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MicroReview

Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour

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Summary

In many γ-proteobacteria, the conserved GacS/GacA (BarA/UvrY) two-component system positively controls the expression of one to five genes specifying small RNAs (sRNAs) that are characterized by repeated unpaired GGA motifs but otherwise appear to belong to several independent families. The GGA motifs are essential for binding small, dimeric RNA-binding proteins of a single conserved family designated RsmA (CsrA). These proteins, which also occur in bacterial species outside the γ-proteobacteria, act as translational repressors of certain mRNAs when these contain an RsmA/CsrA binding site at or near the Shine-Dalgarno sequence plus additional binding sites located in the 5′ untranslated leader mRNA. Recent structural data have established that the RsmA-like protein RsmE of *Pseudomonas fluorescens* makes specific contacts with an RNA consensus sequence 5′-$^{A}/_{U}$CANGGANG$^{U}/_{A}$-3′ (where N is any nucleotide). Interaction with an RsmA/CsrA protein promotes the formation of a short stem supporting an ANGGAN loop. This conformation hinders access of 30S ribosomal subunits and hence translation initiation. The output of the Gac/Rsm cascade varies widely in different bacterial species and typically involves management of carbon storage and expression of virulence or biocontrol factors. Unidentified signal molecules co-ordinate the activity of the Gac/Rsm cascade in a cell population density-dependent manner.

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Introduction

Bacteria respond to changing environments by adjusting the cellular levels of mRNAs, stable RNAs (that is, rRNAs and tRNAs) and small RNAs (sRNAs). Whereas the regulation of transcription initiation is crucial in this adaptation, subsequent control of translation initiation can be just as important. Recent studies have shown that two major classes of sRNAs influence the rate of translation initiation in bacteria (Majdalani *et al.*, 2005; Storz *et al.*, 2005). sRNAs of the first class interact with 5′ leader regions of target mRNAs by base pairing. Such interactions interfere with ribosome binding when they occur at or near the Shine-Dalgarno (SD) sequence of mRNAs. The opposite effect, stimulation of ribosome binding, can also be observed in situations where sRNAs change the secondary structure of target mRNAs by base pairing with an upstream region. The RNA chaperone Hfq facilitates these base pairing interactions in Gram-negative bacteria, but seems to be dispensable in Gram-positive bacteria (Heidrich *et al.*, 2006; Bohn *et al.*, 2007). sRNAs of the second class, which have a high affinity for RNA-binding proteins of the RsmA/CsrA family, can relieve translational repression owing to these proteins by sequestering them (Majdalani *et al.*, 2005; Storz *et al.*, 2005; Babitzke and Romeo, 2007). RsmA and CsrA are acronyms for regulator of secondary metabolism and carbon storage regulator respectively. In pseudomonads, sRNAs that bind RsmA/CsrA proteins are typically produced under the positive control of a two-component system, termed GacS/GacA (for global activation of antibiotic and cyanide synthesis). Other γ-proteobacteria also have GacS/GacA homologues, many of which bear different names (see Table 1). The general characteristics of the Gac/Rsm signal transduction pathway are outlined in Fig. 1. The target genes that are translationally regulated by this regulatory cascade, and hence the output, vary considerably among various bacteria (Table 1). However, as we wish to point out in this review, two features are conserved: in general, mutants blocked in this regulatory pathway are impaired in social behaviour and there appears to exist a common molecular basis of the RNA-RsmA/CsrA protein

Table 1. Mutants affected in *gacS* and *gacA* homologues in γ-proteobacteria.

Species	GacS/GacA homologues	Major GacS/GacA-controlled phenotypes	GacS/GacA-dependent sRNAs	References
Acinetobacter baumannii	GacS/GacA	Citrate utilization	RsmX, RsmY, RsmZ[a]	Dorsey *et al.* (2002)
Azotobacter vinelandii	GacS/GacA	Alginate, poly-β-hydroxy-butyrate, encystment	?	Castañeda *et al.* (2001)
Erwinia carotovora ssp. *carotovora*	GacS/GacA (ExpS/ExpA)	Extracellular pectinases, cellulase, protease, virulence, motility	RsmB	Cui *et al.* (2001)
Erwinia chrysanthemi	?/GacA	Extracellular pectinases, cellulase, protease, TTSS, virulence	?	Lebeau *et al.* (2008)
Escherichia coli	BarA/UvrY	Central carbon metabolism, biofilm, virulence[b], motility	CsrB, CsrC	Suzuki *et al.* (2002); Weilbacher *et al.* (2003); Tomenius *et al.* (2006)
Legionella pneumophila	LetS/LetA	Cytotoxicity, virulence, motility	RsmY, RsmZ[a]	Hammer *et al.* (2002); Molofsky and Swanson (2003)
Pseudomonas aeruginosa	GacS/GacA	AHL, HCN, pyocyanin, lipase, elastase, biofilm, virulence, motility	RsmY, RsmZ	Rahme *et al.* (1995); Reimmann *et al.* (1997); Parkins *et al.* (2001); Kay *et al.* (2006)
Pseudomonas chlororaphis (aureofaciens)	GacS/GacA	AHL, phenazines, HCN, surfactants, 2,3-butanediol, protease, biocontrol	?	Chancey *et al.* (1999); Schmidt-Eisenlohr *et al.* (2003); Han *et al.* (2006); Girard *et al.* (2006)
Pseudomonas entomophila	GacS/GacA	Protease, hemolysin, virulence	RsmY, RsmZ[c]	Vodovar *et al.* (2006)
Pseudomonas fluorescens	GacS/GacA	DAPG, HCN, pyoluteorin, pyrrolnitrin, protease, phospholipase, biocontrol, H_2O_2 resistance, motility	RsmX, RsmY, RsmZ	Haas and Défago (2005); Kay *et al.* (2005); Heeb *et al.* (2005); Dubuis *et al.* (2007)
Pseudomonas marginalis	LemA/GacA	Pectinases, virulence	?	Liao *et al.* (1997)
Pseudomonas syringae pv. *syringae*	GacS(LemA)/ GacA	Syringomycin, syringolin, AHL, alginate, protease, virulence	RsmX, RsmY, RsmZ[c]	Willis *et al.* (2001); Quinones *et al.* (2004)
Pseudomonas syringae pv. *tomato*	GacS/GacA	Coronatine, AHL, TTSS, virulence	RsmX[a], RsmY, RsmZ	Chatterjee *et al.* (2003)
Pseudomonas tolaasii	PheN(RtpA)/?	Tolaasin, protease, virulence, motility	?	Grewal *et al.* (1995); Murata *et al.* (1998)
Pseudomonas viridiflava	RepA/RepB	Extracellular pectinase, protease, alginate, virulence	?	Liao *et al.* (1996)
Salmonella enterica ssp. Typhimurium	BarA/SirA	TTSS, invasion, motility	CsrB, CsrC	Altier *et al.* (2000); Fortune *et al.* (2006)
Serratia marcescens	PigW/PigQ	Prodigiosin	?	Williamson *et al.* (2006)
Serratia plymuthica	GrrS/GrrA	Extracellular protease, pyrrolnitrin, biocontrol	?	Ovadis *et al.* (2004)
Vibrio cholerae	VarS/VarA	HapR-dependent virulence factors	CsrB1[a], CsrB2[a] (= CsrC), CsrB3[a] (= CsrD)	Lenz *et al.* (2005)
Vibrio fischeri	GacS/GacA	Bioluminescence, squid colonization	CsrB1[a], CsrB2[a]	Whistler and Ruby (2003)

a. Predicted by Kulkarni *et al.* (2006).
b. In uropathogenic strains.
c. Predicted by BLASTN.
AHL, *N*-acyl-homoserine lactone; DAPG, 2,4-diacetylphloroglucinol; TTSS, type III secretion system; ? indicates that GacS/GacA-dependent sRNAs or regulators have not yet been identified.

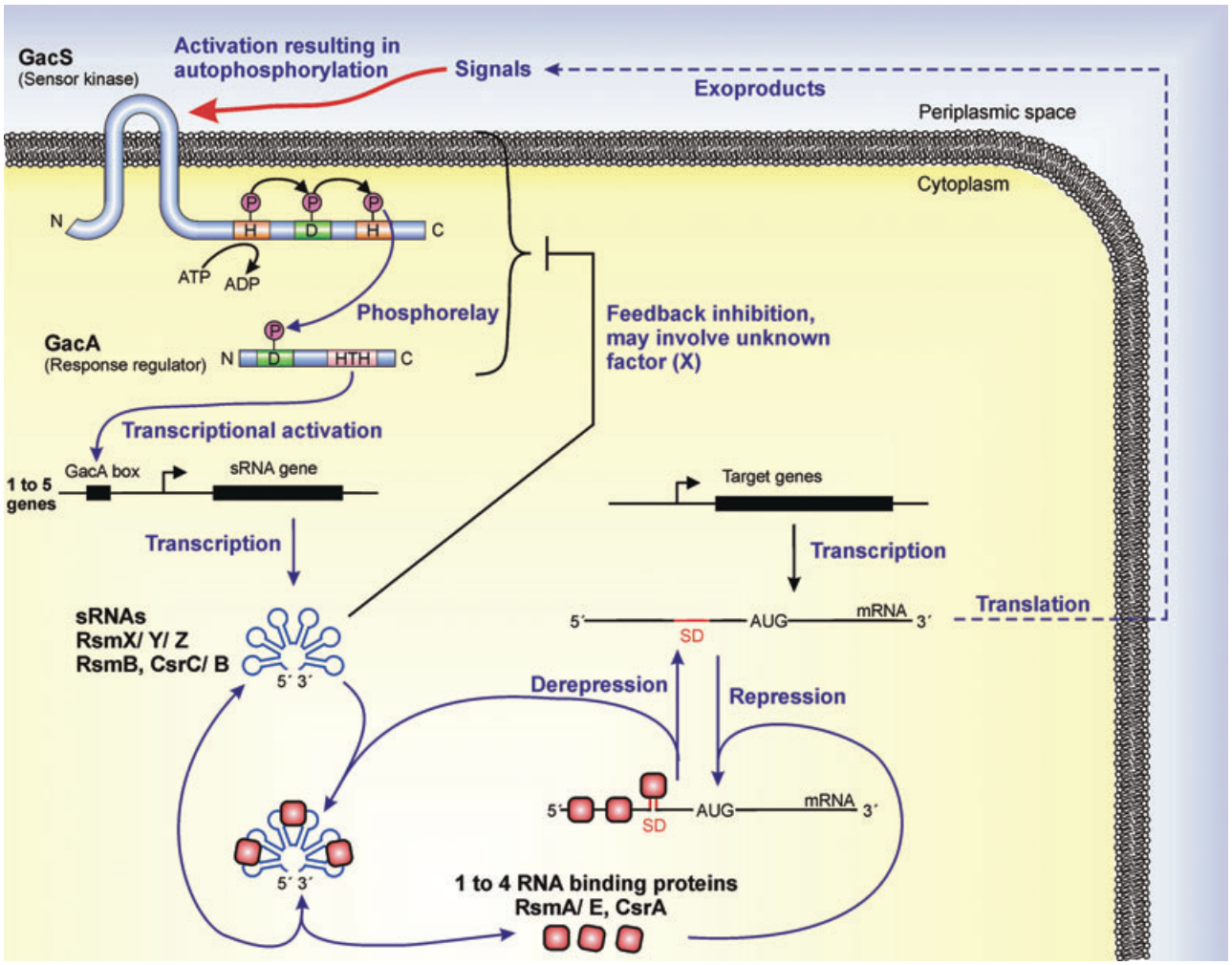

Fig. 1. General characteristics of the Gac/Rsm signal transduction pathway in γ-proteobacteria. The highest number of sRNA genes (five) is predicted in *Photobacterium profundum* (Kulkarni *et al.*, 2006). The highest number of genes for small RNA-binding proteins (four) appears to occur in *P. syringae* pv. *tomato* (Rife *et al.*, 2005). ↓, positive effect; ⊥, negative effect; dotted line, positive feedback loop; X, unknown hypothetical component.

interaction, which ultimately determines the output of the regulatory pathway. The emphasis of this review will be on the mechanisms that regulate target gene expression in the Gac/Rsm cascade, on common features of target genes and on recent insight gained by structural analysis of a complex formed between an RsmA/CsrA-type protein and a target RNA.

Putting the pieces of a jigsaw together

When the components of the Gac/Rsm pathway were first described in various bacteria, the researchers had widely different objectives. The sensor kinase GacS (originally designated LemA) was found in the plant pathogen *Pseudomonas syringae* pv. *syringae* as a key regulator of pathogenicity (Hrabak and Willis, 1992) and its homologue BarA as a multicopy suppressor of an osmotically compromised *envZ* mutant of *Escherichia coli* (Nagasawa *et al.*, 1992). The response regulator GacA was discovered as a master regulator of antifungal metabolites in the biocontrol bacterium *Pseudomonas fluorescens* CHA0 (Laville *et al.*, 1992). Evidence that GacS and GacA form a two-component system came from genetic studies in *P. syringae* (Rich *et al.*, 1994) and later from *in vitro* phosphotransfer experiments with the GacS/GacA homologues BarA/UvrY in *E. coli* (Pernestig *et al.* 2001). At the hierarchical level below GacS/GacA, two genes that encode GacA-controlled sRNAs were initially found as a multicopy suppressor (TRR) of a phaseolotoxin-negative, presumably GacA-defective mutant of *P. syringae* pv. *phaseolicola* (Rowley *et al.*, 1993) and as an activator of extracellular virulence factor production (*aepH*) in *Erwinia carotovora* ssp. *carotovora* (Murata *et al.*, 1994). However, at the time of their discovery, the sRNA products

of these genes were not recognized simply because sRNAs were not on the agenda. The findings that the RNA-binding protein CsrA is a global post-transcriptional regulator of carbon metabolism (Romeo *et al*., 1993; Liu and Romeo, 1997) and that its biological activity is antagonized by the sRNA CsrB in *E. coli* (Liu *et al*., 1997) eventually opened up a new perspective. The TRR and *aepH* loci were found to encode the sRNAs RsmY and RsmB, respectively; both RsmY and RsmB have high affinity for RsmA/CsrA-like proteins (Liu *et al*., 1998; Valverde *et al*., 2003). The mechanistic link between the GacS/GacA two-component system and the post-transcriptional regulator RsmA/CsrA with its antagonistic sRNAs was recognized in *P. fluorescens* (Blumer *et al*., 1999; Aarons *et al*., 2000). Thus, it was possible to assemble the backbone of the Gac/Rsm signal transduction pathway (Fig. 1) from pieces of evidence obtained in bacteria that differ widely with respect to their activities and habitats. This illustrates well that the Gac/Rsm regulatory cascade is conserved in bacterial evolution but fulfils diversified functions. A detailed picture of this regulatory pathway has been obtained mainly in *E. carotovora*, *E. coli*, *Pseudomonas aeruginosa*, *P. fluorescens*, *Salmonella enterica* ssp. Typhimurium, *Legionella pneumophila* and *Vibrio* spp. and is discussed in several recent reviews (Babitzke and Romeo, 2007; Bejerano-Sagie and Xavier, 2007; Toledo-Arana *et al*., 2007; Valverde and Haas, 2008).

Gac/Rsm control is mostly positive

Upon activation, the GacS/GacA two-component system switches on the transcription of sRNA genes termed *csrB*, *csrC* (in enteric bacteria), *rsmB* (in *E. carotovora*) and *rsmX*, *rsmY* and *rsmZ* (in pseudomonads and related bacteria) (Fig. 1). The expression of the sRNA genes and, hence, that of many target genes increases strongly with increasing cell population densities in *E. coli* (Suzuki *et al*., 2002; Dubey *et al*., 2003; Weilbacher *et al*., 2003), *S. enterica* (Johnston *et al*., 1996), *E. carotovora* (Eriksson *et al*., 1998; Cui *et al*., 2001), *Vibrio cholerae* (Lenz *et al*., 2005), *P. fluorescens* (Heeb *et al*. 2002; Valverde *et al*., 2003; Kay *et al*., 2005) and *P. aeruginosa* (Heurlier *et al*., 2004; Burrowes *et al*., 2005; Kay *et al*., 2006). The activated (phosphorylated) GacA response regulator is suspected to bind to a conserved upstream element termed the GacA box (consensus TGTAAGN_6 CTTACA, where N is any nucleotide) in the promoters of the sRNA genes mentioned above (Valverde *et al*., 2003; Kay *et al*., 2005; Lenz *et al*., 2005; Kulkarni *et al*., 2006). This interaction remains to be demonstrated by *in vitro* experiments, however. By producing the sRNAs, the Gac/Rsm signal transduction pathway upregulates the production of numerous proteins whose production is repressed by RsmA/CsrA proteins (Fig. 1). Mechanistically, this outcome is now well understood and will be discussed in detail below. The alternative, less well-documented scenario is that the Gac/Rsm cascade downregulates the expression of certain genes, e.g. those involved in the synthesis of flagella in *P. fluorescens* or *E. coli* (Wei *et al*., 2001; Sánchez-Contreras *et al*., 2002). Here, RsmA/CsrA proteins formally act as activators but how they do this is not entirely clear. They might negatively regulate some repressors or they might exert a favourable influence on mRNA stability (Wei *et al*., 2001).

gacA mutants lack specific social activities

From the foregoing section it follows that mutants defective in the GacS/GacA two-component system and its homologues typically lack a range of functions, whereas gain of function is less prominent. A non-exhaustive survey of *gacS/gacA* mutants and their phenotypes in various bacteria (Table 1) reveals several interesting points. (i) All mutants described belong to the γ-proteobacteria. Furthermore, a bioinformatic search for sRNAs that bind RsmA/CsrA and are controlled by GacA homologues predicts such sRNAs only in γ-proteobacteria (Kulkarni *et al*., 2006). This suggests that the Gac/Rsm pathway may be a specialty of γ-proteobacteria, at least in the form depicted in Fig. 1. (ii) Under laboratory conditions, especially in rich media, *gacS/gacA* mutants grow well and may even have a temporary advantage over the wild type (Eriksson *et al*., 1998; Bull *et al*., 2001). (iii) In animal- and plant-pathogenic bacteria, *gacS/gacA* mutants show reduced production of virulence factors and are less virulent than the wild type in a variety of host-pathogen systems (Ahmer *et al*., 1999; Rahme *et al*., 2000). In biocontrol bacteria (e.g. *P. fluorescens* and *Serratia plymuthica*), which protect plant roots from pathogens (fungi, nematodes), *gacS/gacA* mutants produce only low amounts of biocontrol factors (secondary metabolites, lytic enzymes) and have reduced biocontrol ability (Table 1). In such biocontrol interactions, the host plant derives a benefit, while fungi and nematodes experience biocontrol as an act of virulence. It is therefore not surprising that many biocontrol factors and virulence factors have similar properties (Haas *et al*., 2004). In γ-proteobacteria, the virulence and biocontrol factors controlled by the Gac/Rsm pathway depend strongly on population sizes and hence can be regarded as manifestations of social behaviour (or quorum sensing). When bacteria lose these functions, they lose competitiveness in nature, but remain fit under laboratory conditions. The signals that modulate the activity of the Gac/Rsm pathway will be discussed in the section on signalling and cross-talk.

In several *Pseudomonas* species, e.g. *P. aeruginosa*, *P. syringae* and *P. chlororaphis*, the Gac/Rsm system exerts positive control on the synthesis of *N*-acyl-homoserine lactones, the classical quorum sensing signals in these organisms. However, many mechanistic details of this regulation are still unclear (Reimmann *et al*., 1997; Chancey *et al*., 1999; Quinones *et al*., 2004; Girard *et al*., 2006; Kay *et al*., 2006). In *V. cholerae*, the VarS/VarA (= GacS/GacA) – CsrA pathway is a branch of three quorum sensing pathways, which converge at the central transcriptional regulator LuxO (Lenz *et al*., 2005). In *P. fluorescens* CHA0, where *N*-acyl-homoserine lactones have not been found, it is the Gac/Rsm system that accounts for cell population density-dependent expression of exoproducts (Laville *et al*., 1992; Zuber *et al*., 2003; Kay *et al*., 2005).

rsmA/csrA mutants are also socially handicapped

As *gacA* mutants are defective in virulence, one might expect that *rsmA/csrA* mutations would have the opposite effect and would result in hypervirulence. This is indeed the case in the soft rot pathogen *E. carotovora* (Chatterjee *et al*., 1995) and, to some extent, in the human pathogen *L. pneumophila*, where a *csrA* mutant is more highly infectious for macrophages than the wild type. However, a *csrA* mutant of *L. pneumophila* is impaired in subsequent intracellular growth in macrophages (Table 2) (Molofsky and Swanson, 2003). In other bacteria, the situation is even more complex (Table 2). For instance, in *S. enterica*, both *sirA* (*gacA*) and *csrA* mutants are unable to invade epithelial cells (Ahmer *et al*., 1999; Altier *et al*., 2000), suggesting that a balance of positive and negative regulatory effects of CsrA is important for infection (Fortune *et al*., 2006). In *P. aeruginosa*, GacA negatively and RsmA positively regulates the type III secretion system (TTSS). As a consequence, an *rsmA* mutant shows reduced cytotoxicity for epithelial cells (Mulcahy *et al*., 2006; Soscia *et al*., 2007). A *gacA* mutant is nevertheless attenuated for virulence in a number of host organisms because GacA positively controls a range of virulence factors, especially those secreted via type II secretion (Rahme *et al*., 2000).

For several reasons, the analysis of *rsmA/csrA* mutants can be less straightforward than that of *gacA* mutants. (i) In several bacterial species, it is difficult to isolate *rsmA/csrA* null mutants, as they tend to show strong cell–cell aggregation and/or slow growth (Romeo *et al*., 1993; Lawhon *et al*., 2003; Molofsky and Swanson, 2003). For instance, suppressor mutations of unknown nature arise at high frequencies in a *S. enterica csrA* mutant (Altier *et al*., 2000). In *L. pneumophila*, the *csrA* gene could only be deleted in a strain that carried an additional functional copy of this gene *in trans* (Molofsky and Swanson, 2003). In *E. coli*, the *csrA* mutant commonly used carries a resistance cassette insertion near the 3′ end of the *csrA* gene (Romeo and Gong, 1993), which therefore might conserve residual function. In *P. aeruginosa*, clumping restricts growth of an *rsmA* mutant and results in a small colony phenotype (Heurlier *et al*., 2004). (ii) Some bacteria contain two or more *rsmA* alleles. For instance, in *P. fluorescens* CHA0, single mutations in *rsmA* or its homologue *rsmE* have little effect, and an *rsmA rsmE* double mutation is required for derepressed production of biocontrol factors (Reimmann *et al*., 2005). *P. syringae* pv. *tomato* even contains four functional *rsmA* homologues (Rife *et al*., 2005). (iii) Some bacteria, e.g. *P. fluorescens* CHA0, appear to have a safeguard function that puts a ceiling on the induced expression of Gac/Rsm-controlled

Table 2. Mutants affected in *rsmA*/*csrA* in bacteria.

Species	RsmA/CsrA homologues	Major phenotypic effects of *rsmA/csrA* mutation	References
Bacillus subtilis	*csrA*	Flagella, motility	Yakhnin *et al.* (2007)
Erwinia carotovora ssp. *carotovora*	*rsmA*	Extracellular pectinases, protease, cellulase, virulence	Chatterjee *et al.* (1995); Cui *et al.* (2001)
Escherichia coli	*csrA*	Central carbon metabolism, Adherence, motility	Romeo *et al.* (1993); Wang *et al.* (2005)
Helicobacter pylori	*csrA*	Virulence, motility	Barnard *et al.* (2004)
Legionella pneumophila	*csrA*	Cytotoxicity, virulence, motility	Molofsky and Swanson (2003)
Proteus mirabilis	*rsmA*	Hemolysin, protease, motility	Liaw *et al.* (2003)
Pseudomonas aeruginosa	*rsmA*	HCN, pyocyanin, elastase, lipase, adherence, motility	Pessi *et al.* (2001); Heurlier *et al.* (2004)
Pseudomonas fluorescens	*rsmA*, *rsmE*	DAPG, HCN, protease, adherence	Reimmann *et al.* (2005)
Salmonella enterica ssp. Typhimurium	*csrA*	1,2-propanediol, TTSS, motility	Altier *et al.* (2000); Lawhon *et al.* (2003)
Serratia marcescens	*rsmA*	Motility	Ang *et al.* (2001)
Vibrio cholerae	*csrA*	HapR-dependent factors	Lenz *et al.* (2005)

Abbreviations are the same as in Table 1.

traits, potentially to avoid intoxication by excessive concentrations of extracellular metabolites (Lapouge *et al*., 2007). In practice, it is often convenient to assess the function of the *rsmA/csrA* genes either by overexpressing them (which will mimic GacA-deficiency) or by expressing them from the LacI-controlled *tac* promoter (Molofsky and Swanson, 2003; Lapouge *et al*., 2007).

Genomic studies have revealed *rsmA/csrA* homologues outside the γ-proteobacteria, e.g. in δ-proteobacteria (*Desulfovibrio*, *Geobacter*), ε-proteobacteria (*Helicobacter*, *Campylobacter*), spirochetes (*Borrelia*, *Treponema*), low GC Gram-positive bacteria (*Bacillus*, *Clostridium*) and *Thermotoga* (Rife *et al*., 2005; Kulkarni *et al*., 2006). Mutants defective in *csrA* have been reported in *Helicobacter pylori* and in *Bacillus subtilis* where they are characterized by attenuation of virulence and derepressed synthesis of flagellar protein, respectively (Barnard *et al*., 2004; Yakhnin *et al*., 2007). An open question is how the activity of RsmA/CsrA is regulated outside the γ-proteobacteria. Are sRNAs involved and, if so, how are they regulated?

Several families of GacA-controlled sRNAs

The size of GacA-regulated sRNAs varies between about 100 and 479 nt, the largest known being RsmB of *E. carotovora*. They all share multiple unpaired GGA motifs, which are mostly located in loops or between stems of stem-loop structures (Babitzke and Romeo, 2007). These motifs are most often embedded in an ANGGA (55%) or AGGA (45%) context in pseudomonads, whereas in *E. coli* AGGA (67%) is more frequent than ANGGA (33%). According to sequence comparison, the sRNAs belong to several families typified by CsrB, CsrC (of enteric bacteria), RsmB (of *E. carotovora*) and RsmX, RsmY and RsmZ (of pseudomonads and related bacteria). It is not clear whether these families have common ancestors. Until this question is settled, we prefer to consider them as functional homologues rather than as homologues. The sRNAs feedback inhibit the transcription of their own genes by interfering with the function of the GacS/GacA system (Fig. 1), e.g. in *E. coli* (Suzuki *et al*., 2002), *P. aeruginosa* (Heurlier *et al*., 2004; Kay *et al*., 2006) and *P. fluorescens* (Heeb *et al*. 2002; Valverde *et al*., 2003; Kay *et al*., 2005). The simplest assumption – the sRNAs allosterically inhibit GacA phosphorylation or GacA binding to the putative GacA box – lacks experimental support. From mutant studies it appears that the RsmA/CsrA proteins act as positive control elements on the sRNA promoters in the bacteria mentioned. The mechanism involved is obscure. Possibly, the RsmA/CsrA proteins might translationally repress unknown transcriptional repressors of the sRNA genes. In *E. carotovora*, where such a feedback regulation does not operate (Chatterjee *et al*., 2002), negative control of *rsmB* expression is exerted by three transcriptional repressors, RsmC, KdgR and HexA (Mukherjee *et al*., 2000).

Knocking out all GacA-controlled sRNA genes in a bacterium results in phenotypes that are similar to those of a *gacA* mutant. This has been observed for *rsmB* and *gacA* mutants in *E. carotovora* (Cui *et al*., 2001), *csrB csrC* and *uvrY* mutants in *E. coli* (Weilbacher *et al*., 2003), *csrB csrC* and *sirA* mutants in *S. enterica* (Fortune *et al*., 2006), *rsmY rsmZ* and *gacA* mutants in *P. aeruginosa* (Kay *et al*., 2006) and *rsmX rsmY rsmZ* and *gacA* mutants in *P. fluorescens* (Kay *et al*., 2005). These observations suggest that the GacS/GacA system and its homologues mainly drive the expression of sRNA genes. However, the possibility that the GacS/GacA system directly regulates other types of genes cannot be excluded.

Signalling and cross-talk

Pseudomonads and Vibrios growing to high population densities excrete signal molecules that activate the GacS/GacA system; both intraspecies and interspecies signalling have been observed (Dubuis and Haas, 2007; Dubuis *et al*., 2007). The signals appear to be unrelated to well-known quorum sensing signals such *N*-acyl-homoserine lactones or autoinducer 2, and their chemical structures remain to be elucidated. The signals might interact with the GacS sensor. Circumstantial evidence for this hypothesis comes from a signal-blind *gacS* mutant of *P. fluorescens* in which the Gac/Rsm pathway is constitutively switched on (Zuber *et al*., 2003). Very little signal activity is present in culture supernatants of *P. fluorescens* and *P. aeruginosa gacA* mutants (Kay *et al*., 2005; Dubuis and Haas, 2007). This suggests that the signals act as autoinducers of the Gac/Rsm system, via a positive feedback loop (Fig. 1). Currently, no mutants are available that are affected specifically in structural genes for signal biosynthesis.

Depending on the bacterial species, the activity of the GacS/GacA system can be modulated by accessory regulators. In *P. aeruginosa*, two sensor kinases, RetS and LadS, have a negative and a positive influence, respectively, on GacA-dependent expression of *rsmZ* (Goodman *et al*., 2004; Laskowski and Kazmierczak, 2006; Ventre *et al*., 2006). The simplest interpretation is that RetS might prevent phosphorylation of GacA, whereas LadS might favour phosphorylation. Whether RetS and LadS respond to specific signals remains to be seen. Additionally, in *P. aeruginosa*, the sigma factor RpoN affects GacA expression negatively (Heurlier *et al*., 2003). In *E. coli*, YhdA, a protein predicted to be inserted in the cytoplasmic membrane, modulates UvrY (GacA)-

mediated expression of *csrB* and *csrC* (Jonas *et al.*, 2006).

Recognition of sRNAs and mRNAs by proteins of the RsmA/CsrA family

In striking contrast to the existence of several families of GacA-dependent sRNAs, there appears to be one conserved family of RsmA/CsrA proteins that bind these sRNAs. The small RsmA/CsrA proteins have a monomer size of about 7 kDa; in solution, they are present as dimers (Dubey *et al.*, 2003). The structures of *E. coli* CsrA and of RsmA from *P. aeruginosa* and *Yersinia enterocolitica* show that each monomer contains five β-strands and a C-terminal α-helix (Gutiérrez *et al.*, 2005; Rife *et al.*, 2005; Heeb *et al.*, 2006). Alanine-scanning substitution analysis of *E. coli* CsrA revealed two regions, i.e. strands β1 and β5, which are important for RNA binding *in vivo* (Mercante *et al.*, 2006). Two approaches have been useful to define the interactions of RsmA/CsrA proteins with sRNAs. First, RNA ligands with high affinity for *E. coli* CsrA were enriched *in vitro* by SELEX (systematic evolution of ligands by exponential enrichment). The RNAs obtained have a fully conserved ACANGGANGU consensus sequence in which the central GGA motif is part of a loop placed on variable short stems. Substitution mutations of conserved nucleotides greatly diminish affinity for CsrA (Dubey *et al.*, 2005). Second, extensive mutational analysis of the RsmY sRNA (Valverde *et al.*, 2004) and of the untranslated 5′ leader of *hcnA* mRNA (Lapouge *et al.*, 2007) revealed critical contacts between these RNAs and the RsmA protein and its homologue RsmE in *P. fluorescens.* The *hcnA* gene is the first of the *hcnABC* operon that encodes hydrogen cyanide (HCN) synthase. The *hcnABC* operon is positively regulated by GacA and is involved in the biosynthesis of the biocontrol factor HCN (Blumer *et al.*, 1999). RsmY, which is predicted to have six unpaired GGA motifs, forms four discrete complexes with RsmA in gel mobility assays. An RsmY mutant in which five of the GGA motifs have been altered by mutation retains the ability to form one complex *in vitro*, but is inactive as a regulator *in vivo* (Valverde *et al.*, 2004). The *hcnA* 5′ leader has five GGA motifs, all of which contribute to regulation by GacA, RsmA and RsmE *in vivo*; moreover, they allow RsmE to form at least three distinct complexes with the *hcnA* 5′ leader RNA *in vitro* (Lapouge *et al.*, 2007). The most distal GGA motif overlaps the SD sequence and occurs in a sequence (UCACGGAUGA) that matches the SELEX-derived consensus (underlined) except for the flanking nucleotides, which are inverted but still contribute to the ability of the sequence to form a short stem. Point mutations in the conserved nucleotides strongly diminish the regulation of *hcnA* expression by GacA, RsmA and RsmE; point mutations in the variable nucleotides have less marked effects (Lapouge *et al.*, 2007). Taken together, these observations can be interpreted as showing that RsmA/CsrA proteins bind to SD regions that resemble the SELEX-derived consensus; strong binding is favoured by additional upstream GGA motifs in the mRNA 5′ leader. Together, these interactions hinder ribosome access and translation initiation. GacA-controlled sRNAs prevent the translational roadblock by virtue of their multiple GGA motifs (Fig. 2).

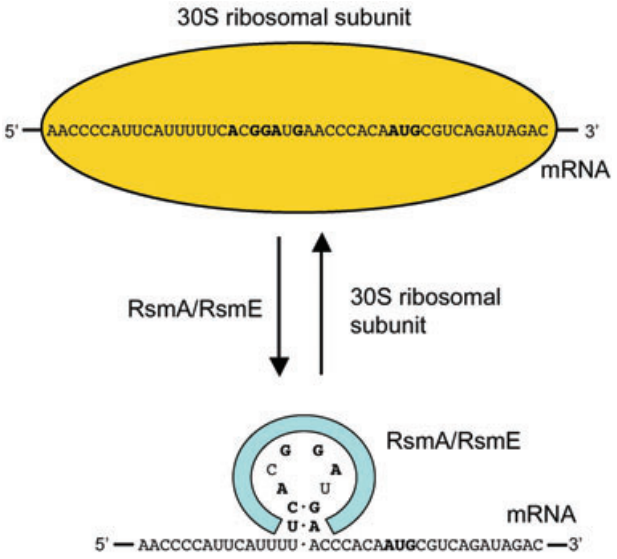

Fig. 2. Model for regulation of translation initiation, partly based on recent work on *hcnA* mRNA expression in *P. fluorescens* (Lapouge *et al.*, 2007; Schubert *et al.*, 2007). The *hcnA* 5′ leader mRNA adopts either of two conformations. Translation initiation is favoured when the SD sequence (bold face) is free to base pair with the 3′ end of 16S rRNA in the 30S ribosomal subunit. Binding of the RsmA or RsmE protein to an extended GGA motif (bold face) in the ribosome binding site as well as to GGA motifs further upstream (not shown) results in a conformational change that hinders the access of ribosomes and hence translation initiation. The AUG translation start codon is also indicated in bold face.

Structure of an RNA–RsmE complex

The solution structure of RsmE in complex with a 12-nucleotide fragment of the *hcnA* 5′ leader mRNA of *P. fluorescens* was determined recently by NMR spectroscopy. The RNA fragment used contains the most distal GGA motif, which is part of the SD sequence and participates in RsmE binding *in vitro* (Schubert *et al.*, 2007). The structure shows that the RsmE dimer binds two RNA molecules (Fig. 3A). The mode of binding is unusual as the main RNA binding surfaces of RsmE are not the planes of two β-sheets, as would be common in other RNA recognition motifs (Maris *et al.*, 2005), but rather RsmE makes contacts with the target RNA at the edges of two β-sheets, i.e. at the edge of the β1 strand in one monomer and at the edge of the β5 strand in the other

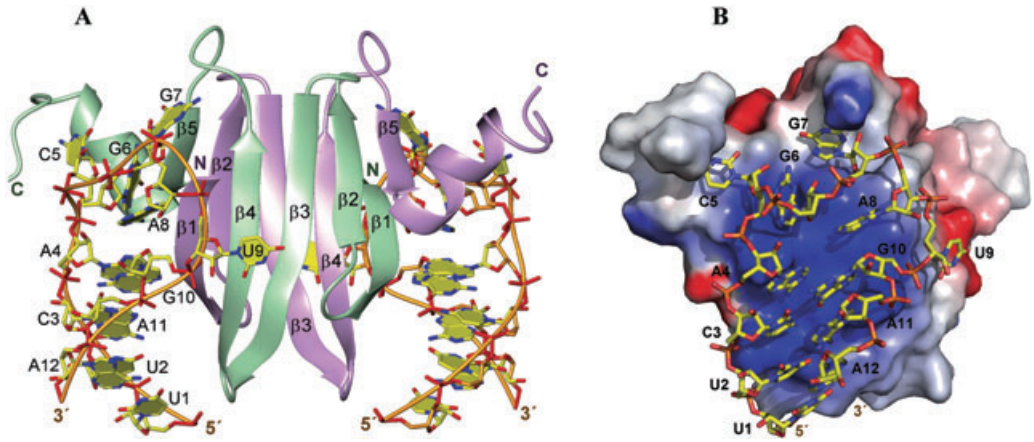

Fig. 3. NMR solution structure of an RsmE–*hcnA* RNA complex.
A. A representative structure shows the 2:2 complex between the RsmE protein and a 12-nucleotide *hcnA* mRNA fragment that contains the most distal of five GGA motifs and encompasses the SD sequence. Protein ribbons for each monomer are shown in green and violet. Heavy atoms of the two RNAs are shown in yellow (carbon), blue (nitrogen) and red (oxygen and phosphorus). The linking phosphates in the backbone are designated by an orange ribbon.
B. A surface representation of the RsmE dimer is shown in complex with one 12-nucleotide *hcnA* RNA molecule; the second RNA in the background is omitted for clarity. The protein is colour-coded for the electrostatic potential (blue, positive; red, negative); a representative structure is shown.

monomer (Fig. 3A). When bound to the positively charged RsmE interface, the 5′-UUCACGGAUGAA-3′ *hcnA* sequence adopts a stem-loop conformation with the 5′-UUC and GAA-3′ termini forming three base pairs (Fig. 3B). Of these, the U2–A11 and C3–G10 pairs are recognized by protein side-chains from both RsmE subunits (Fig. 3A). Among the six central nucleotides, the two adenines (A4 and A8) and the two guanines (G6 and G7) are coplanar and specifically interact with the β-strands β1 and β5, whereas the cytosine (C5) and the uracil (U9) are spread out and interact non-sequence specifically with the C-terminal α-helix and the β-sheets β3/β4, respectively (Fig. 3A). Quite strikingly, the sequence-specific recognition is almost solely mediated by the protein main-chains of β1 (for A4 and A8) and β5 (for G6 and G7), indicating that it is the fold of the protein rather than its side-chain arrangement that mediates the sequence-specific recognition of the RNA (Schubert *et al*., 2007). Details of the RsmE–RNA structure are given in Fig. S1. The structure rationalizes well the RNA consensus sequence found by SELEX (ACANGGANGU) and by footprinting experiments for the RsmA/CsrA family of proteins. Moreover, the structure explains how binding of RsmE (or RsmA) to the *hcnA* mRNA sequesters the ribosome binding site, as almost all the nucleotides of the SD sequence are in contact with the protein and therefore unavailable for base pairing with 16S rRNA.

RsmA/CsrA effects on mRNA stability and a caveat

An arrest of translation initiation usually results in enhanced mRNA decay in *E. coli* (Kaberdin and Bläsi, 2006). Thus, mRNAs may become more susceptible to degradation when they are repressed by RsmA/CsrA proteins, and more stable in the absence of RsmA/CsrA. Such effects have been observed in an *E. coli csrA* mutant, in which the half-lives of the *glgC* and *pgaA* target mRNAs are significantly longer than those in the wild type (Liu *et al*., 1995; Wang *et al*., 2005). Further work is needed in bacteria other than *E. coli* before a generalization of these findings can be offered. In this context, we note that several researchers have used transcriptional reporter fusions to monitor regulation of target gene expression by the Gac/Rsm cascade. Sometimes this approach works, sometimes it does not. A transcriptional reporter that is fused to a distal part of a target gene may fortuitously pick up any mRNA instability caused by arrested translation in the upstream target gene fragment. Therefore, reporter expression might be lowered by a *gacA* mutation, giving the erroneous impression that GacA control of target mRNA expression is transcriptional. However, if a transcriptional reporter is joined directly to the promoter of a target gene (as it should be), such a construct will not monitor direct regulation of the target mRNA by the Gac/Rsm cascade. Instead, it is advisable to use translational (*lacZ* or *gfp*) reporter fusions for testing regulatory effects of the Gac/Rsm cascade.

Regulation *versus* modulation

We have pointed out the important roles of the Gac/Rsm cascade in the regulation of virulence factors and cellular adherence properties. How do these considerations apply

to a non-pathogenic bacterium such as *E. coli* K12? In this strain, the most profound influence of the UvrY/CsrA system has been observed on the biosynthesis of the storage compound glycogen (Romeo *et al*., 1993; Baker *et al*., 2002). An intermediate influence is reported for the biosynthesis of poly β-1,6-*N*-acetyl-D-glucosamine, an extracellular polysaccharide and adhesin (Wang *et al*., 2005) and a weak influence for the CstA peptide transporter (Dubey *et al*., 2003) and the Hfq protein (Baker *et al*., 2007). In *P. fluorescens* CHA0, the effects of the GacA/RsmA+RsmE system on exoproduct formation are more pronounced, with typical GacA induction factors of ≥ 50 for the *hcnA* and *aprA* (alkaline protease) genes (Blumer *et al*., 1999; Kay *et al*., 2005). We have the impression that the UvrY/CsrA system of *E. coli* mainly serves to modulate gene expression, whereas the GacA/RsmA+RsmE system of *P. fluorescens* fulfils a more decisive regulatory function. The amplitude of regulation in both bacterial species correlates positively with the number and the sequence conservation of RsmA/CsrA binding sites on target mRNAs, as determined by footprint and toeprint analyses (Baker *et al*., 2002; 2007; Dubey *et al*., 2003; Wang *et al*., 2005; Lapouge *et al*., 2007). In *P. fluorescens*, the fact that one of the two RNA-binding proteins (RsmE) is itself regulated by the Gac/Rsm system also contributes to a highly effective regulation (Reimmann *et al*., 2005).

Outlook

While several important features of the Gac/Rsm cascade are now understood in molecular detail, further questions remain to be solved. For instance: How have the sRNAs evolved and to how many phylogenetically distinct families do they belong? How do the sRNAs control the promoters of their structural genes? What is the significance of the sRNA redundancy, and does this redundancy allow fine-tuning in response to environmental or metabolic stimuli? What determines the stability of mRNAs and sRNAs, in addition to the recently discovered CsrD decay factor of *E. coli*, which targets CsrB and CsrC for degradation (Suzuki *et al*., 2006)? What is the optimal spacing between RsmA/CsrA binding sites, allowing tight binding of these dimeric proteins, and what are the stoichiometries of typical complexes? What is the role of the Gac/Rsm system in carbon metabolism of bacteria other than *E. coli*? Last but not least, what are the chemical structures of the activating signals and with which sensors (GacS, LadS, RetS, etc.) do they interact?

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Supplementary material

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