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Regulatory RNA as Mediator in GacA/RsmA-Dependent Global Control of Exoproduct Formation in *Pseudomonas fluorescens* CHA0

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In *Pseudomonas fluorescens* CHA0, an antagonist of root-pathogenic fungi, the GacS/GacA two-component system tightly controls the expression of antifungal secondary metabolites and exoenzymes at a posttranscriptional level, involving the RNA-binding protein and global regulator of secondary metabolism RsmA. This protein was purified from *P. fluorescens*, and RNA bound to it was converted to cDNA, which served as a probe to isolate the corresponding chromosomal locus, *rsmZ*. This gene encoded a regulatory RNA of 127 nucleotides and a truncated form lacking 35 nucleotides at the 3′ end. Expression of *rsmZ* depended on GacA, increased with increasing population density, and was stimulated by the addition of a solvent-extractable extracellular signal produced by strain CHA0 at the end of exponential growth. This signal appeared to be unrelated to *N*-acyl-homoserine lactones. A conserved upstream element in the *rsmZ* promoter, but not the stress sigma factor RpoS, was involved in *rsmZ* expression. Overexpression of *rsmZ* effectively suppressed the negative effect of *gacS* and *gacA* mutations on target genes, i.e., *hcnA* (for hydrogen cyanide synthase) and *aprA* (for the major exoprotease). Mutational inactivation of *rsmZ* resulted in reduced expression of these target genes in the presence of added signal. Overexpression of *rsmA* had a similar, albeit stronger negative effect. These results support a model in which GacA upregulates the expression of regulatory RNAs, such as RsmZ of strain CHA0, in response to a bacterial signal. By a titration effect, RsmZ may then alleviate the repressing activity of RsmA on the expression of target mRNAs.

The GacS/GacA two-component system controls the expression of secondary metabolism and protein secretion in a wide variety of bacterial species (19, 39). In the biocontrol strain *Pseudomonas fluorescens* CHA0, which protects plant roots from pathogenic fungi, a functional GacS/GacA system is strictly required for the production of extracellular antimicrobial agents such as 2,4-diacetylphloroglucinol, pyoluteorin, and hydrogen cyanide (HCN) and the production of enzymes such as tryptophan side chain oxidase, phospholipase C, and exoprotease (24, 25, 32, 37, 48, 52).

Structural genes whose expression is controlled by GacA in strain CHA0 include *hcnABC* for HCN synthase (6, 8, 25), *aprA* for the major exoprotease (8, 48), and *phlACBDE* for the 2,4-diacetylphloroglucinol biosynthesis enzymes (52). Translational *hcnA′-′lacZ* and *aprA′-′lacZ* fusions have proved to be useful reporter constructs to monitor signal transduction mediated by the GacS/GacA two-component system. By contrast, a transcriptional *hcnA-lacZ* fusion and the *hcnA* promoter are not subject to control by GacA, indicating that GacA regulates expression of target genes by a posttranscriptional mechanism in *P. fluorescens* (5, 8).

In *Erwinia carotovora* subsp. *carotovora*, the production of exoenzymes and virulence are posttranscriptionally controlled by the RsmA/RsmB system (11, 30). The RNA-binding protein RsmA and its homolog CsrA in *Escherichia coli* and *Salmonella enterica* can act as repressors of target genes at the mRNA level and as factors favoring mRNA decay (28, 29, 45). RsmB of *E. carotovora* and CsrB of *E. coli* are regulatory RNAs that complex the RsmA and CsrA proteins and thereby are assumed to titrate these repressors, allowing the translation of target mRNAs to proceed (18, 45). Searches in the GenBank databases (including sequences from unfinished microbial sequencing projects) show that homologs of RsmA/CsrA can be found in a large number of gram-negative bacteria as well as some gram-positive bacteria, e.g., *Bacillus subtilis* (12, 45).

In *P. fluorescens* CHA0, RsmA is an important negative control element involved in the posttranscriptional control of secondary metabolism regulated by the GacS/GacA system (7, 8). Therefore, it could be postulated that an RNA analogous to RsmB/CsrB would be required to counteract the repressing effects of RsmA on target genes and that the expression of this regulatory RNA would be directly or indirectly controlled by GacA. This hypothesis has received support from the recent finding that the GacS/GacA system positively controls the expression of RsmB in *E. carotovora* (12, 22). However, whereas the RsmA/CsrA protein sequences are well conserved in different bacteria, such sequence conservation is not observed for the antagonistic regulatory RNAs. No homologs of RsmB/CsrB could be detected in genomic databases of bacteria not belonging to the *Enterobacteriaceae*.

Aarons et al. (1) have reported the isolation of a *gacS* and *gacA* multicopy suppressor in *P. fluorescens* strain F113. This suppressor, PrrB, is a 133-nucleotide regulatory RNA whose sequence is unrelated to that of RsmB and CsrB. However, whether PrrB interacts with RsmA and how PrrB is regulated in strain F113 have not been reported.

The aims of the present study are to characterize a regulatory RNA that binds to RsmA in *P. fluorescens* CHA0 and to

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study the regulation of this RNA, which we designated RsmZ, by the GacS/GacA system, by the stress sigma factor RpoS, and by a novel bacterial signal.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains, vectors, and oligonucleotides used in this study are listed in Table 1. Strains were routinely grown in nutrient yeast broth (NYB; 2.5% [wt/vol] nutrient broth, 0.5% [wt/vol] yeast extract) with shaking, or on nutrient agar (NA; 4% [wt/vol] blood agar base, 0.5% [wt/vol] yeast extract) amended with the following antibiotics, when required: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; streptomycin, 20 μg/ml; tetracycline, 25 μg/ml (125 μg/ml for *P. fluorescens*); and gentamicin, 10 μg/ml. Chloramphenicol was used at a concentration of 10 μg/ml to select *P. fluorescens* and to counterselect *E. coli* in mating experiments. 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 (e.g., in transformation or triparental matings with *E. coli*).

DNA manipulations. Small-scale plasmid extractions were done by the cetyltrimethylammonium bromide method (13), and large-scale preparations were done by using the Qiagen Plasmid midi kit (Qiagen Inc.). Chromosomal DNA from *Pseudomonas* spp. was prepared as previously described (17). RNA was extracted with the High Pure RNA isolation kit (Roche Diagnostics) according to the manufacturer's recommendations. DNA manipulations were carried out as described (50). DNA fragments were purified from agarose gels with the Geneclean II Kit (Bio101, La Jolla, Calif.). Restriction sites shown in brackets indicate treatment of DNA ends with T4 DNA polymerase in the presence of deoxynucleoside triphosphates (dNTPs). DNA sequencing was performed with the Big Dye Terminator Cycle sequencing kit and an ABI-Prism 373 automatic sequencer (Applied Biosystems).

PCRs were typically carried out with 2.5 U of thermostable DNA polymerase (Extra-Pol II; Eurobio) in a reaction mixture containing 100 ng of target DNA, a 250 μM concentration of each of the four dNTPs (Roche), 10 pmol of two primers, 5 mM $MgCl_2$, and 1× Extra-Pol buffer in a final volume of 20 μl. For the amplification reaction, 25 cycles (1 min at 94°C, 1 min at 50 to 55°C [depending on the G+C content of the primers], and 1 min at 72°C) were followed by a final elongation step of 5 min at 72°C.

Southern and Northern blots. DNA was blotted from agarose gels to Hybond-N nylon membranes (Amersham). RNA for Northern blots was electrophoretically separated on denaturing urea-polyacrylamide gels (8.3 M urea, 8% acrylamide, 0.4% bisacrylamide) in 1× TBE buffer (50 mM Tris-borate [pH 8.3], 1 mM EDTA). The lane corresponding to the molecular weight markers (low range RNA ladder; Fermentas) was cut out, stained with TBE containing 5 μg of ethidium bromide per ml for 5 min, and photographed under UV light beside a scale reference; the rest of the gel was electroblotted at 150 mA (15 to 25 V) onto a Hybond-N membrane in TBE buffer for 10 min. All membranes were washed with 20× SSC (50) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before nucleic acids were cross-linked by exposing the membranes to UV for 5 min. Southern and Northern hybridizations were done with digoxigenin (DIG)-labeled DNA probes or DIG-labeled oligonucleotides (Table 1; obtained from Microsynth, Switzerland) according to recommended protocols (Roche's DIG System User's Guide for Filter Hybridization). Probes were produced by replacing dNTPs in a standard PCR with DIG DNA labeling mix (Roche) before purifying the product by phenol-chloroform extraction and ethanol precipitation.

Cloning and overexpression of *rsmA* from *P. fluorescens* CHA0. The 311-bp *Bam*HI-*Sma*I insert of pME3849 carrying the *Pseudomonas aeruginosa rsmA* gene was used as a probe to clone a hybridizing 1.2-kb *Pst*I genomic fragment from *P. fluorescens* CHA0 into pBLS, resulting in pME6071. The 0.54-kb *Pst*I-*Pvu*II *rsmA* fragment of pME6071 was inserted into *Pst*I- and *Pvu*II-digested pME6001. This produced, in pME6072, a translational fusion between the N-terminal part of the LacZα′ peptide from pME6001 and RsmA. To allow translation of *rsmA* from its natural initiation codon, pME6072 was digested with *Pst*I and treated with T4 DNA polymerase prior to ligation. The resulting plasmid, pME6073, had a 4-bp deletion interrupting *lacZ*α′ translation with a stop codon 32 bp upstream of the start codon of *rsmA*.

Chromosome walking upstream of *rsmA*. The *Pst*I fragment of pME6071 was subcloned into the *Pst*I-digested suicide plasmid pME3087 to produce pME6074. After integration of pME6074 into the chromosome of strain CHA0 by triparental mating using *E. coli* HB101/pME497 as the mobilizing strain (60), the integrated plasmid was rescued by digesting the chromosomal DNA with *Bam*HI and ligation. The resulting plasmid, pME6075, had an additional 6.8-kb segment upstream of the initial 1.2-kb *Pst*I *rsmA* fragment; the chromosomal insert was subcloned as an 8.0-kb *Bam*HI-*Hin*dIII fragment into pBLS, resulting in pME6079. A 6.0-kb *Sac*II deletion of this plasmid resulted in *rsmA* being bordered by 0.9 kb of upstream and 1.0 kb of downstream chromosomal DNA in pME6080. This 2.1-kb *Sac*II-*Pst*I fragment was sequenced (GenBank accession no. AF136151, Fig. 1).

Chromosomal inactivation of *rsmA*. The 2.0-kb ΩKm^r kanamycin resistance cassette of pHP45ΩKm was inserted into the *rsmA* gene at the eighth codon, i.e., at the *Fsp*I site, resulting in the pME3088 derivative pME6081. *rsmA*::ΩKm^r mutants were obtained by integration of the suicide plasmid pME6081 into the chromosome of strains CHA0, CHA19 (Δ*gacS*), and CHA806 (*aprA′-′lacZ* Δ*gacS*) after triparental mating as above, with selection for Km^r, tetracycline-resistant (Tc^r), chloramphenicol-resistant (Cm^r) recombinants. After a second crossing-over, Km^r Tc^s recombinants were obtained, producing strains CHA809, CHA808, and CHA807, respectively (Table 1).

Purification of histidine-tagged RsmA and cDNA synthesis from its associated RNA. RsmA was histidine-tagged by PCR using primers T3 and RSMA7 with pME6072 as the template. This was done by adding five histidine codons to the 3′ end of *rsmA* (the wild-type protein already has a histidine residue at its C terminus). The purified PCR product was treated with T4 DNA polymerase to produce blunt ends, digested with *Pst*I, and subcloned in the expression vector pME6032 (Table 1) to produce pME6078. Purification of the RsmA6H protein was performed as previously described (28): CHA0/pME6078 was grown in 200 ml of NYB at 30°C with shaking. When the optical density at 600 nm (OD_{600}) of the culture reached 0.5, 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added. The culture was further grown to an OD_{600} of ≈1.6. After centrifugation, RsmA6H was purified by Qiagen Ni-nitrilotriacetic acid (NTA)-agarose chromatography as recommended by the manufacturer (Qiagen QIAexpressionist handbook). The 50 mM potassium phosphate buffer, pH 8.0, containing 300 mM NaCl had the following imidazole concentrations: lysis, 10 mM; wash, 20 mM; and elution, 250 mM. The eluate from the Ni-NTA columns typically contained about 200 μg of protein, as determined by the method of Bradford (9), and about 120 μg of nucleic acids, as determined by absorption at 260 nm.

RNA was purified from the eluate by two extractions with phenol-chloroform (1:1) and once with chloroform. Salts were removed from the RNA with the High Pure RNA isolation kit (Roche) according to the manufacturer's manual. cDNA synthesis was performed as previously described (28) after polyadenylation of RNA with poly(A) polymerase (Gibco-BRL) and by using the Universal Riboclone cDNA synthesis system (Promega). The resulting blunt-ended cDNA fragments were subcloned into *Sma*I-digested, calf intestinal phosphatase-treated pBLS. In this way, pRSMCDNA7 and six other clones were obtained.

Cloning and overexpression of *rsmZ* from *P. fluorescens* CHA0. A DIG probe was made from plasmid pRSMCDNA7 by PCR using oligonucleotides T3 and T7. Southern hybridization of chromosomal DNA of CHA0 digested with *Xho*I and *Pst*I revealed a single 2.3-kb band, which was isolated and inserted into *Xho*I- and *Pst*I-digested pBLS, producing pME6084. The fragment of interest (*rsmZ*) was located in pME6084 on a 673-bp *Xho*I-*Eco*RI fragment (see Fig. 3A), which was subcloned into pBLS, resulting in pME6085. A PCR product obtained from pME6085 with primers PRSMCHA03 and PRSMCHA04 was digested with *Eco*RI and *Xho*I and subcloned into *Eco*RI- and *Xho*I-cut pME6032, producing pME6096. In this plasmid, transcription from P_{tac} starts 37 nucleotides upstream from the *Eco*RI cutting site (3).

To make the RsmZ transcript exactly from the +1 transcription start of P_{tac}, a 41-bp deletion was created in pME6096 by PCR using oligonucleotides TETA1 and TACSALI. Primer TACSALI joins the −1 nucleotide of P_{tac} to the first seven nucleotides of *rsmZ*, six of which naturally form a *Sal*I site (see Fig. 3A). Primer TETA1 is complementary to a region in the *tetA* tetracycline resistance marker in pME6096, annealing upstream of a *Sal*I site. The resulting 3.4-kb product was digested with *Sal*I and used to replace the corresponding 3.4-kb *Sal*I fragment in pME6096, producing pME6359. Since this construct was deleterious to the growth of *E. coli* strains even when the *tac* promoter was not induced, the last subcloning step was done by transforming *P. fluorescens* CHA806, in which this construct did not have a detrimental effect. The P_{tac}-*rsmZ* fusion on pME6359 was checked by sequencing.

Construction of *rsmZ-lacZ* fusions. Reporter plasmids were constructed carrying transcriptional *rsmZ-lacZ* fusions in which the +1 nucleotide of *lacZ* corresponds to the +1 nucleotide of the *rsmZ* promoter. First, the *rsmZ* promoter was amplified by PCR from pME6085 with primers T3 and PRSMCHA02. This fragment was digested with *Xho*I (located upstream of the *rsmZ* promoter) and *Pst*I (artificially introduced by PRSMCHA02 in the +1 region) before being ligated to the *Pst*I-*Cla*I *lacZ′* cassette of pME6522 and to *Cla*I- and *Sal*I-digested vector pUC6S. The *lacZ′* fusion to the *rsmZ* promoter was verified by sequencing

TABLE 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Description	Reference or source
E. coli		
HB101, DH5α, JM105	Laboratory strains	50
TR1-5BW3414	Δ*lacU169 csrA*::Km^r	46
P. fluorescens		
CHA0	Wild type	58
CHA19	Δ*gacS*	F. Carruthers
CHA89	*gacA*::Km^r	25
CHA207	Chromosomal *hcnA′-′lacZ* fusion	8
CHA89.207	Chromosomal *hcnA′-′lacZ* fusion, *gacA*::Km^r	8
CHA805	Chromosomal *aprA′-′lacZ* fusion	8
CHA806	Chromosomal *aprA′-′lacZ* fusion, Δ*gacS*	This study
CHA807	Chromosomal *aprA′-′lacZ* fusion, Δ*gacS rsmA*::ΩKm^r	8
CHA808	Δ*gacS rsmA*::ΩKm^r	This study
CHA809	*rsmA*::ΩKm^r	This study
CHA810	Δ*rsmZ*	This study
CHA811	Chromosomal *hcnA′-′lacZ* fusion, Δ*rsmZ*	This study
CHA812	Chromosomal *aprA′-′lacZ* fusion, Δ*rsmZ*	This study
CHA813	Chromosomal *aprA′-′lacZ* fusion, Δ*rpoS*	This study
CHA814	Chromosomal *hcnA′-′lacZ* fusion, Δ*rpoS*	This study
CHA815	Δ*rpoS*	This study
Plasmids		
pBLS	pBluescript II KS+ cloning vector, ColE1 replicon, Ap^r	Stratagene
pHP45ΩKm	ColE1 replicon, Ap^r Km^r	14, 43
pJF118EH	*lacI*^{q}-P$_{tac}$ expression vector, ColE1 replicon, Ap^r	16
pME497	Mobilizing plasmid; IncP-1, Tra; RepA(Ts); Ap^r	60
pME3087, pME3088	Suicide vectors; ColE1 replicon, IncP-1-Mob, Tc^r	58
pME3849	pME6001 with a 311-bp *Bam*HI-*Sma*I *rsmA* fragment of *P. aeruginosa* PAO1	42
pME6001	Cloning vector, pBBR1MCS Gm^r derivative	8, 33
pME6014	pVS1-p15A shuttle vector for translational *lacZ* fusions, Tc^r	52
pME6016	pVS1-p15A shuttle vector for transcriptional *lacZ* fusions, Tc^r	52
pME6031	pVS1-p15A shuttle vector, Tc^r	20
pME6032	*Nru*I-*Eco*RI *lacI*^{q}-P$_{tac}$ fragment of pJF118EH subcloned in [*Bam*HI]-*Eco*RI-digested pME6031; *lacI*^{q}-P$_{tac}$ expression vector	This study
pME6071	pBLS with a 1.2-kb *Pst*I fragment of strain CHA0 carrying *rsmA*	This study
pME6072	pME6001 with a 0.54-kb *Pst*I/*Pvu*II fragment of pME6071, with *lacZ′-rsmA* fusion	This study
pME6073	pME6072 with deletion of the *Pst*I site; *rsmA* under P$_{lac}$ promoter control	8
pME6074	pME3087 with 1.2-kb *Pst*I fragment of pME6071	This study
pME6075	*Bam*HI plasmid rescued from CHA0::pME6074, with 6.8-kb genomic fragment upstream of 1.2-kb *Pst*I fragment	This study
pME6078	*rsmA*, histidine tagged (RsmA6H) by PCR using primers T3 and RSMA7, subcloned in *Xho*I-[*Eco*RI]-cut pME6032	This study
pME6079	8.0-kb *Bam*HI/*Hin*dIII fragment of pME6075 in pBLS	This study
pME6080	6.0-kb *Sac*II deletion of pME6079	This study
pME6081	pME3088 carrying *rsmA* with ΩKm at its eighth codon	This study
pME6084	pBLS with a 2.3-kb *Xho*I-*Pst*I genomic fragment of strain CHA0, hybridizing with a probe from pRSMCDNA7	This study
pME6085	pBLS with the 673-bp *Xho*I-*Eco*RI fragment of pME6084	This study
pME6086	673-bp *Xho*I-*Eco*RI *rsmZ* fragment of pME6084 in pME3088	This study
pME6087	Plasmid rescued from CHA0::pME6086 cut with *Hin*dIII, with 1.8-kb genomic fragment upstream of *rsmZ*	This study
pME6090	pUC6S with an *rsmZ-lacZ′* fusion at the +1 site	
pME6091	*Bam*HI-*Cla*I fragment of pME6090 with P$_{rsmZ}$-*lacZ′* subcloned into *Bam*HI-*Cla*I-digested pME6016	This study
pME6092	pME6090 deleted for box (nucleotides −196 to −157) of *rsmZ* promoter	This study
pME6093	*Bam*HI-*Cla*I fragment of pME6092 with P$_{rsmZ\Delta box}$-*lacZ* subcloned into *Bam*HI-*Cla*I-digested pME6016	This study
pME6094	pUC6S with an *rsmZ-lacZ′* fusion at the *Ssp*I site of the *rsmZ* promoter	This study
pME6095	*Bam*HI-*Cla*I fragment of pME6094 with P$_{rsmZSspI}$-*lacZ′* subcloned in *Bam*HI-*Cla*I-digested pME6016	This study
pME6096	*rsmZ* under the P$_{tac}$ promoter at position +37 in pME6032	This study
pME6097	pME6085 deleted for nucleotides −243 to +125 of *rsmZ*	This study
pME6099	pME3087 containing *Hin*dIII-*Xho*I insert of pME6089 + *Xho*I-*Pst*I insert of pME6097	This study
pME6352	pME3088 containing a 945-bp in-frame deletion of *rpoS*	
pME6359	Deletion of pME6096 creating a P$_{tac}$-*rsmZ* fusion at the +1 site	This study
pME6522	Source of promoterless *lacZ* gene	8
pRSMCDNA7	pBLS, with a 90-bp cDNA made from RNA copurifying with RsmA6H	This study
pUC6S	Cloning vector, ColE1 replicon, Ap^r	57
Oligonucleotides[a] (5′→3′)		
DIGRSMZ1	TGATGGCTGTGTCTATCCGTCGAC (5′ DIG-labeled), 5′-specific probe for RsmZ	
DIGRSMZ2	AGGGGCGGTATGACCCGCCCACATT (5′ DIG-labeled), 3′-specific probe for RsmZ	
PRSMCHA02	CGACACTGCAGTGATATTAGAGAGTTCCC, reverse *rsmZ, Pst*I site at −2	
PRSMCHA03	TCAGAATTCTGTCGACGGATAGACACAG, forward *rsmZ, Eco*RI site at −6	
PRSMCHA04	AACTCGAGCGGGACTTTTCGACAGACG, reverse *rsmZ, Xho*I site at +147	
PRSMCHA05	ACGAATTCGGTTCTCGGCTACTTCTGCG, forward *rsmZ, Eco*RI site at −162	
PRSMCHA06	CATGAATTCTGAGAGTTAAGCCAACTTC, reverse *rsmZ, Eco*RI site at −196	
PRSMCHA07	CCACAAGCTTCGTGCAATAAAAAGCC, reverse *rsmZ, Hin*dIII site at −247	
PRSMCHA08	GCCCCTAAGCTTCGTCTGTCGAAAAGTCCCG, forward *rsmZ, Hin*dIII site at +122	
RPOSCHA03	CAAACTCGAGCACTTCTTTACTGAGAGCCATTG, reverse *rpoS, Xho*I site at the eighth codon	
TACSALI	CGCTGTCGACACCACACATTATACGAGCCGA, reverse P$_{tac}$, natural *Sal*I site at +7	
RSMA7	TTAGTGATGGTGATGGTGATGGCTTGGTTCTTCGTCC, reverse *rsmA*, adding 5 supplementary histidine codons to RsmA to produce RsmA6H	
TETA1	AACCCAAAGGAAAGGCGCTGTC, anneals between the *Eco*RV and *Sal*I sites of *tetA* in pME6032, oriented 5′ to 3′ toward the multiple cloning site	
T3, T7	pBLS primers	Stratagene

[a] Specified restriction sites are underlined.

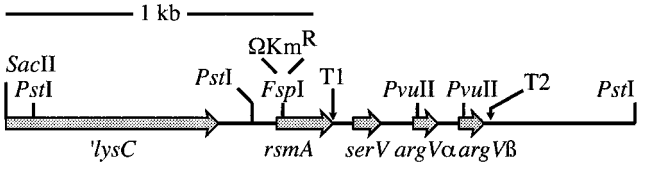


FIG. 1. *lysC-rsmA* region in strain CHA0. ΩKm^r, insertion in *rsmA* mutants CHA807, CHA808, and CHA809; T1 and T2, potential rho-independent terminators.

the resulting plasmid, pME6090. To complete the β-galactosidase open reading frame, the *Bam*HI-*Cla*I *rsmZ-lacZ'* cassette from pME6090 was subcloned into *Bam*HI- and *Cla*I-digested vector pME6016, resulting in plasmid pME6091. To delete nucleotides −196 to −157 of the *rsmZ* promoter, an inverse PCR was performed on pME6090 with primers PRSMCHA05 and PRSMCHA06 containing artificial *Eco*RI sites; the product was digested with *Eco*RI and ligated. The insert of the resulting plasmid, pME6092, was subcloned as a *Bam*HI-*Cla*I fragment as above, producing pME6093.

To construct a transcriptional *lacZ* fusion at position −94 at the *Ssp*I site (see Fig. 3A), the *Xho*I-*Ssp*I 253-bp fragment of pME6084, the [*Pst*I]-*Cla*I *lacZ'* cassette of pME6522, and pUC6S digested with *Cla*I and *Sal*I were joined in pME6094. The *Bam*HI-*Cla*I insert of pME6094 was then subcloned into pME6016 digested by the same enzymes to produce pME6095.

Chromosome walking upstream of *rsmZ*. The *Xho*I-*Eco*RI (*rsmZ*) fragment of pME6084 was subcloned into *Xho*I- and *Eco*RI-digested pME3088 to produce pME6086. Plasmid rescue of pME6086 integrated in the CHA0 chromosome with *Hin*dIII resulted in the formation of plasmid pME6087, which had an additional 1.9 kb upstream of the initial 673-bp *Xho*I-*Eco*RI fragment. The *Hin*dIII-*Xho*I fragment carrying *rpoS'* of pME6087 was sequenced together with the contiguous 2.3-kb *Xho*I-*Pst*I insert of pME6084 (GenBank accession number AF245440; see Fig. 3A)

Chromosomal deletion of *rsmZ*. After inverse PCR on pME6085 with primers PRSMCHA06 and PRSMCHA07, the product was digested with *Hin*dIII (sites generated by the primers) and ligated. In the resulting plasmid, pME6097, 374 bp were replaced by a *Hin*dIII site, producing a deletion spanning the *rsmZ* promoter as well as the whole *rsmZ* gene but leaving intact the flanking *rpoS* and *fdxA* transcription terminators (see Fig. 3A). To produce the suicide plasmid for gene replacement, pME6099, a triple ligation was performed between pME3087 digested with *Pst*I and *Hin*dIII, the *Hin*dIII-*Xho*I insert of pME6087, and the *Xho*I-*Pst*I insert of pME6097. The *rsmZ*-negative mutants CHA810, CHA811, and CHA812 were obtained by gene replacement in strains CHA0, CHA207, and CHA805, respectively, as above for inactivation of *rsmA*; the introduced *Hin*dIII site in *rsmZ* allowed detection of the mutation by Southern blot with an *Xho*I-*Eco*RI *rsmZ* probe.

In-frame deletion of *rpoS*. PCR with oligonucleotides RPOSCHA03 and T7 was done to amplify a 0.96-kb *rpoS'* fragment from pME6087. RPOSCHA03 creates an artificial *Xho*I site at the eighth codon of *rpoS*. The PCR product was digested with *Hin*dIII and *Xho*I and combined with the *Xho*I-*Eco*RI *'rpoS-rsmZ* fragment of pME6084 in suicide plasmid pME3088, resulting in an *rpoS* in-frame deletion of 315 codons (Δ*rpoS*) out of 336 in pME6352. Gene replacement of *rpoS* by Δ*rpoS* was done by double crossing-over of pME6352 in strains CHA805, CHA207, and CHA0, producing strains CHA813, CHA814, and CHA815, respectively, and verified by Southern hybridization.

Extraction of signal from cell-free culture supernatants. Strain CHA0 was grown in 500 ml of glycerol-Casamino Acids medium (52) in a 2-liter Erlenmeyer flask for 24 h with shaking to an OD_{600} of 2.0 to 2.5. Cells were removed by centrifugation. The supernatant was passed through a 0.45-μm-pore-size filter, adjusted to pH 5.0, and extracted once with 200 ml and twice with 150 ml of dichloromethane. The extracts were pooled, dried with anhydrous Na_2SO_4, filtered through Whatman paper, evaporated to dryness, and stocked at −20°C. Extracts dissolved in 500 μl of methanol were added at 2 μl per ml of culture (see Fig. 7).

Qualitative assays of protease, tryptophan side chain oxidase, and HCN. Proteolytic activity was detected on King's medium B agar (2% [wt/vol] Oxoid proteose peptone, 1% [vol/vol] glycerol, 6 mM $MgSO_4$, 6 mM K_2HPO_4, 1.5% [wt/vol] Oxoid agar No. 1) supplemented with 0.5% Blue Gelaspher, obtained from Ivo Safarik (49). HCN production was detected as previously described (59). Tryptophan side chain oxidase was assayed as described (38).

β-Galactosidase assays. β-Galactosidase activities were quantified by the Miller method (35), using cells permeabilized with 5% toluene.

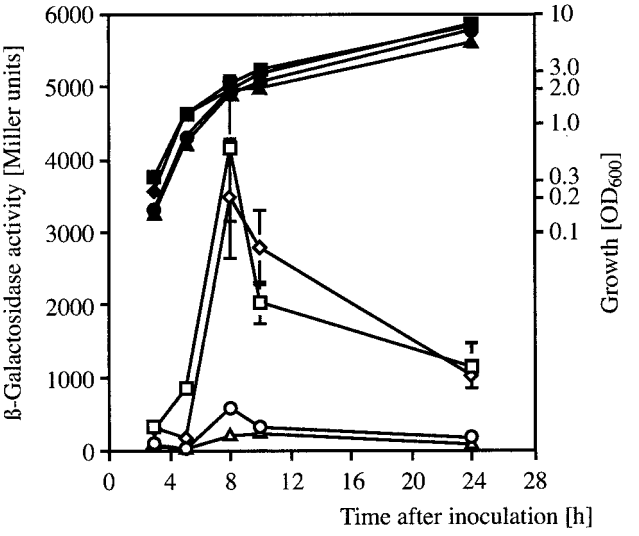


FIG. 2. Regulation of *aprA'-'lacZ* and *hcnA'-'lacZ* fusions in strains overexpressing the translational repressor RsmA from pME6073 or harboring the vector pME6001 alone in NYB. □, ■, CHA207 (*hcnA'-'lacZ*)/pME6001; ◇, ◆, CHA805 (*aprA'-'lacZ*)/pME6001; ○, ●, CHA207/pME6073; △, ▲, CHA805/pME6073. Open symbols, β-galactosidase activity; solid symbols, OD_{600}. Each value is the average from three different cultures ± standard deviation.

RESULTS

Characterization of the chromosome region containing *rsmA* in *P. fluorescens* CHA0. Previously we briefly reported the isolation of the *rsmA* gene from *P. fluorescens* CHA0 (8). When expressed from the multicopy plasmid pME6073 (8), this gene phenotypically complemented an *E. coli csrA*::Km^r mutant (46) for repression of glycogen synthesis (data not shown), demonstrating functional conservation of RsmA and CsrA. Using a genome walking approach (described in Materials and Methods), we found that the *rsmA* gene was flanked upstream by *lysC* (aspartokinase) and downstream by *serV* (seryl-tRNA, anticodon GCT) and *argV*α and *argV*β (two arginine-tRNAs, anticodons ACG) (Fig. 1). In *P. aeruginosa*, the genetic neighborhood is similar (42, 54), whereas in *E. coli*, *csrA* is located upstream of the same tRNA genes, but downstream of *alaS* (46).

Role of RsmA in the expression of GacS/GacA-regulated genes. A kinetic experiment (Fig. 2) illustrates that the cell density-dependent expression of a chromosomal *hcnA'-'lacZ* fusion in strain CHA207 was repressed about sixfold by overexpressed *rsmA*. Expression of the exoprotease gene *aprA* in strain CHA805 (also measured by a translational *lacZ* fusion) paralleled that of the *hcnA* gene (Fig. 2), establishing a repressive effect of RsmA throughout the growth cycle. By comparison, inactivation of either *gacS* or *gacA* reduces the expression of *hcnA* and *aprA* more strongly, about 50- to 100-fold (8). When the chromosomal *rsmA* gene was inactivated by insertion of an ΩKm cassette (Fig. 1) in a Δ*gacS* background (CHA807), the effect of the *gacS* mutation on a chromosomal *aprA'-'lacZ* reporter fusion was suppressed to about 30% during the late exponential phase (corresponding to OD_{600} of 2.0 to 2.5) (8). Furthermore, a Δ*gacS rsmA* double mutant

(CHA808) produced significant amounts of exoprotease, HCN, and tryptophan side chain oxidase by comparison with strain CHA19 (Δ*gacS*), which was negative for these phenotypes (data not shown). These results extend and corroborate the role of RsmA as a global negative control element acting downstream of GacA (8).

Characterization of an RsmA-associated RNA. To characterize RNAs that interact with RsmA in *P. fluorescens* CHA0, this protein was histidine tagged (RsmA6H) and purified by affinity chromatography. Following affinity chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the eluate revealed that most (about 90%) of the protein was represented by a band of about 7.0 kDa, corresponding to the expected size of RsmA6H (data not shown). RsmA6H was biologically active in vivo; strain CHA805 (*aprA′-′lacZ*) containing pME6078 (*lacI*^{q} P*tac-rsmA6H*) formed blue colonies on X-Gal plates, whereas on the same plates amended with 1 mM IPTG, the colonies were white to light blue. Nucleic acids separated from protein by phenol-chloroform extraction could be detected by ethidium bromide staining after agarose gel electrophoresis and were resistant to a 15-min treatment with RNase-free DNase at 37°C, but were completely digested by an equivalent treatment with DNase-free RNase (data not shown), suggesting that most of the nucleic acids associated with RsmA6H were composed of RNA.

cDNA was synthesized from this RNA and cloned into pBLS, as described in Materials and Methods. A clone having an insert of 90 bp (including a 10-bp stretch of adenines at the 3′ end) showed 77% identity to the regulatory RNA PrrB recently described in *P. fluorescens* F113 (1). Southern hybridization of the chromosome of strain CHA0 with a DIG probe made from this insert revealed a single *Xho*I-*Pst*I band of about 2.3 kb. This band was subsequently subcloned in pME6084. In this construct, the fragment of interest was located on a 679-bp *Xho*I-*Eco*RI fragment (Fig. 3A). Nucleotides 1 to 176 (starting from the *Xho*I side) show 100% identity with the 3′ end of the *P. fluorescens* Pf-5 *rpoS* gene (GenBank accession no. U34203) (51), and nucleotides 554 to 679 encode a polypeptide showing 85% identity and 93% similarity with the 41 C-terminal residues of the *Azotobacter vinelandii fdxA* gene product (GenBank accession no. J03521 and M63007) (26, 36). The *rpoS-fdxA* intergenic region shows 72% identity over 248 nucleotides with the *prrB* locus of *P. fluorescens* F113 (Fig. 3A). We conclude that the RNA bound to RsmA protein in *P. fluorescens* CHA0 is encoded by a gene which is related to *prrB* and which we designate *rsmZ*, to highlight the substantial sequence divergence between the two loci and the affinity of RsmZ RNA for RsmA protein.

No significant sequence similarity could be found between *rsmZ* and *rsmB* of various *Erwinia* strains or *csrB* of *E. coli* and *S. enterica*. Secondary-structure predictions, however, do reveal common features of RsmZ of strain CHA0 (Fig. 3B), PrrB of strain F113 (1), RsmB of *E. carotovora* (30), CsrB of *E. coli* (28), and CsrB of *S. enterica* (2). All these regulatory RNAs have several stem-loop structures, with AGG(G)A motifs in the loops. An alignment of the mapped *prrB* promoter with the corresponding *rsmZ* sequence shows sufficient similarity to predict the *rsmZ* transcription start (+1) site (Fig. 3A). A potential rho-independent transcription terminator (T1; Fig. 3A) is located downstream of the *rpoS* gene of *P. fluorescens* CHA0, analogous to the situation in *P. aeruginosa* PAO (56). A second terminator (T2) appears to be an integral part of the *rsmZ* transcript (see below); another terminator (T3) follows the *fdxA* gene (Fig. 3A).

Evidence for two RsmZ transcripts. Northern blot analysis of RNA extracted from strain CHA0 at various cell densities was done with a DIG-labeled probe hybridizing to the full-length *rsmZ*. Two bands of about 90 and about 130 nucleotides were revealed (Fig. 4A). The intensities of both RsmZ bands increased with increasing cell densities (Fig. 4A); the highest amount was found at an OD_{600} of 3.4, corresponding to the transition from exponential to stationary phase (Fig. 2). No signal was detected in RNA preparations from either an *rsmZ* deletion mutant (used as a control) or the *gacA* mutant CHA89 (Fig. 4A).

To answer the question of whether the two bands both originated from the same gene (*rsmZ*), Northern hybridizations were performed with three different probes: one covering the whole RsmZ transcript, and two oligonucleotides hybridizing to the 5′ or the 3′ end of the transcript. The probe specific for the 5′ end gave the same signals as did the full-length probe, while the probe specific for the 3′ end hybridized only to the larger band (Fig. 4B). This result and sequence analysis suggest that RsmZ is present in the cells as a 127-nucleotide RNA, together with a 92-nucleotide molecule lacking the last 35 nucleotides of the transcript, i.e., the part corresponding to the T2 rho-independent terminator (Fig. 3B). RsmZ may be first transcribed as a single RNA molecule together with its terminator, and then this terminator may be truncated at the 3′ end by a processing mechanism. Alternatively, transcription of *rsmZ* may stop at two sites lying 35 nucleotides apart.

Overexpression of *rsmZ* suppresses *gacA* and *gacS* mutations. The *rsmZ* gene was placed under the control of the inducible *tac* promoter in pME6359, so that the +1 site of the promoter coincided with the first *rsmZ* nucleotide. This construct, which was deleterious to *E. coli* strains such as DH5α and JM105, was obtained in *P. fluorescens*. In strain CHA806 (Δ*gacS aprA′-′lacZ*), pME6359 had no significant effect on growth, although some slight aggregation of cells on glass walls was observed. In this strain, even without IPTG induction, β-galactosidase activities were similar to those in the corresponding wild-type strain CHA805 (*gacS*$^+$ *aprA′-′lacZ*), indicating that excess RsmZ can restore *aprA′-′lacZ* expression in a *gacS* mutant to wild-type levels (Fig. 5A). When pME6359 was introduced into strain CHA89.207 (*gacA hcnA′-′lacZ*), β-galactosidase activities were much higher and occurred earlier than in the *gacA*$^+$ strain CHA207 carrying the same chromosomal reporter (Fig. 5B). Thus, a surplus of RsmZ can elevate *hcnA′-′lacZ* expression in a *gacA* mutant to levels above those present in the wild type.

Expression of *rsmZ* depends on GacS/GacA but not on RpoS. Results obtained by Northern hybridization (Fig. 4) suggest that *rsmZ* transcription depends on GacA and the growth phase. To quantify the regulation by GacA, the *lacZ* gene was placed directly under the control of the *rsmZ* promoter in plasmid pME6091 (Fig. 6). The resulting *rsmZ-lacZ* fusion was controlled by GacA, with an induction factor of about 35, after subtraction of the background activity of the vector control (pME6016; Fig. 6). The vector used in this

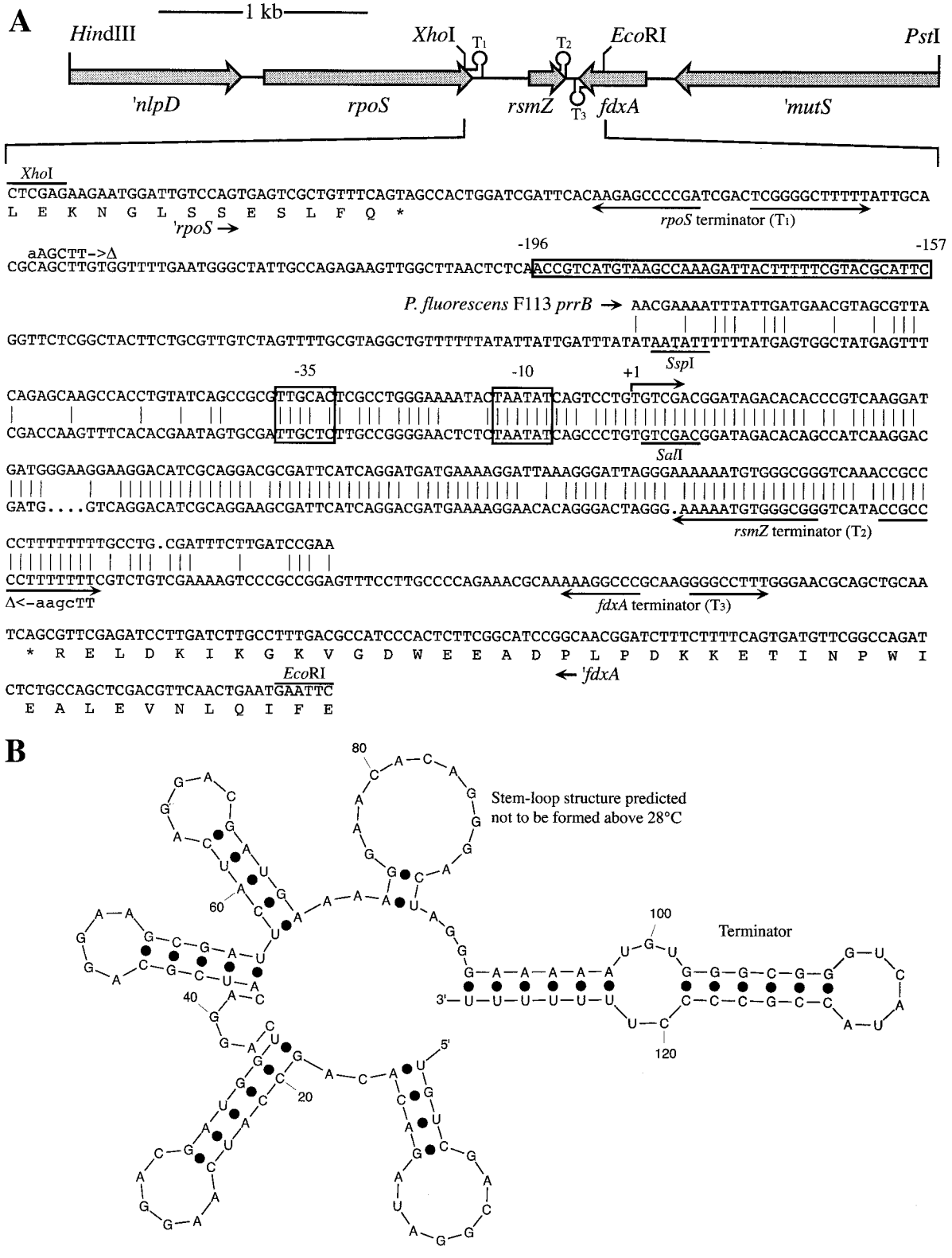


FIG. 3. (A) The 4.1-kb region of *P. fluorescens* CHA0 with *rpoS*, *rsmZ*, and *fdxA*. The *Hin*dIII sites introduced to produce the *rsmZ* deletion mutant are indicated by Δ. The published *prrB* sequence of *P. fluorescens* F113 and its −35, −10, and +1 sites (1) are aligned above the *rsmZ* sequence. Rho-independent terminators T1, T2, and T3, predicted by the method of Brendel and Trifonov (10), are indicated by arrows beneath the sequence. The nucleotides deleted in the pME6093 reporter plasmid are shown in a box. (B) Predicted secondary structure of RsmZ using the Mfold program (63).

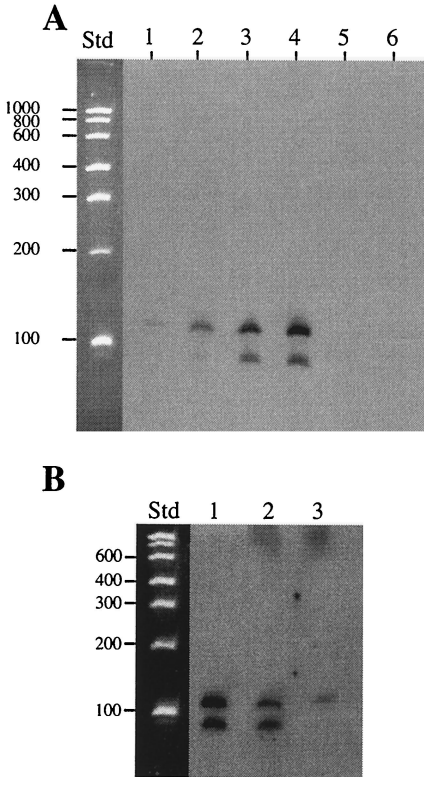


FIG. 4. (A) Northern blot of RsmZ. Total RNA was extracted from strains CHA0 (lanes 1 to 4), CHA810 (*rsmZ*) (lane 5), and CHA89 (*gacA*) (lane 6) grown in NYB and hybridized with a DIG-labeled probe covering the full-length *rsmZ* transcript. In each lane, 3 μg of RNA was loaded. OD_{600} values at the time of harvesting were, in lanes 1 to 6, 0.5, 1.0, 1.5, 3.4, 2.4, and 2.6, respectively. (B) Northern blot of total RNA extracted from strain CHA0 at an OD_{600} of 3.4 was hybridized with the same probe as in panel A (lane 1), with the DIG-labeled oligonucleotide DIGRSMZ1, specific for the 5′ end of the transcript (lane 2), or with the DIGRSMZ2 probe, specific for the 3′ end (lane 3). X-ray film exposure for chemiluminescent detection was 2 min for lane 1 and 40 min for lanes 2 and 3. Std, RNA standards; sizes are shown in bases.

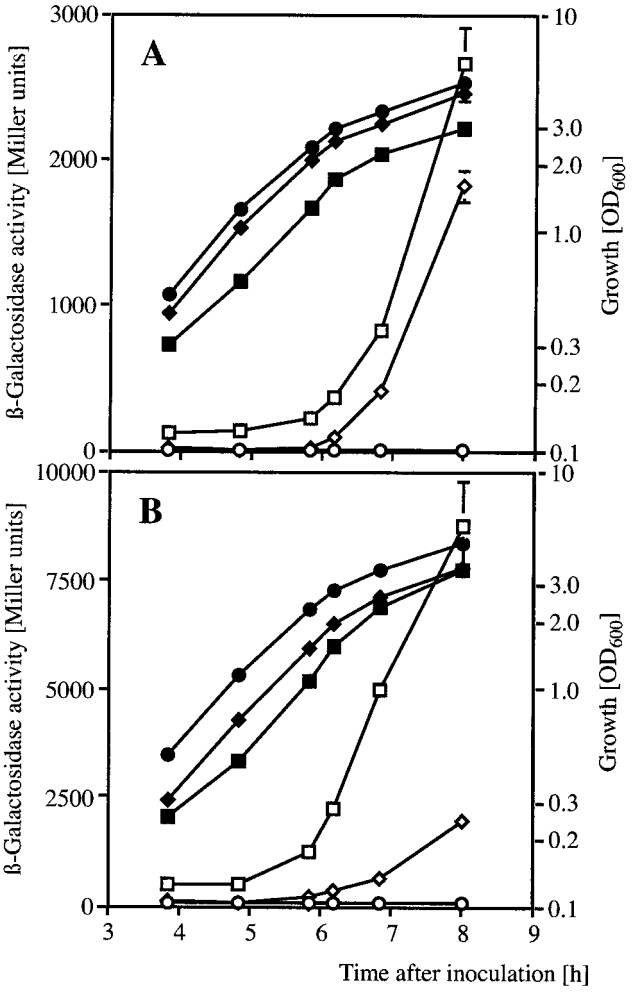
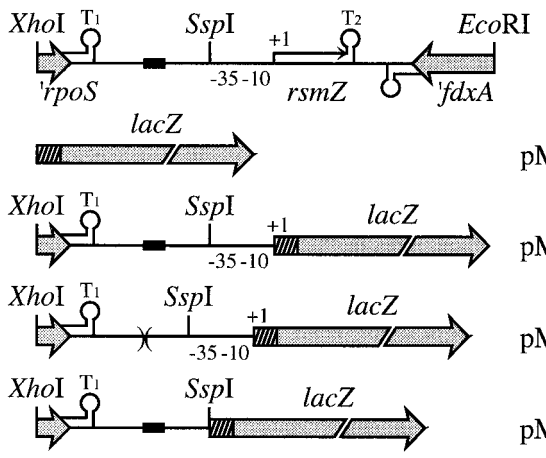


FIG. 5. Suppression of *gacS* and *gacA* mutations by overexpressed RsmZ. (A) Expression of a chromosomal *aprA′-′lacZ* translational fusion and growth were determined in a wild-type context (◇, ◆, CHA805/pME6032), in a *gacS* mutant (○, ●, CHA806/pME6032), and in a *gacS* mutant overexpressing *rsmZ* (□, ■, CHA806/pME6359). (B) Expression of a chromosomal *hcnA′-′lacZ* translational fusion and growth in a wild-type context (◇, ◆, CHA207/pME6032), in a *gacA* mutant (○, ●, CHA89.207/pME6032), and in a *gacA* mutant overexpressing *rsmZ* (□, ■, CHA89.207/pME6359). Open symbols, β-galactosidase; solid symbols, OD_{600}. Each value is the average from three different cultures ± standard deviation.

experiment has about 5 to 7 copies (20). A transcriptional *lacZ* fusion constructed at −90 upstream of the transcription start was devoid of activity (pME6095; Fig. 6), indicating that there is no readthrough transcription from the upstream *rpoS* gene. The *rpoS* gene itself was not required for *rsmZ-lacZ* expression, in that an *rpoS* in-frame deletion had no effect on this reporter system (Fig. 6). The β-galactosidase activities resulting from chromosomal *aprA′-′lacZ* and *hcnA′-′lacZ* fusions were 20 to 30% higher in a Δ*rpoS* mutant (CHA813 and CHA814, respectively) than in the wild-type background grown to early stationary phase (data not shown), suggesting that the effects of RpoS on *aprA* and *hcnA* expression are not mediated by modulation of RsmZ expression.

Examination of the *rsmZ* promoter region reveals an upstream sequence (positions −192 to −161; Fig. 3A) which is remarkably well conserved (≥53% identity) in *P. aeruginosa*, *Pseudomonas syringae* pv. phaseolicola, *P. syringae* pv. syringae, and two strains of *Pseudomonas putida* (see Discussion). To investigate the role of this conserved sequence in the expression of *rsmZ* of *P. fluorescens* CHA0, pME6093 was constructed, carrying an *rsmZ-lacZ* fusion in which nucleotides −196 to −157 upstream of the promoter were deleted (Fig. 3A). This deletion caused a marked reduction in the expression of the reporter fusion and completely abolished the control by GacA (Fig. 6). This suggests that either GacA itself or a regulatory element controlled by GacA interacts with this upstream sequence, to activate the *rsmZ* promoter.

Evidence for quorum-sensing control of *rsmZ* expression. The *rsmZ* gene was expressed most strongly at the end of exponential growth, at cell densities above 2×10^9 cells/ml ($OD_{600} \approx 2.0$). This was seen in Northern blots (Fig. 4A) as well as with an *rsmZ-lacZ* fusion (Fig. 7). This regulatory pattern could be the consequence of some type of quorum sensing (cell density-dependent) control involving a diffusible



	ß-Galactosidase activity (Miller units)		
	wild-type	*gacA*	*rpoS*
pME6016	318 ± 6	262 ± 39	ND
pME6091	2692 ± 337	330 ± 70	2817 ± 172
pME6093	620 ± 169	338 ± 40	ND
pME6095	195 ± 8	137 ± 5	ND

FIG. 6. Regulation of *rsmZ* expression. β-Galactosidase activities of transcriptional *rsmZ-lacZ* fusions containing the full promoter (pME6091), the promoter with the conserved upstream sequence (black box) deleted (pME6093), or no promoter (pME6095) in strains CHA0 (wild type), CHA89 (*gacA*), and CHA815 (*rpoS*). As a background control, the reporter vector pME6016 carrying the *lacZ* gene with its ribosome-binding site (dashed box) was used. ND, not determined. Each value is the average ± standard deviation from three different overnight cultures grown to an OD_{600} of ≈3.0

signal. Therefore, we searched for signal activity in cultures of the wild-type CHA0 grown to early stationary phase in NYB or rich glycerol-Casamino Acids medium. A dichloromethane extract from CHA0 culture supernatants was found to advance and to stimulate markedly the expression of the *rsmZ* gene (Fig. 7). Uninoculated medium did not contain the signal, and little inducing activity was found in exponentially growing cultures.

Signal activity was not affected by treatment of culture supernatants at pH 12 and 30°C for 30 min. Under these conditions, *N*-acyl-homoserine lactones (AHLs), which are typical

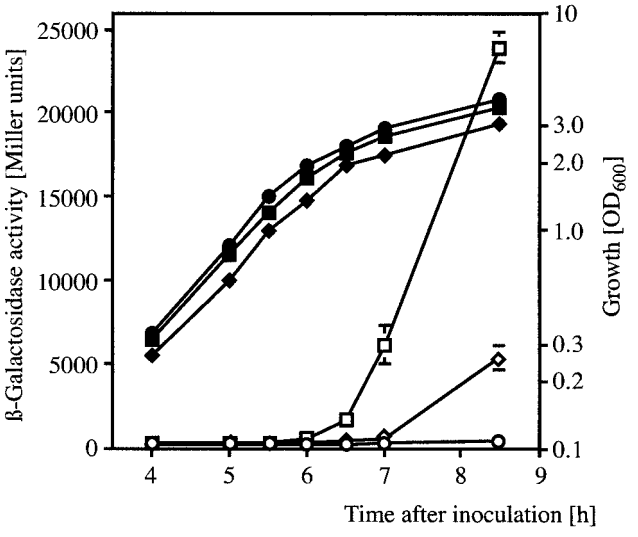


FIG. 7. Regulation of *rsmZ* expression and stimulation by a signal from spent medium. β-Galactosidase activities of a transcriptional *rsmZ-lacZ* fusion carried by pME6091 were determined in strain CHA0 (◇, ◆), in strain CHA0 amended with extract from spent medium (□, ■), and in the *gacA* mutant CHA89 (○, ●). Open symbols, β-galactosidase activity; solid symbols, OD_{600}. Each value is the average from three different cultures ± standard deviation.

cell density-related signal molecules in a range of gram-negative bacteria (55), are inactivated. Moreover, the *hcnA'-'lacZ* reporter strain CHA207 was not induced by addition of various AHL compounds at concentrations of 1 to 70 μM (data not shown). Furthermore, two reporter systems that detect a wide spectrum of AHLs, *Chromobacterium violaceum* CV026 (34) and *Agrobacterium tumefacien*s with a *traG-lacZ* fusion (53), failed to show any reaction with extracts from CHA0 culture supernatants. By contrast, when the *P. aeruginosa rhlI* gene encoding *N*-butanoyl-homoserine lactone synthase is expressed in strain CHA0, the corresponding AHL product is readily detectable (40), ruling out the possibility that some CHA0 exoproducts might mask intrinsic AHL activity. Together, these results suggest that the inducing signal of strain CHA0 is not of the AHL type. Furthermore, the apparent absence of *luxS* genes from pseudomonads argues against the possibility that the signal molecule of strain CHA0 could be related to autoinducer 2 (AI-2) of *Vibrio harveyi* and other bacteria (55).

Effect of an *rsmZ* null mutation on *aprA* and *hcnA* expression. Since *rsmZ* expression is controlled by GacA (Fig. 4A) and since RsmZ RNA can bind RsmA, an *rsmZ* null mutation should lower the expression of target genes such as *aprA* and *hcnA*. Expression of *aprA'-'lacZ* and *hcnA'-'lacZ* is maximal during late exponential phase, corresponding to an OD_{600} of 2.0 to 2.5 (Fig. 2). However, during this phase, GacA-dependent expression of *rsmZ* is weak (Fig. 4A) unless the inducing signal is added (Fig. 7). With these considerations in mind, we deleted the chromosomal *rsmZ* gene in strain CHA810, resulting in the complete absence of RsmZ RNA (Fig. 4A). In strains carrying either a chromosomal *aprA'-'lacZ* fusion (CHA812) or an *hcnA'-'lacZ* fusion (CHA811), deletion of *rsmZ* had a very slight effect on the expression of these reporters at an OD_{600} of ≥2.0 (Fig. 8). However, when the RsmZ inducing signal was added, a clear difference of about 20% was observed between the Δ*rsmZ* mutant and the *rsmZ*$^+$ parent at an OD of ≥2.0 (Fig. 8). These results suggest that, under

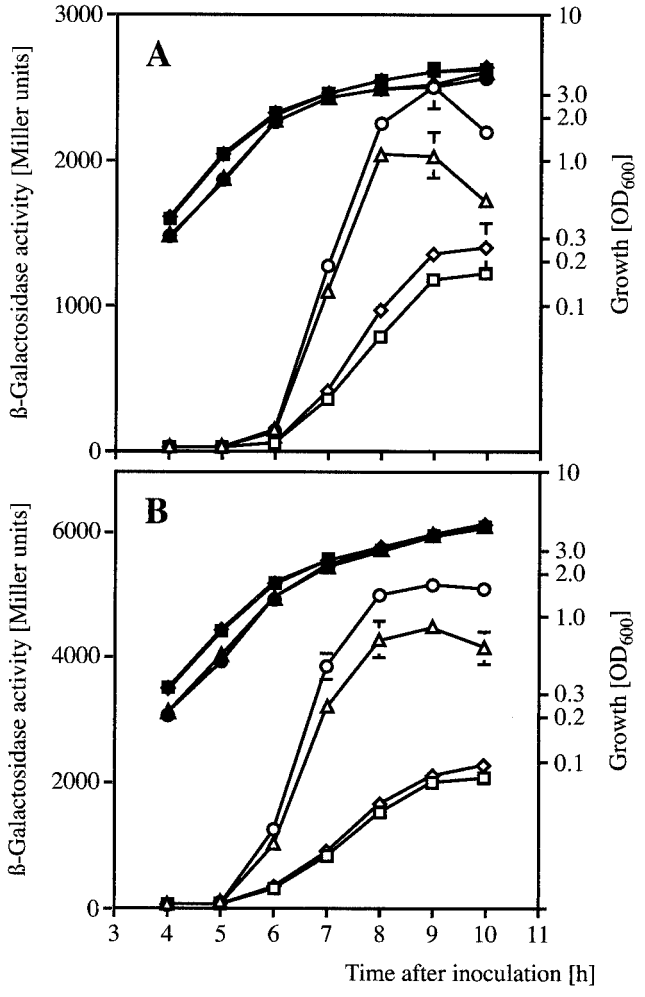


FIG. 8. Effect of an *rsmZ* deletion on expression of the *aprA* and *hcnA* genes. (A) β-Galactosidase activities of a chromosomal *aprA'-'lacZ* translational fusion and growth were measured in the *rsmZ*$^+$ strain CHA805 without (◇, ◆) and with extract (○, ●) and in the *rsmZ* mutant CHA812 without (□, ■) and with extract (△, ▲). (B) β-Galactosidase activities of a chromosomal *hcnA'-'lacZ* translational fusion and growth were determined in the *rsmZ*$^+$ strain CHA207 without (◇, ◆) and with extract (○, ●) and in the *rsmZ* mutant CHA811 without (□, ■) and with extract (△, ▲). Open symbols, β-galactosidase; solid symbols, OD_{600}. Each value is the average from three different cultures ± standard deviation.

inducing conditions, RsmZ can alleviate RsmA-mediated posttranscriptional repression of the two target genes.

DISCUSSION

In many gram-negative bacteria, the GacS/GacA two-component system has important regulatory functions in the synthesis of extracellular products and in the adaptation to diverse environmental conditions. In animal and plant pathogens, the GacS/GacA system controls the expression of virulence factors (2, 12, 20a, 22, 39). In plant-beneficial, root-colonizing bacteria such as *P. fluorescens* CHA0 and Pf-5, the GacS/GacA system is required for the production of antifungal secondary metabolites and exoenzymes (1, 25, 48, 52, 58, 62). The structural genes for HCN biosynthesis (*hcnABC*) and exoprotease (*aprA*) have proved useful for studying the regulatory cascade initiated by the GacA response regulator in *P. fluorescens* CHA0.

We have previously obtained genetic evidence that GacA indirectly regulates the expression of these structural genes at a posttranscriptional level, involving the RNA-binding protein RsmA as a downstream regulatory element (5–8). In the present study, we have characterized a regulatory RNA termed RsmZ, which was found to bind to RsmA and, when overproduced, caused strong derepression of *aprA* and *hcnA* expression. By analogy with the RsmA(CsrA)/RsmB(CsrB) system in enteric bacteria, this derepression is thought to result from sequestration of RsmA (12, 18, 30, 45). While this work was in progress, a *gacS*/*gacA* multicopy suppressor, PrrB, was reported in *P. fluorescens* F113 (1). PrrB RNA has 91% sequence identity with RsmZ of *P. fluorescens* CHA0 (Fig. 3A).

There are several arguments for RsmZ being part of the GacA regulon. (i) Expression of *rsmZ* strongly depended on *gacA* (Fig. 4A and 6). (ii) Overexpression of *rsmZ* suppressed *gacS* and *gacA* mutations by >100% (Fig. 5). (iii) Expression of *rsmZ* was stimulated markedly by a solvent-extractable signal produced by the bacteria at high cell densities (Fig. 7). This non-AHL signal is produced under GacS/GacA control and requires a functional GacS/GacA system to exert its positive effect on secondary metabolism in strain CHA0 (our unpublished results). (iv) In the presence of added signal, an *rsmZ*-negative mutant showed reduced expression of the *hcnA* and *aprA* genes (Fig. 8).

These findings lend support to a model (8, 20a) whose validity has recently also been assessed in *E. carotovora* (12, 22). The GacS/GacA system upregulates the synthesis of the regulatory RNA RsmZ during late exponential phase; in strain CHA0 this occurs under the influence of a diffusible bacterial signal. RsmZ then binds to and titrates RsmA, preventing this protein from acting as a translational repressor and/or mRNA decay factor (45). The sigma factor RpoS does not seem to upregulate *rsmZ* expression (Fig. 6). Similarly, RpoS does not activate transcription of CsrB in *E. coli* (18). Our simplified linear model explains the facts observed in this study, but may have to be refined later for the following reasons. First, an *rsmA* mutation suppressed a *gacS* defect only to 30 to 60%, depending on the reporter construct used (8), and added signal retained a weak inducing effect in an *rsmA* mutant (data not shown). Therefore, it is likely that additional negative control elements exist in the GacA regulon. Second, an *rsmZ* mutation caused a relatively small loss of *hcnA* and *aprA* expression. Similarly, mutational inactivation of *prrB* in strain F113 reduces the synthesis of 2,4-diacetylphloroglucinol but has no detectable effect on HCN and exoprotease production (1), and in *S. enterica csrB* is not required for the expression of invasion genes but has a dramatic effect when overproduced (2).

It seems possible that RsmZ might be only one among several regulatory RNAs. Indeed, recent studies on *E. coli* have uncovered at least 10 new regulatory RNAs whose abundance increases towards the end of exponential growth (4, 61). Similar RNAs may exist in pseudomonads as well, and such RNAs may be elements of the GacA regulon. Our preliminary unpublished results suggest that strain CHA0 contains at least one additional RNA which copurifies with RsmA and is positively regulated by GacA. Third, the mRNA target specificity has been addressed only in the case of the *hcnA* 5′ untranslated

leader sequence, where a stretch of about 10 nucleotides surrounding the ribosome-binding site determines regulation by GacA and RsmA (8). Although a similar sequence motif can be found in the *aprA* leader (8), not all GacA-controlled genes appear to have this motif. Additional specificity determinants are likely to exist in the GacA regulon.

The gene organization *rpoS-rsmZ-fdxA-mutS* found in *P. fluorescens* CHA0 (Fig. 3A) is maintained in *P. aeruginosa* PAO (GenBank accession no. AE004782 and D26134) (15), *P. syringae* pv. syringae B728a (GenBank AF208000), and *P. putida* strains WCS358 (GenBank Y19122) (23) and Corvallis (GenBank AF178851), but not in enteric bacteria such as *E. coli* or *Salmonella* spp., in which the *rpoS-mutS* intergenic region is highly variable in composition and size and does not include *csrB* (21, 27). In *Azotobacter vinelandii, fdxA* is also located downstream of *mutS* (GenBank M63007) (26), but the genes lying further downstream are not yet known. An alignment of the *rpoS-rsmZ* intergenic region in fluorescent pseudomonads shows very little sequence conservation, with one exception, the *rsmZ* upstream box (−196 to −157; Fig. 3A). This box contains some conserved motifs (notably CNTGTAAG) which also occur upstream of the TRR locus in *P. syringae* pv. phaseolicola (47). TRR overexpression overrides thermoregulation of phaseolotoxin synthesis in this bacterium (47) by an unknown mechanism. It is conceivable that the TRR product is a regulatory RNA. The conserved box upstream of *rsmZ* in *P. fluorescens* CHA0 is essential for *rsmZ* expression and regulation by GacA (Fig. 6).

Regulatory RNAs interacting with RsmA in various bacteria appear to lack conservation of size and primary sequence, which makes detection of the corresponding genes difficult in genomic sequence databases. For example, in *Enterobacteriaceae*, RsmB and CsrB primary transcripts are relatively large (260 to 480 nucleotides), whereas PrrB and RsmZ of fluorescent pseudomonads are much smaller (about 130 nucleotides). Perhaps a combination of characteristic secondary structures—multiple stems—with AG(G)GAs motif in loops (1, 29, 31) accounts for the interaction of these regulatory RNAs with RsmA.

In some gram-negative bacteria, e.g., *E. carotovora* and *P. aeruginosa*, GacA positively controls the production of AHLs. By influencing the concentrations of these quorum-sensing signals, GacA can modulate the expression of exoproduct genes (11, 41, 44). However, in the same organisms, GacA control of exoproduct genes also follows an AHL-independent signal transduction pathway (11, 41). Such an AHL-independent GacA cascade appears to be responsible for the regulation of exoproducts in *P. fluorescens* CHA0. This strain's non-AHL signal stimulates the expression of *rsmZ* (Fig. 7 and 8) as well as that of the structural genes *aprA*, *hcnA*, and *phlA*, but does not influence housekeeping gene expression (S. Heeb, C. Gigot-Bonnefoy, M. Péchy, C. Reimmann, and D. Haas, unpublished results). The chemical nature of this signal and its mode of action are currently under investigation.

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