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Posttranscriptional Repression of GacS/GacA-Controlled Genes by the RNA-Binding Protein RsmE Acting Together with RsmA in the Biocontrol Strain *Pseudomonas fluorescens* CHA0

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In the plant-beneficial soil bacterium *Pseudomonas fluorescens* CHA0, the production of biocontrol factors (antifungal secondary metabolites and exoenzymes) is controlled at a posttranscriptional level by the GacS/GacA signal transduction pathway involving RNA-binding protein RsmA as a key regulatory element. This protein is assumed to bind to the ribosome-binding site of target mRNAs and to block their translation. RsmA-mediated repression is relieved at the end of exponential growth by two GacS/GacA-controlled regulatory RNAs RsmY and RsmZ, which bind and sequester the RsmA protein. A gene (*rsmE*) encoding a 64-amino-acid RsmA homolog was identified and characterized in strain CHA0. Overexpression of *rsmE* strongly reduced the expression of target genes (*hcnA*, for a hydrogen cyanide synthase subunit; *aprA*, for the main exoprotease; and *phlA*, for a component of 2,4-diacetylphloroglucinol biosynthesis). Single null mutations in either *rsmA* or *rsmE* resulted in a slight increase in the expression of *hcnA*, *aprA*, and *phlA*. By contrast, an *rsmA rsmE* double mutation led to strongly increased and advanced expression of these target genes and completely suppressed a *gacS* mutation. Both the RsmE and RsmA levels increased with increasing cell population densities in strain CHA0; however, the amount of RsmA showed less variability during growth. Expression of *rsmE* was controlled positively by GacA and negatively by RsmA and RsmE. Mobility shift assays demonstrated specific binding of RsmE to RsmY and RsmZ RNAs. The transcription and stability of both regulatory RNAs were strongly reduced in the *rsmA rsmE* double mutant. In conclusion, RsmA and RsmE together account for maximal repression in the GacS/GacA cascade of strain CHA0.

Pseudomonas fluorescens CHA0 is a root-colonizing biocontrol strain which suppresses soil-borne plant diseases caused by phytopathogenic fungi (19, 22). Disease suppression is mainly due to the antifungal metabolites 2,4-diacetylphloroglucinol, pyoluteorin, and hydrogen cyanide (HCN) which are produced by the bacterium at the end of exponential growth (23, 24, 32, 43). Biosynthesis of these compounds and of the exoenzymes phospholipase C and the exoprotease AprA strictly depends on the GacS/GacA two-component system, which operates a switch from primary to secondary metabolism in various gram-negative bacteria and which can also be involved in pathogenicity to plants and animals, in ecological fitness, and in stress tolerance (18).

GacS/GacA control of secondary metabolites and exoenzymes was shown to occur in strain CHA0 at the posttranscriptional level involving the RNA-binding protein RsmA as a key regulatory element (7). RsmA is presumed to interact with specific ribosome-binding sites present in target genes and thereby prevent translation. Such translational repression can be alleviated by the action of the small regulatory RNAs RsmY and RsmZ, whose expression is controlled by the GacS/GacA system in response to signal molecules produced by CHA0 at the end of exponential growth (19, 47). RsmY and RsmZ bind multiple copies of the RsmA protein (47, 48) and, by a titration effect, may thus render the ribosome-binding site of target genes accessible for the translation machinery.

RsmA-like proteins are highly conserved in various eubacteria (2, 4, 6, 7, 12, 14, 25, 26, 36, 38, 40, 53, 54). In *Escherichia coli,* for instance, the RsmA homolog CsrA regulates carbon flux, biofilm formation, and motility, and its effect is antagonized by the UvrY (= GacA)-controlled regulatory RNAs CsrB and CsrC (28, 38, 52). Interestingly, CsrA can act as a repressor or as an activator of translation, depending on the target mRNA. Binding of CsrA to the untranslated leader of the *glgCAP* mRNA prevents ribosome binding and promotes mRNA decay (5, 27, 28), whereas binding of CsrA to the 5′ segment of *flhDC* mRNA increases messenger stability and expression (51). In *Salmonella enterica* serovar Typhimurium, CsrA controls genes involved in cell invasion, and its effect is antagonized by the SirA (= GacA)-regulated CsrB RNA (2, 3). The RsmA protein of the plant pathogen *Erwinia carotovora* subsp. *carotovora* controls the production of several virulence factors, including pectolytic enzymes, proteases, and cellulases (8). RsmA-mediated repression is relieved by the small regulatory RNA RsmB expressed under ExpS/ExpA (= GacS/GacA) control (10, 30). In the opportunistic human pathogen *Pseudomonas aeruginosa*, RsmA posttranscriptionally controls the production of secondary metabolites directly as well as indirectly by modulating the quorum-sensing circuitry (35, 36). A GacA-dependent regulatory RNA closely related to RsmZ of *P. fluorescens* strain CHA0 (19) and to PrrB of *P. fluorescens* F113 (1) antagonizes the RsmA effect (21).

Genetic evidence indicates that, in *P. fluorescens* CHA0,

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RsmA is not the only negative control element in the GacS/GacA cascade: whereas mutational inactivation of *gacS* or *gacA* drastically reduces the expression of genes required for HCN, AprA, and 2,4-diacetylphloroglucinol production, inactivation of the *rsmA* gene suppresses the *gacS* defect only partially (7). Furthermore, genomic data indicate that in the closely related strain *P. fluorescens* Pf-5, two *rsmA*-like genes exist (http://pseudo.bham.ac.uk). In the present study, we describe RsmE, a homolog of RsmA, which participates in posttranscriptional control of GacS/GacA-dependent genes in *P. fluorescens* CHA0.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. Bacteria were usually grown on nutrient agar and in nutrient yeast broth (44). When indicated, Triton X-100 was added to liquid cultures at a final concentration of 0.05% to avoid cell aggregation. Antibiotics and mercuric chloride, when required, were added to the growth medium at the following concentrations: tetracycline, 25 μg/ml for *E. coli* and 100 μg/ml for *P. fluorescens*; ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; gentamicin, 10 μg/ml; mercuric chloride, 10 μg/ml for *E. coli*; and kanamycin, 25 μg/ml for *E. coli* and 50 μg/ml for *P. fluorescens*. To counterselect *E. coli* donor cells in matings with *P. fluorescens*, chloramphenicol was used at 10 μg/ml. When relevant, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to plates at a final concentration of 0.02%. Routine incubation temperatures were 37°C for *E. coli* and 30°C for *P. fluorescens*. *P. fluorescens* was grown at 35°C to improve its capacity to accept heterologous DNA, i.e., in electrotransformation or triparental matings with *E. coli*.

DNA manipulation and cloning procedures. DNA manipulations were carried out as described by Sambrook and Russell (39). Small-scale preparations of plasmid DNA were carried out by the cetyltrimethylammonium bromide method (11), large-scale preparations were performed with JetStar-Tips (Genomed, Basel, Switzerland). Chromosomal DNA was prepared according to Gamper et al. (15). DNA fragments were purified from agarose gels with the GeneClean DNA extraction kit (Bio 101, La Jolla, Calif.). Transformation of *E. coli* and *P. fluorescens* was carried out by electroporation (13). Nucleotide sequences were determined with a Big Dye Terminator Cycle sequencing kit and an ABI-Prism 373 automatic sequencer (Applied Biosystems). Nucleotide sequences were analyzed with the programs of the University of Wisconsin Genetics Computer Group package (version 9.1).

RNA manipulations and Northern blots. RNA preparations from *P. fluorescens* strains and Northern blots were done as described previously (48). RNA half-lives were estimated after addition of 200 μg of rifampin per ml to cultures. RNA was extracted at given time points and analyzed by Northern blotting.

Construction of a gene bank from *P. fluorescens* CHA0 and isolation of the *rsmE* gene. Chromosomal DNA from CHA0 was digested partially with Sau3A. Fragments of 2 to 4 kb were gel purified and ligated to pME6000, digested with BamHI, and treated with alkaline phosphatase. To avoid destruction by the host's restriction system, pME6000 DNA was prepared from CHA0. Ligation mixtures were purified as described (46) and stored in aliquots at −20°C until used to electro-transform strain CHA805. Among ca. 5,000 transformants obtained, 6 which showed a white colony phenotype on nutrient agar containing X-Gal were retained. Sequence analysis showed that these clones had a common gene (*rsmE*) in the pME6000 insert. One clone, pME6834, was kept for further study.

Construction of plasmids and gene replacement mutants. The *rsmE* subclones pME6850 and pME6851 were generated by PCR-amplifying *rsmE* from pME6834 with primers MB20.1 and MB21.1. The 0.53-kb PCR product was blunted with T4 DNA polymerase and cloned into the SmaI site of pME6001 to give pME6850, which carries *rsmE* in the opposite orientation from that of the vector promoter P_{lac}. To place the gene under P_{lac} control, *rsmE* was excised from pME6850 on a 0.53-kb BamHI-EcoRV fragment and recloned into pME6001 between the XbaI, made blunt by T4 DNA polymerase treatment, and BamHI sites. This generated pME6851. For overexpression and purification of RsmE, a histidine tag was added at its C terminus by PCR with primers RSMEHIS6 and T7 with pME6850 as the template. The 0.4-kb PCR product was blunted with T4 DNA polymerase, digested with BamHI, cloned into pBLS II KS between the BamHI and HincII sites, excised with XbaI and XhoI, and inserted into pET28a under the control of the T7 promoter. The resulting overexpression construct was named pME7013.

Chromosomal mutations in *rsmE*, *rsmA*, and *gacS* were generated by gene replacement as described previously (24, 42). The suicide plasmid pME6879 used to generate an *rsmE*::Ω-Hg mutation was constructed as follows. Two PCR products flanking the *rsmE* gene were obtained from pME6834 with primers RSMEUP plus T3 and RSMEDOWN plus T7, respectively. The resulting 1.1-kb upstream fragment and the 1.7-kb downstream fragment were digested with HindIII and BamHI and with BamHI and EcoRI, respectively, ligated with a 5-kb BamHI-BamHI fragment carrying the Ω-Hg cassette from pHP45Ω-Hg, and cloned into pUK21. The Ω-Hg cassette flanked by the *rsmE* up- and downstream regions was excised from this pUK21 derivative on an 8-kb SpeI-SpeI fragment and cloned into the XbaI site of pME3087 to produce pME6879. Plasmid pME6879 was introduced into CHA0, CHA805, and CHA207 to generate the *rsmE*::Ω-Hg mutants CHA1003, CHA1005, and CHA1025, respectively. The *rsmA* gene was mutated with pME6081 (7) in strains CHA1003, CHA805, CHA1005, CHA207, CHA1025, and CHA0 to give the corresponding *rsmA*::Ω-Km mutants CHA1009, CHA1020, CHA1021, CHA1023, CHA1027, and CHA1076, respectively. Finally, the suicide plasmid pME3274 (55) served to delete the *gacS* gene in strains CHA1021, CHA1009, CHA207, and CHA1027, resulting in CHA1007, CHA1008, CHA1022, and CHA1028, respectively (Table 1).

Expression of *rsmE* was measured with a translational *rsmE′-′lacZ* fusion, which was constructed as follows. The first two codons of *rsmE* and 2.3 kb of its upstream region were PCR amplified from CHA0 chromosomal DNA with primers RSME-1 and RSME-2. The resulting fragment was cleaved with BamHI and HindIII and cloned into pNM481. From the resulting construct, a 5.4-kb BamHI-XhoI fragment carrying *rsmE′-′lacZ* was excised, blunted with T4 DNA polymerase, and ligated to the SmaI-linearized vector pME3280a, which delivers a mini-Tn*7* with passenger DNA into the unique Tn*7* attachment site of the *P. fluorescens* chromosome. The resulting plasmid, pME7545, was used as described previously (55) to integrate its mini-Tn*7* with *rsmE′-′lacZ* and a gentamicin resistance gene into the chromosome of strains CHA0, CHA89, CHA1003, CHA1076, and CHA1009 to give strains CHA1134, CHA1136, CHA1138, CHA1161, and CHA1162, respectively.

Detection of RsmE and RsmA by Western blotting. Erlenmeyer flasks containing 20 ml of NYB amended with 0.05% Triton X-100 were inoculated 1:100 and grown at 30°C with shaking. At given time points after inoculation, cells equivalent to an optical density at 600 nm of 0.4 U per ml were centrifuged, washed with 0.9% (wt/vol) NaCl, resuspended in 20 μl of loading buffer (50 mM Tris-HCl, pH 6.8, 2% [wt/vol] sodium dodecyl sulfate, 0.1% [wt/vol] bromophenol blue, 15% [vol/vol] glycerol, 5% [vol/vol] β-mercaptoethanol) and immediately treated at 100°C for 10 min. Of each sample, 15 μl was loaded on a 16% acrylamide-bisacrylamide gel containing Tricine and sodium dodecyl sulfate (41). After electrophoresis at 70 V during 4 h, proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore) at 50 mA and 4°C for 1 h. The RsmA and RsmE proteins were detected with polyclonal antibodies raised against purified *Yersinia enterocolitica* RsmA (36) and a secondary antibody coupled to peroxidase. Membranes were developed with the ECL Western blotting analysis system (Amersham-Pharmacia) following the manufacturer's instructions.

Purification of histidine-tagged protein fusions of RsmE (RsmE6H) and RsmA (RsmA6H). The RsmE6H and RsmA6H proteins were overexpressed in *E. coli* BL21/pME7013 and *E. coli* DH5α/pME6078, respectively, and purified by Ni-nitrilotriacetic acid affinity chromatography (Qiagen) as described previously (19). The protein eluates were dialyzed against 10 mM Tris-acetate (pH 8.0) at 4°C and stored at −20°C. Protein contents were estimated with the Bradford method with bovine serum albumin as the standard. The purity of the preparations was ≥90% as judged from sodium dodecyl sulfate-Tricine-polyacrylamide gel electrophoresis.

Mobility shift assays. Radioactively labeled transcripts of RsmY and RsmZ were synthesized from linearized pME6919 and pME6920, respectively, with a T7 transcription kit (Fermentas) in the presence of [α-^{33}P]UTP, following the manufacturer's instructions. Unlabeled competitor RNAs (RsmY, RsmZ, or *carA* leader) were synthesized following the same protocol but with unlabeled UTP from linearized pME6919, pME6920, or pME6926, respectively. RNA was purified by phenol-chloroform extraction and desalted with Sephadex G-25 mini-columns (Amersham Biosciences). RNA concentrations were estimated by UV absorption at 260 nm. Binding reactions contained [α-^{33}P]UTP-labeled RsmY or RsmZ RNA and purified RsmA6H or RsmE6H at various concentrations (see the legend to Fig. 6 for details). Assays were also carried out in the presence of various unlabeled RNA competitors (see the legend to Fig. 7 for details). In this case, RsmA6H or RsmE6H was added last to the binding reaction containing competing RNAs. The reaction mixtures (10 μl) were incubated at 30°C for 30 min to allow complex formation. Samples were then fractionated on native 10%

TABLE 1. Bacterial strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Relevant characteristics[a] or sequence (5′ → 3′)	Source or reference
E. coli		
BL21(DE3)	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-)*gal dcm* (λDE3)	Novagen
DH5α	*recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d*lac*ZΔM15)	39
HB101	*hsdS recA proA2 leu-6 ara-14 galK2 lacY1 xyl-5 mtl-1 rpsL20 thi-1 supE44*	39
P. fluorescens		
CHA0	Wild type	50
CHA19	Δ*gacS*	55
CHA89	*gacA*::Km^r	23
CHA207	*hcnA′-′lacZ*	7
CHA805	*aprA′-′lacZ*	7
CHA806	Δ*gacS, aprA′-′lacZ*	19
CHA1003	*rsmE*::Ω-Hg Hg^r	This study
CHA1005	*rsmE*::Ω-Hg *aprA′-′lacZ*, Hg^r	This study
CHA1007	*rsmA*::Ω-Km *rsmE*::Ω-Hg Δ*gacS aprA′-′lacZ*, Km^r Hg^r	This study
CHA1008	*rsmA*::Ω-Km *rsmE*::Ω-Hg Δ*gacS*, Km^r Hg^r	This study
CHA1009	*rsmA*::Ω-Km *rsmE*::Ω-Hg, Km^r Hg^r	This study
CHA1020	*rsmA*::Ω-Km *aprA′-′lacZ,* Km^r	This study
CHA1021	*rsmA*::Ω-Km *rsmE*::Ω-Hg *aprA′-′lacZ,* Km^r Hg^r	This study
CHA1022	Δ*gacS hcnA′-′lacZ*	This study
CHA1023	*rsmA*::Ω-Km *hcnA′-′lacZ,* Km^r	This study
CHA1025	*rsmE*::Ω-Hg *hcnA′-′lacZ,* Hg^r	This study
CHA1027	*rsmA*::Ω-Km *rsmE*::Ω-Hg *hcnA′-′lacZ,* Km^r Hg^r	This study
CHA1028	*rsmA*::Ω-Km *rsmE*::Ω-Hg Δ*gacS, hcnA′-′lacZ,* Km^r Hg^r	This study
CHA1076	*rsmA*::Ω-Km, Km^r	This study
CHA1134	mini-Tn7 Gm^r *rsmE′-′lacZ*	This study
CHA1136	*gacA*::Km^r mini-Tn7 Gm^r *rsmE′-′lacZ*	This study
CHA1138	*rsmE*::Ω-Hg mini-Tn7 Gm^r *rsmE′-′lacZ,* Hg^r	This study
CHA1161	*rsmA*::Ω-Km mini-Tn7 Gm^r *rsmE′-′lacZ,* Km^r	This study
CHA1162	*rsmA*::Ω-Km *rsmE*::Ω-Hg mini-Tn7 Gm^r *rsmE′-′lacZ,* Km^r Hg^r	This study
Plasmids		
pET28a	Expression vector, P_{T7}, Km^r	Novagen
pBLS II KS, SK	pBluescript cloning vectors, Ap^r	Stratagene
pHP45ΩSm/Sp	Source of transcription/translation stop cassette; Ap^r Sm^r Sp^r	37
pHP45ΩHg	Source of transcription/translation stop cassette; Ap^r Hg^r	37
pME497	Mobilizing plasmid, Ap^r	7
pME3087	Suicide vector, MCS, Tc^r	50
pME3274	Suicide plasmid derived from pME3087 for deletion of *gacS* by gene replacement	55
pME3280a	Chromosomal integration vector, mini-Tn7 Gm^r, MCS, Ap^r	55
pME6000	Cloning vector, pBBR1MCS derivative, Tc^r	32
pME6001	Cloning vector, pBBR1MCS derivative, Gm^r	7
pME6078	*P. fluorescens* CHA0 *rsmA* (encoding RsmA6H) in pME6032	19
pME6081	Suicide plasmid for gene replacement containing *rsmA*::Ω-Km, Tc^r Km^r	7
pME6091	Transcriptional *rsmZ-lacZ* fusion	19
pME6359	P_{tac}-*rsmZ* fusion at the +1 site; Tc^r	19
pME6702	P_{tac}-*phlA′-′lacZ,* Tc^r	C. Gigot-Bonnefoy and D. Haas, unpublished data
pME6834	pME6000 derivative carrying *rsmE* in 3-kb insert; Tc^r	This study
pME6850	pME6001 derivative carrying *rsmE,* Gm^r	This study
pME6851	pME6001 derivative carrying *rsmE* under P_{lac} control, Gm^r	This study
pME6879	Suicide plasmid for gene replacement containing *rsmE*::Ω-Hg Tc^r Hg^r	This study
pME6916	Transcriptional *rsmY-lacZ* fusion	47
pME6918	P_{tac}-*rsmY* fusion at the +1 site; Tc^r	47
pME6919	Template for in vitro transcription with *rsmY* under P_{T7}	47
pME6920	Template for in vitro transcription with *rsmZ* under P_{T7}	47
pME6926	Template for in vitro transcription with *carA* under P_{T7}	47
pME7013	*P. fluorescens* CHA0 *rsmE* (encoding RsmE6H) in pET28a	This study
pME7545	pME3280a derivative containing *rsmE′-′lacZ,* Gm^r Ap^r	This study
pNM481	*′lacZ* fusion vector, Ap^r	34
pUK21	Cloning vector, MCS, Km^r	49
Oligonucleotides		
MB20.1	GGCTCGCTATCGCGAAAGG	
MB21.1	GATATTTATTGCCTATGT	
RSMEHIS6	TTAAATGGTGATGGTGATGGTGGGGGGTTTCGCGTTTGTCC	
RSMEUP	ACGTGGATCCATGATCTTCTCCTTGAT (BamHI site underlined)	
RSMEDOWN	ACGTGGATCCTGAACAGCATGAGCAC (BamHI site underlined)	
RSME-1	ACGTGGATCCTGACCATGAACGATGAAATC (BamHI site underlined)	
RSME-2	ACGTAAGCTTCAGCATGATCTTCTCCTTG (HindIII site underlined)	

[a] Ap, ampicillin; Gm, gentamicin; Hg, mercuric chloride; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; MCS, multiple cloning site.

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RsmA    MLILTRRCAESLIIGDGEITVTVLGVKGNQVRIGVNAPKEVAVHREEIYLRIKK-----EKDEEPSH
RsmE    MLILTRKVGESINIGD-DITITILGVSGQQVRIGINAPKDVAVHREEIYQRIQAGLTAPDKRETP--
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FIG. 1. Alignment of the deduced amino acid sequences of RsmA (19) and RsmE (this study). Asterisks indicate identical amino acids.

polyacrylamide gels (4 h at 10 mA), and radioactive bands were visualized by autoradiography after drying the gels (47, 48).

β-Galactosidase assays. *P. fluorescens* strains were grown in 20 ml of NYB (in 50-ml Erlenmeyer flasks) with shaking at 180 rpm and 30°C; 0.05% Triton X-100 was routinely added to avoid cell aggregation. β-Galactosidase activities were quantified by the Miller method (33), with cells permeabilized with 5% (vol/vol) toluene.

Nucleotide sequence accession number. The nucleotide sequence of *rsmE* has been assigned GenBank accession number AY547272.

RESULTS

Discovery of *rsmE*, a homolog of the posttranscriptional regulator gene *rsmA*. To identify new regulatory elements involved in the GacS/GacA signal transduction pathway of *P. fluorescens* CHA0, we searched for genes which, when present on a multicopy plasmid, would downregulate the expression of typical GacS/GacA-controlled genes, such as the translational reporter gene fusions *aprA'-'lacZ* and *hcnA'-'lacZ* in strains CHA805 and CHA207, respectively. To this end, a CHA0 gene expression library was constructed in the broad-host-range vector pME6000 and introduced into CHA805. Among several thousand transformants, six were identified which strongly reduced the expression of *aprA'-'lacZ* as judged by their white color on medium containing X-Gal.

Sequence analysis revealed that these clones had a common gene in their pME6000 insert. The deduced amino acid sequence of this gene, which we named *rsmE*, had 71% identical amino acids with the posttranscriptional regulator RsmA of CHA0 (Fig. 1). To ensure that *rsmE* was indeed responsible for repression of *aprA'-'lacZ* in CHA805, a subclone, pME6851, which carries only the *rsmE* gene under P_{lac} control was constructed (see Materials and Methods for details). The expression of both *aprA'-'lacZ* and *hcnA'-'lacZ* was strongly reduced in CHA805/pME6851 and CHA207/pME6851 compared to CHA805 and CHA207 transformed with the empty vector pME6001, in that both reporter genes were repressed more than 10-fold (data not shown).

To test if strain CHA0 encodes additional RsmA/E homologs, Southern blots were performed under nonstringent conditions with *rsmA* and *rsmE* as molecular probes. However, we found no evidence for further homologs of these genes (data not shown). Moreover, in the total genomic sequence of the closely related *P. fluorescens* strain Pf-5 (http://pseudo.bham.ac.uk), we identified two proteins which are 100% identical to RsmA and RsmE but found no additional homologs. Taken together, these data strongly suggest that the genome of *P. fluorescens* CHA0 encodes two, but not more, closely related regulators of the RsmA family.

Complete suppression of a *gacS* mutation by an *rsmA rsmE* double mutation. Inactivation of either *gacS* or *gacA* strongly reduces the expression of *hcnA*, *aprA*, and *phlA* (7, 55). When the chromosomal *rsmA* gene is inactivated in a Δ*gacS* background, the effect of the *gacS* mutation on *aprA'-'lacZ* is suppressed only partially, indicating that RsmA is not the only negative regulator operating in the Gac/Rsm cascade (7). To test whether RsmE acts as an additional negative regulator, we evaluated the effect of an *rsmE* mutation on the expression of GacS/GacA-controlled genes. As expected, expression of *hcnA'-'lacZ* was strongly reduced in the *gacS* mutant

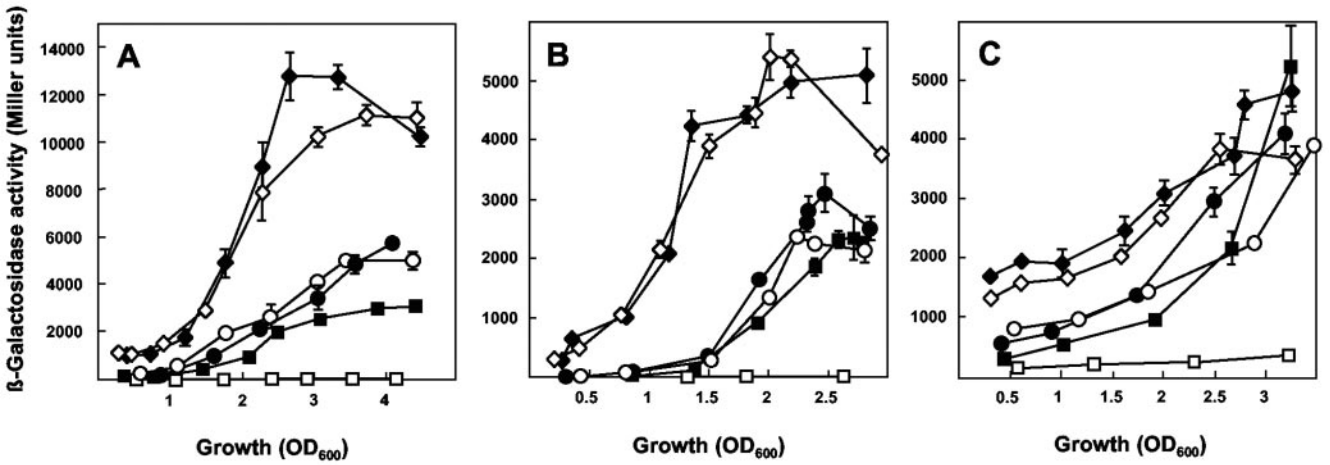

FIG. 2. Impact of *rsmE, rsmA*, and *gacS* mutations on the expression of *hcnA*, *aprA*, and *phlA*. (A) β-Galactosidase expression of a chromosomal *hcnA'-'lacZ* fusion was determined in CHA207 (solid squares, wild-type context), CHA1022 (open squares, *gacS* mutant), CHA1023 (open circles, *rsmA* mutant), CHA1025 (solid circles, *rsmE* mutant), CHA1027 (solid diamonds, *rsmA rsmE* double mutant), and CHA1028 (open diamonds, *rsmA rsmE gacS* triple mutant). (B) Expression of a chromosomal *aprA'-'lacZ* fusion in CHA805 (solid squares), CHA806 (open squares), CHA1020 (open circles), CHA1005 (solid circles), CHA1021 (solid diamonds), and CHA1007 (open diamonds). (C) Expression of a *phlA'-'lacZ* fusion on pME6702 in CHA0 (solid squares), CHA19 (open squares), CHA1076 (open circles), CHA1003 (solid circles), CHA1009 (solid diamonds), and CHA1008 (open diamonds). Each value is the average from three different cultures ± standard deviation.

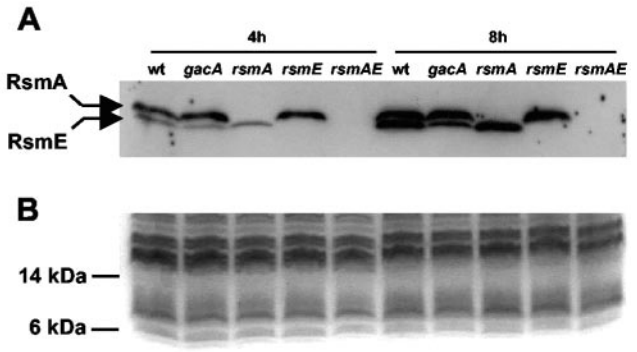

FIG. 3. Production of the RsmE and RsmA proteins in *P. fluorescens* mutants. (A) Western blot detection of the RsmE and RsmA proteins from 20-ml cultures grown in 125-ml flasks. Samples from strains CHA0 (wild type, wt), CHA89 (*gacA*), CHA1076 (*rsmA*), CHA1003 (*rsmE*), and CHA1009 (*rsmA rsmE*) were taken in late exponential phase (4 h after inoculation) and in stationary phase (8 h after inoculation) for gel electrophoresis and immunodetection. (B) Control of protein load. A portion of the gel showing polypeptides of 6 to 20 kDa, after protein transfer and staining with Coomassie blue. Note that under the conditions used, the slightly larger but more hydrophobic RsmE protein migrated ahead of RsmA.

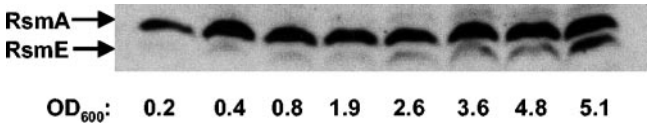

FIG. 4. Cellular levels of RsmE and RsmA proteins in *P. fluorescens* CHA0 during growth in 50-ml flasks containing 20 ml of NYB and 0.05% Triton X-100. Samples for immunoblot analysis were taken at increasing cell densities. The protein load in each well was similar (not shown).

CHA1022 compared to the expression of this chromosomal fusion in the wild-type CHA207 (Fig. 2A). Inactivation of *rsmA* or *rsmE* in strains CHA1023 and CHA1025, respectively, resulted in a slight increase in *hcnA* expression. However, when both *rsmA* and *rsmE* were mutated, *hcnA* expression was fourfold higher than in the wild type, irrespective of the presence (CHA1027) or the absence (CHA1028) of a functional *gacS* gene (Fig. 2A).

Similar results were found with the *aprA'-'lacZ* reporter (Fig. 2B). The expression of *aprA* was only marginally increased in the *rsmA* (CHA1020), and the *rsmE* mutant (CHA1005) compared with the wild-type CHA805 and was very low in the Δ*gacS* mutant CHA806. Again, inactivation of both *rsmA* and *rsmE* resulted in a three- to fourfold increase in a *gacS*-positive (CHA1021) as well as in a *gacS*-negative (CHA1007) background (Fig. 2B).

Finally, expression of a plasmid-borne *phlA'-'lacZ* fusion (driven by the *tac* promoter) in the different genetic backgrounds followed a pattern similar to that described above for the chromosomal *hcnA'-'lacZ* and *aprA'-'lacZ* fusions, although the effects of the *rsmA* and *rsmE* mutations were less pronounced, possibly due to the particular *phlA* construct used. Importantly, the *rsmA rsmE* double mutation fully suppressed a *gacS* defect with respect to *phlA* expression (Fig. 2C).

We conclude from these data that in the absence of both the RsmA and RsmE proteins, the presence of a functional GacS/GacA system is no longer required for *hcnA*, *aprA*, or *phlA* expression.

Expression of RsmA and RsmE. Although the calculated molecular masses of RsmA (6.95 kDa) and RsmE (7.01 kDa) are similar, the two proteins could be separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in Tricine buffer, visualized in Western blots with polyclonal antibodies against RsmA (purified from *Yersinia enterocolitica*), and identified by the use of *rsmA* and *rsmE* mutants (Fig. 3). This allowed us to study the expression of the two proteins during growth. The expression of both RsmA and RsmE increased with increasing cell density; however, the amount of RsmA showed considerably less variation (Fig. 4). The expression of RsmE was also followed by measuring the expression of a chromosomal *rsmE'-'lacZ* fusion (integrated at the Tn*7* attachment site); throughout growth, expression in the *gacA* mutant CHA1136 was lower than that in the wild-type background CHA1134 (Fig. 5). Inactivation of *rsmA* or *rsmE* in mutants CHA1161 and CHA1138, respectively, resulted in a moderate increase in *rsmE'-'lacZ* expression, whereas expression was highest in the *rsmA rsmE* double mutant CHA1162 (Fig. 5). The Western blot data (Fig. 3) are in agreement with the regulatory effects of GacA and RsmA on RsmE levels.

Interaction of RsmE6H and RsmA6H with RsmY and RsmZ. To investigate whether RsmE can be captured by RsmY and RsmZ, we prepared in vitro transcripts of both RNAs and performed mobility shift assays with RsmE6H and, in parallel, with RsmA6H. Both proteins were overexpressed in *E. coli* and purified as histidine-tagged fusions. Binding of RsmA6H and RsmE6H to RsmY (5 nM) was detected as two distinct complexes at 34 to 69 nM (Fig. 6A and B) and higher-order complexes became visible at ≥550 nM RsmA6H and

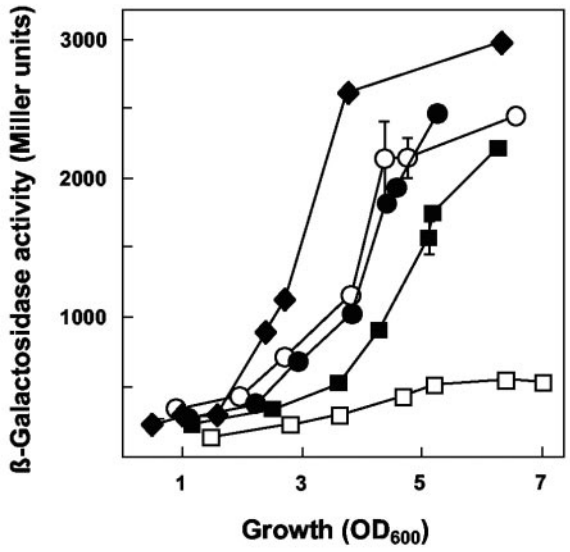

FIG. 5. Expression of a chromosomal *rsmE'-'lacZ* fusion in the wild-type context (CHA1134, solid squares), in a *gacA* mutant (CHA1136, open squares), in an *rsmA* mutant (CHA1161, open circles), in an *rsmE* mutant (CHA1138, solid circles), and in an *rsmA rsmE* double mutant (CHA1162, solid diamonds). Each value is the average from three different cultures ± standard deviation.

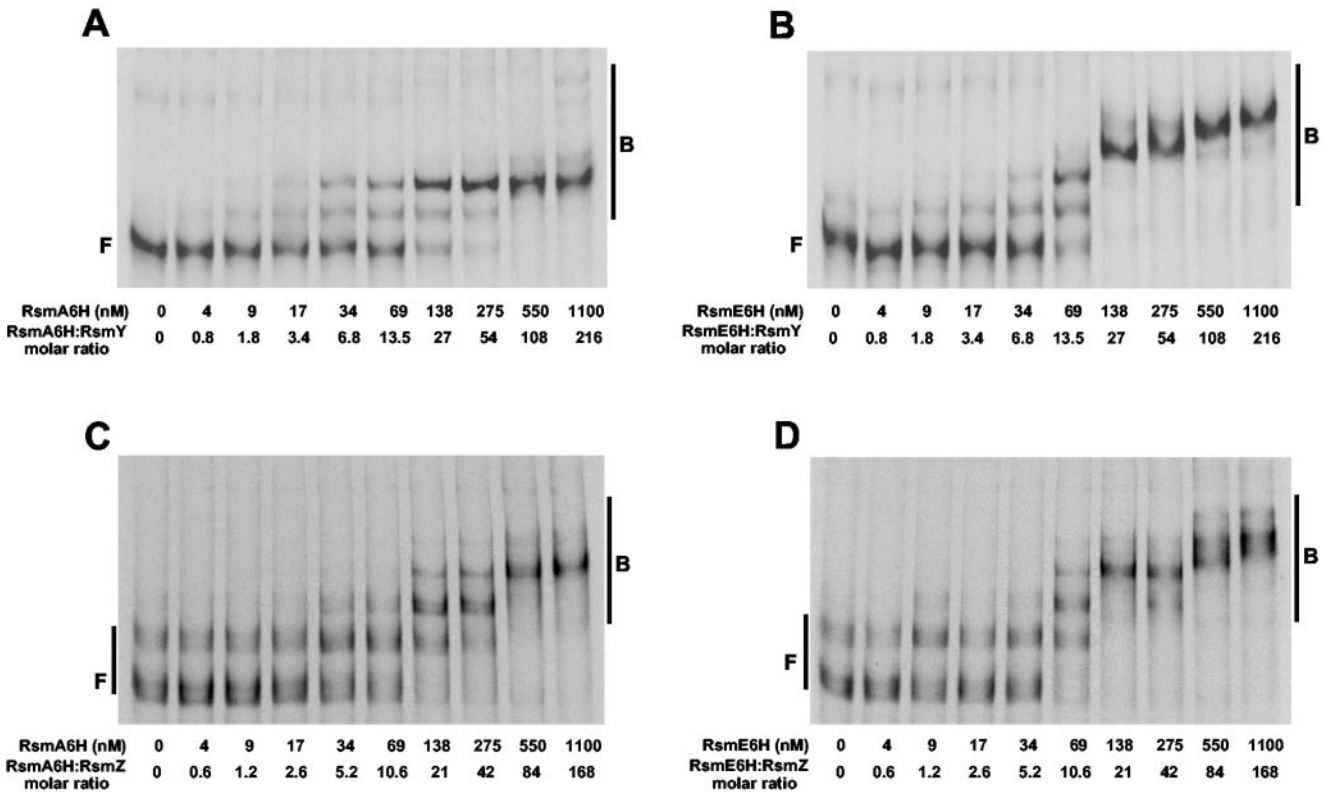

FIG. 6. Interaction of RsmA6H and RsmE6H with the regulatory RNAs RsmY and RsmZ. [α-^{33}P]UTP-labeled RsmY (5 nM) and RsmZ (6.5 nM) were incubated with different concentrations of purified RsmA6H or RsmE6H before fractionation on nondenaturing gels and autoradiography. The positions of free (F) and bound (B) RNA species are indicated. (A) RsmY versus RsmA6H. (B) RsmY versus RsmE6H. (C) RsmZ versus RsmA6H. (D) RsmZ versus RsmE6H.

≥138 nM RsmE6H, suggesting that RsmY may have a somewhat higher binding capacity for RsmE6H than for RsmA6H. The interactions of RsmY with RsmA6H and RsmE6H were also compared (Fig. 6C and D). As observed previously (47), in vitro transcription of *rsmZ* gave two different-sized transcripts. Again, binding of both proteins to RsmZ (6.5 nM) was similar, and the formation of higher-order complexes appears to occur more readily with RsmE6H than with RsmA6H.

Competition experiments were performed to assess the specificity of RsmE6H binding to RsmY and RsmZ. As shown in Fig. 7, unlabeled RsmY and RsmZ transcripts were both able to compete with RsmE6H-RsmY and RsmE6H-RsmZ complexes, whereas the same amount of a similarly sized RNA lacking the putative RsmA/E binding elements (i.e., the untranslated leader of the *P. fluorescens carA* gene) did not modify the binding of RsmE6H to RsmY or RsmZ. We conclude from these experiments and from data obtained earlier with RsmA6H (47) that both regulatory RNAs bind RsmA and RsmE specifically and with similar affinity.

RsmA and RsmE positively control RsmY and RsmZ levels. To assess the effects of RsmA and RsmE on the transcription of the two regulatory RNAs, we measured their expression with plasmid-encoded transcriptional *lacZ* fusions made at the +1 transcription start site (Fig. 8). Expression of *rsmY-lacZ* and *rsmZ-lacZ* was similar in the wild type and in the *rsmA* and *rsmE* mutants CHA1076 and CHA1003, respectively. In agreement with previous results (19, 47), both fusions gave strongly reduced expression in the *gacA* mutant CHA89. Interestingly, in the *rsmA rsmE* double mutant CHA1009 as well as in the *rsmA rsmE gacS* triple mutant CHA1008, the expression of both fusions was equally low. We conclude from this that RsmA and RsmE are required together with GacA for the transcription of both RNAs.

RsmA and RsmE also affected the stability of RsmY and RsmZ (Fig. 9). The estimated half-lives of both RNAs were >20 min in the wild type and <10 min in the *rsmA rsmE* double mutant, indicating that RsmY and RsmZ are protected from decay by the RsmA and RsmE proteins in vivo.

DISCUSSION

In the present work, we have searched for novel negative regulators involved in the expression of the *hcnA* and *aprA* genes in the biocontrol strain *P. fluorescens* CHA0, and this has led to the discovery of the RNA-binding protein RsmE. This protein belongs to the RsmA/CsrA family of posttranscriptional regulators, which control a large spectrum of physiological processes ranging from carbon flux to the expression of bacterial traits important for beneficial or deleterious host-microbe interactions (2, 6, 7, 8, 14, 25, 26, 36, 38, 40). In *P. fluorescens* CHA0, the RsmA protein posttranscriptionally represses the expression of exoproduct genes during exponential growth, probably by obstructing ribosome access to the Shine-Dalgarno sequences of target mRNAs. This repression is re-

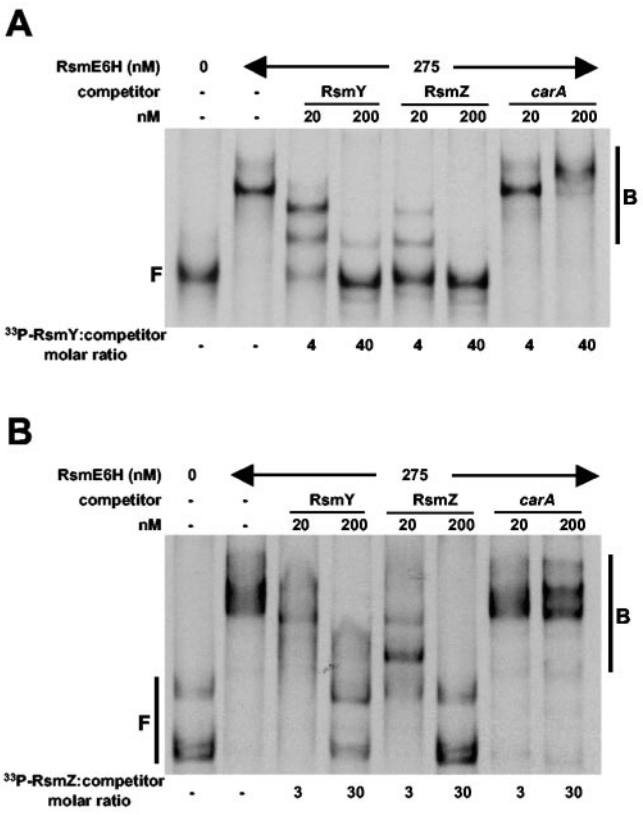

FIG. 7. Competition of RsmY and RsmZ RNAs for binding to RsmE6H. [α-^{33}P]UTP-labeled RsmY (5 nM) and RsmZ (6.5 nM) and different unlabeled RNA competitors (RsmY, RsmZ, and the leader of *carA* mRNA) were incubated with RsmE6H (275 nM) before fractionation on nondenaturing gels and autoradiography. (A) Competition of unlabeled RNAs with RsmY-RsmE6H complexes. (B) Competition of unlabeled RNAs with RsmZ-RsmE6H complexes. F, free transcripts; B, transcripts bound to RsmE6H.

lieved by GacS/GacA-dependent regulatory RNAs such as RsmY and RsmZ, which sequester the RsmA regulator (19, 47).

What is the role of RsmE in this regulatory network? Several lines of evidence indicate that the overall function of RsmE is similar to that of RsmA and that both proteins are required together for maximal translational repression of GacS/GacA-controlled target genes. (i) Chromosomal deletion of either *rsmA* or *rsmE* resulted in slightly increased expression of three target genes tested, i.e., *hcnA, aprA,* and *phlA* (Fig. 2). (ii) Deletion of both *rsmA* and *rsmE* strongly increased and advanced expression of the target genes (Fig. 2). (iii) Overexpression of *rsmA* or *rsmE* from a vector promoter (*plac*) strongly reduced target gene expression (7; K. Starke and D. Haas, unpublished data). (iv) RsmA and RsmE specifically bound to the regulatory RNAs RsmY and RsmZ in vitro (Fig. 6 and 7). However, the expression profiles of RsmA and RsmE are different. RsmA was present in considerable amounts throughout growth, whereas little RsmE was made at low cell densities (Fig. 4). Moreover, *rsmE* expression was regulated positively by GacA and negatively by RsmA and RsmE (Fig. 3 and 5). The *rsmA* gene appears to be cotranscribed with the upstream *lysC* (aspartokinase) gene and possibly also with the *alaS* (alanyl-tRNA synthetase) gene (our unpublished observations), complicating detailed analysis of *rsmA* expression. The observation that RsmE levels were highest at the end of growth (Fig. 4) suggests that RsmE could play a role in termination of GacA-controlled gene expression.

RsmA and RsmE stabilize both RsmY and RsmZ in vivo (Fig. 9), probably by protecting them from degradation by cellular RNases. In *Erwinia carotovora* subsp. *carotovora,* RsmA also increases the half-life of the RsmB riboregulator (9), whereas the stability of the CsrB RNA in *E. coli* does not

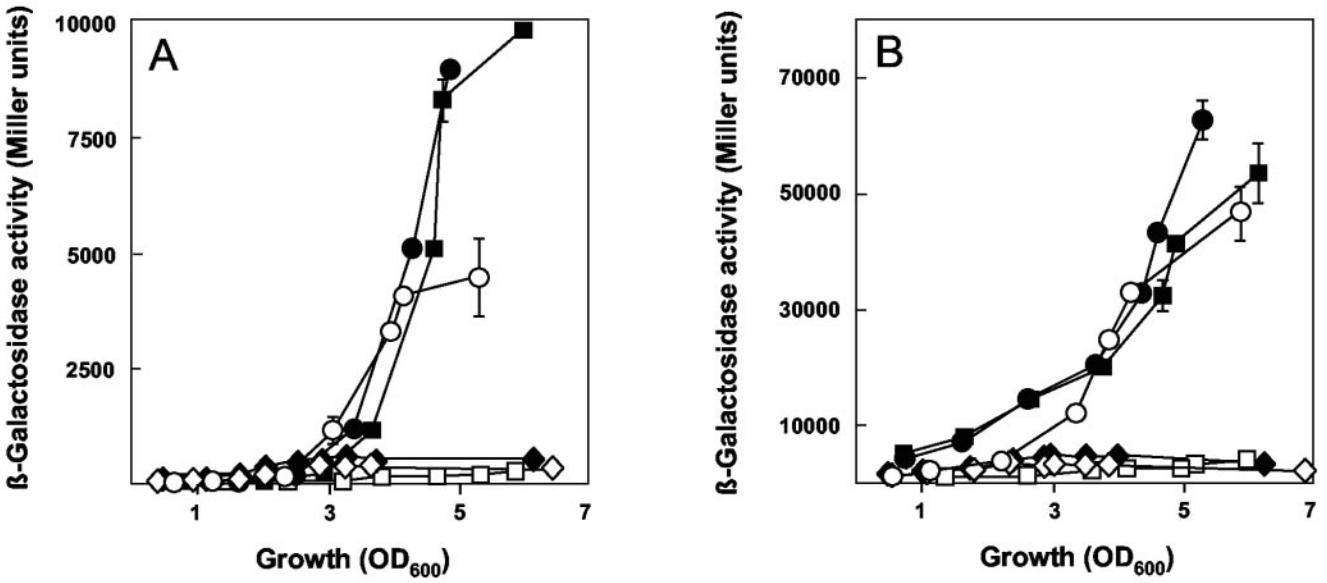

FIG. 8. Impact of *rsmE, rsmA*, and *gacA* mutations on *rsmY* and *rsmZ* transcription. (A) β-Galactosidase expression of an *rsmY-lacZ* transcriptional fusion on pME6916 in the wild-type CHA0 (solid squares), the *gacA* mutant CHA89 (open squares), the *rsmE* mutant CHA1003 (solid circles), the *rsmA* mutant CHA1076 (open circles), the *rsmA rsmE* double mutant CHA1009 (solid diamonds), and the *rsmA rsmE gacS* triple mutant CHA1008 (open diamonds). (B) β-Galactosidase expression of an *rsmZ-lacZ* transcriptional fusion on pME6091 in CHA0 (solid squares), CHA89 (open squares), CHA1003 (solid circles), CHA1076 (open circles), CHA1009 (solid diamonds), and CHA1008 (open diamonds).

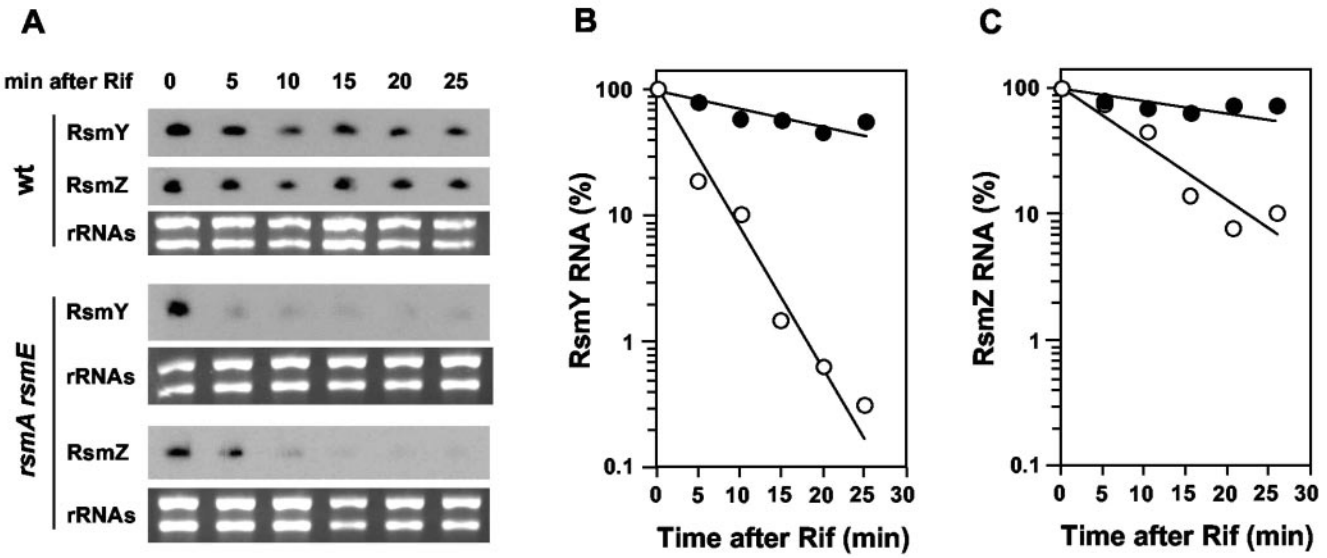

FIG. 9. RsmA and RsmE proteins stabilize RsmY and RsmZ RNAs. (A) RsmY and RsmZ transcript decay in the wild-type strain *P. fluorescens* CHA0 (wt) and in the *rsmA rsmE* double mutant CHA1009 was determined by Northern blotting after blocking transcription with rifampin. The amount of RNA loaded was 0.5 μg for the wild type and 3 μg for the *rsmA rsmE* double mutant. As both *rsmY* and *rsmZ* are poorly expressed in the *rsmA rsmE* background, the stability of RsmY and RsmZ was studied in CHA1009 expressing *rsmY* or *rsmZ* from the *tac* promoter of pME6918 (*rsmY*) or pME6359 (*rsmZ*). (B) Densitometric analysis of RsmY stability in CHA0 (solid circles) and CHA1009 (open circles). (C) Densitometric analysis of RsmZ stability in CHA0 (solid circles) and CHA1009 (open circles).

appear to be affected by the RNA-binding protein CsrA (16). In *Pseudomonas aeruginosa*, RsmZ RNA is absent from stationary-phase cells (21). The half-lives of these regulatory RNAs are critical parameters in the GacS/GacA cascade, as they will determine the duration of the "on" phase.

RsmA and RsmE not only affect the stability of RsmY and RsmZ but are also required for good promoter activity of the *rsmY* and *rsmZ* genes (Fig. 8). Similarly, the *E. coli* RsmA homolog CsrA controls the transcription of the *csrB* and *csrC* riboregulator genes. CsrA appears to have a positive regulatory effect on the expression of the *gacS* homolog *barA*, suggesting that CsrA control of *csrB* and *csrC* may, at least in part, be indirect, via GacS and GacA (45). It is currently unknown whether *gacA* expression is controlled by RsmA and RsmE in *P. fluorescens*. In *P. aeruginosa*, however, RsmA positively controls *rsmZ* transcription without affecting *gacA* expression (21).

The circuit diagram presented in Fig. 10 summarizes our current understanding of the regulatory interactions operating in the Gac/Rsm cascade of *P. fluorescens* CHA0. Genetic evidence strongly suggests that GacS activates GacA by a phosphorelay mechanism in response to a bacterial signal (55). Although GacA is crucial for *rsmY* and *rsmZ* expression, GacA binding to the promoter regions of *rsmY* and *rsmZ* has not been demonstrated yet. RsmY and RsmZ bind multiple copies of RsmA and RsmE in vitro and antagonize the regulatory effects of these RNA binding proteins on secondary metabolite production in vivo (19, 47) (Fig. 2, 6, and 7). RsmE expression is regulated negatively by RsmA and RsmE and positively by GacA (Fig. 5). RsmA and RsmE are required for RsmY and RsmZ transcription and stability (Fig. 8 and 9). It will be of particular interest to analyze how this transcriptional activation by RsmA and RsmE is achieved.

RsmA/CsrA redundancy in some bacterial species is evident from genomic data, but the present study is the first to examine this phenomenon experimentally. Whereas in enteric bacteria (e.g., *E. coli*, *Salmonella enterica*, and *Erwinia* spp.) and in *P. aeruginosa* there is only a single *rsmA* gene (20), in silico analysis reveals two *rsmA/E* homologs in *P. fluorescens* Pf-5, three in *P. fluorescens* SBW25, *P. fluorescens* Pf0-1, *P. putida* KT2440, and *P. syringae* pv. *syringae* B728a, four in *P. syringae* pv. *tomato* DC3000, and at least five in *P. syringae* pv. *phaseolicola* 1448A (http://pseudo.bham.ac.uk; http://pseudomonas-syringae.org). The genomic neighborhood of *rsmA* appears to be conserved in pseudomonads and consists of *lysC* located upstream and several tRNA genes located downstream. By contrast, the neighborhood of *rsmE*-like genes is variable, de-

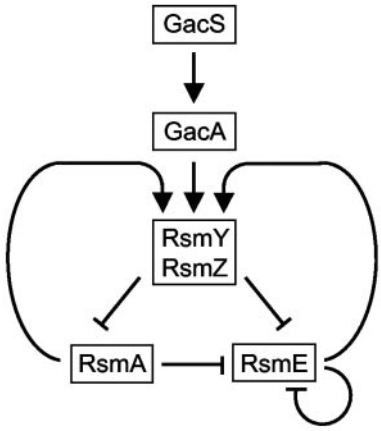

FIG. 10. Regulatory interactions operating in the Gac/Rsm cascade of *P. fluorescens* CHA0. See text for details. →, positive effect; ⊣, negative effect.

pending on the bacterial species. Thus, *rsmE* might have been recruited later in evolution and might reinforce the posttranscriptional control machinery steered by GacS/GacA. The roles of multiple *rsmA*/*E* homologs in some *Pseudomonas* spp. remain to be determined.

In conclusion, the fact that an *rsmA rsmE* double mutation fully suppresses a *gacS* defect (Fig. 2) indicates that RsmA and RsmE together represent the major negative control elements in the GacS/GacA cascade of *P. fluorescens* CHA0.

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