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The Global Regulatory Role of the RNA Binding Protein CsrA

by

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Doctor of Philosophy

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The Global Regulatory Role of the RNA Binding Protein CsrA

by

Adrianne N. Edwards
B.S., University of Georgia, 2004

Advisor: Tony Romeo, Ph.D.

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Abstract

The Global Regulatory Role of the RNA Binding Protein CsrA
By Adrianne N. Edwards

CsrA influences several important cellular processes by binding to target mRNAs and altering their translation and/or stability. CsrA activity is antagonized by two sRNAs, CsrB and CsrC. To elucidate the global role of CsrA, we combined a biochemical method with high throughput cDNA sequencing to determine the identities of mRNAs that directly bound to *Escherichia coli* CsrA in vivo. Among the 721 transcripts discovered were *relA*, *spoT* and *dksA*, which encode proteins that mediate the stringent response. RelA and SpoT are involved in guanosine tetraphosphate (ppGpp) metabolism, while DksA typically functions synergistically with ppGpp to potentiate the stringent response. CsrA directly repressed expression of *relA* and indirectly repressed *spoT* transcript levels, suggesting that CsrA may affect cellular ppGpp levels in certain conditions. CsrA directly activated *dksA* expression, but this effect was masked by DksA negative autoregulation. In turn, DksA and ppGpp activated *csrA*, *csrB* and *csrC* transcription. CsrA and DksA were both required for full expression of *csrB* and *csrC*, which required the response regulator UvrY. These complex direct and indirect regulatory interactions between the Csr and stringent response systems suggest that these systems function to finely tune gene expression in response to carbon metabolism and nutrient availability. In addition, a screen designed to find novel CsrA targets in *E. coli* using a microarray-based approach revealed that CsrA affected the expression of several GGDEF and EAL domain proteins. GGDEF and EAL domains are associated with c-di-GMP metabolism, a second messenger that positively influences biofilm formation and represses motility. CsrA directly repressed the expression of two GGDEF domain proteins in a post-transcriptional manner while modestly influencing the expression of five additional GGDEF/EAL domain proteins through unknown mechanisms. Cellular c-di-GMP concentrations were increased modestly in the *csrA* mutant, signifying that CsrA globally affects c-di-GMP synthesis. Finally, analysis of effects of CsrA on GGDEF/EAL domain proteins in *Salmonella* Typhimurium demonstrated that CsrA has both direct and indirect effects on regulating the switch between motility and biofilm formation. These studies have expanded the regulatory role of CsrA and suggest that CsrA governs cellular physiology and behavior on a global scale.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Chapter 1.

Chapter 2.

Chapter 3.

Chapter 4.

Chapter 5.

Chapter 1. Introduction

To quickly sense and adapt to their environment, bacteria rely on regulatory systems that coordinate physiological processes. Several global regulatory networks are often required for efficient detection of multiple inputs to produce an appropriate response. Responses involve global changes in gene expression resulting in metabolic and physiological changes.

Frequently, bacteria use second messenger signaling molecules in the form of modified nucleotides to efficiently counter nutrient limitations and environmental stressors. These second messengers are quickly synthesized and diffused, and their synthesis and degradation are tightly controlled. The most well studied second messenger, cyclic AMP (cAMP), serves to alert cells to the status of the available carbon source, while others, such as a quanosine tetraphosphate (ppGpp) and cyclic di-GMP (c-di-GMP), are synthesized in response to a variety of conditions. Here, we describe the interconnections between the Csr global regulatory system and stringent response, which synthesizes ppGpp during nutritional stress, and how the Csr system regulates the metabolism of c-di-GMP in both *E. coli* and *Salmonella*, creating a complex regulatory network that controls motility and biofilm formation.

Csr/Rsm regulatory system

The Csr system is a global regulatory network that is found in many eubacteria, including pathogens, and has multiple pleiotropic effects on metabolism and cellular physiology. CsrA (carbon storage regulator), or its homolog RsmA (repressor of

secondary metabolites), is the central regulatory component of gene expression in this system and post-transcriptionally activates exponential phase processes such as glycolysis (Sabnis *et al.*, 1995), acetate metabolism (Wei *et al.*, 2000) and motility (Wei *et al.*, 2001), while repressing several stationary phase processes including glycogen metabolism (Romeo *et al.*, 1993), gluconeogenesis (Romeo *et al.*, 1993) and biofilm formation (Wang *et al.*, 2005). CsrA frequently exhibits opposite effects on opposing cellular processes; for example, CsrA inversely regulates motility and biofilm formation.

CsrA is a 61-amino acid RNA-binding protein that binds to the 5' untranslated region of messenger RNA to repress or activate expression by altering mRNA stability (Liu *et al.*, 1995) and/or by competing for binding to the Shine-Dalgarno and blocking efficient translation (Baker *et al.*, 2002). Other components of the Csr system include the small, noncoding RNAs, CsrB and CsrC, which possess multiple CsrA binding sites that titrate out CsrA activity by sequestering the protein (Liu *et al.*, 1997; Weilbacher *et al.*, 2003); the two component signal transduction system, BarA-UvrY, which is required for expression of CsrB and CsrC (Suzuki *et al.*, 2002); and CsrD, a specificity factor that targets both CsrB and CsrC for degradation by RNase E (Suzuki *et al.*, 2006).

CsrA mechanism and regulation

Systematic evolution of ligands by exponential enrichment (SELEX) revealed that CsrA recognizes RNA containing an almost invariant GGA sequence that is frequently located in the loop of RNA hairpins (Dubey *et al.*, 2005). The RNA secondary structure, often provided by conserved flanking nucleotides, is beneficial but not essential for high affinity binding. Stuctural studies of CsrA homologs demonstrate that CsrA forms a

symmetrical homodimer composed of interdigitated β-strands and represents a novel class of RNA-binding proteins (Gutierrez *et al.*, 2005; Rife *et al.*, 2005; Heeb *et al.*, 2006). Scanning alanine mutagenesis revealed that the amino acids critical for CsrA function are located within two β-strands that lie parallel to each other on either side of the homodimer, indicating that each CsrA dimer contains two identical RNA binding surfaces on opposite sides of the protein (Mercante *et al.*, 2006). Two residues within the RNA binding pocket, R44 and V24, come directly into contact with the critical GG motif of the target RNA (Schubert *et al.*, 2007).

Characterized CsrA mRNA targets contain from one (e.g. *hfq*) up to six (e.g. *pga*) binding sites (Wang *et al*., 2005; Baker *et al.*, 2007). In the mechanism of CsrA-mediated negative regulation, CsrA binds with high affinity and specificity to the 5' untranslated region of transcripts at or near the Shine-Dalgarno (SD) sequence and competes for binding of the 30S ribosomal subunit (Baker *et al.*, 2002). In most cases, CsrA binding leads to rapid degradation of target transcripts either by passively affecting mRNA turnover due to the inhibition of translation or by directly increasing ribonucleolytic cleavage and turnover due to the presence of bound CsrA (Liu and Romeo, 1997). In transcripts that contain multiple binding sites, it is likely that CsrA first binds to the higher affinity binding site which then increases the local concentration to facilitate CsrA binding to the SD sequence or other sites (Mercante *et al.*, 2009). These studies also indicated that CsrA can simultaneously bind at two target binding sites within a single transcript, allowing for bridging between these two sites, and demonstrated that this feature is needed for full regulation in vitro (Mercante *et al*., 2009). This suggests that CsrA dimers might tether non-adjacent binding sites within the

CsrA-CsrB complex, allowing for the globular form observed and the 1:2 ratio of CsrA dimers to binding sites within CsrB (Liu *et al*., 1997).

Historically, the mechanism of positive regulation by CsrA has been less well understood than repression. Previous observations determined that CsrA binding stabilizes *flhDC* mRNA, leading to increased expression; however, this mechanism was not elucidated in detail (Wei *et al*., 2001). Recent data demonstrate that CsrA activates *flhDC* expression by protecting the transcript from RNase E-mediated endonucleolytic cleavage (Baker *et al.*, 2010). RNase E preferentially interacts with RNA containing a 5'-monophosphate end before it initiates endonucleolytic cleavage (Mackie, 1998). CsrA binds at two sites within the 5' UTR of *flhDC*, one of which is located at the extreme 5' end, suggesting that CsrA inhibits the initial RNase E interaction with target transcripts (Baker *et al*., 2010).

Finally, recent results reveal the ability of CsrA to interact with the intergenic region within a transcript (Yang *et al*., 2010; A. Pannuri, A. N. Edwards, and T. Romeo, unpublished data), indicating a novel mechanism for CsrA regulation. CsrA binds to and represses the expression of *cel*, the third gene in the Colicin E7 operon of *E. coli*, which encodes the lysis gene. This study also reported the first account of a role for CsrA in regulating expression from an extrachromosomal element (Yang *et al*., 2010). Additionally, CsrA binds to the intergenic region of the *nhaA-nhaR* transcript with high affinity and specificity but does not bind to the 5' UTR of *nhaA*. Thus, CsrA represses expression of *nhaR* while having little effect on expression of *nhaA* (A. Pannuri, A. N. Edwards and T. Romeo, unpublished data). These studies suggest that the mechanistic

role of CsrA is still not fully explored and that there may be other ways in which CsrA interacts with RNA to mediate its regulatory effects.

Other components of the Csr regulatory system

CsrB was originally identified as RNA that copurified with histidine-tagged CsrA (Liu *et al*., 1997) while CsrC was discovered during a genetic screen for additional factors that affect glycogen synthesis (Weilbacher *et al*., 2003). Both of these noncoding small RNAs contain multiple GGA motifs: CsrB (369 nt) contains approximately 18-22 putative binding sites while CsrC (245 nt) is predicted to have approximately nine. Together, CsrB and CsrC antagonize CsrA activity by binding multiple CsrA dimers and reducing the availability of CsrA to interact with target mRNAs. The effects of CsrB and CsrC are CsrA-dependent, although each might be expressed differently under certain conditions, allowing for complex regulation of CsrA activity (Weilbacher *et al*., 2003). CsrB and CsrC have relatively short half-lives (≈2 minutes), which permits their levels to be rapidly altered in response to changes in their synthesis (Gudapaty *et al.*, 2001; Weilbacher *et al*., 2003).

CsrB and CsrC transcription is activated indirectly by CsrA through the BarA-UvrY two-component signal transduction system (Suzuki *et al*., 2002). BarA is a tripartite sensor kinase and initiates a His-Asp-His phosphorelay in response to accumulation of acetate, the physiological stimulus for BarA activity (Chavez *et al.*, 2010). BarA phosphorylates its cognate response regulator, UvrY, which, in turn, activates transcription of *csrB* and *csrC* (Pernestig *et al.*, 2001; Suzuki *et al*., 2002). BarA-UvrY is important for growth in low pH conditions and for switching between

glycolytic and gluconeogenic carbon sources (Pernestig *et al.*, 2003; Mondragon *et al.*, 2006). *csrB* and *csrC* expression is also upregulated in minimal media in a CsrA-, BarA- and UvrY-dependent manner, and the addition of tryptone, casamino acids or amino acids repressed mRNA levels of *csrB*, although the exact mechanism of the response was unidentified (Jonas and Melefors, 2009). Additionally, BarA-UvrY homologs in *Bordetella bronchiseptica* and *Legionella pneumophila* appear to act as rheostat-like switches rather than on/off switches, allowing for several phenotypic states depending upon the ratio of phosphorylated to unphosphorylated UvrY and the affinity of each UvrY-regulated promoter (Cotter and Miller, 1997; Edwards *et al.*, 2010). In *L. pneumophila*, modulation of the phosphorylation state of LetS, the UvrY homolog, results in delayed expression of both *rsmY* and *rsmZ* (CsrB/CsrC homologs) in the postexponential phase (Edwards *et al*., 2010). While more than two phenotypic states in regards to UvrY-dependent promoters have not been identified in *E. coli*, it is tempting to speculate that this rheostat-like behavior could play a role in regulating CsrB and CsrC levels and, ultimately, CsrA activity.

CsrC transcription appears to be less dependent upon UvrY compared to CsrB (Suzuki *et al*., 2002; Weilbacher *et al*., 2003). Interestingly, deletion of one sRNA results in a compensatory effect, increasing the levels of the other (Weilbacher *et al*., 2003), revealing an important feedback loop within the Csr circuit.

Finally, CsrD, a degenerate GGDEF-EAL domain protein, regulates the turnover of CsrB and CsrC (Suzuki *et al*., 2006; Jonas *et al.*, 2006). Both *csrB* and *csrC* transcripts were significantly stabilized in a *csrD* mutant, and CsrD bound to these sRNAs in vitro with high affinity but no specificity (Suzuki *et al*., 2006). It appears that

CsrD is required for targeting CsrB and CsrC degradation by RNase E (Suzuki *et al*., 2006). CsrD was the first example of a GGDEF-EAL domain protein that is not involved in metabolism of c-di-GMP (Suzuki *et al*., 2006).

The Csr circuitry is characterized by negative feedback loops that regulate CsrA activity. In one feedback loop, CsrA represses expression of *csrD* (Suzuki *et al*., 2006), reducing turnover of CsrB and CsrC. In another, CsrA activates glycolysis (Sabnis *et al*., 1995), a major source of acetate. Acetate, the physiological sensor for BarA activity (Chavez *et al*., 2010), upregulates *csrB* and *csrC* expression. Recent data reveal that CsrA negatively regulates its own expression by directly binding to its mRNA leader (H. Yakhnin and P. Babitzke, unpublished data). These negative feedback loops provide a mechanism of fine-tuning CsrA expression and activity.

CsrA activity is regulated by the small RNAs, CsrB and CsrC, and is subject to transcriptional regulation as well. PmrA, the response regulator of the PmrAB two-component system, activates *csrA* expression, likely directly influencing transcription since a putative PmrA-binding site was identified in the *csrA* regulatory region (Rasis and Segal, 2009; P. Babitzke, personal communication). CsrA indirectly regulates its own transcription by inducing RpoS expression; transcription of *csrA* occurs from at least three promoters, one of which is σ^S-dependent (H. Yakhnin and P. Babitzke, unpublished data). These results suggest that CsrA and RpoS are two global regulatory proteins that participate in a positive regulatory circuit. Altogether, many regulatory inputs on multiple levels provide tightly controlled expression and activity of CsrA, highlighting the importance of precisely controlling CsrA activity in the cell.

CsrA regulated genes in E. coli *K-12*

CsrA was originally discovered as a regulator of the *glgCAP* operon by screening for transposon mutants that accumulated high levels of glycogen (Romeo *et al*., 1993). CsrA directly represses expression of the three enzymes that are essential for glycogen biosynthesis, ADP-glucose pyrophosphorylase, glycogen synthase and glycogen branching enzyme, encoded by *glgC*, *glgA* and *glgB* (Liu *et al*., 1995; Yang *et al.*, 1996). In addition, CsrA negatively regulates *glgP*, glycogen phosphorylase, and *glgS*, whose gene product promotes glycogen synthesis (Yang *et al*., 1996). It has been further elucidated that CsrA plays an extensive role in regulating intracellular carbon flux by activating expression of glucose-6-phosphate isomerase, triose-phosphate isomerase and enolase, which are all glycolytic enzymes, along with repressing fructose-1,6-bisphosphatase, PEP carboxykinase and phosphoenolpyruvate synthetase, which are gluconeogenic enzymes (Sabnis *et al*., 1995). Additionally, CsrA upregulates expression of *acs*, which encodes acetyl-coenzyme A synthetase, along with increasing the activity of the glyoxylate shunt enzyme, isocitrate lyase (Wei *et al*., 2000). CsrA also has the potential to regulate transportation and utilization of amino acids through repression of *cstA* (Dubey *et al*., 2003). These studies have revealed an important role that CsrA has in carbon flux and nutrient acquisition.

CsrA inversely controls motility and biofilm formation within *E. coli*. As mentioned above, CsrA activates motility by positively regulating the expression of *flhDC* (Wei *et al*., 2001), which encodes for the two major subunits of the master regulator that induces the flagellar transcriptional cascade (Liu and Matsumura, 1994). In contrast, CsrA directly represses expression of *pgaABCD*, which encodes for the gene

products necessary to produce and secrete poly-β-1,6-N-acetyl-D-glucosamine (PGA), inhibiting biofilm formation (Wang *et al*., 2005; Itoh *et al.*, 2008). There is evidence that CsrA negatively regulates biofilm production on multiple levels (A. Pannuri, A. N. Edwards and T. Romeo, unpublished data). CsrA directly represses expression of *nhaR*, whose gene product is an important transcriptional activator for *pgaABCD* expression (Goller *et al.*, 2006), and directly binds to the 5'UTR of *glmS*, which encodes the protein that catalyzes a rate-limiting step in production of UDP-GlcNAc, the precursor required for PGA biosynthesis. Additionally, CsrA represses expression of *ycdT* and *ydeH*, two GGDEF domain-containing proteins that synthesize c-di-GMP (Chapter 3). c-di-GMP has positive effects on biofilm production (see below) and has been shown to activate *pgaABCD* expression (Tagliabue *et al.*, 2010; M. Kumar, C. C. Goller and T. Romeo, unpublished data) and affect the level of PgaD protein (Boehm *et al.*, 2009; M. Kumar, C. C. Goller and T. Romeo, unpublished data).

While CsrA often influences biofilm and motility in other species, the effects are sometimes oppositely regulated by CsrA. In the Gram-positive *B. subtilis*, CsrA directly represses the expression of *hag*, encoding flagellin, employing a very similar mechanism of negative regulation as described above (Yakhnin *et al.*, 2007), while in *Campylobacter jejuni*, an ε-proteobacteria, CsrA activates biofilm formation and represses motility, although the molecular mechanisms for these phenotypic effects are not known (Fields and Thompson, 2008). In addition, RsmA represses swarming in *Proteus mirabilis* (Liaw *et al*., 2003).

Interestingly, the *csrA* mutant allele (*csrA*::*kan*) that is frequently used for in vivo studies in *E. coli* still possesses some activity since the transposon insertion only

eliminated the last 11 amino acids of the original C-terminus, creating a 62 amino acid fusion protein (Romeo *et al*., 1993; Timmermans and Van Melderen, 2009). In vitro data confirmed that the purified CsrA::Kan fusion protein still retains some RNA binding activity (C. Baker and P. Babitzke, unpublished data). These studies suggest that the regulatory role of CsrA may have been underestimated since the mutant allele utilized in many strains retains residual activity. A recent study indicates that the essentiality of a full *csrA* knockout grown on LB medium can be alleviated by the addition of pyruvate to the media, which enters the carbon cycle after glycolysis, or by eliminating carbon flux to glycogen biosynthesis by deleting the *glgCAP* operon, which encodes proteins involved in the primary steps for glycogen biosynthesis (Timmermans and Van Melderen, 2009). Here, the carbon flux either bypasses the glycolytic pathway or allows carbon to be redirected to glycolysis.

Despite the apparent global role of CsrA in both metabolism and physiology, little is known about the overall global influence of CsrA on gene expression. Two studies of global regulation by CsrA gave some insight through microarray analysis in *Salmonella enteric* serovar Typhimurium (Lawhon *et al.*, 2003) and *Pseudomonas aeruginosa* (Burrowes *et al.*, 2006). However, these types of studies do not exclude indirect effects of CsrA, probe for intergenic regions, nor account for altered metabolism in the *csrA* mutant. In addition, there is evidence that CsrA can inhibit translation without affecting transcript stability (Baker *et al*., 2007), and these cases would be overlooked in array studies. A recent study used an approach similar to ours (described in Chapter 2) in which RNA that copurified with histidine-tagged RsmA was enriched, converted into cDNA, cloned, and subsequently sequenced to determine the identities of RNAs that

interact with RsmA in vivo (Brencic and Lory, 2009). These types of studies have elucidated many new putative targets of CsrA/RsmA and have also uncovered new potential mechanisms of CsrA regulation (A. Pannuri, A. N. Edwards and T. Romeo, unpublished data; L. Patterson-Fortin and T. Romeo, unpublished data).

The Csr/Rsm system in other organisms

The Csr circuit has been most carefully studied in *E. coli*; however, orthologous Csr systems and components are widely distributed in eubacteria, and some species appear to encode more than one CsrA homolog (Mercante *et al*., 2006). While CsrA and the other components in this circuitry exist in many organisms, the influence that the Csr system has on physiological functions varies considerably, and there are notable differences from *E. coli* in several of the systems described below.

The Csr system of *Salmonella enterica* serovar Typhimurium is very similar to the *E. coli* system. The CsrA proteins of these species exhibit the same deduced amino acid sequence, and the two antagonizing sRNAs, CsrB and CsrC, are activated by the BarA-SirA TCS (BarA-UvrY in *E. coli*) (Teplitski *et al.*, 2003; Teplitski *et al.*, 2006; Fortune *et al.*, 2006). The Csr system plays a role in virulence through SirA activation of two key regulators of invasion, *hilA* and *hilC* (Teplitski *et al*., 2003), along with regulating expression of the *Salmonella* pathogenicity island 1 (SPI1) (Altier *et al.*, 2000; Lawhon *et al*., 2003). Similar to *E. coli*, a *csrA* mutant is amotile; however, microarray analysis suggests that CsrA could regulate approximately 8% of the genome, including some metabolic pathways not present in *E. coli* (Lawhon *et al*., 2003). As evidenced by phenotypic observations in other organisms, it is apparent that CsrA levels must be

tightly controlled as both deletion and overexpression of *csrA* lead to a defect in invasion by *Salmonella* (Altier *et al*., 2000).

RsmA (CsrA) and the GacS-GacA (BarA-UvrY) TCS have been studied in several species of *Pseudomonads*. Originally, the GacS-GacA system was discovered to control pathogenicity of the plant pathogen, *Pseudomonas syringae* pv. *syringae* (Hrabak and Willis, 1992; Rich *et al.*, 1994). However, more extensive studies with this system have been done in *P. fluorescens*, a plant biocontrol species important for protection against phytopathogenic fungi, and in *P. aeruginosa*, an opportunistic pathogen that frequently infects the lungs of immunocompromised and cystic fibrosis patients. The Rsm system in several *Pseudomonas* species represses hydrogen cyanide and extracellular protease production (Blumer *et al.*, 1999; Pessi *et al.*, 2001; Heeb *et al.*, 2002). In addition, the GacS-GacA TCS system positively regulates synthesis of *N*-acyl-homoserine lactones (AHL) (Reimmann *et al.*, 1997; Chancey *et al.*, 1999; Kay *et al.*, 2006), a quorum-sensing signal, while RsmA has negative effects on AHL production (Pessi *et al*., 2001; Burrowes *et al.*, 2005). In *P. fluorescens*, three RsmA/E-regulating sRNAs activate the synthesis of an unknown quorum-sensing signal, which is unrelated to AHLs (Kay *et al.*, 2005).

Studies in *P. fluorescens* have demonstrated the presence of two CsrA homologs, RsmA (Blumer *et al.*, 1999) and RsmE (Haas *et al.*, 2002; Reimmann *et al.*, 2005), which are negatively regulated by three redundant sRNAs, RsmX, RsmY and RsmZ (Heeb *et al.*, 2002; Valverde *et al.*, 2003; Kay *et al.*, 2005). RsmA and RsmE indirectly activate transcription of the three sRNAs through the response regulator GacA, creating an autoregulatory loop similar to *E. coli*. Interestingly, all three sRNAs do not show the

same expression patterns; *rsmX* and *rsmY* gradually increase during growth while *rsmZ* expression is delayed (Kay *et al*., 2005). Recent studies found that PsrA, a transcriptional regulator that activates *rpoS* expression and represses fatty acid degradation (Kang *et al.*, 2009), activates expression of *rsmZ* (Humair *et al.*, 2010), revealing an additional regulatory component within the Rsm system. In contrast to *E. coli*, RsmA represses expression of *rpoS*, although it is unknown if this regulation is direct or indirect (Heeb *et al.*, 2005).

P. aeruginosa strains possess a CsrA homolog (RsmA) (Pessi *et al*., 2001), the GacS-GacA TCS (Kay *et al*., 2006) and two sRNAs, RsmY (Valverde *et al*., 2003; Burrowes *et al*., 2005) and RsmZ (Heurlier *et al.*, 2004). Recently, additional regulators have been discovered. The sensor kinase RetS indirectly controls sRNA expression by modulating the phosphorylation state of GacS (Goodman *et al.*, 2004; Goodman *et al.*, 2009). LadS is another sensor kinase within this signaling network and functions in a manner opposite of RetS (Ventre *et al.*, 2006). Hfq has been reported to directly bind two regions of RsmY and stabilize the sRNA by protecting the transcript from endonucleolytic cleavage by RNase E (Sonnleitner *et al.*, 2006; Sorger-Domenigg *et al.*, 2007).

In *Vibrio cholerae*, the Csr system acts within the quorum sensing network and indirectly regulates the master regulator of quorum sensing, HapR (Lenz *et al.*, 2005). The VarS-VarA (BarA-UvrY) TCS is activated in a growth-dependent manner and induces expression of the three sRNAs, *csrB, csrC* and *csrD* at high cell density. The sRNA-mediated inhibition of CsrA indirectly reduces LuxO activity, reducing the expression of four sRNAs, *qrr1-4*, which work with Hfq in an antisense mechanism to

destabilize the *hapR* transcript. Thus, in low cell density, CsrA indirectly coordinates repression of HapR; in high cell density, the upregulation of the CsrA-regulating sRNAs indirectly stabilizes the *hapR* transcript, allowing for quorum-sensing-mediated regulation to occur. In support of this model, CsrA affects the expression of two quorum-sensing-regulated virulence genes, *vpsT* and *vspL* (Lenz *et al*., 2005), while mutations in the VarS-VarA-CsrA/CsrBCD pathway affect another HapR-regulated gene, *hapA* (Lenz *et al*., 2005; Jang *et al.*, 2010). While these two studies suggest that the Csr system plays an integral role in controlling quorum sensing, further studies are needed to clarify the precise interactions between the components in *V. cholerae* Csr system.

Legionella pneumophila, an intracellular pathogen, depends upon the Csr system to coordinate differentiation between replicative and transmissive phases (Molofsky and Swanson, 2003). Preliminary data suggest that CsrA-mediated regulation depends upon ppGpp accumulation. During the intracellular replication phase, induction of the stringent response occurs after nutrient exhaustion (Hammer *et al.*, 2002), and subsequent ppGpp synthesis activates the LetS-LetA (BarA-UvrY homologs) TCS system in an unknown manner (Hammer *et al.*, 2002). LetA then directly induces transcription of *rsmY* and *rsmZ* (*csrB* and *csrC*) (Rasis and Segal, 2009; Sahr *et al*., 2009), which reduces free CsrA and relieves repression of the transmission phase traits (Molofsky and Swanson, 2003). In addition, two studies indicate that RpoS induces expression of *rsmY* and *rsmZ* (Hovel-Miner *et al*., 2009; Sahr *et al*., 2009), providing another possible link between stress and induction of sRNA expression. However, more studies are needed to definitively establish this regulatory cascade. Interestingly, all sequenced strains of *L.*

pneumophila possess at least three *csrA* genes, perhaps highlighting the critical importance of CsrA in regulating *Legionella* life cycle.

The RsmA-GacS-GacA (CsrA-BarA-UvrY) system plays a crucial role in regulating synthesis of extracellular enzymes and polysaccharides, quorum sensing, secondary metabolite production, plant-microbe interactions and motility in the plant pathogen, *Erwinia caratovora* (Chatterjee *et al.*, 1995; Cui *et al.*, 1995; Cui *et al.*, 2001; Chatterjee *et al.*, 2010). This organism apparently possesses only a single sRNA, RsmB (Liu *et al.*, 1998). This system in *E. caratovora* is characterized by additional multiple regulators. RsmC is a transcriptional adaptor that inhibits FlhDC-dependent activation of *gacA* expression, thus reducing *rsmB* expression (Cui *et al.*, 1999; Cui *et al.*, 2008; Chatterjee *et al.*, 2009). KdgR also negatively regulates *rsmB* by binding within the transcribed region, perhaps by blocking efficient transcription (Liu *et al.*, 1999). HexA, a homolog to LhrA in *E. coli*, represses both *rsmB* and *rpoS* expression (Mukherjee *et al.*, 2000). Similarly to *E. coli*, RpoS activates expression of *rsmA* (Mukherjee *et al.*, 1998). Finally, both the Rsm and the quorum sensing network are interconnected (Koiv and Mae, 2001). Two receptors of AHL, ExpR1 and ExpR2 (homologous to LuxR in *E. coli*), directly activate *rsmA* transcription at low cell density, and this interaction is abolished in the presence of AHLs (Cui *et al.*, 2005; Chatterjee *et al.*, 2005; Cui *et al.*, 2006; Sjoblom *et al.*, 2006).

At present it is not clear that all bacterial species that possess homologs of CsrA contain homologs of the sRNAs, CsrB and CsrC, and/or the BarA-UvrY TCS. Homologs of *csrB* and *csrC* are notoriously difficult to identify since they are not easily recognized by homology searches (Weilbacher *et al*., 2003). A bioinformatics study identified all

previously discovered CsrA-regulating sRNAs and predicted a number of similar sRNAs in a variety of bacteria (Kulkarni *et al.*, 2006). This study suggested that the use of sRNAs in regulating CsrA activity is somewhat conserved; however, CsrA-regulating sRNAs were not identified outside of γ-proteobacteria. In addition, BarA-UvrY homologs have only been described in γ-proteobacteria. It is unknown whether CsrA is regulated similarly in other bacteria or whether the Csr circuitry varies qualitatively in other bacterial species.

Stringent Response

The stringent response is characterized by a downshift in transcription of stable RNAs (i.e. rRNA and tRNA) and by the upregulation of transcription of a number of operons, such as those that encode proteins that mediate amino acid biosynthesis and transport, all in response to nutritional starvation. This adaptive mechanism, first described in 1961 (Stent and Brenner, 1961), couples inhibition of translation with a reduction in transcription, and leads to global changes within the transcriptome of starved cells. The effector of this response is guanosine tetraphosphate (ppGpp) or guanosine pentaphosphate (pppGpp) (collectively referred to here as ppGpp), a nucleotide alarmone that is common in eubacteria and plants (Mittenhuber, 2001). ppGpp was discovered over forty years ago as derivatives of GTP and GDP that appeared on two-dimensional thin layer chromatography from extracts of *E. coli* undergoing amino acid starvation and were termed then as “magic spots” (Cashel and Gallant, 1969). ppGpp is produced not only in response to amino acid starvation, but from other stresses and nutritional limitations including carbon (Hernandez and Bremer, 1991; Murray and Bremer, 1996;

Cashel *et al*., 1996), iron (Vinella *et al.*, 2005) and fatty acid starvation (Seyfzadeh *et al.*, 1993; Gong *et al.*, 2002).

Metabolism and regulation of ppGpp

Levels of ppGpp are regulated by two classes of enzymes: monofunctional synthetase-only enzymes (RelA) and bifunctional synthetase/hydrolase enzymes (SpoT or RSH [RelA/SpoT homologue]). With the exception of *Neisseria* and *Bordetella* species, which belong to the β-proteobacteria subdivision, only *E. coli* and other γ-proteobacteria species possess the paralogues, RelA and SpoT (Mittenhuber, 2001). These enzymes synthesize ppGpp from GTP or GDP and use ATP to catalyze the addition of a pyrophosphate onto the ribose 3'-OH (Cochran and Byrne, 1974; Hernandez and Bremer, 1991; Xiao *et al.*, 1991), although SpoT exhibits weak synthetase activity and serves primarily as a manganese-dependent phosphohydrolase, degrading pppGpp or ppGpp to GTP or GDP and pyrophosphate (PP_i) (Heinemeyer and Richter, 1977; Sy, 1977; An *et al.*, 1979; Murray and Bremer, 1996, Cashel *et al*., 1996). pppGpp can subsequently be converted to ppGpp by guanosine pentaphosphate hydrolase, GPP (Keasling *et al.*, 1993). Although the amino acid sequences of RelA, SpoT and RSH proteins are interrelated (Metzger *et al.*, 1989), RelA contains only synthetase activity because of the lack of the conserved histidine-aspartate (HD) domain required for the phosphohydrolase activity found in SpoT and RSH proteins (Aravind and Koonin, 1998). The synthetase and hydrolase domains overlap within the N-terminal portion of SpoT and RSH proteins (Gentry and Cashel, 1996; Avarbock *et al.*, 2005). In vivo and biochemical analyses, along with recent X-ray crystallography of the N-terminal fragment of Rel_{Seq} (RSH in

Streptococcus dysgalactiae subsp. *equisimilis*), have revealed reciprocal regulation of the two antagonistic activities (hydrolase-OFF/synthetase-ON and hydrolase-ON/synthetase-OFF) which prevents futile cycling of ppGpp synthesis and hydrolysis (Mechold *et al.*, 2002; Hogg *et al.*, 2004).

Unlike γ-proteobacteria and the few exceptions, most other organisms encode a single bifunctional RSH protein, often known as Rel or RelA (Mittenhuber, 2001). *Streptococcus mutants* (Lemos *et al.*, 2007), *Enterococcus faecilis* (Abranches *et al.*, 2009), *Bacillus subtilis* (Nanamiya *et al.*, 2008) and *Vibrio cholerae* (Das *et al.*, 2009), along with other predicted organisms, encode one or more monofunctional, RelA-like synthetase fragments, known as small alarmone synthetases (SASs), in addition to a single RSH protein. These proteins (termed RelP, RelQ, and RelV) lack both the hydrolase and the regulatory C-terminal domains but appear to be primarily responsible for basal level production of ppGpp in some organisms (Lemos *et al.*, 2007). SASs are hypothesized to function in ppGpp synthesis in a mechanism unique from RelA and SpoT, perhaps by sensing extracellular inputs. Unlike in *E. coli*, which encodes no SASs, accumulation of ppGpp in *V. cholerae* occurs in a *relA spoT* double mutant in response to glucose and fatty acid starvation in a RelV-dependent manner (Das *et al*., 2009), demonstrating that these closely related bacteria have evolved different modes of regulation to respond to similar nutritional stresses. Importantly, in some organisms, but not all, that encode SASs, deletion of the bifunctional enzyme (RSH) is not lethal (Wendrich and Marahiel, 1997; Lemos *et al.*, 2004); this is in contrast to *spoT* mutants in *E. coli*, *V. cholerae* and other organisms that contain both RelA and SpoT (Xiao *et al*., 1991; Das and Bhadra, 2008). The hydrolase activity of SpoT is essential in *relA*$^{+}$ strains

probably because accumulation of RelA-derived ppGpp inhibits growth (Xiao *et al*., 1991). This suggests that ppGpp synthesis by SASs is tightly governed in a variety of mechanisms to prevent overproduction of ppGpp and subsequent growth arrest.

Enzyme activities for RelA, SpoT, and RSH proteins are modulated to allow coordination of global transcription patterns in response to distinct nutrient stimuli, and it seems that the N-terminal and C-terminal domains of these proteins contribute to regulation of their activity in a variety of mechanisms. RelA is associated with the ribosome and synthesizes ppGpp in response to accumulation of uncharged (deacylated) tRNA in the ribosomal acceptor site (A-site) during amino acid starvation (Haseltine and Block, 1973; Ramagopal and Davis, 1974). RelA activation is dependent upon the proline-rich 36-amino acid N-terminal segment of L11, a 50S ribosomal protein (Friesen *et al.*, 1974; Yang and Ishiguro, 2001; Wendrich *et al.*, 2002). A proposed model for the mechanism of RelA-mediated ppGpp synthesis involves RelA detection of and binding to blocked ribosomes containing a 3' extension of mRNA, and the subsequent stimulation of RelA activity, which requires ribosomal protein L11 (Wendrich *et al*., 2002). Upon ppGpp synthesis, RelA, but not the deacylated tRNA, is released from the ribosome, and data suggest that RelA moves from blocked ribosome to blocked ribosome which would correlate the amount of ppGpp synthesized to the number of blocked ribosomes within the cell (Wendrich *et al*., 2002). Charged tRNAs have a much higher affinity for the acceptor site of the ribosome (Schilling-Bartetzko *et al.*, 1992) and, in post-starvation conditions, these charged tRNAs can easily replace the deacylated tRNAs, rescuing blocked ribosomes. Studies have indicated that bifunctional RSH proteins respond to amino acid starvation (Mechold *et al.*, 1996; Wendrich and Marahiel, 1997) and are

activated in a similar ribosome-dependent mechanism (Martinez-Costa *et al.*, 1998; Mechold *et al*., 2002) with hydrolase activity decreasing significantly in the presence of uncharged tRNAs (Avarbock *et al.*, 2000).

The activities of RelA and bifunctional proteins are controlled through the C-terminal domains (CTD) of these proteins. CTD point mutations and deletions abolish activation of RelA in *E. coli* and the RSH homolog (Rel_{Mtb}) in *Mycobacterium tuberculosis* (Gropp *et al.*, 2001; Avarbock *et al.*, 2005). These studies have also demonstrated that the CTD is required for oligomerization of RelA and Rel_{Mtb}. This suggests a potential regulatory role for the CTD in relaying the activation signal from the ribosome to the N-terminal catalytic domain and that this signal transfer possibly involves oligomerization. Indeed, it has been proposed that the CTD could act as a regulator of ppGpp synthesis and may be controlled by ligand or protein binding (Mechold *et al*., 2002).

SpoT has been implicated in responding to a variety of nutritional stresses, including fatty acid and carbon starvation. Several reports have demonstrated that the ppGpp hydrolase activity of SpoT cofractionates with the ribosome and is inhibited by uncharged tRNA (Heinemeyer and Richter, 1977; Sy, 1977; Richter, 1980) while a recent study found that SpoT copurifies with the 50S ribosomal subunit (Jiang *et al.*, 2007). In *E. coli*, a specific, physical interaction between SpoT and the acyl carrier protein (ACP), the central cofactor in fatty acid and lipid metabolism, has been characterized and implicated as a signal for SpoT-dependent ppGpp synthesis (Battesti and Bouveret, 2006). ACP interacts with the TGS (for "ThrRS, GTPase, and SpoT") domain within the CTD domain of SpoT, and this binding may be influenced by the ratio of unacylated to

acylated ACP. However, the TGS domain does not determine the specificity of the interaction as this domain is highly conserved between SpoT and RelA. The N-terminal domain of RelA prevents interaction with ACP. The ACP and SpoT interaction was subsequently shown to be restricted to bacteria containing both RelA and SpoT and to ACPs encoded by genes located in fatty acid synthesis operons (Battesti and Bouveret, 2009), highlighting the diversity of ppGpp regulation and mechanisms in bacteria. The SpoT-ACP interaction was also shown to be necessary for *Legionella pneumophila* to respond to perturbations in fatty acid biosynthesis as a single amino acid substitution that is required for ACP interaction in SpoT (Battesti and Bouveret, 2006) eliminated SpoT-dependent ppGpp synthesis (Dalebroux *et al.*, 2009). A working model proposes that ACP relays the lipid metabolic state of the cell to SpoT and regulates the switch between SpoT-dependent ppGpp hydrolysis and synthesis activity.

The essential Obg (*spo0B*-associated GTP-binding protein) GTP-binding protein family (also known as CgtA (conserved GTPase) or YhbZ but will be referred to here as Obg for clarity) has recently been suggested to play a role in stringent response. Obg is important in sporulation in *Bacillus subtilis* (Kok *et al.*, 1994; Vidwans *et al.*, 1995) and morphological development in *Streptomyces coelicolor* (Okamoto and Ochi, 1998) and has been implicated in responding to DNA replication fork arrest in *E. coli* (Foti *et al*., 2005). Obg proteins typically have high guanosine nucleotide exchange rates but relatively low GTP hydrolysis rates (Welsh *et al.*, 1994; Lin *et al.*, 1999). Several working models propose that rather than serve as GTPases, the nucleotide bound state of Obg proteins reflects the GDP:GTP ratio within the cell and ultimately affects general stress response and stationary phase survival (Okamoto and Ochi, 1998; Lin *et al.*, 1999;

Wout *et al.*, 2004). Interestingly, numerous studies have revealed that Obg cofractionates with the 50S ribosomal subunit and is required for ribosome assembly, stability and maturation (Scott *et al.*, 2000; Lin *et al.*, 2004; Wout *et al*., 2004; Sato *et al.*, 2005; Sikora *et al.*, 2006), but this function may not be the cause for essentiality of Obg since ribosome assembly is not further impaired in a temperature sensitive allele of *Caulobacter crescentus* at the non-permissive temperature (Datta *et al.*, 2004). Most recently, Obg has been speculated to play a role in stringent response. Surprisingly, the crystal structure of Obg from *B. subtilis* was found to contain ppGpp (Buglino *et al.*, 2002). Obg in *E. coli* was shown to bind ppGpp in vitro with a similar affinity as GDP and alter the ratio of pppGpp to ppGpp within the cell during stringent response (Persky *et al.*, 2009). In *E. coli* and *V. cholerae*, Obg directly interacts with SpoT (Wout *et al*., 2004; Raskin *et al.*, 2007) and affects ppGpp levels during exponential growth (Raskin *et al*., 2007; Jiang *et al*., 2007), suggesting that Obg promotes the ppGpp hydrolase activity of SpoT to maintain low ppGpp levels in normal growth conditions. However, Obg remains essential for cell growth in mutants that cannot synthesize ppGpp (Jiang *et al*., 2007; Shah *et al.*, 2008) which has led to the speculation that Obg has a role on the output of the stringent response that is possibly ppGpp-independent (Persky *et al*., 2009). Studies in *B. subtilis* have revealed interactions between Obg and several regulators necessary for activation of σ^B, the general stress sigma factor (Scott and Haldenwang, 1999) and have also determined that Obg and these σ^B regulators cofractionate with ribosomes (Scott *et al*., 2000). The fact that SpoT in *E. coli* and the σ^B regulators in *B. subtilis* are ribosome associated suggests that the role of Obg proteins in stress response is coupled to their ribosome association. Finally, two different mutant alleles of Obg in

B. subtilis were discovered to exhibit separate effects for growth-promotion and for general stress response (Kuo *et al.*, 2008), clarifying that other phenotypes attributed to Obg deficiencies are not indirect consequences of the underlying growth defects but are rather direct regulation by Obg. Altogether, these data indicate a complex model in which Obg activity is possibly regulated by guanosine nucleotide interaction and subsequently plays a role in regulating ppGpp metabolism, stress response and ribosome biogenesis.

DksA, a cofactor for ppGpp regulation

An additional mediator of stringent response, DksA (DnaK suppressor A), was first discovered as a multicopy suppressor of the temperature sensitive growth and filamentous phenotypes of a *dnaK* (encoding Hsp70) mutant (Kang and Craig, 1990). Since then, DksA has been associated with many pleiotropic effects within the cell, producing profound changes in amino acid biosynthesis (Kang and Craig, 1990), cell division (Yamanaka *et al.*, 1994; Ishii *et al.*, 2000), quorum sensing (Branny *et al.*, 2001) and virulence (Webb *et al.*, 1999; Mogull *et al.*, 2001). Eventually, DksA was demonstrated to affect ppGpp-mediated regulation of RpoS (Brown *et al.*, 2002). Further studies revealed that DksA is necessary for the stringent response and augments ppGpp regulation for both the inhibition of rRNA transcription (Paul *et al.*, 2004a) and the activation of amino acid biosynthetic promoters (Paul *et al.*, 2005). The discovery of a ppGpp cofactor resolved the long-standing discrepancy in the field where the extremely strong effects of ppGpp on transcription of target promoters in vivo were not reproducible in vitro.

DksA binds within the secondary channel of RNA polymerase (Perederina *et al.*, 2004; Rutherford *et al.*, 2007) which is both the entry point for nucleotide triphosphate precursors (NTPs) and the exit point for backtracked RNA. X-ray crystallography of DksA revealed a coiled-coil motif with two highly conserved, aspartic acids (Asp) at the finger tip (Vassylyeva *et al.*, 2004; Perederina *et al.*, 2004) that are required for DksA function (Perederina *et al.*, 2004). The DksA structure is related to that of GreA and GreB, although they share no sequence homology (Perederina *et al.*, 2004). GreA and GreB are transcription elongation factors that rescue arrested RNA polymerases by cleaving backtracked RNA and restoring a transcription complex that is able to continue RNA synthesis (Hsu *et al.*, 1995). The Gre factors contain two conserved acidic residues, similar to DksA, at the finger tip, and these coordinate a Mg^{2+} ion required for hydrolysis of the backtracked RNA (Laptenko *et al.*, 2003); however, DksA does not possess enzymatic activity. While DksA and the Gre factors display independent and even antagonistic functions in vivo (Potrykus *et al.*, 2006), there have been indications that there are common effects of protein binding within the secondary channel because GreB can replace DksA for stringent regulation of rRNA promoters in vitro; however, the low concentration of GreB prevents any measureable effects in vivo, and GreB is unable to rescue activation of transcription nor rescue the ability of RNAP lacking the ω subunit to respond to ppGpp (see below) (Rutherford *et al.*, 2007).

In studies that were designed to gain insight into DksA function, random mutagenesis of *dksA* revealed two independent mutations that were able to restore growth of a *relA spoT dksA* triple mutant on minimal media (Blankschien *et al.*, 2009). These mutations, N88I and L15F, were able to increase DksA activity in vivo and bypass the

requirement for ppGpp. These mutants result in shorter half-lives for open complex formation between RNAP and promoters and bind more tightly to RNAP than wild-type DksA. This study, along with a report that DksA has higher affinity for free RNAP versus RNAP in an open complex formation with DNA (Lennon *et al.*, 2009), suggests that the affinity of DksA to RNAP plays a role in its function. The super DksA mutants also identify putative surface areas of DksA that might interact with RNAP.

Mechanism of ppGpp and DksA

Originally, identification of suppressors of *relA spoT* double mutants (also known as $ppGpp^0$ mutant) in *E. coli* provided insights into the role of ppGpp function (Hernandez and Cashel, 1995; Bartlett *et al.*, 1998; Barker *et al.*, 2001a; Murphy and Cashel, 2003; Trautinger and Lloyd, 2002; Szalewska-Palasz *et al.*, 2007). The majority of suppressors mapped to *rpoBC*, the genes that encode β and β‘ subunits of RNAP. These mutations destabilized promoter-RNAP interactions, mimicking the stringent response. Similar results were obtained with *dksA* mutants, suggesting that DksA hinders the conformational changes in RNAP and DNA during the transition to the open complex intermediate (Rutherford *et al.*, 2009). Meanwhile, ppGpp and DksA have been demonstrated to directly interact with RNA polymerase and influence transcription in a promoter-dependent mechanism (Barker *et al.*, 2001b; Paul *et al*., 2004a). ppGpp and DksA reduce the half-life of the open complex of promoters with RNAP, in turn, inhibiting transcription from promoters that already have intrinsically short-lived open complexes (e.g. rRNA promoters) (Zhou and Jin, 1998; Barker *et al*., 2001b; Paul *et al*., 2004a). Promoters that have long-lived open complexes are presumably not negatively

affected by ppGpp and DksA because RNAP clears the promoter before dissociation occurs. Negatively regulated promoters are characterized by the presence of a GC-rich discriminator sequence located between the -10 and the transcription start site and suboptimal sequences for sigma factor recognition (Travers, 1980; Travers, 1984; Josaitis *et al.*, 1995; Park *et al.*, 2002; Haugen *et al.*, 2006). Importantly, DksA reduces the lifetimes of open complexes even in the absence of ppGpp, indicating that DksA can function independently of ppGpp (Paul *et al*., 2004a). However, DksA protein levels remain constant in all conditions tested (Paul *et al*., 2004a; Rutherford *et al*., 2007), supporting the hypothesis that the varying concentrations of ppGpp and inititating NTPs are the modulators of rRNA transcription (Murray *et al.*, 2003; Dalebroux *et al.*, 2010).

The positive effects of ppGpp and DksA most likely occur in both direct and indirect mechanisms. While ppGpp and DksA are responsible for directly activating transcription of some amino acid promoters by increasing the isomerization rate constant (k_i) on the pathway to open complex formation (Paul *et al*., 2005), some of these promoters are possibly activated indirectly. One hypothesis suggests that the concentration of free RNAP can be increased by reducing that amount of RNAP involved in transcription of stable RNA promoters (Barker *et al*., 2001a; Paul *et al*., 2005). In support, positively regulated promoters that are activated indirectly have low affinity for RNAP and require high concentrations of RNAP in vitro (Barker *et al*., 2001a). Additionally, increased levels of free core RNAP could also allow competition by alternative sigma factors (e.g. σ^S, σ^E, σ^N, *etc*.), indirectly promoting alternative sigma factor-dependent gene regulation (Jishage *et al.*, 2002). One study demonstrated that RpoS-dependent promoters are not efficiently transcribed in a $ppGpp^0$ strain, even in the

presence of high concentrations of RpoS, perhaps because of a reduction of free RNAP core in the absence of ppGpp (Kvint *et al.*, 2000). Several studies have presented evidence that ppGpp and/or DksA are required for alternative sigma factor-dependent transcription in vivo and have also shown that mutations in RNAP that mimic the stringent response allow for efficient sigma factor competition in the absence of ppGpp accumulation (Jishage *et al*., 2002; Laurie *et al.*, 2003; Bernardo *et al.*, 2006; Szalewska-Palasz *et al*., 2007; Costanzo *et al.*, 2008), suggesting that the stringent response employs both direct and indirect mechanisms to alter global transcription.

Although there is direct evidence that ppGpp and DksA can inhibit or activate transcription from target promoters, the exact molecular mechanism remains unclear. It is thought that ppGpp could increase DksA activity, that ppGpp and DksA work cooperatively to induce common conformational changes in RNAP, or that each factor induces different conformational changes in RNAP that are synergistic (Blankschien *et al*., 2009). Cocrystal structures of the *Thermus thermophilus* RNA polymerase-ppGpp complex determined that ppGpp binds in two different orientations near the active site (Artsimovitch *et al.*, 2004). However, this structural data was challenged by a study that systematically made amino acid substitutions in ten residues that were near the putative ppGpp binding site and found no effect in ppGpp-mediated regulation (Vrentas *et al.*, 2008). Futhermore, *T. thermophilus* RNA polymerase does not respond to ppGpp directly in vitro (Vrentas *et al*., 2008) or in vivo (Kasai *et al.*, 2006), suggesting an indirect mechanism for stringent control, similar to *B. subtilis* (see below).

Finally, the ω subunit of RNAP has been implicated in stringent response. First, the ω subunit plays a structural role in assembly of RNAP. ω aids the proper folding of

the β' subunit and directs the association of β' with the $\alpha_2\beta$ subunits (Gentry and Burgess, 1993; Mukherjee *et al.*, 1999; Ghosh *et al.*, 2001). Encoded in the same operon as *spoT* (Gentry and Burgess, 1989), an initial study concluded that the ω protein was required for RNAP sensitivity to ppGpp in vitro (Igarashi *et al.*, 1989) while another study demonstrated that it was not necessary for stringent response in vivo (Gentry *et al.*, 1991). Further studies revealed that RNAP could regain sensitivity to ppGpp in vitro upon the addition of ω, indicating that ω is needed for an appropriate response to ppGpp by RNAP (Vrentas *et al.*, 2005). The discrepancy between the in vivo and in vitro studies was resolved upon the discovery that DksA can rescue RNAP sensitivity to ppGpp in the absence of the ω subunit in vitro (Vrentas *et al*., 2005). This most likely accounts for the ability of *rpoZ* mutants to retain sensitivity to ppGpp in vivo; however, more studies are needed to further elucidate the role that ω has on the ppGpp-responsiveness of RNAP.

Interestingly, *B. subtilis* and *T. thermophilus* employ a different mechanism for ppGpp regulation than *E. coli* and other γ-proteobacteria. In *B. subtilis*, ppGpp does not directly inhibit rRNA promoters; instead, synthesis of ppGpp depletes the intracellular pools of GTP, reducing the concentration of the initiating NTP for all rRNA promoters (Krasny and Gourse, 2004). In agreement with this hypothesis, there is no longer a ppGpp-dependent reduction of transcription when the initiating nucleotide of rRNA promoters is mutated from the native G (Krasny and Gourse, 2004). ppGpp also reduces the synthesis of GTP by inhibiting inosine monophosphate (IMP) dehydrogenase, the enzyme that catalyzes the first reaction toward GTP biosynthesis (Lopez *et al.*, 1981; Ochi *et al.*, 1982; Ochi, 1987; Kasai *et al*., 2006). Although ppGpp strongly inhibits IMP

dehydrogenase in *E. coli* (Gallant *et al.*, 1971), many of the rRNA operons initiate transcription with ATP, making these promoters insensitive to changes in the GTP pool (Gaal *et al.*, 1997). However, direct interaction of ppGpp with RNAP associated with rRNA promoters apparently allows efficient regulation in *E. coli*. Importantly, there are no homologs in *B. subtilis* with strong homology to DksA, supporting the hypothesis that ppGpp regulation is indirect (Krasny and Gourse, 2004).

An additional factor involved in the stringent response of *B. subtilis* and other low G+C Gram-positive bacteria is CodY, a DNA-binding transcriptional repressor (Serror and Sonenshein, 1996b). The CodY regulon encompasses operons that regulate sporulation (Molle *et al.*, 2003) and competence (Serror and Sonenshein, 1996a) and that are involved in branched-chain amino acid (BCAA) synthesis (Molle *et al.*, 2003). CodY is directly activated by GTP (Ratnayake-Lecamwasam *et al.*, 2001; Handke *et al.*, 2008) and the BCAAs, isoleucine and valine (Shivers and Sonenshein, 2004). Activity of CodY has been correlated with ppGpp levels in several organisms, in that many CodY-repressed genes are transcribed following RelA activation, ppGpp synthesis and the reduction of intracellular GTP concentrations (Inaoka and Ochi, 2002; Malke *et al.*, 2006; Lemos *et al.*, 2008). These studies indicate that CodY is a global sensor that responds to intracellular levels of GTP which reflect nutritional limitations and allow for complete alteration of global transcription.

Regulatory targets of ppGpp and DksA

The effects of ppGpp and DksA on bacterial physiology are quite broad. In early studies, the stringent response was linked to growth rate control (Bremer and Dennis,

1996). Here, basal level changes of ppGpp, not NTP substrate concentrations as previously thought, are inversely correlated with growth rate and the number of ribosomes per cell (Schneider and Gourse, 2004). In addition to regulating metabolic processes, ppGpp and DksA are responsible for regulating a number of cellular processes involved in survival under various environmental stresses and also play a role in colonization and virulence in pathogenic bacteria.

In *E. coli*, the stringent response induces expression of the stationary phase sigma factor, RpoS (Gentry *et al.*, 1993), and both ppGpp and DksA appear to exert effects at multiple levels. Transcription of *rpoS* depends upon ppGpp (Lange *et al.*, 1995). Further studies revealed that ppGpp has an effect on basal expression of *rpoS*, although there is a slight delay in *rpoS* expression in a $ppGpp^0$ strain (Hirsch and Elliott, 2002). DksA is also necessary for the ppGpp-dependent activation of *rpoS* at the translational level; however, it is unknown if this is direct or indirect (Brown *et al*., 2002; Hirsch and Elliott, 2002). ppGpp also controls RpoS protein stability. During phosphate starvation, SpoT-derived ppGpp positively regulates *iraP* transcription, which encodes an anti-adaptor protein that inhibits proteolysis of RpoS by interfering with the RssB-specific targeted degradation by ClpXP (Bougdour and Gottesman, 2007). Another RpoS anti-adaptor protein, IraD, is also transcriptionally upregulated by ppGpp and requires both RelA and SpoT synthetase activities (Merrikh *et al.*, 2009).

Recent data suggest that ppGpp and DksA can function independently and even antagonistically. An initial study revealed that while ppGpp activated the expression of type 1 fimbriae in uropathogenic *E. coli*, expression was increased in the absence of DksA (Aberg *et al.*, 2008). Further analysis of *fimB* and other similarly regulated targets

identified via microarray studies, including targets important for flagellum synthesis and chemotaxis, demonstrated that the increased access of the Gre factors to the secondary channel of RNAP was responsible for the upregulation of expression (Aberg *et al*., 2008; Aberg *et al.*, 2009). In fact, strains lacking both GreA and GreB along with DksA no longer exhibited an increase in *fimB* expression (Aberg *et al*., 2008). In contrast to the in vivo results, ppGpp and DksA exhibit the same inhibitory effect in vitro (Aberg *et al*., 2008). Because GreA and GreB are present in very low concentrations compared to DksA (Rutherford *et al*., 2007), the ability for the Gre factors to effectively compete for binding to the secondary channel of RNAP in the presence of DksA is questionable; however, these studies suggest that the competition for occupancy of the secondary channel potentially plays a role in transcriptional regulation.

Other studies have found opposing effects of ppGpp and DksA in *E. coli*. One study found that DksA is required for upregulation of the λ pR promoter both in vivo and in vitro while ppGpp represses expression (Lyzen *et al.*, 2009). Because the effect of DksA is positive in this case, the Gre factors likely do not play a role. Transcription of *iraD*, mentioned above, is positively regulated by ppGpp but is repressed by DksA (Merrikh *et al.*, 2009). While these studies are intriguing, more work is needed to clarify how ppGpp and DksA exhibit antagonistic and independent effects at the same promoter and whether these effects are physiologically relevant.

In addition to effects mediated on transcriptional and post-transcriptional levels, ppGpp and DksA influence DNA replication during amino acid starvation (Levine *et al.*, 1991). In *B. subtilis*, ppGpp inhibits replication elongation by directly inhibiting primase, an essential component of the replication machinery, and reducing the ability to recruit

RecA to replication forks (Wang *et al.*, 2007). In *E. coli*, ppGpp blocks replication initiation and appears to also prevent chromosome segregation; these effects require both Dam and SeqA (Ferullo and Lovett, 2008). Independently of ppGpp, DksA, along with GreA, GreB and TraR, inhibits stalled transcription complexes from interfering with replication, likely by promoting transcriptional elongation (Tehranchi *et al.*, 2010). As previously mentioned, *B. subtilis* does not contain a DksA homolog and appears to resolve the conflict between transcription and replication through genome organization by having a high percentage of genes codirectionally transcribed and replicated (Rocha, 2004; Srivatsan *et al.*, 2010). *B. subtilis* and *E. coli* utilize the stringent response through different mechanisms to coordinate nutritional status and other stresses with DNA replication and protect the genome from possible DNA damage.

Regulation of biofilm formation and c-di-GMP

A biofilm is an aggregate of bacteria that is encased in a matrix composed primarily of exopolysaccharides and may contain proteins and extracellular DNA (Costerton, 1995). The formation of biofilms is distinct from motile, planktonic cells, and the phenotypes of biofilm and planktonic cells are vastly different. 3',5'-cyclic diguanylic acid (c-di-GMP) has been characterized as a nearly ubiquitous second messenger that plays a role in regulation of several physiological processes, including signaling the transition between sessility and motility. c-di-GMP was originally discovered as a modified nucleotide that allosterically activates cellulose synthase in *Gluconacetobacter xylinus* (formerly *Acetobacter xylinus*; Ross *et al.*, 1987). Since then, c-di-GMP has been implicated in promoting synthesis of adhesions and

exopolysaccharide matrix components (reviewed in Jonas *et al.*, 2009), inhibiting motility (reviewed in Wolfe and Visick, 2008), controlling long-term survival (Kumar and Chatterji, 2008) and response to environmental stresses (Klebensberger *et al.*, 2009; Guo and Rowe-Magnus, 2010), as well as regulating virulence (reviewed in Cotter and Stibitz, 2007), synthesis of secondary metabolites (Fineran *et al.*, 2007) and cell cycle progression (Duerig *et al.*, 2009).

Metabolism of c-di-GMP

c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGCs) and degraded to linear pGpG or GMP by phosphodiesterases (PDEs). GGDEF domains possess DGC activity (Ausmees *et al.*, 2001; Paul *et al.*, 2004b; Ryjenkov *et al.*, 2005) while PDE activity is conferred by either the EAL (Bobrov *et al.*, 2005; Schmidt *et al.*, 2005) or the HD-GYP domains (Ryan *et al.*, 2006). These motifs, named for their conserved amino acids, are critical for DGC and PDE activity. Frequently, c-di-GMP metabolizing proteins contain sensory and signal transduction domains (e.g. CheY-like (REC), PAS and GAF; Hecht and Newton, 1995; Aravind and Ponting, 1999; Galperin *et al.*, 2001), which may allow activity to be regulated through environmental signals (Jenal and Malone, 2006; see below).

PleD, an unusual response regulator required for pole development in *Caulobacter crescentus*, was the first GGDEF domain protein that was characterized biochemically to contain DGC activity (Paul *et al.*, 2004b). A crystal structure revealed that PleD forms a dimer with a c-di-GMP molecule binding to each aligned GGDEF domain (Chan *et al.*, 2004), and subsequent studies indicated that phosphorylation-

mediated dimerization via the CheY-like phosphoacceptor (or receiver [REC]) domains is necessary for catalytic activity of PleD (Paul *et al.*, 2007; Wassmann *et al.*, 2007). However, some DGC proteins are not dependent upon phosphorylation for regulatory control. WspR from *P. aeruginosa* switches from inactive to active states through an intermediate tetrameric form via non-modified REC domains (De *et al.*, 2008). Recent observations suggest that DgcA and DgcB from *C. crescentus* are permanent dimers and are constitutively active (Schirmer and Jenal, 2009), highlighting a variety of mechanisms for DGC activation and regulation.

The PleD and DgcA crystal structures revealed an additional binding site (I-site) for c-di-GMP, allowing for allosteric noncompetitive product inhibition of DGC activity (Chan *et al*., 2004; Christen *et al.*, 2006). Additional experiments defined an RXXD binding motif within the GGDEF domain (Christen *et al.*, 2006). This motif is highly conserved within many characterized and predicted GGDEF domain proteins (Christen *et al*., 2006), suggesting that this mechanism of feedback inhibition is important for regulatory control of c-di-GMP synthesis. An additional mode of feedback inhibition entails c-di-GMP-mediated crosslinking between each DGC domain within the dimer, resulting in dimer immobilization (Wassmann *et al*., 2007; De *et al.*, 2009).

Several observations hinted that the EAL domain may possess PDE activity (Tal *et al.*, 1998; Galperin *et al.*, 1999; Chang *et al.*, 2001), and in vivo experiments indicated that this domain was responsible for repressing biofilm formation in *V. cholerae* (Tischler and Camilli, 2004) and for reducing c-di-GMP levels in *Salmonella* (Simm *et al.*, 2004). Subsequently, purified EAL domains and intact proteins were demonstrated to possess PDE activity (Christen *et al.*, 2005; Schmidt *et al*., 2005; Bobrov *et al*., 2005; Tamayo *et*

al., 2005). In addition to the conserved EAL domain, an extended motif (DDFG(T/A)GYSS) that forms a conserved loop (known as loop 6) is important for dimerization of EAL domain proteins and for c-di-GMP binding (Rao *et al.*, 2008; Rao *et al.*, 2009). Interestingly, sequence analysis revealed that approximately half of the known EAL domain proteins contain a degenerate loop, suggesting that two classes of EAL domain proteins may exist (Rao *et al*., 2009; Romling, 2009).

HD-GYP domain proteins are part of the HD superfamily of metal-dependent phosphohydrolases and were hypothesized to function as phosphodiesterases because of the association of the HD-GYP domain with the GGDEF domain (Galperin *et al*., 1999; Galperin *et al*., 2001). Additionally, several species, most notably *Thermatoga maritima*, were found to contain numerous GGDEF domains, but no EAL domains, suggesting that these bacteria could contain phosphodiesterase activity mediated by an alternative domain (Galperin *et al*., 1999). The first example of the HD-GYP domain as a c-di-GMP phosphodiesterase was from *Xanthamonas campestris* pv. *campestris*, a plant pathogen responsible for black rot (Ryan *et al*., 2006). An isolated HD-GYP domain degraded c-di-GMP to GMP via a linear pGpG substrate, and mutation of the conserved HD residues eliminated the regulatory and enzymatic activities of the protein (Ryan *et al*., 2006). Subsequent studies have revealed that the phosphodiesterase activity of HD-GYP domain proteins is required for quorum sensing-mediated biofilm production in *V. cholerae* El Tor (Hammer and Bassler, 2009) and for the synthesis and/or secretion of virulence factors and swarming capabilities of *P. aeruginosa* (Ryan *et al.*, 2009). In addition, the GYP residues have been shown to play a role in a physical interaction with a subset of

GGDEF domain proteins in *X. campestris*, influencing pilus-dependent motility (Ryan *et al.*, 2010).

Although these three characterized domains can occur separately, they are frequently found as composite proteins containing both GGDEF and EAL or HD-GYP domains. Bifunctional enzymes, reminiscent of SpoT and RSH proteins, can possess DGC and PDE activities. The regulation of DGC versus PDE activity can be constitutive (Tarutina *et al.*, 2006), regulated by accessory proteins (Ferreira *et al.*, 2008), or demonstrate both enzymatic events simultaneously (Kumar and Chatterji, 2008). There are also a number of GGDEF-EAL composite proteins that contain degenerative GGDEF and/or EAL domains. The best characterized example, PdeA from *C. crescentus*, possesses a degenerative GGDEF domain that lacks DGC activity but is able to increase PDE activity through an allosteric mechanism of sensing GTP availability (Christen *et al.*, 2005). The *V. cholerae* CdpA appears to function similarly as the GGDEF domain is necessary for PDE activity in vivo (Tamayo *et al.*, 2008). Supporting the above studies, computational genomics revealed that there are predominantly two classes of composite proteins: those that contain conserved GGDEF and EAL domains and are possibly regulated by an additional domain and those that only possess an intact EAL domain that is typically associated with a signal-sensing domain (Seshasayee *et al.*, 2010).

Some recently characterized GGDEF-EAL domain proteins lack both DGC and PDE activity and have adopted other roles. The crystal structure of FimX, a degenerate GGDEF-EAL protein that regulates twitching motility and biofilm in *P. aeruginosa*, revealed that FimX is capable of interacting with c-di-GMP within the EAL domain (Navarro *et al.*, 2009). This study suggests that c-di-GMP sensing through degenerate

GDDEF and EAL domains may provide additional regulatory mechanisms beyond c-di-GMP metabolism (see below). Indeed, CsrD, a specificity factor necessary for the turnover of CsrB and CsrC sRNAs, was the first GGDEF-EAL domain protein assigned a function not involving c-di-GMP metabolism (Suzuki *et al*., 2006), providing a possible explanation for the large number of GGDEF and/or EAL domain proteins found in some species.

The number of encoded GGDEF and/or EAL domain proteins is highly variable among various eubacteria (Galperin *et al.*, 2001). A few species have none or few while *E. coli* and other γ-proteobacteria contain a large number of GGDEF and/or EAL domain proteins (Galperin *et al*., 2001). It is surprising that a single species would possess multiple proteins containing the same enzymatic activities since most appear to retain catalytic activity and thus be involved in c-di-GMP signaling. This high redundancy could tightly control c-di-GMP levels in response to a variety of conditions and, thus, raises questions about how the specificity of the signal is achieved and how the cell could avoid cross-talk. Do different GGDEF/EAL domain proteins act independently and affect different targets or do all of the proteins control a common, diffusible pool of c-di-GMP? Recent studies have suggested that not all of the GGDEF and EAL domain proteins are present and active at the same time and in the same place.

Temporal regulation of expression and activity of GGDEF and/or EAL domain proteins in a variety of organisms allows c-di-GMP regulation to occur only under specific conditions. In *E. coli*, only a few GGDEF and/or EAL domain proteins are expressed during exponential growth while the majority of these are expressed during stationary phase under the control of the stationary sigma factor, σ^S (Sommerfeldt *et al.*,

2009). Several GGDEF and/or EAL domain-encoding genes in *V. cholerae* are regulated by cAMP-CRP (Fong and Yildiz, 2008) and the quorum-sensing regulator HapR (Waters *et al.*, 2008). In *Yersinia pestis*, the GGDEF domain protein HmsT is degraded by ClpXP and Lon proteases (Kirillina *et al.*, 2004).

In addition to transcriptional and post-translational mechanisms of regulation, GGDEF and EAL domain proteins are regulated post-transcriptionally by CsrA in *E. coli* and *Salmonella* Typhimurium. CsrA directly represses the expression of two GGDEF domain proteins, YcdT and YdeH, in *E. coli*, preventing accumulation of c-di-GMP (Chapter 3). In *Salmonella* Typhimurium, CsrA plays a complex role in directly and indirectly mediating the expression of eight GGDEF and/or EAL domain proteins, generally promoting motility and downregulating biofilm formation (Chapter 4).

Additionally, bioinformatics data suggest that the activities of DGCs and PDEs may be frequently regulated by associated accessory domains, which allow the integration of environmental and cellular signals. A comparative genomics study found that over half of the identified GGDEF and EAL domain proteins contain an additional domain (Seshasayee *et al.*, 2010). The oxygen-sensing PAS and blue-light sensing BLUF domains have been suggested to regulate PDE activity of EAL domain proteins in *G. xylinus* (Chang *et al.*, 2001; Qi *et al.*, 2009), *B. subtilis* (Minasov *et al.*, 2009), *Klebsiella pneumonia* (Wu and Gardner, 2009; Barends *et al.*, 2009) and *E. coli* (Hasegawa *et al.*, 2006; Schroeder *et al.*, 2008). However, the YcgF protein in *E. coli* that contains an EAL domain associated with a BLUF domain does not function as a c-di-GMP phosphodiesterase, but instead serves as an anti-repressor, releasing the transcriptional repressor YcgE in response to blue-light irradiation (Tschowri *et al.*,

2009). In addition to the regulatory function of the REC domain of PleD in dimerization (Paul *et al*., 2004b; Wassmann *et al*., 2007), the REC domain is important for two-component and phosphorelay signaling pathways. For example, the *V. cholerae* EAL domain protein and transcription factor, VieA, contains a REC domain that activates transcription of the *vieSAB* operon upon phosphorylation by VieS, creating a positive feedback loop that increases the concentration of VieA and, potentially, PDE activity (Martinez-Wilson *et al.*, 2008). The REC domain also appears to be necessary for localization of certain GGDEF and EAL domain proteins to the cell poles (Paul *et al*., 2004b; Kazmierczak *et al.*, 2006; Guvener and Harwood, 2007). Some GGDEF and EAL proteins contain multiple sensory domains, suggesting that complex signal integration may be involved in the regulation of these proteins.

Several reports suggest that some of the GGDEF and/or EAL domain proteins can be functionally sequestrated (reviewed in Hengge, 2009, Jonas *et al.*, 2009), allowing c-di-GMP signaling to occur within "microcompartments" of the cell. Recent data suggest that specific protein-protein interactions between GGDEF domains and EAL and HD-GYP domains can occur (Andrade *et al.*, 2006; Bobrov *et al.*, 2008), perhaps allowing local confinement of c-di-GMP signaling. Functional sequestration also includes spatial separation, exemplified by the extensively characterized cell cycle of *C. crescentus*, which requires the localization of specific DGCs, PDEs and a newly identified effector protein for cell cycle progression (Paul *et al*., 2004b; Huitema *et al.*, 2006; Duerig *et al.*, 2009).

Regulation by c-di-GMP

The production of c-di-GMP allows for a quickly synthesized signal to interact with downstream targets to relay environmental cues. c-di-GMP regulation occurs on multiple levels including transcriptional, translational and posttranslational mechanisms. The first enzyme shown to directly bind c-di-GMP and undergo allosteric activation was cellulose synthase of *G. xylinus* (BscA) (Ross *et al*., 1987; Weinhouse *et al.*, 1997). Subsequently, the PilZ domain was suggested to interact with c-di-GMP through a bioinformatics analysis that revealed PilZ domains were sometimes found C-terminally of GGDEF and EAL domain proteins and have a similar phyletic distribution as GGDEF and EAL domain proteins (Amikam and Galperin, 2006). In addition, this study revealed that the cellulose synthase of *G. xylinus* contains a putative PilZ domain, providing evidence that the PilZ domain is a c-di-GMP receptor. The PilZ domain protein YcgR of *E. coli* was found to directly bind c-di-GMP stoichiometrically (Ryjenkov *et al.*, 2006), confirming that the PilZ domain is a conserved c-di-GMP-binding domain. Subsequent studies have shown that PilZ domain proteins play a broad regulatory role in biofilm, motility and virulence (Pratt *et al.*, 2007; Merighi *et al.*, 2007; Christen *et al.*, 2007; Ramelot *et al.*, 2007). It appears that PilZ domain proteins may function primarily through protein-protein interactions. Structural studies have revealed that various PilZ domain proteins exhibit different c-di-GMP binding stoichiometry and quarternary structure (Ramelot *et al.*, 2007; Benach *et al.*, 2007; Ko *et al.*, 2010).

The PilZ domain protein YcgR was recently hypothesized to inhibit motility by interfering with the flagellum switch complex, a structure located at the base of the flagellum (Wolfe and Visick, 2008). Indeed, YcgR was discovered to directly interact

with FliG subunit of the switch complex, and the affinity for YcgR binding to FliG was increased upon binding to c-di-GMP (Paul *et al.*, 2010; Fang and Gomelsky, 2010). This interaction biases flagellar rotation to the counter-clockwise direction (Paul *et al*., 2010; Fang and Gomelsky, 2010). However, another study reported that YcgR interacts with the motor protein MotA in response to c-di-GMP binding and that a gradient of c-di-GMP levels affects swimming velocity (Boehm *et al.*, 2010). While more studies are required to eliminate these inconsistencies, both studies reveal that YcgR negatively influences motility upon c-di-GMP binding.

A PilZ domain protein could not be attributed to a c-di-GMP-dependent phenotype of PEL polysaccharide production in *P. aeruginosa*, suggesting that another c-di-GMP-sensing domain might mediate c-di-GMP effects. PelD, encoded in the *pel* operon, was found to directly bind c-di-GMP. Binding of c-di-GMP required an RxxD motif, similar to the I-site of the GGDEF domain protein PleD of *C. crescentus* (Lee *et al.*, 2007). PEL synthesis requires binding to c-di-GMP to PelD, perhaps functioning, in turn, as an allosteric activator, similar to the PilZ domain of the BscA1 protein in *G. xylinus*. Other than PelD homologs in closely related bacteria, it appears that no other proteins contain the PelD domain for c-di-GMP binding (Lee *et al.*, 2007).

To determine the mechanism of c-di-GMP effects on motility in *P. aeruginosa*, analysis of transcriptional regulation of flagellum biosynthesis operons was investigated. These studies revealed that FleQ, the master regulator of flagella gene expression and repressor of exopolysaccharide (EPS) biosynthesis operons, directly bound to c-di-GMP (Hickman and Harwood, 2008). Cyclic-di-GMP binding to FleQ relieves transcriptional repression of the EPS operons, upregulating biofilm formation (Hickman and Harwood,

2008). Perhaps c-di-GMP binding promotes a conformational change in FleQ, reducing DNA binding. Interestingly, c-di-GMP binding did not have a significant effect on positively regulated promoters, suggesting that the mechanisms for FleQ activation and repression differ. Further studies are needed to elucidate the FleQ regulon and other potential c-di-GMP regulatory targets.

Transcriptional regulators may be a common target for c-di-GMP signaling. The cAMP receptor-like protein (CLP) in *X. campestris* is no longer able to interact with DNA when bound by c-di-GMP (Chin *et al.*, 2010). The proposed c-di-GMP binding site of this protein has limited similarity to the RxxD motif (Chin *et al.*, 2010), and further analysis is required to determine if CLP contains a novel c-di-GMP binding motif. VpsT of *V. cholerae* contains a unique, conserved c-di-GMP binding site and undergoes a conformational change upon c-di-GMP binding that is required for DNA binding (Krasteva *et al.*, 2010). The latter study also demonstrated that VpsT inversely coordinates biofilm and motility in a c-di-GMP-dependent mechanism (Krasteva *et al.*, 2010).

Degenerate GGDEF and EAL domains have been hypothesized to serve as c-di-GMP receptors. LapD, an inner membrane protein that functions as a c-di-GMP effector in *P. aeruginosa*, relays intracellular c-di-GMP levels to the membrane localized attachment machinery required for biofilm formation (Newell *et al.*, 2009). Here, c-di-GMP binds the degenerate EAL domain of LapD, creating a signal that is transferred through the periplasmic domain to control biofilm formation via the cell surface adhesin LapA (Newell *et al.*, 2009). In *V. cholerae*, a degenerate GGDEF domain protein, CdgG,

contains a conserved I-site (the RxxD motif) that is essential for its function, indicating that this motif may interact with c-di-GMP (Beyhan *et al.*, 2008).

Recently, PopA, a PleD paralog in *C. crescentus* that contains a degenerate GGDEF motif, was demonstrated not to possess DGC activity, but to bind c-di-GMP at the conserved I-site (RxxD motif) (Duerig *et al*., 2009). The binding of c-di-GMP sequesters PopA at the cell pole where it recruits the replication initiation inhibitor CtrA and proteases of CtrA to positively regulate cell cycle progression (Duerig *et al*., 2009). This finding established a novel role for c-di-GMP in regulation.

Interestingly, c-di-GMP can serve as a ligand for a highly conserved RNA motif, GEMM, which functions as a riboswitch (Sudarsan *et al.*, 2008). Bioinformatic searches determined that this riboswitch paradigm is present in a variety of organisms, including in the genome of a bacteriophage, and suggested that the c-di-GMP regulon could include riboswitch regulation of pilus formation, flagellum biosynthesis and virulence gene expression (Sudarsan *et al*., 2008). A recent study describes a second class of riboswitches in *Clostridium difficile* which regulates a cis-encoded ribozyme (Lee *et al.*, 2010). c-di-GMP binding to the riboswitch alters RNA processing by the ribozyme, allowing for translation of the downstream gene (Lee *et al*., 2010). These findings have mechanistic implications for additional roles of c-di-GMP as a secondary messenger by exerting effects on anti-termination or translation control.

Interestingly, in *Staphylococcus epidermidis* and other representatives of *Firmicutes*, only one conserved and one modified GGDEF domain proteins are encoded, and no EAL /HD-GYP domain nor PilZ domain proteins are present (Amikam and Galperin, 2006). Furthermore, one study found that the conserved GGDEF domain

protein of *S. epidermidis* appears to regulate biofilm formation in a c-di-GMP-independent fashion (Holland *et al.*, 2008). This study suggests that the physiological roles of GGDEF domain proteins may be conserved while the precise molecular mechanism may vary greatly.

Global regulatory networks and global regulons in prokaryotes

Global regulatory systems allow the conversion of extracellular cues and environmental signals to defined responses by the bacterium. These responses create global changes in gene expression and permit the bacteria to survive conditions of nutritional starvation and environmental stress. Recent advances in transcriptomics and proteomics have allowed computational and systems biology approaches to produce comprehensive models of regulatory networks and interactions in *E. coli* (Barabasi and Oltvai, 2004; Gama-Castro *et al.*, 2008).

Global regulatory networks have traditionally been characterized at the transcriptional level. The role that other types of regulators perform in global regulatory networks has not been thoroughly studied, but it is clear that post-transcriptional, translational and post-translational regulators influence gene expression on a global level. Two post-transcriptional regulators, Hfq and CsrA, have been discovered to participate in regulatory circuits containing transcription factors (Beisel and Storz, 2010; Suzuki *et al.*, 2002). Hfq and CsrA also utilize sRNAs for their regulatory processes; Hfq is an RNA chaperone that aids in base pairing between sRNAs and their target mRNAs (reviewed in Waters and Storz, 2009). These two regulators have potential advantages over transcriptional regulators in that they provide a faster regulatory speed by modulating

gene expression at a point closer to translation and afford “layered regulation” to tightly control expression (Beisel and Storz, 2010). The sRNAs of each system also have a reduced metabolic cost in that they are not translated.

Recent advances in high-throughput sequencing technologies have allowed global regulons to be elucidated for Hfq (Sittka *et al.*, 2008; Sittka *et al.*, 2009) and CsrA (Chapter 2). Additionally, systematic evolution of ligands by exponential enrichment (SELEX) and Genomic SELEX have uncovered a consensus binding site for CsrA (Dubey *et al*., 2005) and additional targets for Hfq (Lorenz *et al.*, 2010), respectively, which would have not been easily elucidated by traditional screens. Discovery of preferred binding sites via these techniques also provide data for bioinformatics and computational tools to predict novel targets (Baker *et al*., 2007). In addition, these studies provide data which can refine predictions and models of complex regulatory networks.

Global regulatory systems frequently employ feedback regulation to modulate the response to a stimulus. Feedback regulation occurs when a regulator influences the expression of its own gene, establishing a closed, autoregulatory loop (Beisel and Storz, 2010). Positive and negative feedback loops possess opposite regulatory properties. The response time of gene expression in system containing a positive feedback circuit is decreased compared to a system lacking a positive feedback loop (Maeda and Sano, 2006). In addition, cell-cell variability (bistability) increases since a small level of activation of a regulatory factor is amplified by further activation of expression (Becskei *et al.*, 2001; Maeda and Sano, 2006). Negative autoregulation can accelerate the regulatory response because an intermediate concentration of the regulatory factor

represses its own expression (Rosenfeld *et al.*, 2002). A negative feedback circuit tends to decrease cell-cell variability and bistability (Becskei and Serrano, 2000) and produces graded responses (Nevozhay *et al.*, 2009). These features contained within networks allow bacteria to robustly detect and amplify specific signals. As mentioned previously, the Csr system is characterized by three defined negative feedback loops, which potentially provide these features to the Csr system and its regulated targets. Indeed, two negative feedback loops within the quorum-sensing LuxO-sRNA signaling network of *V. harveyi* have been demonstrated to contain these characteristics and play a role in mediating the transition between low-cell-density and high-cell-density states (Tu *et al.*, 2010).

The Csr, stringent response, and c-di-GMP systems create an interconnected signaling network, which can be functionally separated into its separate motifs. However, its modularity allows diverse signals to precisely regulate the expression of target genes in an efficient and appropriate manner to benefit the cell as a whole (Chapters 2 and 4). The interconnection between the Csr, stringent response, and c-di-GMP systems defined in this study suggests that overlapping regulons may be a common theme for global networks in *E. coli* and *Salmonella* Typhimurium.

Conclusion

All of the regulatory networks described herein sense specific stimuli and coordinate transcriptional, post-transcriptional, and translational responses that ultimately affect hundreds of gene products and provide global gene reprogramming. The comprehensive regulation and control of the Csr, stringent response and c-di-GMP

signaling networks highlight the importance of providing precise temporal and spatial regulation by incorporating a variety of signals to fine-tune global gene expression. In complex environments, such as nutrient poor soils or within hosts, multiple signals are integrated to allow for rapid adaptation. Determining the interactions between regulators clarifies the characteristics of cellular networks and aids in predicting the way in which a defined biological process may respond to multiple signals.

Understanding the impact and response of global regulatory networks in the model prokaryote *E. coli* provides insights for how systems may be regulated in related species and perhaps even higher organisms. Despite the fact that there appears to be unlimited diversity of networks, their nature is governed by principles that apply to most networks (Barabasi and Oltvai, 2004). The characteristics of regulatory networks in *E. coli* appear to be fundamental for functional organization and may be applicable to systems in eukaryotes. There are a number of signaling motifs that are conserved in both prokaryotes and eukaryotes, including negative and positive feedback loops, crosstalk and modularity (reviewed in Kiel *et al.*, 2010), as well as architectural and mechanistic similarities in signaling proteins (reviewed in Aravind *et al.*, 2003). In addition, ppGpp signaling has been described in plants (van der Biezen *et al.*, 2000; Givens *et al.*, 2004) and appears to function similarly to the *E. coli* stringent response system, as chloroplast RNA polymerase is inhibited by ppGpp in vitro (Takahashi *et al.*, 2004) and ppGpp synthetase activity copurified with the 70S ribosomes of chloroplasts (Kasai *et al.*, 2004). Similar regulatory properties of the stringent response system may apply to the mechanisms by which plants sense and respond to environmental and nutritional stresses. It has also been suggested that the use of ubiquitous second messengers (e.g. ppGpp and

cAMP) in synthetic biology may be useful in engineering novel signal transduction pathways in prokaryotes and eukaryotes (Kiel *et al.*, 2010).

To appropriately coordinate gene expression to allow the most efficient use of energy sources, these global regulatory systems not only perform independent roles but also interact with each other to provide regulatory fine-tuning, which likely confers a competitive advantage for the organism during exposure to the constantly changing conditions of the environment. We are only beginning to understand the impact that CsrA has on controlling bacterial regulation. Continuing to determine the direct targets of CsrA regulation will help us to understand and predict the responses of bacteria to a variety of environments.

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Chapter 2. Circuitry Linking the Csr and Stringent Response Global Regulatory Systems

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This chapter consists of a manuscript submitted to *Molecular Microbiology*. The manuscript was written by Adrianne N. Edwards and Tony Romeo. Laura Patterson-Fortin constructed the CRIM plasmids used for construction of *lacZ* fusions. Christopher A. Vakulskas performed the CsrB and CsrC Northern blots. Jeffery W. Mercante provided technical assistance. Katarzyna Potrykus performed the *dksA* primer extension. All remaining experiments were performed by Adrianne N. Edwards.

ABSTRACT

CsrA protein regulates several important cellular processes by binding to target mRNAs and altering their translation and/or stability. In *Escherichia coli*, CsrA binds to sRNAs, CsrB and CsrC, which sequester CsrA and antagonize its activity. In this study, RNA that copurified with CsrA (CsrA-His_6) was analyzed by high throughput sequencing. Among the 721 transcripts identified were *relA*, *spoT* and *dksA* of the stringent response system. Gel shift assays confirmed specific, high affinity binding of CsrA to the *relA* mRNA leader and weaker interactions with *dksA* and *spoT*. Studies using reporter fusions, qRT-PCR, and Western blotting revealed that CsrA posttranscriptionally repressed *relA*. CsrA did not affect a reporter fusion containing the *spoT* mRNA leader, although it lowered *spoT* transcript levels. While CsrA activated a *dksA'-'lacZ* translational fusion, *dksA* transcription was negatively autoregulated via a feedback loop that masked the effects of CsrA. DksA and ppGpp modestly activated CsrA, but robustly activated CsrB/C RNAs. Epistasis studies indicated that DksA and CsrA were both required for transcription of *csrB* and *csrC* by the response regulator UvrY. Our findings suggest that this composite network tightens the regulation of target genes responding to both systems and that the Csr system fine-tunes the stringent response.

INTRODUCTION

Bacteria utilize genetic regulatory mechanisms to adapt, compete and survive in response to changing environmental and physiological conditions. Moreover, global regulatory networks permit bacteria to coordinate expression of large sets of genes in multiple operons (Gottesman, 1984, Beisel and Storz, 2010). Two global regulatory

networks, Csr (carbon storage regulator) and stringent response, provide mechanisms for sensing end products of carbon metabolism and nutrient availability, and regulating translation and transcription, respectively (Babitzke and Romeo, 2007, Chavez *et al.*, 2010, Potrykus and Cashel, 2008).

CsrA (and its orthologs, RsmA/E) is a small, dimeric RNA binding protein that post-transcriptionally coordinates expression of a diverse set of genes by positively or negatively regulating the translation and/or stability of target transcripts. In this way, CsrA activates exponential phase processes while repressing several stationary phase functions (Babitzke and Romeo, 2007). CsrA is widely distributed among eubacteria (White *et al.*, 1996, Mercante *et al.*, 2006) and regulates expression of genes for virulence factors (Fortune *et al.*, 2006, Bhatt *et al.*, 2009b), quorum sensing (Cui *et al.*, 1995, Lenz *et al.*, 2005), motility (Wei *et al.*, 2001, Yakhnin *et al.*, 2007), carbon metabolism (Romeo *et al.*, 1993, Sabnis *et al.*, 1995), biofilm formation (Jackson *et al.*, 2002, Wang *et al.*, 2005), cyclic di-GMP synthesis (Jonas *et al.*, 2008) and peptide uptake (Dubey *et al.*, 2003).

CsrA directly regulates gene expression by interacting with the 5' untranslated leaders of target mRNAs at sites characterized by a GGA sequence that is often located within the loop of a short stem-loop structure (Liu *et al.*, 1997, Dubey *et al.*, 2005, Schubert *et al.*, 2007). CsrA typically represses translation initiation by binding at sites that overlap the Shine-Dalgarno sequence (SD), thus competing with the 30S ribosomal subunit and accelerating mRNA degradation (Liu and Romeo, 1997, Baker *et al.*, 2002, Dubey *et al.*, 2003). CsrA can also activate gene expression by stabilizing a bound transcript, as exemplified by the *E. coli flhDC* mRNA (Wei *et al.*, 2001).

The *E. coli* Csr system includes other important regulatory components. CsrB and CsrC are noncoding RNAs that contain multiple CsrA binding sites, which permit them to sequester and antagonize CsrA (Liu *et al.*, 1997, Weilbacher *et al.*, 2003). Transcription of these sRNAs is activated by the two-component signal transduction system (TCS), BarA-UvrY (Suzuki *et al.*, 2002, Weilbacher *et al.*, 2003). Recently, acetate was identified as a physiological stimulus for BarA-dependent signaling (Chavez *et al.*, 2010). This finding suggested a negative feedback loop whereby CsrA activates glycolysis (Sabnis *et al*., 1995), a major source of acetate, and thus indirectly activates *csrB* and *csrC* transcription (Suzuki *et al*., 2002, Weilbacher *et al*., 2003). CsrA participates in at least two other negative feedback loops. CsrA represses expression of *csrD*, which encodes a GGDEF-EAL domain protein that functions along with RNase E and PNPase to mediate CsrB/C turnover (Suzuki *et al.*, 2006). CsrA also binds to the *csrA* mRNA leader and represses its own translation (H. Yakhnin and P. Babitzke, unpublished data).

The stringent response defines another global regulatory network of eubacteria. It is characterized by a rapid downshift in synthesis of stable RNAs, such as rRNA and tRNA, and the upregulation of a number of operons, such as those for amino acid biosynthesis, in response to starvation for amino acids or other nutrients (Potrykus and Cashel, 2008). The effector of this response is the nucleotide secondary messenger guanosine tetraphosphate (ppGpp) (Cashel and Gallant, 1969), which binds to RNA polymerase and positively or negatively affects transcription, depending upon promoter characteristics (Barker *et al.*, 2001). In *E. coli*, ppGpp levels are regulated by two enzymes: RelA and SpoT. RelA is a monofunctional pppGpp/ppGpp synthetase, which

responds to the presence of uncharged tRNA in the ribosomal A-site (Wendrich *et al.*, 2002). SpoT is a bifunctional synthetase/hydrolase, which exhibits weak ppGpp synthetase activity and serves primarily to degrade ppGpp (Cashel *et al.*, 1996). However, SpoT synthesizes ppGpp in response to carbon starvation (Cashel *et al*., 1996), fatty acid starvation (Seyfzadeh *et al.*, 1993, Gong *et al.*, 2002) and other stresses, such as iron limitation (Vinella *et al.*, 2005).

In most cases, regulation by ppGpp requires the transcription factor DksA (Paul *et al.*, 2004a, Paul *et al.*, 2005), which interacts with the secondary channel of RNA polymerase (Paul *et al*., 2004a, Perederina *et al.*, 2004). Together, ppGpp and DksA regulate the expression or activity of a number of global regulators, including the stationary phase sigma factor RpoS (Brown *et al.*, 2002), the extracytoplasmic sigma factor RpoE (Costanzo *et al.*, 2008) and the master regulator of the motility cascade, $FlhD_4C_2$ (Lemke *et al.*, 2009).

Here, we used high throughput sequencing to search for novel, direct targets of CsrA regulation. Among the transcripts identified by our screen were *relA*, *spoT* and *dksA*, which encode proteins that mediate the stringent response. In addition, we developed a reporter system for monitoring post-transcriptional regulation and used this and other approaches to reveal regulatory connections between the Csr and stringent response systems. Our findings reveal complex interactions between the Csr and stringent response systems, which likely allow fine tuning of gene expression by coupling global regulatory responses to substrate availability and end product accumulation. These interconnections may be designed in part to reinforce the direct effects of DksA and ppGpp on transcription of target genes by mediating indirect effects on the post-

transcriptional regulation of these genes by the Csr system. Our data also raise the possibility that the Csr system affects the magnitude of stringent response under certain conditions.

RESULTS

Screening for novel targets of CsrA binding. Several studies suggest that CsrA regulates expression of a large number of transcripts (Lawhon *et al.*, 2003, Burrowes *et al.*, 2006, Brencic and Lory, 2009). Data from these studies were primarily derived from transcriptome analyses, which in large part, did not exclude effects of indirect regulation or examine expression from intergenic regions. To screen for putative direct targets of CsrA binding, recombinant His-tagged CsrA (CsrA-His_6) was ectopically expressed and purified from a *csrA csrB csrC* triple mutant. RNA that was noncovalently bound to CsrA-His_6 was isolated, converted to cDNA, and analyzed by 454 sequencing (Margulies *et al.*, 2005). The results of this analysis suggested that CsrA binds to the RNAs of at least 721 genes (Appendix, Table 1), representing many fundamental physiological and regulatory processes, including the stringent response (Table 2-1). As an additional screen for potential targets of CsrA regulation, proteins from *csrA* mutant and wild-type strains were harvested under four growth conditions and identified by two-dimensional PAGE and MALDI-ToF/ToF mass spectrometry (Appendix, Table 2). Although some of these abundant proteins may not represent directly regulated targets of CsrA, transcripts of several of these proteins also copurified with CsrA (Appendix, Table 3).

CsrA binds to *relA* and *dksA* mRNA leaders with high affinity and specificity. We found that CsrA-His_6 copurified with *relA*, *spoT*, and *dksA* transcripts. The mRNA

leader of *relA* contains six putative CsrA binding sites, while the mRNA leader of *dksA* contains two (Fig. 1A). To determine whether CsrA binds directly to *relA* and *dksA* transcripts, gel shift assays were performed with CsrA protein and in vitro synthesized transcripts containing the 5' untranslated region and part of the coding region of *relA* (+1 to +200 relative to the start of transcription) and *dksA* (+1 to +64 relative to the start of transcription). CsrA binding was initially observed at 5 to 10 nM CsrA for *relA* and at 10 nM CsrA for *dksA* (Fig. 1C, E). As the concentration of CsrA was increased further, additional shifted species were observed for the CsrA-*relA* interaction, suggesting that multiple CsrA proteins were bound to each *relA* transcript at the higher concentrations of CsrA. A nonlinear least-squares analysis of these data yielded apparent K_d values of 17 ± 1 nM CsrA for *relA* and 66 ± 4 nM CsrA for *dksA*.

The specificity of the CsrA-RNA interactions was investigated by performing competition experiments with specific (*relA* and *dksA*) and nonspecific (*Bacillus subtilis trp* leader) unlabeled RNA competitors. *relA* was an effective competitor, whereas the *B. subtilis trp* leader RNA did not compete with the CsrA-*relA* RNA interaction, confirming that CsrA binds to the *relA* mRNA leader with high affinity and specificity (Fig. 1C, D). *dksA* was also an effective competitor for the CsrA-*dksA* RNA interaction. However, in this case, the *B. subtilis trp* leader RNA exhibited weak competition, indicating that the affinity and specificity of the CsrA-*dksA* RNA interaction is not as strong (Fig. 1F).

Analyses of CsrA binding to *gmk* and *rpoZ* mRNA leaders. *spoT* is located within the five gene *spo* operon, *gmk-rpoZ-spoT-trmH-recG*, which is transcribed by three mapped promoters (Gentry *et al.*, 1993; Fig. 1B). Sequence analysis identified a possible CsrA binding site, with modest similarity to consensus, overlapping the *gmk* SD

sequence, and two putative CsrA binding sites in *rpoZ*, one of which overlaps the SD sequence (Fig. 1A, B). Gel shift assays with transcripts containing the 5' untranslated leader and part of the coding region of *gmk* (-42 nt to +25 nt) and *rpoZ* (-28 nt to +33 nt) were used to assess binding. CsrA did not interact with the *gmk* transcript (Fig. 1G) but bound to the *rpoZ* transcript, beginning at 5 to 10 nM CsrA, resulting in an apparent K_d value of 66 ± 4 nM (Fig. 1H).

Experiments with specific (*rpoZ*) and nonspecific (*B. subtilis trp*) unlabeled RNA competitors revealed that *rpoZ* RNA competed effectively for the CsrA-*rpoZ* interaction (Fig. 1I). *trp* leader RNA was also able to compete, although not as effectively as *rpoZ* RNA (Fig. 1I), implying that CsrA interacts with modest specificity to the *rpoZ* mRNA leader.

Repression of *relA* and activation of *dksA* expression by CsrA. To determine whether CsrA regulates *relA* expression, β-galactosidase specific activity from a chromosomal *relA*'-'*lacZ* translational fusion containing the upstream non-coding region through the first three codons was monitored in wild-type and *csrA* mutant strains. Disruption of *csrA* increased expression ~40%, and activity was restored to wild-type levels by complementation when *csrA* was expressed from its native promoter on a low-copy number plasmid (Fig. 2-2A). The promoters driving *relA* expression have been previously mapped (Metzger *et al.*, 1988, Nakagawa *et al.*, 2006), so to further examine CsrA-mediated repression of *relA*, expression from a transcriptional fusion containing the entire upstream non-coding region through the upstream transcriptional start was tested and found to be unaltered by the *csrA* mutation (Fig. 2-2B). In addition, the 5' untranslated region and first three codons of *relA* were cloned downstream from the

constitutive *lacUV5* promoter and in frame with *'lacZ* and integrated into the chromosome. We refer to this and other reporter fusions driven by the *lacUV5* promoter as post-transcriptional or leader fusions (Materials and Methods). Expression from the *relA* leader fusion was increased in the stationary phase in the *csrA* mutant compared to the wild-type strain (Fig. 2-2C). These data imply that CsrA affects *relA* expression post-transcriptionally and that the 5' untranslated region of the *relA* gene mediates this regulation.

In contrast, CsrA had little to no effect on the expression of a P*lacUV5-rpoZ-spoT*'-'*lacZ* leader fusion (Fig. 2-2D). Translational fusions for *gmk*, *rpoZ* or *spoT* containing the native promoters and ribosome binding sites produced extremely low levels of β-galactosidase and were not quantitated.

Expression of the *dksA*'-'*lacZ* fusion was decreased ~30% in the *csrA* mutant and was complemented by ectopic expression of *csrA* (Fig. 2-2E), indicating that CsrA activates *dksA* expression. This effect is examined in more detail below.

Effect of CsrA on *relA*, *spoT* and *dksA* steady-state transcript levels. Because CsrA frequently affects the stability and steady-state levels of its target RNAs (Liu *et al.*, 1995, Wei *et al*., 2001, Wang *et al*., 2005), quantitative real-time reverse transcription polymerase chain reactions (RT-qRT-PCR) on *relA*, *spoT* and *dksA* transcripts were performed with Taqman probes, using 16S rRNA as an internal control. In the *csrA* mutant, *relA* mRNA was increased 1.4-fold in exponential phase (OD_{600} = ~0.5), and 2.0-fold in stationary phase (Fig. 2-3), confirming that repression of *relA* involves an alteration in mRNA levels. In contrast to the leader fusion results, steady-state transcript levels of *spoT* RNA were increased 1.5-fold in exponential phase and 1.8-fold in

stationary phase in the *csrA* mutant (Fig. 2-3), suggesting that CsrA has an indirect effect on *spoT* transcript levels. *dksA* transcript levels remained unchanged between the *csrA* mutant and wild type strains (Fig. 2-3).

RelA accumulates in a *csrA* mutant while GMK and DksA remain unchanged. The effects of CsrA on steady state levels of RelA, GMK and DksA proteins were examined by western blotting. RelA was increased by the *csrA* mutation in both mid-exponential and stationary phases (Fig 4A), confirming the results obtained with the translational and leader reporter fusions. Steady-state protein levels of GMK, the product of the first gene of the *spo* operon (Fig. 2-1A), were unchanged in the *csrA* mutant, indicating that CsrA has no effect on *gmk* expression (Fig. 2-4B). Similar to *dksA* transcript levels, DksA protein levels were comparable between wild-type and *csrA* mutant strains (Fig. 2-4C). A possible explanation for this result is that while CsrA directly binds *dksA* mRNA and activates *dksA* expression, another factor(s) may compensate for these effects. Analyses of SpoT protein levels had technical complications.

Because CsrA bound specifically to the *relA* mRNA leader, repressed *relA* expression post-transcriptionally, and appeared to indirectly repress *spoT* expression, we examined ppGpp synthesis and accumulation in *csrA* mutant and wild-type strains using thin-layer chromatography. There was no significant difference in basal or serine hydroxamate-induced ppGpp levels in *csrA* mutant and wild-type strains (Appendix, Fig. 1). We suspect that the relatively modest stringent response that was triggered by serine hydroxamate in EZ Rich Defined Medium was insufficient to fully utilize the excess RelA protein present in the *csrA* mutant. However, growth defects of the *csrA* mutant in

minimal media prevented us from testing ppGpp levels under minimal media conditions, which would likely induce a more robust stringent response.

DksA negatively regulates its own transcription. Because CsrA bound specifically to *dksA* mRNA and a *dksA'-'lacZ* fusion responded modestly to CsrA, yet *dksA* transcript and protein levels were similar in the *csrA* mutant and wild-type strains, we sought to clarify the influence of CsrA on *dksA* expression. We reasoned that many global regulators are autoregulatory (H. Yakhnin and P. Babitzke, unpublished data), which might have complicated these analyses. We examined expression of a *dksA'-'lacZ* translational fusion and observed a ~2.5-fold increase in β-galactosidase activity in a *dksA* mutant, suggesting that DksA negatively regulates its own expression (Fig. 2-5A). β-galactosidase activity from the *dksA'-'lacZ* fusion was reduced to wild-type levels when *dksA* was ectopically expressed from an IPTG-inducible promoter and was further reduced upon addition of IPTG (Fig. 2-5A).

To determine if DksA negative autoregulation influences the effect of CsrA on *dksA* expression, activity of the *dksA'-'lacZ* translational fusion was compared in isogenic wild-type, *dksA*, *csrA* and *dksA csrA* strains. The *dksA csrA* double mutant showed ~50% less β-galactosidase activity compared to the *dksA* single mutant (Fig. 2-5B), confirming that CsrA activates DksA expression and indicating that this effect can be masked by DksA autoregulation. The *dksA csrA* double mutant exhibited an increase in β-galactosidase activity compared to the *csrA* single mutant (Fig. 2-5B), indicating that DksA negative autoregulation occurs independently of CsrA regulation.

To assess the levels at which DksA and CsrA regulate *dksA* expression, a *dksA-lacZ* transcriptional fusion and a P*lacUV5-dksA'-'lacZ* leader fusion were examined.

Expression from the transcriptional fusion was increased ~2.5-fold in the *dksA* mutant, similar to the translational fusion (compare Fig. 2-5C and 2-5B), revealing that negative autoregulation is mediated at the level of transcription and that the promoter DNA in this construct is sufficient for this regulation. Expression from this fusion in the *dksA* mutant was complemented by ectopic expression of *dksA* from an IPTG-inducible promoter (Appendix, Fig. 2A). In contrast, there was no significant difference in expression of the *dksA-lacZ* transcriptional fusion between the *csrA* mutant and wild-type strains or the *dksA* and *dksA csrA* double mutant strains, suggesting that CsrA regulates *dksA* post-transcriptionally (Fig. 2-5C). Furthermore, we observed a slight decrease in β-galactosidase activity from the P*lacUV5-dksA'-'lacZ* leader fusion in the *dksA* mutant (Fig. 2-5D), demonstrating that the 5' leader of *dksA* mRNA does not support negative autoregulation, and confirming that DksA autoregulation is mediated at the level of transcription. Similar to expression of the translational fusion, β-galactosidase activity from the P*lacUV5-dksA'-'lacZ* was decreased ~50% in a *csrA* mutant, confirming that CsrA activates *dksA* post-transcriptionally via the 5' leader of *dksA*, and that CsrA regulation occurs independently of DksA negative autoregulation (Fig. 2-5D). As expected from these findings, ectopic expression of *dksA* did not substantially alter expression from the P*lacUV5-dksA'-'lacZ* leader fusion (Appendix, Fig. 2B).

Primer extension analysis of *dksA* mRNA was conducted to further examine DksA negative autoregulation. Transcription of *dksA* from the chromosome was below the level of detection in our hands, even after long exposures (Fig. 2-6B, Lanes 1, 4-5). Using a *dksA* mutant, we examined ectopic expression of *dksA* transcripts from strains containing plasmids that expressed either the wild-type *dksA* allele or a *dksA* allele that

contained amino acid substitutions in the two aspartic acid residues (D71N D74N) that are critical for DksA activity (Perederina *et al*., 2004). Both alleles were expressed from the native *dksA* promoters present in these plasmids. The wild-type *dksA* allele produced two transcripts, P1 and P2, in low abundance (Fig. 2-6B, Lane 3), which were increased in the strain expressing the defective *dksA* D71N D74N allele (Fig. 2-6B, Lane 2). This finding confirmed that a functional DksA protein is required for negative autoregulation and that the P1 and P2 transcripts are both subject to this regulation. The P1 promoter has been characterized previously (Kang and Craig, 1990), and in vitro transcription analysis resulted in two identically mapped transcripts (data not shown).

To confirm that DksA directly repressed transcription from the putative P1 and P2 promoters, we attempted in vitro transcription using a linear template and purified DksA and/or ppGpp, but were unable to demonstrate DksA-mediated inhibition (data not shown), suggesting that an additional factor may be required or that this effect is indirect.

DksA and ppGpp modestly activate *csrA* expression. The Csr and stringent response systems share a number of regulatory targets, e.g. *glgCAP* (Romeo and Preiss, 1989; Romeo *et al*., 1990; Liu and Romeo, 1997) and *flhDC* (Wei *et al*., 2001, Lemke *et al*., 2009). Furthermore, the Csr system possesses multiple feedback loops in which its components both control and are controlled by other factors. Thus, we reasoned that the stringent response components might regulate expression of the genes of the Csr system. Western blot analysis demonstrated that CsrA protein levels were reduced ~50% in *dksA* mutant and ppGpp^0 strains, and CsrA protein levels in the *dksA* mutant were restored to wild-type or higher levels by ectopic expression of *dksA* (Fig. 2-7A), suggesting that DksA and ppGpp activate *csrA* expression. β-galactosidase activity from a *csrA*'-'*lacZ*

translational fusion was reduced in the *dksA* and *relA spoT* (ppGpp^0) mutants (Fig. 2-7B). Activity was restored to wild-type levels when *dksA* was expressed ectopically from an IPTG-inducible plasmid in the *dksA* mutant, and activity was further increased by the addition of IPTG (Fig. 2-7B).

We recently found that *csrA* transcription is directly activated by RpoS (σ^S), the stationary phase and general stress response sigma factor (H. Yakhnin and P. Babitzke, unpublished data). Furthermore, ppGpp and DksA are required for full expression of RpoS (Brown *et al*., 2002, Hirsch and Elliott, 2002). We therefore asked whether DksA activation of *csrA* expression requires RpoS. Expression of the *csrA'-'lacZ* fusion was reduced ~60% in the *rpoS* and *dksA* single mutant strains, while the *rpoS dksA* double mutant exhibited a further reduction in β-galactosidase activity (Fig. 2-7C). Ectopic expression of *dksA* from an IPTG-inducible plasmid partially restored β-galactosidase activity in the double mutant, but not to wild-type levels, suggesting that activation of *csrA* expression by DksA depends in part on RpoS.

Transcription of *csrA* is driven by at least three promoters (H. Yakhnin and P. Babitzke, unpublished data). RpoS directs *csrA* transcription from P3, while P1 and P5 are transcribed by the housekeeping sigma factor, σ^{70}. Using transcriptional *lacZ* fusions for each promoter, we found that β-galactosidase activity from the P1-*csrA*-*lacZ* and P5-*csrA*-*lacZ* fusions were unaffected by the *dksA* mutant, whereas there was a threefold reduction in activity from the P3-*csrA*-*lacZ* fusion in the *dksA* mutant (Fig. 2-7D-F). These data further indicate that DksA activates *csrA* expression primarily through RpoS-driven transcription of P3. Interestingly, ppGpp was required for full activity of the P1-

csrA-lacZ and P3-*csrA-lacZ* fusions (Fig. 2-7D-F), suggesting that ppGpp likely influences *csrA* expression through RpoS as well as through an additional pathway.

DksA and ppGpp activate *csrB* and *csrC* expression. To further examine effects of DksA and ppGpp on the Csr system, steady-state CsrB and CsrC RNA levels were determined by Northern blotting. Both RNAs were reduced 10-fold in *dksA* and ppGpp^0 mutant strains (Fig. 2-8A). Additionally, *csrB* and *csrC* transcript levels were reduced in the *csrA* mutant, as previously determined (Fig. 2-8A, Suzuki *et al*., 2002, Weilbacher *et al*., 2003). To further characterize this regulation, we measured β-galactosidase activity from *csrB-lacZ* and *csrC-lacZ* transcriptional fusions. Activity from both fusions was decreased substantially in the *dksA* mutant and ppGpp^0 strains (Fig. 2-8B, C), indicating that DksA and ppGpp activate *csrB* and *csrC* transcription. Furthermore, activity was restored to near wild-type levels in the *dksA* mutant strain by complementation of *dksA* using an IPTG-inducible plasmid, and activity was increased further by the addition of IPTG (Fig. 2-8B, C).

CsrA indirectly activates expression of CsrB and CsrC sRNAs, through the BarA-UvrY TCS, which directly activates their transcription (Suzuki *et al.*, 2002, Weilbacher *et al.*, 2003). BarA is a membrane bound tripartite sensor kinase, while UvrY functions as its cognate response regulator (Pernestig *et al.*, 2001). Epistasis studies were conducted to determine whether the effects of DksA on *csrB* and *csrC* expression were dependent upon UvrY. Ectopic expression of *uvrY* from a multicopy plasmid restored *csrB-lacZ* and *csrC-lacZ* expression in a *dksA uvrY* double mutant while ectopic expression of *dksA* did not, implying that *dksA* lies upstream of *uvrY* in this signaling pathway (Fig. 2-8D, E). Expression from a *uvrY*'-'*lacZ* translational fusion was unaffected in the *dksA* mutant

strain (Appendix, Fig. 3), suggesting that DksA affects UvrY activity, not *uvrY* expression. CsrA is required for normal signaling through the BarA-UvrY TCS (Suzuki *et al*., 2002), and since DksA activates CsrA, epistasis studies were performed to determine if DksA activated *csrB* and *csrC* expression through CsrA. In this case, neither overexpression of CsrA nor DksA fully restored *csrB-lacZ* or *csrC-lacZ* expression in a *dksA csrA* double mutant (Fig. 2-8F, G). These findings reveal that both CsrA and DksA are necessary for full expression of *csrB* and *csrC*, and suggest that their effects are not mediated in series (i.e. sequentially).

DksA, ppGpp and CsrA effects on acetate production. Because acetate acts as a stimulus for BarA-UvrY signaling, we asked whether CsrA, DksA or ppGpp might affect *csrB* and *csrC* expression via effects on acetate accumulation under our growth conditions (LB). Acetate levels were monitored through the growth curve in isogenic wild-type, *csrA*, *dksA*, *csrA dksA*, and ppGpp^0 strains. We observed no significant difference in acetate levels in any of these strains (Appendix, Fig. 4A), suggesting that under these conditions, ppGpp, DksA and CsrA signaling may affect *barA* expression or UvrY activity through an unknown factor. Because CsrA activates glycolysis (Sabnis *et al*., 1995), acetate accumulation was also monitored in a medium requiring glycolytic metabolism for growth (Kornberg medium; Appendix, Fig. 4B). These studies revealed that the *csrA*, *dksA* and ppGpp^0 mutants accumulated less acetate than the parent strain. However, the effects were apparently insufficient to account for strong regulation of *csrB* and *csrC* expression by these genes. A *barA'-'lacZ* translational fusion was constructed to monitor effects of ppGpp, DksA and CsrA on *barA* expression, but expression from this fusion was too low to permit quantitation.

DISCUSSION

The motivation for these studies was our observation that the mRNAs for the three stringent response genes, *relA*, *spoT* and *dksA*, copurified with a recombinant CsrA protein. While the Csr and stringent response systems were known to govern a number of the same genes and processes, the present study revealed novel regulatory interactions within and among the components of these global regulatory systems (summarized in Fig. 2-9). As discussed below, we propose that these interactions provide a fine-tuning role for the Csr system during stringent response, and more importantly, they downregulate CsrA activity upon induction of the stringent response. Thus, the direct transcriptional effects of stringent response, mediated by DksA and ppGpp, on a subset of its target genes are reinforced by indirect effects on their translation and/or mRNA stability via the Csr system.

Despite its strong binding interactions with *relA* and *dksA* transcripts (Fig. 2-1B-E), the effects of CsrA on *relA* and *dksA* expression were modest (Figs. 2-2 though 2-5). These results differ from the much stronger effects of CsrA on various structural genes, e.g. for glycogen and poly-β-1,6-*N*-acetyl-glucosamine biosynthesis, which are mediated via CsrA binding affinities that are similar to *relA* mRNA (Romeo *et al*., 1993, Baker *et al*., 2002, Wang *et al*., 2005). Such high affinity binding in the context of modest regulation is indicative of a fine-tuning role for CsrA in stringent response. We envision a role for the Csr system in enhancing stringent response under conditions that require strong regulation, triggered by an abundance of uncharged tRNAs. Activation of *csrB* and *csrC* transcription (Fig. 2-8) under this condition should greatly increase CsrB and CsrC RNA levels, thus relieving CsrA repression of *relA*. The additional RelA protein

that accumulates through these effects should poise the cell for an enhanced response to abundant uncharged tRNAs.

In contrast to the modest to negligible effects of CsrA on stringent response components, ppGpp and DksA exhibited robust regulation (10-fold) of CsrB and CsrC levels via the response regulator UvrY (Fig. 2-8). Because DksA and ppGpp modestly activated *csrA* expression (Fig. 2-7), the net effect of increased ppGpp accumulation through the induction of stringent response should be to inhibit CsrA activity. Furthermore, one molecule of CsrB or CsrC RNA is capable of sequestering ~10 or 5 CsrA dimers, respectively (Babitzke and Romeo, 2007), thus magnifying the indirect inhibitory effects of ppGpp on CsrA activity. The implication of these findings is that the regulation of genes that respond oppositely to CsrA versus ppGpp should be enhanced indirectly by decreased CsrA activity due to elevated CsrB and CsrC levels during stringent response. Previous studies demonstrated that CsrB and CsrC RNA levels are greatly elevated in minimal media and decline drastically upon amino acid supplementation, while CsrA exhibited only modest effects under these conditions (Jonas and Melefors, 2009b). These physiological observations are fully consistent with our genetic findings on the effects of ppGpp and DksA on CsrA, CsrB, and CsrC levels, and strengthen this model for the workings of this composite network.

Clearly, there is substantial regulatory overlap by the stringent response and Csr global regulatory systems. Altogether, 40% of the genes whose transcripts copurified with CsrA (Table 2-1) were found previously to respond to ppGpp and/or DksA (Aberg *et al*., 2009). Even more striking, 68% of the genes of COG C, energy production and conversion, were found in to be common in these two studies (Table 2-1). These values

represent minimum estimates of the overlap, as only exact gene matches in the two data sets were tabulated.

To illustrate our model with specific examples, glycogen synthesis and *glgCAP* expression are directly activated by ppGpp (Romeo and Preiss, 1989, Romeo *et al*., 1990) and directly repressed by CsrA (Romeo *et al*., 1993, Baker *et al*., 2002). During stringent response, increased CsrB and CsrC levels should relieve CsrA repression, reinforcing the direct effects of ppGpp on *glgCAP* expression. Indeed, CsrA also represses *cstA* translation (Dubey *et al*., 2003), which is a carbon starvation response gene, required for efficient peptide uptake (Schultz and Matin, 1991). Thus, derepression of CsrA-mediated *cstA* translation during stringent response may facilitate nutrient scavenging. Furthermore, many mRNAs of amino acid and peptide uptake and metabolism genes copurified with CsrA (Table 2-1; Appendix, Table 1), raising the possibility that CsrA plays global regulatory role in peptide and amino acid uptake.

In a similar fashion, expression of *flhDC*, which encodes the master regulator of the motility cascade, is transcriptionally repressed by DksA and ppGpp (Lemke *et al*., 2009) and activated post-transcriptionally by CsrA binding to and stabilizing *flhDC* mRNA (Wei *et al*., 2001). In this case, the indirect effects of stringent response on the Csr system should reinforce its direct negative effects on motility by decreasing CsrA activity. Thus, Csr and ppGpp are proposed to function together under starvation conditions to promote nutrient acquisition and sequestration of intracellular carbon and energy, while repressing energy intensive flagellum synthesis and motility, under starvation conditions.

The effects of DksA and ppGpp on expression and levels of CsrB and CsrC RNAs required CsrA and the response regulator UvrY (Fig. 2-8), which directly stimulates *csrB* and *csrC* transcription (Suzuki *et al*., 2002, Weilbacher *et al*., 2003). Because DksA and ppGpp did not affect *uvrY* expression (Appendix, Fig. 3), and ectopic expression of *uvrY* restored *csrB* and *csrC* expression in a *dksA uvrY* mutant background, it is apparent that DksA somehow affects UvrY activity. In *Pseudomonas aeruginosa* , the BarA sensor-kinase ortholog (GacS), as well as two other sensors that do not have orthologs in *E. coli*, RetS (Goodman *et al.*, 2004, Goodman *et al.*, 2009) and LadS (Ventre *et al.*, 2006), regulate the activity of the UvrY ortholog (GacA). Whether BarA alone or other regulatory factors in *E. coli* mediate DksA and ppGpp effects on *csrB* and *csrC* expression will require additional investigation.

Previous studies revealed that DksA protein levels remain relatively constant throughout growth (Brown *et al*., 2002, Paul *et al*., 2004a, Rutherford *et al.*, 2007). A recent stringent response model suggests that since DksA levels are constant, ppGpp levels mediate stringent control upon nutritional stress (Dalebroux *et al.*, 2010). Our results further demonstrated that DksA levels are regulated by a negative feedback loop, whereby DksA represses its own transcription. Autoregulation of *dksA* tended to mask the opposing effect of CsrA (Fig. 2-5). Such an effect is consistent with negative feedback loops in promoting homeostasis (Becskei and Serrano, 2000), and is not unexpected for a gene whose product is maintained within a relatively narrow range.

We previously demonstrated that the Csr system of *E. coli* contains multiple negative feedback loops (Suzuki *et al*., 2002, Weilbacher *et al*., 2003, Suzuki *et al*., 2006, Fig. 2-9), which may provide distinct advantages for this regulatory network. Negative

feedback loops produce graded responses, thus reducing cell-cell variability (Nevozhay *et al.*, 2009) and stochastic events and noise (Becskei and Serrano, 2000), and can also accelerate regulatory responses (Rosenfeld *et al.*, 2002). The critical negative feedback loop of the Csr system involves a multistep pathway from CsrA to *csrB* and *csrC* expression via the BarA-UvrY TCS (Fig. 2-9). The recent finding that acetate is a stimulus for BarA signaling (Chavez *et al*., 2010), coupled with previous studies showing that CsrA activates glycolysis (Sabnis *et al*., 1995), suggested that glycolysis might provide the link between CsrA and BarA activity. However, we determined here that mutations affecting CsrA, DksA or ppGpp have negligible or modest affects on acetate accumulation under gluconeogenic or glycolytic growth conditions, respectively (Appendix, Fig. 4). Thus, effects of these genes on acetate accumulation cannot account for their impact on *csrB* and *csrC* expression, and additional studies will be necessary to define this negative feedback loop, which provides a central link between the Csr and stringent response systems.

A surprising observation was that CsrA had opposite effects on *relA* and *dksA* expression (e.g. Fig. 2-2). Although ppGpp and DksA often potentiate each other's effects, there have been several reports of independent and even antagonistic effects of ppGpp and DksA (Magnusson *et al.*, 2007, Aberg *et al.*, 2008, Aberg *et al.*, 2009, Lyzen *et al.*, 2009, Merrikh *et al.*, 2009). Indeed, $ppGpp^0$ and *dksA* mutant strains exhibit multiple, but not identical, amino acid auxotrophies (Brown *et al*., 2002, Potrykus *et al.*, 2010b), perhaps signifying alternative gene expression roles for ppGpp and DksA. Presently, it is not clear how the modest effect of CsrA on *dksA* expression impacts such

differential genetic regulation or whether negative feedback by DksA itself overshadows CsrA effects under all physiological conditions.

Given that stringent response was only one of many transcriptional regulatory systems whose mRNAs copurified with CsrA (Table 2-1, Appendix, Table 1), the complexity of the regulatory circuitry surrounding the Csr system is undoubtedly vast. Supporting this hypothesis, transcripts for regulatory factors necessary for critical cellular processes were identified, including alternative sigma factors (*rpoE* and *rpoH*), universal stress proteins (*uspA*, *uspB* and *uspD*) and the proteins that mediate catabolite repression (*crp* and *cyaA*). A recent study reveals additional complexity within the Csr system, consisting of direct feedback repression by CsrA of its own translation and indirect activation of its transcription via RpoS (H. Yakhnin and P. Babitzke, unpublished data). We expect that the high-throughput sequencing of CsrA-bound transcripts from the present study will spur additional research on the complex circuitry and global regulatory role of Csr system.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids and growth conditions. All *E. coli* K-12 strains, plasmids and bacteriophage used in this study are listed in Appendix, Table 4. Unless otherwise indicated, bacteria were grown at 37°C, shaking at 250 rpm, in Luria-Bertani (LB) medium (Miller, 1972). Media were supplemented with antibiotics, as needed, at the following concentrations: kanamycin, 100 μg/ml; ampicillin, 25 μg/ml; spectinomycin, 25 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 10 μg/ml, except

that ampicillin was used at 100 μg/ml during the construction of *lacZ* fusion plasmids. P1*vir* transduction was performed as previously described (Miller, 1972).

Construction of transcriptional, translational and leader fusions and integration into the chromosome. Plasmids pRELZ and pDKSZ were constructed by PCR amplification of an 888 bp fragment containing the upstream regulatory region through the first three codons of *relA* and 628 bp fragment containing the upstream regulatory region through the first three codons of *dksA* using the primer pairs relA-F/relA-R and dksA-F/dksA-R. Table S5 lists primer sequences. The products were digested with *Eco*RI and *Bam*HI and cloned into the same sites of pMLB1034 to create *relA*'-'*lacZ* and *dksA*'-'*lacZ* translational fusions. Both fusions were moved into the *E. coli* CF7789 chromosome using λInCh1 and confirmed by PCR analysis, as previously described (Boyd *et al.*, 2000).

A series of plasmids using the pAH125 plasmid backbone (Haldimann and Wanner, 2001) were created for the generation of various *lacZ* fusions. pLFT was generated to replace the kanamycin resistance marker of pAH125 with ampicillin resistance. The *bla* PCR product was generated using the primer pair AmpRF/AmpRR and plasmid pUC19 DNA as template. Purified PCR product was digested with *Cla*I and *Not*I. *Cla*I/*Not*I digestion of pAH125 generated three fragments; the 1.2 kb and 3.5 kb DNA fragments were purified from 1X TAE agarose gels and ligated with the *bla* PCR product to generate the *lacZ* transcriptional fusion vector, pLFX. pLFX was used to generate a *lacZ* translational fusion vector. *BssH*II/*Eco*RI digestion of pLFX and pMLB1034 liberated 4.3 kb and 1.5 kb fragments, respectively. These fragments were gel purified and ligated to generate the *lacZ* translation fusion vector, pLFT. pLFT was also

used to create a post-transcriptional (or leader) fusion vector containing the constitutive *lacUV5* promoter. The *lacUV5* promoter was amplified using the primer pair LPF-19/LPF-20 and pUV5moaA (L. Patterson-Fortin and T. Romeo, unpublished data) plasmid DNA as template, and the purified PCR product was digested with *Pst*I and *Eco*RI and cloned into *Pst*I/*Eco*RI digested pLFT plasmid DNA. The resulting plasmid pUV5 allows construction of *lacZ* translational fusions expressed from the constitutive *lacUV5* promoter.

Plasmid pPFINT was constructed to replace the ampicillin resistance marker of the parent plasmid, pINT-ts (Haldimann and Wanner, 2001), with tetracycline resistance. The *tet* PCR product was generated using the primer pair LPF-21/LPF-22 and pBR322 plasmid DNA as template, and the purified PCR product was digested with *Cla*I. pINT-ts was digested with *Bgl*I, blunt-ended using DNA polymerase I, and digested with *Cla*I. The resulting 4 kb fragment was gel purified and ligated with the *Cla*I-digested *tet* PCR product to generate pPFINT.

The plasmids pRELZtxn and pDKSZtxn were constructed by PCR amplification of a 255 bp fragment containing the upstream regulatory region of *relA* from –880 to –625 relative to the translational start and a 66 bp fragment containing the upstream regulatory region of *dksA* from -118 to -52 relative to the translational start using the primer pairs relA-F-txn/relA-R-txn and dksA-F-txn/dksA-R-txn, respectively. The products were digested with *Pst*I and *Bam*HI and cloned into the same sites of pLFX to create *relA-lacZ* and *dksA-lacZ* transcriptional fusions. Plasmids pRELZplac, pDKSZplac, pGMKZplac, and pSPOZplac were constructed by PCR amplification of the 5’ untranslated mRNA leaders of *relA*, *dksA*, *gmk*, and *rpoZ-spoT* using the primer pairs

relA-F-plac/relA-R, dksA-F-plac/dksA-R, gmk-F-plac/gmk-R-plac, and spoT-F-plac/spoT-R-plac, respectively, to create leader fusions under the control of the *lacUV5* promoter. These products were digested with *Eco*RI and *Bam*HI and cloned into the same sites of pUV5. All fusions were integrated into the CF7789 chromosome as previously described (Haldimann and Wanner, 2001). All oligonucleotide primers used in this study (Table 5) were synthesized by Integrated DNA Technologies Inc., Coralville, Iowa, and all cloned DNA inserts were sequenced to confirm the absence of mutations.

Cloning of the *csrA* gene. The plasmid pCsrA, encoding the *csrA* gene including 400 bp upstream from the start of *csrA* translation through the coding region, was constructed by PCR amplifying the *csrA* gene with primers csrA-F-pGB2 and csrA-R-pGB2. The PCR product was digested with *Hind*III and *Eco*RI and cloned into the same sites of pGB2 (Churchward *et al.*, 1984).

RNA Electrophoretic 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 *relA* and *gmk* transcript were PCR-amplified from MG1655 genomic DNA using the primer pairs relA-F-T7/relA-R-T7 and gmk-F-T7/gmk-R-T7. DNA templates for *rpoZ* and all *dksA* transcripts were produced by annealing primer pairs rpoZ-T7/GC-rpoZ-T7, dksA-T7/GC-dksA-T7, dksA-BS1/GC-dksA-BS1, dksA-BS2/GC-dksA-BS2, and dksA-BS1-2/GC-dksA-BS1-2. RNA was synthesized *in vitro* using the MEGAshortscript kit (Ambion, Austin, TX) and purified PCR products (for *relA* and *rpoZ*), annealed DNA primers (*gmk* and all *dksA* transcripts) or linearized plasmid pPB77 (for nonspecific *trp*

leader RNA from *B. subtilis*) (Babitzke *et al.*, 1994) as templates, and RNA was gel purified. 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 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 10% native TBE polyacrylamide gels, and radioactive bands were visualized with a Molecular Dynamics phosphorimager. Free and bound RNA species were quantified with Quantity One (Bio-Rad, Hercules, CA), 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).

β-galactosidase and total protein assays. β-galactosidase activity was determined as described previously (Romeo *et al*., 1990), except that 100 µl chloroform and 50 µl 0.01% SDS were used for cell membrane permeabilization. Total cellular protein was measured by the bicinchoninic acid (BCA) assay with bovine serum albumin as the protein standard (Pierce Biotechnology, Rockford, IL). All analyses compared isogenic strains, derived from CF7789.

rt-qRT-PCR. To measure steady-state levels of *relA*, *spoT* and *dksA* transcripts, wild-type (MG1655) and *csrA* mutant strains were grown at 37°C in LB medium, and

cells were harvested in exponential ($OD_{600} \approx 0.5$) and early stationary phases. Total RNA was isolated using the Ribo-pure Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA was quantified by its absorbance at 260 and 280 nM, and rRNA integrity was analyzed on formaldehyde agarose gels. Real-time quantitive reverse-transcription PCR (rt-qRT-PCR) was performed using the iScript one-step RT-PCR Kit (Bio-Rad, Hercules, CA), the primer pairs relA-F-taqman and relA-R-taqman, spoT-F-taqman and spoT-R-taqman, and dksA-F-taqman and dksA-R-taqman and the probes relA-6FAM-BHQ1, spoT-6FAM-BHQ1, and dksA-6FAM-BHQ1, which were 5'-end labeled with 6-carboxyfluorescein (6FAM) and 3'-end labeled with Black Hole Quencher 1(BHQ1). Reactions were conducted using the Lightcyler 480 (Roche Diagnostics) under the following conditions: 50°C for 10 min, 95°C for 5 min, and 40 cycles of 95°C for 15 sec and 60°C for 30 seconds with real time measurements taken at the 60°C step. Each reaction was performed in triplicate in two independent experiments, each time with 100 ng and 10 ng template RNA, and the mean values of the two experiments were determined. A reaction lacking reverse transcriptase was included for each sample, which served as a control for DNA contamination. For normalization of *relA*, *spoT* and *dksA* transcript levels, rt-qRT-PCRs were performed with each sample for 16S rRNA quantitation using the primer pair 16S-Fw and 16S-Rw and the probe 16S-6FAM-BHQ1 (Baker *et al*., 2007). The reaction conditions for rt-qRT-PCR of 16S rRNA were identical to the other transcripts except that 1 ng and 0.1 ng of RNA were used for each example. The PCR product identities were confirmed by electrophoresis on 1% agarose gels with ethidium bromide staining. The $2^{-\Delta\Delta CT}$ method was used to calculate relative transcript levels (Livak and Schmittgen, 2001).

Western blotting. Cultures for western blot analyses were grown at 37°C with shaking, and at indicated time points, cells from 1 ml of culture were concentrated and resuspended in Lysis Buffer A (90 mM Tris-HCl, 2% SDS, pH 6.8). Samples were boiled for 3 min, cell debris was removed by centrifugation, and the supernatant saved and assayed for total protein using the BCA assay with bovine serum albumin as the protein standard (Pierce Biotechnology, Rockford, IL). 10 μg total protein was applied to 8-15% SDS-polyacrylamide gels and transferred to Immunoblot PVDF membrane (Bio-Rad, Hercules, CA) after separation. RelA, DksA and CsrA proteins were detected as previously described (Brown *et al*., 2002, Gudapaty *et al.*, 2001). GMK antibody was obtained from the Cashel lab and was raised against GMK protein purified as previously described (Gentry *et al*., 1993). Protein bands were quantified with Quantity One (Bio-Rad, Hercules, CA).

Primer Extension. Primer extension was carried out as previously described (Potrykus *et al.*, 2010a) except that 10 ug of total RNA was used. RNA was isolated from the following strains: CF9239 (MG1655 dksA::kan), CF9239/pJK537 and CF9239/pHM1684.

Northern Blotting. Bacterial cells were grown in LB at 37°C with shaking, and cellular RNA was stabilized by the addition of 2 vol of the RNAprotect Bacteria Reagent (Qiagen). Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen) and the resulting RNA (5 μg) was separated on 5% polyacrylamide gels containing 7 M urea, and transferred to positively charged nylon membranes (Roche Diagnostics) by electroblotting using the Mini Trans-Blot Cell (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. RNA was cross-linked to nylon membranes by exposure to

UV light (120,000 μJ) followed by baking at 80° C for 30 min. Membranes were then blotted with DIG-labeled anti-sense RNA probes using the DIG Northern Starter Kit (Roche Diagnostics) according to the manufacturer's instructions. Blots were developed using the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA) and densitometry was performed using Quantity One image analysis software (Bio-Rad, Hercules, CA).

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Table 2-1. COG assignments for RNAs that copurified with CsrA-His$_6$

COGs	Description	Number	Percentage of genes in COG	Overlap with *dksA* and ppGpp responsive genes[a]
J	Translation, ribosomal structure and biogenesis	37	20.3	16
K	Transcription	40	12.2	13
L	DNA replication, recombination and repair	27	11.4	7
D	Cell division and chromosome partitioning, cell cycle control	7	19.4	1
O	Posttranslational modification, protein turnover, chaperones	34	24.3	14
M	Cell wall, membrane and envelope biogenesis	39	15.9	5
N	Cell motility and secretion	8	7.0	3
P	Inorganic ion transport and metabolism	31	10.9	16
T	Signal transduction mechanisms	20	9.5	9
C	Energy production and conversion	69	22.2	47
G	Carbohydrate transport and metabolism	61	14.3	25
E	Amino acid transport and metabolism	68	15.2	34
F	Nucleotide transport and metabolism	13	14.1	7
H	Coenzyme transport and metabolism	23	15.1	6
I	Lipid transport and metabolism	19	17.6	7
V	Defense mechanisms	3	6.0	0
U	Intracellular trafficking and secretion	5	3.7	3

Q	Secondary metabolites biosynthesis, transport and catabolism	6	7.4	1
R	General function prediction only	42	8.1	15
S	Function unknown	25	7.7	9
	COG Unassigned; Uncharacterized	136		46
RNAs	Regulatory RNAs, tRNAs	8		NA
Total		721		284

[a]Data for genes within each COG, which also respond to ppGpp, *dksA*, or both, were from Aberg *et al*., 2009. Only exact gene matches (not operons) were tabulated.

FIGURE LEGENDS

Fig. 2-1. Gel shift analyses of CsrA binding to mRNA leaders of *relA*, *dksA*, *gmk* and *rpoZ*. (A) The nucleotide sequences of *relA*, *gmk*, *rpoZ* and *dksA* mRNA leaders are shown with putative CsrA binding sites underlined. Positions of the experimentally determined transcriptional starts are depicted as capital letters, and positions of the Shine-Dalgarno (SD) sequences and initiation codons (Met) are shown. (B) The structure of the *spo* operon; arrows denote promoters while asterisks indicate potential CsrA binding sites. (C-I) RNA gel shifts: 5'-end-labeled transcripts (80 pM) were incubated with CsrA at the concentration indicated below each lane. Reactions were performed in the absence (C, E, G and H) or presence (D, F and I) of specific or nonspecific (*trp* from *B. subtilis*) unlabeled RNA competitors, at the concentrations shown. The positions of free (F) and bound (B) RNA are shown. (C, D) CsrA-*relA* interaction. (E, F) CsrA-*dksA* interaction. (G) CsrA-*gmk* interaction. (H, I) CsrA-*rpoZ* interaction.

Fig. 2-2. Effects of *csrA* on expression of *relA*, *spoT* and *dksA* reporter fusions. Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420} / mg protein). The values represent the average of two independent experiments. Error bars depict standard error of the mean. (A-E) Activity of indicated fusions in wild-type, ■; *csrA*, ▲; *csrA* pGB2 (empty vector), ▼; and *csrA* pCsrA (*csrA*$^+$), ●. (E) Strain identities were identical, except pBR322 (empty vector, ▼) and pCRA16 (*csrA*$^+$, ●) were used for complementation. Growth curves are represented by open symbols.

Fig. 2-3. Effect of *csrA* on *relA*, *spoT* and *dksA* transcript levels, measured by real-time qRT-PCR, using strains MG1655 (wild-type) and TRMG1655 (*csrA* mutant). Samples were taken at exponential phase (OD_{600} = 0.5) and stationary phase (at 8 hours of growth). The values represent the average of two independent experiments. Error bars depict standard error of the mean (** $P < 0.001$).

Fig. 2-4. Effect of *csrA* disruption and complementation on RelA, GMK and DksA protein levels by western blot analyses. Shown below each representative blot, the PVDF membrane was stained using the MemCode™ Reversible Protein Stain Kit (Pierce Biotechnology, Rockford, IL) and imaged as an internal loading control. Protein was harvested at exponential (OD_{600} = 0.5) and stationary phases (after 8 hours of growth). (A) RelA; Lane 1,6 MG1655; Lane 2,7 *csrA*::*kan*; Lane 3,8 *csrA*::*kan* pGB2; Lane 4,9 *csrA*::*kan* pCsrA; Lane 5, *relA::kan spoT::cat*. (B) GMK; Lane 1,5 MG1655; Lane 2,6 *csrA*::*kan*; Lane 3,7 *csrA*::*kan* pGB2; Lane 4,8 *csrA*::*kan* pCsrA; and Lane 9, CF80005 (pGMK). (C) DksA; Lane 1,6 MG1655; Lane 2,7 *csrA*::*kan*; Lane 3,8 *csrA*::*kan* pGB2; Lane 4,9 *csrA*::*kan* pCsrA; Lane 5, *dksA*::*kan*. Fold differences in protein levels (relative to wild-type) are indicated and represent an average of three independent experiments. The standard deviation for all values was less than 10% from the mean.

Fig. 2-5. Effects of *dksA* and *csrA* on expression of chromosomally-encoded *dksA* translational, transcriptional, and leader fusions. (A-D) Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420} / mg protein). Values represent the average of two independent experiments. Error bars depict

standard error of the mean. (A) Activity from a *dksA'-'lacZ* fusion in wild-type, ■; *dksA*, ▲; *dksA* pHM1883 (empty vector), ▼; *dksA* pHM1506 (*dksA*$^{+}$), ♦; *dksA* pHM1506 + 0.1 mM IPTG, ●; and *dksA* pHM1506 + 1 mM IPTG, *. Growth curves are represented by open symbols except for *dksA* pHM1506 + 1 mM IPTG (+). (B-D) Activity from the indicated fusions in wild-type, ■; *csrA*, ▲; *dksA*, ▼; and *csrA dksA*, ●. Growth curves are represented by open symbols.

Fig. 2-6. Effect of *dksA* on *dksA* transcription. (A) Nucleotide sequence of the *dksA* promoters. Positions of the mapped P1 and putative P2 transcriptional starts are depicted as capital letters, and predicted -35 and -10 promoter elements are shown. Numbering is with respect to the start of *dksA* translation. (B) Primer extension analysis of the *dksA* 5' end. Lane 1, *dksA* (disrupted with *kan*); Lane 2, *dksA* pHM1684 (*dksA* containing the D71N and D74N mutations); Lane 3, *dksA* pJK537 (wild-type *dksA*); Lane 4, WT; Lane 5, *dksA* (disrupted with *tet* downstream from the AUG translational start). The dideoxynucleotide sequencing ladder (G, A, T and C) was generated with the same primer (pdksA2) used for the primer extension analysis.

Fig. 2-7. Effects of *dksA*, ppGpp and *rpoS* on *csrA* expression. (A) Western blot of CsrA protein levels in MG1655 (wild-type) and *dksA* mutant strains (upper panel) and PVDF membrane stained with the MemCode^{TM} Reversible Protein Stain Kit (Pierce Biotechnology, Rockford, IL) and imaged as an internal loading control (lower panel). Cells were harvested at exponential phase (OD_{600} = 0.5) and stationary phase (at 8 hours of growth). Lane 1, MG1655; Lane 2, *dksA*::*kan*; Lane 3, *dksA*::*kan* pHM1883 (empty

vector); Lane 4, *dksA*::*kan* pHM1506 (*dksA*$^{+}$); Lane 5, *dksA*::*kan* pHM1506 + 0.1 mM IPTG; Lane 6, *relA spoT* (ppGpp^{0}). Fold differences in protein levels (relative to wild-type) are indicated and represent an average of three independent experiments. The standard deviation for these values was < 10% from the mean. (B-F) Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420} / mg protein). The values represent the average of two independent experiments. Error bars depict standard error of the mean. (B) Activity from a chromosomal *csrA'-'lacZ* translational fusion: CF7789 (wild-type), ■; *dksA*, ▲; *dksA* pHM1883 (empty vector), ▼; *dksA* pHM1506 (*dksA*$^{+}$), ♦; *dksA* pHM1506 + 0.1 mM IPTG, ●; and *relA spoT* (ppGpp^{0}), *. (C) Activity from a chromosomal *csrA'-'lacZ* translational fusion: CF7789 (wild-type), ■; *dksA*, ▲; *rpoS*, ▼; *dksA rpoS*, ♦; *dksA rpoS* pHM1883 (empty vector), ●; and *dksA rpoS* pHM1506 (*dksA*$^{+}$) + 0.1 mM IPTG, *. Growth curves are represented by open symbols except for *dksA rpoS* pHM1506 + 1 mM IPTG (+). (D-E) Activity from the indicated fusions in wild-type, ■; *dksA*, ▲; and *relA spoT* (ppGpp^{0}), *. Growth curves are represented by open symbols.

Fig. 2-8. Effects of *dksA* and ppGpp on CsrB and CsrC RNA levels and gene expression. (A) A representative Northern blot of RNA from MG1655 (WT) and isogenic mutants is shown. A plasmid vector (pHM1883) and a *dksA* expression plasmid (pHM1506) were used for *dksA* complementation analysis. Fold differences in RNA levels (relative to wild-type) are indicated and represent an average of three independent experiments. The standard deviation for all values was <10% from the mean. (B, C) Cells were harvested at various times throughout growth and assayed for β-galactosidase specific activity (A_{420}

/ mg protein). The values represent the average of two independent experiments, and error bars depict the standard error of the mean. Activity from indicated fusions in wild-type, ■; *dksA*, ▲; *dksA* pHM1883 (empty vector), ▼; *dksA* pHM1506 (*dksA*$^{+}$), ♦; *dksA* pHM1506 (*dksA*$^{+}$) + 0.1 mM IPTG, ●; and *relA spoT* (ppGpp^{0}), *. Growth curves are represented by open symbols except for *relA spoT* (ppGpp^{0}) (+). (D, E) Epistasis studies with *csrB-lacZ* (D) and *csrC-lacZ* transcriptional fusions (E) in *dksA*, *uvrY* and *dksA uvrY* backgrounds with ectopic expression of *uvrY* (pUY14) or *dksA* (pHM1506) at 8 hours of growth. The vector controls were pBR322 and pHM1883, respectively. (F, G) Epistasis studies with *csrB-lacZ* (F) and *csrC-lacZ* transcriptional fusions (G) in *dksA*, *csrA* and *dksA csrA* backgrounds with ectopic expression of *csrA* (pCsrA) or *dksA* (pHM1506) at 8 hours of growth. The vector controls were pGB2 and pHM1883, respectively.

Fig. 2-9. Regulatory interactions within and between the Csr and stringent response systems in *E. coli*. CsrA activates *csrB* and *csrC* expression through the BarA-UvrY two component signal transduction system (Suzuki *et al*., 2002; Weilbacher *et al*., 2003). In turn, CsrB and CsrC RNAs sequester and antagonize CsrA (Liu *et al*., 1997; Weilbacher *et al*., 2003). CsrA represses expression of CsrD, which mediates the RNase E-dependent turnover CsrB and CsrC (Suzuki *et al*., 2006). Here, we show that DksA and ppGpp also activate transcription of CsrB and CsrC RNAs via the UvrY response regulator. The effects of CsrA, ppGpp and DksA on CsrB and CsrC may be partly mediated via effects on acetate accumulation and BarA signaling, but predominantly involve other uncharacterized mechanism(s). DksA and ppGpp also activate *csrA* expression in RpoS-dependent and independent mechanisms, although their effects on

CsrA levels are weaker than on CsrB and CsrC RNAs. CsrA binds tightly to *relA* mRNA and represses *relA* expression post-transcriptionally. CsrA binds with lower affinity to *dksA* mRNA and activates *dksA* expression post-transcriptionally. However, DksA negative autoregulation tends to mask the effects of CsrA. CsrA appears to indirectly repress *spoT* transcript levels through an unknown factor (X). Broken lines emphasize uncertain mechanisms.

Fig. 2-1.

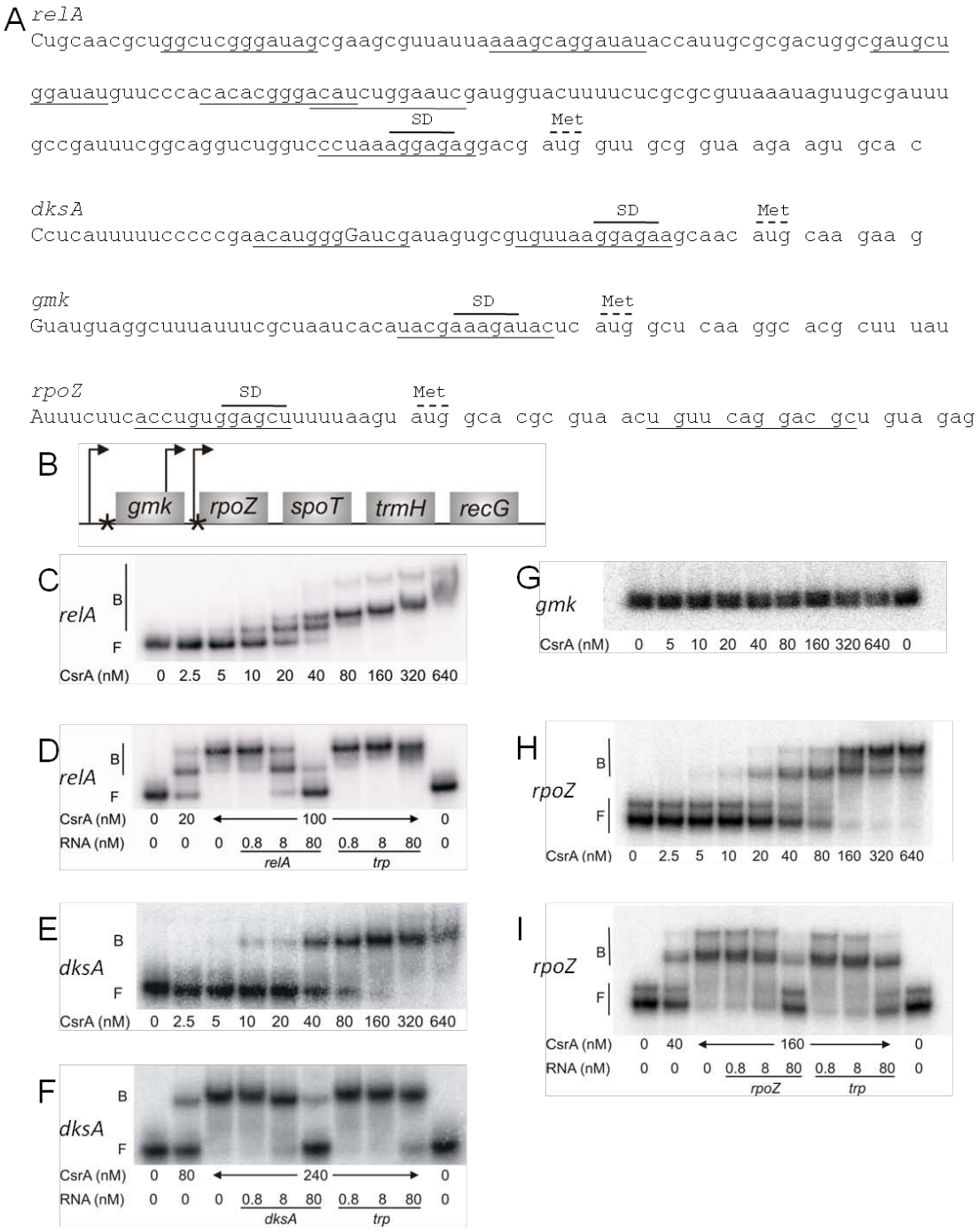

A
relA
Cugcaacgcuggcucgggauagcgaagcguuauuaaaagcaggauauaccauugcgcgacuggcgaugcu
ggauauguucccacacacgggacaucuggaaucgaugguacuuuucucgcgcguuaaauaguugcgauuu
SD
Met
gccgauuucggcaggucuggucccuaaaggagaggacg aug guu gcg gua aga agu gca c
dksA
SD
Met
CcucauuuuuccccccgaacaugggGaucgauagugcguguuuaaggagaagcaac aug caa gaa g
gmk
SD
Met
Guauguaggcuuuauuucgcuaaucacauacgaaagauacuc aug gcu caa ggc acg cuu uau
rpoZ
SD
Met
Auuucuucaccuguggagcuuuuuaagu aug gca cgc gua acu guu cag gac gcu gua gag
B
gmk
rpoZ
spoT
trmH
recG
C
relA
B
F
CsrA (nM) 0 2.5 5 10 20 40 80 160 320 640
G
gmk
CsrA (nM) 0 5 10 20 40 80 160 320 640 0
D
relA
B
F
CsrA (nM) 0 20 100 0
RNA (nM) 0 0 0 0.8 8 80 0.8 8 80 0
relA
trp
H
rpoZ
B
F
CsrA (nM) 0 2.5 5 10 20 40 80 160 320 640
E
dksA
B
F
CsrA (nM) 0 2.5 5 10 20 40 80 160 320 640
I
rpoZ
B
F
CsrA (nM) 0 40 160 0
RNA (nM) 0 0 0 0.8 8 80 0.8 8 80 0
rpoZ
trp
F
dksA
B
F
CsrA (nM) 0 80 240 0
RNA (nM) 0 0 0 0.8 8 80 0.8 8 80 0
dksA
trp

Fig. 2-2.

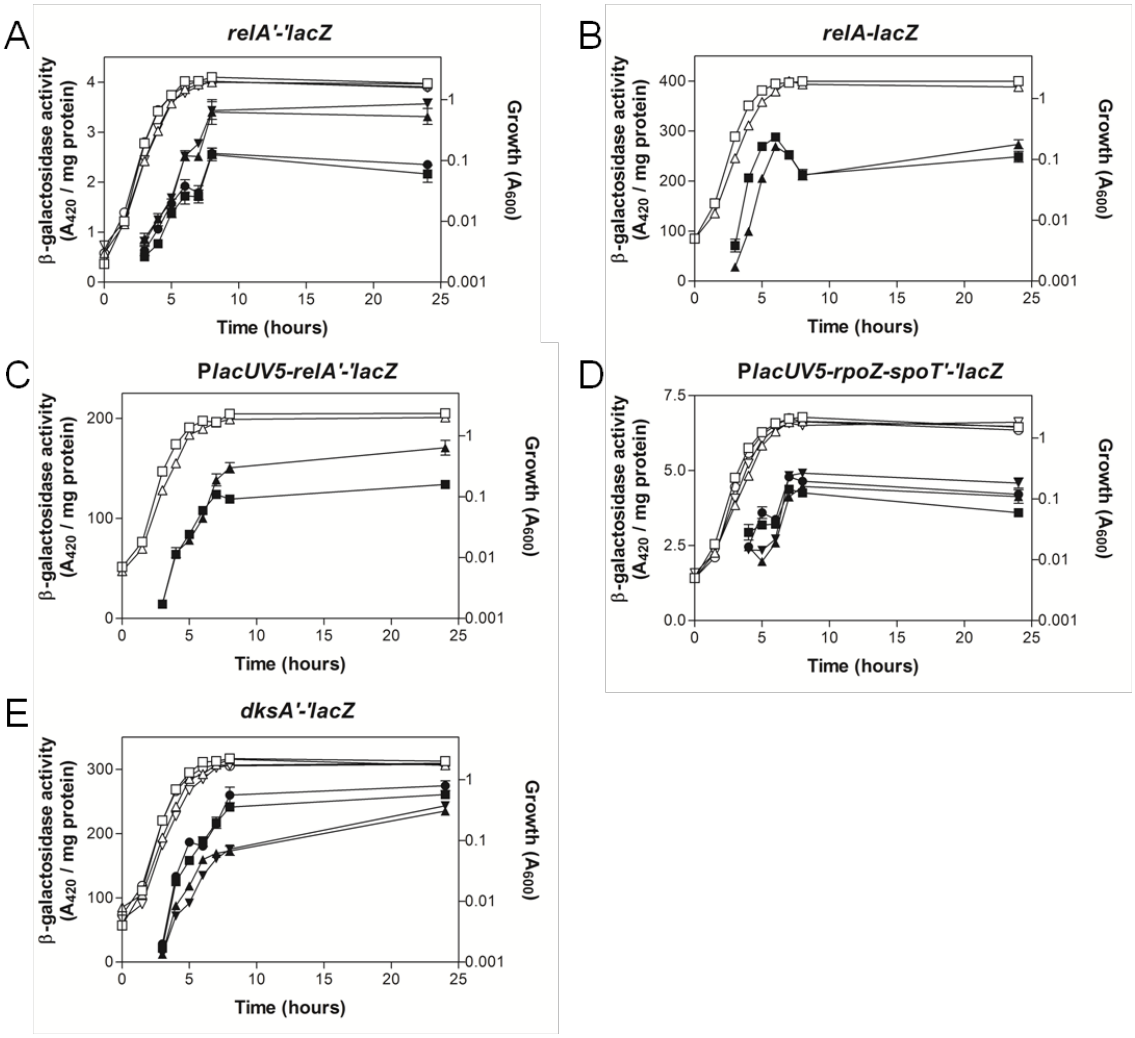

A
relA'-'lacZ
B
relA-lacZ
C
PlacUV5-relA'-'lacZ
D
PlacUV5-rpoZ-spoT'-'lacZ
E
dksA'-'lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)

Fig. 2-3.

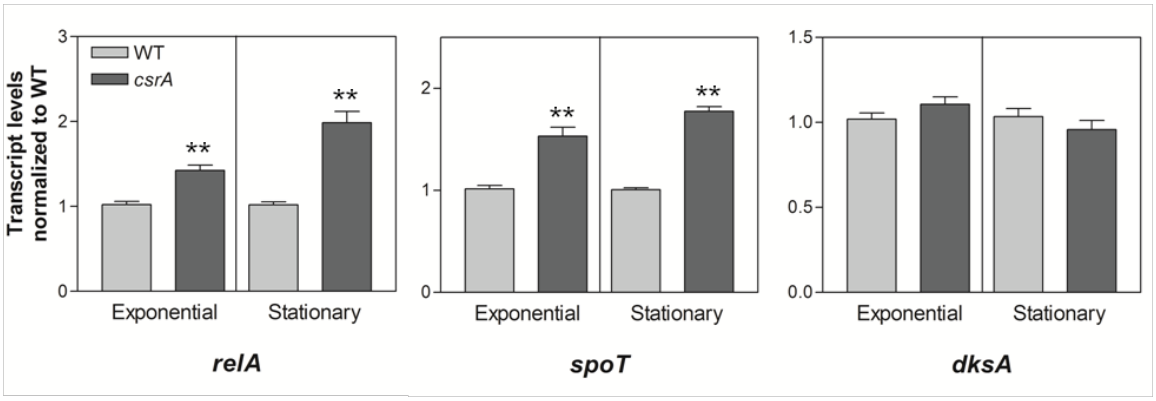

Transcript levels normalized to WT
WT
csrA
**
Exponential
Stationary
relA
spoT
dksA

Fig. 2-4.

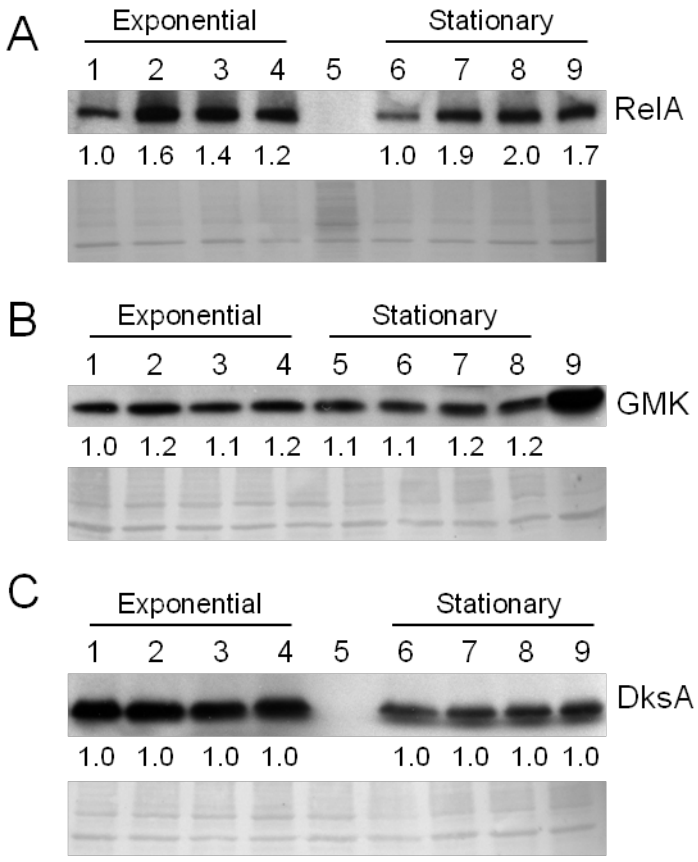

A
Exponential
Stationary
1 2 3 4 5 6 7 8 9
RelA
1.0 1.6 1.4 1.2 1.0 1.9 2.0 1.7
B
Exponential
Stationary
1 2 3 4 5 6 7 8 9
GMK
1.0 1.2 1.1 1.2 1.1 1.1 1.2 1.2
C
Exponential
Stationary
1 2 3 4 5 6 7 8 9
DksA
1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0

Fig. 2-5.

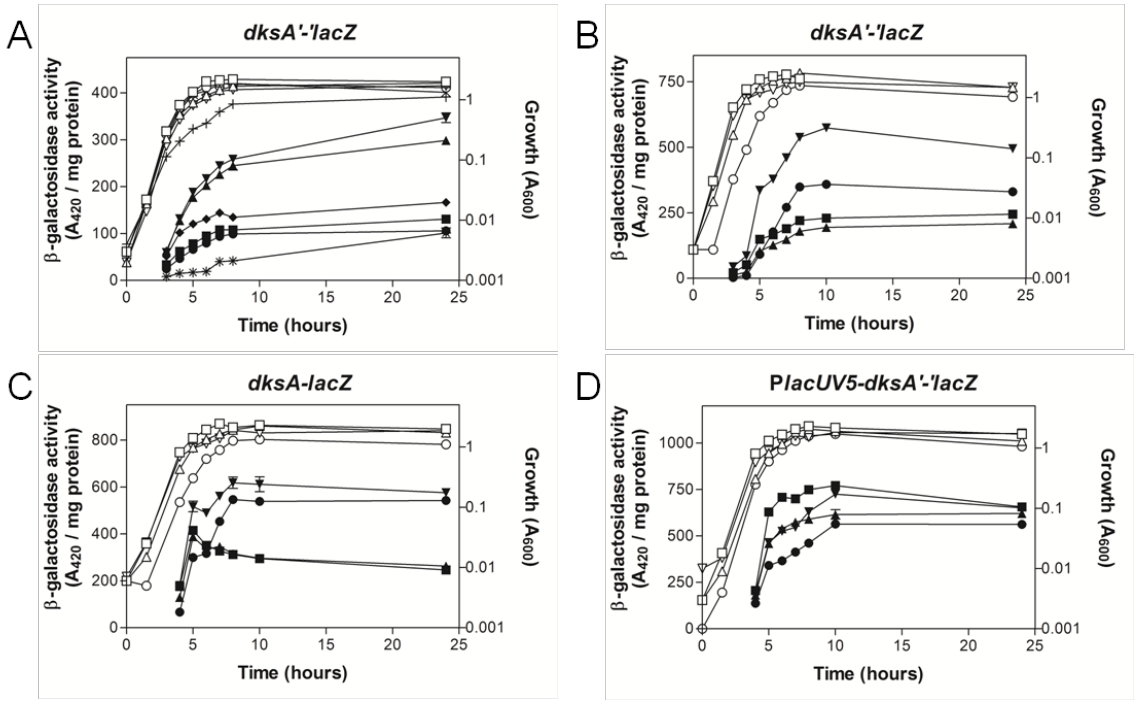

A
dksA'-'lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)
B
dksA'-'lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)
C
dksA-lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)
D
PlacUV5-dksA'-'lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)

Fig. 2-6.

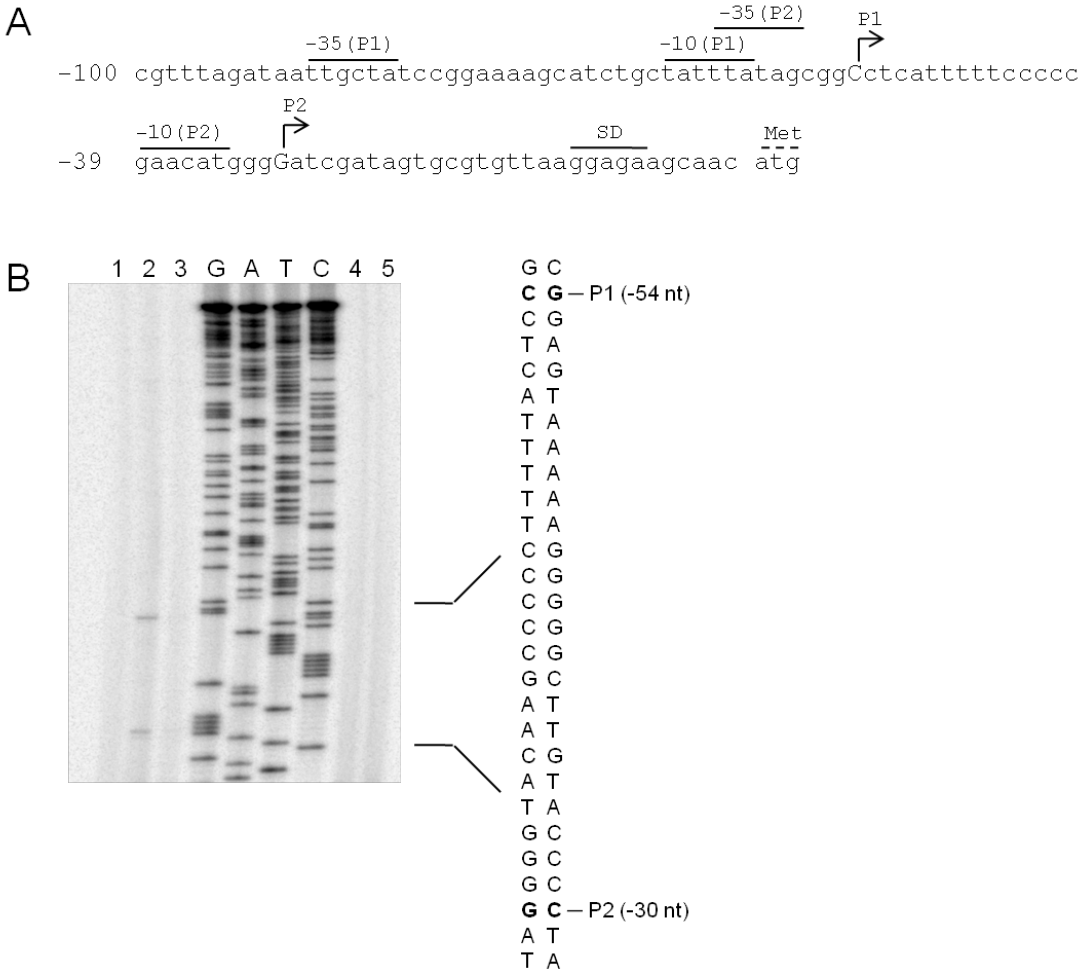

A
-35(P1)
-35(P2)
-10(P1)
P1
-100 cgtttagataattgctatccggaaaagcatctgctatttatagcggCctcatttttccccc
P2
-10(P2)
SD
Met
-39 gaacatgggGatcgatagtgcgtgttaaggagaagcaac atg
B
1 2 3 G A T C 4 5
G C
C G — P1 (-54 nt)
C G
T A
C G
A T
T A
T A
T A
T A
T A
C G
C G
C G
C G
C G
G C
A T
A T
C G
A T
T A
G C
G C
G C
G C — P2 (-30 nt)
A T
T A

Fig. 2-7.

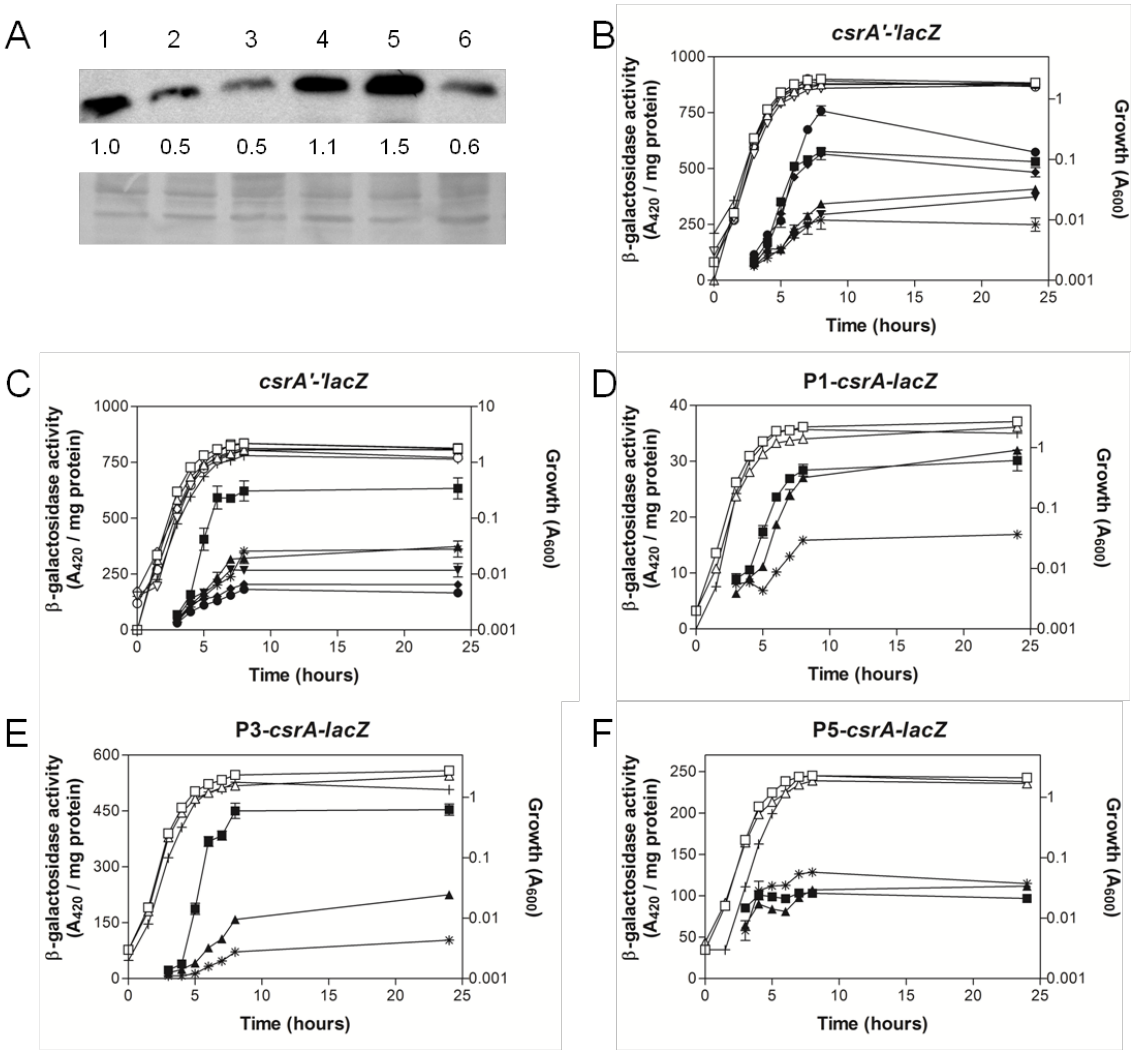

A
1 2 3 4 5 6
1.0 0.5 0.5 1.1 1.5 0.6
B
csrA'-'lacZ
C
csrA'-'lacZ
D
P1-csrA-lacZ
E
P3-csrA-lacZ
F
P5-csrA-lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)

Fig. 2-8.

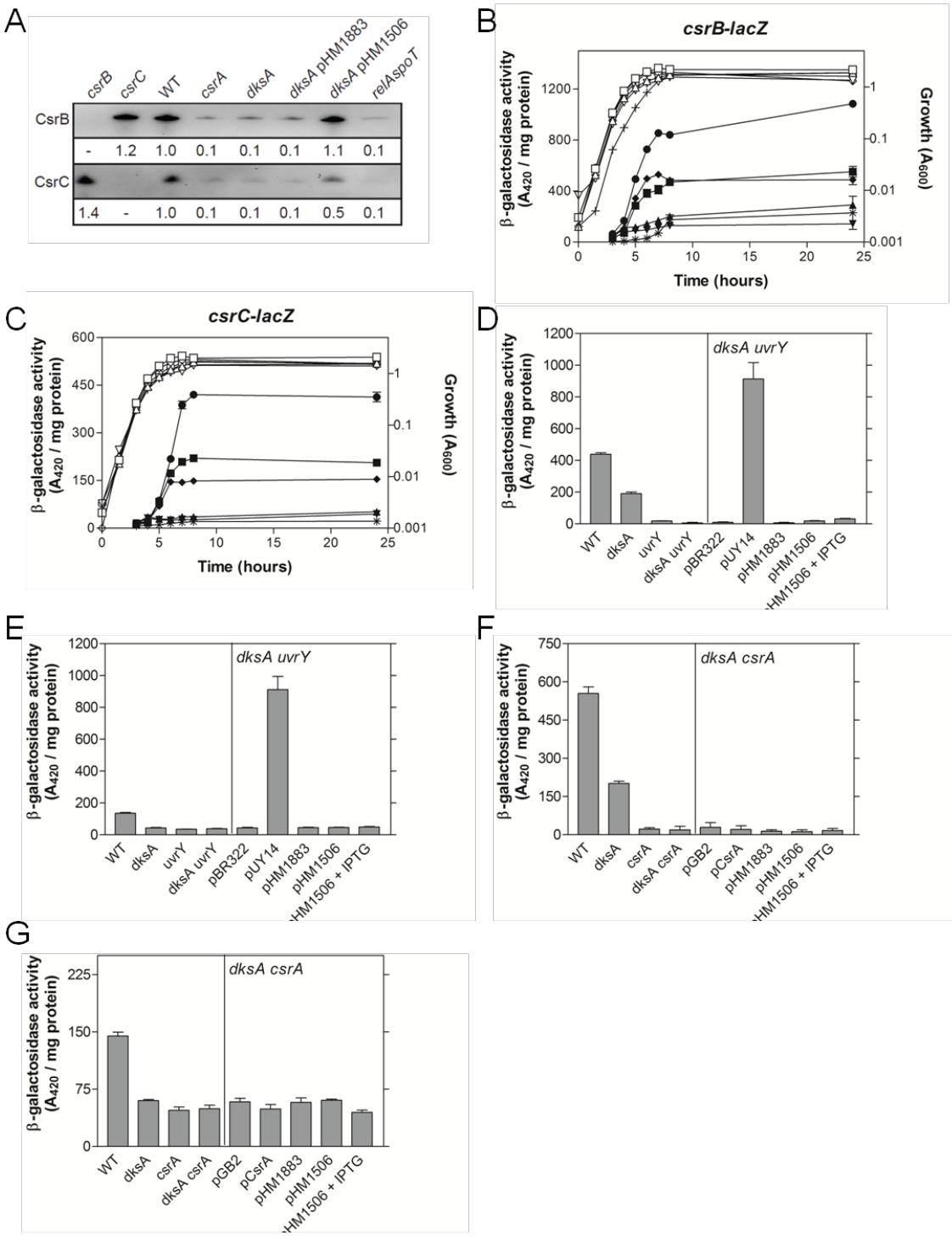
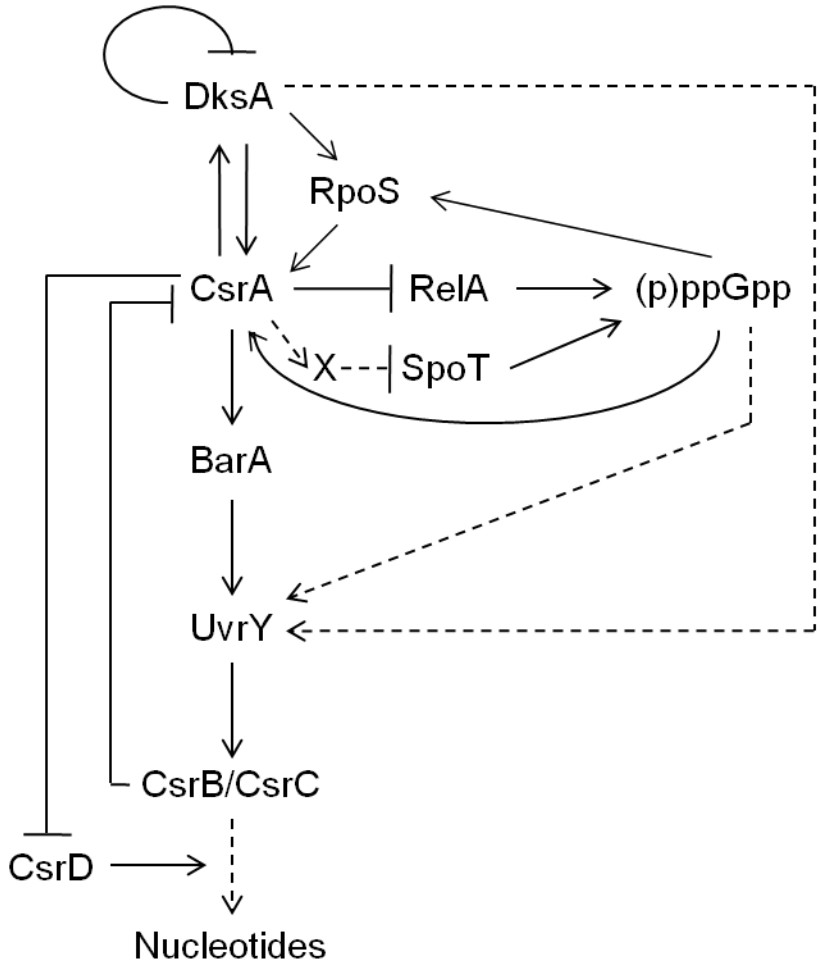

A
csrB
csrC
WT
csrA
dksA
dksA pHM1883
dksA pHM1506
relAspoT
CsrB
- 1.2 1.0 0.1 0.1 0.1 1.1 0.1
CsrC
1.4 - 1.0 0.1 0.1 0.1 0.5 0.1
B
csrB-lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)
C
csrC-lacZ
β-galactosidase activity (A420 / mg protein)
Growth (A600)
Time (hours)
D
dksA uvrY
β-galactosidase activity (A420 / mg protein)
WT
dksA
uvrY
dksA uvrY
pBR322
pUY14
pHM1883
pHM1506
pHM1506 + IPTG
E
dksA uvrY
β-galactosidase activity (A420 / mg protein)
WT
dksA
uvrY
dksA uvrY
pBR322
pUY14
pHM1883
pHM1506
pHM1506 + IPTG
F
dksA csrA
β-galactosidase activity (A420 / mg protein)
WT
dksA
csrA
dksA csrA
pGB2
pCsrA
pHM1883
pHM1506
pHM1506 + IPTG
G
dksA csrA
β-galactosidase activity (A420 / mg protein)
WT
dksA
csrA
dksA csrA
pGB2
pCsrA
pHM1883
pHM1506
pHM1506 + IPTG

Fig. 2-9.


DksA
RpoS
CsrA
RelA
(p)ppGpp
X
SpoT
BarA
UvrY
CsrB/CsrC
CsrD
Nucleotides

Chapter 3. The RNA binding protein CsrA controls cyclic-di-GMP levels by directly regulating expression of GGDEF proteins

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This manuscript was published in *Mol Micro*. 2008 Oct;70(1):236-57. Adrianne N. Edwards performed the RNA electrophoretic mobility shift assays in this study.

ABSTRACT

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 cyclic di-GMP (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.

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 (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 homologues 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 (Sabnis *et al*., 1995; Baker *et al*., 2002), 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; Heeb and Haas, 2001; Barnard *et al*., 2004). CsrA is a homodimer containing two identical RNA binding surfaces located on opposite sides of the protein, whose structure and function recently have 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; Storz *et al*., 2005; Romby *et al*., 2006). 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 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) (Simm *et al*., 2004; Ryjenkov *et al*., 2005; Schmidt *et al*., 2005), 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 activities, indicating that CsrD is involved neither 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 min) to *E. coli* KJ157 (KSB837 *csrA*::*kan*), carrying the arabinose-inducible vector pBADcsrA, resulted in a strong upregulation of *csrA* expression within 2 min (Fig. 3-1A). Consistent with our prediction mRNA levels of the direct target *pgaA* dramatically decreased within less than 10 min (Fig. 3-1B). 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 min (Fig. 3-1C). 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. As *pgaA* mRNA was downregulated within less than 12 min, we compared the transcriptional profiles 4 and 12 min after arabinose addition with the profile before arabinose induction (0 min). To eliminate genes downregulated in a CsrA-independent manner, we normalized the observed signal ratios against the signal ratios resulting from induction of the vector control pBAD28.

Four of the genes showing the strongest repression (more than sevenfold) 12 min after pBADcsrA expression belonged to the *pga* operon (Fig. 3-1D). 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. 3-1E and F). Among the most downregulated genes in response to CsrA overexpression we found another GGDEF protein encoding open reading frame, *ydeH* (Fig. 3-1D and G). *ydeH* is predicted to encode a cytoplasmic protein, not containing any known domains involved in signalling (Fig. 3-1G).

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. 3-2A). The mRNA level of *ycdT* was halved within 4 min and reached a minimum of 3% between 12 and 24 min. *ydeH* mRNA decreased to 50% within 5 min and continued to decrease to approximately 22% after 24 min. Similar results were observed when arabinose induction was performed earlier during growth at OD_{600} 0.5 (Fig. 3-2B). In contrast, addition of arabinose to a strain carrying the empty vector pBAD28 did not affect the levels of *ycdT* and *ydeH* transcripts (Fig. 3-2C).

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. 3-2D), whereas *ydeH* mRNAs remained at constant levels (Fig. 3-2E). 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 with later time points (Fig. 3-2D), whereas the transcript levels of *ydeH* were approximately 10-fold higher throughout the growth (Fig. 3-2E). Monitoring *csrA* transcript levels over time in the wild-type strain shows that *csrA* expression rapidly decreased between 8 and 24 h (Fig. 3-2F), 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; 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 (approximately 70%) compared with the wild type (Fig. 3-3). 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 threefold increased (Fig. 3-3). 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. As no information about the *ycdT* promoter was available in the database, 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. 3-4A). 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. 3-4A). 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 were

found, one of which overlaps the Shine–Dalgarno sequence and the other one is close to the 5' end of the transcript (Fig. 3-4B).

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. 3-4C and 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. 3-4C). 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. 3-4D). 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 non-linear 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. 3-4E and 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 nt 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. 3-4G). 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. 3-4G). 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 D'Argenio and Miller, 2004; Jenal, 2004; Romling *et al*., 2005; Romling and Amikam, 2006; Cotter and Stibitz, 2007). To characterize 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. 3-5A). The same effect was observed in a *Salmonella*

enterica serovar Typhimurium (*S.* Typhimurium) background, although *S.* Typhimurium do not contain orthologues of these proteins (Fig. 3-5B). Mutations in *ydeH* and *ycdT* led to slightly increased swimming ability compared with the wild type. A *ydeH ycdT* double mutant was, however, not more motile than the wild type (Fig. 3-5C).

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 DGCs, 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 carrying the empty vector (Fig. 3-5A and 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. 3-5D), 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 characterized 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 influences curli production under the conditions tested (data not shown).

Atomic force microscopy 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. 3-6A). Overexpression of pBADydeH resulted in a clear reduction in the abundance of flagella (Fig. 3-6A and 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. 3-6A and 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. 3-6), 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; Simm *et al*., 2004; Schmidt *et al*., 2005). So far, proteins with 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. 3-7A). Together with the finding that site-directed mutations of the GGDEF domains of YcdT and YdeH disrupted the effect on motility (Fig. 3-5), 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 high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). Expression of *ydeH* resulted in

clearly increased c-di-GMP levels (2215.3 fmol mg^{-1} cells) compared with 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. 3-7B). These data provide strong evidence that both of these proteins function as 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 with the wild type (74.5 fmol mg^{-1} cells) (Fig. 3-7C). 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 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. 3-7D) 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 (less than twofold) in c-di-GMP levels were observed by HPLC and MALDI-TOF, when *ycdT* was overexpressed in *Salmonella*, grown at 28°C on Luria–Bertani (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. 3-7E). 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 min 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 min: *yddV* (GGDEF), *yliF* (GGDEF), *dos* (GGDEF-EAL), *yhjK* (GGDEF-EAL), *csrD* (GGDEF-EAL), *yliE* (EAL) and *yjcC* (EAL) (Fig. 3-8A and E). CsrA-dependent repression of these genes was, however, not as strong (1.5–2.5 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. 3-8B), but remained constant or increased upon induction of the vector control (Fig. 3-8C). In the *csrA* mutant strain, expression of these genes was moderately increased (between two- and sixfold) compared with the wild type (Fig. 3-8D). 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. 3-8F and G). The expression patterns of *yliE* and *yliF* as well as *yddV* and *dos* followed almost identical kinetics (Fig. 3-8B and 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 (twofold) 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 them 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, 39, 40 and 38 nM respectively) (Baker *et al*., 2002; 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. As CsrA homologues 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 signaling. 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 overrepresented 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. 3-9). 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 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 of *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. 3-1E). A comprehensive bioinformatics study has recently demonstrated that chromosomal proximity indicates gene co-regulation in prokaryotes independent of relative gene orientation and that adjacent bidirectionally transcribed genes with conserved gene orientation are strongly co-regulated (Korbel *et al*., 2004). Furthermore, the *ycdT* homologue 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 (Itoh *et al*., 2005; Bobrov *et al*., 2008). 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 synthesis of the PEL polysaccharide in *Pseudomonas aeruginosa* is regulated by c-di-GMP. Here, the PelD protein serves as the 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.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 3-1. Chromosomal *ydeH*::*cat* and *csrB*::*cat* mutations were constructed using the Datsenko method (Datsenko and Wanner, 2000). The *cat* gene was amplified from pKD3 by PCR using primers ydeHKOFor2 and ydeHKORev2 or csrBKOFor and csrBKORev (Table 3-2) 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 3-2). 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 3-1. 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 3-2). 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 nt 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 3-2) 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 linearized 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 3-2) 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 vols of RNAprotect Bacterial Reagent (Qiagen) and incubated for 5 min 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 Bioanalyser.

Microarray analysis. Microarray analysis was performed at the Bioinformatics and Expression Analysis Core Facility 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 (http://www.affymetrix.com). The absolute signals from the samples, taken at

0 min (before arabinose induction), were compared with the signals from the 4 and 12 min samples. The signal ratios resulting from pBADcsrA overexpression were then normalized 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.

Q**uantitative real-time RT-PCR.** Five hundred nanograms of total RNA was 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 3-2. 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 artefacts of amplification, a dissociation curve was run immediately after completion of the real-time PCR. Individual gene expression profiles were normalized 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 min 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 micrograms of total RNA was 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 to +20) and GC ycdT-T7 (−36 to +20) and ydeH-T7 (−29 to +25) and GC ydeH-T7 (−29 to +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-labelled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. Radiolabelled RNA was gel-purified and re-suspended 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 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 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 microlitres of bacterial culture, grown to an OD_{600} of 1.5, was 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 re-suspended in ice-cold water, heated to 95°C for 10 min, before nucleotides were extracted by ethanol treatment. For c-di-GMP extraction from plate-grown bacteria, approximately 100 mg of cells was harvested and immediately suspended in ice-cold 0.19%

formaldehyde, before being boiled for 10 min and treated with ethanol. Nucleotide extracts of 10 or 50 mg of cells (wet weight) were fractioned by HPLC using a reversed-phase column (Hypersil ODS 5 μ; 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 re-suspended in 10 μl of water. Fractions containing c-di-GMP were pinpointed by 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 of LB medium without NaCl. After incubation the mica slides were dipped three to four 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^{-1}. 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 were counted at five different locations on the microscope slide for each strain. The ratio of flagella per 10 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 analyse 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 CR (40 µg ml^{-1}) and Coomassie brilliant blue (20 µg ml^{-1}) or CF (fluorescence brightener 28; 50 µg ml^{-1}). Plates were incubated at 28°C or 37°C for 20 h or 24 h respectively. The colony morphology and dye binding were 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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Table 3-1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description or genotype	Reference
Strains		
Escherichia coli		
MG1655	$F^-\lambda^-$	Michael Cashel
BW25141	*lacI* ^{q} *rrnB*T14 *lac*ZWJ16 *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	MG1655Δ*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*::*cat*^{r}	Pernestig *et al*. (2003)
XWMGΔT	MG1655 *ycdT*::*cat*^{r}	Wang *et al*. (2005)
KJ295	MG1655 *ydeH*::*cat*^{r}	This study
KJ311	MG1655Δ*ydeH ycdT*::*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
S. Typhimurium		
MAE103	*ΔcsgBA102 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	This study

	the plasmid-borne P_{BAD} promoter	
pBADydeH	YdeH expression plasmid, *ydeH* is controlled by the plasmid-borne P_{BAD} promoter	This study
pBADycdT-mut	mutagenized pBADycdT (G359A, G360A)	This study
pBADydeH-mut	mutagenized 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 3-2. Primers used in this study.

Primer name	Primer sequence (5' to 3')
Genetic approaches	
ydeHKOFor2	ATGGACTGTGCCAGTTTGGTCGGTGGATTGATCA TCTGGGGCCACTCGTGTAGGCTGGAGCTGCTTC
ydeHKORev2	CGGTTTGCTTACCCTCATACATTGCCCGGTCCGC TCTTCCAATGACCATATGAATATCCTCCTTAG
ydeHKOtestFor	ACAAGGAACTGTGAAAAAG
ydeHKOtestRev	ATCGTTGACACAGTAGCA
csrBKOFor	GAGTCAGACAACGAAGTGAACATCAGGATGATG ACACTTCTGCGTAGGCTGGAGCTGCTTC
csrBKORev	AATAAAAAAAGGGAGCACTGTATTCACAGCGCT CCCGGTTCGTTTATATGAATATCCTCCTTAG
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**CGAAGAATTT ATCATTATTG
YdeH-Mut-Rev	CAATAATGATAAATTCTTCG**G**CC**G**CGTAGCGAT AAACCGTTTC
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
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	TAATACGACTCACTATAGGGAAAGGGATCTACA ACCTACAGATTGGTGTAGCTTTATGGAAAAAGA CTATTTGAG
GC ycdT-T7	CTCAAATAGTCTTTTTCCATAAAGCTACACCAAT CTGTAGGTTGTAGATCCCTTTCCCTATAGTGAGT CGTATTA
ydeH-T7	TAATACGACTCACTATAGGGCACAAGGAACTGT GAAAAAGGAGTGGCAATGATCAAGAAGACAAC GGAAATTG
GC ydeH-T7	CAATTTCCGTTGTCTTCTTGATCATTGCCACTCCT TTTTCACAGTTCCTTGTGCCCTATAGTGAGTCGT ATTA

Bold letters indicate the sites, where point mutations have been introduced. Italic letters indicate the recognition sites for restriction enzymes.

FIGURE LEGENDS

Fig. 3-1. Identification of *ycdT* and *ydeH* as novel targets for CsrA. Plasmid-encoded *csrA* (pBADcsrA) was expressed in KJ157 (*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 min. D. A genome-wide screen for genes, whose transcript levels decrease within 4 and 12 min (more than threefold compared with 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 eight transmembrane regions and is predicted to contain a GGDEF motif. G. YdeH contains a GGDEF motif and is predicted to be cytoplasmic.

Fig. 3-2. 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 with 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*.

Fig. 3-3. 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.

Fig. 3-4. 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 and 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 and 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.

Fig. 3-5. 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 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. A. MG1655 (wt), carrying pBAD28, pBADycdT, pBADycdT-mut, pBADydeH, pBADydeH-mut or pBADcsrA. B. *S.* 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*).

Fig. 3-6. 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.

Fig. 3-7. 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), *S.* Typhimurium (ST), *Y. 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 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,

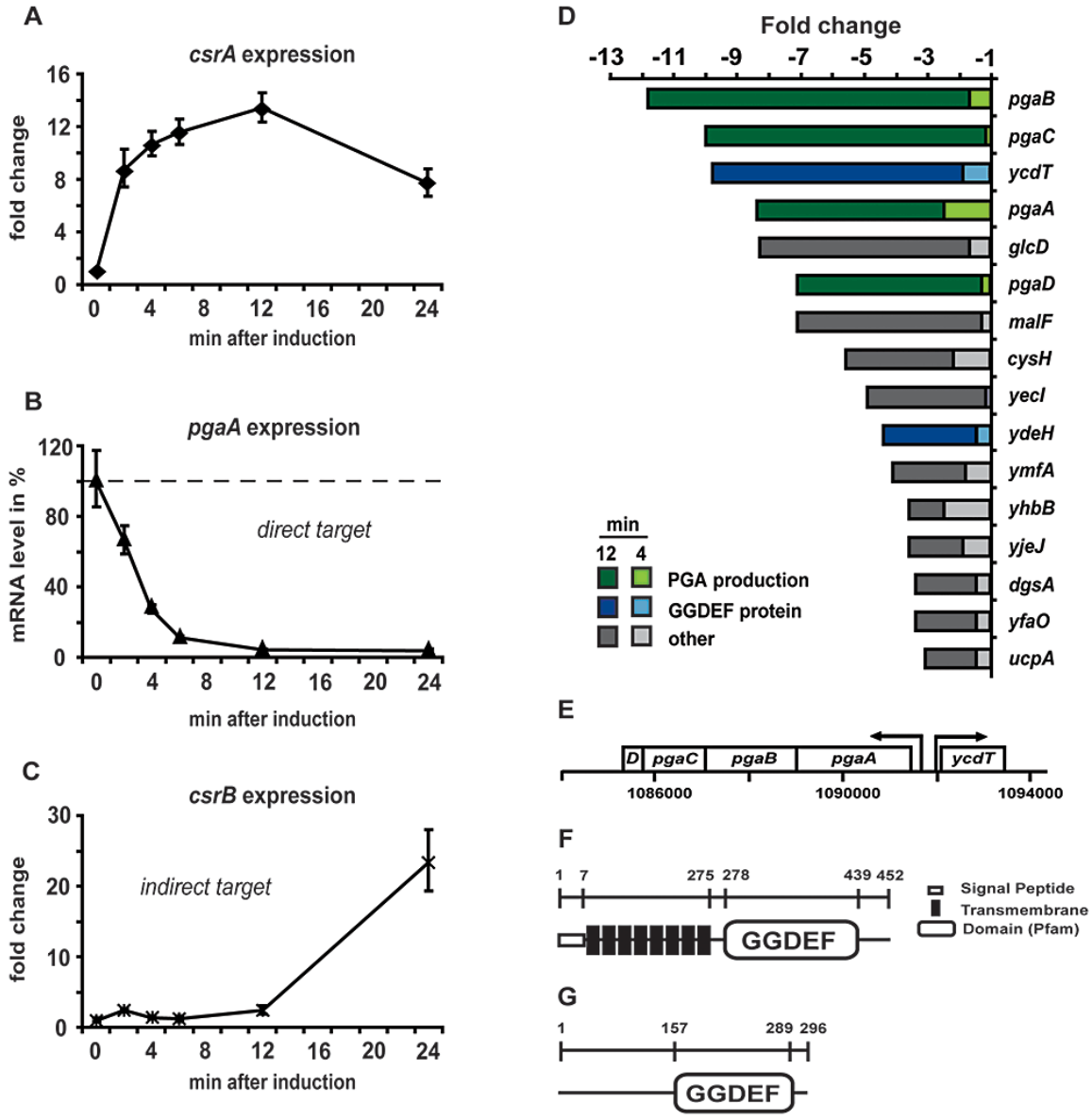
carrying pBAD28, pBADydeH and pBADycdT, grown for 20 h at 28°C on LB agar with 0.1% arabinose, but without salt.

Fig. 3-8. 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.

Fig. 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 styles 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.

Fig. 3-1.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csrA expression
fold change
16
12
8
4
0
0 4 8 12 16 20 24
min after induction
B
pgaA expression
mRNA level in %
120
80
40
0
direct target
0 4 8 12 16 20 24
min after induction
C
csrB expression
fold change
30
20
10
0
indirect target
0 4 8 12 16 20 24
min after induction
D
Fold change
-13 -11 -9 -7 -5 -3 -1
pgaB
pgaC
ycdT
pgaA
glcD
pgaD
malF
cysH
yecI
ydeH
ymfA
yhbB
yjeJ
dgsA
yfaO
ucpA
min
12 4
PGA production
GGDEF protein
other
E
D
pgaC
pgaB
pgaA
ycdT
1086000
1090000
1094000
F
1 7
275 278
439 452
GGDEF
Signal Peptide
Transmembrane
Domain (Pfam)
G
1
157
289 296
GGDEF

Fig. 3-2.

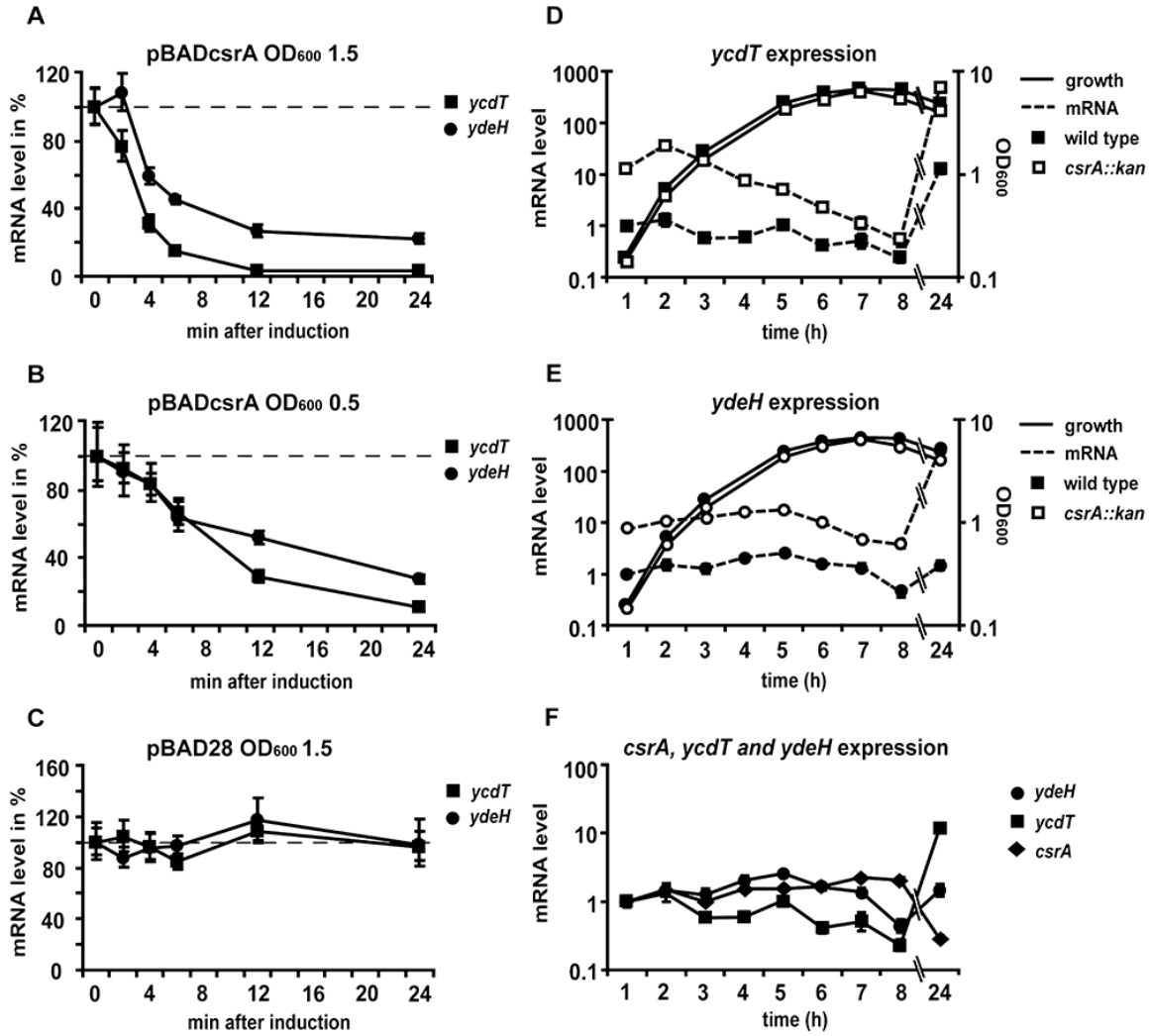

A
pBADcsrA OD600 1.5
mRNA level in %
min after induction
ycdT
ydeH
B
pBADcsrA OD600 0.5
C
pBAD28 OD600 1.5
D
ycdT expression
mRNA level
time (h)
OD600
growth
mRNA
wild type
csrA::kan
E
ydeH expression
F
csrA, ycdT and ydeH expression
csrA

Fig. 3-3.

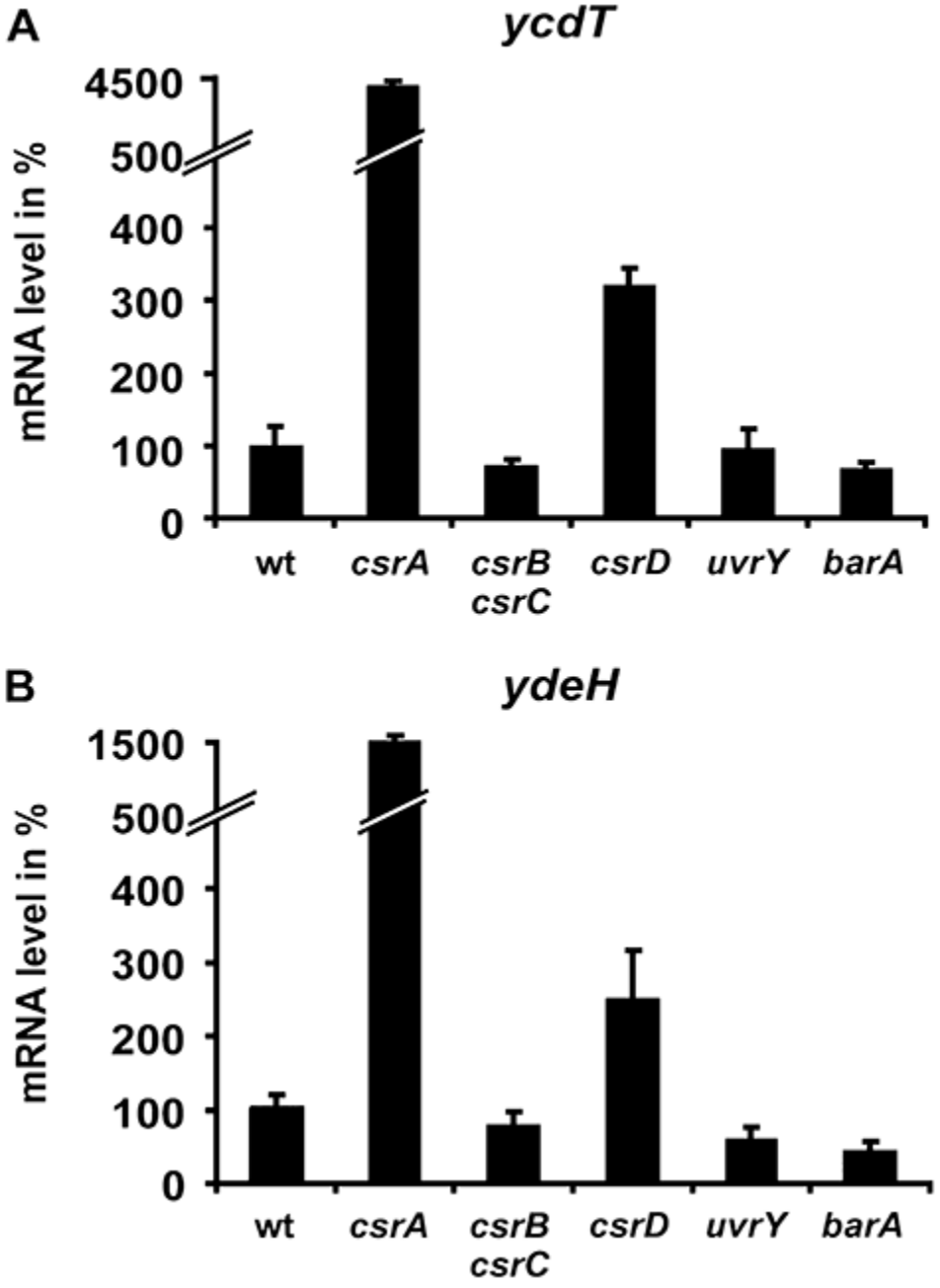

A
ycdT
mRNA level in %
4500
500
400
300
200
100
0
wt
csrA
csrB csrC
csrD
uvrY
barA
B
ydeH
mRNA level in %
1500
500
400
300
200
100
0
wt
csrA
csrB csrC
csrD
uvrY
barA

Fig. 3-4.

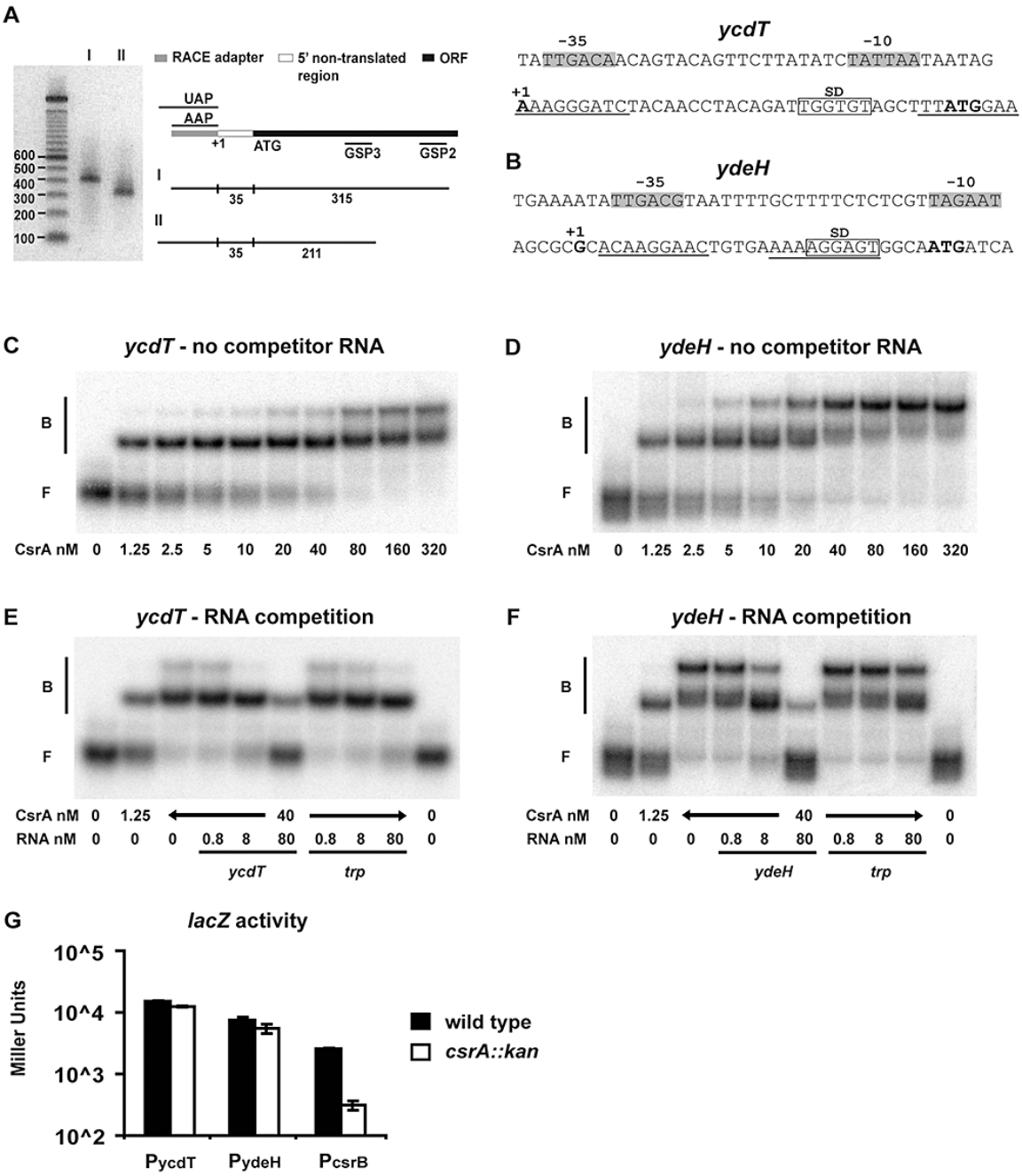

A
I
II
RACE adapter
5' non-translated region
ORF
600
500
400
300
200
100
UAP
AAP
+1
ATG
GSP3
GSP2
I
35
315
II
35
211
ycdT
-35
-10
TATTGACAACAGTACAGTTCTTATATCTATTAATAATAG
+1
SD
AAAGGGATCTACAACCTACAGATTGGTGTAGCTTTATGGAA
B
ydeH
-35
-10
TGAAAATATTGACGTAATTTTGCTTTTCTCTCGTTAGAAT
+1
SD
AGCGCGCACAAGGAACTGTGAAAAAGGAGTGGCAATGATCA
C
ycdT - no competitor RNA
B
F
CsrA nM 0 1.25 2.5 5 10 20 40 80 160 320
D
ydeH - no competitor RNA
B
F
CsrA nM 0 1.25 2.5 5 10 20 40 80 160 320
E
ycdT - RNA competition
B
F
CsrA nM 0 1.25 40 0
RNA nM 0 0 0 0.8 8 80 0.8 8 80 0
ycdT
trp
F
ydeH - RNA competition
B
F
CsrA nM 0 1.25 40 0
RNA nM 0 0 0 0.8 8 80 0.8 8 80 0
ydeH
trp
G
lacZ activity
Miller Units
10^5
10^4
10^3
10^2
wild type
csrA::kan
PycdT
PydeH
PcsrB

Fig. 3-5.

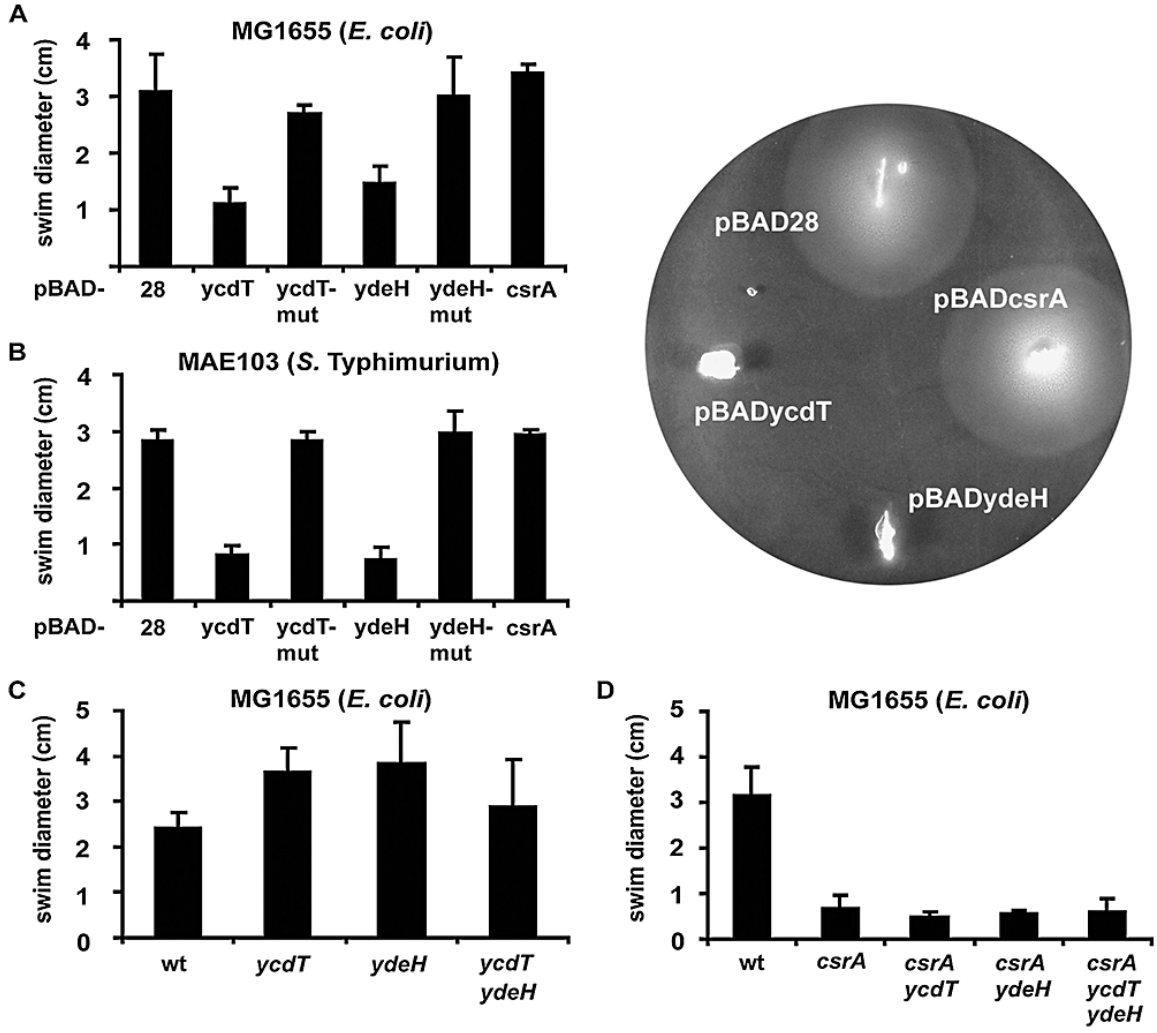

A
MG1655 (E. coli)
swim diameter (cm)
pBAD-
28
ycdT
ycdT-mut
ydeH
ydeH-mut
csrA
B
MAE103 (S. Typhimurium)
swim diameter (cm)
pBAD-
28
ycdT
ycdT-mut
ydeH
ydeH-mut
csrA
pBAD28
pBADcsrA
pBADycdT
pBADydeH
C
MG1655 (E. coli)
swim diameter (cm)
wt
ycdT
ydeH
ycdT ydeH
D
MG1655 (E. coli)
swim diameter (cm)
wt
csrA
csrA ycdT
csrA ydeH
csrA ycdT ydeH

Fig. 3-6.

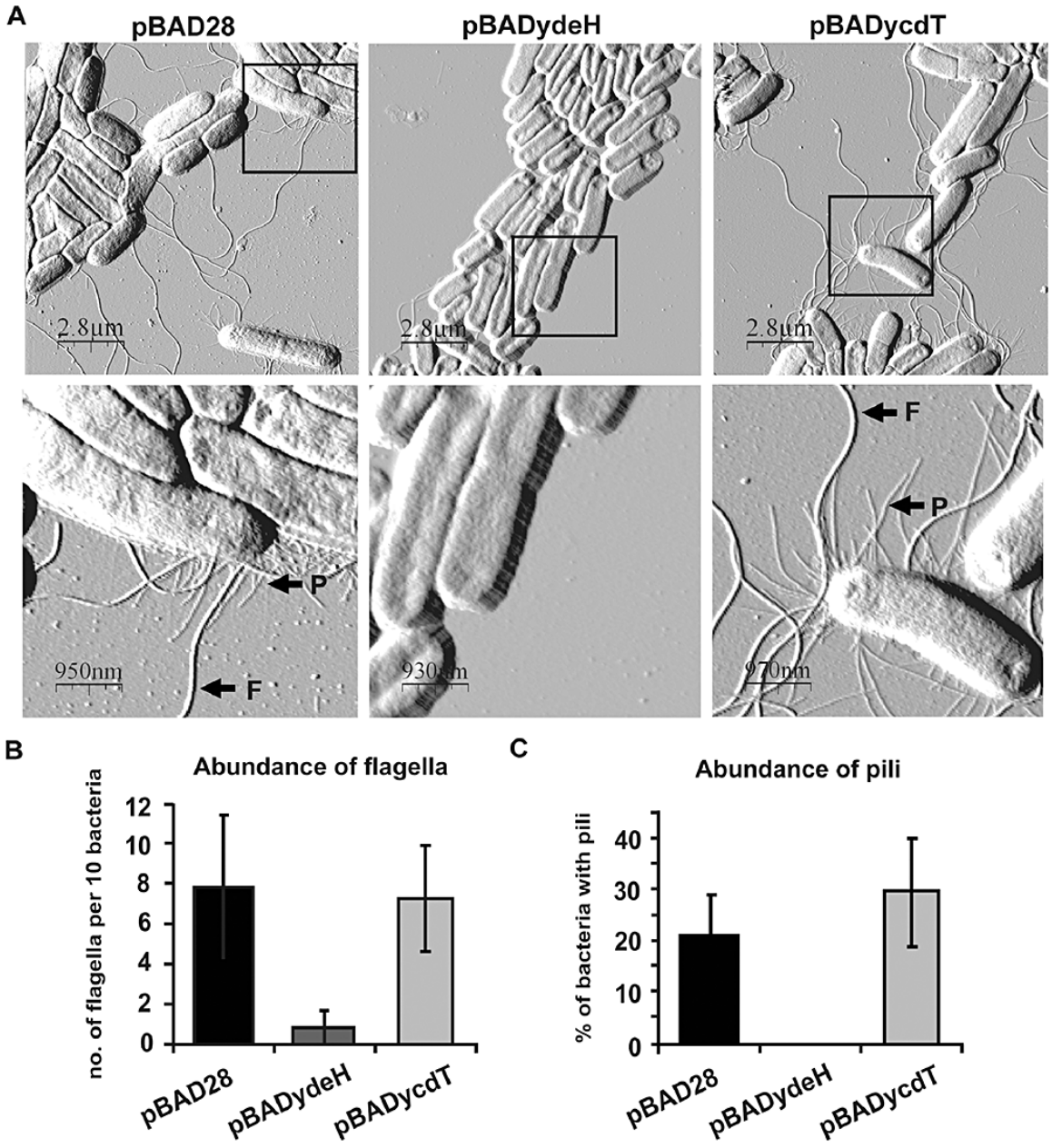

A
pBAD28
pBADydeH
pBADycdT
2.8μm
2.8μm
2.8μm
P
F
950nm
930nm
F
P
970nm
B
Abundance of flagella
no. of flagella per 10 bacteria
12
10
8
6
4
2
0
pBAD28
pBADydeH
pBADycdT
C
Abundance of pili
% of bacteria with pili
40
30
20
10
0
pBAD28
pBADydeH
pBADycdT

Fig. 3-7.

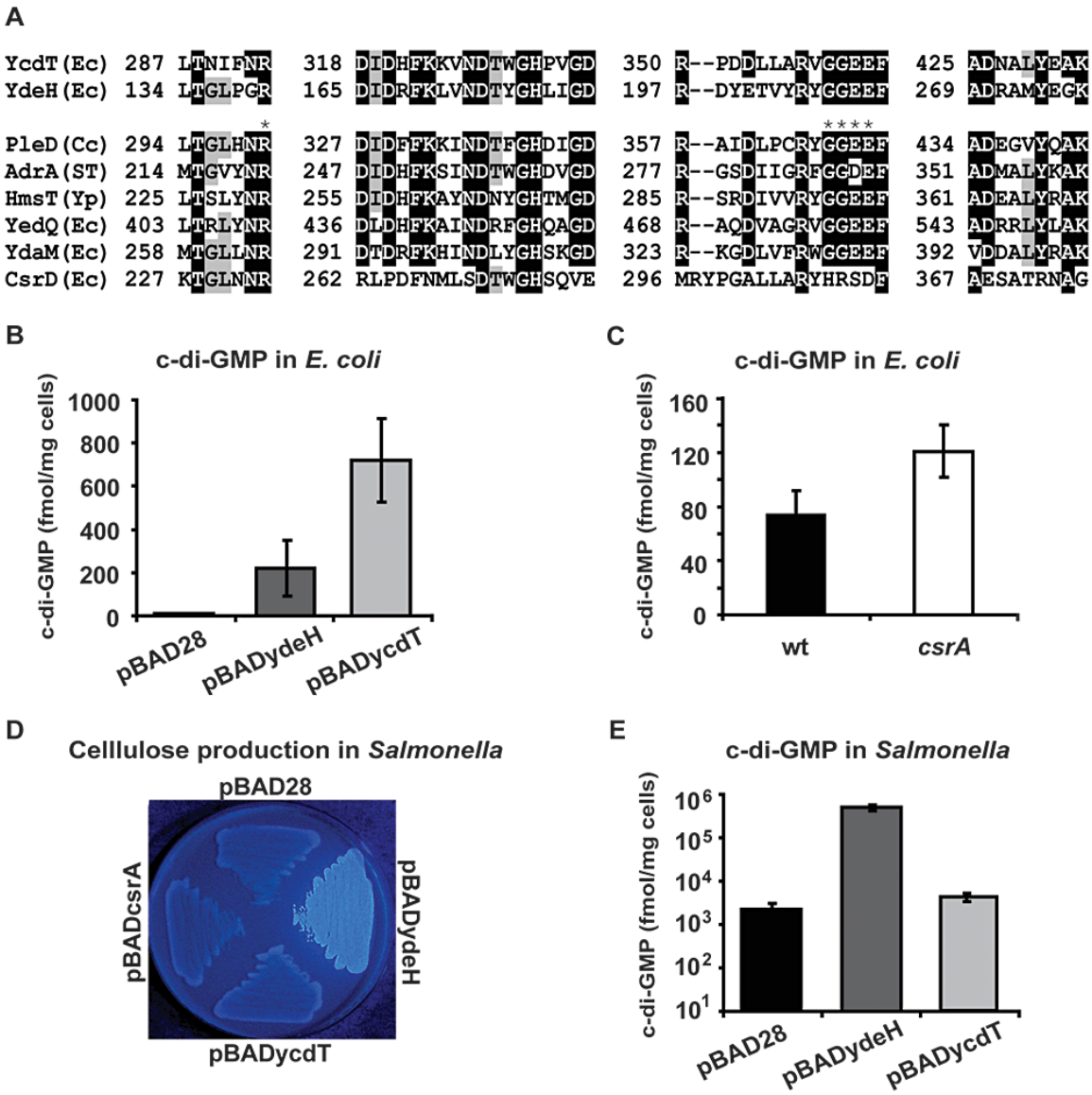

A
YcdT(Ec) 287 LTNIFNR 318 DIDHFKKVNDTWGHPVGD 350 R--PDDLLARVGGEEF 425 ADNALYEAK
YdeH(Ec) 134 LTGLPGR 165 DIDRFKLVNDTYGHLIGD 197 R--DYETVYRYGGEEF 269 ADRAMYEGK
PleD(Cc) 294 LTGLHNR 327 DIDFFKKINDTFGHDIGD 357 R--AIDLPCRYGGEEF 434 ADEGVYQAK
AdrA(ST) 214 MTGVYNR 247 DIDHFKSINDTWGHDVGD 277 R--GSDIIGRFGGDEF 351 ADMALYKAK
HmsT(Yp) 225 LTSLYNR 255 DIDHFKAYNDNYGHTMGD 285 R--SRDIVVRYGGEEF 361 ADEALYRAK
YedQ(Ec) 403 LTRLYNR 436 DLDHFKAINDRFGHQAGD 468 R--AQDVAGRVGGEEF 543 ADRRLYLAK
YdaM(Ec) 258 MTGLLNR 291 DTDRFKHINDLYGHSKGD 323 R--KGDLVFRWGGEEF 392 VDDALYRAK
CsrD(Ec) 227 KTGLNNR 262 RLPDFNMLSDTWGHSQVE 296 MRYPGALLARYHRSDF 367 AESATRNAG
B
c-di-GMP in E. coli
c-di-GMP (fmol/mg cells)
1000
800
600
400
200
0
pBAD28
pBADydeH
pBADycdT
C
c-di-GMP in E. coli
c-di-GMP (fmol/mg cells)
160
120
80
40
0
wt
csrA
D
Celllulose production in Salmonella
pBAD28
pBADcsrA
pBADydeH
pBADycdT
E
c-di-GMP in Salmonella
c-di-GMP (fmol/mg cells)
10^6
10^5
10^4
10^3
10^2
10^1
pBAD28
pBADydeH
pBADycdT

Fig. 3-8.

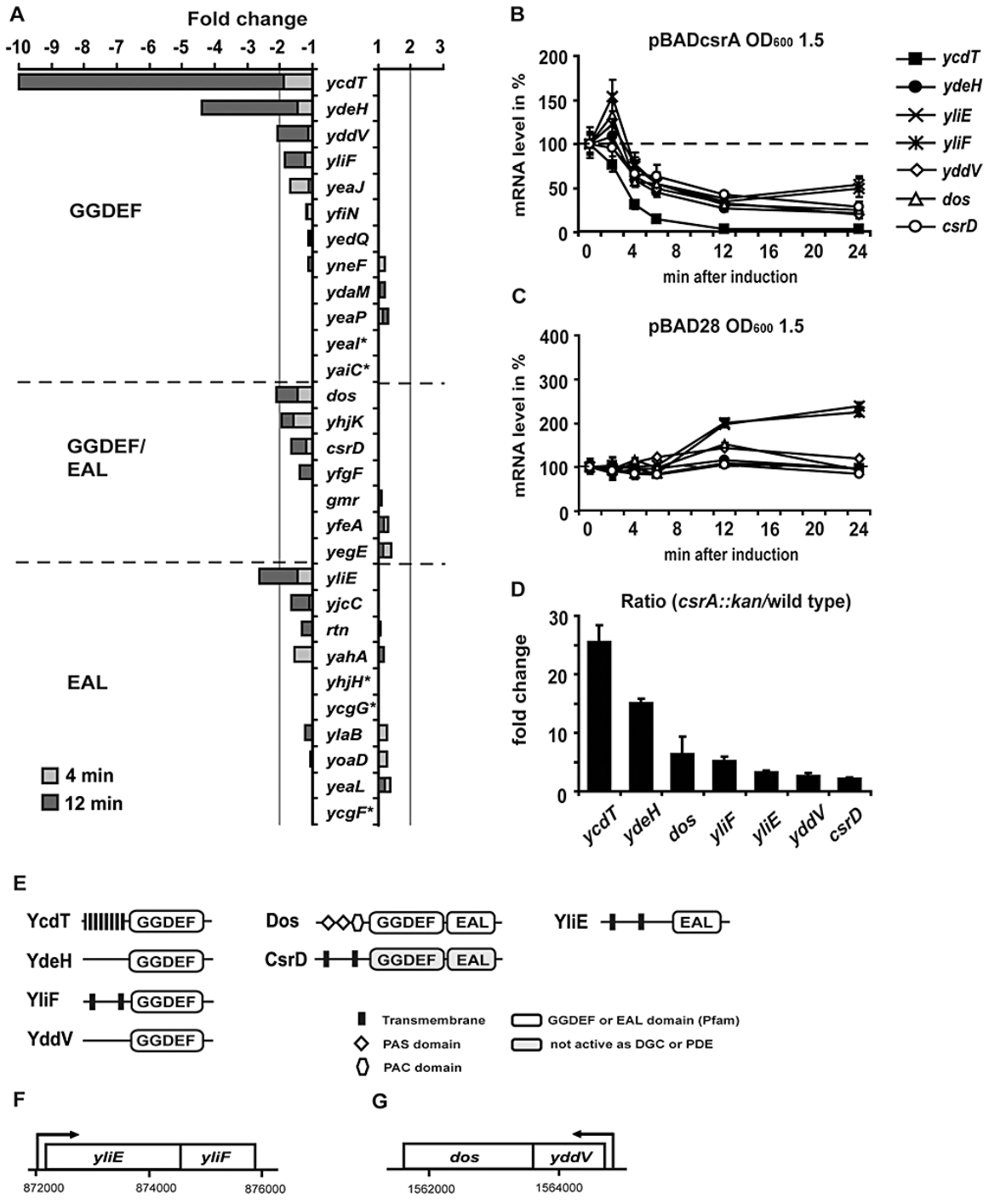
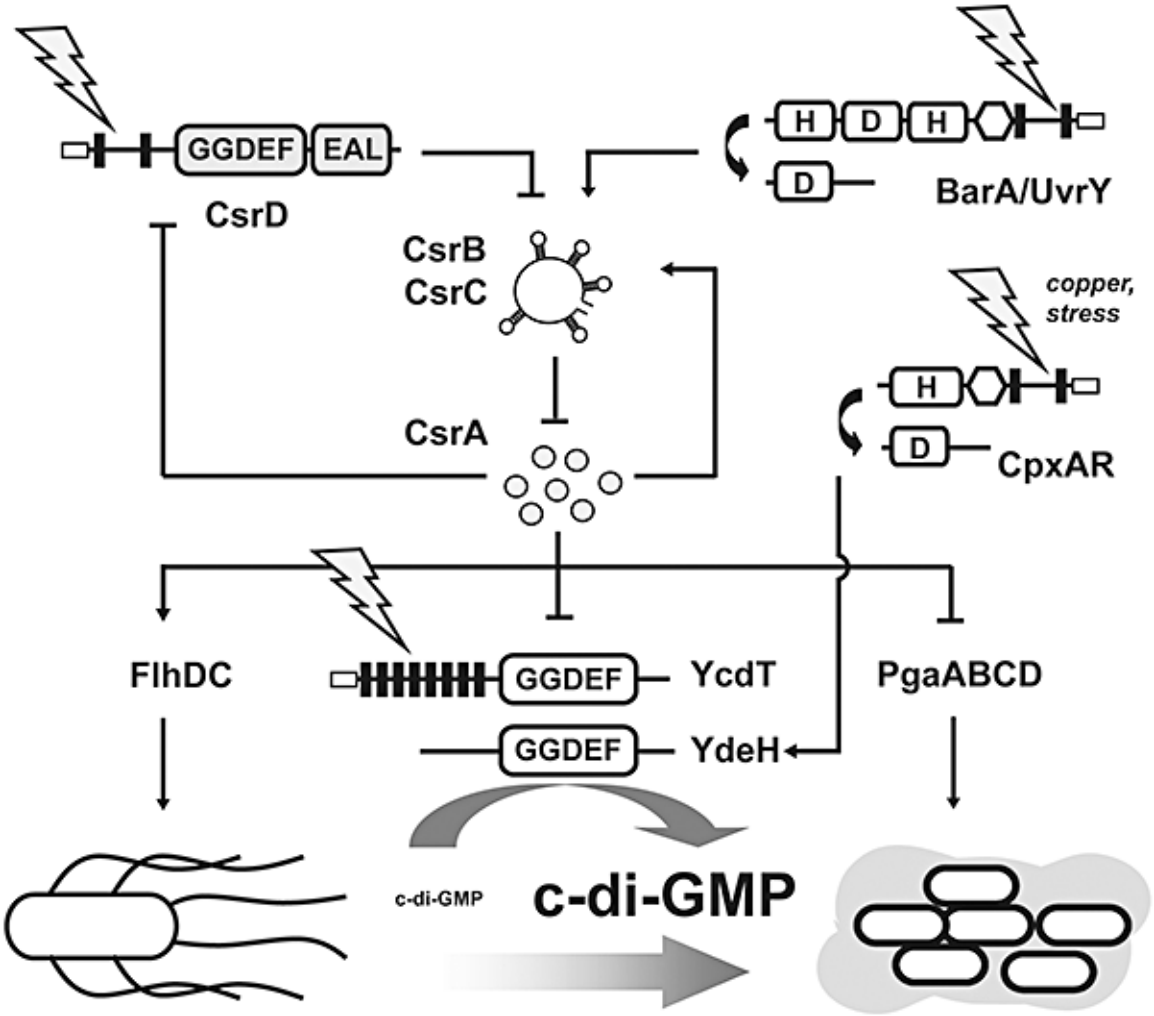

A
Fold change
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 1 2 3
ycdT
ydeH
yddV
yliF
yeaJ
yfiN
yedQ
yneF
ydaM
yeaP
yeaI*
yaiC*
dos
yhjK
csrD
yfgF
gmr
yfeA
yegE
yliE
yjcC
rtn
yahA
yhjH*
ycgG*
ylaB
yoaD
yeaL
ycgF*
GGDEF
GGDEF/
EAL
EAL
4 min
12 min
B
pBADcsrA OD600 1.5
mRNA level in %
min after induction
ycdT
ydeH
yliE
yliF
yddV
dos
csrD
C
pBAD28 OD600 1.5
mRNA level in %
min after induction
D
Ratio (csrA::kan/wild type)
fold change
ycdT
ydeH
dos
yliF
yliE
yddV
csrD
E
YcdT
YdeH
YliF
YddV
Dos
CsrD
YliE
GGDEF
EAL
Transmembrane
PAS domain
PAC domain
GGDEF or EAL domain (Pfam)
not active as DGC or PDE
F
yliE
yliF
872000
874000
876000
G
dos
yddV
1562000
1564000

Fig. 3-9.


GGDEF
EAL
CsrD
H
D
H
D
BarA/UvrY
CsrB
CsrC
copper,
stress
CsrA
H
D
CpxAR
FlhDC
GGDEF
YcdT
PgaABCD
GGDEF
YdeH
c-di-GMP
c-di-GMP

Chapter 4. Complex regulatory network encompassing the Csr, c-di-GMP and motility systems of *Salmonella* Typhimurium

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ABSTRACT

Bacterial survival depends on the ability to switch between sessile and motile lifestyles 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) (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 (Tamayo *et al.*, 2007, Cotter and Stibitz, 2007). 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 (Christen *et al.*, 2005, Paul *et al.*, 2004b, Ryjenkov *et al.*, 2005, Schmidt *et al.*, 2005, Simm *et al.*, 2004, Ryan *et al.*, 2006). Notably, individual bacterial genomes frequently encode numerous GGDEF and EAL/HD-GYP proteins (Galperin, 2004, Galperin *et al.*, 2001), 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 (Simm *et al.*, 2007, Solano *et al.*, 2009, Kader *et al.*, 2006, Garcia *et al.*, 2004). 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 (Lapouge *et al.*, 2008, Romeo, 1998, Lucchetti-Miganeh *et al.*, 2008, Babitzke and Romeo, 2007). In γ-proteobacteria CsrA proteins are antagonized by small non-coding RNAs (sRNAs) that bind and sequester multiple copies of CsrA (Lapouge *et al*., 2008, Babitzke and Romeo, 2007). 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 (Lapouge *et al*., 2008, Babitzke and Romeo, 2007).

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 (Lawhon *et al*., 2003, Altier *et al.*, 2000a), no direct mRNA targets have been identified in *Salmonella*. Here, we show that CsrA 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 4-1). 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 4-1). 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. 4-1). 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 (Wang *et al*., 2005, Wei *et al*., 2001). Previous studies determined an optimal site [5'-(A/U)CA-GGA-G(U/A)-3'] for high affinity CsrA/RsmA binding (Schubert *et al*., 2007, Valverde *et al.*, 2004, Dubey *et al*., 2005). 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 (Wang *et al*., 2005, Dubey *et al*., 2003, Baker *et al*., 2002, Baker *et al*., 2007, Jonas *et al*., 2008, Weilbacher *et al*., 2003, Liu *et al*., 1997, Bhatt *et al.*, 2009a). 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*, *STM3375*, *STM1703*, *STM3611*, *STM1344* and *STM1697* contained at least one (*STM1703*) and at most 11 (*STM1987*) GGA motifs (Table 4-2), 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 4-2). 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. 4-2A). 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 4-2). 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. 4-2A), 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. 4-2A). 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) (Dubey *et al*., 2003, Baker *et al*., 2007, Baker *et al*., 2002, Wang *et al*., 2005, Bhatt *et al*., 2009a). In contrast, CsrA binding to the transcripts of *ycdT*, *ydeH* (K_ds ≈ 2.5 nM) and *grlR* (K_d ≈ 6 nM) was stronger (Bhatt *et al*., 2009a, Jonas *et al*., 2008).

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. 4-2B, S. 2B). Unlabelled *STM3611* RNA exhibited complex interactions (Fig. 4-2B). As the concentration of this RNA was increased, slower migrating bands were observed, the major species of which was also seen in the 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. 4-2B). 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. 4-1). Consistent with this finding, the level of STM3611 protein was strongly decreased in the *csrA* mutant as observed by Western Blot analysis (Fig. 4-3A). *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, Frye *et al.*, 2006, Wang *et al.*, 2004, 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 (Lawhon *et al*., 2003, Wei *et al*., 2001). 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. 4-3B). 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, Frye *et al*., 2006, Wang *et al*., 2004, 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. 4-3C). 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. 4-3D).

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. 4-3E). 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.

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. 4-2A,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 4-2), 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. 4-4A), 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. 4-4A).

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. 4-4B).

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. 4-1, Fig. 4-5A). 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. 4-5B). 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. 4-5C), 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 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. 4-5D). 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. 4-5E). 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. 4-5E). 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 suggests 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 (Wang *et al*., 2005, Wei *et al*., 2001, Jonas *et al*., 2008, Jackson *et al*., 2002, Romeo, 1998). In agreement, CsrA was found to directly or indirectly downregulate the c-di-GMP producing enzymes STM1987 and STM4551, respectively, in *S.* Typhimurium (Figs. 4-1 and 4-2). 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 flagella motor by degrading a local pool of c-di-GMP (Wolfe and Visick, 2008, Romling and Amikam, 2006). 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 (Wolfe and Visick, 2008, Romling and Amikam, 2006). 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. 4-3) and STM1344 (Fig. 4-5), respectively, and by a direct, presumably post-transcriptional mechanism (Figs. 4-2 and 4-4). 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. 4-1), 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. 4-1, 4-2 and 4-5), 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. 4-6). 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. 4-1 and 4-2). 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. 4-1, Fig. 4-2). The *E. coli* STM3375 orthologue CsrD (YhdA) is itself a component of the Csr system (Suzuki *et al*., 2006, Jonas *et al.*, 2006), which facilitates RNaseE-dependent 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. 4-6), which activates the transcription of *csrB* and *csrC* (Altier *et al.*, 2000b, Teplitski *et al.*, 2003, Weilbacher *et al*., 2003, Suzuki *et al*., 2002). 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 (Jonas and Melefors, 2009a, Pernestig *et al.*, 2003, Mondragon *et al.*, 2006). 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. 4-6) 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, 2009a). 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. 4-6). 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.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 4-1. 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 4-3) 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 4-1. 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 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. 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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Table 4-1. 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	(Solano *et al*., 2009, Garcia *et al*., 2004)
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	(Simm *et al*., 2007, Garcia *et al*., 2004)
STM3611 (YhjH)	positive, direct and indirect	ELL	PDE	Upregulation of motility, downregulation of the rdar morphotype	(Simm *et al*., 2007, Simm *et al*., 2004)
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	(Simm *et al*., 2007, Simm *et al*., 2009, Wozniak *et al*., 2008, Hisert *et al*., 2005)
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 4-2. 5’ untranslated leader sequences of CsrA regulated genes.

Gene	5’ UTR
STM1987	**U**UUCAUCUGAUACA**GGA**GGCAGGUAUGGUCUUUUCUGUCAGUGA**GGA**AGUUACCGUGAAAGAGGGUG GGCCCCGGAUGAUCGUCACCGGUUAUUCCAGC**GGA**AUGGUAGAGUGUCGCUGGUAUGAUGGUUUUGG CGUAAAGCG**GGA**GGCAUUCCAUGAGAAUGAGCUCGUGCCAGGCAA**GGA**AAGGCGCGUGCG**GGA**CGAA GCCCGAUGACAAAACGCCCGGUUAAUGCCGGGCGUUUUGCUGU**GGA**GAAAAAGAGUCGUCGCGCUGC GACAUGCUGCUACCUGGUUAUGCCA**GGA**UUGAGUGGUAUCCCUUGAUGAGGUCUGGCUACCGUAAGC CAUCAGGG**GGA**GUUGUAUCAAUAACCA**GGA**GUUACCAGG**GUG**
STM4551	**G**UUAACGUAAAAACAACAACACCTCGCUUUAGGCGACCUUUUCUCAUACGUUAUUUUCAUGUUGGCGC CCGAUUCCGUGUAUAUCACCGAUAAAUAACGACAAUUACCC**AUG**
STM3375	**U**UCAGGCU**GGA**CCAGU**GGA**CAUCCACCGUCACAUCACCUGCCGGCAGUUGACUCUCUUCGAGAUGUUG CAC**GGA**UGCGAGGGUUUUACCGUCCUGCUGUUCUAAGAUCAACGCCUGCAUAACCUGUCCUCACUUUA CAUGGUAGAUU**GGA**AAAAUAGCUACGAAGACUAUACUCGCUAAU**U**AAAACGUGAUGCCGAUGCAACG CAAUAAAUUGCCAGAUAGAUCCAUUUUGGUAGUAUGCCUGCUUCAUUGCGCGCCGUGGCGAAUUCCGC CUUCAGAUUCGCUUUUUCAUACUGUUUAUAACCGUCGGAGUUAACUCAA**GGAUG**
STM1703	**A**UAGAUAAAACAAUACGUUCAGCCAUAAUUACCCUUAUUUAUAACAUGGCUAACCUUUCUUUUCGGG CACCUGGCGCUUAAAUGGGAUGGUACGC**AUG**
STM3611	**A**GUCC**GGA**CAGUCACACUCCCAUUAACA**GGA**CAACUGAG**AUG**
STM1827	**U**CGCGGUUGGCUUUUGUCGCCAACGCUUAUUGGGCGGCCCGC**AUG**
STM1344	**A**ACCGUAGCAUUUACAAACGTACUUUUAUUAAAAACGGGCGUGUUCACGCCCUGUGAAAAGUACAAGA UUGAAUAUUGGUUUAUAAUCAGAG**U**AAGUUAAAAAAA**GGA**ACCGGUUCACCGGUAAACAAGAAAAAU GAUGACGCUUCAAUUAGUAUCUGAAGUUACGUCUGUUUCCGUCCGUAGUGACU**GGA**UGGCGAAUAGC GCCCUAACCAUG**GGA**CUGGCGUAA**AUG**
STM1697	**U**UUUAUUGAUUGAUUUGCGUUAAAAGCGCAUUAUCAG**GGA**GUUAAGAUCAAGU**GGA**GUGGUCAC**AU G**

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

Table 4-3. Strains and plasmids used in this study.

Strain or plasmid	Description or genotype	Reference
Strains		
Salmonella enterica serovar Typhimurium ATCC 14028		
UMR1	ATCC 14028-ls 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)

FIGURE LEGENDS

Figure 4-1. 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 (** $P < 0.01$; * $P < 0.05$).

Figure 4-2. 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.

Figure 4-3. 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 4-4. 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.

Figure 4-5. 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.

Figure 4-6. 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 (Solano *et al*., 2009, Garcia *et al*., 2004), whereas STM1703, STM1827 and STM3611 act as PDEs (Simm *et al*., 2007, Simm *et al*., 2004). 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.

Fig. 4-1.

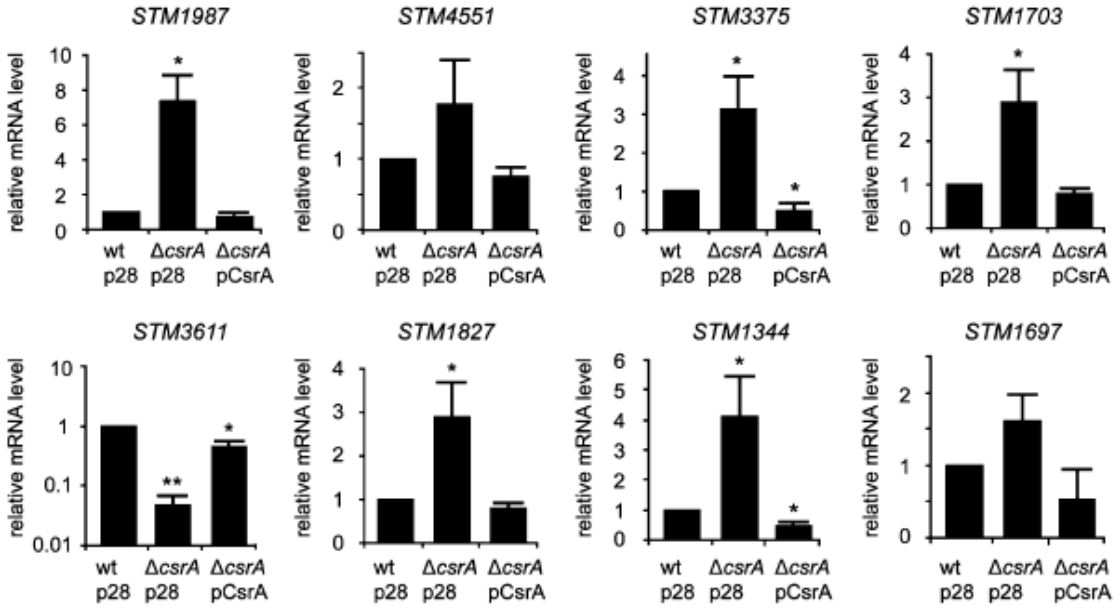

STM1987
STM4551
STM3375
STM1703
STM3611
STM1827
STM1344
STM1697
relative mRNA level
wt p28
ΔcsrA p28
ΔcsrA pCsrA
*
**

Fig. 4-2.

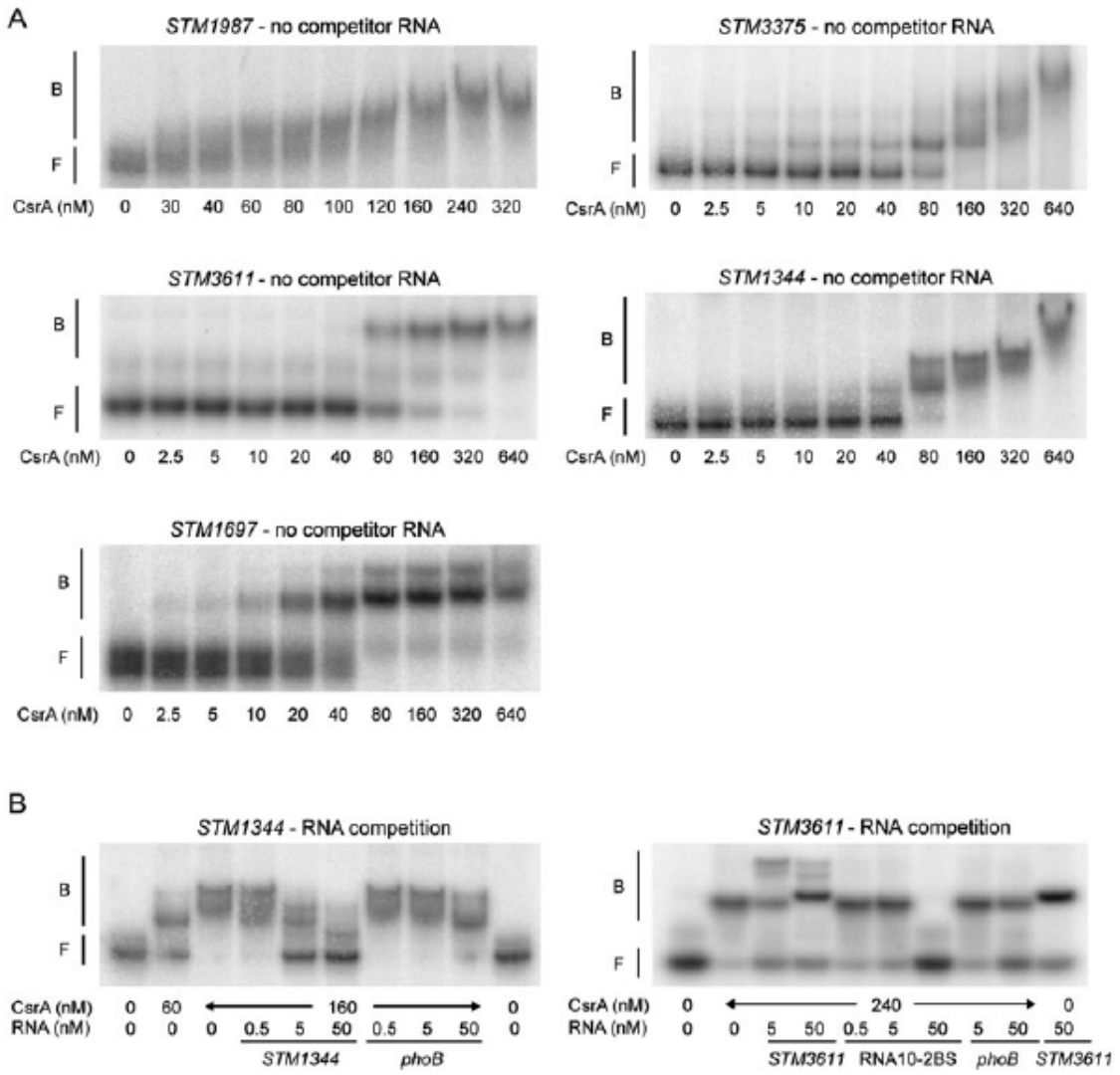

A
STM1987 - no competitor RNA
B
F
CsrA (nM) 0 30 40 60 80 100 120 160 240 320
STM3375 - no competitor RNA
CsrA (nM) 0 2.5 5 10 20 40 80 160 320 640
STM3611 - no competitor RNA
CsrA (nM) 0 2.5 5 10 20 40 80 160 320 640
STM1344 - no competitor RNA
CsrA (nM) 0 2.5 5 10 20 40 80 160 320 640
STM1697 - no competitor RNA
CsrA (nM) 0 2.5 5 10 20 40 80 160 320 640
B
STM1344 - RNA competition
CsrA (nM) 0 60 160 0
RNA (nM) 0 0 0 0.5 5 50 0.5 5 50 0
STM1344
phoB
STM3611 - RNA competition
CsrA (nM) 0 240 0
RNA (nM) 0 0 5 50 0.5 5 50 5 50 50
STM3611
RNA10-2BS
phoB
STM3611

Fig. 4-3.

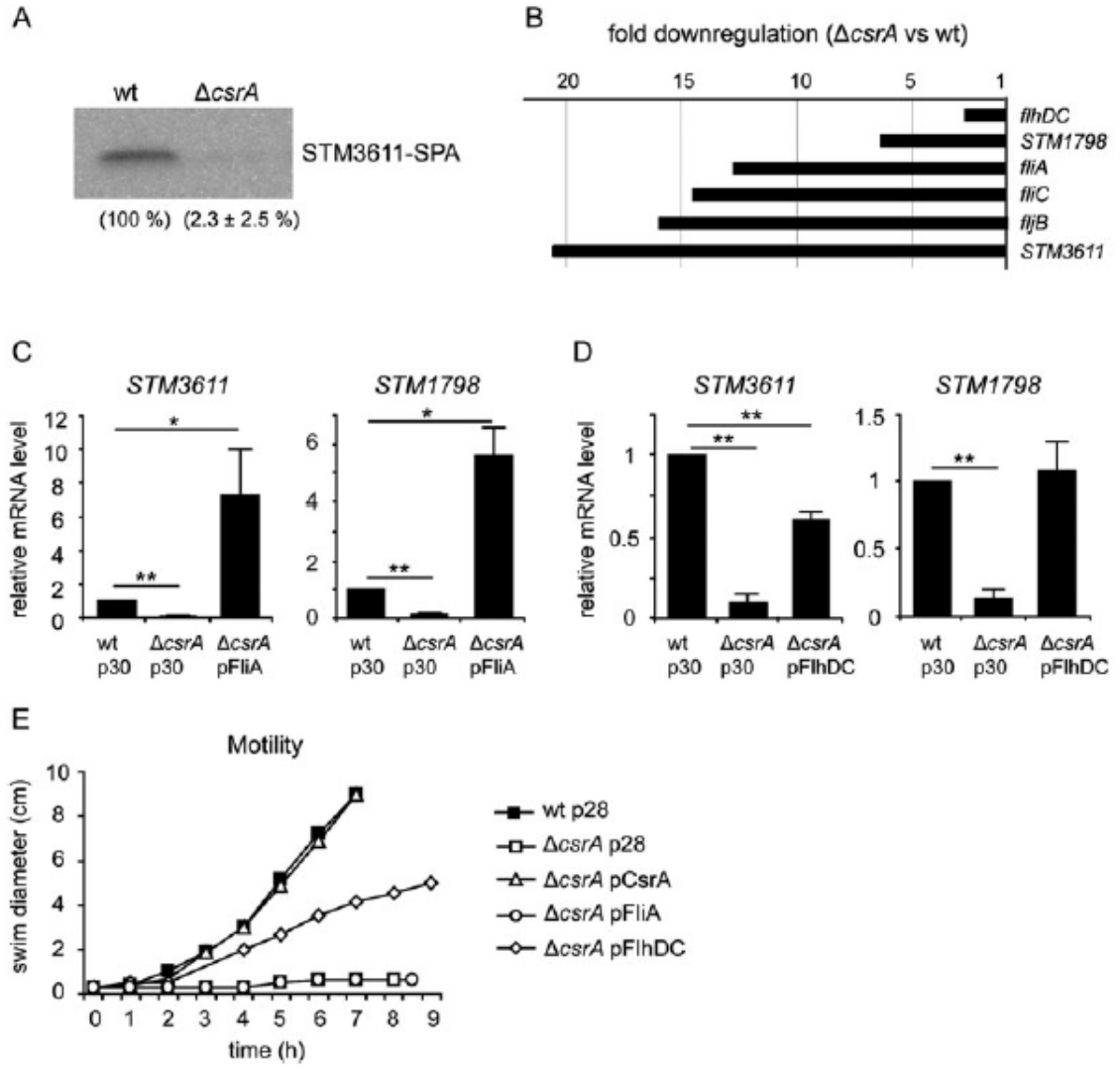

A
wt
ΔcsrA
STM3611-SPA
(100 %) (2.3 ± 2.5 %)
B
fold downregulation (ΔcsrA vs wt)
20
15
10
5
1
flhDC
STM1798
fliA
fliC
fljB
STM3611
C
STM3611
STM1798
relative mRNA level
wt p30
ΔcsrA p30
ΔcsrA pFliA
D
STM3611
STM1798
relative mRNA level
wt p30
ΔcsrA p30
ΔcsrA pFlhDC
E
Motility
swim diameter (cm)
time (h)
wt p28
ΔcsrA p28
ΔcsrA pCsrA
ΔcsrA pFliA
ΔcsrA pFlhDC

Fig. 4-4.

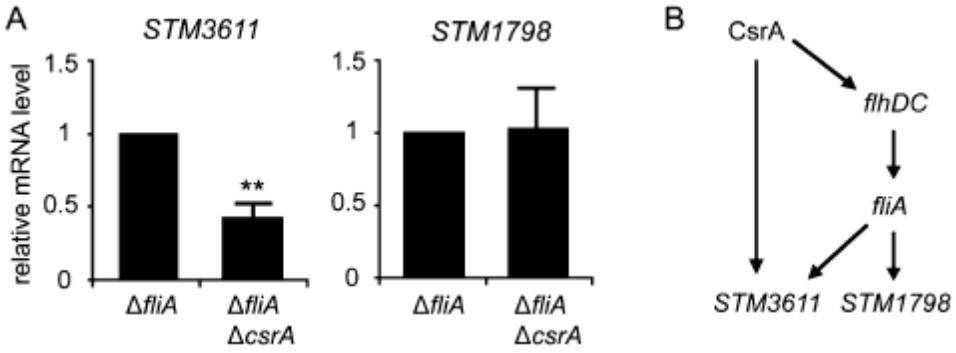

A
STM3611
STM1798
relative mRNA level
1.5
1
0.5
0
**
ΔfliA
ΔfliA ΔcsrA
B
CsrA
flhDC
fliA
STM3611
STM1798

Fig. 4-5.

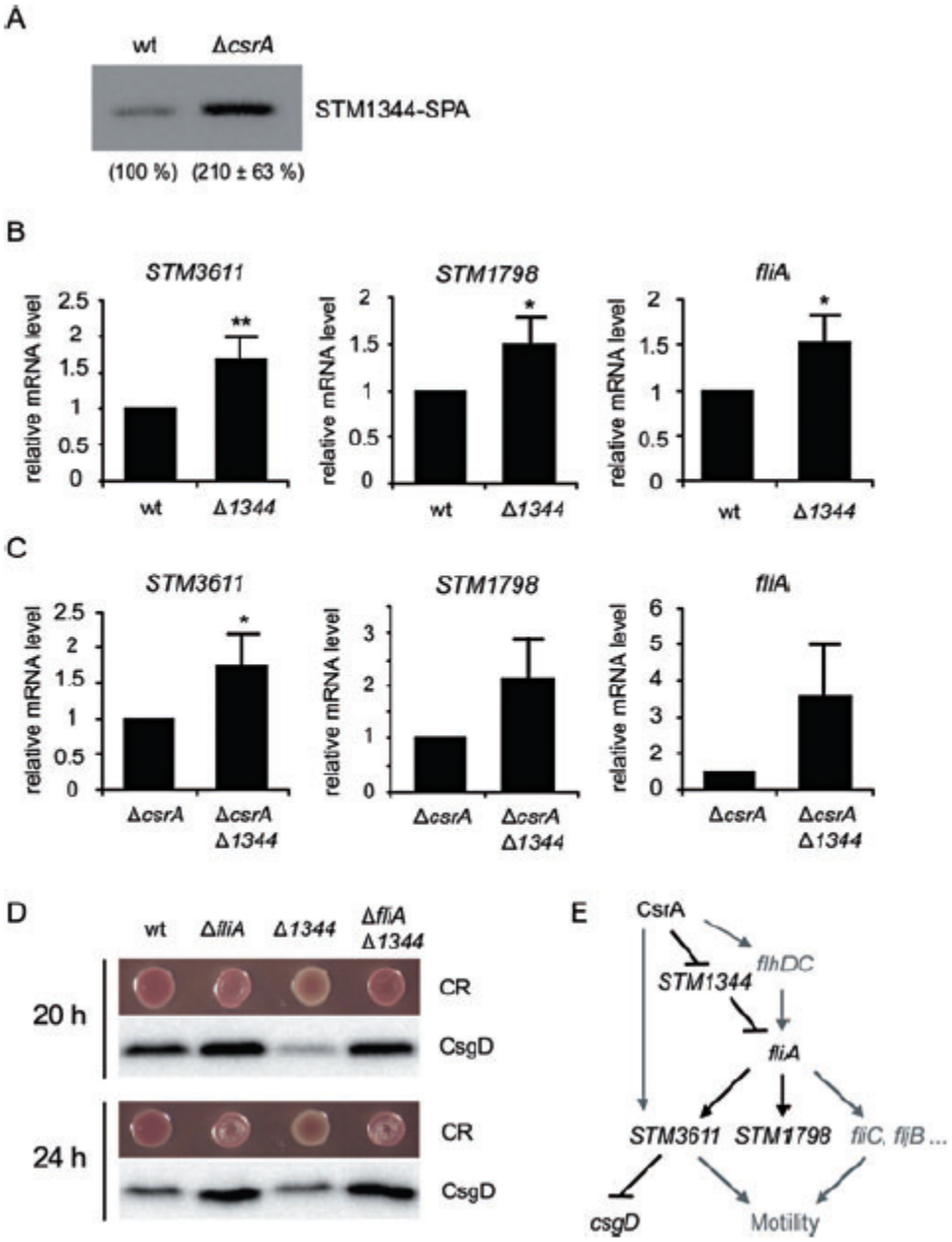
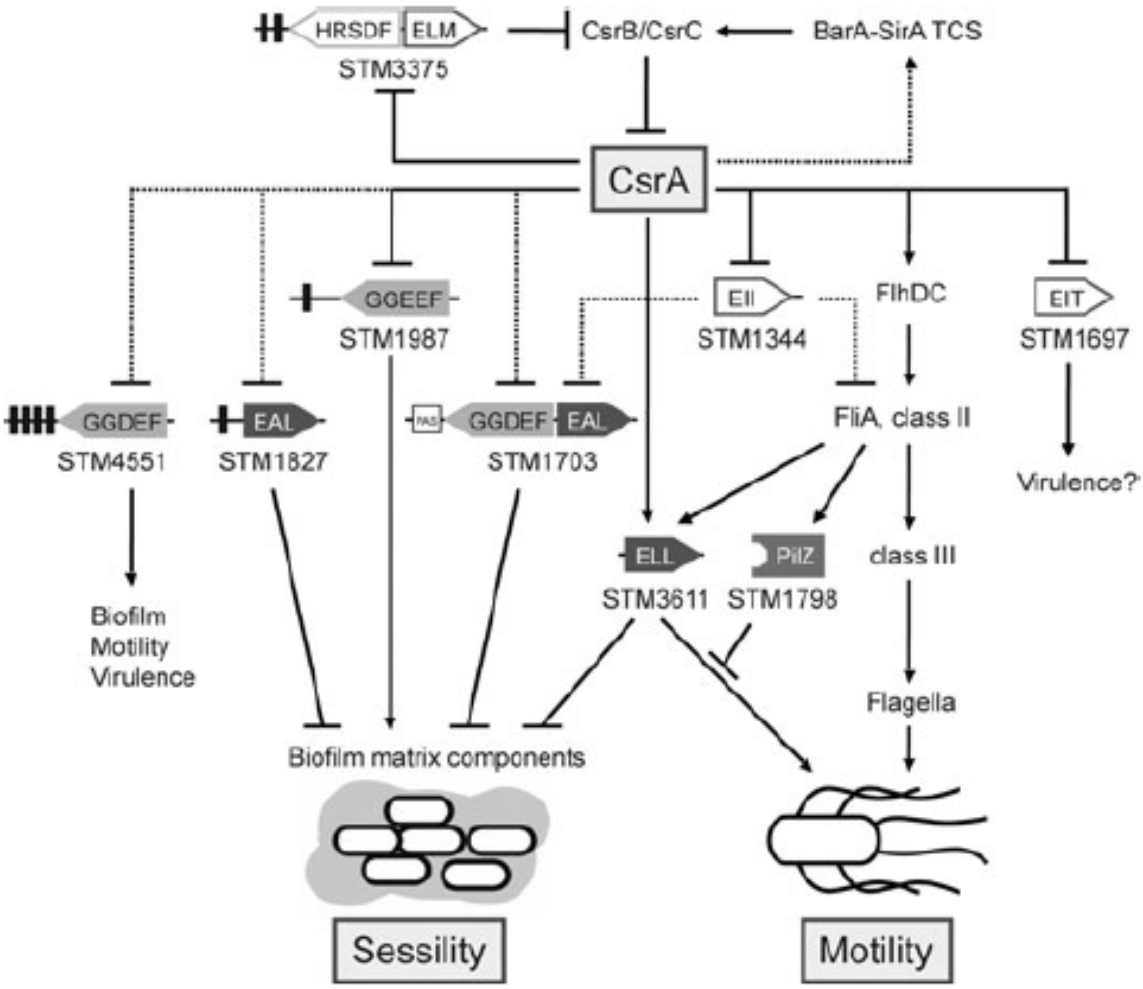

A
wt
ΔcsrA
STM1344-SPA
(100 %) (210 ± 63 %)
B
STM3611
STM1798
fliA
relative mRNA level
wt
Δ1344
C
ΔcsrA
ΔcsrA Δ1344
D
wt
ΔfliA
Δ1344
ΔfliA Δ1344
20 h
24 h
CR
CsgD
E
CsrA
STM1344
flhDC
fliA
STM3611
STM1798
fliC, fljB ...
csgD
Motility

Fig. 4-6.


HRSDF
ELM
STM3375
CsrB/CsrC
BarA-SirA TCS
CsrA
GGEEF
STM1987
EII
STM1344
FlhDC
EIT
STM1697
GGDEF
STM4551
EAL
STM1827
PAS
GGDEF
EAL
STM1703
FliA, class II
Virulence?
ELL
STM3611
PilZ
STM1798
class III
Biofilm
Motility
Virulence
Flagella
Biofilm matrix components
Sessility
Motility

Chapter 5. Discussion

Global role of CsrA in *Escherichia coli*

CsrA has been implicated as a global regulator in a variety of prokaryotes, coordinating gene expression for many physiological processes (Romeo *et al.*, 1993; Romeo, 1998; Lawhon *et al.*, 2003; Timmermans and Van Melderen, 2010). Corroborating this hypothesis, the work presented here determined that CsrA in *Escherichia coli* K-12 has a potential regulon of 721 targets and regulates components of the stringent response and the metabolism of c-di-GMP. Although the CsrA-His_6 copurification screen was not saturated, the number of genes whose transcripts copurified with CsrA was comparable to a recent study that estimates that the regulon of CsrA (RsmA) of *Pseudomonas aeruginosa* PAK encompasses over 500 genes (Brencic and Lory, 2009).

Evidence that CsrA directly interacts with the majority of these transcripts in vivo is as follows: i) Few representatives of highly abundant classes of cellular RNAs, e.g. antisense sRNAs and tRNAs, were identified in these experiments, with the exception of rRNA (discussed below). ii) Low abundance transcripts, which have been previously found to be targets of CsrA binding, were identified (e.g. *flhDC* (Wei *et al*., 2001)). iii) Less than 1% of the amount of RNA that was recovered by nickel affinity purification from cells expressing recombinant CsrA-His_6 protein was isolated from control cells lacking this protein. iv) Gel shift assays using the leaders of 25 of these transcripts revealed that CsrA bound to 80% with high affinity and specificity (unpublished data, A. N. Edwards and T. Romeo). Because CsrA can also interact with intervening noncoding

segments of polycistronic mRNAs (Yang *et al.*, 2010), we estimate that the maximal occurrence of false positives in the copurification experiments was 20%. We also recovered abundant rRNAs in our copurification experiments. These may have originated from polysomes of mRNAs that were bound specifically to CsrA, as CsrA was not identified in a blind screen for proteins that interact directly with ribosomes (Jiang *et al.*, 2007) nor was CsrA found to interact with ribosomal proteins in a comprehensive protein-protein interaction study (Butland *et al.*, 2005).

Two dimensional protein gel analysis revealed some novel potential targets of CsrA regulation (Appendix, Table 2). While some of these effects could be indirect, several transcripts that copurified with CsrA-His_6 were also identified in this analysis (Appendix, Table 3), strongly suggesting that these are direct targets of CsrA. Indeed, CsrA was already known to regulate phosphoenolpyruvate carboxykinase (encoded by *pckA*) and phosphoglucomutase (encoded by *pgm*) (Appendix, Table 3; Romeo *et al.*, 1993; Sabnis *et al.*, 1995). Additionally, *gatZ* was discovered through both analyses. Gel shift analyses confirmed a specific, high affinity interaction between CsrA and the 5' mRNA leader of *gatY* (unpublished data, A. N. Edwards and T. Romeo), the first gene in the *gatYZABCDR'* operon, which encodes the enzymes required for galactitol metabolism (Nobelmann and Lengeler, 1996). The *csrA* mutant also grew better than the wild-type strain on minimal media with galactitol as the only carbon source (unpublished data, A. N. Edwards and T. Romeo), suggesting that CsrA may directly repress expression of the *gatYZABCDR'* operon and that the upregulation of the *gat* operon in the *csrA* mutant may allow for more efficient utilization of this carbon source. Together, both of these global analyses indicated that a large number of metabolic and physiological processes are

included in the Csr regulon and confirms the critical role CsrA has in regulating carbon flux. Additionally, because the stringent response regulatory system is only one of many transcriptional regulatory systems whose mRNAs copurified with CsrA-His_6 (Appendix, Table 1), these data suggest that the extent of the complete regulon and circuitry of the Csr system is considerable and underappreciated.

Our data have not only revealed a more definitive role for CsrA as a global regulator, but have also indicated that CsrA may exert its effects on multiple levels to efficiently regulate a single cellular process. CsrA directly and post-transcriptionally represses *pgaABCD* expression (Wang *et al.*, 2005), which encodes the structural genes necessary for the synthesis of the polysaccharide adhesion, poly-β-1,6-*N*-acetyl-glucosamine, and is essential for biofilm formation in *E. coli* (Wang *et al*., 2004). In addition, c-di-GMP promotes biofilm formation through regulating the expression of the *pgaABCD* operon in an unknown mechanism (Boehm *et al.*, 2009; unpublished data, M. Kumar, C. C. Goller, A. Pannuri and T. Romeo). We discovered that CsrA directly binds to the mRNA leaders and strongly represses the expression of the GGDEF domain proteins, *ydeH* and *ycdT*, and modestly affects expression of five other GGDEF domain proteins in *E. coli* (Chapter 3). Together, with the finding that the cellular concentration of c-di-GMP is increased approximately 2-fold in a *csrA* mutant (Chapter 3), these findings highlight an important regulatory connection between the Csr system, c-di-GMP metabolism and biofilm formation.

Our global analysis revealed an additional connection between CsrA and the expression of the *pgaABCD* operon. The *nhaR* transcript, encoding the transcriptional activator NhaR, which is required for transcription of *pgaABCD* (Goller *et al.*, 2006),

copurified with CsrA-His_6. Gel shift assays confirmed that CsrA directly binds to the *nhaR* mRNA leader with high affinity and specificity, while reporter fusions demonstrated that CsrA post-transcriptionally represses *nhaR* expression ~5-fold (unpublished data, A. Pannuri, A. N. Edwards and T. Romeo). Altogether, these data present a complex circuitry in which multiple effects of CsrA regulation converge to control PGA production and biofilm formation in *E. coli*. CsrA regulates biofilm formation through both c-di-GMP-independent and dependent mechanisms, allowing a variety of specific adaptive signals to trigger biofilm formation. These multiple effects of CsrA also provide a redundancy of regulation that likely affords robust regulation.

Our results further demonstrate that CsrA is a key regulator for controlling the switch between motility and biofilm formation in *E. coli* and *S.* Typhimurium. In *E. coli*, CsrA directly activates expression of *flhDC* (Wei *et al.*, 2001), which encodes the master regulator for the flagellar cascade. In *S.* Typhimurium, CsrA appears to possess a conserved role in promoting motility as CsrA positively regulates expression of *flhDC* and *yhjH*, which encodes a phosphodiesterase (Chapter 4). It is likely that CsrA may regulate *yhjH* expression in *E. coli* as well, but this has not been experimentally determined.

Although CsrA inhibits biofilm formation in both *E. coli* and *S.* Typhimurium, the mechanisms differ. *S.* Typhimurium does not produce PGA; here, CsrA likely inhibits biofilm formation by downregulating production of curli and cellulose (Chapter 4). Moreover, the set of GGDEF/EAL domain proteins regulated by CsrA differs between *E. coli* and *S.* Typhimurium. For example, CsrA directly represses expression of the two GGDEF domain proteins, YcdT and YdeH, which are present in *E. coli* but do not have

homologs in *S.* Typhimurium. While the mechanisms of regulation vary, CsrA plays a conserved global role in controlling these physiological processes, and it is possible that similar regulation occurs in most bacteria possessing a CsrA homolog.

Global regulators besides CsrA have been demonstrated to influence biofilm formation and motility (e.g. RpoS and cAMP-CRP; Karatan and Watnick, 2009; Sahr *et al.*, 2009; Fong and Yildiz, 2008). Similarly to CsrA, many of these directly and indirectly regulate the expression of GGDEF and EAL domain proteins (Weber *et al.*, 2006; Fong and Yildiz, 2008; Waters *et al.*, 2008). In addition, ppGpp has been implicated in regulating both biofilm formation (Boehm *et al*., 2009) and motility (Lemke *et al.*, 2009) in *E. coli*. This multilayered control and interconnected regulation by and within the Csr, stringent response and c-di-GMP global regulatory systems allow for the incorporation of a variety of environmental stimuli and is likely a common manner for controlling physiological processes in bacteria.

Potential role of CsrA in regulation of secondary messenger metabolism

We have defined the regulatory interactions CsrA has with transcripts that encode proteins required for the metabolism of guanosine tetraphosphate (ppGpp) (Chapter 2) and cyclic di-GMP (Chapters 3 and 4). Our data demonstrate that the Csr system regulates the expression of ppGpp synthetases in *E. coli* and suggest that CsrA may control the synthesis of ppGpp in certain conditions in which a strong induction of stringent response occurs. Additionally, the Csr circuitry regulates the expression and activity of GGDEF and EAL domain proteins in *E. coli* and *S.* Typhimurium. Altogether,

these studies suggest that CsrA may be an important mediator of global second messenger metabolism.

With the modest effects of Csr on the expression of *relA, spoT* and *dksA*, it is likely that the Csr system may function to fine tune gene expression, rather than serving as an on/off switch. It is possible that CsrA-mediated regulation of *relA* and *spoT* expression affects levels of ppGpp. There were no significant changes between the wild-type and *csrA* mutant strains in basal levels of ppGpp, the kinetics of ppGpp synthesis upon amino acid starvation or in accumulation of ppGpp after the induction of stringent response in one dimensional TLC analysis (Appendix, Fig. 2). The relatively poor growth of the *csrA* mutant on minimal media prevented us from monitoring ppGpp levels under starvation conditions, which are necessary for maximum ppGpp synthesis. It is possible that under rich growth conditions, the amount of RelA protein in the wild-type strain is sufficient to fully respond to amino acid starvation. However, there may be other conditions that generate a stronger stringent response in which ppGpp levels between the wild-type and *csrA* mutant strains differ. Careful studies are needed to clarify the role CsrA may have in regulating ppGpp accumulation and the stringent response.

As previously mentioned, CsrA represses global c-di-GMP levels in *E. coli* by directly regulating expression of GGDEF domain proteins (Chapter 3) and may play a similar role in *S.* Typhimurium (Chapter 4). Interestingly, our data also implicate CsrD, a degenerate GGDEF/EAL domain protein that is not involved in c-di-GMP metabolism, as possessing a role as a regulator within the c-di-GMP network. CsrD may affect the metabolism of c-di-GMP through mediating the decay of the sRNAs, CsrB and CsrC,

thus, regulating CsrA activity. Furthermore, CsrA represses expression of *csrD* in both *E. coli* and *S.* Typhimurium, producing an autoregulatory loop to provide precise regulation of CsrA activity and likely c-di-GMP accumulation.

Preliminary data suggest that CsrA may also affect the synthesis and regulation of an additional secondary messenger, cyclic AMP (cAMP). The transcripts for *crp* and *cyaA*, which encode the catabolite repressor protein and adenylate cyclase respectively, copurify with CsrA-His_6 (Appendix, Table 1). Gel shift analyses confirm that CsrA directly bound to the *crp* transcript with high affinity and specificity (unpublished data, A. N. Edwards and T. Romeo), suggesting that CsrA may regulate expression of *crp* in vivo. CsrA also interacted with the *cyaA* mRNA leader but exhibited modest affinity and specificity. In addition, preliminary studies examining steady state *crp* transcript and CRP protein levels suggest that CsrA represses *crp* expression (unpublished data, A. N. Edwards and T. Romeo). Further studies are needed to determine the effect CsrA may have on *crp* and *cya* expression, but it is plausible that CsrA may influence catabolite repression. Because CsrA has a central role in regulating carbon metabolism and flux, regulation of catabolite repression would create an additional layer of control over the response to carbon availability in the environment. Overall, our data indicate that the Csr system may be a global regulator of secondary messenger metabolism in *E. coli* and other closely related organisms.

Complex interconnections between global regulatory networks

Our studies reveal that the Csr and stringent response networks possess direct and indirect regulatory interactions within and among the components of these systems.

While the influence of CsrA on the stringent response appears modest, ppGpp and DksA exhibited strong effects on the Csr components. This suggests that under nutrient starvation, it may be necessary to reduce CsrA activity by strong upregulation of the small regulatory RNAs, CsrB and CsrC. Indeed, *csrB* and *csrC* expression is significantly increased in minimal media, and a rapid reduction in CsrB levels is observed upon the addition of tryptone, casamino acids, or amino acids (Jonas and Melefors, 2009). DksA- and ppGpp -mediated reduction of CsrA activity may allow the upregulation of CsrA-repressed genes that are necessary for survival during nutrient starvation. Indeed, CsrA represses expression of a carbon starvation response gene, *cstA* (Dubey *et al.*, 2003), which is necessary for efficient peptide uptake (Schultz and Matin, 1991). Derepression of *cstA* expression during nutrient limitation may be needed for nutrient scavenging.

It is possible that the interconnection between these regulatory systems allows for efficient regulation of shared regulatory targets. CsrA directly and post-transcriptionally represses expression of *glgCAP* (Baker *et al.*, 2002), which encodes the structural genes for glycogen biosynthesis, while ppGpp directly activates *glgCAP* transcription (Romeo, 1989; Romeo *et al*., 1990). Upon the induction of stringent response, ppGpp increases *glgCAP* transcription, while the reduction in CsrA activity through the DksA and ppGpp-mediated upregulation of *csrB* and *csrC* transcription allows for stabilization of the *glgCAP* transcript and, thus, efficient translation. Upregulating glycogen production is common during conditions in which an excess carbon source is available and other nutrients are limiting (Preiss and Romeo, 1994). In addition, CsrA activates motility by stabilizing the *flhDC* transcript (Wei *et al*., 2001) while ppGpp and DksA directly repress

expression of *flhDC* (Lemke *et al*., 2009). During the stringent response, downregulation of motility is mediated directly through ppGpp and DksA repression of *flhDC* transcription and the reduction of CsrA activity. This prevents further transcription of *flhDC* while also destabilizing the *flhDC* transcript, eliminating translation by facilitating mRNA turnover. Reducing the production of flagella during nutrient limitation allows the cell to conserve energy and nutrients. These data imply that under nutrient limiting conditions, DksA and ppGpp may indirectly upregulate *csrB* and *csrC* expression, reduce CsrA activity, and alter the expression of co-regulated genes in response to nutrient limitation. Additionally, this dual regulation provides control of gene expression at both the transcriptional and the post-transcriptional levels.

Our data indicate that DksA affects *csrA* expression primarily through the RpoS-dependent promoter, P3 (Chapter 2). It is possible that ppGpp is also needed for this regulation through RpoS as β-galactosidase activity from a P3 transcriptional fusion was decreased in a $ppGpp^0$ strain. However, additional studies are needed to confirm whether ppGpp directly activates *csrA* expression through the P1 promoter independently of DksA. Additionally, characterizing the response of *csrA, csrB* and *csrC* expression and target genes that are co-regulated by stringent response and the Csr systems upon artificial induction of the stringent response by serine hydroxamate will provide further information for the importance of the signaling between these global networks.

Interestingly, both DksA and CsrA are necessary for full expression of *csrB* and *csrC* through the response regulator, UvrY. Although acetate is a physiological stimulus for BarA activity, we found that acetate accumulation is not altered in *dksA*, *csrA*, or *dksA csrA* mutants (Appendix, Fig. 4). It is possible that both DksA and CsrA may affect *barA*

expression and/or activity. However, preliminary data indicate that DksA activates *barA* expression 2-fold in exponential phase but both DksA and CsrA repress *barA* expression 11-fold and 4-fold, respectively, in stationary phase (unpublished data, A. N. Edwards and T. Romeo). Due to a reduction of competition for access to the secondary channel of RNA polymerase, the anti-termination factors GreA and GreB can upregulate transcription in a *dksA* mutant (Aberg *et al.*, 2008). Further studies are required to confirm that derepression of *barA* expression in a *dksA* mutant also occurs in the absence of GreA and/or GreB.

Altogether, our data suggest that both DksA and CsrA signal through regulating UvrY activity, although additional studies are needed to determine if BarA activity is necessary or if an unidentified factor regulates UvrY activity. Activity of GacA, the UvrY ortholog in *Pseudomonas* species, is regulated by the BarA homolog GacS, as well as two other sensors, RetS (Goodman *et al.*, 2004; Goodman *et al.*, 2009) and LadS (Ventre *et al.*, 2006), suggesting that alternative pathways for UvrY activation may also exist in *E. coli*.

Regulatory feedback loops provide distinct advantages for complex regulatory networks. Negative feedback loops reduce stochasticity and noise (Becskei and Serrano, 2000) while also decreasing bistability (Nevozhay *et al.*, 2009). Additionally, negative autoregulation, a regulatory element controlling both *dksA* (Chapter 2) and *csrA* (unpublished data, H. Yahknin and P. Babitzke) expression, accelerates regulatory responses since an intermediate level of the regulator represses its own expression (Rosenfeld *et al.*, 2002). Because multiple feedback loops are integrated within the Csr and stringent response regulatory networks, these advantages and characteristics may be

applied to these systems. Additionally, since both systems share some genetic targets, this creates a regulatory circuit known as a dense overlapping regulon (DOR), which allows the integration of multiple signals (Beisel and Storz, 2010). The regulatory impact of each of these feedback loops within both systems is not fully defined, and further studies are necessary to determine how environmental signals are efficiently translated into a singular response for downstream regulatory targets of only one or both systems.

Outlook

Our studies provide a foundation for understanding the impact that CsrA may have on global gene expression and have implicated CsrA as an important regulator of many cellular processes that were previously unknown. Because only two global regulatory systems of many were studied here, it is likely that the Csr circuitry functions within a broad genetic network. Indeed, *csrA* transcription is driven by three defined promoters, one of which is an RpoS-dependent promoter (unpublished data, H. Yahknin and P. Babitzke), and CsrA post-transcriptionally represses *hfq* expression (Baker *et al.*, 2007). These studies highlight the global interconnections between the Csr and other regulatory systems. Furthermore, our studies define a complex regulatory network in prokaryotes to include metabolic signaling and transcriptional and post-transcriptional regulatory interactions along with protein-RNA interactions. The combination of conventional molecular genetic studies and systems biology will provide a deeper understanding of the importance of biological networks and the control these have on the behavior of the cell. This understanding of natural networks may present guidelines for building synthetic networks for specific biotechnology functions or applications.

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Appendix. Supporting Information for Chapter 2

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This partial manuscript was submitted to *Molecular Microbiology* as supporting information for Chapter 2. Martha I. Camacho and Dimitris Georgellis performed the acetate accumulation studies. Joshua A. Fields and Stuart A. Thompson performed the two dimensional protein gels and annotation. Adrianne N. Edwards performed all other experiments and analysis.

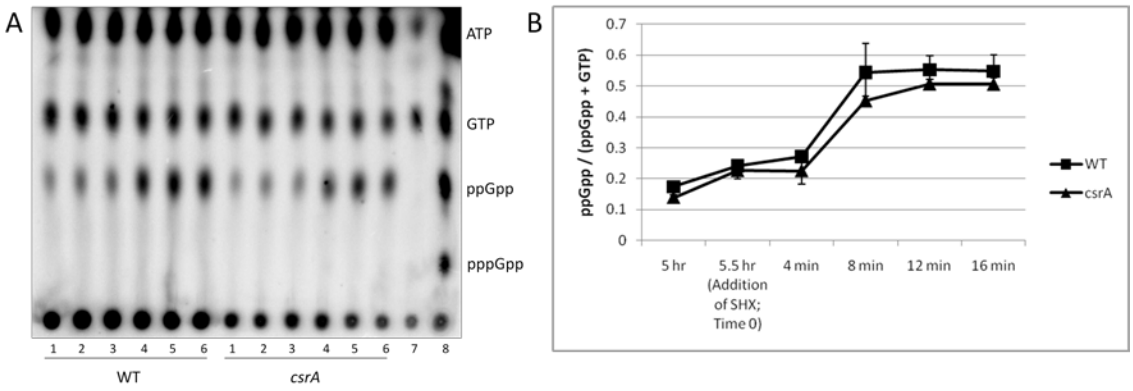

Figure 1

ppGpp quantification with one dimensional thin-layer chromatography (TLC). MG1655 (wild-type) and *csrA* strains were grown in EZ Rich Defined Medium (EZ RDM; Teknova, Hollister, CA) containing 0.33 mM K_2HPO_4 and 20 μCi ml^{-1} ^{32}P orthophosphate (Perkin-Elmer). After taking two time points in mid-exponential phase, 100 μg ml^{-1} serine hydroxamate (SHX) was added and aliquots were sampled every four minutes up to 16 minutes. Nucleotide extraction was performed by adding 200 μl of culture to 40 μl of cold formic acid and incubating on ice for approximately 20 minutes. Following centrifugation, 16 μl of supernatant was spotted onto PEI-F cellulose TLC plates and separated in 1.5 M KH_2PO_4 (pH 3.4). (A) ppGpp analysis. Lanes 1, 5 hours; Lanes 2, 5.5 hours (addition of serine hydroxamate = Time 0); Lanes 3, 4 minutes; Lanes 4, 8 minutes; Lanes 5, 12 minutes; Lanes 6, 16 minutes; Lane 7, *relA spoT* ($ppGpp^0$) in EZ RDM and 100 μg ml^{-1} serine hydroxamate; Lane 8, MG1655 (wild-type) in MOPS minimal media and 100 μg ml^{-1} serine hydroxamate. Cold ATP and GTP were also spotted and visualized by UV shadowing to confirm migration products. (B) Ratio of ppGpp to total guanine nucleotide (ppGpp and GTP) for both the wild-type (■) and *csrA* mutant (▲). Error bars represent standard deviation.

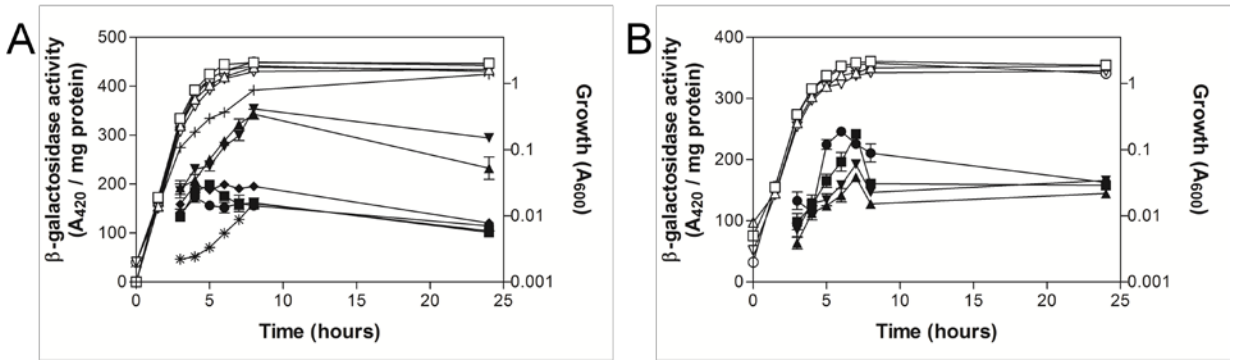

Figure 2

Complementation of *dksA* mutant on *dksA-lacZ* and P*lacUV5-dksA'-'lacZ* expression. Symbols for β-galactosidase activity corresponding to each strain are: CF7789 (wild-type), ■; *dksA*, ▲; *dksA* pHM1883 (empty vector), ▼; *dksA* pHM1506 (*dksA*$^{+}$), ♦; *dksA* pHM1506 + 0.1 mM IPTG, ●, and *dksA* pHM1506 + 1 mM IPTG, *. Growth curves are represented by open symbols except for *dksA* pHM1506 + 1 mM IPTG (+). The values represent the average of two independent experiments. Error bars depict the standard error of the mean. (A) β-galactosidase specific activity from a *dksA-lacZ* transcriptional fusion. (B) β-galactosidase specific activity from a P*lacUV5dksA'-'lacZ* leader fusion.

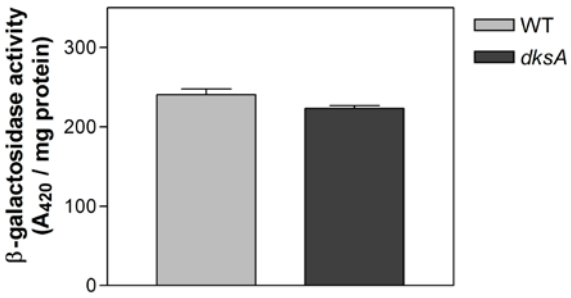

Figure 3

Effect of *dksA* on *uvrY* expression. β-galactosidase specific activity (A_{420} / mg protein) was measured at 8 hours of growth from a chromosomal *uvrY'-'lacZ* translational fusion. The values represent the average of two independent experiments, and error bars depict the standard error of the mean.

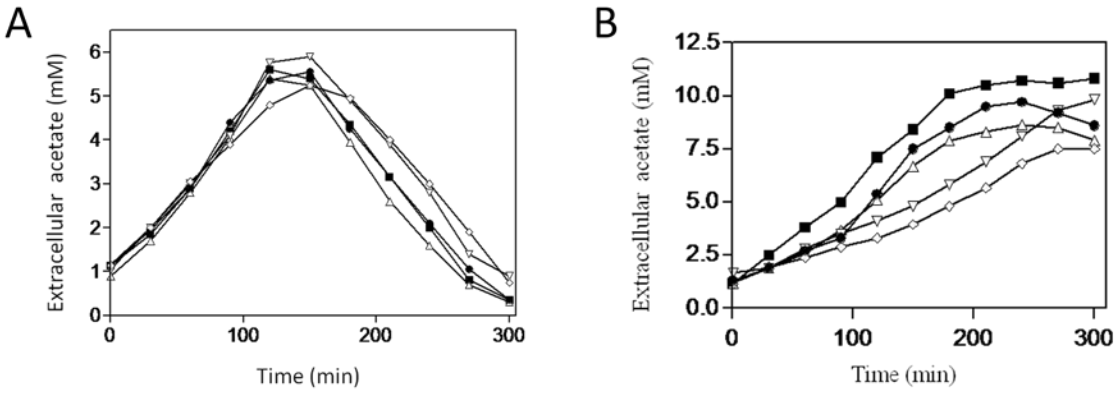

Figure 4

Acetate accumulation in wild-type (■), *csrA* (Δ), *dksA* (▽), *csrA dksA* (●), and *relA spoT* (◊) strains grown in (A) the gluconeogenic LB medium (pH 7.0) or (B) the glycolytic Kornberg medium with 0.5% glucose (pH 6.8). Samples were withdrawn at the indicated times, and the concentration of extracellular acetate was determined using the R-Biopharm Acetic acid determination kit (Boehringer Mannheim). The averages of three independent experiments are presented (standard deviations were less than 5% from the mean).

Table 1. Identities of RNAs that copurified with CsrA-His_6.

Gene Name [a]	Pull-down Condition [b]
J – Translation, ribosomal structure and biogenesis	
yadB	KB – Exponential phase
leuS	KB – Exponential phase
glnS	KB – Exponential phase
serS	KB – Exponential phase
rpsA	KB – Exponential phase
yciO	KB – Exponential phase
pheT (R)	KB – Exponential phase
infC	KB – Exponential phase
thrS	KB – Exponential phase; LB – Exponential phase
aspS	KB – Exponential phase
yeiP	KB – Exponential phase
truA	KB – Exponential phase
raiA (*yfiA*)	KB – Exponential phase; LB – Exponential phase
alaS	KB – Exponential phase
lysS	KB – Exponential phase
pnp	KB – Exponential phase
prmA	KB – Exponential phase
rpsK	KB – Exponential phase
rpsM	KB – Exponential phase
rpsE	KB – Exponential phase
rplR	KB – Exponential phase
rplE	KB – Exponential phase
rplX	KB – Exponential phase
rpsC	KB – Exponential phase
hslR	KB – Exponential phase
glyS	KB – Exponential phase
rph	KB – Exponential phase
trmH (*spoU*)	KB – Exponential phase
lysU	KB – Exponential phase; LB – Exponential phase
rne	LB – Exponential phase
fmt	KB – Exponential phase
rplW	KB – Exponential phase
selB	KB – Exponential phase
smpA	LB – Exponential phase
truC	LB – Exponential phase
rimN	LB – Exponential phase
tufB	LB – Exponential phase
K – Transcription	
nhaR	KB – Exponential phase
hepA (L)	KB – Exponential phase
yafY	KB – Exponential phase
mhpR	KB – Exponential phase
ybdO	KB – Exponential phase
rutR (*ycdC*)	KB – Exponential phase

uidR	KB – Exponential phase
ydhM	KB – Exponential phase
rpoE	KB – Exponential phase
yfiE	KB – Exponential phase
ascG	KB – Exponential phase
rpoD	KB – Exponential phase
ebgR	KB – Exponential phase
yhaJ	KB – Exponential phase
sohA (*prlF*)	KB – Exponential phase
agaR (G)	KB – Exponential phase
nanK (G)	KB – Exponential phase
fusA	KB – Exponential phase
rpsG	KB – Exponential phase
yhgF	KB – Exponential phase
malT	KB – Exponential phase
rpoH	KB – Exponential phase
nikR	KB – Exponential phase
dgoR	KB – Exponential phase
rho	KB – Exponential phase
metR	KB – Exponential phase
yihW (G)	KB – Exponential phase
cytR	KB – Exponential phase; LB – Exponential phase
fabR	KB – Exponential phase
nusG	KB – Exponential phase
rpoB	KB – Exponential phase
iclR	KB – Exponential phase
dcuR (T)	KB – Exponential phase
allR	LB – Exponential phase
dicA	LB – Exponential phase
hyfR (T)	KB – Exponential phase
nsrR	KB – Exponential phase
cdaR (T)	LB – Exponential phase
fhlA (T)	LB – Exponential phase
ycfQ	LB – Stationary phase
L – DNA replication, recombination and repair	
yafM	KB – Exponential phase
recG (K)	KB – Exponential phase
yagA	KB – Exponential phase
sbcC	KB – Exponential phase
ybcK	KB – Exponential phase
nohB	KB – Exponential phase
holA	KB – Exponential phase
helD	KB – Exponential phase ; LB – Exponential phase
ycfH	KB – Exponential phase
topB	KB – Exponential phase
ligA	KB – Exponential phase
intA	KB – Exponential phase
recC	KB – Exponential phase

parE	KB – Exponential phase
smf (U)	KB – Exponential phase
yhhF	KB – Exponential phase
mutM	KB – Exponential phase
ligB	KB – Exponential phase
dnaN	KB – Exponential phase
dnaA	KB – Exponential phase
uvrD	KB – Exponential phase
dnaB	KB – Exponential phase
sbcD	KB – Exponential phase
insH-6	KB – Exponential phase
insH-7	KB – Exponential phase
insH-3	LB – Exponential phase
insB	LB – Exponential phase
insH-1	KB – Stationary phase
D – Cell division and chromosome partitioning, cell cycle control	
ftsA	KB – Exponential phase
mukB	KB – Exponential phase; LB – Exponential phase
mreB	KB – Exponential phase
gidA	KB – Exponential phase
ftsN	KB – Exponential phase
ftsZ	KB – Exponential phase; LB – Exponential phase
etk (M)	LB – Exponential phase
O – Posttranslational modification, protein turnover, chaperones	
dnaJ	KB – Exponential phase
dapB	KB – Exponential phase
glnD	KB – Exponential phase
tig	KB – Exponential phase
clpP (U)	KB – Exponential phase
clpX	KB – Exponential phase
ppiD	KB – Exponential phase
dsbG	KB – Exponential phase
yliJ	KB – Exponential phase
ydfT	KB – Exponential phase
htpX	KB – Exponential phase
yedF	KB – Exponential phase
hscA	KB – Exponential phase
hscB	KB – Exponential phase
hypB (K)	KB – Exponential phase
hypC	KB – Exponential phase
hypE	KB – Exponential phase
ygcF	KB – Exponential phase
hybG	KB – Exponential phase
ftsH (*hflB*)	KB – Exponential phase
yhfA	KB – Exponential phase; LB – Exponential phase
trxA (C)	KB – Exponential phase
hslU	KB – Exponential phase
nrfE	KB – Exponential phase

nrfF	KB – Exponential phase
trxB	LB – Exponential phase
gst	LB – Exponential phase
fkpB	KB – Exponential phase; LB – Exponential phase
cyoE	KB – Exponential phase; LB – Exponential phase
dsbC	KB – Exponential phase
yggG	KB – Exponential phase
lon	LB – Exponential phase
groL	KB – Stationary phase
smpB	LB – Stationary phase
M – Cell wall, membrane and envelope biogenesis	
caiT	KB – Exponential phase
imp	KB – Exponential phase
ddlB	KB – Exponential phase
ftsQ	KB – Exponential phase; LB – Exponential phase
yaeT	KB – Exponential phase
acrA	KB – Exponential phase
rhsD	KB – Exponential phase; LB – Exponential phase
ompT	KB – Exponential phase
cusC (U)	KB – Exponential phase
rlpA	KB – Exponential phase
pal	KB – Exponential phase; LB – Exponential phase; LB – Stationary phase
ompA	KB – Exponential phase; LB – Exponential phase
lolE	KB – Exponential phase
ycgV (U)	KB – Exponential phase
kdsA	KB – Exponential phase
msbB (*lpxM*)	KB – Exponential phase
cld	KB – Exponential phase
gif	KB – Exponential phase
yehZ	KB – Exponential phase
pbpG	KB – Exponential phase
ompC	KB – Exponential phase; LB – Exponential phase
yfdH	KB – Exponential phase
mreC	KB – Exponential phase
mrcA	KB – Exponential phase
yhiI	KB – Exponential phase; LB – Stationary phase
yhjG	KB – Exponential phase
hldD (G)	KB – Exponential phase
waaU (*rfaK*)	KB – Exponential phase; LB – Exponential phase
waaR (*rfaJ*)	KB – Exponential phase
waaO (*rfaI*)	KB – Exponential phase
glmU	KB – Exponential phase
rffG	KB – Exponential phase; LB – Exponential phase
skp	LB – Exponential phase
ompF	LB – Exponential phase
csgG	LB – Exponential phase
ompX	LB – Exponential phase
ydeU (U)	LB – Exponential phase

murB	LB – Exponential phase
glmS	LB – Stationary phase
N – Cell motility and secretion	
ybgD (U)	KB – Exponential phase
flgE	KB – Exponential phase
fliK	KB – Exponential phase
yqiG (U)	KB – Exponential phase
yraJ (U)	KB – Exponential phase
gspD (U)	KB – Exponential phase
gspF (U)	KB – Exponential phase
aer (T)	LB – Exponential phase
P – Inorganic ion transport and metabolism	
kefC	KB – Exponential phase
thiP	KB – Exponential phase
metN	KB – Exponential phase
cynX	KB – Exponential phase
tauA	KB – Exponential phase
ybeX	KB – Exponential phase; LB – Exponential phase
modA	KB – Exponential phase
cvrA	KB – Exponential phase
ydaN	KB – Exponential phase
cysW	KB – Exponential phase
yfeX	KB – Exponential phase
focB	KB – Exponential phase
cysI	KB – Exponential phase
ygjE	KB – Exponential phase
yheM	KB – Exponential phase
zntA	KB – Exponential phase
yieL	KB – Exponential phase
corA	KB – Exponential phase
katG	KB – Exponential phase; LB – Exponential phase
nrfA	KB – Exponential phase
copA	KB – Exponential phase; LB – Exponential phase
ydeN	LB – Exponential phase
fecB	LB – Exponential phase
yfbS	KB – Exponential phase
feoB	KB – Exponential phase
yjbB	KB – Exponential phase
fecR (T)	KB – Exponential phase
citT	LB – Exponential phase
sodB	LB – Exponential phase
dps	KB – Stationary phase
cysQ	LB – Stationary phase
T – Signal transduction mechanisms	
dksA	KB – Exponential phase; LB – Exponential phase
phoR	KB – Exponential phase
cusS	KB – Exponential phase
ybeZ	KB – Exponential phase

narL (K)	KB – Exponential phase
uspE	KB – Exponential phase; LB – Exponential phase
baeR (K)	KB – Exponential phase
yfhK	KB – Exponential phase
ptsP	KB – Exponential phase
ecfG (*htrG*)	KB – Exponential phase
crp	KB – Exponential phase; LB – Exponential phase
ompR (K)	KB – Exponential phase
uspA	KB – Exponential phase
typA (*bipA*)	KB – Exponential phase
uspD (*yiiT*)	KB – Exponential phase
dcuS	KB – Exponential phase
relA (K)	KB – Exponential phase; LB – Exponential phase
rseA	LB – Exponential phase
ylaB	LB – Stationary phase
basS	LB – Stationary phase
C – Energy production and conversion	
leuB (E)	KB – Exponential phase
yagR	KB – Exponential phase
yajO	KB – Exponential phase
sdhD	KB – Exponential phase
sucB	KB – Exponential phase
galT	KB – Exponential phase
cydD (O)	KB – Exponential phase
dmsA	KB – Exponential phase
pflB	KB – Exponential phase
hyaA	KB – Exponential phase
icd	KB – Exponential phase
narG	KB – Exponential phase; LB – Exponential phase
narH	KB – Exponential phase
narJ	KB – Exponential phase
narI	KB – Exponential phase; LB – Exponential phase
ydbK	KB – Exponential phase
paaZ (I)	KB – Exponential phase
ydiJ	KB – Exponential phase
ydjA	KB – Exponential phase
torZ	KB – Exponential phase
dld	KB – Exponential phase
napH	KB – Exponential phase
napA	KB – Exponential phase; LB – Exponential phase
glpA	KB – Exponential phase; LB – Exponential phase
glpC	KB – Exponential phase
nuoH	KB – Exponential phase; LB – Exponential phase
nuoG	KB – Exponential phase
ackA	KB – Exponential phase
maeB	KB – Exponential phase; LB – Exponential phase
hyfE	KB – Exponential phase
gabD	KB – Exponential phase

hydN	KB – Exponential phase
hycG	KB – Exponential phase
hycE	KB – Exponential phase
ygcN	KB – Exponential phase
fldB	KB – Exponential phase; LB – Exponential phase
glcB	KB – Exponential phase; LB – Stationary phase
glcD	KB – Exponential phase
tdcE	KB – Exponential phase; LB – Exponential phase
tdcD	KB – Exponential phase
mdh	KB – Exponential phase
yhdH (R)	KB – Exponential phase; LB – Exponential phase
prkB	KB – Exponential phase; LB – Exponential phase
pckA	KB – Exponential phase
yidS (cbrA)	KB – Exponential phase
atpF	KB – Exponential phase
fdoI	KB – Exponential phase
fdoG	KB – Exponential phase
fpr	KB – Exponential phase; LB – Exponential phase
ppc	KB – Exponential phase
fumB	KB – Exponential phase
ykgE	LB – Exponential phase
frdB	LB – Exponential phase
aceE	LB – Exponential phase
cydB	KB – Exponential phase
adhE	KB – Exponential phase
cyoB	LB – Exponential phase
putA	LB – Exponential phase; LB – Stationary phase
fdnI	LB – Exponential phase
yeaU (E)	LB – Exponential phase
napG	LB – Exponential phase
napF	LB – Exponential phase
glpQ	LB – Exponential phase
nuoB	LB – Exponential phase
nirB	LB – Exponential phase
aldA	LB – Stationary phase
yfiQ	LB – Stationary phase
aceB	LB – Stationary phase
acnB	LB – Stationary phase
G – Carbohydrate transport and metabolism	
fruB	KB – Exponential phase
yadI	KB – Exponential phase
yagG	KB – Exponential phase
nagB	KB – Exponential phase
pgm	KB – Exponential phase
dhaM (S)	KB – Exponential phase; LB – Exponential phase
ycjT	KB – Exponential phase
yniA	KB – Exponential phase
chbC	KB – Exponential phase

gnd	KB – Exponential phase; LB – Exponential phase
gatC	KB – Exponential phase; LB – Exponential phase
gatA (T)	KB – Exponential phase
gatZ	KB – Exponential phase; LB – Exponential phase
gatY	KB – Exponential phase; LB – Exponential phase
fruA	KB – Exponential phase
ypdE	KB – Exponential phase
ptsH	KB – Exponential phase
ptsI	KB – Exponential phase; LB – Exponential phase
yphD	KB – Exponential phase
fbaA	KB – Exponential phase
agaZ (*kbaZ*)	KB – Exponential phase
agaY (*kbaY*)	KB – Exponential phase
glmM	KB – Exponential phase
nanE	KB – Exponential phase
malP	KB – Exponential phase; LB - Exponential phase
glgA	KB – Exponential phase
glgC	KB – Exponential phase
glgB	KB – Exponential phase
ugpE	KB – Exponential phase
ugpA	KB – Exponential phase
ugpB	KB – Exponential phase
yiaO	KB – Exponential phase
sgbU	KB – Exponential phase
pgmI	KB – Exponential phase
glvC	KB – Exponential phase
rbsA	KB – Exponential phase
rbsK	KB – Exponential phase
yihP	KB – Exponential phase
yihS	KB – Exponential phase
yihT	KB – Exponential phase
yihV	KB – Exponential phase
frvB	KB – Exponential phase
rhaB	KB – Exponential phase
pfkA	KB – Exponential phase
tpiA	KB – Exponential phase
ptsA (T)	KB – Exponential phase
malE	KB – Exponential phase; LB - Exponential phase
alsC	KB – Exponential phase
glpT	LB – Exponential phase
rpe	LB – Exponential phase
yihR	LB – Exponential phase
gapA	KB – Exponential phase; LB - Exponential phase
rbsB	KB – Exponential phase
yigM (ER)	KB – Exponential phase
pgi	KB – Exponential phase
treA	LB – Exponential phase
tktA	LB – Exponential phase
gpmM	LB – Exponential phase

idnT (E)	KB – Stationary phase
kdgK	LB – Stationary phase
araG	LB – Stationary phase
E – Amino acid transport and metabolism	
carB (F)	KB – Exponential phase
dapD	KB – Exponential phase
dinJ	KB – Exponential phase
proB	KB – Exponential phase
yagE (M)	KB – Exponential phase
argF	KB – Exponential phase
yahI	KB – Exponential phase
oppC (P)	KB – Exponential phase
oppD (P)	KB – Exponential phase
puuP	KB – Exponential phase
ydcT	KB – Exponential phase
gatD (R)	KB – Exponential phase
yehY	KB – Exponential phase
lysP	KB – Exponential phase
yfbQ	KB – Exponential phase
hisM	KB – Exponential phase
hisJ (T)	KB – Exponential phase
usg	KB – Exponential phase
mnmC (*trmC*) (S)	KB – Exponential phase
eutJ	KB – Exponential phase
dapE	KB – Exponential phase
iscS	KB – Exponential phase
yphC (R)	KB – Exponential phase
yfhB	KB – Exponential phase
proV	KB – Exponential phase
proW	KB – Exponential phase
sdaC	KB – Exponential phase; LB – Exponential phase
gcvH	KB – Exponential phase
gcvT	KB – Exponential phase
pepP	KB – Exponential phase
yggP (R)	KB – Exponential phase
gltB	KB – Exponential phase
argD	KB – Exponential phase
asd	KB – Exponential phase
livF	KB – Exponential phase
livH	KB – Exponential phase
nikC (P)	KB – Exponential phase
nikD (P)	KB – Exponential phase
nikE (P)	KB – Exponential phase
dppF	KB – Exponential phase
ddpD (*ydpP*) (P)	KB – Exponential phase; LB – Exponential phase
selA	KB – Exponential phase; LB – Exponential phase
ilvC (H)	KB – Exponential phase
metE	KB – Exponential phase

pepQ	KB – Exponential phase
metL	KB – Exponential phase
argC	KB – Exponential phase
argH	KB – Exponential phase
metH	KB – Exponential phase
yjdL	KB – Exponential phase
aspA	KB – Exponential phase; LB – Exponential phase
oppA	LB – Exponential phase
trpB	LB – Exponential phase
trpC	LB – Exponential phase
ydcT	LB – Exponential phase
tdcC	LB – Exponential phase
tdcB	LB – Exponential phase
tnaA	LB – Exponential phase
proA	KB – Exponential phase
potD	KB – Exponential phase
asnA	KB – Exponential phase; LB – Exponential phase
glnK	LB – Exponential phase
glpB	LB – Exponential phase
speA	LB – Exponential phase
tdh (R)	LB – Exponential phase
gadA	KB – Stationary phase
ydiB	LB – Stationary phase
F – Nucleotide transport and metabolism	
dgt	KB – Exponential phase
adk	KB – Exponential phase
purE	KB – Exponential phase
purB	KB – Exponential phase
nrdB	KB – Exponential phase
upp	KB – Exponential phase
purL	KB – Exponential phase
pyrE	KB – Exponential phase
ade	KB – Exponential phase
gpp (P)	KB – Exponential phase
purD	KB – Exponential phase
guaA	LB – Exponential phase
ygfU	LB – Exponential phase
H – Coenzyme transport and metabolism	
ribF	KB – Exponential phase
nadC	KB – Exponential phase
hemL	KB – Exponential phase
mhpA (C)	KB – Exponential phase
dxs (I)	KB – Exponential phase
thiI (P)	KB – Exponential phase
moaA	KB – Exponential phase
rimK (H)	KB – Exponential phase
hemA	KB – Exponential phase
pdxH	KB – Exponential phase

folE	KB – Exponential phase
menC	KB – Exponential phase
menB	KB – Exponential phase
gshA	KB – Exponential phase
ubiH (C)	KB – Exponential phase
gshB (J)	KB – Exponential phase
ispB	LB – Exponential phase
coaE	KB – Exponential phase
kbl	KB – Exponential phase
moeA	LB – Exponential phase
pdxB (E)	LB – Exponential phase
hemY	LB – Exponential phase
ubiF (C)	LB – Stationary phase
I – Lipid transport and metabolism	
ispU	KB – Exponential phase
accA	KB – Exponential phase
prpE	KB – Exponential phase
aes	KB – Exponential phase
fabA	KB – Exponential phase
plsX	KB – Exponential phase
acpP (Q)	KB – Exponential phase
fadD (Q)	KB – Exponential phase
ucpA (QR)	KB – Exponential phase; LB – Exponential phase
idi	KB – Exponential phase
sbm (*yliK*)	KB – Exponential phase
garR	KB – Exponential phase
yhbT	KB – Exponential phase
accB	KB – Exponential phase
fadB	KB – Exponential phase
yihU	KB – Exponential phase
ygfF (QR)	LB – Exponential phase
ispD	KB – Exponential phase
accC	KB – Exponential phase
V – Defense mechanisms	
ybhF	KB – Exponential phase
rbbA	KB – Exponential phase
yibH	KB – Exponential phase
U – Intracellular trafficking and secretion	
tolB	KB – Exponential phase
secA	KB – Exponential phase
secG	KB – Exponential phase
secE	KB – Exponential phase
yidC	LB – Exponential phase
Q – Secondary metabolites biosynthesis, transport and catabolism	
mhpF	KB – Exponential phase
entF	KB – Exponential phase
entE	KB – Exponential phase
entB	KB – Exponential phase

smtA (R)	KB – Exponential phase
dhaR (K)	KB – Exponential phase
R – General function prediction only	
ybbB	KB – Exponential phase
ybfF	KB – Exponential phase
gsiA (*yliA*)	KB – Exponential phase
ybjI	KB – Exponential phase; LB – Exponential phase
yncB	KB – Exponential phase
ydfG	KB – Exponential phase
ynjD	KB – Exponential phase
yobA	KB – Exponential phase
yecA	KB – Exponential phase
yedE	KB – Exponential phase
mqo	KB – Exponential phase
yfcH	KB – Exponential phase
ypfH	KB – Exponential phase
ypfI	KB – Exponential phase
hcaD	KB – Exponential phase
yfiC	KB – Exponential phase
ecfD (*yfiO*)	KB – Exponential phase
yfjP	KB – Exponential phase
ygaF	KB – Exponential phase
yqaB	KB – Exponential phase; LB – Exponential phase
dkgA	KB – Exponential phase
nlpI	KB – Exponential phase
yheT	KB – Exponential phase
yrfG	KB – Exponential phase
glpG	KB – Exponential phase
bcsC	KB – Exponential phase; LB – Exponential phase
yicO	KB – Exponential phase
yieG	KB – Exponential phase
yigL	KB – Exponential phase
ubiB	KB – Exponential phase
yihA	KB – Exponential phase
actP	KB – Exponential phase
dcuA	KB – Exponential phase; LB – Exponential phase
ycjS	LB – Exponential phase
yhhX	LB – Exponential phase
ybjL	KB – Exponential phase
viaA	KB – Exponential phase
ybbM	LB – Exponential phase
ybcS	LB – Exponential phase
ybhL	LB – Exponential phase
tldD	LB – Exponential phase
yhiN	LB – Exponential phase
S – Function unknown	
yaaA	KB – Exponential phase
yaaH	KB – Exponential phase

yadR	KB – Exponential phase
ybgI	KB – Exponential phase; LB – Exponential phase
ychA	KB – Exponential phase
ydgA	KB – Exponential phase
ydhK	KB – Exponential phase
ydhS	KB – Exponential phase
ynhG	KB – Exponential phase
dedD	KB – Exponential phase
ygiD (*zupT*)	KB – Exponential phase
yqiK	KB – Exponential phase
ygjQ	KB – Exponential phase
ygjD	KB – Exponential phase
yrbK	KB – Exponential phase
yhdP	KB – Exponential phase
yhhQ	KB – Exponential phase
yicC	KB – Exponential phase
rmuC	KB – Exponential phase
yihF	KB – Exponential phase; LB – Exponential phase
yjbJ	KB – Exponential phase
yafQ	LB – Exponential phase
ycfD	KB – Exponential phase
yjeF (G)	KB – Exponential phase
ytfN	LB – Exponential phase
Not on COG List	
araC	KB – Exponential phase
ftsW	KB – Exponential phase
lpxC	KB – Exponential phase
panC	KB – Exponential phase
yadM	KB – Exponential phase
sfsA	KB – Exponential phase
tsf	KB – Exponential phase
yagV	KB – Exponential phase
yaiL	KB – Exponential phase
yaiA	KB – Exponential phase
hupB	KB – Exponential phase; LB – Exponential phase
nrfB	KB – Exponential phase
hokE	KB – Exponential phase
cspE	KB – Exponential phase
ybfE	KB – Exponential phase
iaaA	KB – Exponential phase
ybjJ	KB – Exponential phase
ybjN	KB – Exponential phase
ybjQ	KB – Exponential phase
somA (*ybjX*)	KB – Exponential phase
mgsA	KB – Exponential phase
cspG	KB – Exponential phase
yceH	KB – Exponential phase
yceG	KB – Exponential phase

ychQ	KB – Exponential phase
yciQ	KB – Exponential phase; LB – Exponential phase; KB – Stationary phase
recE	KB – Exponential phase
ydcA	KB – Exponential phase
ydcP	KB – Exponential phase
yneG	KB – Exponential phase
yneH	KB – Exponential phase
ynfD	KB – Exponential phase
ihfA	KB – Exponential phase
ydjY	KB – Exponential phase
edd	KB – Exponential phase; LB – Exponential phase
zwf	KB – Exponential phase
yecM	KB – Exponential phase
flhC	KB – Exponential phase
flhD	KB – Exponential phase
yecH	KB – Exponential phase; LB – Exponential phase
yehQ	KB – Exponential phase
yeiM	KB – Exponential phase
yfcZ	KB – Exponential phase
yfdN	KB – Exponential phase
yfdX	KB – Exponential phase
eutC	KB – Exponential phase
hcaT	KB – Exponential phase
yphG	KB – Exponential phase
yfiL	KB – Exponential phase
yfjO	KB – Exponential phase
ypjC	KB – Exponential phase
csrA	KB – Exponential phase; LB – Exponential phase
hypD	KB – Exponential phase
ppdC	KB – Exponential phase
xdhB	KB – Exponential phase
ygfB	KB – Exponential phase
ygfA	KB – Exponential phase
yggD	KB – Exponential phase
yggF	KB – Exponential phase
galP	KB – Exponential phase
yggN	KB – Exponential phase
yggL	KB – Exponential phase
yqhC	KB – Exponential phase
glgS	KB – Exponential phase
yqiJ	KB – Exponential phase
ygjE	KB – Exponential phase
agaW	KB – Exponential phase
rpsO	KB – Exponential phase
yrbL	KB – Exponential phase
nanT	KB – Exponential phase
rplM	KB – Exponential phase
def	KB – Exponential phase

rplN	KB – Exponential phase
rplD	KB – Exponential phase
rtcA	KB – Exponential phase
bcsG	KB – Exponential phase
yhjX	KB – Exponential phase
yiaF	KB – Exponential phase
glyQ	KB – Exponential phase
ecfI (*yidQ*)	KB – Exponential phase
mdtL	KB – Exponential phase
ilvD	KB – Exponential phase
cyaA	KB – Exponential phase; LB – Exponential phase
yihI	KB – Exponential phase
glnA	KB – Exponential phase
yiiD	KB – Exponential phase
rhaA	KB – Exponential phase
kdgT	KB – Exponential phase
cdh	KB – Exponential phase
menG (*rraA*)	KB – Exponential phase
pagB (*yjbD*)	KB – Exponential phase
yjbE	KB – Exponential phase
yjbF	KB – Exponential phase
phnH	KB – Exponential phase
proP	KB – Exponential phase
yacL	LB – Exponential phase
frsA	LB – Exponential phase
yaiC (*adrA*)	LB – Exponential phase
yaiI	LB – Exponential phase
ybgJ	LB – Exponential phase
yliE	LB – Exponential phase
ydgD	LB – Exponential phase
ydiU	LB – Exponential phase
cvpA	LB – Exponential phase
eutB	LB – Exponential phase
gudP	LB – Exponential phase
yqcC	LB – Exponential phase
ygjR	LB – Exponential phase
garP	LB – Exponential phase
lamB	LB – Exponential phase
yjeI	LB – Exponential phase
yjfM	LB – Exponential phase
ybjL	KB – Exponential phase
ydiM	KB – Exponential phase
yebC	KB – Exponential phase
ppk	KB – Exponential phase
yghJ	KB – Exponential phase
rpsL	KB – Exponential phase
setA	LB – Exponential phase
can	LB – Exponential phase
mhpB	LB – Exponential phase

agp	LB – Exponential phase
yecR	LB – Exponential phase
yedD	LB – Exponential phase
yfjQ	LB – Exponential phase
ansB	LB – Exponential phase
agaC	LB – Exponential phase
gspH	LB – Exponential phase
yhjE	LB – Exponential phase
yhjY	LB – Exponential phase
ytfJ	LB – Exponential phase
msrA	LB – Exponential phase
ybgS	KB – Stationary phase
rmf	KB – Stationary phase; LB – Stationary phase
yohK	KB – Stationary phase
yciN	LB – Stationary phase
RNA	
ssrA	KB – Exponential phase; LB – Exponential phase
pheV	KB – Exponential phase
rnpB	KB – Exponential phase
sraG	KB – Exponential phase
selC	KB – Exponential phase
gcvB	LB – Exponential phase
ffs	KB – Exponential phase; LB – Exponential phase
valT	LB – Exponential phase

[a] Gene name indicates the corresponding coding region that the cDNA was annotated to in *E. coli* K-12 MG1655 (Accession number U00096).

[b] Pulldown condition abbreviations: KB = Kornberg broth (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, 0.5% glucose, pH 6.8); LB = Luria broth, pH 7.4.

Table 2. Identities of proteins from two dimensional protein gel analysis.

Accession No.	MG1655 Gene No.	Gene Name	Fold Change
Luria Broth, Exponential Phase			
gi|26250243	b3602	*yibL*	2.79
gi|26246978	b0957	*ompA*	2.54
gi|26246978	b0957	*ompA*	2.20
gi|16128831	b0863	*artI*	1.99
gi|16131280	b3403	*pckA*	1.90
gi|3212630	b0811	*glnH*	1.90
gi|49176218	b2426	*ucpA*	1.90
gi|1311293	b4034	*malE*	1.83
gi|32329155	b0957	*ompA*	1.82
gi|26246978	b0957	*ompA*	1.78
gi|16131280	b3403	*pckA*	1.72
gi|1311293	b4034	*malE*	1.67
gi|16130152	b2215	*ompC*	1.65
gi|19568909	b0231	*dinB*	-1.53
gi|26248857	b2498	*upp*	-1.79
gi|4902909	b0169	*rpsB*	-2.47
gi|42842	b0169	*rpsB*	-2.92
Luria Broth, Stationary Phase			
gi|16130152	b2215	*ompC*	2.16
gi|49176218	b2426	*ucpA*	1.68
gi|16129718	b1764	*selD*	1.50
gi|42900	b0911	*rpsA*	-1.52
gi|26249817	b3236	*mdh*	-1.53
gi|1314675	b2417	*crr*	-1.54
gi|563868	b2779	*eno*	-1.59
gi|16130062	b2124	*yehS*	-1.61
gi|26248187	b1920	*fliY*	-1.64
gi|16128279	b0294	*matA*	-1.64
gi|24111953	b0605	*ahpC*	-1.65
gi|26246721	b0755	*gpmA*	-1.72
gi|26249339	b2926	*pgk*	-1.73
gi|26248857	b2498	*upp*	-1.90
gi|26249339	b2926	*pgk*	-1.94
gi|563868	b2779	*eno*	-2.09
gi|12516470	b2150	*mglB*	-2.20

gi|16132194	b4376	*osmY*	-2.40
gi|41287	b2133	*dld*	-2.60
Kornberg Broth + 0.5% glucose, Exponential Phase			
gi|16128885	b0918	*kdsB*	5.00
gi|13360177	b0688	*pgm*	3.25
gi|56480572	b4139	*aspA*	2.22
gi|2147837	b0957	*ompA*	2.18
gi|56413951	b0930	*asnS*	2.00
gi|24111996	b0728	*sucC*	1.98
gi|16129622	b1664	*ydhQ*	1.87
gi|16130033	b2095	*gatZ*	1.83
gi|16129718	b1764	*selD*	1.81
gi|39654499	b4291	*fecA*	-1.83
gi|26246488	b0473	*htpG*	-1.83
gi|405895	b2114	*metG*	-1.84
gi|54294026	(b3832)??	(*yigN*)??	-1.85
gi|1805568	b2508	*guaB*	-1.87
gi|33383753	b2499	*purM*	-2.02
gi|56480489	b3942	*katG*	-2.03
gi|27574023	b0903	*pflB*	-2.05
gi|1805568	b2508	*guaB*	-2.20
gi|27574023	b0903	*pflB*	-2.29
gi|147478	b2780	*pyrG*	-2.31
gi|43237	b3962	*udhA*	-2.33
gi|42595	b4006	*purH*	-5.21
Kornberg Broth + 0.5% glucose, Stationary Phase			
gi|1742120	b1299	*ycjC*	1.96
gi|26250394	b2003	*yeeT*	1.60
gi|440007	b3236	*mdh*	1.50
gi|22035193	b1790	*yeaM*	-1.57
gi|1736707	b2028	*udg*	-1.59
gi|26250945	NT01EC5045		-1.59
gi|26250243	b3602	*yibL*	-1.60
gi|16129077	b1114	*mfd*	-1.63
gi|38703945	NT01EC0673		-2.26

Table 3. Genes that copurified with CsrA-His6 and identified in two dimensional protein gel analysis.

Gene	Condition in pull down	Condition in protein analysis
ompA	LB[a], exponential phase KB[b], exponential phase	LB, exponential phase KB, exponential phase
pckA	KB, exponential phase	LB, exponential phase
malE	LB, exponential phase KB, exponential phase	LB, exponential phase
upp	KB, exponential phase	LB, exponential phase LB, stationary phase
ompC	LB, exponential phase KB, exponential phase	LB, exponential phase LB, stationary phase
ucpA	LB, exponential phase KB, exponential phase	LB, exponential phase LB, stationary phase
dld	KB, exponential phase	LB, stationary phase
mdh	KB, exponential phase	LB, stationary phase KB, stationary phase
pflB	KB, exponential phase	KB, exponential phase
pgm	KB, exponential phase	KB, exponential phase
aspA	LB, exponential phase KB, exponential phase	KB, exponential phase
katG	LB, exponential phase KB, exponential phase	KB, exponential phase
gatZ	LB, exponential phase KB, exponential phase	KB, exponential phase

[a] Luria broth, pH 7.4

[b] Kornberg broth (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, 0.5% glucose, pH 6.8)

Table 4. Strains, phage and plasmids used in this study.

Strain, plasmid or phage	Genotype or Description	Source
Strain		
MG1655	$F^-\lambda^-$	Michael Cashel
TRMG1655	MG1655 *csrA*::*kan*	(Romeo *et al.*, 1993)
CF7789	MG1655 Δ*lacIZ* (*Mlu*I)	Michael Cashel
TRCF7789B$^-$C$^-$	CF7789 *csrA*::*kan* Δ*csrB*::*cam* Δ*csrC*::*tet*	(Mercante *et al.*, 2006)
CF1693	MG1655 Δ*relA*251::*kan* Δ*spoT*207::*cam*	(Xiao *et al.*, 1991)
CF9239	MG1655 *dksA*::*kan*	Michael Cashel
CF9240	MG1655 *dksA*::*tet*	(Brown *et al.*, 2002)
RH MG1655	*rpoS*::Tn*10*	(Gudapaty *et al.*, 2001)
UY CF7789	CF7789 *uvrY*::*cam*	(Suzuki *et al.*, 2002)
CAG18642	*zfh-3131*::Tn*10* (near *csrA*)	(Singer *et al.*, 1989)
KSA712	CF7789 ϕ(*csrA'-'lacZ*)	(Jackson *et al.*, 2002)
KSB837	CF7789 ϕ(*csrB-lacZ*)	(Gudapaty *et al.*, 2001)
GS1114	CF7789 ϕ(*csrC-lacZ*)	(Weilbacher *et al.*, 2003)
KSY009	CF7789 ϕ(*uvrY'-'lacZ*)	(Suzuki *et al.*, 2002)
CF80005	BL21(λDE3) pGN1	(Gentry *et al.*, 1993)
DHB6521	SM551 λInCh1 (Kan^r)	(Boyd *et al.*, 2000)
SM551 (DHB6501)	$F^-\lambda^-\lambda^s$ Δ*lac*(*MS265*)*mel* $NalA^r$ *supF58*	(Boyd *et al.*, 2000)
dH5α λpir	F^-Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17 suupE44thi-1 gyrA96 relA1* λ*::pir*	(Bourassa and Camilli, 2009)
Plasmids		
pBR322	Cloning vector; Amp^r Tet^r	(Sambrook *et al.*, 1989)
pUC19	Cloning vector; Amp^r	(Yanisch-Perron *et al.*, 1985)
pCRA16	*csrA* in blunt-ended *Vsp*I site of pBR322	(Suzuki *et al.*, 2002)
pUY14	*uvrY* in blunt-ended *Vsp*I site of pBR322	(Suzuki *et al.*, 2002)
pGB2	Cloning vector; Spc/Str^r	(Churchward *et al.*, 1984)
pCsrA	*csrA* cloned into *Hind*III and *Eco*RI of pGB2	This study
pHM1883	pGB2 with P*tac* promoter	(Potrykus *et al.*, 2006)
pHM1506	*dksA* expressed from P*tac* promoter in pGB2	(Potrykus *et al.*, 2006)
pJK537	*dksA* wild-type allele in pBR322	(Kang and Craig, 1990)
pHM1684	pJK537 *dksA* D71N D74N	This study

pMLB1034	Vector for *lacZ* translation fusions; Amp^r	(Silhavy *et al*., 1984)
pRELZ	pMLB1034 ϕ(*relA'-'lacZ*)	This study
pDKSZ	pMLB1034 ϕ(*dksA'-'lacZ*)	This study
pAH125	Vector for *lacZ* transcriptional fusions; Kan^r	(Haldimann and Wanner, 2001)
pLFT	Vector for *lacZ* transcriptional fusions; Amp^r	This study
pRELZtxn	pLFT ϕ(*relA-lacZ*)	This study
pDKSZtxn	pLFT ϕ(*dksA-lacZ*)	This study
pLFX	Vector for *lacZ* translational fusions; Amp^r	This study
pUV5	Vector for *lacZ* leader fusions; Amp^r	This study
pRELZplac	pUV5 ϕ(P*lacUV5relA'-'lacZ*)	This study
pDKSZplac	pUV5 ϕ(P*lacUV5dksA'-'lacZ*)	This study
pSPOZplac	pUV5 ϕ(P*lacUV5rpoZ-spoT'-'lacZ*)	This study
pBARAZ	pLFX ϕ(*barA'-'lacZ*)	This study
pINT-ts	Vector for integration; Amp^r	(Haldimann and Wanner, 2001)
pPFINT	Tet^r cloned into pINT-ts	This study
pPB77	contains *trp* leader (-1 to +111) of *B. subtilis*	(Babitzke *et al*., 1994)
Bacteriophages		
P1*vir*	Strictly lytic P1	Carol Gross
λInCh1	For genomic insertions; Kan^r	(Boyd *et al*., 2000)

Table 5. Primers used in this study.

Primer name	Primer sequence (5' to 3')
Genetic Techniques	
relA-F	GTC*GAATTC*TCGACGTCAAACAATGCCC
relA-R	GTC*GGATCC*ACCGCAACCATCGTCCTCTC
dksA-F	GTC*GAATTC*GACGAAAGAGGCTATCCTTA
dksA-R	GTC*GGATCC*TCTTCTTGCATGTTGCTTCTC
relA-F-txn	GTC*CTGCAG*TCGACGTCAAACAATGCCC
relA-R-txn	GTC*GGATCC*CGGTTTCGAGTATCTCACTA
dksA-F-txn	GTC*CTGCAG*CACAGTTGTCAAGTGTT
dksA-R-txn	GTC*GGATCC*GGCCGCTATAAATAGCA
relA-F-plac	GTC*GAATTC*GTCTCTGGTGAGATGCCCTG
dksA-F-plac	GTC*GAATTC*CCTCATTTTTCCCCCGA
rpoZspoT-F-plac	GTC*GAATTC*CATTTCTTCACCTGTGGAGC
rpoZspoT-R-plac	GTC*GAATTC*ACAAGGGCGACCCGCTTTG
barA-F	GACG*CTGCAG*GCCATTCCAACGCACGCGCT
barA-R	GAC*GGATCC*TTGGTCATGGAGTTCCGTTATG
csrA-F-pGB2	GTC*GAATTC*CACAGATCGTGTGAAAGCAG
csrA-R-pGB2	GTC*AAGCTT*TTAGTAACTGGACTGCTGGG
AmpRF	AGCTCG*ATCGAT*TGGTTTCTTAGACGTCAGGTGGCA
AmpRR	AGCTCG*GCGGCCGC*GGTCTGACGCTCAGTGGAACGAAA
LPF-19	TAAT*CTGCAG*CTCATTAGGCACCCC
LPF-20	GGC*GAATTC*TCCACACATTATACGAG
LPF-21	AGCTCG*ATCGAT*AATAGGCGTATCACGAGGCCCTTT
LPF-22	TGGTTTGCGCATTCACAGTTCTCC
RNA EMSAs	
relA-F-T7	TAATACGACTCACTATAGGCTGCAACGCTGGCTCGGGAT
relA-R-T7	GTGCACTTCTTACCGCAACCAT
dksA-T7	TAATACGACTCACTATAGGGCCTCATTTTTCCCCCGAACA TGGGGATCGATAGTGCGTGTTAAGGAGAAGCAACATGCA AGAAG
GC-dksA-T7	CTTCTTGCATGTTGCTTCTCCTTAACACGCACTATCGATCC CCATGTTCGGGGGAAAAATGAGGCCCTATAGTGAGTCGT ATTA
gmk-F-T7	TAATACGACTCACTATAGGGTATGTAGGCTTTATTTCGC
gmk-R-T7	CAATATAAAGCGTGCCTTGA

rpoZ-T7	TAATACGACTCACTATAGGATTTCTTCACCTGTGGAGCTT TTTAAGTATGGCACGCGTAACTGTTCAGGACGCTGTAGAG
GC-rpoZ-T7	CTCTACAGCGTCCTGAACAGTTACGCGTGCCATACTTAAA AAGCTCCACAGGTGAAGAAATCCTATAGTGAGTCGTATTA
rt-qRT-PCR	
relA-F-taqman	CGGCGTGCGTGATATGG
relA-R-taqman	TTCGGAGGAAACAGAATCAGTGT
relA-6FAM-BHQ1	CGATCCGCCAGCTGAAAGCG
spoT-F-taqman	TGCAGGATATCCGCGTCAT
spoT-R-taqman	CCCAGCGTGCGCATGT
spoT-6FAM-BHQ1	CTCATCAAACTTGCCGACCGT
dksA-F-taqman	CGGGCGAAGAGTATATGAATGAA
dksA-R-taqman	GATTACGCCATGCTTCCAGAA
dksA-6FAM-BHQ1	CCCAGCTGGCGCACTTCCGT
Primer Extension	
pdksA2	CCCCAGGGATGGCGAGAATACTCAGC
Removal of rRNA from CsrA-bound RNA fractions	
SSUrRNA1F	GAAGAGTTTGATCATGGCTCAG
SSUrRNA2R	CTTAACAAACCGCCTGCGTGCGC
SSUrRNA3F	GCACCGGCTAACTCCGTGCCAGC
SSUrRNA4R	GGTTGCGCTCGTTGCGGGACTTAAC
SSUrRNA5F	GGCTGTCGTCAGCTCGTGTTGTGA
SSUrRNA6R	AAGGAGGTGATCCAACCGCA
LSUrRNA1F	GCGACTAAGCGTACACGGTGGATG
LSUrRNA2R	GCCACAAGTCATCCGCTAATTTTTC
LSUrRNA3F	CTGGAGGACCGAACCGACTAATG
LSUrRNA4R	CACCTTCACAGGCTTACAGAACGC
LSUrRNA5F	CCATGCACCGAAGCTGCGGCAGCG
LSUrRNA6R	CTTCGACTGATTTCAGCTCCACG
LSUrRNA7F	GGCACGCTGATATGTAGGTGAAGC
LSUrRNA8R	CGTCACGCTCGCAGTCAAGCTGGC
LSUrRNA9F	CCTGGTCGGACATCAGGAGGTTAG
LSUrRNA10R	AAGCCTCACGGTTCATTAGTACCG

MATERIALS AND METHODS

Preparation of CsrA-bound RNA for 454 Sequencing. *E. coli* CsrA-His_6 protein was purified as described previously (Mercante *et al*., 2006) from TRCF7789B$^-$C$^-$ grown at 37°C to exponential (OD_{600} = 0.5) or stationary phase (8 hours) in either LB medium or Kornberg broth (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, 0.5% glucose, pH 6.8). The protein-containing fractions were pooled, and RNA was extracted three times by acid phenol (pH 4.5) and twice by chloroform. Isolated RNA was recovered by ethanol precipitation. Purified RNA was fractionated on 1.5% formaldehyde-agarose gels, and most products were approximately 100-200 nt in length. Because large quantities of ribosomal RNA had been identified through trial experiments, rRNA was removed by annealing biotinylated ssDNA complementary to rRNA sequences and subsequently removing these complexes. Briefly, dsDNA was synthesized using primers SSUrRNA1F through LSUrRNA10R (Table S5) to generate three PCR products complementary to 16S rRNA and five PCR products complementary to 23S rRNA. An additional PCR reaction containing only the reverse primer was performed to create antisense ssDNA, which was then labeled with biotin using the Biotin 3' End DNA Labeling Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of biotinylated ssDNA resuspended in TE buffer were added to purified RNA, and the mixture was heated up to 80°C for 5 min and allowed to cool to 4°C over 45 min. The biotinylated ssDNA-rRNA complexes were removed per manufacturer's instructions using the Streptavidin MagneSphere® Paramagnetic Particles (Promega, Madison, WI) and a magnetic separation stand. Assuming 90% of the original purified RNA fraction was rRNA, this process removed 49% of rRNA. Subsequent RNA was converted to cDNA using random hexamer primers and the Universal RiboClone cDNA Synthesis System

(Promega, Madison, WI) according to the manufacturer's instructions. Because random hexamer primers were used, the resulting cDNA did not necessarily possess a CsrA binding site, but may encode flanking sequences. The resulting cDNA was blunt-ended using T4 DNA polymerase (NEB) and phosphorylated by T4 polynucleotide phosphorylase (NEB). Before sequencing, cDNA was checked for integrity by efficient cloning into the *Hinc*II site of pUC19. Because cDNA quantities were limited, the Genomics Core of the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR) performed limited PCR amplification of the cDNA library. cDNA was sequenced using the 454 Life Sciences GS-20 Sequencer and annotated against the MG1655 genome (Accession number U00096). The identified transcripts were collated according to function into Clusters of Orthologous Groups of proteins (COGs) (Tatusov *et al.*, 1997).

Two-dimensional protein gels. Proteomics experiments were performed using differential in-gel electrophoresis technology (GE Biosystems, Piscataway, NJ) and *E. coli* MG1655 or TRMG1655 (*csrA*::*kan*) cells grown at 37°C to exponential (OD_{600} = 0.5) or stationary phase (8 hours) in either LB medium or Kornberg broth (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, 0.5% glucose, pH 6.8). Cell pellets were washed three times in wash buffer (10 mM Tris, pH 8.0, 5 mM magnesium acetate) at 4°C for 4 min at 12,000 g. The pellets were then resuspended and lysed in 1 ml of lysis buffer (8 M urea, 20 mM Tris, pH 8.5, 5 mM magnesium acetate, 4% (w/v) CHAPS) for 30 min on ice followed by sonication (6 x 10s bursts). The lysate was then centrifuged for 10 min at 12,000 g at 4°C and the supernatant was retained for the next steps. After determining the protein concentrations of each sample (Bicinchoninic acid protein assay, Pierce

Biotechnology, Rockford, IL), 25 μg of each sample was labeled (for 10 min in the dark) at lysine residues with 1μl of either Cy2 (MG1655 samples) or Cy3 (TRMG1655 samples) dye conjugates. The labeling reactions were stopped with the addition of lysine to a final concentration of 1 mM. Following Cy2 and Cy3 labeling, each sample was mixed with an equal amount of unlabeled protein and paired samples (ie. MG1655 and TRMG from KB-0.5) were mixed together and then applied to an IPGPhor IEF strip (24 cm, non-linear, pH 3-11) and subjected to isoelectric focusing (IEF) to separate the proteins by pI. After IEF, the strips were washed in equilibration buffer (6 M urea, 10 mM Tris pH 6.8, 30% glycerol, 1% SDS) and applied to the top of a 12% SDS-polyacrylamide gel to separate the proteins by size. After electrophoresis, the gel was scanned on a Typhoon fluorescent scanner (GE Biosystems) at the appropriate wavelengths for each dye (Cy2, 488 nm excitation, 520 nm emission; Cy3, 532 nm excitation, 580 nm emission). The subsequent images were then overlaid and analyzed with Decyder Differential In-Gel Analysis (DIA) software (version 4.0, GE Biosystems). Differentially expressed proteins were then excised by a robotic spot picker, digested with trypsin, and tryptic peptides were analyzed using a matrix-associated laser desorption ionization-time-of-flight/time-of-flight (MALDI-ToF/ToF) spectrometer (Applied Biosystems). Protein IDs were obtained by querying protein databases with both the tryptic fingerprint data as well as primary amino acid sequence data obtained from the MALDI-ToF/ToF.

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