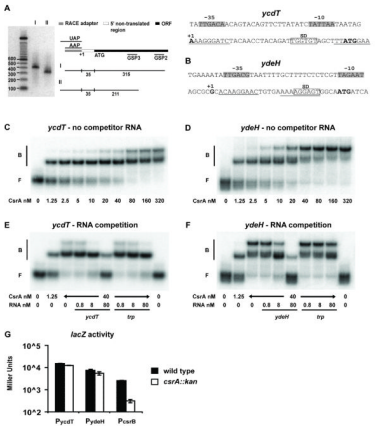

Figure 4.

Physical interaction between CsrA and *ycdT* and *ydeH* transcripts. (A) The transcription start site for *ycdT* was determined by 5′RACE. An approximately 410 nt PCR product was detected after amplification of reverse transcribed cDNA with the gene specific primer GSP2 and the adapter primer AAP (lane 1 (I)). The product of a second nested PCR reaction with primers GSP3 and UAP was approximately 100 nt shorter (lane 2 (II) – as labelled in figure). Sequencing of the shorter fragment identified A (35 nt upstream of ATG) as transcription start site. Analysis of the 5′ leader of *ycdT* suggests two potential CsrA binding sites (underlined). (B) Sequence analysis of the intergenic region of *ydeH* suggests two potential CsrA binding sites (underlined), one of them overlapping the Shine-Dalgarno sequence (SD). (C, D) Gel mobility shift analyses of CsrA-*ycdT* and CsrA-*ydeH* interactions in the absence of RNA competitor. 5′ end-labelled *ycdT* or *ydeH* transcripts (80 pM) were incubated with CsrA at the indicated concentrations. The positions of free (F) and bound (B) RNA are shown. (E, F) Competition reactions using specific (*ycdT*, *ydeH*) or non-specific (*trpL* from *B. subtilis*) unlabelled RNA competitors. The concentration of competitor RNA is shown at the bottom of each lane. (G) β-galactosidase activity for plasmid encoded transcriptional *ycdT*-, *ydeH*- and *csrB*-*lacZ* fusions in wild type MG1655 and its *csrA* mutant. Bacteria containing pPYCDT, pPYDEH or pCBZ1, respectively, were grown until OD_{600} 1.5. The mean values and the standard deviations were calculated for each strain from two parallel experiments.

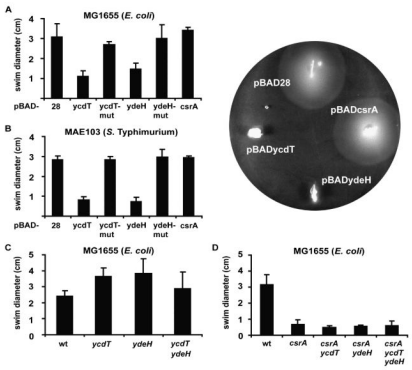

Figure 5.

The effects of YcdT and YdeH on motility. Motility was analysed by measuring the diameter of the swimming zone on 0.3 % agar plates, supplemented with 0.1 % arabinose, if necessary. (A) MG1655 (wt), carrying pBAD28, pBADycdT, pBADycdT-mut, pBADydeH, pBADydeH-mut or pBADcsrA. (B) *Salmonella* Typhimurium strain MAE103, carrying pBAD28, pBADycdT, pBADycdT-mut, pBADydeH, pBADydeH-mut or pBADcsrA. (C) MG1655 (wt) and mutants XWMGΔT (*ycdT*), KJ295 (*ydeH*) and the double mutant KJ311 (*ycdT ydeH*). (D) MG1655 and mutants TRMG (*csrA*), KJ331 (*csrA ycdT*), KJ330 (*csrA ydeH*) and KJ369 (*csrA ycdT ydeH*). A representative image of a motility agar plate for MAE103 carrying pBAD28, pBADycdT, pBADydeH or pBADcsrA is illustrated. All motility assays were repeated at least two times independently and the mean and the standard deviation were calculated.

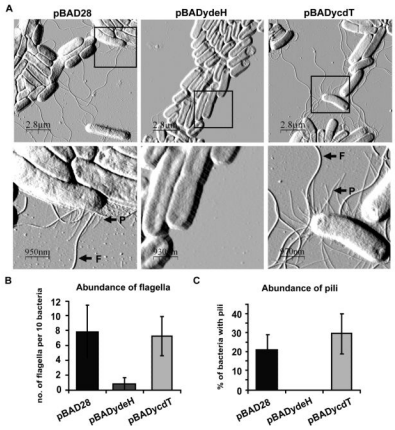

Figure 6.

High-resolution AFM analysis of cell morphology. Bacteria were grown on mica surfaces for 24 h at 28 °C in LB medium containing 0.1 % arabinose, but no salt. Afterwards the samples were air-dried and analysed with the AFM in contact mode. Representative images were chosen for presentation. (A) Lower (first row) and higher magnification (second row) AFM images of MG1655 (wt), carrying pBAD28, pBADydeH or pBADycdT. The arrows highlight the appearance of flagella (F) and pili (P). (B) Flagella expression was quantified by counting the number of flagella per total number of bacteria at five different locations on the microscope slide. The mean values and the standard deviations were calculated for each strain. (C) Pili expression was quantified by counting the number of bacteria expressing pili per total number of bacteria at five independent sites, from which the mean and standard deviation were determined.

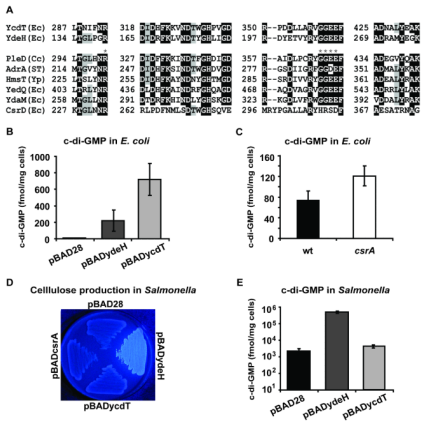

Figure 7.

Functional characterization of YcdT and YdeH. (A) The amino acid sequences of YcdT and YdeH were compared with other GGDEF domain proteins from *Caulobacter crescentus* (Cc), *Salmonella* Typhimurium (ST), *Yersinia pestis* (Yp) and *E. coli* (Ec), proven to synthesize c-di-GMP (PleD, AdrA, HmsT, YedQ and YdaM) or demonstrated not to be involved in c-di-GMP metabolism (CsrD). Dark background indicates a high level of similarity between the proteins. The stars (*) depict amino acid residues that have been demonstrated to be critical for substrate binding or catalysis (Chan *et al.*, 2004). (B) c-di-GMP concentrations were determined in MG1655 carrying pBAD28, pBADydeH or pBADycdT grown to OD_{600} 1.5 in LB medium with 0.1 % arabinose at 37 °C. (C) c-di-GMP concentrations of wt MG1655 and its isogenic *csrA* mutant TRMG, cultivated under equal conditions as (B), but without arabinose. (D) Expression of pBADydeH in the *Salmonella adrA* mutant MAE103 successfully restored cellulose production as visualized on calcofluor (CF), when grown for 20 h at 28 °C on LB agar with 0.1 % arabinose, but without salt. No dye binding was observed for MAE103 carrying pBADycdT, pBADcsrA or the control vector pBAD28. (E) c-di-GMP measurements in *Salmonella* MAE103, carrying pBAD28, pBADydeH and pBADycdT, grown for 20 h at 28 °C on LB agar with 0.1 % arabinose, but without salt.

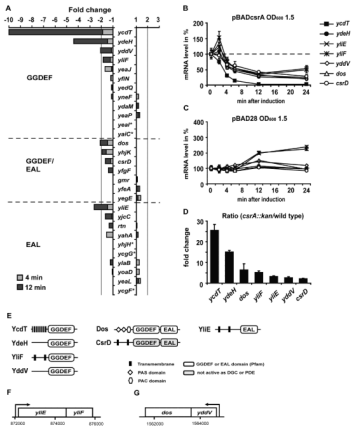

Figure 8.

The global effect of CsrA on GGDEF/EAL proteins. (A) All 29 *E. coli* genes encoding GGDEF, GGDEF-EAL or EAL proteins were analysed for CsrA-dependent changes in gene expression using the array data. Genes expressed at levels too low for microarray detection are indicated with a star (*). (B) The kinetics of CsrA-dependent downregulation of *ycdT*, *ydeH*, *yliE*, *yliF*, *yddV*, *dos* and *csrD* upon induction of pBADcsrA were confirmed by RT-PCR. (C) Expression of pBAD28 did not lead to a decrease in *ycdT*, *ydeH*, *yliE*, *yliF*, *yddV*, *dos* and *csrD* expression. (D) The ratio in mRNA levels between TRMG (*csrA*::*kan*) and MG1655 (wt) was determined for *ycdT*, *ydeH*, *yliE*, yliF, *yddV*, *dos* and *csrD*, indicating that expression of these genes is increased in the *csrA* mutant. (E) Four of the CsrA regulated genes encode GGDEF proteins (*ycdT*, *ydeH*, *yddV* and *yliF*), two GGDEF-EAL proteins (*dos*, *csrD*) and one of them encodes an EAL protein (*yliE*). (F) *yliE* and *yliF* are organized as an operon. (G) Likewise, *yddV* and *dos* are present in an operon.

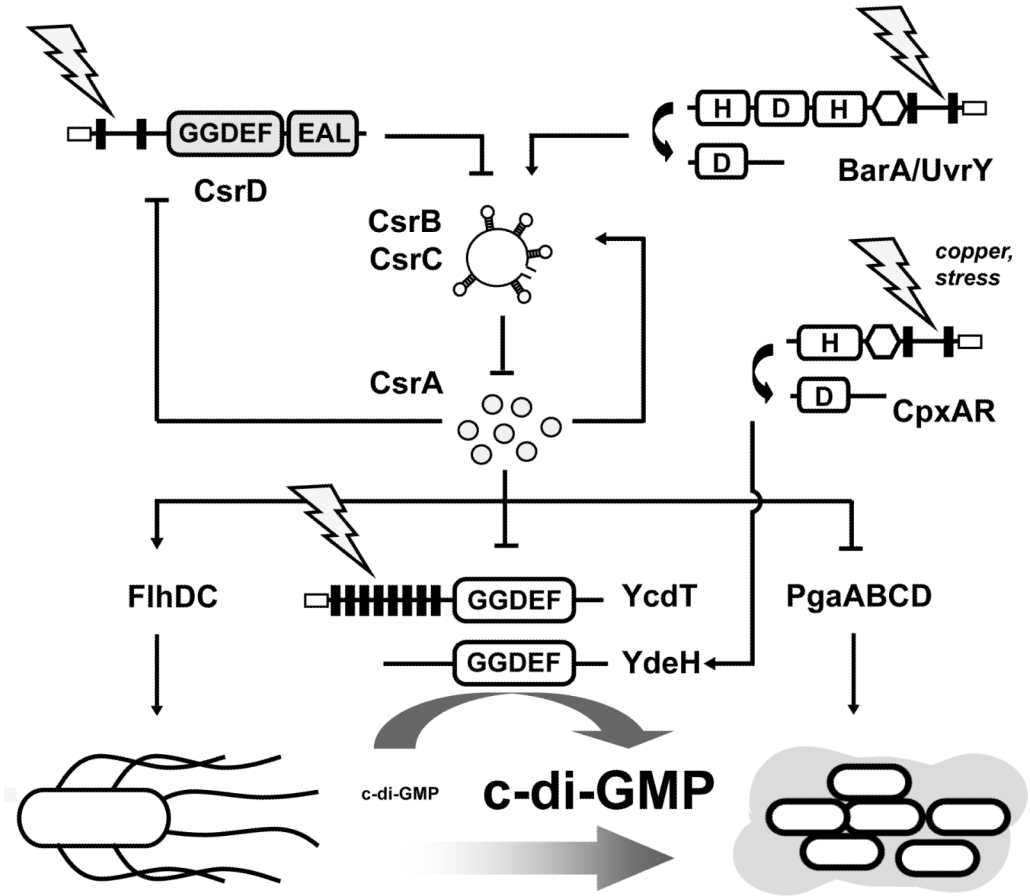

Figure 9.

Schematic view of the interconnection between Csr and c-di-GMP signalling in *E. coli*. The activity of the central player CsrA is controlled by the sRNAs CsrB and CsrC, which are regulated by the BarA-UvrY two-component system and CsrD, a GGDEF-EAL protein not involved in c-di-GMP metabolism. CsrA directly acts on motility and biofilm formation, by controlling mRNA levels of *flhDC* and *pga*A, respectively. In addition, CsrA controls indirectly the switch between a motile and a sessile life style by regulating the levels of c-di-GMP through post-transcriptional regulation of the GGDEF proteins YcdT and YdeH and possibly additional proteins with GGDEF or EAL domains. Signals from the outside controlling the CsrA and c-di-GMP specific adaptive responses are integrated through the BarA-UvrY TCS and possibly through CsrD and YcdT. Transcription of *ydeH* was shown to be controlled by the CpxAR two-component system in response to cell envelope stress and external copper.

Table 1

Bacterial strains and plasmids used in this study.

Strain or plasmid	Description or genotype	Reference
Strains		
Escherichia coli		
MG1655	F^- λ^-	Michael Cashel
BW25141	*lacI*^{q} *rrnB*T14 *lacZ*WJ16 *phoBR580 hsdR514 araBAD* AH33 *rhaBAD*LD78 *galU95 endA*BT333 *uidA* (MluI)::pir$^+$ recA1	(Datsenko and Wanner, 2000)
TRMG	MG1655 *csrA::kan*	(Romeo *et al.*, 1993)
KSB837	CF7789 Δ(λ*att-lom*)::*bla* φ (*csrB-lacZ*)*1* (Hyb) *amp*^{r}	(Suzuki *et al.*, 2002)
KJ157	KSB837 *csrA::kan*^{r}	This study
KJ227	MG1655 *csrB::cat*^{r}	This study
TWMG1655	MG1655 *csrC::tet*^{r}	(Weilbacher *et al.*, 2003)
KJ230	MG1655 Δ*csrB csrC::tet*^{r}	This study
KJ27	KSB837 *yhdA::cat*^{r}	(Jonas *et al*., 2006)
KJ205	MG1655 *yhdA::cat*^{r}	This study
AKP199	MG1655 *barA::kan*^{r}	(Pernestig *et al.*, 2003)
AKP200	MG1655 *uvrY::catr*	(Pernestig *et al.*, 2003)
XWMGΔT	MG1655 *ycdT::cat*^{r}	(Wang *et al.*, 2005)
KJ295	MG1655 *ydeH::cat*^{r}	This study
KJ311	MG1655 Δ*ydeHycdT::cat*^{r}	This study
KJ331	KJ295 *csrA::kan*^{r}	This study
KJ330	XWMGΔT *csrA::kan*^{r}	This study
KJ369	KJ311 *csrA::kan*^{r}	This study
Salmonella enterica serovar Typhimurium		
MAE103	Δ*csgBA102 adrA101::MudJ*	(Romling *et al.*, 2000)
Plasmids		
pKD46	temperature sensitive λ red recombinase expression vector	(Datsenko and Wanner, 2000)
pKD3	template for mutant construction, carries chloramphenicol resistance cassette	(Datsenko and Wanner, 2000)
pBAD28	pBAD expression plasmid	(Guzman *et al.*, 1995)
pBADcsrA	CsrA expression plasmid, *csrA* is controlled by the plasmid-borne P_{BAD} promoter	This study
pBADycdT	YcdT expression plasmid, *ycdT* is controlled by the plasmid-borne P_{BAD} promoter	This study
pBADydeH	YdeH expression plasmid, *ydeH* is controlled by the plasmid-borne P_{BAD} promoter	This study
pBADycdT-mut	mutagenised pBADycdT (G359A, G360A)	This study
pBADydeH-mut	mutagenised pBADydeH (G206A, G207A)	This study
pCBZ1	pGE593, Φ(*csrB-lacZ*)	(Gudapaty *et al.*, 2001)
pPYCDT	LacZ reporter plasmid, containing a *ycdT-lacZ* transcriptional fusion	This study
pPYDEH	LacZ reporter plasmid, containing a *ydeH-lacZ* transcriptional fusion	This study

Table 2

Primers used in this study.

Primer name	Primer Sequence (5′ to 3′)
Genetic approaches	
ydeHKOFor2	ATGGACTGTGCCAGTTTGGTCGGTGGATTGATCATCTGGGGCCACTCGTGTAGGCTGGAGCTGCTTC
ydeHKORev2	CGGTTTGCTTACCCTCATACATTGCCCGGTCCGCTCTTCCAATGACCATATGAATATCCTCCTTAG
ydeHKOtestFor	ACAAGGAACTGTGAAAAAG
ydeHKOtestRev	ATCGTTGACACAGTAGCA
csrBKOFor	GAGTCAGACAACGAAGTGAACATCAGGATGATGACACTTCTGCGTAGGCTGGAGCTGCTTC
csrBKORev	AATAAAAAAAGGGAGCACTGTATTCACAGCGCTCCCGGTTCGTTTATATGAATATCCTCCTTAG
csrBKOtestFor	GTAGGAGATCGCCAGGAAAT
csrBKOtestRev	CACGCAGTAACGCTTCAAGC
CsrAForBAD	ACC*TCTAGA*TCTTTCAAGGAGCAAAGAATG
CsrARevBAD	ACC*AAGCTT*GATGAGACGCGGAAAGATTA
pBADydeHFor	ACC*TCTAGA*GTGAAAAAGGAGTGGCAATG
pBADydeHRev	ACC*AAGCTT*TGAATGTTAAACGGAGCTTA
pBADycdTFor2	ACC*GAGCTC*AGATTGGTGTAGCTTTATG
pBADycdTRev2	ACC*TCTAGA*AGGATCAAAATGCCGCTTTA
YcdT-Mut-For	TAGCGCGCGTCG**C**CG**C**CGAAGAGTTTGGC
YcdT-Mut-Rev	GCCAAACTCTTCG**G**CG**G**CGACGCGCGCTA
YdeH-Mut-For	GAAACGGTTTATCGCTACG**C**GG**C**CGAAGAATTTATCATTATTG
YdeH-Mut-Rev	CAATAATGATAAATTCTTCG**G**CC**G**CGTAGCGATAAACCGTTTC
PydeHFor-EcoRI	ACC*GAATTC*TAAATTAGCCTGATGGCCTG
PydeHRev-BamHI	ACC*GGATCC*TGCGCGCTATTCTAACGAG
PycdTFor-EcoRI	ACC*GAATTC*TATTACTCCATGTATTGCC
PycdTRev-BamHI	ACC*GGATCC*TTCTATTATTAATAGATATAAG
Real-Time PCR	
RTrrnDFor	AGTTCCAGTGTGGCTGGTCAT
RTrrnDRev	GCTCACCAAGGCGACGAT
RTcsrAFor	TGGTGAGACCCTCATGATTGG
RTcsrARev	CGTACCTGGTTGCCCTTTACC
RTcsrBFor	CAAGGATGAGCAGGGAGCAA
RTcsrBRev	CGCTCCCGGTTCGTTTC
RTpgaAFor	TCGAACGTGAACCGCAAGA
RTpgaARev	ATGTACATCAACCGCACGTTTT
RTycdTFor	ACGCCTTATTGCGTCATGATT
RTycdTRev	CCCCAGGTGTCGTTGACTTT
RTydeHFor	AATAAGGCTATCGATGCCCACTAC
RTydeHRev	CGCGACCACGCTGTGA
RTyddVFor	TGCCCAGGTTGACGATGTC
RTyddVRev	ACTTCCGCGACGGTATGC
RTdosFor	CGCCGATGGCATTTTTTT

Primer name	**Primer Sequence (5′ to 3′)**
RTdosRev	ATTAACACCGCACCCATCATATT
RTyliEFor	TCGGTGGCTTCAGATGACTCT
RTyliERev	GGACGATCAAAGCAATTGTATGC
RTyliFFor	CCTGGACGACCTGACCAAA
RTyliFRev	GCGCTTTTAAATCTTCGTCAAAG
RTyhdAFor	GCCACGCTCACCGTTTAAGA
RTyhdARev	GCCGGGCAAGAATTGCT
RTyhjKFor	AGCCGGGAACACTGATTCTG
RTyhjKRev	GCATGAGGGTCGTCAATACGT
RTyjcCFor	GGCGCTGAAGCGTTGTTAC
RTyjcCRev	TCTGCCGGATTCATTATTTGC
5′ RACE	
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
UAP	CUACUACUACUAGGCCACGCGTCGACTAGTAC
YcdTGSP1	CTGACGAAACAAATAAT
YcdTGSP2HindIII	ACC*AGGCTT*GCTTGTCAAACGCTCCTCAATAA
YcdTGSP3HindIII	ACC*AGGCTT*ATTGCCTACGGTCATAAATGAAAT
RNA Gel Mobility Shift Assays	
ycdT-T7	TAATACGACTCACTATAGGGAAAGGGATCTACAACCTACAGATTGGTGTAGCTTTATGGAAAAAGACTATTTGAG
GC ycdT-T7	CTCAAATAGTCTTTTTCCATAAAGCTACACCAATCTGTAGGTTGTAGATCCCTTTCCCTATAGTGAGTCGTATTA
ydeH-T7	TAATACGACTCACTATAGGGCACAAGGAACTGTGAAAAAGGAGTGGCAATGATCAAGAAGACAACGGAAATTG
GC ydeH-T7	CAATTTCCGTTGTCTTCTTGATCATTGCCACTCCTTTTTCACAGTTCCTTGTGCCCTATAGTGAGTCGTATTA