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Positive Control of Swarming, Rhamnolipid Synthesis, and Lipase Production by the Posttranscriptional RsmA/RsmZ System in *Pseudomonas aeruginosa* PAO1†

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In *Pseudomonas aeruginosa*, the small RNA-binding, regulatory protein RsmA is a negative control element in the formation of several extracellular products (e.g., pyocyanin, hydrogen cyanide, PA-IL lectin) as well as in the production of *N*-acylhomoserine lactone quorum-sensing signal molecules. RsmA was found to control positively the ability to swarm and to produce extracellular rhamnolipids and lipase, i.e., functions contributing to niche colonization by *P. aeruginosa*. An *rsmA* null mutant was entirely devoid of swarming but produced detectable amounts of rhamnolipids, suggesting that factors in addition to rhamnolipids influence the swarming ability of *P. aeruginosa*. A small regulatory RNA, *rsmZ*, which antagonized the effects of RsmA, was identified in *P. aeruginosa*. Expression of the *rsmZ* gene was dependent on both the global regulator GacA and RsmA, increased with cell density, and was subject to negative autoregulation. Overexpression of *rsmZ* and a null mutation in *rsmA* resulted in quantitatively similar, negative or positive effects on target genes, in agreement with a model that postulates titration of RsmA protein by RsmZ RNA.

Pseudomonas aeruginosa is a ubiquitous saprophyte and an opportunistic human pathogen which synthesizes numerous extracellular products including elastase, LasA protease, alkaline protease, phospholipase C, lipase, exotoxin A, rhamnolipids, hydrogen cyanide (HCN), and pyocyanin (25, 63). The production of these exoproducts, most of which can act as virulence factors, is positively controlled by two quorum-sensing signal molecules, *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-homoserine lactone (C4-HSL), which activate the transcription factors LasR and RhlR, respectively (20, 26, 59). The *las* and *rhl* systems are organized in a hierarchical manner such that the *las* system exerts transcriptional control over both *rhlR* and *rhlI* (26). A third signal molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone, the synthesis and activity of which is linked to the *las* and *rhl* circuitry, is also required for virulence factor production and, in particular, for *rhl*-dependent exoproducts including pyocyanin and PA-IL lectin (41).

Motility helps *P. aeruginosa* to colonize niches (10); three types of motility are observed, i.e., swimming, twitching, and swarming (24, 44). Whereas swimming in liquid media depends on flagella, twitching on solid media requires type IV pili. Swarming on semisolid media results from a combination of both types of motility and also requires rhamnolipid production (24, 46). Rhamnolipids are biosurfactants which not only enhance bacterial surface translocation by virtue of their wetting properties but also stimulate solubilization and degradation of hydrocarbons (35) and act as heat-stable hemolysins (21).

The production of *N*-acyl-homoserine lactones (AHLs) and the expression of many virulence determinants in *P. aeruginosa* is negatively controlled at a posttranscriptional level by the small RNA-binding protein RsmA (42, 43). This regulator is a homolog of CsrA in *Escherichia coli* and *Salmonella enterica* (27, 47) and of RsmA in *Erwinia* spp. and *Pseudomonas fluorescens* CHA0 (3, 7). When RsmA and CsrA exert a negative effect on gene expression, they are assumed to prevent translation initiation by binding at or near the ribosome binding site, and this can favor mRNA decay. A well-documented example for this regulation is provided by CsrA-mediated posttranscriptional repression of glycogen biosynthesis in *E. coli*, where CsrA binding to the 5′ leader transcript of the *glgG* gene inhibits translation (2, 28). In a number of cases, CsrA can also exert positive effects on gene expression in *E. coli*, directly or indirectly (39). For instance, flagellar motility of *E. coli* is positively regulated by CsrA at the level of the *flhDC* master operon. The binding of CsrA to a 5′ segment of *flhDC* mRNA stimulates its translation and enhances its half-life (57). The repressive action of CsrA and RsmA can be relieved by small regulatory RNAs such as CsrB and CsrC in *E. coli*, RsmB in *Erwinia carotovora*, or RsmY and RsmZ (PrrB) in *P. fluorescens* (1, 18, 29, 30, 55, 58). The present model postulates that the regulatory RNAs antagonize the effect of CsrA/RsmA by sequestering multiple copies of these proteins, thereby titrating

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their activity (17, 29, 47). This kind of posttranscriptional control may facilitate a rapid but potentially reversible regulation of diverse cellular functions.

RsmA/RsmY/RsmZ of *P. fluorescens*, RsmA/RsmB of *E. carotovora*, and CsrA/CsrB/CsrC of *E. coli* and *S. enterica* are all part of the GacS/GacA signal transduction pathway which operates an important metabolic switch from primary to secondary metabolism in many gram-negative bacteria and can also pleiotropically affect central carbon metabolism and enzyme secretion (3, 17, 19, 27, 39, 52, 58). In *P. aeruginosa*, the global response regulator GacA positively regulates the quorum-sensing machinery and the expression of several virulence factors via a mechanism involving the participation of RsmA as a negative control element (42, 43, 45). In this study, we demonstrate that RsmA of *P. aeruginosa* can also be a positive control element, as it is required for swarming motility, rhamnolipid synthesis, and lipase production. Both positive and negative effects of RsmA were found to be antagonized by the small regulatory RNA RsmZ, a homolog of *P. fluorescens* RsmZ.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. Both *E. coli* and *P. aeruginosa* strains were routinely grown in nutrient yeast broth (NYB) or on nutrient agar plates at 37°C (45). Where required, antibiotics were added to media at the following concentrations: tetracycline, 25 μg ml^{-1} (*E. coli*) or 125 μg ml^{-1} (*P. aeruginosa*); gentamicin, 10 μg ml^{-1}; chloramphenicol, 250 μg ml^{-1}; spectinomycin, 1,000 μg ml^{-1} (*P. aeruginosa*). To counterselect *E. coli* S17-1 donor cells in matings with *P. aeruginosa*, chloramphenicol was used at a concentration of 10 μg ml^{-1}; enrichment for tetracycline-sensitive strains was performed with carbenicillin (2,000 μg ml^{-1}) and tetracycline (20 μg ml^{-1}) (43, 45). Flagellar swimming was examined as described by Rashid and Kornberg (44) on NYB solidified with 0.3% (wt/vol) agar. Swarming was evaluated on plates containing 0.5% (wt/vol) agar, 8 g of nutrient broth (Oxoid) liter^{-1}, and 5 g of glucose liter^{-1} (44). Twitching motility was assayed on 1% (wt/vol) agar supplemented with Luria broth (24).

DNA manipulation and cloning procedures. Small-scale preparations of plasmid DNA were carried out by the cetyltrimethylammonium bromide method (8), and large-scale preparations were performed by using JetStar-Tips (Genomed, Basel, Switzerland). Chromosomal DNA was extracted from *P. aeruginosa* and purified as described elsewhere (14). Restriction enzyme digestions, ligations, and agarose gel electrophoresis were performed by standard methods (48). Restriction fragments were purified from agarose gels with the Gene Clean II kit (Bio 101). Transformation of *E. coli* and *P. aeruginosa* strains was carried out by electroporation (12). Cloned PCR products were sequenced with the Big Dye terminator cycle sequencing kit and an ABI-PRISM 373 automatic sequencer (Applied Biosystems). Alignment of nucleotide and deduced amino acid sequences was performed by using the Genetics Computer Group program GAP. Oligonucleotides used in this study are listed in Table 1.

Plasmid constructions. For the construction of plasmid pME3838, a 369-bp fragment containing the promoter of *rhlA* was amplified by PCR from chromosomal DNA of *P. aeruginosa* with primer DS5-EcoRI, carrying an artificial restriction site for EcoRI, and primer DS6-PstI, annealing to the +1 region of the *rhlA* gene (32) and carrying an artificial restriction site for PstI. The PCR product digested with EcoRI and PstI was inserted into pME6016 carrying the *lacZ* gene with its own ribosome binding site. The resulting *rhlA-lacZ* transcriptional fusion contains 353 bp of the *rhlA* promoter region and lacks the *rhlA* translational control region, which is present in the translational *rhlA′-′lacZ* fusion on plasmid pECP60. To overexpress the rhamnolipids, we constructed plasmid pME3839 as follows: a 2.6-kb fragment containing the *rhlA* and *rhlB* genes was amplified by PCR from *P. aeruginosa* chromosomal DNA with primer RhlAB-KH3, annealing to the 5′ region of the *rhlA* gene and carrying an artificial restriction site for EcoRI, and primer RhlAB-KH4, carrying the natural restriction site for ClaI located 108 bp after the stop codon of *rhlB*. The PCR product was digested with EcoRI and ClaI and inserted into pME6032 cut with the same enzymes. The resulting plasmid carries the *rhlAB* genes under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible (1 mM) *tac* promoter, such that the ATG start codon of *rhlA* was located 41 bp downstream of the transcription start. This region contains the ribosome binding site of *lacZ*, which is not controlled by RsmA (3). Thus, pME3839 allows RsmA-independent overexpression of *rhlAB*.

Cloning, overexpression, and mutation of the *P. aeruginosa rsmZ* gene. A 0.70-kb fragment containing *rsmZ* was amplified by PCR from *P. aeruginosa* chromosomal DNA with primers PRSMPAO2 and PRSMPAO4 (Table 1), digested with HindIII and XhoI, and inserted into pBluescript KS cut with the same enzymes. A 0.73-kb BamHI-HindIII fragment from pDB18R, carrying the upstream *rpoS* gene and part of the *rsmZ* promoter, was inserted into this plasmid, resulting in pME3328 (see Fig. 3). To overexpress the *rsmZ* gene, a 1.1-kb EcoRI-BamHI fragment from pME3328 carrying *rsmZ* with its promoter was subcloned into two multicopy plasmids, pME6000 and pME6001, cut with the same restriction enzymes, resulting in pME3337.1 and pME3337.3, respectively. To obtain an *rsmZ* deletion, an inverse PCR with the primers PRSMPAO8 and PRSMPAO9 (Table 1) was carried out with pME3328 as the template. The amplified fragment was digested with HindIII and recircularized. This produced a 250-bp deletion of *rsmZ*. The resulting 1.15-kb KpnI-BamHI *′rpoS* Δ*rsmZ ′fdxA* fragment was inserted into the suicide plasmid pME3087, producing pME3332. Biparental conjugation with PAO1 cultivated overnight at 43°C as the recipient and *E. coli* S17-1/pME3332 as the donor allowed selection of chromosomal integration of the plasmid on nutrient agar containing chloramphenicol and tetracycline. Plasmid excision by a second crossover event was obtained after enrichment with carbenicillin. The *rsmZ* deletion in the recombinant strain was verified by PCR with the primers PRSMPAO4 and PRSMPAO7 (Table 1). For the construction of a transcriptional *rsmZ-lacZ* fusion, a 289-bp fragment containing the *rsmZ* promoter fragment was amplified from pME3328 by PCR with the primers PRSMPAO7 and PRSMPAO1 (Table 1), digested with XhoI and PstI, and ligated to a 1-kb PstI-ClaI fragment containing the 5′ end of *lacZ* from pME6522. The resulting 1.3-kb BamHI-ClaI fragment carrying *rsmZ-lacZ′* was inserted into pME6016 cut with the same enzymes, resulting in pME3331 (Table 1). A chromosomal *gacA*::ΩSm/Sp mutation was introduced into PAZH13 and PAO6354 as previously described (45), with the ColE1-based suicide vector pME6111, resulting in PAO6343 and PAO6385, respectively.

Semiquantitative determination of rhamnolipid concentrations by the orcinol method. Strains were grown at 37°C in 50-ml Erlenmeyer flasks containing 10 ml of M9 medium supplemented with glycerol (2% vol/vol), glutamate (0.05%), and Triton X-100 (0.05%) with shaking for 18 h. Rhamnolipids were extracted with three volumes of diethyl ether from culture supernatants filtered through a 0.22-μm-pore-size membrane. Rhamnolipid contents were quantified by the orcinol method as described by Pearson et al. (38), with an extract from the rhamnolipid-negative *rhlA* mutant PT712 as a blank.

Northern blot analysis. RNA was prepared with the High Pure RNA isolation kit (Roche) according to the manufacturer's recommendations. RNA (4 μg) was electrophoretically separated on a denaturing urea-polyacrylamide gel in 1× Tris-borate-EDTA buffer and electrophoretically transferred to a Hybond-N membrane. Prehybridization, hybridization, and detection were performed as previously described (18). A 120-bp DNA probe for *rsmZ* RNA was produced by PCR (95°C for 5 min; 30 cycles of 95°C for 1 min, 48°C for 40 s, 72°C for 30 s; and 72°C for 5 min) where standard deoxynucleoside triphosphates were replaced with digoxigenin-labeled deoxynucleoside triphosphates (Roche), with primers PRSMZ1 and PRSMZ2 (Table 1) which amplify *rsmZ* from the +1 transcription start to the terminator. The *rsmZ* probe was purified by using a QIAquick purification kit (Qiagen).

β-Galactosidase assay. *P. aeruginosa* reporter strains were routinely cultivated, in triplicate, in 50-ml Erlenmeyer flasks containing 20 ml of 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 (33).

Lipase assay. Lipase was quantified in *P. aeruginosa* cultures grown in triplicate in 50-ml Erlenmeyer flasks containing 20 ml of YEA medium (61) for 18 h. Culture samples were centrifuged, and 10 to 100 μl of the supernatant was assayed for soluble lipase activity with *p*-nitrophenol palmitate as the substrate (51). Cell-associated lipase was extracted and assayed as previously described (61). Lipase activities are the totals of soluble and cell-associated enzyme. Specific lipase activities are expressed as nanograms of enzyme per 10^9 cells. The following conversions were used: 1.0 A_{410} unit (absorption of *p*-nitrophenol) equals 0.212 ng of pure lipase, and 1.0 OD_{600} unit (optical density of cell cultures at 600 nm) corresponds to 10^9 cells ml^{-1}.

Assays for lytic activities. Total proteolytic activity was assayed as casein hydrolyzing activity (4). Elastolytic activity (LasB) of bacterial supernatants was determined with the elastin Congo red (Sigma) assay (37) as previously described (9). Staphylolytic protease activity (LasA) was assayed as previously described by Kessler et al. (23) by determining the ability of *P. aeruginosa* supernatants from

TABLE 1. Bacterial strains, plasmids, and oligonucleotides

Strain, plasmid, or primer	Genotype, phenotype, or sequence (5′–3′) and description	Reference or origin
Strains		
P. aeruginosa		
PAO1	Wild type	ATCC 15692
PAO6281	*gacA*::Ω-Sp/Sm	45
PAO6327	*gacS*::Ω-Sp/Sm	C. Reimmann, unpublished data
PAO6343	Δ*rsmA, gacA′*::Ω-Sp/Sm	This study
PAO6354	Δ*rsmZ*	This study
PAO6385	Δ*rsmZ*, *gacA′*::Ω-Sp/Sm	This study
PAZH13	Δ*rsmA*	43
PT712	*rhlA*::Ω-Gm	24
E. coli		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 *deoR* λ(ϕ80d*lacZ*ΔM15)	48
S17-1	*pro thi hsdR recA* Tp^r Sm^r; chromosome::RP4-2 Tc::Mu-Km::Tn7	49
Plasmids		
pBLS-II KS	Cloning vector; ColE1 replicon, Ap^r	Stratagene
pDB18R	pTZ18R, *rpoS*, Ap^r	54
pECP60	*rhlA′-′lacZ* translational fusion on pSW205, Ap^r	40
pME3087	Suicide vector, polylinker of pMMB67, ColE1-replicon, Tc^r	56
pME3280b	Mini-Tn7 gene delivery vector based on pUX-BF5 with a HindIII-PstI-MluI-SpeI MCS, ColE1-replicon Gm^r Ap^r	64
pME3328	pBLS-II KS, with a 1.43-kb BamHI-XhoI fragment containing *′rpoS rsmZ* and *′fdxA*, Ap^r	This study
pME3331	pME6016 derivative, containing the *rsmZ* promoter fused at +1 to *lacZ*, Tc^r	This study
pME3332	pME3087 carrying a 1.15-kb KpnI-BamHI *′rpoS-′fdxA* insert with a 250-bp deletion in *rsmZ*, Tc^r	This study
pME3337.1	pME6000, with a 1.1-kb EcoRI-BamHI fragment of pME3328 containing *rsmZ*, Tc^r	This study
pME3337.3	pME6001, with a 1.1-kb EcoRI-BamHI fragment of pME3328 containing *rsmZ*, Gm^r	This study
pME3838	pME6016 derivative, containing the *rhlA* promoter fused 2 bp after the +1 site to *lacZ*, Tc^r	This study
pME3839	pME6032 carrying the *rhlAB* genes under P_{tac} control, Tc^r	This study
pME3849	pME6001 with *rsmA*, Gm^r	43
pME6000	Cloning vector derived from pBRR1MCS, Tc^r	31
pME6001	Cloning vector derived from pME6000, Gm^r	16
pME6016	Cloning vector for transcriptional *lacZ* fusions, pVS1-p15A replicon, Tc^r	18
pME6032	Cloning vector for overexpression under the IPTG-inducible *tac* promoter, Tc^r	16
pME6111	pME3088 suicide vector carrying a 4.8-kb EcoRI-KpnI fragment with *gacA*::ΩSm/Sp, Tc^r, Cm^r	45
pME6313	Mini-Tn7 gene delivery vector based on pUX-BF5 with a HindIII-PstI-SmaI-SpeI MCS, Gm^r Ap^r	H. Winteler and D. Haas, unpublished data
pSB536	AHL biosensor, *ahyR″-luxCDABE* in pAHP13, Ap^r	53
pSB1075	AHL reporter plasmid, *P. aeruginosa lasRI* fused with *luxCDABE* from *Photorhabdus luminescens*, Ap^r	60
Primers		
DS5-EcoRI	AAAAGAATTCCAATACCACCAACC, with an underlined EcoRI restriction site, located in *rhlA* promoter	
DS6-PstI	AAAACTGCAGA**T**GAACACTTTTTAGCC, with an underlined PstI restriction site, annealing to the +1 site (bold) region of *rhlA*	
RhlAB-KH3	AAAAGAATTC**ATG**CGGCGCGAAAGTC, with an underlined EcoRI restriction site, located in the start codon (bold) region of *rhlA*	
RhlAB-KH4	CCCTGATCGATAAAATGC, with an underlined ClaI restriction site, located 108 bp after the stop codon of *rhlB*	
PRSMPAO1	CCCTGTACGCTGCAGTGATATTAGCGATTCCC, with an underlined PstI restriction site, located in *rsmZ* promoter (Fig. 3)	
PRSMPAO2	AAACGCTCGGTGAATTCAAGTAACTTATTG, located in *rsmZ* promoter region (Fig. 3)	
PRSMPAO4	GATGAATTCTCGAGGATCAATC, with an underlined XhoI restriction site, located upstream of *fdxA*	
PRSMPAO7	GGCCTCTCGAGTGACGCGCTGTTCC, with an underlined XhoI restriction site, located at the 3′ end of *rpoS* (Fig. 3)	
PRSMPAO8	AATAAGCTTAATGCTTACAAGAGCAGACAC, with an underlined HindIII restriction site, located in the upstream activation sequence of *rsmZ* (Fig. 3)	
PRSMPAO9	AATAAGCTTAGGGACTGAAGAGTGGGCGG, with an underlined HindIII restriction site, located 75 bp after *rsmZ* start (Fig. 3)	
PRSMZ1	CGTACAGGGAACACGCAACC, corresponding to the +1 and the first 20 bases of *rsmZ* gene	
PRSMZ2	AAAAAAAGGGGCGGGGTATT, located in the terminator of *rsmZ* gene	

NYB cultures, grown at 37°C with shaking to an OD_{600} of 2.5, to lyse boiled *Staphylococcus aureus* cells.

Lectin detection. PA-IL lectin was detected by Western blotting with polyclonal antibodies raised against the purified *P. aeruginosa* lectin (62) in cells grown in NYB at 37°C with shaking, as previously described (43).

Assays for pyocyanin and HCN. Pyocyanin was extracted with chloroform from culture supernatants of strains grown in 50-ml Erlenmeyer flasks containing 20 ml of glycerol-alanine medium (13) with shaking at 37°C for 20 h. Pyocyanin was quantified spectrophotometrically at 520 nm (11). HCN was quantified (15) in culture supernatants of strains grown in tightly closed 125-ml bottles containing 60 ml glycine minimal medium (5); samples were taken after 10 h of incubation (at ~10^9 cells ml^{-1}).

AHL detection and analysis. Samples (900 μl) were taken from culture supernatants of *P. aeruginosa* strains grown in NYB at 37°C with shaking to OD_{600}s of 0.6 and 2.5, filter sterilized, and treated for AHL quantification as described by Diggle et al. (9). Detection and quantification of 3-oxo-C12-HSL or C4-HSL

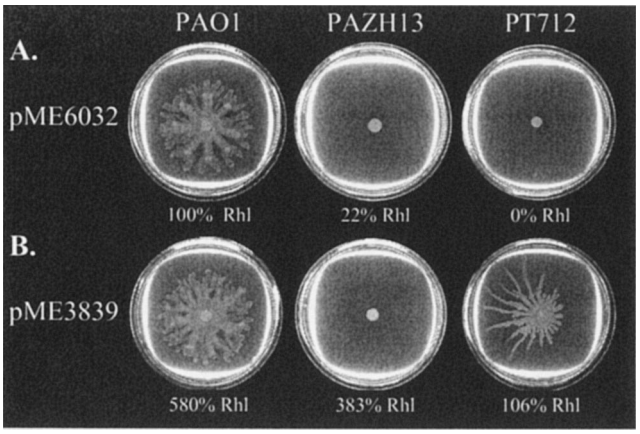

FIG. 1. Influence of an *rsmA* mutation on swarming ability in the absence or presence of rhamnolipids. Inocula of 2 μl from overnight cultures of PAO1 (wild type), PAZH13 (Δ*rsmA*), or PT712 (*rhlA'*::Ω-Gm) with the control vector pME6032 (A) or plasmid pME3839 carrying the *rhlAB* genes under the control of the inducible *tac* promoter (B) were spotted onto 0.5% agar supplemented with 1 mM IPTG and incubated at 37°C overnight. The amounts of rhamnolipids (Rhl) produced by each strain were assayed in a separate experiment as described in Materials and Methods and are indicated below each swarming plate. The value obtained for PAO1/pME6032 (1.21 ± 0.04 μg/ml) was set at 100%.

were done after separation by normal- or reverse-phase thin-layer chromatography (silica gel 60 F_{254}, or RP-18 F_{245}; Merck), respectively. *E. coli* AHL biosensor strains with reporter plasmid pSB1075 (for 3-oxo-C12-HSL detection) or pSB536 (for C4-HSL detection) were used, and bioluminescence was quantified with a Luminograph LB 980 photon video camera (EG & G Berthold) (9). AHL concentrations were estimated by comparison with standards, i.e., 0.13, 0.25, 0.50, or 1.00 μM for 3-oxo-C12-HSL and 1.56, 3.13, 6.25, or 12.50 μM for C4-HSL.

RESULTS

RsmA can act as a positive control element. When compared with the wild type *P. aeruginosa* PAO1, the *rsmA* mutant PAZH13 was unable to swarm (Fig. 1A; Table 2). Swarming of *P. aeruginosa* requires flagella, pili, and rhamnolipids (24). The absence of rhamnolipids in strain PT712 (*rhlA*) sufficed to abolish swarming (Fig. 1A); the *rhlA* gene is proximal in the *rhlAB* rhamnolipid biosynthetic operon (36). The fact that the *rsmA* mutation in strain PAZH13 had no marked effect on swimming and twitching motility (data not shown), but resulted in a fivefold reduction of rhamnolipid synthesis compared to the wild type (Fig. 1A), is consistent with the concept that rhamnolipids sustain swarming motility. However, when pME3839, a *rhlAB*$^+$ plasmid allowing RsmA-independent rhamnolipid production (see Materials and Methods), was introduced into strains PT712 and PAZH13, swarming ability was restored to the *rhlA* mutant but not to the *rsmA* mutant (Fig. 1B). This finding suggests that lack of swarming of the *rsmA* mutant is not solely a consequence of reduced rhamnolipid synthesis but may also be caused by cellular clumping.

The expression of a translational *rhlA'-'lacZ* fusion on pECP60 was reduced two- to threefold in the *rsmA* mutant PAZH13 compared to the wild type, PAO1 (Fig. 2A), confirming the observed reduction of rhamnolipid production in the mutant. By contrast, a transcriptional *rhlA-lacZ* fusion on pME3838 was expressed almost equally well in the wild type and in the *rsmA* mutant (Fig. 2B). This result indicates that the positive effect of RsmA on *rhlA* expression occurs essentially at a posttranscriptional level.

The production of extracellular lipase was also shown to be positively controlled by RsmA in *P. aeruginosa*, as lipase specific activity in a culture supernatant was reduced about twofold in the *rsmA* mutant PAZH13 compared to the wild type (Table 2). This parallel positive regulation of rhamnolipids and lipase is striking and will be considered in Discussion.

Cloning and mutational inactivation of the *rsmZ* gene of *P. aeruginosa* PAO1. Since in other gram-negative bacteria the effects of RsmA are antagonized by small noncoding RNAs such as RsmY and RsmZ of *P. fluorescens* CHA0 (18, 55), we carried out a nucleotide sequence alignment of the conserved *rpoS-rsmZ-fdxA* region of different *Pseudomonas* species, i.e., *P. putida* Corvallis and WCS358, *P. syringae* pv. s*yringae* B728a,

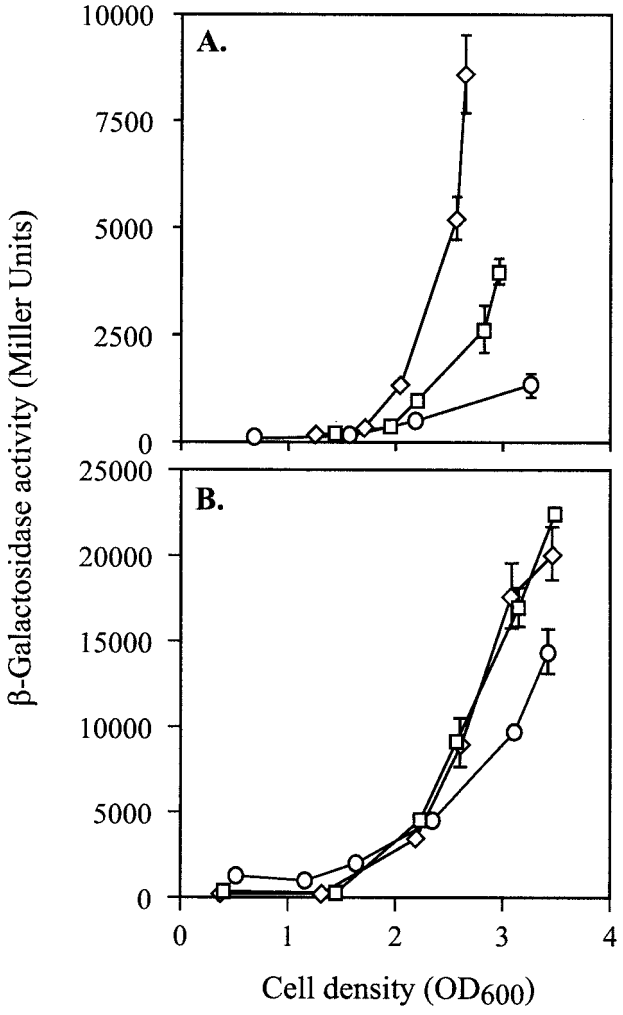

FIG. 2. Influence of *rsmA* and *rsmZ* mutations on *rhlA* expression. β-Galactosidase expression from a translational *rhlA'-'lacZ* fusion on pECP60 (A) and a transcriptional fusion on pME3838 (B) was determined in PAO1 (□), PAO6354 (*rsmZ*) (◇), and PAZH13 (*rsmA*) (○). Each result is the mean ± standard deviation of the results from three measurements. Bacterial growth in NYB medium reached a plateau at an OD_{600} of about 3.

TABLE 2. RsmA- and RsmZ-dependent production of lipase, pyocyanin, and HCN and swarming ability in *P. aeruginosa* strains

Strain (genotype)	Swarming[a]	Lipase[b] (ng/10^9 cells)	Pyocyanin[c] (μg/10^9 cells)	HCN[d] (μM)
PAO1 (wild type)	+	1.48 ± 0.52	1.24 ± 0.21	6.25 ± 0.42
PAZH13 (*rsmA*)	−	0.77 ± 0.19	4.89 ± 0.13	32.50 ± 1.88
PAO6354 (*rsmZ*)	+	1.69 ± 0.39	0.92 ± 0.26	5.57 ± 0.92
PAO1/pME6000 (control for pME3337.1)	+	1.18 ± 0.39	0.76 ± 0.08	5.28 ± 1.32
PAO1/pME3337.1 (*rsmZ*$^{++}$)	−	0.21 ± 0.02	5.92 ± 0.98	16.53 ± 0.98

[a] Swarming ability was tested on semisolid medium incubated at 37°C, as described in Materials and Methods. +, ability to swarm and completely invade the plate within 24 h; −, no swarming.
[b] Concentrations of lipase were measured for strains grown in YEA medium to about 4×10^9 cells ml^{-1} (see Materials and Methods).
[c] Pyocyanin was extracted and quantitated for *P. aeruginosa* strains grown in glycerol-alanine medium (see Materials and Methods).
[d] Concentrations of HCN were determined for strains grown under semianaerobic conditions in MMC medium to about 1.1×10^9 cells ml^{-1}, as described in Materials and Methods.

P. fluorescens F113 and CHA0, and *P. aeruginosa* PAO (data not shown). Although within the *rsmZ* gene of these organisms, nucleotide sequence conservation was only about 45%, a repeated GGA motif, which is also characteristic of CsrB, CsrC, RsmB, and RsmY, clearly stands out, as illustrated in RsmZ of *P. fluorescens* CHA0 and *P. aeruginosa* PAO1 (Fig. 3A). Moreover, despite primary sequence differences, the predicted secondary structures of RsmZ RNAs from both bacterial strains are highly conserved. In particular, both RsmZ RNAs show four stem-loop structures with GGA motifs in the loops and an additional hairpin structure formed by the 3′ region (Fig. 3B) (18). Using sequence information from the complete genome of *P. aeruginosa* PAO, we PCR amplified and cloned the *rsmZ* region including its promoter and its terminator, and a chromosomal 250-bp deletion in *rsmZ* was constructed in strain PAO6354 (as described in Materials and Methods). RsmZ RNA was revealed as a single band corresponding to ca. 120 nucleotides in the wild type, PAO1, and the absence of this RNA from strain PAO6354 was confirmed in the same Northern blot experiment (Fig. 4A).

RsmZ as an antagonist of RsmA. We studied the phenotypic effects of deletion and overexpression of *rsmZ* and compared them with those observed for deletion of *rsmA*, to verify the prediction that RsmZ RNA antagonizes RsmA activity in *P. aeruginosa*. The *rsmZ* deletion mutant PAO6354 grew as well as the wild type, PAO1, and did not exhibit any morphological changes in colony phenotype after growth on nutrient agar. Production of RsmA protein, as determined by Western blotting, was similar in the *rsmZ* mutant and in the wild type (data not shown). For overexpression, *rsmZ* was cloned with its promoter into the multicopy vector pME6000, giving pME3337.1. Overproduction of RsmZ RNA was confirmed by Northern blotting (Fig. 4B). We examined the role of RsmZ in the regulation of rhamnolipid and lipase production, which in this study, have been shown to be positively controlled by RsmA. We also determined the importance of RsmZ for extracellular enzymes and AHLs whose production is known to be negatively regulated by RsmA in *P. aeruginosa* (43). These experiments gave the following results.

Overexpression of *rsmZ* in PAO1/pME3337.1 resulted in loss of swarming ability, comparable with that of an *rsmA* mutant, whereas an *rsmZ* deletion had no visible effect on swarming (Table 2). The expression of a translational *rhlA′-′lacZ* fusion in strain PAO1 was enhanced about twofold in the *rsmZ* mutant PAO6354, at an OD_{600} of ≥2.5, compared to the wild type, PAO1 (Fig. 2A).

Whereas the *rsmZ* mutant PAO6354 was not different from the wild type for lipase production (Table 2), the *rsmZ*-overexpressing strain PAO1/pME3337.1 produced about six-times-less lipase than did the control PAO1/pME6000 (Table 2). In this case, overproduction of RsmZ RNA resulted in an effect that was even greater than that of an *rsmA* mutation (Table 2); the reasons for this difference are not yet evident.

The *rsmZ*-overexpressing strain PAO1/pME3337.1, in common with the *rsmA* mutant PAZH13, produced more pyocyanin than did the wild type, PAO1 (Table 2). In contrast, the *rsmZ* mutation in strain PAO6354 did not exert any significant effect on pyocyanin levels (Table 2).

Cyanogenesis was compared in the same strains. Both the *rsmA* mutant and the *rsmZ*-overexpressing strain produced more HCN than did the PAO1 control or the *rsmZ* mutant (Table 2).

The cytotoxic internal lectin PA-IL (LecA) followed the same expression pattern as that observed for pyocyanin and HCN. Mutation of *rsmA* or overexpression of *rsmZ* resulted in strongly enhanced production compared to the wild type and the *rsmZ* mutant (Fig. 5).

Thus, loss of RsmA and overexpression of RsmZ had similar effects on the formation of these exoproducts, and this RsmA-RsmZ antagonism was observed for both positive and negative control exerted by RsmA. In only one case (*rhlA* expression) did the *rsmZ* mutation result in a measurable effect; in the other examples studied, mutational loss of *rsmZ* did not have significant consequences, suggesting a possible redundancy of genes encoding small regulatory RNAs able to interact with RsmA.

With respect to AHL production and to expression of the quorum-sensing genes *lasR*, *lasI*, *rhlR*, and *rhlI*, the consequences of *rsmZ* overexpression were slight (induction factors of ≤2) (data not shown). Furthermore, *rsmZ* overexpression had only minor effects on total extracellular protease, elastase (LasB), and staphylolytic enzyme (LasA). All of these phenotypes revealed no apparent change in an *rsmZ* null mutant (data not shown).

Regulation of *rsmZ* expression. We constructed a transcriptional fusion of the *lacZ* reporter gene to the *rsmZ* promoter on pME3331, in which the +1 nucleotide of *lacZ* corresponds to the transcription start site of the *rsmZ* promoter (Fig. 3A), as described in Materials and Methods. Expression of this *rsmZ-lacZ* fusion in the wild type, PAO1, was cell density dependent, with optimal transcription of the *rsmZ* gene at the end of exponential growth phase (Fig. 6A). This result was

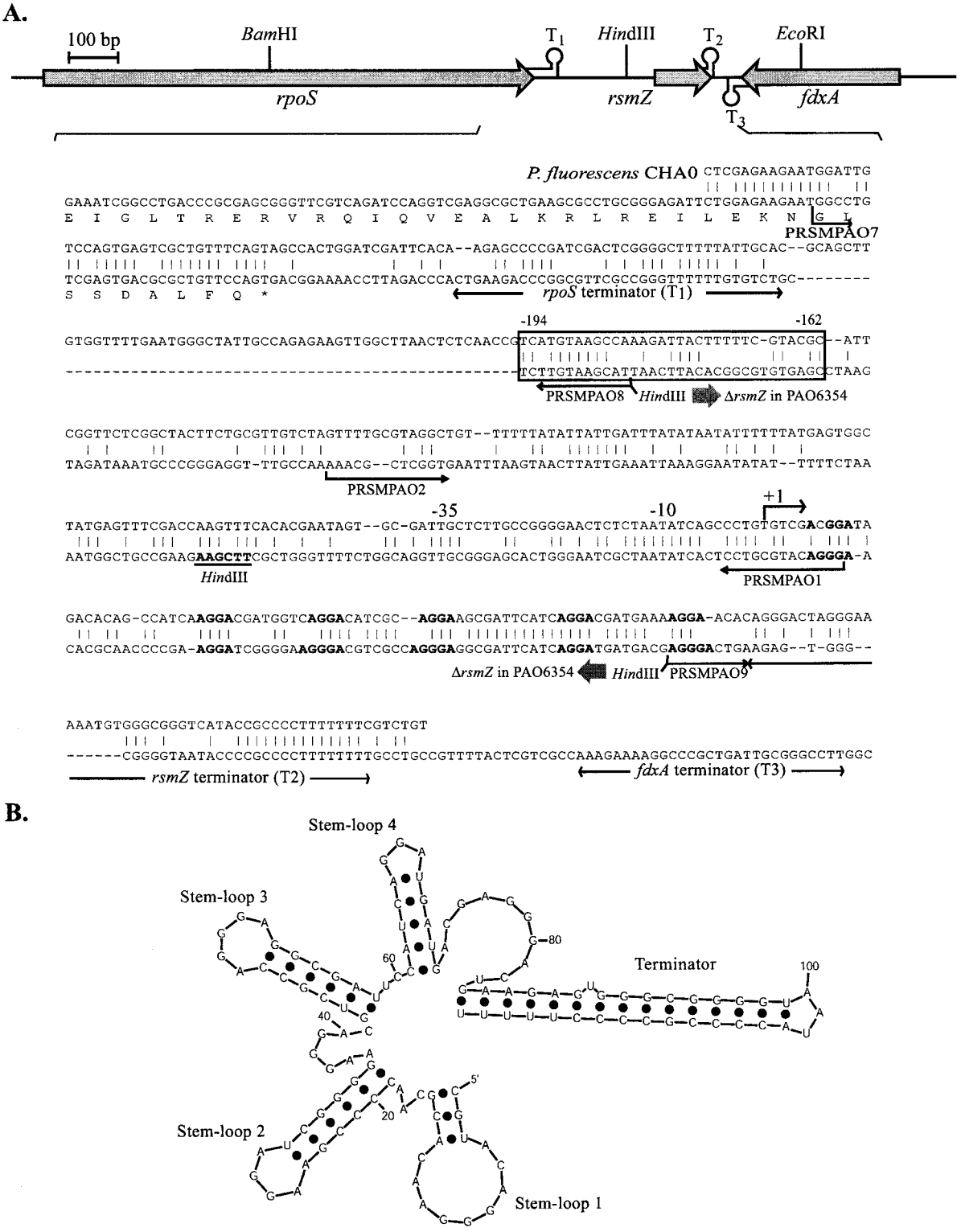

FIG. 3. (A) The 1.8-kb region of *P. aeruginosa* PAO1 with *rpoS*, *rsmZ*, and *fdxA* (50). Nucleotide sequence identities with the *rsmZ* region of *P. fluorescens* CHA0 (18) are represented. The transcription start site (+1) and the −35/−10 promoter sequences are deduced from the similar *prrB* (*rsmZ*) gene of *P. fluorescens* F113 (1). T1 and T2 are rho-independent terminators. A well-conserved upstream activation sequence pointed out by Heeb et al. (18) located around position −180 is boxed. The *rsmZ* deletion of strain PAO6354 extends from −177 to +70 and was created between the two artificial HindIII restriction sites in primers PRSMPAO8 and PRSMPAO9 indicated below the sequence. (B) Predicted secondary structure of RsmZ at 37°C obtained by using the M-fold program (65).

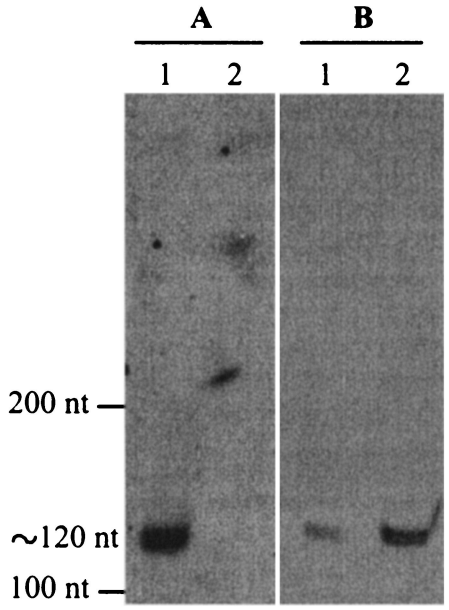

FIG. 4. Northern blot revealing RsmZ RNA. Total RNA (4 μg) from *P. aeruginosa* PAO1 and PAO6354 was electrophoresed in a urea–8% acrylamide–0.4% bisacrylamide gel and hybridized with an *rsmZ* digoxigenin-labeled probe (see Materials and Methods). (A) Lane 1, PAO1; lane 2, PAO6354 (Δ*rsmZ*). (B) Lane 1, PAO1/pME6000 vector control; lane 2, PAO1/pME3337.1 (*rsmZ*$^{++}$). nt, nucleotides.

corroborated by a Northern blot showing increasing RsmZ RNA concentrations in parallel with increasing cell densities. Interestingly, in the stationary phase (after 24 h of growth), most of the RsmZ RNA was degraded (Fig. 6B). When either *gacA* or *rsmA* was inactivated in the mutants PAO6281 and PAZH13, respectively, expression of the *rsmZ-lacZ* fusion on pME3331 was abolished, indicating that both GacA and RsmA have positive effects on the *rsmZ* promoter, directly or indirectly (Fig. 6A). Again, a Northern blot confirmed the positive regulatory roles of GacA and RsmA. Overexpression of RsmA resulted in overexpression of RsmZ (Fig. 6C). This *rsmZ* expression pattern prompted us to examine the possibility that *rsmZ* might be controlled via AHL-dependent quorum sensing; however, addition of 5 μM 3-oxo-C12-HSL or 10 μM C4-HSL had no effect on the expression of *rsmZ-lacZ* in strain PAO1 (data not shown).

Deletion of *rsmZ* in the mutant PAO6354 resulted in a threefold-enhanced expression of *rsmZ-lacZ* (Fig. 6A), suggesting that *rsmZ* can negatively regulate its own expression.

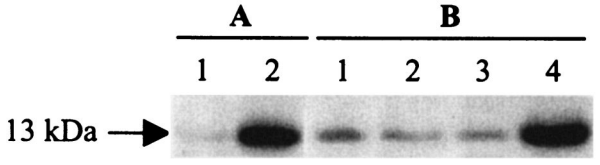

FIG. 5. Influence of an *rsmZ* mutation on lectin production. Cells were grown in NYB to an OD_{600} of 2.5, and lectin was visualized by Western blotting as previously described (43). (A) Lane 1, PAO1 (wild type); lane 2, PAZH13 (Δ*rsmA*). (B) Lane 1, PAO1 (wild type); lane 2, PAO6354 (Δ*rsmZ*); lane 3, PAO1/pME6000 (control for pME3337.1); lane 4, PAO1/pME3337.1 (*rsmZ*$^{++}$).

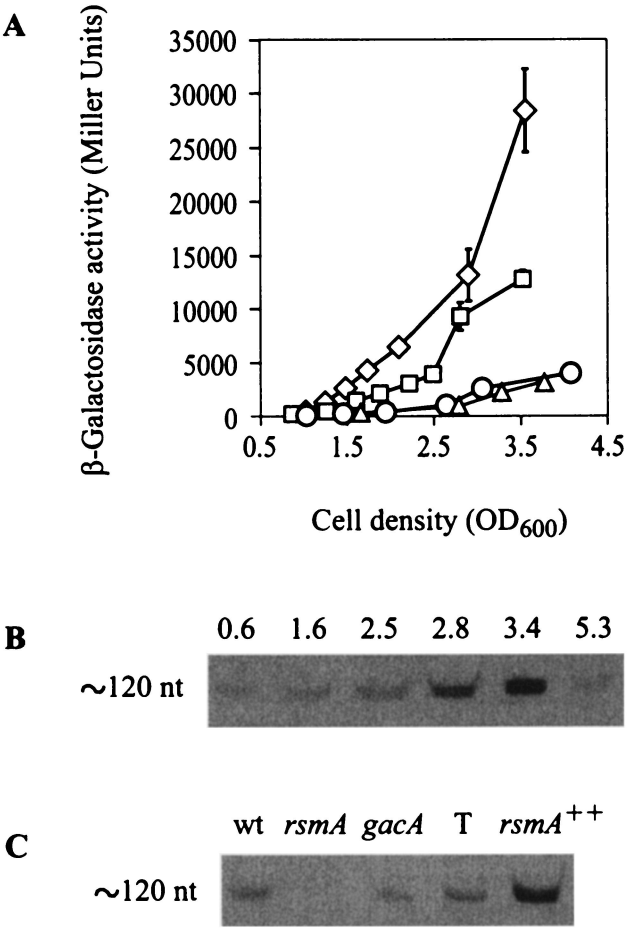

FIG. 6. Influence of different mutations on *rsmZ* expression. (A) β-Galactosidase expression from the transcriptional fusion *rsmZ-lacZ* on pME3331 was determined in the wild type, PAO1 (□), the *rsmZ* mutant PAO6354 (◇), the *rsmA* mutant PAZH13(○), and the *gacA* mutant PAO6281 (△). Each result is the mean ± standard deviation of the results from three measurements. Bacterial growth reached a plateau at an OD_{600} of 2 to 3. (B) Northern blot showing RsmZ RNA. Total RNA (4 μg) from *P. aeruginosa* PAO1 was prepared from cultures grown to different densities, as indicated by OD_{600} values. (C) Northern blot showing RsmZ RNA in different genetic contexts. T, pME6001 vector control; nt, nucleotides; wt, wild type.

The double mutants PAO6343 (*gacA rsmA*) and PAO6385 (*gacA rsmZ*) both showed the same low levels of *rsmZ* expression, as did the single *gacA* mutant PAO6281 (data not shown). We considered the possibility that RsmA might exert its positive effect on *rsmZ* expression via GacA, similar to the situation described for *E. coli* where the RsmA homolog CsrA appears to regulate the GacA homolog UvrY (58). However, a chromosomal translational *gacA′-′lacZ* fusion showed no significant difference in its expression when inserted into either the wild type, PAO1, or the *rsmA* mutant PAZH13 (Fig. 7). Moreover, the *gacA′-′lacZ* fusion was expressed normally in the *gacA* mutant PAO6281 and the *gacS* mutant PAO6327 (data not shown). Together, these data indicate that RsmZ and RsmA have opposite effects on the expression of *rsmZ* and that these effects are not mediated by the GacS/GacA two-component system.

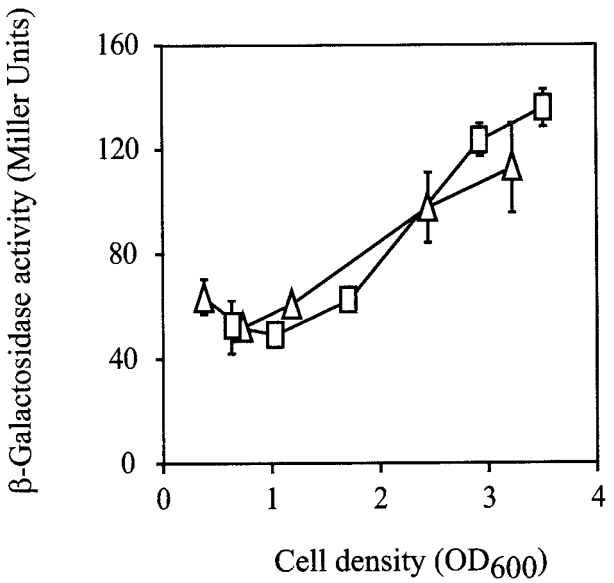

FIG. 7. Influence of an *rsmA* mutation on *gacA* expression. β-Galactosidase activities from a translational *gacA′-′lacZ* fusion inserted at the Tn*7* attachment site of the chromosome were determined in the wild type, PAO1 (□), and the *rsmA* mutant PAZH13 (△). Each result is the mean ± standard deviation of the results from three measurements. Bacterial growth reached a plateau at an OD_{600} of about 3.

DISCUSSION

In *P. aeruginosa*, the small RNA-binding protein RsmA has previously been shown to control negatively the expression of several genes involved in the production of extracellular virulence factors, e.g., HCN, pyocyanin, lectin (LecA), elastase (LasB), and staphylolytic enzyme (LasA) (43). Moreover, RsmA modulates negatively the synthesis of 3-oxo-C12-HSL and C4-HSL, by exerting some kind of transient repression on the *lasI* and *rhlI* genes (43). Here we report positive effects of RsmA on swarming and on lipase and rhamnolipid production in *P. aeruginosa* (Fig. 2; Table 2). The positive effect on swarming may involve the production of rhamnolipids (Fig. 2), i.e., surfactants which lower the surface tension and facilitate the spreading of bacteria on semisolid surfaces. However, the inability of rhamnolipid production in strain PAZH13 (*rsmA*) carrying pME3839 ($rhlA^+B^+$) to restore swarming suggests that an *rsmA* mutation may cause cell-cell aggregation that rhamnolipids cannot dissolve. Whether RsmA regulates the formation of flagella and pili, which are also required for swarming (24), is uncertain. However, judging from the swimming and twitching abilities of an *rsmA* mutant, we believe that a major involvement of RsmA in the control of these processes is unlikely. By contrast, the RsmA homolog CsrA positively regulates the synthesis of flagella and swimming in *E. coli* (57), whereas in *Erwinia* spp., RsmA negatively affects motility (34).

Negative control exerted by RsmA on pyocyanin, HCN, and lectin formation in *P. aeruginosa* (43) was confirmed in the present study (Table 2; Fig. 5). The positive and negative effects of RsmA were revealed to be antagonized by overexpression of a small noncoding RNA termed RsmZ. This regulatory RNA is similar to RsmZ (alternatively designated PrrB) of *P. fluorescens* strains (1, 18). Although nucleotide sequence identities between RsmZ of *P. aeruginosa* PAO1 and RsmZ of *P. fluorescens* CHA0 are only 58%, the conserved neighborhood of the *rsmZ* genes and the conserved secondary structures of the RNAs encoded in both organisms (Fig. 3) clearly indicate homology and suggest an analogous mode of action, i.e., sequestration of the RNA-binding protein RsmA. This general model is supported by data for CsrA/CsrB/CsrC in *E. coli*, RsmA/RsmB in *Erwinia* spp., and RsmA/RsmY/RsmZ in *P. fluorescens* (3, 6, 18, 47, 55, 58). Moreover, akin to the situation in *E. coli*, *E. carotovora*, and *P. fluorescens* (18, 19, 52), the GacS/GacA two-component system is strictly required for expression of *rsmZ* in *P. aeruginosa* (Fig. 6A and C).

Expression of the *rsmZ* gene strongly depends on RsmA and is derepressed in an *rsmZ* mutant of *P. aeruginosa* (Fig. 6A). As these effects were observed with a construct consisting of only the *rsmZ* promoter fused to the *lacZ* reporter, a possible effect of RsmA on RsmZ RNA stability cannot explain the observed regulation. Given the strict GacA dependence of the promoter (Fig. 6A and C), we considered the possibility that RsmA might have a positive feedback regulation effect on the GacS/GacA system. However, using a *gacA′-′lacZ* fusion, we could not detect any significant stimulation of *gacA* expression by RsmA (Fig. 7). It could be hypothesized that the *rsmZ* promoter might additionally be under negative control mediated by one or several regulatory proteins, which in turn would be repressed by RsmA. The model (Fig. 8) which we derive from this study and previous work (42, 43, 45) is a variant of similar models that have been proposed for the BarA/UvrY (= GacS/GacA)-CsrA/CsrB and the GacS/GacA-RsmA/RsmB regulatory circuitries of *E. coli* and *Erwinia* spp., respectively (6, 52).

The role of the quorum-sensing machinery in the GacS/GacA-RsmZ/RsmA regulatory network of *P. aeruginosa* deserves a closer look. Production of C4-HSL and, to a lesser extent, of 3-oxo-C12-HSL is controlled positively by GacA (45) and negatively by RsmA (43). Extracellular products whose synthesis is positively regulated by AHLs may therefore be expected to be under positive control by GacA and under negative control by RsmA. Such is indeed observed in a number of examples, e.g., in the case of the *hcnABC* genes encoding HCN synthase (42). However, the GacA-RsmA 3-oxo-C12-HSL/C4-HSL regulatory pathway contributes <30% of the observed cell density-dependent regulation of HCN synthesis (42). A second regulatory pathway involving direct RsmA-mediated posttranscriptional repression of the *hcnA* 5′ leader mRNA has a more pronounced effect and accounts for >70% of the positive effect of GacA (42). This dual GacA control may also apply to the regulation of rhamnolipid and lipase formation, where an RsmA-stimulated direct pathway appears to have a stronger overall impact than the RsmA-repressed quorum-sensing branch (Fig. 8).

The mechanism by which RsmA brings about a positive effect on rhamnolipid and lipase synthesis remains to be elucidated. By analogy with positive CsrA control of flagellar mobility in *E. coli*, which results from a stabilization of the *flhDC* mRNA by CsrA binding (57), we propose that RsmA might stabilize the *rhlA* and *lipA* mRNAs and/or facilitate their translation initiation. However, an alternative possibility is that RsmA might act indirectly. Previously, the product of the *dksA* gene has been found to exert positive posttranscriptional control on the expression of *rhlI*, *rhlAB*, and *lasB* in *P. aeruginosa* (22).

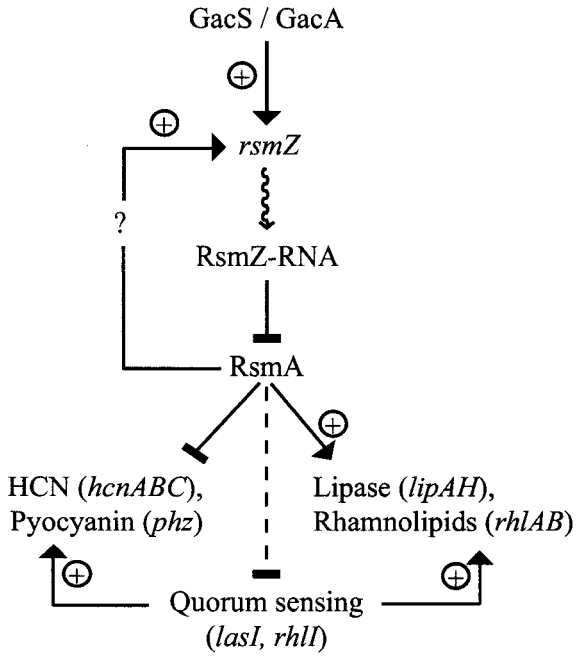

FIG. 8. Model of the GacA/RsmA signal transduction pathway in *P. aeruginosa* PAO1. Expression of the untranslated regulatory RNA RsmZ depends on the presence of GacA. The function of RsmZ is to antagonize the action of the small RNA-binding protein RsmA. RsmA positively controls *rsmZ* expression, thus forming a negative autoregulatory circuit whose mechanism is not understood at present. RsmA also negatively controls AHL-dependent quorum sensing as well as a number of quorum-sensing-dependent genes, some of which code for secondary metabolites and virulence determinants; these are regulated indirectly at the transcriptional level via quorum sensing but probably also directly at the translational level, as is the case for *hcnA* (42). Lipase and rhamnolipid production are controlled positively by RsmA, independently of the quorum-sensing control. Dotted line, modulating negative effect; solid bar, negative effect; arrow, positive effect.

The parallel positive impact of RsmA on rhamnolipid and lipase production may be beneficial to *P. aeruginosa*, as rhamnolipids improve the availability of hydrophobic compounds such as lipids to the bacteria, resulting in accelerated degradation of such compounds (35).

The *rsmZ* mutant PAO6354 was phenotypically similar to the wild type, PAO1, in most assays conducted (Table 2) and also with respect to AHL production. Overexpression of *rsmZ* from the multicopy plasmid pME6000, by contrast, had significant effects on the formation of several exoproducts, similar to those caused by an *rsmA* mutation (Table 2). These results suggest that RsmZ might not be the only regulatory RNA in the GacS/GacA cascade of *P. aeruginosa*. The fact that *rsmZ* expression is subject to negative autoregulation in wild-type *P. aeruginosa* should be a reason for cautious interpretation of the *rsmZ* overexpression data. Whereas these data support the model of RsmA sequestration by RsmZ, they do not reflect the natural, finely balanced situation in vivo.

The small regulatory RNAs CsrB and CsrC of *E. coli*, CsrB of *Erwinia* spp., RsmY and RsmZ of *P. fluorescens* (1, 18, 29, 55, 58), and RsmZ of *P. aeruginosa* (Fig. 3B) all have elaborate secondary structures, and they share limited nucleotide sequence identities. As a common denominator, single-stranded GGA motifs stand out and might be important for binding of RsmA/CsrA (58).

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