Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites

Caroline Blumer, Stephan Heeb, Gabriella Pessi, and Dieter Haas*

Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne, Switzerland

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The conserved two-component regulatory system GacS/GacA determines the expression of extracellular products and virulence factors in a variety of Gram-negative bacteria. In the biocontrol strain CHA0 of *Pseudomonas fluorescens*, the response regulator GacA is essential for the synthesis of extracellular protease (AprA) and secondary metabolites including hydrogen cyanide. GacA was found to exert its control on the hydrogen cyanide biosynthetic genes (*hcnABC*) and on the *aprA* gene indirectly via a posttranscriptional mechanism. Expression of a translational *hcnA′-′lacZ* fusion was GacA-dependent whereas a transcriptional *hcnA-lacZ* fusion was not. A distinct recognition site overlapping with the ribosome binding site appears to be primordial for GacA-steered regulation. GacA-dependence could be conferred to the *Escherichia coli lacZ* mRNA by a 3-bp substitution in the ribosome binding site. The gene coding for the global translational repressor RsmA of *P. fluorescens* was cloned. RsmA overexpression mimicked partial loss of GacA function and involved the same recognition site, suggesting that RsmA is a downstream regulatory element of the GacA control cascade. Mutational inactivation of the chromosomal *rsmA* gene partially suppressed a *gacS* defect. Thus, a central, GacA-dependent switch from primary to secondary metabolism may operate at the level of translation.

translational control | two-component regulatory system | hydrogen cyanide | biocontrol | virulence

Microorganisms that live in association with plant or animal cells rely extensively on the production of extracellular proteins, secondary metabolites, and siderophores to establish themselves in their habitats. In many Gram-negative bacteria, a conserved two-component regulatory system consisting of the sensor kinase GacS and the cognate response regulator GacA has a decisive role in the control of extracellular products. The sensor GacS (originally designated LemA) was discovered in the plant pathogen *Pseudomonas syringae* pv. syringae as a factor being necessary for the manifestation of spot lesions on bean leaves (1, 2). The GacA response regulator was first described as a global activator of secondary metabolism and biocontrol activity in the plant-beneficial strain CHA0 of *Pseudomonas fluorescens* (3, 4). There is ample genetic evidence that the GacS and GacA proteins form a functional pair in a variety of bacterial species (5–7).

In animal pathogens such as *Pseudomonas aeruginosa* (8, 9), *Salmonella typhimurium* (10, 11), uropathogenic *Escherichia coli* (12), and *Vibrio cholerae* (13), the *gacS* and *gacA* homologs are important for virulence. Similarly, in plant pathogens, e.g., *P. syringae* (1, 2), *Pseudomonas tolaasii* (14), *Erwinia carotovora* subsp. carotovora (6, 15), and *Xanthomonas campestris* pv. campestris (16), mutations in the *gacS* and *gacA* homologs result in a nonpathogenic phenotype. Interestingly, a *gacA* mutant of *P. aeruginosa* has attenuated virulence in both animals and plants (8, 9). In root-colonizing biocontrol strains of *P. fluorescens*, the GacS/GacA system determines the expression of extracellular antifungal compounds and the protection of plant roots from fungal pathogens (3, 7, 17, 18).

On interaction with unknown signals, the GacS sensor is presumed to activate the GacA response regulator by phosphorylation. Activated GacA, by virtue of its typical C-terminal helix-turn-helix DNA binding motif (3), is thought to regulate the transcription of target genes. Although the direct GacA targets remain to be identified, it has been shown that the GacS/GacA system exerts a positive effect on cell density-dependent gene regulation mediated by *N*-acylhomoserine lactones, in at least three bacterial species, *P. aeruginosa* (19), *P. syringae* (2), and *Pseudomonas aureofaciens* (20). However, the GacS/GacA system also operates effectively in some Gram-negative bacteria that are not known to produce *N*-acylhomoserine lactones (21). For example, in *P. fluorescens* CHA0, there is no evidence for these signal molecules, and yet the GacS/GacA system strictly controls the expression of extracellular products (antibiotics, exoenzymes, and hydrogen cyanide) when cells are in the transition from exponential to stationary phase (ref. 3; data not shown).

In this study, we show that GacA control of the synthesis of two different exoproducts manifests itself strongly at a posttranscriptional level in *P. fluorescens.* We present evidence that GacA and RsmA (repressor of secondary metabolism), an RNA binding protein previously studied in enteric bacteria (22, 23), have opposite regulatory effects on the translational expression of target genes. From these results, a model is deduced, according to which the GacS/GacA system can determine virulence or biocontrol activities at the level of translation in Gram-negative bacteria.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. *P. fluorescens* CHA0 (wild-type), CHA89 (*gacA*::Km), and CHA21 (*anr*::ΩKm) have been previously described (3, 24). The construction of strains CHA510 (*gacS*::Tn*5*), CHA805 (chromosomal *aprA′-′lacZ* fusion) and CHA806 (*gacS*, *aprA′-′lacZ*) will be reported elsewhere. Strain CHA807 was obtained as described below. Strains CHA207 (chromosomal *hcnA′-′lacZ* fusion), CHA89.207 (*gacA*, *hcnA′-′lacZ*), CHA213 (chromosomal *hcn-lacZ* fusion), and CHA89.213 (*gacA*, *hcn-lacZ*) were constructed by transferring the translational and transcriptional fusions of pME3219 and pME6521, respectively, to the chromosome of strains CHA0 and CHA89, using the suicide vector pME3088 as described (24, 25). Recombinant plasmids were constructed in the vectors pME6000 (26), pME6001 (a gentamicin-resistant pME6000 derivative), pME6010 (GenBank accession no. AF118810), and the cosmid pVK100 (27) and were

Abbreviation: RBS, ribosome binding site(s).

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF136151 (*rsmA*) and AF118810 (pME6010)].

*To whom reprint requests should be addressed: E-mail: Dieter.Haas@lbm.unil.ch.

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introduced into *P. fluorescens* by electroporation (28). *P. fluorescens* and *E. coli* strains were grown at 30°C and 37°C, respectively, in nutrient yeast broth or on nutrient agar plates (24). Tetracycline was used at 25 μg/ml (*E. coli*) or 125 μg/ml (*P. fluorescens*), and gentamicin at 10 μg/ml.

DNA Manipulation and Cloning Procedures. DNA cloning and plasmid preparations were performed according to standard methods (29). The promoter probe vector pME6522 carrying the promoterless *E. coli lacZ* gene (on a 3.3-kilobase *Pst*I-*Dra*I fragment) was derived from pME3533 (30) and the vector pME6010 (Fig. 1). Plasmid pME6521 was obtained by PCR-amplification of the *hcn* promoter region (260 bp) by using *Eco*RI-tagged primer 1 (24) and primer 12 (5′-CATGCCTGCAGCAATCTAGTCGGTTTGTCGG-3′), which anneals at the +1 transcription start site and creates an artificial *Pst*I site (underlined). This fragment was fused to *lacZ* of pME6522 (Fig. 1). Plasmid pME6060–3 (Fig. 1) was constructed by fusing, in frame, the *aprA* gene on a 3.0-kilobase genomic *Xho*I-*Sac*I fragment of *P. fluorescens* CHA0 (S.H., unpublished work) with a *′lacZ* fragment (31). Plasmids pME6530 and pME3843 (Fig. 1) were obtained as follows: Plasmid pME3219, which carries a translational *hcnA′-′lacZ* fusion of strain CHA0 (24), and pME3826, which carries an analogous fusion of *P. aeruginosa* (G.P., unpublished work), were used as templates to amplify the respective *hcnA′* fragments by the use of primers homologous to the first 20 nucleotides of each 5′ leader sequence (5′-GCTCGGTTCTGACAACAGC-3′ and 5′-GCCCGGCCCGACTCCTAGTGT-3′, respectively) and a primer annealing within the *lacZ* sequence (5′-TGCTGCAAGGCGATTAAGTGGG-3′). The *tac* promoter of pJF118 (32) was PCR-amplified on a 930-bp fragment by primer 15 (5′-CGAGACAGATCTTAATGGGC-3′), which anneals within the *lacI* sequence and contains an artificial *Bgl*II site (underlined), and primer 16 (5′-GCTCGGTACCCACACATTATACGAGCCGA-3′), which anneals at the +1 transcriptional start and creates a *Kpn*I site (underlined). The *tac* promoter fragment obtained was inserted into pUK21 (33). This construct was cleaved with *Kpn*I, was treated with T4 DNA polymerase to remove the 3′ overhang, and was linked to either of the blunt-end *hcnA′* fragments, which had been cut with *Pst*I at their 3′ ends. The *Bgl*II-*Pst*I fragments of the resulting constructs were reintroduced into pME3219 and pME3826, giving pME6530 and pME3843, respectively. This placed the authentic 5′ *hcnA′* regions under *tac* promoter control. The prototype plasmid pME6533 (Fig. 4), which served as a starting point for mutagenesis of the ribosome binding site (RBS), was identical with the translational *hcnA′-′lacZ* fusion plasmid pME3219, except for the *Kpn*I and *Sph*I restriction sites. These were introduced into pME3219 (Fig. 1) by PCR, using primers 1 (24) and 17 (5′-ATGAATGGGGTACCCGGCGTCCCG-3′), which anneals to the RBS region of *P. fluorescens hcnA* and contains two nucleotide changes resulting in a *Kpn*I site (underlined). This 340-bp PCR fragment was joined to a 70-mer, which was bordered by a 5′ *Kpn*I and a 3′ *Pst*I site and consisted of the *hcnA* sequence, except for two nucleotides creating a *Sph*I site (Fig. 4), and was inserted into pME3219 cleaved with *Eco*RI and *Pst*I. The derivatives of pME6533 were constructed by exchanging 32-mer *Kpn*I-*Sph*I linkers carrying different sequence modifications. The three-base substitution in the plasmid pME6544, which is otherwise identical with pME6521, was generated by the overlap extension method (34), using primer 19 (5′-TTCACGGATGAAACAGCTATGACCA-3′) to introduce the mutation (underlined). The *rsmA* gene of *P. fluorescens* was cloned as explained in Fig. 5. This gene was inactivated by insertion of the Ω-Km element (35) into the unique *Fsp*I site and marker exchanged (24) with the chromosomal *rsmA* gene of strain CHA806 (*gacS*, *aprA′-′lacZ*), resulting in strain CHA807.

Southern Hybridization and Nucleotide Sequence Analyses. Southern blotting of *P. fluorescens* genomic DNA was performed as described (19). The nucleotide sequence of the *rsmA* gene of *P. fluorescens* (GenBank accession no. AF136151) as well as the products of all PCR and linker constructs were determined and verified by using the Dye Terminator Kit (Perkin–Elmer,

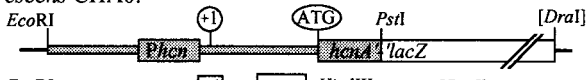
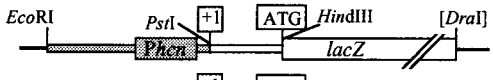
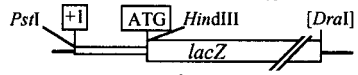
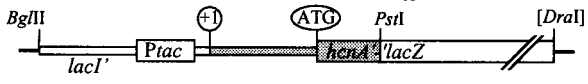
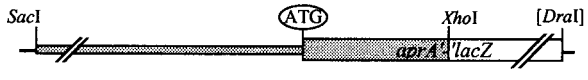
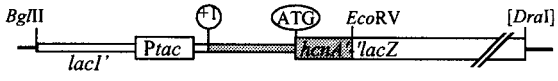
Plasmids		ß-Galactosidase activity*	
		CHA0 (wild type)	CHA89 (*gacA*)
hcnA of *P. fluorescens* CHA0:			
pME3219		25800 ± 3100	440 ± 120
pME6521		15200 ± 1700	12100 ± 2100
pME6522		210 ± 50	180 ± 20
pME6530		35400 ± 4200	1200 ± 130
aprA of *P. fluorescens* CHA0:			
pME6060-3		900 ± 70	7 ± 0.7
hcnA of *P. aeruginosa* PAO1:			
pME3843		5400 ± 70	1350 ± 130

Fig. 1. Posttranscriptional, *gacA*-dependent regulation of the *Pseudomonas hcnA* and *aprA* genes in *P. fluorescens*. All constructs were made in the vector pME6010. *, β-galactosidase expression (Miller units) of the *lacZ* fusions shown was tested in the *P. fluorescens* strains CHA0 and CHA89 when cells reached an OD_{600} of 2.0–2.5. Activities are mean values of triplicate experiments ± standard deviation.

Table 1. Regulation of a translational *hcnA′-′lacZ* fusion on pME3219 by the *gacS*/*gacA* and *anr* genes in *P. fluorescens*

Host*	Relevant genotype	β-galactosidase activity, (Miller units)†
CHA0	wild type	25,800 ± 3,100
CHA89	*gacA*::Km	440 ± 120
CHA510	*gacS*::Tn*5*	490 ± 100
CHA21	*anr*::Ω-*Km*	5 ± 0.5

*Each host strain carried pME3219. β-galactosidase activity (Miller units) was determined when cells were mildly oxygen-limited at an OD_{600} of 2.0–2.5. Under these conditions, the *hcnA* promoter is partially induced.
†Means of three or more independent experiments (± standard deviation).

Table 2. Expression of the chromosomal *hcn* genes of *P. fluorescens*

Strain	Genotype	β-galactosidase activity, Miller units*
CHA207	Translational *hcnA′-′lacZ* fusion	1,515 ± 140
CHA89.207	Translational *hcnA′-′lacZ* fusion, *gacA*::Km	35 ± 2
CHA213	Transcriptional *hcn-lacZ* fusion	2,065 ± 89
CHA89.213	Transcriptional *hcn-lacZ* fusion, *gacA*::Km	1,825 ± 52

*Conditions were the same as in Table 1; measurements were taken at an OD_{600} of 2.

402080) and the Applied Biosystems PRISM 373 sequencer. Nucleotide and deduced amino acid sequences were analyzed with the programs BLAST, GAP, BESTFIT, and PILEUP by using the Genetics Computer Group (Madison, WI) package.

β-Galactosidase Assay. For β-galactosidase measurements, *P. fluorescens* cells were grown with shaking in 50-ml flasks containing 20 ml of nutrient yeast broth at 30°C. β-Galactosidase specific activities were determined by the Miller method (29).

Results

Regulation of the *hcnABC* Operon in *P. fluorescens* CHA0. The expression of the hydrogen cyanide biosynthetic genes *hcnABC* requires the transcription factors GacA and ANR in *P. fluorescens* (3, 24). The anaerobic regulator ANR activates transcription of the *hcnABC* genes by binding to the −40 region of the *hcnA* promoter (24). The sites of GacA control have not been determined previously. A translational *hcnA′-′lacZ* fusion on plasmid pME3219 (Fig. 1) was used to quantify the regulatory effects of GacS/GacA and ANR in *P. fluorescens* CHA0. In both a *gacS* and a *gacA* mutant, *hcnA′-′lacZ* expression was reduced ≈50-fold, compared with the level in the wild-type CHA0. In an *anr* mutant, *hcnA′-′lacZ* expression was very low, at the detection limit (Table 1). To see whether GacS/GacA and ANR might be elements of a common regulatory cascade, we tested translational *gacA′-′lacZ* and *anr′-′lacZ* fusions in the wild type, in an *anr* mutant, and in a *gacA* mutant. The expression of both fusions was similar in the three strains (data not shown), suggesting that ANR and GacA are not in a common cascade and show little, if any, autoregulation. We (C.B. and D.H., unpublished work) have obtained evidence that transcriptional regulation of *hcnABC* by oxygen and iron availability depends on ANR and the −40 promoter region and that GacA (or a regulator controlled by GacA) activates cyanogenesis independently of ANR.

GacS/GacA Control of the *hcn* Biosynthetic Genes Manifests Itself at a Posttranscriptional Level. The promoter probe vector pME6522 was used to construct a transcriptional *hcn-lacZ* fusion, in which the *hcn* promoter was linked, at the transcription start site, to the *E. coli lacZ* gene with its own 5′ leader sequence and ribosome binding site (RBS) (Fig. 1). Expression of the transcriptional *hcn-lacZ* fusion (on pME6521) was similar in the wild-type CHA0 and in the *gacA* mutant CHA89 whereas the translational *hcnA′-′lacZ* fusion (on pME3219) strongly depended on GacA function (Fig. 1). β-galactosidase expression of the *lacZ* reporter pME6522 was negligibly low and not influenced by GacA (Fig. 1). To rule out copy-number artifacts (the vector pME6010 has about six copies in *P. fluorescens*), we transferred the translational and transcriptional *lacZ* fusions of pME3219 and pME6521, respectively, to the chromosome of strains CHA0 and CHA89 (*gacA*). The same pattern of GacA-dependent *hcn* regulation was observed (Table 2). Furthermore, the plasmid-borne translational *hcnA′-′lacZ* fusion remained essentially under GacA control when the anaerobically inducible *hcn* promoter was replaced by the constitutively expressed *tac* promoter (on pME6530; Fig. 1). In this construct, the 5′ untranslated *hcnA* leader sequence was the same as in the native *hcnABC* operon. Irrespective of the promoter used, the *hcnA′-′lacZ* translational fusions on both pME3219 and pME6530 showed similar growth phase-dependent expression, with maximal levels reached at the end of exponential growth phase (Fig. 2). Taken together, these results suggest that the GacS/GacA two-component system regulates *hcn* expression at a posttranscriptional level, in response to the growth phase and/or cell density.

Evidence for a General GacS/GacA Control Mechanism. In *P. fluorescens* CHA0, the expression of the major extracellular protease is under strict GacA control (4). The structural gene, *aprA*, for this metalloprotease was cloned (S.H., unpublished data). A translational *aprA′-′lacZ* fusion (on pME6060-3) was tightly regulated by GacA in *P. fluorescens*, with an induction factor of >100 (Fig. 1). We also included in this analysis the *P. aeruginosa hcnABC* cluster, which is 77% identical to the *hcnABC* operon of *P. fluorescens* (G.P., unpublished results). The *hcnA* promoter of *P. aeruginosa* is expressed under the control of *N*-butyryl-homoserine lactone, whose level is regulated by GacA in the native host (19). In strain CHA0, the *hcnA* promoter of *P. aeruginosa* was poorly expressed (data not shown). Therefore, we replaced this promoter by the *tac* promoter. Unexpectedly, the *P. aeruginosa hcnA′-′lacZ* fusion (expressed from p*tac*, on

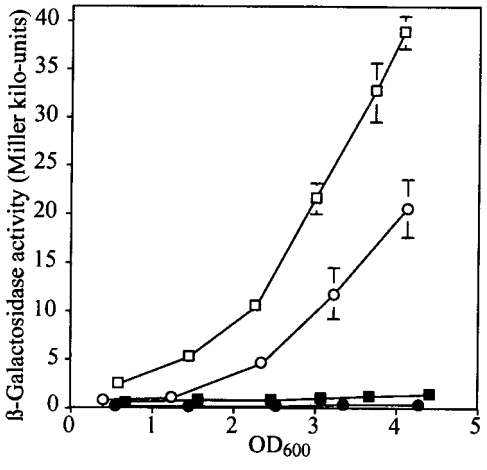


Fig. 2. Cell density-dependent expression of a translational *hcnA′-′lacZ* fusion from the *hcn* promoter on pME3219 (circles) or from the *tac* promoter on pME6530 (squares) in CHA0 (open symbols) and CHA89 (closed symbols). Bacterial growth reached a plateau at OD_{600} 4–5.

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Fig. 3. Alignment of the regions containing the RBS of the *hcnA* genes of *P. fluorescens* CHA0 and *P. aeruginosa* PAO1, the *aprA* gene of *P. fluorescens* CHA0, and the *E. coli lacZ* gene. In each case, translation is initiated at the ATG codon shown at the 3′ end.

pME3843) was still regulated, albeit weakly, by GacA in *P. fluorescens* CHA0 (Fig. 1). A sequence alignment of the 5′ untranslated leader regions of the *hcnA* genes of *P. fluorescens* and *P. aeruginosa* and of the *aprA* gene of *P. fluorescens* revealed some similarity in the vicinity of the RBS (Fig. 3). We therefore postulated that this region could be a common target for GacA control. The *E. coli lacZ* gene, whose expression was not modulated by GacA in *P. fluorescens* (Fig. 1), was included in the alignment (Fig. 3), to narrow down the most important zone of similarity among the three *Pseudomonas* sequences.

Mutational Analysis of the RBS Region of the *P. fluorescens hcnA* Gene. Guided by the sequence comparison (Fig. 3), we introduced specific mutations into a 33-bp segment carrying the RBS of the *P. fluorescens hcnA* gene, using the translational *hcnA′-′lacZ* fusion on pME3219 (Fig. 1) as a reporter. To facilitate mutagenesis, we first created artificial restriction sites for *Kpn*I and *Sph*I bracketing the target region. The resulting construct, pME6533, remained under GacA control (Fig. 4). However, when the *hcn* target region was substituted by the equivalent *lac* segment (on pME6536), no GacA-dependent regulation was observed (Fig. 4). (The induction factor of 1.5 measured for pME6536 was considered to be insignificant.) Insertion of three strategically placed nucleotides (ACA), which are characteristic of the *lacZ* leader, sufficed to abolish GacA control of the *hcnA′-′lacZ* fusion in pME6537 (Fig. 4). The ACA insertion altered the spacing between the presumed RBS (GGA) and several conserved pyrimidines located upstream (Fig. 4). Next, a critical substitution was introduced into the *lacZ* leader carried by pME6538: the *lac*-specific nucleotides ACAG were exchanged for the *hcn*-specific tetrad GGAT. This substitution installed GacA control (Fig. 4), presumably because the correct spacing between the conserved pyrimidines and the RBS (GGA) was restored.

Several further constructs were made to confirm these observations. Addition of a single base (A) to the *hcn* sequence changed the critical spacing (on pME6539) and abolished GacA control (Fig. 4). In the construct pME6540, two bases were exchanged (CA→AC) to disrupt a potential secondary mRNA structure. However, this change had no influence on GacA control (Fig. 4). Another substitution (C→A), which created an RBS typical of *aprA* (Fig. 3), did not affect GacA control either (on pME6541; Fig. 4). By contrast, a substitution (CA→AT) that modified the central conserved CA.GGA motif (Fig. 3) abolished GacA control (on pME6542; Fig. 4). Two further changes of the central motif, i.e., the deletions in pME6549 and pME6550, similarly interfered with GacA control. The conserved pyrimidines upstream of the central motif appear to make some contribution to GacA control, as evidenced by pME6543 (Fig. 4). Although the translational *′lacZ* fusions of the constructs shown in Fig. 4 were expressed at widely different levels because of variable translation efficiencies, induction factors of >30 or <2 clearly indicated the presence or absence of GacA control, respectively.

Is the segment lying between the *Kpn*I and *Sph*I sites sufficient to promote GacA control? To answer this question, the GacA-independent *hcn-lacZ* transcriptional fusion on pME6521 (Fig. 1) was mutated at the RBS by exchanging the *lac*-specific nucleotides ACAG by the *hcn* counterpart GGAT (on pME6544). This substitution of three nucleotides in the *lacZ* 5′ leader sequence was enough to establish regulation by GacA (Fig. 4). A preliminary and tentative consensus sequence based on the alignments of Figs. 3 and 4 is postulated for GacA control (Fig. 4).

Plasmid	5' leader sequence with RBS*	ß-Galactosidase activity (Miller Units)†		
		CHA0 (wild type)	CHA89 (*gacA*)	Induction factor
pME3219	GGCAACCCCATTCATTTTTCACGGATGAACCCACAATGCGT	25800 ± 3100	440 ± 120	59
	*Kpn*I *Sph*I			
pME6533	GGGTACCCCATTCATTTTTCACGGATGAACCCAGCATGCGT	21100 ± 5600	640 ± 40	33
pME6536	GGGTACCGGATAACAATTTCACACAGGAAACAGCATGCGT	63500 ± 8700	42900 ± 8800	1.5
pME6537	GGGTACCCCATTCATTTTTCACACAGGATGAACCCAGCATGCGT	52400 ± 5700	38100 ± 2600	1.4
pME6538	GGGTACCGGATAACAATTTCACGGATGAAACAGCATGCGT	3300 ± 740	73 ± 38	45
pME6539	GGGTACCCCATTCATTTTTCACAGGATGAACCCAGCATGCGT	33600 ± 5500	30300 ± 5900	1.1
pME6540	GGGTACCCCATTACTTTTTCACGGATGAACCCAGCATGCGT	15600 ± 1500	390 ± 170	40
pME6541	GGGTACCCCATTCATTTTTCAAGGATGAACCCAGCATGCGT	19100 ± 3900	540 ± 2	35
pME6542	GGGTACCCCATTCATTTTTATCGGATGAACCCAGCATGCGT	41400 ± 3200	33200 ± 3100	1.2
pME6543	GGGTACCCCATTCAGACAGCACGGATGAACCCAGCATGCGT	4880 ± 580	1700 ± 100	2.9
pME6549	GGGTACCCCATTCATTTTTCAAG··T·AACCCAGCATGCGT	1280 ± 10	1170 ± 135	1.1
pME6550	GGGTACCCCATTCATTTTTCAAGG·T·AACCCA·CATGCGT	45900 ± 7800	39100 ± 1300	1.2
pME6521	TGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACC	15200 ± 1700	12100 ± 2100	1.3
pME6544	TGTGAGCGGATAACAATTTCACGGATGAAACAGCTATGACC	5700 ± 600	125 ± 35	46
Tentative consensus	$(Py)_nCA.GGA$			

Fig. 4. Posttranscriptional, GacA-dependent regulation of a translational *hcnA′-′lacZ* fusion (derived from the *P. fluorescens hcn* operon): influence of mutations in the 5′ leader sequence. *, Nucleotides of importance are designated as follows: doubly underlined, RBS; underlined, artificial restriction sites; shaded boxes, *E. coli lacZ* sequence; open box, ATG initiation codon of *hcnA*; encircled, base substitutions; ●, deletion. †, β-galactosidase expression (Miller units) was determined in strains CHA0 and CHA89, when cells reached an OD_{600} of 2.0–2.5. Activities are mean values from triplicate experiments ± standard deviation.

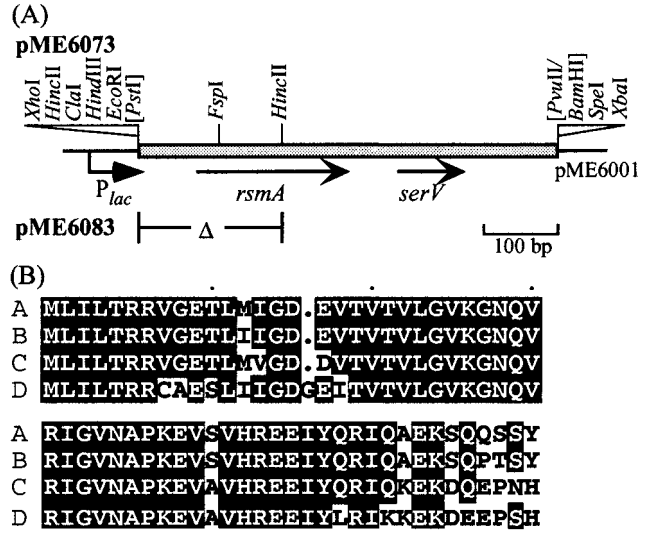

Fig. 5. (*A*) Cloning of the *rsmA* gene of *P. fluorescens* CHA0. The *P. aeruginosa rsmA* gene was isolated by PCR and was used as a probe to identify the homolog in *P. fluorescens* by Southern hybridization. A 1.25-kilobase genomic *Pst*I fragment containing *rsmA* of *P. fluorescens* was cloned. In pME6073, *rsmA* on a 0.55-kilobase *Pst*I/*Pvu*II fragment is expressed from the *lac* promoter of the vector pME6001; in pME6083, *rsmA* is deleted. The *serV* gene, which encodes a serine tRNA, appears to belong to a separate transcription unit. (*B*) Alignment of RsmA and CsrA amino acid sequences. A, CsrA of *E. coli* (GenBank accession no. L07596); B, RsmA of *E. carotovora* subsp. *carotovora* 71 (L40173); C, RsmA of *P. aeruginosa* (AF061757); D, RsmA of *P. fluorescens*. Amino acid residues that are conserved in at least three of the four proteins are shown in black boxes.

Cloning of the Global Translational Repressor RsmA of *P. fluorescens*. In *E. carotovora*, RsmA is a global negative regulator of genes encoding extracellular products and virulence factors (36). The small RNA-binding protein RsmA, like its homolog CsrA of *E. coli*, is assumed to recognize a region at or close to the RBS of susceptible mRNA molecules (22, 23). Strains overexpressing *rsmA* or being devoid of *expA* (=*gacA*) have similar pleiotropic phenotypes in *Erwinia* spp. (6, 36). To see whether the same holds true for *P. fluorescens*, we cloned and sequenced the *rsmA* gene of strain CHA0 (Fig. 5). The RsmA proteins of *P. fluorescens, P. aeruginosa*, and *E. carotovora* and CsrA of *E. coli* were found to be highly conserved (Fig. 5), suggesting a common mode of action.

GacA and Overexpressed RsmA Act on the Same Specific RBS. The *rsmA* gene of *P. fluorescens* was placed under the control of the *lac* promoter in the multicopy plasmid pME6073 (Fig. 5). This plasmid repressed the expression of chromosomal *hcnA′-′lacZ* and *aprA′-′lacZ* fusions in *P. fluorescens* 7-fold and 20-fold, respectively. A pME6073 derivative (pME6083) in which most of the *rsmA* gene had been deleted (Fig. 5) did not repress the *hcnA′-′lacZ* and *aprA′-′lacZ* fusions (data not shown). To test whether the overexpressed *rsmA* gene could mimick a *gacA* defect, we chose 10 representative *hcnA′-′lacZ* fusion plasmids containing different RBS modifications (Fig. 4) and assayed β-galactosidase activities with or without extra *rsmA* copies (pME6073 or vector pME6001, respectively; Table 3). Each construct that was controlled by GacA was repressible at least 3-fold by overexpressed RsmA; conversely, each GacA-independent construct had an RsmA repression factor of <1.5 (Table 3). This provides strong evidence that regulation by GacA and RsmA acts on essentially the same specific RBS and that both regulators are elements of the same regulatory cascade.

Given that RsmA is a negative control element, it can be predicted that an *rsmA* mutation should suppress a defect in the *gacS* or *gacA* gene. Therefore, the chromosomal *rsmA* gene of the *gacS* mutant CHA806, which contains a chromosomal *aprA′-′lacZ* fusion, was inactivated by the insertion of a resistance cassette. The resulting mutant CHA807 grown in nutrient yeast broth to an OD_{600} of 2–2.5 expressed β-galactosidase activity (180 ± 20 Miller units) whereas no activity (<5 Miller units) was detected in the parental strain CHA806. In strain CHA805 (*gacS*$^+$) the chromosomal *aprA′-′lacZ* fusion gave a 3-fold higher β-galactosidase expression (550 ± 90 Miller units). Thus, an *rsmA* mutation can compensate, at least in part, for the effect of a *gacS* mutation on *aprA* expression in *P. fluorescens*, supporting our model according to which the GacS/GacA system can antagonize the repression by RsmA (see *Discussion*). However, additional unidentified regulatory elements under GacS/GacA control might be involved in the regulation of the *aprA* and *hcn* genes.

Table 3. Strict correlation between GacA- and RsmA-dependent regulation in *P. fluorescens*

Strain/plasmid*	β-galactosidase activity† + pME6001, vector control	β-galactosidase activity† + pME6073, *rsmA*$^{++}$	RsmA repression factor	GacA induction factor‡
CHA0/pME3219	12,400 ± 1,000	2,600 ± 500	4.8	59
CHA0/pME6530	27,200 ± 1,500	6,400 ± 450	4.0	30
CHA0/pME6533	14,400 ± 700	2,600 ± 80	5.5	33
CHA0/pME6536	46,800 ± 1,500	36,000 ± 2,300	1.3	1.5
CHA0/pME6537	39,300 ± 1,900	29,700 ± 3,000	1.3	1.4
CHA0/pME6538	4,300 ± 200	830 ± 40	5.2	45
CHA0/pME6539	29,400 ± 900	20,000 ± 1,200	1.5	1.1
CHA0/pME6541	12,400 ± 500	2,100 ± 140	6.0	35
CHA0/pME6521	7,800 ± 510	7,600 ± 290	1.0	1.3
CHA0/pME6544	3,400 ± 70	1,000 ± 30	3.4	46

*The *hcnA′-′lacZ* fusion constructs (Figs. 1 and 4) were tested in the presence (pME6073) or absence (pME6001) of overexpressed RsmA. Cells were grown in 20 ml of nutrient yeast broth with gentamicin (10 μg/ml) to an OD_{600} of 2.0–2.5.

†β-galactosidase activities (Miller units) were determined in triplicate; mean values ± standard deviation are given.

‡Values are from Fig. 4.

Discussion

The most important result of this study is that the widely conserved GacS/GacA two-component system determines the production of an extracellular protein and a secondary metabolite (hydrogen cyanide) at a posttranscriptional level and that small mutational changes in the RBS of target transcripts are sufficient to install or abolish GacA-mediated control. For instance, substitution of three nucleotides in the *E. coli lacZ* leader mRNA near the RBS sufficed to place the expression of the *lacZ* gene under GacA control in *P. fluorescens* (Fig. 4). After this change, *lacZ* behaved like the *aprA* and *hcn* genes. Conversely, substitution or insertion of single strategically located nucleotides in the *hcn* leader mRNA of *P. fluorescens* could completely eliminate GacA control (Fig. 4). Thus, a few nucleotides can operate a critical switch from primary to secondary metabolism, and it is likely that this situation applies not only to *P. fluorescens* but to a wide range of Gram-negative bacteria. In pathogenic species, the same switch may be vital for the regulation of virulence factors.

Because the region responsible for GacA control overlaps with the RBS of *hcnA* and *aprA*, this region could comprise a binding site for a regulatory protein: e.g., a translational repressor such as RsmA of *E. carotovora* and CsrA of *E. coli* (22, 23). By cloning and overexpressing the *rsmA* gene of *P. fluorescens*, we have

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obtained evidence that regulation by GacA and RsmA depend on essentially the same specific RBS regions, which could constitute RsmA binding sites. This leads to a model that, in its simplest form, predicts that GacA could up-regulate a regulatory macromolecule alleviating RsmA-mediated translational repression. It is possible that relief of repression could be exerted by an RsmB-like RNA molecule. It is known that the action of the translational repressors RsmA in *E. carotovora* and CsrA in *E. coli* is antagonized by the regulatory, noncoding RNAs RsmB and CsrB, respectively. These RNAs bind and sequester the repressor proteins (22, 23). An *rsmB*-negative mutant of *E. carotovora* has a pleiotropic phenotype resembling that of an *expA* (*gacA*)-negative strain (6, 22). In *P. fluorescens*, binding of RsmA to target mRNAs remains to be demonstrated. Whereas the RsmA/CsrA proteins are very similar in different bacteria (Fig. 5; ref. 23), the RsmB/CsrB RNAs appear to be much less conserved. For instance, our homology searches have failed to reveal an *rsmB* gene in *P. aeruginosa*.

Several extensions of our basic model can be envisaged. First, it is possible that RsmA recognition sites could also be located outside the RBS, in coding or noncoding sequences of target genes. Second, in enteric bacteria, RsmA and CsrA can enhance the degradation of target mRNAs (23), and RsmA of *P. fluorescens* might have the same effect. Third, the GacS/GacA system might directly affect *rsmA* expression.

Regulatory elements intervening between GacA and target genes can complicate the regulatory cascade. For instance, in *P. aeruginosa*, the *rhlI* gene encoding *N*-butyryl-homoserine lactone synthase appears to be posttranscriptionally regulated by GacA and RsmA (G.P., unpublished data). In turn, *N*-butyryl-homoserine lactone activates the transcription of *hcnABC* and other target genes, presumably by binding to the transcriptional regulator RhlR (19, 37). As an overall consequence, *hcn* expression would seem to be GacA-regulated at the transcriptional level. Such complications might explain some results obtained in *P. aureofaciens* (20) and *E. carotovora* (6) in which GacA appears to influence the transcription of target genes. However, our present results do not rule out direct transcriptional control of certain target genes by GacA. In *P. fluorescens* CHA0, this possibility has been excluded in the case of the *hcnA* promoter whose expression is totally independent of GacA (C.B. and D.H., unpublished work).

As a rule in Gram-negative bacteria, typical genes of primary metabolism are expressed during different growth phases but especially in the course of exponential growth. By contrast, typical genes of secondary metabolism are predominantly expressed during the idiophase: i.e., the transition from the exponential to stationary phase (Fig. 2; ref. 6). By manipulating the RBS sequences of selected genes, as demonstrated in Fig. 4, it has become possible to target translational gene expression specifically to the idiophase.

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