Review

The post-transcriptional regulator CsrA plays a central role in the adaptation of bacterial pathogens to different stages of infection in animal hosts

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The importance of Csr post-transcriptional systems is gradually emerging; these systems control a variety of virulence-linked physiological traits in many pathogenic bacteria. This review focuses on the central role that Csr systems play in the pathogenesis of certain bacteria and in the establishment of successful infections in animal hosts. Csr systems appear to control the 'switch' between different physiological states in the infection process; for example switching pathogens from a colonization state to a persistence state. Csr systems are controlled by two-component sensor/regulator systems and by non-coding RNAs. In addition, recent findings suggest that the RNA chaperone Hfq may play an integral role in Csr-mediated bacterial adaptation to the host environment.

Introduction

In order to compete and survive in nature, bacteria routinely alter their physiological state to tolerate or exploit local environments. Pathogenic bacteria often undergo cellular changes that enable them to initiate and establish infection. Complex regulatory networks orchestrate these cellular changes and control the expression of virulence factors necessary to colonize and persist in the host. An increasing amount of evidence suggests that post-transcriptional regulators play a central role in host–pathogen interactions. Amongst these post-transcriptional systems are homologues of the Csr (carbon storage regulator) system in *Escherichia coli*. The CsrA protein belongs to a new class of RNA-binding proteins that act on the translation of target genes and/or the stability of target mRNA transcripts (Baker *et al.*, 2002; Dubey *et al.*, 2003; Romeo, 1998). Homologues of *csrA* are found throughout the prokaryotic world and have been identified in more than 176 different eubacterial species, including proteobacteria or firmicutes; several bacteria, such as *Legionella pneumophila*, *Pseudomonas fluorescens*, *Coxiella burnetii* and *Pirellula* sp., encode more than one CsrA homologue (Mercante *et al.*, 2006).

The Csr system was first described in 1993 by T. Romeo and colleagues: transposon mutagenesis of the *csrA* gene caused the strain to accumulate increased levels of glycogen

compared to wild-type *E. coli* (Romeo *et al.*, 1993). In *E. coli*, the Csr system is composed of the 61 amino acid CsrA protein and two small, non-coding regulatory RNAs (ncRNAs), CsrB and CsrC (Romeo, 1998). CsrA is an RNA-binding protein that could prevent translation of target mRNA by binding to a site near the Shine–Dalgarno sequence, thus blocking ribosome binding and facilitating mRNA decay. CsrA has also been shown to act as a positive regulator by stabilizing and subsequently increasing the translation of certain target mRNAs. This positive regulation increases translation by increasing target transcript levels, not by increasing translational efficiency (Wei *et al.*, 2001). CsrB and CsrC regulate the activity of the CsrA protein by sequestering up to 18 molecules of CsrA, thus preventing it from binding to its target mRNAs (Romeo, 1998; Weilbacher *et al.*, 2003). Transcription of these two small RNAs is regulated by the BarA/UvrY two-component signal transduction system (TCS) in *E. coli* or by homologous systems such as GacS/GacA in other bacteria (Burrowes *et al.*, 2005; Heurlier *et al.*, 2004; Suzuki *et al.*, 2002; Weilbacher *et al.*, 2003). A recent review describes the interaction of ncRNAs with Csr regulatory circuitries, with a particular focus on the importance of the 'ANGGA' CsrA-binding motif for CsrA activity (Babitzke & Romeo, 2007).

The Csr/BarA/UvrY system and its homologous Rsm/GacS/GacA systems are involved in the regulation of numerous widespread cellular functions including carbon metabolism (Baker *et al.*, 2002), motility (Barnard *et al.*, 2004; Lawhon *et al.*, 2003), biofilm formation (Goodman *et al.*, 2004;

Abbreviations: ncRNA, non-coding RNA; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing; TCS, two-component signal transduction system.

Wang *et al.*, 2005), secondary metabolite production (Lawhon *et al.*, 2003), environmental stress resistance (Barnard *et al.*, 2004; Molofsky & Swanson, 2003) and cytotoxic factor production (Goodman *et al.*, 2004; Molofsky & Swanson, 2003). Numerous functions controlled by CsrA are important during interactions with both animal and plant hosts (Cui *et al.*, 1995; Goodman *et al.*, 2004; Molofsky & Swanson, 2003) and, taken together, these features suggest an important virulence-related evolutionary role for CsrA.

This review aims (i) to emphasize the role that Csr systems play in mediating adaptive physiology and timed virulence trait expression in animal pathogens, such as extracellular pathogens (*Pseudomonas aeruginosa*, *Vibrio cholerae*, *Helicobacter pylori*) and intracellular pathogens (*Legionella pneumophila*, *Salmonella enterica* serovar Typhimurium), at different stages of infection (colonization, persistence and pathogenicity), and (ii) to unravel the complex interactions between CsrA-like proteins and other regulators, such as TCSs and ncRNAs. *E. coli* K-12 is frequently cited throughout this review as it is the model organism for CsrA-mediated gene regulation to date.

Mode of action of CsrA and known direct targets

Despite the important role played by CsrA regulators in controlling bacterial physiology, not many direct targets of CsrA, namely transcripts that physically interact with the protein, have been identified to date. Moreover, the precise mechanism by which CsrA activates or represses target genes is not always known. It is possible that CsrA post-transcriptionally controls target mRNAs via two modes of action: (i) by preventing translation and activating mRNA decay by endonuclease attack or (ii) by stabilizing mRNA (Fig. 1).

Studies have shown that loss of CsrA alters the mRNA transcript levels of certain genes, but for the most part, direct interaction between CsrA and target mRNA has not been demonstrated. To date, only a few direct targets (i.e. mRNA targets that CsrA physically binds to and prevents translation of by blocking the ribosome-binding site) of CsrA have been identified and these are found in *E. coli*, *Bacillus subtilis* and *P. fluorescens*; direct CsrA targets in animal pathogens have yet to be identified. Direct CsrA targets in *E. coli* are (i) the *glg* operon, encoding glycogen biosynthetic proteins (Baker *et al.*, 2002); (ii) *pgaA* mRNA, which encodes a biofilm polysaccharide adhesin (Wang *et al.*, 2005); (iii) the *cstA* gene, which encodes a peptide transporter (Dubey *et al.*, 2003); and (iv) the RNA chaperone protein gene *hfq* (Baker *et al.*, 2007). In addition, CsrA was found to bind to and stabilize the *flh* operon mRNA, which is required for flagellum biosynthesis (Wei *et al.*, 2001). In *B. subtilis*, CsrA binds to *hag* mRNA, which encodes the flagellin protein, and prevents its translation by blocking the ribosome-binding site (Yakhnin *et al.*, 2007). Recently it was found that in *P. fluorescens*, CsrA physically interacts with *hcnA* mRNA,

which encodes the cyanide synthase subunit A, and inhibits its translation (Schubert *et al.*, 2007).

The way in which CsrA represses glycogen biosynthesis has been elucidated: free CsrA binds the *glgCAP* mRNA untranslated leader sequence and overlaps the *glgC* ribosome-binding site, preventing translation initiation and thereby accelerating the rate of decay of *glg* transcripts (Baker *et al.*, 2002; Liu & Romeo, 1997). Studies on *cstA* and *pgaABC* showed that these genes are regulated by CsrA in a similar manner (Dubey *et al.*, 2003; Wang *et al.*, 2005). Recently, it was demonstrated that CsrA inhibits translation initiation of *hfq* by binding to a site overlapping the ribosome-binding site (Baker *et al.*, 2007). Each of the CsrA target transcripts has multiple CsrA-binding sites (e.g. *cstA* has three or four predicted sites) (Dubey *et al.*, 2003), except for *hfq*, which only has one CsrA-binding site (Baker *et al.*, 2007).

In *E. coli*, CsrA is required for full motility and flagellar biosynthesis. RNA gel mobility shift assays were used to show that CsrA bound specifically to *flhDC* transcripts and that binding of the CsrA protein to *flhDC* transcripts resulted in their stabilization and subsequent translation (Wei *et al.*, 2001). Wei *et al.* (2001) suggested that CsrA prevents endonucleolytic attack of bound target mRNA, thus acting as a positive regulator.

In some cases, however, the mechanism by which CsrA controls the expression of its targets is rather confusing. In *S. enterica* serovar Typhimurium, both the loss of *csrA* and its overexpression repress the same set of genes required for cell invasion (Altier *et al.*, 2000). The regulation of SPI1 genes (encoding components of a type III secretion apparatus) depends on the concentration of free CsrA, which also depends on the concentration of the two small RNA regulators, CsrB and CsrC, and of the BarA/SirA TCS. This is described below in the section 'Regulation of CsrA activity'.

In *P. aeruginosa*, RsmA seems to have a pleiotropic role. Microarray analysis showed that expression of 9 % of the *P. aeruginosa* genome was altered in an *rsmA* mutant compared to wild-type PAO1 (Burrowes *et al.*, 2006). RsmA is involved in the regulation of many functions in the cell, such as carbon metabolism, QS, biofilm formation, motility, stress resistance and virulence. Not all of these effects can be explained through the established regulatory roles of RsmA. Given that several cellular functions are greatly affected by inactivation of RsmA, we believe that CsrA-like regulators control multiple targets, including transcriptional regulators, which in turn affect the transcription of a number of genes.

The three-dimensional structures of free CsrA/RsmA proteins have been solved, revealing a novel protein fold consisting of two interdigitated monomers (Gutierrez *et al.*, 2005; Rife *et al.*, 2005) with functional domains important for RNA binding. Recently, comprehensive alanine-scanning mutagenesis of CsrA in *E. coli* revealed two CsrA

Fig. 1. Interactions between CsrA, ncRNA, Hfq and target transcripts. This figure combines several modes of action of CsrA/ncRNA/Hfq systems from distinct organisms and does not purport to propose a unifying model that would be accurate for all bacterial species. Examples for each process or current hypotheses (question marks) are given below. CsrA- and Hfq-mediated processes are in red and blue, respectively. CsrA has two potential modes of action to post-transcriptionally control target mRNAs. It prevents translation by blocking the ribosome-binding site [e.g. *glgCAP* mRNA of *E. coli* (Baker *et al.*, 2002); *hag* mRNA of *B. subtilis* (Yakhnin *et al.*, 2007)] and activates mRNA decay by endonuclease attack [e.g. *glgC* mRNA of *E. coli* (Liu & Romeo, 1997)] or stabilizes target mRNA [e.g. *flhDC* transcript of *E. coli* (Wei *et al.*, 2001)]. ncRNAs inhibit CsrA-mediated regulation by titrating free CsrA protein via repeated 5'-ACGGA(U,C,A)G-3' sequences located in predicted hairpin loops of the ncRNA. Hfq may control the CsrA system by (i) stabilizing *csrA* mRNA [e.g. suggested for *L. pneumophila* (McNealy *et al.*, 2005)], (ii) mediating the turnover of ncRNA (not experimentally proven to date) via formation of a complex with RNase E (Morita *et al.*, 2005), (iii) stabilizing the ncRNA structure [e.g. RsmY of *P. aeruginosa* (Sonnleitner *et al.*, 2006)], (iv) protecting ncRNA from degradation by masking AU-rich sequences preferentially cleaved by RNase E [e.g. RsmY (Sorger-Domenigg *et al.*, 2007)], or (v) binding concomitantly to CsrA overlapping sites on ncRNA resulting in a decreased capacity to sequester CsrA [e.g. RsmY (Sonnleitner *et al.*, 2006)].

regions critical for regulation of glycogen accumulation, motility and biofilm formation and for RNA binding. One region is located within the first β -strand of CsrA while the second region includes the last β -strand of CsrA. These regions may possibly contribute to the creation of functional domains in CsrA dimers, as proposed and discussed by Mercante *et al.* (2006).

Regulation of CsrA activity

CsrA-like regulators play a role in both host-to-pathogen and pathogen-to-host communication, and are involved in the adaptation of pathogenic bacteria to different stages of

infection. CsrA activity is regulated mainly by ncRNAs and TCSs. In addition, other components involved in this regulation have recently been identified.

Regulation by non-coding RNAs

Recent studies have revealed the importance of ncRNAs in the adaptive response of pathogens to the host environment, controlling several processes such as QS (Bejerano-Sagie & Xavier, 2007) and pathogenicity (Toledo-Arana *et al.*, 2007). Recent reviews have focused on the importance of ncRNA in bacteria (Livny & Waldor, 2007; Storz & Haas, 2007).

CsrA proteins interact with ncRNAs (Table 1), which act as sequestering molecules (reviewed by Babitzke & Romeo, 2007). When the *E. coli* CsrA protein was purified it was found to be non-covalently bound to the RNA molecule CsrB (Liu *et al.*, 1997). The small (369 nt) ncRNA CsrB (Babitzke & Romeo, 2007) has a multiple stem-loop predicted structure, with the repeat 5'-CAGGA(U,C,A)G-3' sequence located in its predicted hairpin loops. This sequence is reminiscent of Shine-Dalgarno sequences involved in the initiation of translation of mRNA. CsrB modulates the activity of CsrA by sequestering multiple copies of the protein via these binding sites (Liu *et al.*, 1997) and it is estimated that CsrB binds to approximately nine CsrA dimers; CsrB has 22 potential CsrA-binding sites (Babitzke & Romeo, 2007). A second small ncRNA belonging to the Csr system, CsrC, was discovered by Weilbacher *et al.* (2003). Its predicted secondary structure is similar to that of CsrB, with the repeated sequence in its predicted hairpin loops, but CsrC has only 13 potential CsrA-binding sites (Babitzke & Romeo, 2007). In *P. aeruginosa*, two small ncRNAs, RsmZ (also called RsmB) and RsmY, were found (Table 1), both of which antagonize the CsrA homologue, RsmA (Burrowes *et al.*, 2005; Heurlier *et al.*, 2004; Kay *et al.*, 2006). Both ncRNAs

contain seven 'GGA' motifs in their single-stranded regions (Babitzke & Romeo, 2007).

The CsrA-binding site was refined by Dubey *et al.* (2005), using an *in vitro* procedure termed SELEX (systematic evolution of ligands by exponential enrichment). SELEX was performed with various lengths of RNA sequence containing single CsrA-binding sites. A high-affinity binding site consensus was defined as RUACAR-GGAUGU, with the GGA and ACA motifs (underlined) completely conserved and GU residues (in bold) present in all but one of the selected ligands. Mutational analysis of SELEX-derived RNA targets demonstrated that both the ncRNA secondary structure and the GGA motif were important for CsrA-ncRNA interaction (Dubey *et al.*, 2005).

By combining bioinformatics (Kulkarni *et al.*, 2006) and experimental approaches (Kay *et al.*, 2006; Lenz *et al.*, 2005; Liu *et al.*, 1997; Weilbacher *et al.*, 2003), numerous genes encoding ncRNAs have been identified in bacteria that have *csrA* homologues (Table 1). Small, cognate ncRNA regulators have mostly been found or predicted in γ -proteobacteria (summarized in Table 1), but they are also present in ε -proteobacteria and firmicutes: two ncRNAs,

Table 1. Csr/Rsm regulatory systems and their role in animal host-bacteria interactions

E. coli is included in this table as a model organism.

Bacterium	Components*	TCS†	Functions	Response‡	References
<i>Escherichia coli</i>	CsrA	BarA/UvrY	Motility	+	Wei <i>et al.</i> (2001)
	CsrB/C		Biofilm formation	–	Wang <i>et al.</i> (2005)
			Carbon metabolism	+/-	Romeo (1998)
<i>Pseudomonas aeruginosa</i>	RsmA	GacS/GacA	Quorum sensing	–	Heurlier <i>et al.</i> (2004)
	RsmY/Z		Motility	+	Burrowes <i>et al.</i> (2006)
			Virulence	+/-	Goodman <i>et al.</i> (2004)
<i>Salmonella enterica</i> serovar Typhimurium	CsrA	BarA/SirA	Pathogenesis	+/-	Altier <i>et al.</i> (2000); Fortune <i>et al.</i> (2006)
	CsrB/C		Chemotaxis, aerotaxis	+	Lawhon <i>et al.</i> (2003)
			Flagellar synthesis	+	
<i>Helicobacter pylori</i>	CsrA	?	Motility	+	Barnard <i>et al.</i> (2004)
	CsrB1/B2		Oxygen resistance	+	
			Virulence	+	
<i>Legionella pneumophila</i>	CsrA (3 homologues) <i>RsmY/Z</i>	LetS/LetA	Motility	–	Molofsky & Swanson (2003)
			Cytotoxicity	–	
			Stress resistance	–	
			Sodium sensitivity	–	
			Macrophage infection	–	
			Intracellular replication	+	
<i>Vibrio fischeri</i>	CsrA CsrB1/B2	?	?	?	Kulkarni <i>et al.</i> (2006)
<i>Vibrio cholerae</i>	CsrA CsrB/C/D	VarS/VarA	Quorum sensing	–	Lenz <i>et al.</i> (2005)

*Known or predicted components of post-transcriptional regulatory systems. Protein components are in bold; ncRNAs in italics are predicted by computer analysis.

†Known or predicted two-component systems.

‡–, Repression by free CsrA; +, induction by free CsrA.

CsrB1 and CsrB2, have been predicted in *H. pylori* and an RsmY-like RNA in *B. subtilis* (Kulkarni *et al.*, 2006). These small RNAs, like their Gram-negative counterparts, are found in intergenic regions of the genome and their predicted secondary structures have the GGA repeat sequence in the stem-loops and single-stranded regions. As is the case in *E. coli* and *P. aeruginosa*, some bacteria have more than one ncRNA species (e.g. *S. enterica* serovar Typhimurium, *V. cholerae*, *V. fischeri*; Table 1). Why bacteria have several partially redundant ncRNA regulatory systems is not understood. Weilbacher *et al.* (2003) suggested that these RNAs may be produced at different phases of growth for fine tuning of the Csr regulatory system. It has also been suggested recently that redundant ncRNA genes may exist in some bacterial pathogens to respond to different signalling pathways and TCSs (Babitzke & Romeo, 2007).

In addition, it was found that CsrA controls transcription of ncRNAs, which represents a form of autoregulation, possibly through the cognate response regulator (see below, 'Regulation by two-component systems') in *E. coli* (Gudapaty *et al.*, 2001; Weilbacher *et al.*, 2003), *S. enterica* (Fortune *et al.*, 2006) and *P. aeruginosa* (Heurlier *et al.*, 2004). CsrA is also known to increase the stability of the CsrC ncRNA in *Salmonella* (Fortune *et al.*, 2006).

Regulation by two-component systems

TCSs mediate the link between external stimuli, most of which are unknown signals, and the transcription programme of bacteria (Beier & Gross, 2006; Heeb & Haas, 2001). The TCS is a ubiquitous mechanism for sensing and responding to various environmental stimuli in bacteria. A typical TCS consists of a sensor kinase and a response regulator (some hybrid proteins contain both domains), where the sensor kinase monitors environmental signals, and, through a phospho-relay system, the response regulator modulates gene expression.

The GacS/GacA (global activator of antibiotic and cyanide synthesis) system is used by bacteria to modulate virulence, stress tolerance, secondary metabolism and motility in response to changing environmental conditions (Goodman *et al.*, 2004; Heeb & Haas, 2001; Venturi, 2006). While the nature of the signal stimulating the membrane sensor is unknown, GacS/GacA systems seem to respond to a wide range of environmental stimuli, including host signals or signals produced by the bacterial population itself, and are therefore ideally suited to serve as hierarchical regulators in host-pathogen communication.

In many micro-organisms, CsrA activity is indirectly controlled by GacA-like response regulators, which directly regulate the levels of CsrA-sequestering ncRNAs (Goodman *et al.*, 2004; Molofsky & Swanson, 2003; Suzuki *et al.*, 2002; Teplitski *et al.*, 2003; Ventre *et al.*, 2006). However, while CsrA-like proteins have been identified in all proteobacterial species examined

(Mercante *et al.*, 2006), to date GacS/GacA homologues have only been described in γ -proteobacteria.

In *E. coli*, the BarA/UvrY TCS regulates carbon flow and the switch between different metabolic pathways via control of CsrA activity (Pernestig *et al.*, 2003). The ability to switch efficiently between different carbon sources (i.e. a functional UvrY regulator) was proven to be important for bacterial fitness in a monkey cystitis model (Tomenius *et al.*, 2006). BarA/UvrY was shown to activate the expression of *csrB* and *csrC* and the resulting sequestration of CsrA led to a derepression of genes involved in gluconeogenesis and a concomitant repression of glycolysis. Expression of a *csrB-lacZ* transcriptional fusion was dependent on both CsrA and UvrY, and the effect of UvrY on *csrC* expression was less significant than that on *csrB* expression (Weilbacher *et al.*, 2003).

A similar regulatory cascade exists in *P. aeruginosa*. GacA negatively regulates the activity of RsmA by controlling the level of its partner ncRNA molecule, RsmZ (Burrowes *et al.*, 2005). Expression of *rsmZ* was shown to increase with cell density, and was subject to negative autoregulation (Burrowes *et al.*, 2005; Heurlier *et al.*, 2004). Both overexpression of *rsmZ* and loss of RsmA resulted in quantitatively similar, negative or positive effects on target genes (Heurlier *et al.*, 2004). The GacA/RsmA/RsmZ signal transduction pathway in *P. aeruginosa* was refined by including RsmY, the newly discovered ncRNA that acts in parallel with RsmZ and in a similar manner. It has been demonstrated that both GacA and RsmA are required for *rsmY* transcription (Kay *et al.*, 2006).

In *S. enterica* serovar Typhimurium, CsrB and CsrC ncRNAs are controlled by the BarA/SirA TCS (Fortune *et al.*, 2006; Teplitski *et al.*, 2003, 2006), as is the case in *E. coli* (Weilbacher *et al.*, 2003), and seem to play redundant roles in the control of invasion. SirA was found to directly bind the *csrB* promoter, but a direct interaction between *csrC* and SirA has not been identified (Teplitski *et al.*, 2003, 2006). Both ncRNAs are also regulated by other members of the Csr system. The loss of CsrB increases *csrC* expression, which also applies vice versa, and overexpression of *csrA* increases both the levels and stability of CsrB and CsrC (Fortune *et al.*, 2006).

In *V. cholerae*, seven small RNA molecules have been identified, including four named Qrr for quorum regulatory RNAs, which regulate virulence gene expression (Lenz *et al.*, 2005) and three ncRNAs (CsrB/CsrC/CsrD) similar to the Csr ncRNA molecules described in *E. coli* (Lenz *et al.*, 2005). These three RNAs are regulated by the VarS/VarA TCS (GacS/GacA homologues) and act by controlling CsrA activity. They also have an impact on the expression of the Qrr RNAs: single or triple mutations in *varS/varA* or *csrB/csrC/csrD* increased the levels of Qrr RNAs (Lenz *et al.*, 2005).

Interestingly, most of the bacterial species studied that have ncRNA homologues also have BarA/UvrY TCS

homologues (Table 1), suggesting that the mechanisms for CsrA regulation could be conserved in bacteria.

Other components involved in the Csr regulatory network

Several bacteria such as *L. pneumophila* have more than one CsrA-like regulator; for example *L. pneumophila* subsp. *pneumophila* Philadelphia 1 possesses three homologues of CsrA (TIGR, <http://cmr.tigr.org/>) (Chien *et al.*, 2004). As is the case for ncRNA system redundancy, the role of multiple CsrA homologues remains to be determined.

Recent studies have revealed a new protein involved in the regulation of Csr systems in *L. pneumophila*: LetE. In a *csrA* mutant strain of *L. pneumophila*, levels of *letE* transcript were high, indicating that CsrA acts as a negative regulator of *letE* expression, in a direct or indirect manner (Forsbach-Birk *et al.*, 2004). The LetE protein increases expression of several genes involved in stationary-phase traits, which promote bacterial transmission between host amoebae, including cytotoxicity, motility and macrophage infection (Bachman & Swanson, 2004a, b). It has been suggested that LetE stabilizes transcripts encoding transmission-related genes, which are dependent on LetS/LetA (GacS/GacA homologues) and destabilizes LetS/LetA-independent transcripts, including *letE* itself.

In addition, in *L. pneumophila*, the regulation of the Csr system seems to involve the global alarmone (p)ppGpp (guanosine 3',5'-bispyrophosphate). First described as a component of the stringent response, which occurs during nutritional deprivation, (p)ppGpp controls DNA replication through regulation of RNA synthesis (Szalewska-Palasz *et al.*, 2007) and the synthesis of (p)ppGpp and its accumulation enable bacteria to survive and persist in the host. (p)ppGpp is generally proposed to bind near the catalytic site of RNA polymerase and modulates its activity; however, the precise localization of the (p)ppGpp-binding site on RNA polymerase has not been identified (Touloukhonov *et al.*, 2001). This stress alarmone is also implicated in virulence, QS, biofilm formation (Jain *et al.*, 2006) and regulation of the LetS/LetA TCS (Hammer *et al.*, 2002), which links (p)ppGpp to the CsrA regulatory network. In *L. pneumophila*, when the level of (p)ppGpp is high, the LetS/LetA TCS is activated and represses the activity of CsrA (Molofsky & Swanson, 2003), possibly via the induction of the predicted ncRNA expression (Kulkarni *et al.*, 2006). This potential link between (p)ppGpp and CsrA-mediated post-transcriptional regulation has not been examined in other bacteria.

More recent studies have shown possible links between the Csr regulatory network and other conserved regulatory elements, such as Hfq, in several bacteria (partially summarized in Fig. 1). This molecule governs cellular behaviour and physiological traits, some of which influence bacteria–host interactions. Hfq (or HF-I) is an RNA chaperone that was first identified for its role in the

replication of bacteriophage Q β in *E. coli* (Kajitani *et al.*, 1994). Hfq controls the stability of small regulatory RNAs (Gottesman, 2004; Majdalani *et al.*, 2005), mRNAs (Brennan & Link, 2007; Folichon *et al.*, 2003; Moll *et al.*, 2003) and, depending on the target in question, positively or negatively regulates the translation of target mRNAs via small ncRNAs (Sledjeski *et al.*, 2001). Hfq-mediated regulation seems to be a highly conserved mechanism for controlling bacterial virulence (Ding *et al.*, 2004; Nakao *et al.*, 1995; Sonnleitner *et al.*, 2003). Hfq was shown to stabilize the ncRNA RsmY of *P. aeruginosa* PAO1, thus positively regulating the QS system and inhibiting the repression of target gene translation by RsmA (Sonnleitner *et al.*, 2006); Hfq and RsmA bind concomitantly to overlapping sites on RsmY RNA, so binding of Hfq results in a decreased capacity of RsmY to sequester RsmA. Hfq can also inhibit RNase E cleavage of RsmY. RNase E preferentially cleaves AU-rich sequences, which are found in Hfq-binding sites, so when Hfq is bound to RsmY these RNase E sites are blocked (Sorger-Domenigg *et al.*, 2007). More recently, Baker *et al.* (2007) showed that in *E. coli*, CsrA inhibits Hfq synthesis by binding to a single site that overlaps the Shine–Dalgarno sequence upstream of the *hfq* gene and blocking ribosome binding. Interestingly, the stability of *hfq* mRNA was not altered by the translational repression exerted by CsrA. CsrA also seems to have a negative effect on *hfq* transcription, as *hfq* transcript levels were increased in *csrA* mutant strains (Baker *et al.*, 2007).

In *L. pneumophila*, *csrA* transcript levels are reduced in an *hfq* mutant, suggesting that Hfq regulates either *csrA* transcription or stability in a direct or indirect manner. Moreover, *hfq* is regulated by both LetA and RpoS, an activator of stationary-phase phenotypes, which occurs via the repression of CsrA (McNealy *et al.*, 2005). The *L. pneumophila* *hfq* gene does not complement an *E. coli* *hfq* mutation; this suggests that Hfq is significantly different between these two species, which may explain the differences in Hfq function observed in the two bacteria (McNealy *et al.*, 2005).

Recently, a GGDEF-EAL protein (CsrD) was shown to have a novel function in relation to the CsrA regulatory network in *E. coli* (Suzuki *et al.*, 2006). EAL/HD-GYP and GGDEF domains, associated with cyclic diGMP phosphodiesterase and diguanylate cyclase activities respectively, mediate the synthesis and turnover of cyclic diGMP. Cyclic diGMP is a second messenger involved in exopolymer synthesis, adhesion, motility and biofilm formation (D'Argenio & Miller, 2004), and EAL/HD-GYP and GGDEF domains are widely distributed in bacteria. CsrD was found to regulate decay of the ncRNAs CsrB and CsrC; the authors suggested that CsrD binds CsrB and CsrC RNAs, converting them to a substrate for RNase E degradation (Suzuki *et al.*, 2006). However, the mode of action of CsrD has not been clearly defined and the authors did not uncover a role for CsrD in the synthesis or degradation of cyclic diGMP, although the GGDEF and EAL domains were required for CsrD activity (Suzuki *et al.*, 2006).

Interestingly, the importance of cyclic diGMP phosphodiesterase activity in RsmA-mediated regulation was revealed recently: RpfG, a novel HD-GYP-domain-containing protein of *Xanthomonas campestris*, has the ability to restore swarming motility to an *rsmA* mutant of *P. aeruginosa* (Ryan *et al.*, 2006). Research has only recently focused on cyclic diGMP but it is already obvious that this messenger is used by various bacteria to control multi-cellular behaviour, such as biofilm formation and detachment, motility and virulence traits (Tamayo *et al.*, 2007) which are also controlled by the CsrA/ncRNA regulatory systems. It is likely that the areas of interconnectivity between the two pathways will continue to expand in the future.

Despite our current understanding of the complex regulation of Csr post-transcriptional regulatory systems, many questions still remain unanswered. The mechanisms of activation and of action are only partially understood and it is very likely that additional species-specific players have a role in Csr regulatory schemes.

CsrA controls physiological traits during different stages of infection

See Fig. 2 for a summary of the role of CsrA in host-pathogen interactions.

Regulation by CsrA permits the switch between 'acute' and 'persistent' forms of infection

Important features essential for bacterial virulence include full motility, the capacity to adhere to surfaces, production of cytotoxic factors, toxin delivery/secretion systems and ultimately colonization. Pathogenesis is a multifactorial process, but two main steps that lead to a successful infection can be singled out: rapid colonization (and/or internalization) and persistence. Specific virulence factors contribute to each of these events. Several studies have demonstrated that bacteria involved in acute infections have phenotypic characteristics distinct from those involved in chronic infections, with significant changes in the pattern of gene expression (see below). This differential expression is governed by cues from the host and the environment, as well as communication between bacteria. Csr systems play a central role in coordinating the physiological traits that are relevant to each stage of infection.

Colonization: importance of virulence factor production and motility

P. aeruginosa is a model organism for studying virulence. This ubiquitous Gram-negative bacterium is an important opportunistic human pathogen. Infections with *P. aeruginosa* occur in injured, burned, immunodeficient and immunocompromised patients. *P. aeruginosa* causes acute and chronic respiratory infections in people with cystic

fibrosis (Gomez & Prince, 2007). The sequenced PAO1 genome (<http://www.pseudomonas.com>) revealed that *P. aeruginosa* possesses a large number of genes encoding proteins involved in regulation, including one CsrA-like post-transcriptional regulatory system (the Rsm system) and a large number of TCSs (Stover *et al.*, 2000), which contribute to the remarkable ability of this bacterium to adapt to a wide range of environmental niches (Rodrigue *et al.*, 2000). The sensor-regulator proteins LadS/RetS/GacS and the response regulator GacA are involved in the regulation of gene expression associated with acute or chronic infection (Goodman *et al.*, 2004; Laskowski & Kazmierczak, 2006; Ventre *et al.*, 2006). LadS and RetS share domain organization and downstream targets; LadS and RetS have reciprocal effects on a shared set of virulence genes (Ventre *et al.*, 2006). Environmental signals in acute infection favour activation of RetS, which represses the GacS/GacA pathway and promotes RsmA activity by lowering the transcription of sequestering ncRNAs (Fig. 2). RetS positively controls the levels of free RsmA, by preventing transcription of the genes encoding ncRNAs, and is responsible for the upregulation of virulence genes necessary during acute infection and the repression of other pathways involved in chronic persistence. RetS is required for expression of the type III secretion system and other virulence factors and is involved in repression of genes encoding the exopolysaccharide component of the *P. aeruginosa* biofilm matrix. In contrast, the LadS and GacS signalling cascade brings about decreased levels of free RsmA via increased ncRNA levels, leading to a repression of type III secretion systems and activation of biofilm formation (Laskowski & Kazmierczak, 2006; Ventre *et al.*, 2006). Interestingly, the periplasmic domain of the hybrid sensor kinases RetS and LadS, which regulate ncRNA RsmZ levels in *P. aeruginosa*, belongs to a class of bacterial periplasmic sensor modules (7TMR-DISMED2) predicted to adopt an all-beta-fold secondary structure with structural characteristics reminiscent of certain carbohydrate-binding domains (Anantharaman & Aravind, 2003; Laskowski & Kazmierczak, 2006). This finding suggests that these two proteins may respond to carbohydrates of host or bacterial origin to control the switch from an acute to a chronic infection state via modulation of free RsmA levels.

An initial study carried out in *P. aeruginosa* PAO1 described RsmA as a negative control element in the formation of several extracellular products (e.g. pyocyanin, hydrogen cyanide, PA-IL lectin) as well as in the production of *N*-acylhomoserine lactone QS signal molecules (Pessi *et al.*, 2001). RsmA was found to positively control the ability to swarm and to produce extracellular rhamnolipids and lipase, which are functions known to contribute to niche colonization (Heurlier *et al.*, 2004). Expression of virulence genes, such as *tox*A, *lip*A and those involved in type III secretion, is upregulated by free RsmA. This upregulation is suspected to occur via Vfr, a cAMP-dependent regulatory protein (Goodman *et al.*, 2004). Vfr

Fig. 2. Role of CsrA in several host–pathogen interactions. Regulation of virulence factor expression by the Csr/Rsm system in the pathogens *P. aeruginosa* (Burrowes *et al.*, 2005; Goodman *et al.*, 2004; Ventre *et al.*, 2006), *S. enterica* serovar Typhimurium (Fortune *et al.*, 2006; Lawhon *et al.*, 2003) and *L. pneumophila* (Molofsky & Swanson, 2003, 2004). Arrows indicate activation and bars indicate inhibition. Blue and red lines represent regulation of the Csr/Rsm system in *P. aeruginosa* via the RetS or LadS pathways, respectively. Question marks represent potential interactions that have not been experimentally verified. For the TCSs, the sensor-regulators are underlined. This figure combines the various regulatory cascades found in several organisms as detailed in the text. Although several ncRNAs may be present in the micro-organisms listed (e.g. RsmZ and RsmY in *P. aeruginosa*), only the ncRNA described in the relevant studies is presented in the figure.

has been implicated in the regulation of many processes, including the expression of numerous proteins secreted by the type II pathway such as exotoxin A (Albus *et al.*, 1997; Beatson *et al.*, 2002; Dasgupta *et al.*, 2002; West *et al.*, 1994). These results were confirmed by transcriptomic analysis in which *P. aeruginosa* PAO1 wild-type was compared to its isogenic *rsmA* mutant. This microarray analysis revealed that transcript levels of *vfr* were decreased in the *rsmA* mutant compared to the wild-type strain, thus potentially explaining the upregulation of certain *vfr*-dependent genes (Burrowes *et al.*, 2006).

Salmonella species are the causative agents of typhoid fever and diarrhoeal diseases in humans. Orally ingested bacteria penetrate the intestinal mucosa and migrate via the lymph nodes to the spleen and liver to cause systemic disease. During infection, salmonellae take advantage of the phagocytic nature of macrophages to reside intracellularly within these cells, where they replicate within specialized vacuoles (Richter-Dahlfors *et al.*, 1997). In *S. enterica* serovar Typhimurium, CsrA regulates the expression of genes of the pathogenicity island SPI1 both positively and

negatively (Fig. 2). SPI1, which encodes components of a type III secretion apparatus, is required for penetration of intestinal epithelial cells. In this situation, CsrA acts in a positive or negative manner depending on its concentration (see below).

Inactivation of both the antagonist ncRNAs CsrB and CsrC represses SPI1 gene expression (Fortune *et al.*, 2006) while both overexpression and mutagenesis of the *csrA* gene repress SPI1 genes (Altier *et al.*, 2000). In addition, CsrA is involved in regulation of ethanolamine metabolism, vitamin B12 biosynthesis, maltose transport and motility in *S. enterica* serovar Typhimurium. All of these physiological processes are required for adaptation at the site of bacterial invasion, the intestinal tract of an animal host (Lawhon *et al.*, 2003). The two regulatory RNAs CsrB and CsrC play redundant roles in the control of invasion via the sequestration of CsrA. The levels of both these ncRNAs are positively controlled by (i) the BarA/SirA TCS, (ii) each other and (iii) CsrA (Fortune *et al.*, 2006).

Csr regulatory networks are often linked to QS systems in bacteria, thus adding an additional level of complexity to

the regulation of virulence traits. The relationship between CsrA and QS is complex and depends on the micro-organism in question. CsrA proteins may indirectly control the transcription of numerous genes through regulation of the QS network. In *P. aeruginosa*, the GacS/A TCS positively regulates the production of the QS signal *N*-butanoylhomoserine lactone (C4-HSL), by activating the expression of RsmA-sequestering ncRNAs (Burrowes *et al.*, 2005; Heurlier *et al.*, 2004). Moreover, during late-exponential and stationary phase, cultures of the *rsmA* mutant produce less *Pseudomonas* quinolone signal (PQS) than wild-type (Burrowes *et al.*, 2006). PQS (2-heptyl-3-hydroxy-4-quinolone) is an intercellular signal of *P. aeruginosa*, which also regulates numerous virulence factors and which is linked to the *N*-acylhomoserine lactone QS system (Diggle *et al.*, 2003).

In *V. cholerae*, a non-invasive human pathogen affecting the small intestine, the VarS/VarA TCS and the CsrA system control expression of the regulatory RNAs that control the entire QS regulon (Lenz *et al.*, 2005). In *V. cholerae*, virulence gene expression and biofilm formation occur at low cell densities, in the absence of autoinducers, and are repressed at high cell densities, when QS signal molecules have accumulated to a significant level (Hammer & Bassler, 2003). *V. cholerae* biofilms have been shown to be more acid resistant than planktonic cells, and QS-deficient biofilms had lower colonization capacities (Zhu & Mekalanos, 2003). In this case, it is proposed that QS promotes the detachment of planktonic cells after passage through the acid environment of the stomach (Hammer & Bassler, 2003; Zhu & Mekalanos, 2003). Upon reaching the intestine, a planktonic mode of growth seems required for the efficient colonization of the intestinal surface (Hung *et al.*, 2006; Liu *et al.*, 2006, 2007; Zhu & Mekalanos, 2003).

Motility in bacteria has been shown to be important for initial survival and virulence. Motility not only allows bacteria to seek out ideal local environments, but it enables adhesion of the pathogen to host cells and is integral to the initial steps in biofilm development. CsrA is a regulator of motility in various bacteria, with *csrA* mutants displaying decreased expression of genes involved in motility, as reported in the few following examples.

In *P. aeruginosa*, transcriptome analysis revealed that expression of genes involved in the formation of type IV pili, fimbriae and chemotaxis was decreased in an *rsmA* mutant compared to PAO1 wild-type. Consequently, the *rsmA* mutant was less motile than the wild-type strain (Burrowes *et al.*, 2006). The same phenotype, namely decreased motility compared to wild-type, was observed in *csrA* mutants of *H. pylori* (Barnard *et al.*, 2004) and *E. coli* (Wei *et al.*, 2001). The impact of the loss of CsrA on motility is more drastic in *S. enterica* serovar Typhimurium. A *csrA* mutant has decreased expression of the genes required for the synthesis and assembly of flagella, genes known to be regulated by the flagellar master regulator FhlDC (heterotetrameric transcriptional

regulatory complex), transcriptional regulators of flagellar gene expression such as *flgM* and *fliA*, and genes associated with chemotaxis and aerotaxis (*cheA*, *tsr*, *aer*) (Lawhon *et al.*, 2003). The mutant had no detectable flagella and was non-motile (Lawhon *et al.*, 2003). A recent study showed that SirA and the Csr system also control the *fim* operon, which encodes type 1 fimbriae, important for virulence in *S. enterica* (Teplitski *et al.*, 2006). SirA activates the expression of the CsrA-antagonist RNA genes *csrB* and *csrC*, which in turn promote the translation of the *fim* operon. Previous work has demonstrated that *fimZ* and *fimY* (encoding response-regulatory proteins) also regulate both motility and adherence (Clegg & Hughes, 2002).

It is interesting to note that in *Proteus mirabilis* (Liaw *et al.*, 2003), *Serratia marcescens* (Ang *et al.*, 2001) and *Erwinia carotovora* (Mukherjee *et al.*, 1996) the impact of CsrA on motility was analysed in overexpressing strains rather than mutants. Surprisingly, in all these studies, overexpression of *csrA* led to a repression of motility, the same phenotype observed for *csrA* mutants, as mentioned above.

CsrA-mediated regulation in the intracellular pathogen *L. pneumophila* is complex and is potentially different from that in other proteobacteria studied. *L. pneumophila* is a parasite of aquatic amoebae, which can also replicate within human alveolar macrophages, causing Legionnaires' disease. To persist in an infectious state, it must be proficient at both intracellular replication and transmission. Overproduction of CsrA in *L. pneumophila* led to a reduction of flagellation and pigmentation and an increase in bacterial cell size (Fettes *et al.*, 2001). In this bacterium, CsrA was found to be a repressor of transmissive phase traits. When intracellular nutrients become limiting, a stringent-like response (via (p)ppGpp alarmone accumulation) coordinates the differentiation of *L. pneumophila* to a transmissive and motile form to enable dispersal (Bachman & Swanson, 2001). As described above ('Other components involved in the Csr regulatory network'), this process is mediated by the LetS/LetA TCS, which relieves CsrA repression, possibly by activating the expression of an as yet unidentified ncRNA (Molofsky & Swanson, 2003). CsrA tightly controls the change in gene expression correlated with the phase status, including the motile phenotype essential during the transmissive phase. The motility of an *L. pneumophila* *csrA* mutant increases as cultures progress from the early to the late exponential phase of growth. It was shown that CsrA repression of motility is mediated by both FliA (flagellar sigma factor)-dependent and -independent pathways. A *csrA fliA* double mutant is non-motile and can infect macrophages (Molofsky & Swanson, 2003, 2004).

Persistence: importance of biofilm formation and antibiotic/stress resistance

Some of the strategies developed by bacteria for persistence are (i) the formation of biofilms, which exhibit increased resistance to host defences and act as a reservoir of

pathogenic bacteria involved in nosocomial infections, and (ii) intracellular replication. These forms of life involve distinct morphologies and properties brought about by changes in gene expression.

The favoured mode of growth for bacteria is within sessile, matrix-enclosed communities known as biofilms. Biofilm existence is beneficial for bacteria because it protects them from the host's immune system and leads to increased antibiotic resistance, which often complicates chronic infections (Kharazmi, 1991; Patel, 2005). In several bacteria, CsrA-like regulators modulate the pathogen's ability to resist stress and to persist in the host.

Biofilm formation is increased in a *csrA* mutant of *E. coli* K-12 (Jackson *et al.*, 2002). The effect of CsrA on biofilm formation in *E. coli* is mediated via the regulation of central carbon flux and intracellular glycogen synthesis and catabolism. Moreover, the induction of *csrA* expression within a pre-formed biofilm causes its dispersal. CsrA serves as a repressor of biofilm formation and as an activator of biofilm dispersal under a variety of culture conditions (Jackson *et al.*, 2002). Recent studies have shown that CsrA has a negative effect on the translation of genes encoded by the *pgaABCD* operon, which are responsible for the synthesis of the polysaccharide adhesin PGA (poly- β -1,6-*N*-acetyl-D-glucosamine), necessary for biofilm formation (Wang *et al.*, 2005). Two distinct forms of cell attachment during early biofilm development have been characterized: temporarily attached bacteria mainly associate by a cell pole to the host cell, whereas permanently attached bacteria associate via their lateral cell surface. Interestingly, it was shown that a *csrA* mutant displayed a permanent attachment phenotype, probably due to an overproduction of the PGA adhesin (Agladze *et al.*, 2005; Wang *et al.*, 2005). The implication of this finding for *in vivo* virulence remains to be investigated.

CsrA/RsmA-mediated regulation of biofilm formation has also been studied in *P. aeruginosa*. As mentioned above, RsmA positively controls swarming motility and production of extracellular rhamnolipid and lipase, all of which are known to contribute to niche colonization (Heurlier *et al.*, 2004). As mentioned previously, establishment of chronic infection is linked to an activation of the LadS and/or GacS/GacA signalling pathways, resulting in sequestration of RsmA and in activation of genes involved in biofilm formation and production of AHL autoinducers. As a consequence, a *retS* mutant was found to be hyperadhesive on glass surfaces, and exhibited premature and robust biofilm formation (Goodman *et al.*, 2004). However, other results obtained by Burrowes *et al.* (2006) have shown that the *P. aeruginosa* *rsmA* mutant has significantly decreased ability to form biofilms on PVC compared to the wild-type strain. Nevertheless, these findings are not necessarily contradictory: while RsmA is still present in a *retS* mutant, albeit at reduced levels, an *rsmA* mutant does not produce any RsmA. These data demonstrate the complexity of biofilm development and

point towards a highly sensitive balance between biofilm formation and free-living existence. In addition, biofilm formation is probably highly dependent upon the *in vitro* experimental conditions used.

CsrA-like proteins also play a key role in resistance to various stresses that bacteria encounter in host environments. Many factors change markedly during the transition from the external environment to the host, between different locations in the host, and between different hosts. The first signal frequently encountered by pathogenic bacteria upon entry into a mammalian host is an increase in temperature. Passage from the external environment to the host is also associated with other cellular stresses such as changes in osmolarity (intestine), shifts in pH (stomach), exposure to toxic reactive oxygen species and nitrogen intermediates, and starvation for iron and other nutrients. The bacterium *H. pylori* is responsible for most peptic ulcers and many cases of chronic gastritis (Marshall, 2006). It survives in the acid environment of the stomach and duodenum by 'hiding' in mucus and neutralizing stomach acid in its local environment. The pathogenesis of *H. pylori* depends on its persistence, namely its ability to survive in an acid environment, and to survive attack by phagocytes and their released oxygen species. CsrA is involved in oxidative stress resistance in *H. pylori* and therefore its persistence in hosts. Deletion of *csrA* leads to decreased expression of genes required for oxidative and acid stress resistance (e.g. *nap*, *vacA* and *ahpC*, which encode respectively neutrophil-activating protein, vacuolating cytotoxin and alkyl hydroperoxide reductase, and *cagA*, cytotoxin-associated gene A). The *csrA* mutant is more sensitive than the wild-type strain to stress caused by peroxide and methyl viologen exposure (Barnard *et al.*, 2004). Moreover, CsrA regulation responds to acid and heat stresses, adaptive mechanisms that are a prerequisite for survival in the stomach (Barnard *et al.*, 2004).

While studies have shown that CsrA controls changes in target gene expression, including the activation of a general stress response in bacteria, it has been demonstrated that CsrA functions in a different manner in some intracellular pathogens, where intracellular replication and transmission are important in either colonization or persistence, such as in *L. pneumophila* (Bachman & Swanson, 2001; Molofsky & Swanson, 2004). As described above, in *L. pneumophila*, CsrA is expressed during intracellular replication and inhibits stationary-phase phenotypes (Molofsky & Swanson, 2003). Intracellular pathogens are exposed to more environmental stresses during the transmissive phase. During the intracellular stage, free CsrA decreases the cell's resistance to environmental stresses and favours replicative phenotypes. An *L. pneumophila* *csrA* mutant cannot replicate inside macrophages, although the mutant is highly infectious. The repression of CsrA activates stress resistance and virulence factors that are required during the transmissive phase (Molofsky & Swanson, 2003): the bacteria express a cytotoxin to escape the host, acquire osmotic and heat resistance to survive the external

environment, synthesize pigments that protect them from UV damage, and synthesize the factors required to establish a protected niche within the next host and to avoid lysosomal degradation in macrophages before phagocytosis.

Finally, persistence traits regulated by the Csr system may be linked to the control of multidrug efflux systems. Efflux pumps provide pathogens with a method of resistance to natural substances produced by hosts such as bile, hormones and host defence molecules. In addition, these efflux pumps confer clinically significant multidrug-resistance to pathogens. Transcriptomic analysis showed that in *P. aeruginosa* loss of *rsmA* resulted in increased expression of the genes encoding the MexEF-OprN pump (Burrowes *et al.*, 2006). This pump is involved in the transport of numerous antibiotics and the PQS molecule out of the cell (Kohler *et al.*, 2001). Further studies indicated that the *rsmA* mutant had increased resistance to substrates known to be pumped from the cell by MexEF-OprN: amikacin, nalidixic acid, trimethoprim and ceftazidime (Mulcahy *et al.*, 2006).

Concluding remarks

Csr-like post-transcriptional regulatory systems, first described in regulation of metabolic activities related to carbon sources (glycolysis, gluconeogenesis), are gradually emerging as important control mechanisms for a variety of infection-linked physiological traits in many different pathogenic bacteria. These traits, related to infection mechanisms such as colonization, persistence and pathogenicity, include virulence gene expression, motility, biofilm formation, antibiotic resistance and switching between acute and chronic stages of infection. Indeed, Csr-like post-transcriptional systems play an integral role in the pathogenesis of many bacteria and are pivotal in the establishment of successful infections. The expression/activity of every Gram-negative Csr-like post-transcriptional system discovered to date is linked to and controlled by a two-component sensor-regulator system, sensing as-yet-unidentified environmental signals. The regulation of gene expression is of utmost importance for bacteria that need to adapt to environmental changes and nutritional stresses, especially in cases of infection, and respond to extracellular signals by switching their activities from one state to another. While the regulation of gene expression at the transcriptional level has been the focus of much attention, post-transcriptional control has only recently been recognized as a key mechanism for fine tuning gene expression, adapting the cellular phenotype to suit environmental conditions. For these reasons the Csr network needs to be recognized as an important virulence determinant. The Csr regulatory system seems to rely on a conserved backbone (composed of TCS/ncRNA(s)/CsrA-homologues) associated with various species-specific regulators.

Recent studies have revealed that many of these Csr-like post-transcriptional systems are highly redundant, being composed of two or more RNA-binding proteins and

ncRNA molecules. This degree of redundancy probably exists to ensure the relay of high-fidelity information from both extra- and intracellular signalling to subsequent responsive gene expression, which is extremely important for the success of the pathogen.

In order to refine the Csr cascade, some challenges must be addressed: (i) identification of the direct post-transcriptional targets of CsrA, and (ii) characterization of the signals sensed by Csr-linked TCSs. Moreover, in order to fully understand the function of the Csr system, the regulation of CsrA/RsmA expression needs to be elucidated. The activity of the *csrA* promoter was found to be higher during exponential growth than during stationary phase, which points towards regulation of *csrA* expression. Analysis of transcriptional fusions may suggest transcriptional control of *csrA* gene expression. One last (but not least) question is the exact function of the CsrA/RsmA in micro-organisms where the other components of the Csr system, i.e. regulatory ncRNAs, have not been found. Is there any evolutionary significance in the fact that CsrA/RsmA proteins display variable C-terminal extremities? Generally, CsrA and its homologues have high similarities at the N-terminus, with the same conserved secondary structure, while the C-terminus can vary from a few amino acids to more than 30, sometimes with and sometimes without helix-turn-helix (HTH) motifs. A link between the extended length and the absence/failure to identify Csr-related ncRNAs has not been identified. Another interesting subject is the link between environmental stimuli and the Csr-mediated response in micro-organisms that do not display the two-component regulatory GacS/A system. Once these questions have been answered it is foreseeable that the Csr system can be targeted using drug therapy, to block pathogens at a specific infective stage and thus prevent colonization and/or the establishment of chronic infection. This could prove to be a major breakthrough in the fight against nosocomial and infectious diseases, two areas of enormous human significance.

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