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Two GacA-Dependent Small RNAs Modulate the Quorum-Sensing Response in *Pseudomonas aeruginosa*†

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In *Pseudomonas aeruginosa*, the GacS/GacA two-component system positively controls the quorum-sensing machinery and the expression of extracellular products via two small regulatory RNAs, RsmY and RsmZ. An *rsmY rsmZ* double mutant and a *gacA* mutant were similarly impaired in the synthesis of the quorum-sensing signal *N*-butanoyl-homoserine lactone, the disulfide bond-forming enzyme DsbA, and the exoproducts hydrogen cyanide, pyocyanin, elastase, chitinase (ChiC), and chitin-binding protein (CbpD). Both mutants showed increased swarming ability, azurin release, and early biofilm development.

In many gram-negative bacteria, the GacS/GacA two-component system regulates the expression of extracellular products, and these can be virulence determinants in pathogenic species. Although the signals that activate the GacS/GacA system have not been identified, it is evident from studies in *Escherichia coli*, *Vibrio cholerae*, *Erwinia carotovora*, *Legionella pneumophila*, *Pseudomonas fluorescens*, and other bacteria that activation takes place when cells grow to high densities, particularly during the transition from exponential to stationary phase (9, 13, 14, 16, 21, 45). Therefore, GacS/GacA-dependent gene regulation can be considered part of the quorum-sensing machinery (15, 16). The activated response regulator GacA strongly turns on the transcription of one or several genes encoding small RNAs, termed *csrB*/*csrC* (in *E. coli* and *Salmonella enterica*), *csrB*/*csrC*/*csrD* (in *V. cholerae*), *rsmB* (in *E. carotovora*), and *rsmX*/*rsmY*/*rsmZ* (in *P. fluorescens*) (7, 13, 14, 16, 45). These small regulatory RNAs have a high affinity for certain RNA-binding proteins that act as translational repressors, e.g., CsrA (in *E. coli*, *S. enterica*, and *V. cholerae*), RsmA (in *E. carotovora*), and RsmA/RsmE (in *P. fluorescens*) (7, 16, 17, 29, 30). Repeated ANGGA motifs (where N is any nucleotide) in target mRNAs are binding sites for CsrA, and when the most distal of these motifs coincides with the ribosome-binding site, translation of the mRNA can be repressed (4). The small Csr/Rsm RNAs have flower-like secondary structures with multiple GGA motifs in unpaired regions (10, 14, 16, 45). In the case of RsmY of *P. fluorescens*, it has been shown that the GGA repeats are essential for interaction with RsmA and RsmE (40).

In the opportunistic pathogen *Pseudomonas aeruginosa*, the Gac/Rsm system positively regulates the expression of the quorum-sensing signal *N*-butanoyl-homoserine lactone (C4-HSL) and of extracellular virulence factors, such as hydrogen cyanide (HCN), pyocyanin, and elastase (26, 28). *P. aeruginosa gacA* mutants are less virulent in animal and plant models in comparison with the wild type (27). In the Gac/Rsm signal transduction pathway, GacA is required for the transcription of the small RNA gene *rsmZ*. RsmZ RNA acts as an antagonist of the unique RNA-binding protein RsmA, which negatively controls the expression of quorum sensing and several extracellular products (2, 12, 26). During exoproduct expression, the Gac/Rsm system can have a dual mode of action. In the case of HCN biosynthesis, for example, about 30% of the Gac/Rsm input is due to enhanced expression of the *rhlI* gene encoding C4-HSL synthase. C4-HSL activates the transcription regulator RhlR which, together with the transcription factors LasR and ANR, turns on the transcription of the HCN biosynthetic genes *hcnABC*. About 70% of the Gac/Rsm input bypasses *N*-acyl-homoserine lactone-dependent transcriptional regulation and results directly in enhanced translation of *hcnABC* mRNA (24, 25).

Transcriptional regulation of *rsmZ* in *P. aeruginosa* is complex. In addition to GacS, the sensors RetS and LadS also determine the expression of the *rsmZ* gene. When RetS is deficient, *P. aeruginosa* cells clump and their type III secretion system is switched off. Deficiency of either GacS or LadS results in reduced biofilm maturation and increased type III secretion. GacS and LadS positively regulate *rsmZ* expression, whereas RetS has the opposite effect (8, 41). Furthermore, the *rsmZ* promoter is positively controlled by RsmA; this effect is probably indirect (12).

In *V. cholerae* and *P. fluorescens*, the GacS/GacA system activates the transcription of three functionally redundant small RNAs, whereas in *E. coli* and *S. enterica* two GacA-controlled small RNAs have been described (7, 14, 16, 45). By contrast, only one regulatory RNA that depends on GacS/GacA has been described in *E. carotovora* (17). Here we ex-

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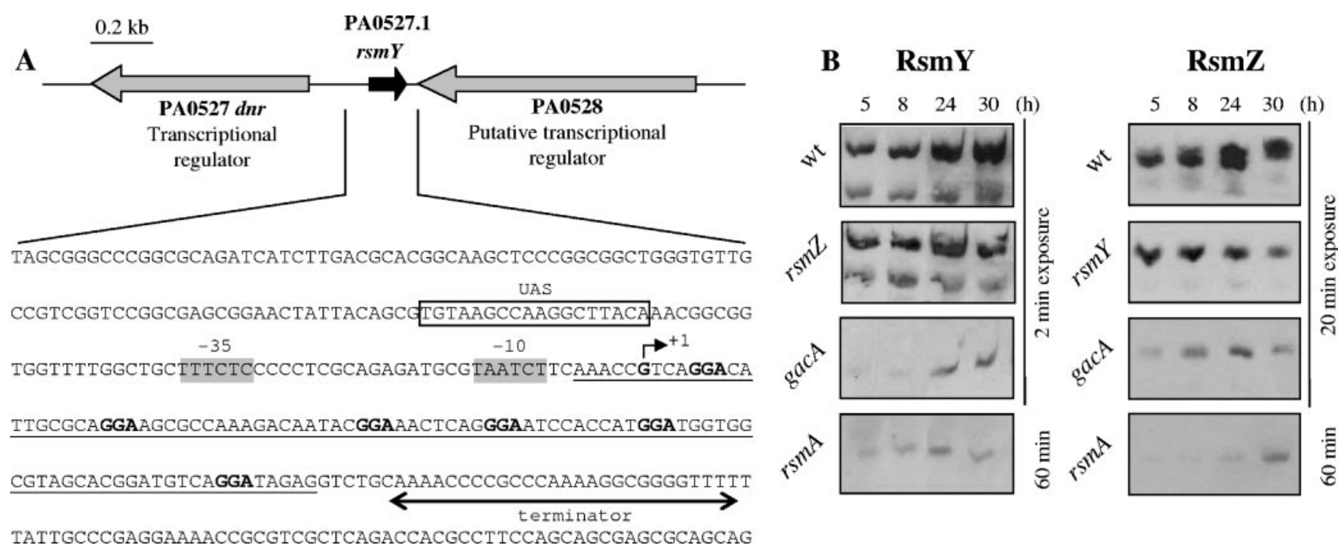

FIG. 1. Expression of the *rsmY* gene in *P. aeruginosa*. (A) Organization of the *rsmY* region in strain PAO1. The deduced −35 and −10 promoter sites are indicated with gray boxes. The palindromic sequence boxed from −75 to −58 denotes an upstream regulatory sequence (UAS), which is highly conserved in GacA-regulated genes (16, 39). Arrows indicate the putative *rsmY* transcription terminator. The 96-bp deletion in the *rsmY* gene (underlined) of the mutants PAO6420 and PAO6421 was verified by PCR and Southern blotting (data not shown). Conserved GGA motifs are shown in boldface. (B) Regulation of *rsmY* and *rsmZ* expression. The Northern blot shows the differential temporal accumulation of RsmY and RsmZ RNAs in PAO1 (wild type), PAO6354 (Δ*rsmZ*), PAO6420 (Δ*rsmY*), PAO6281 (*gacA*), and PAZH13 (*rsmA*). Total RNA was extracted from cells grown at 37°C in 200 ml of NYB, electrophoretically separated on a denaturing urea-polyacrylamide gel (8.3 M urea, 8% [wt/vol] acrylamide, 0.4% [wt/vol] bisacrylamide) in 50 mM Tris-borate, pH 8.3, 1 mM EDTA and transferred to a Hybond N membrane (39). Hybridizations were done as described elsewhere (39) with digoxigenin (DIG)-labeled DNA probes for RsmY and RsmZ, produced by PCR with Hotstar *Taq* polymerase (QIAGEN), DIG-labeled deoxynucleoside triphosphates (Roche), and primer pairs BH1/BH2 and PRSMZ1/PRSMZ2, respectively. Each lane was loaded with 3 μg of total RNA and checked by monitoring the intensities of 23S and 16S rRNAs (not shown).

amine the situation in *P. aeruginosa* and we present evidence that the GacS/GacA system functions with two small RNAs, RsmZ and the newly characterized RsmY regulator (36).

Features of RsmY small RNA and construction of an *rsmY* mutant. The *rsmY* gene of *P. aeruginosa*, which has 69% nucleotide sequence identity with that of *P. fluorescens* CHA0 (10, 39), is located between the *dnr* gene and open reading frame PA0528 on the chromosome of *P. aeruginosa* PAO1 (Fig. 1A). The *rsmY* promoter contains a conserved upstream activating sequence (UAS) which is characteristic for GacA-dependent small RNA genes (16, 39). The probable *rsmY* transcription start site can be deduced from that determined in *P. fluorescens* (10, 39). RsmY RNA (124 nucleotides) is predicted to contain four major stem-loop structures (10) and six GGA motifs (Fig. 1A) in single-stranded parts of the molecule. Unpaired GGA repeats are a hallmark of small RNAs that titrate RsmA/CsrA (10, 16, 30). A 96-bp deletion was introduced into the *rsmY* gene of *P. aeruginosa* PAO1 (Fig. 1A) by homologous recombination (26, 28), using *E. coli* S17-1/pME3087Δ*rsmY* (Table 1) as a donor and PAO1 as a recipient. In nutrient yeast broth (NYB), the resulting mutant PAO6420 had the same growth rate as the wild-type PAO1 (data not shown). According to genomic sequence data, strains PAO1 and PA14 (http://www.pseudomonas.com; http://ausubellab.mgh.harvard.edu/pa14sequencing) contain two genes encoding GacA-dependent small regulatory RNAs, *rsmY* and *rsmZ* (2, 12, 36), but no homolog of the *rsmX* gene, which codes for a third GacA-regulated small RNA in *P. fluorescens* (14).

Expression of RsmY and RsmZ. When hybridized to a specific *rsmY* probe in a Northern blot experiment (Fig. 1B), two bands of RsmY RNA were revealed in a denaturing gel, possibly corresponding to molecules with and without the terminator stem-loop structure. Mutations in *gacA* or *rsmA* markedly reduced the amount of RsmY transcript; the effect was even stronger in the *rsmA* mutant than in the *gacA* mutant. By contrast, deletion of *rsmZ* had little effect (Fig. 1B). The abundance of RsmZ, which was monitored in parallel, showed an analogous pattern (Fig. 1B).

Transcriptional *rsmY-lacZ* and *rsmZ-lacZ* fusions were inserted into the *P. aeruginosa* chromosome of the wild type and of *rsmA*, *rsmY rsmZ*, and *gacA* mutants. This was achieved by transposition (1, 48) of mini-Tn*7* constructs (Table 1) into the unique Tn*7att* site located downstream of the *glmS* gene. The *rsmY-lacZ* and *rsmZ-lacZ* fusions showed positive regulation by GacA and RsmA (Fig. 2A and B). In an *rsmY rsmZ* double mutant, the expression of both fusions was strongly elevated and, at the end of growth, about 40,000 Miller units were measured for the β-galactosidase activity of the *rsmY-lacZ* fusion (Fig. 2B). This value places the induced *rsmY* promoter in the category of the most highly expressed *Pseudomonas* promoters. The *rsmA* gene showed cell density-dependent expression which showed little variation in *gacA*, *rsmA*, or *rsmY rsmZ* mutants (Fig. 2C). In conclusion, transcription of both *rsmY* and *rsmZ* is positively regulated by RsmA and negatively by RsmY and RsmZ together. The mechanism by which the RNA-binding protein RsmA exerts this positive control is not understood. However, the data are consistent with a model in which the positive effects of RsmA on the *rsmY* and *rsmZ* promoters are antagonized by RsmY and RsmZ.

TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Genotype, phenotype, or sequence[a]	Reference or origin
E. coli strain		
S17-1	F^- *pro thi hsdR recA*; chromosome::RP4-2 Tc::Mu Km::Tn*7* Tp^r Sp^r	35
P. aeruginosa strains		
PAO1	Wild type	ATCC 15692
PAO6281	*gacA*::Ω-Sm/Sp; Sm/Sp^r	28
PAO6354	Δ*rsmZ*	12
PAO6420	Δ*rsmY*	This study
PAO6421	Δ*rsmY* Δ*rsmZ*	This study
PAO6554	*rsmZ-lacZ* transcriptional fusion in mini-Tn*7*; Gm^r	This study
PAO6555	*gacA*::Ω-Sm/Sp, *rsmZ-lacZ* fusion in mini-Tn*7*; Sm/Sp^r Gm^r	This study
PAO6556	Δ*rsmY* Δ*rsmZ*, *rsmZ-lacZ* fusion in mini-Tn*7*; Gm^r	This study
PAO6557	Δ*rsmA*, *rsmZ-lacZ* fusion in mini-Tn*7*; Gm^r	This study
PAO6558	*rsmY-lacZ* transcriptional fusion in mini-Tn*7*; Gm^r	This study
PAO6559	*gacA*::Ω-Sm/Sp, *rsmY-lacZ* fusion in mini-Tn*7*; Sm/Sp^r Gm^r	This study
PAO6567	*rsmY* Δ*rsmZ*, *rsmY-lacZ* fusion in mini-Tn*7*; Gm^r	This study
PAO6568	Δ*rsmA*, *rsmY-lacZ* fusion in mini-Tn*7*; Gm^r	This study
PAZH13	Δ*rsmA*	26
Plasmids		
pBluescript II SK, KS	Cloning vectors, ColE1 replicons; Ap^r	Stratagene
pME3087	Suicide vector, ColE1 replicon, IncP-1, Mob; Tc^r	44
pME3087Δ*rsmY*	pME3087 containing insert of pME3830Δ*rsmY*	This study
pME3280a	Mini-Tn*7* gene delivery vector; Gm^r	48
pME3328	pBluescript II KS containing a 1.43-kb BamHI-XhoI fragment with *'rpoS rsmZ fdxA'*; Ap^r	12
pME3331	pME6016 derivative containing a transcriptional *rsmZ-lacZ* fusion; Tc^r	12
pME3830	pBluescript II SK containing a 1.3-kb PstI-StuI PCR fragment with *'dnr rsmY* and PA0528', obtained with primers PAOTRR1 and PAOTRR2; Ap^r	This study
pME3830Δ*rsmY*	pME3830 with a 96-bp deletion in *rsmY*, obtained by inverse PCR with primers Δ1 and Δ2	This study
pME3843	pME6010 containing a translational *hcnA'-'lacZ* fusion under the *tac* promoter	25
pME3859	pME6010 containing a translational *rsmA'-'lacZ* fusion	26
pME3897	pME6182 containing a 3.3-kb BamHI-XhoI fragment of pME3331 with a transcriptional *rsmZ-lacZ* fusion in the SmaI site of mini-Tn*7*; Gm^r	This study
pME3898	pME6182 containing a 3.3-kb EcoRI-XhoI fragment of pME7311 with a transcriptional *rsmY-lacZ* fusion in the SmaI site of mini-Tn*7*; Gm^r	This study
pME6015	Cloning vector for translational *'lacZ* fusions; pVS1- p15A replicon; Tc^r	33
pME6016	Cloning vector for transcriptional *lacZ* fusions, pVS1-p15A replicon; Tc^r	33
pME6182	Mini-Tn*7* gene delivery vector based on pME3280a, HindIII-SmaI-KpnI-NcoI-SphI MCS, ColE1 replicon; Gm^r Ap^r	C. Reimmann
pME7311	pME6016 derivative containing a 216-bp EcoRI-BamHI fragment (amplified with primers PrsmY1 and PrsmY2) carrying the *rsmY* promoter fused at the putative +5 site to the +1 site of *lacZ*; Tc^r	This study
pME7321	pBluescript II KS containing a 0.85-kb EcoRI-BamHI fragment with the *rpsA* upstream region and the first eight *rpsA* codons (amplified with primers PRPSA1 and PRPSA2); Ap^r	This study
pME7322	pME6015 derivative with a 0.85-kb EcoRI-BamHI *rpsA* upstream fragment and the first eight codons fused in frame with the *'lacZ* gene	This study
pUX-BF13	Helper plasmid containing Tn*7* transposition functions, R6K replicon; Ap^r	1
Primers (5' → 3')		
PAOTRR1	CTGTTCACTCGAAGCACTCC located in *dnr*, upstream of the *rsmY* region	
PAOTRR2	TTCGCCAACTCCGCTATTTC located in PA0528, downstream of the *rsmY* region	
PrsmY1	TTCCTGGAGCTGGACGGG located in *dnr*, upstream of the *rsmY* region	
PrsmY2	CGCA<u>GGATCC</u>TGACGGTTTGAAGATTACGC with a BamHI restriction site (underlined), located in the +1 transcription start region of *rsmY*	
Δ1	TAGA<u>GATATC</u>CAAAACCCCGCCCAAAAGGC with an EcoRV restriction site (underlined), located upstream of the *rsmY* terminator	
Δ2	GCGTCTCTACGCATTAGAA<u>GATATC</u>CAGT with an EcoRV restriction site (underlined), located downstream of the *rsmY* transcription start site	
BH1	ATCATCTTGACGCACGGCAAGC located upstream of *rsmY* (−148 to −128)	
BH2	TCTGAGCGACGCGGTTTTCC located downstream of the *rsmY* terminator (+136 to +155)	
PRSMZ1	CTAACAGGGAACACGCAACC, corresponding to the +1 site and the next 20 bp of *rsmZ*	
PRSMZ2	AAAAAAAGGGGCGGGGTATT located in the terminator of *rsmZ*	
PRPSA1	AAAAa<u>GGATCC</u>GAGTTCTGCGAAGCTTTCGCTC, with a BamHI restriction site (underlined), located downstream of the first codon of *rpsA*	
PRPSA2	AAAAA<u>GAATTC</u>CGCGGTCAACCATGGTGTCGAC with an EcoRI restriction site (underlined), located in the *cmk* gene (PA3163) upstream of *rpsA*	

[a] MCS, multiple cloning site; Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

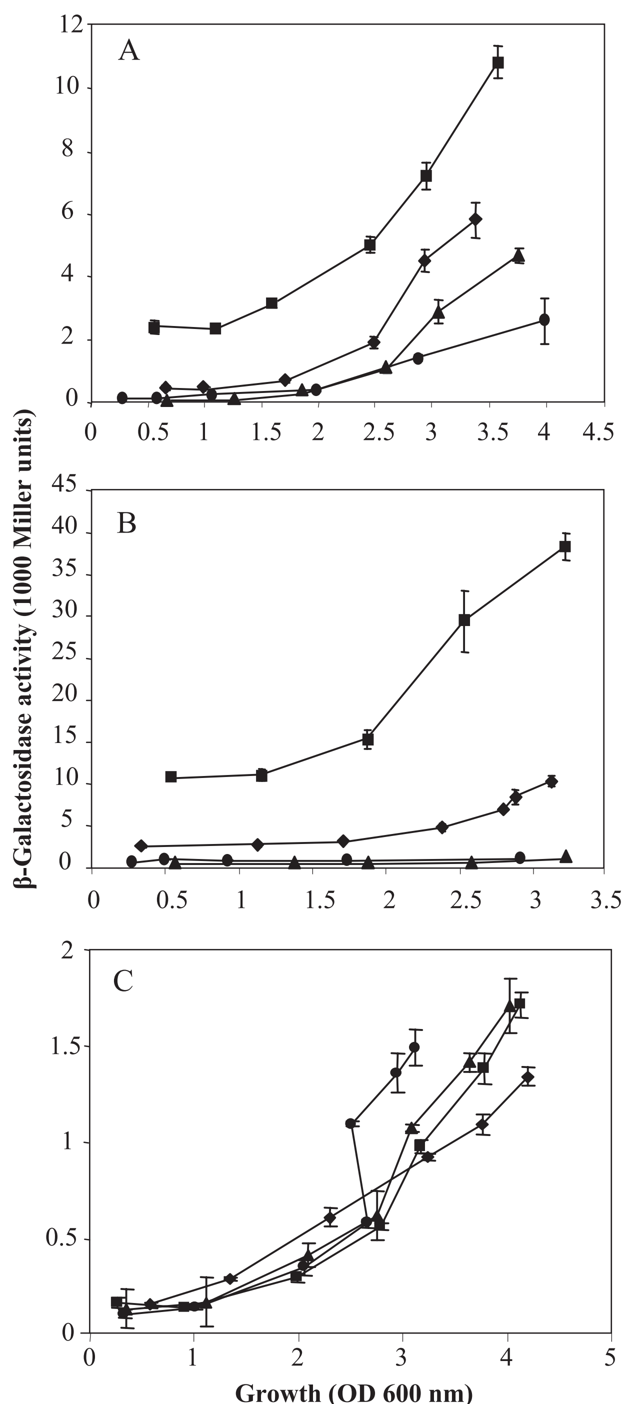

FIG. 2. Regulation of *rsmY*, *rsmZ*, and *rsmA* expression in *P. aeruginosa* PAO1 and mutants. (A) Expression of *rsmZ* was monitored by measuring β-galactosidase activities of a transcriptional *rsmZ-lacZ* fusion in the wild type (PAO6554; diamonds), a *gacA* mutant (PAO6555; triangles), an *rsmY rsmZ* double mutant (PAO6556; squares), and an *rsmA* mutant (PAO6557; circles). (B) Expression of *rsmY* was monitored by determining β-galactosidase activities of a transcriptional *rsmY-lacZ* fusion in the wild type (PAO6558; diamonds), a *gacA* mutant (PAO6559; triangles), an *rsmY rsmZ* double mutant (PAO6567; squares), and an *rsmA* mutant (PAO6568; circles). (C) Expression of *rsmA* was monitored by measuring β-galactosidase activities of a translational *rsmA′-′lacZ* fusion (on pME3859) in the wild type (PAO1; diamonds), a *gacA* mutant (PAO6281; triangles), an *rsmY rsmZ* double mutant (PAO6421; squares), and an *rsmA* mutant (PAZH13; circles). Cells were grown in 50-ml Erlenmeyer flasks containing 20 ml NYB supplemented with 0.05% (vol/vol) Triton X-100 with shaking at 37°C. β-Galactosidase specific activities were determined by the Miller method (20). The experiments were done in triplicate; average values ± standard deviation are shown.

TABLE 2. Differentially expressed proteins[a]

Protein	PA no.[b]	Localization[c]	Fold change[d]	
			gacA/wt	*rsmYZ*/wt
Fructose-1,6-bisphosphate aldolase (Fda)	0555	IC	−3.1	−2.5
Chitin-binding protein (CbpD)	0852	IC	−2.1	−3.6
		SF	−3.3	−5.0
		EC	−5.9	−7.4
N-Succinylglutamate 5-semialdehyde dehydrogenase (AruC)	0895	IC	−2.5	−2.7
Flagellin (FliC)	1092	EC	+7.5	+9.8
Flagellar-capping protein (FliD)	1094	EC	+8.8	+10.2
Chitinase (ChiC)	2300	IC	−8.0	−8.3
Protein with hypothetical function	3021	SF	−4.6	−5.0
Protein with hypothetical function	3022	IC	−2.4	−2.1
Protein with hypothetical function	3088	EC	+4.6	+6.7
Probable peroxidase	3529	SF	+3.4	+2.9
Elastase (LasB)	3724	EC	−3.5	−10.1
Protein with hypothetical function	3785	EC	+4.1	+4.7
Protein with hypothetical function	4453	EC	+2.3	+2.1
Azurin	4922	EC	+2.3	+3.4
Thiol-disulfide interchange protein (DsbA)	5489	SF	−4.0	−7.5

[a] Only those changes in expression that were ≥2.0-fold are shown.
[b] Mass spectrometry data (peptide mass fingerprints and tandem mass spectrometry results) were compared to the complete translated open reading frames of *P. aeruginosa* PAO1 (http://www.pseudomonas.com).
[c] IC, intracellular; SF, surface associated; EC, extracellular.
[d] Average values from six gels. When multiple spots were ascribed to the same protein, average values of all spots are given. Down-regulation in the mutants is indicated by a minus sign; up-regulation in the mutants is indicated by a plus sign.

Proteomic analysis of *gacA* and *rsmY rsmZ* mutants. If the action of GacA is mediated by two small RNAs, RsmY and RsmZ, in *P. aeruginosa*, we expect that the global protein expression pattern should be similar in the *gacA* mutant PAO6281 and in the *rsmY rsmZ* double mutant PAO6421 (Table 1). This was found to be the case (see the supplemental material). Among the intracellular proteins detected, chitinase (the *chiC* product) and chitin-binding protein (the *cbpD* product) were the most strongly down-regulated proteins in both mutants, compared to the wild type (Table 2). The ChiC and CbpD proteins are expressed under quorum-sensing control and accumulate in the cytoplasm before they are slowly secreted (5, 6, 22, 46). Among the cell surface-associated proteins detected, DsbA, an enzyme forming disulfide bonds in periplasmic proteins, was clearly down-regulated in both the *gacA* and *rsmY rsmZ* mutants in comparison with the wild type (Table 2). DsbA function is important for type II secretion; a

TABLE 3. Production of C4-HSL, HCN, and pyocyanin and swarming abilities of *P. aeruginosa* PAO1 and *rsmA*, *gacA*, *rsmY*, *rsmZ*, and *rsmYZ* mutants

Strain	Genotype	C4-HSL[a] (μM)	HCN[b] (nmol/10^9 cells)	Pyocyanin[c] (μg/10^9 cells)	Swarming motility[d]
PAO1	Wild type	2.1 ± 0.1	22.5 ± 2.0	10.4 ± 0.9	+
PAZH13	Δ*rsmA*	ND	56.4 ± 10.0	28.2 ± 4.1	−
PAO6354	Δ*rsmZ*	ND	10.1 ± 2.8	8.6 ± 1.9	+
PAO6420	Δ*rsmY*	ND	8.2 ± 1.3	6.8 ± 0.9	+
PAO6421	Δ*rsmY*Δ*rsmZ*	≤0.5	5.1 ± 0.8	4.7 ± 1.0	++
PAO6281	*gacA*::ΩSp/Sm	≤0.5	6.9 ± 0.8	4.8 ± 1.1	++

[a] C4-HSL concentrations were estimated in culture supernatants of *P. aeruginosa* strains grown in NYB by thin-layer chromatography analysis (11) when cells reached an optical density at 600 nm of ≈2.0. The experiment was performed twice in triplicate. Values are means ± standard deviations. ND, not determined.
[b] HCN was quantified in culture supernatants of strains grown semianaerobically for 24 h in sealed 120-ml flasks containing 60 ml of glycine minimal medium (43) supplemented with 0.05% (wt/vol) Triton X-100. Samples of 50 μl were mixed with 250 μl of 0.09 M NaOH and 1 ml of a reagent containing 0.05 M 1,2-dinitrobenzene and 0.1 M 4-nitrobenzaldehyde in 2-methoxyethanol (43). The reaction product was measured colorimetrically at 578 nm, and cyanide concentrations were estimated using a KCN standard curve. Average values of three measurements ± standard deviations are given.
[c] Pyocyanin was quantified in supernatants of *P. aeruginosa* strains grown in glycerol-alanine medium (28). Supernatants (5 ml) were extracted with 3 ml of chloroform and centrifuged at 8,000 rpm. The chloroform layer was transferred to a new tube containing 1 ml 0.2 M HCl and mixed. After centrifugation, the absorption at 520 nm was measured in the top aqueous layer. Average values of three measurements ± standard deviations are given.
[d] Swarming ability was tested on semisolid medium (12) after 8 h of incubation in high humidity at 37°C. ++, ability to swarm and invade ~50% of the plate; +, ability to swarm and invade ~40% of the plate; −, no swarming.

dsbA null mutant shows reduced secretion of extracellular enzymes such as elastase and lipase (18, 38).

In the extracellular medium (see the supplemental material), CbpD and elastase (LasB protein) were among those proteins that were present in markedly reduced amounts in *gacA* and *rsmY rsmZ* mutants in comparison with the wild type. In both mutants, several extracellular proteins were up-regulated in parallel, notably azurin, flagellin (FliC), and flagellum-capping protein (FliD) (Table 2). Previously, increased expression of flagellin and enhanced motility have been observed in *gacA* mutants of *P. fluorescens* F113 (32). Azurin is a periplasmic copper redox protein that has been implicated in anaerobic electron transport of *P. aeruginosa*. However, the physiological role of azurin is unknown (42). The azurin gene is induced by limited aeration and in stationary phase (42), and the azurin protein is released into the culture medium (47). Interestingly, in cell cultures azurin induces apoptosis of macrophages through binding to tumor suppressor protein p53 (47). The finding that azurin is overproduced in *gacA* and *rsmY rsmZ* mutants was unexpected. If azurin were produced during *P. aeruginosa* infection, one might speculate that these mutants could be more cytotoxic to macrophages than is the wild type. This would add a new facet to regulation of virulence by GacA.

Parallel effects of *gacA* and *rsmY rsmZ* mutations on quorum sensing. The Gac/Rsm system activates the quorum-sensing machinery primarily by stimulating C4-HSL production (25, 28). As expected, the *rsmY rsmZ* double mutant was impaired in C4-HSL production, as was the *gacA* mutant (Table 3). Both mutants produced less HCN and pyocyanin in comparison with the wild type, whereas the single *rsmY* and *rsmZ* mutants showed intermediate levels of these quorum-sensing-regulated exoproducts. The repressing activity of RsmA on HCN and pyocyanin synthesis was lost in an *rsmA* mutant (Table 3), in agreement with the Gac/Rsm model. The negative effect of an *rsmA* mutation on swarming (12) was confirmed; lack of *rsmA* function causes cell clumping (12). Conversely, swarming motility was higher in *gacA* and *rsmY rsmZ* mutants than in the wild type (Table 3). The enhanced motility of the mutants might be facilitated by the absence of a putative clumping factor and by elevated synthesis of flagellar proteins.

β-Galactosidase activities were determined in strains carrying a translational *hcnA'*-*'lacZ* fusion which was expressed from the *tac* promoter, i.e., uncoupled of the natural regulation by *N*-acyl-homoserine lactones and limited aeration. In *gacA* and *rsmY rsmZ* mutants, the activities were similarly low, whereas in an *rsmA*-negative background derepressed levels were found in comparison with the wild type (Fig. 3). Taken together, these findings indicate that in *P. aeruginosa* signal transduction from GacA to target genes for exoproducts is mediated by two small RNAs (RsmY and RsmZ) and one RNA-binding protein (RsmA).

Role of RsmY and RsmZ in biofilm formation. Recent evidence points to an important role of RsmZ in biofilm formation of *P. aeruginosa* strain PAK. The input from three sensors,

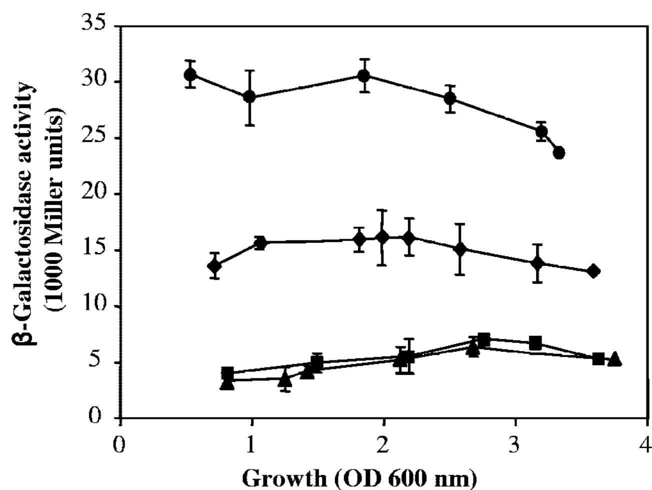

FIG. 3. Expression of HCN biosynthetic genes. β-Galactosidase activities of a translational *hcnA'*-*'lacZ* fusion under the control of the *tac* promoter (carried by plasmid pME3843) were determined in the wild type (PAO1; diamonds), a *gacA* mutant (PAO6281; triangles), an *rsmY rsmZ* double mutant (PAO6421; squares), and an *rsmA* mutant (PAZH13; circles). Cells were grown as indicated for Fig. 2. The experiment was done in triplicate; average values ± standard deviations are shown.

GacS, RetS, and LadS, determines the expression of *rsmZ* and biofilm formation (8, 41). During early colonization of polystyrene microtiter plates by *P. aeruginosa*, mutation in *gacS* weakly enhances the biomass of attached bacteria, whereas mutation in *retS* strongly enhances this biofilm development. Mutation in *ladS* abrogates biofilm formation (8, 41). We tested biofilm formation of our PAO mutants. Cells were grown at 30°C in L broth (31) in polystyrene microtiter plates for 24 h. The medium was then removed, and 100 μl of a 1% (wt/vol) aqueous solution of crystal violet was added (23). Following staining at room temperature for 20 min, the dye was removed and the wells were washed thoroughly. For quantification of surface-attached cells, crystal violet was solubilized in a mixture of 200 μl dimethyl sulfoxide and 800 μl ethanol, and the absorbance was determined at 570 nm against an ethanol blank. In this assay, the relative biofilm masses were 0.5 ± 0.1 (mean ± standard deviation) A_{570} units for the wild-type PAO1, 1.1 ± 0.1 A_{570} units for the *gacA* mutant PAO6281, and 1.0 ± 0.1 A_{570} units for the *rsmY rsmZ* mutant PAO6421. Thus, the positive effect of the *gacA* mutation on biofilm formation is reproduced by the *rsmY rsmZ* double mutation, suggesting that RsmY and RsmZ together influence biofilm formation.

RsmA control of the *rpsA* gene product, ribosomal protein S1. When RsmA acts as a translational repressor, it interferes with the access of ribosomes to mRNA by binding to the Shine-Dalgarno (SD) sequence (4). Ribosomal protein S1, an essential protein in *E. coli* and other gram-negative bacteria, binds to mRNAs upstream of the SD sequence, and this interaction is particularly important for translation initiation from weak or very strong SD sequences (19). The relative concentrations of free RsmA and S1 may therefore critically influence the rate of translation initiation of certain genes. In enteric bacteria, the 5′-untranslated region of *rpsA* mRNA contains two hairpins with GGA motifs in the loops. These hairpins and motifs are essential for translational repression of *rpsA* mRNA, a phenomenon which occurs when ribosomal protein S1 is overproduced (37). Conceivably, these mRNA structures might interact with CsrA/RsmA. Although this type of regulation of the *rpsA* gene is much weaker in *P. aeruginosa* and *Pseudomonas putida* than in enteric bacteria (37), we speculated that RsmA might nevertheless influence the expression of *rpsA* in *P. aeruginosa*. β-Galactosidase activities specified by a translational *rpsA′-′lacZ* fusion were significantly higher in an *rsmA* mutant than in the wild type (Fig. 4), indicating a negative effect of RsmA on *rpsA* expression. In a *gacA* mutant and in an *rsmY rsmZ* mutant, the *rpsA′-′lacZ* levels were slightly (but not significantly) below those of the wild type (Fig. 4).

During growth of *P. aeruginosa*, the intracellular concentration of RsmA protein increases steadily and reaches a high level in stationary phase (26). By contrast, the S1 protein level declines in stationary phase (34). Overall, the RsmA/S1 ratio increases at least 10-fold during growth, and this trend can also be gleaned from the expression of *rsmA′-′lacZ* and *rpsA′-′lacZ* fusions in the wild-type PAO1. RsmA does not appear to regulate its own synthesis (Fig. 2C) but has a negative effect on S1 synthesis (Fig. 4). In this way, high RsmA/S1 ratios may be consolidated during late stages of growth and stationary phase.

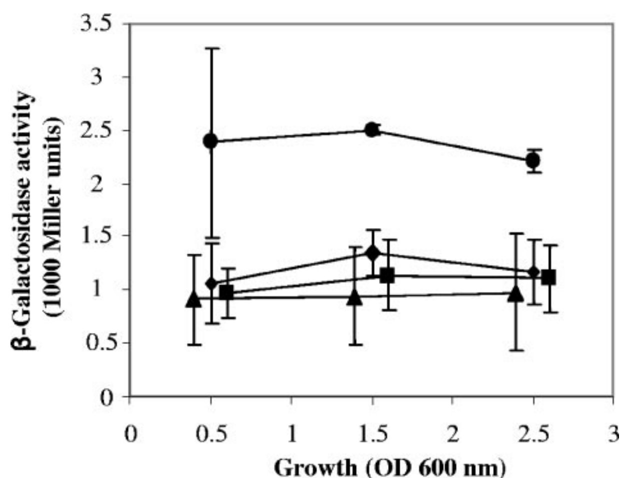

FIG. 4. Expression of the *rpsA* gene. β-Galactosidase activities of a translational *rpsA′-′lacZ* fusion carried by plasmid pME7322 were determined in the wild-type strain (PAO1; diamonds), a *gacA* mutant (PAO6281; triangles), an *rsmY rsmZ* double mutant (PAO6421; squares), and an *rsmA* mutant (PAZH13; circles). Cells were grown as indicated for Fig. 2. Average values ± standard deviations from four experiments are shown.

Concluding remarks. Our previous model of the GacS/GacA signal transduction pathway in *P. aeruginosa* (12) can now be refined by including RsmY. This small RNA has the typical attributes of a GacA-dependent regulatory RNA: it is transcribed from a promoter with a highly conserved palindromic UAS (TGTAAG. . . . . .CTTACA) (Fig. 1A), it has several stem-loop structures with unpaired GGA motifs (10), and it binds RsmA with high affinity (36). RsmY acts in parallel with RsmZ. When both *rsmY* and *rsmZ* are deleted, the phenotypic changes seen in various tests (Tables 2 and 3; Fig. 3 and 4) are very similar to those found in the *gacA* mutant. Both GacA and RsmA are needed for *rsmY* transcription (Fig. 1B and 2B). Whether GacA (in its phosphorylated form) physically interacts with the UAS remains to be demonstrated. The action of the RNA-binding protein RsmA, which is antagonized jointly by RsmY and RsmZ, is likely to be indirect and to involve one or several unknown transcription factors.

GacA is a major positive regulator of virulence in *P. aeruginosa* (27). According to the results of this study, most if not all major effects of GacA can be ascribed to the combined action of RsmY and RsmZ. The signal transduction pathway from GacA to individual virulence genes varies. For instance, the negative effect of *gacA* and *rsmY rsmZ* mutations on HCN production derives from reduced synthesis of the C4-HSL quorum-sensing signal (Table 3), which causes low *hcn* promoter activity, as well as from repressed *hcn* mRNA translation (Fig. 3), in agreement with earlier data (25). Impaired secretion of elastase (LasB protein) in *gacA* and *rsmY rsmZ* mutants can be explained by reduced C4-HSL synthesis (Table 3), which results in down-regulation of type II secretion (3), and by poor expression of DsbA (Table 2), a key enzyme involved in the folding of proteins released by type II secretion (18, 38). To some extent, RsmY and RsmZ appear to have redundant functions. However, they differ in at least one respect: the

global regulator Hfq, a protein that facilitates base-pairing interactions between small RNAs and target mRNAs, stabilizes RsmY, but not RsmZ, and Hfq binds specifically to RsmY (36).

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