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The Global Posttranscriptional Regulator RsmA Modulates Production of Virulence Determinants and *N*-Acylhomoserine Lactones in *Pseudomonas aeruginosa*

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Posttranscriptional control is known to contribute to the regulation of secondary metabolism and virulence determinants in certain gram-negative bacteria. Here we report the isolation of a *Pseudomonas aeruginosa* gene which encodes a global translational regulatory protein, RsmA (regulator of secondary metabolites). Overexpression of *rsmA* resulted in a substantial reduction in the levels of extracellular products, including protease, elastase, and staphylolytic (LasA protease) activity as well as the PA-IL lectin, hydrogen cyanide (HCN), and the phenazine pigment pyocyanin. While inactivation of *rsmA* in *P. aeruginosa* had only minor effects on the extracellular enzymes and the PA-IL lectin, the production of HCN and pyocyanin was enhanced during the exponential phase. The influence of RsmA on *N*-acylhomoserine lactone-mediated quorum sensing was determined by assaying the levels of *N*-(3-oxododecanoyl)homoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL) produced by the *rsmA* mutant and the *rsmA*-overexpressing strain. RsmA exerted a negative effect on the synthesis of both 3-oxo-C12-HSL and C4-HSL, which was confirmed by using *lasI* and *rhlI* translational fusions. These data also highlighted the temporal expression control of the *lasI* gene, which was induced much earlier and to a higher level during the exponential growth phase in an *rsmA* mutant. To investigate whether RsmA modulates HCN production solely via quorum-sensing control, *hcn* translational fusions were employed to monitor the regulation of the cyanide biosynthesis genes (*hcnABC*). RsmA was shown to exert an additional negative effect on cyanogenesis posttranscriptionally by acting on a region surrounding the *hcnA* ribosome-binding site. This suggests that, in *P. aeruginosa,* RsmA functions as a pleiotropic posttranscriptional regulator of secondary metabolites directly and also indirectly by modulating the quorum-sensing circuitry.

Pseudomonas aeruginosa is an opportunistic human pathogen responsible for infections in immunocompromised hosts and in the lungs of individuals with cystic fibrosis (31, 34). Secretion of numerous toxic compounds and degradative enzymes (elastase, LasA protease, phospholipase C, exotoxin A, exoenzyme S, rhamnolipid, hydrogen cyanide [HCN], and pyocyanin) contributes to the pathogenesis of *P. aeruginosa* infections (31, 34). Many of these exoproducts are produced during the late exponential phase of growth, when the cell density is high. In *P. aeruginosa*, cell density-dependent gene expression is coordinated via *N*-acylhomoserine lactone (AHL)-mediated quorum sensing.

P. aeruginosa contains two quorum-sensing systems, termed *las* and *rhl* (13, 21). The *las* system consists of the transcriptional activator protein LasR, the AHL synthase LasI, and *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL). LasR and 3-oxo-C12-HSL (generated via LasI) work in concert to control *lasI* expression as well as expression of the *P. aeruginosa rhl* circuitry, which consists of the regulator RhlR, the AHL synthase RhlI, and *N*-butanoyl homoserine lactone (C4-HSL) (13, 21). The *las* and *rhl* quorum-sensing circuitry operates as a hierarchical cascade responsible for regulating the expression of multiple virulence determinants and secondary metabolites, the type II secretion machinery, and stationary-phase genes (via the alternative sigma factor RpoS) (for reviews, see references 31 and 34). In addition, 3-oxo-C12-HSL may also function directly as a virulence factor, given its immune modulatory and vasorelaxant properties (32).

While the control of secondary metabolites and virulence factors by AHL-dependent quorum sensing in *P. aeruginosa* is known to be mediated at the transcriptional level, secondary metabolite production in certain bacterial species, notably *Erwinia carotovora* and *Pseudomonas fluorescens*, is subject to both transcriptional and posttranscriptional control (1, 2, 6, 15, 17, 20, 25). In *E. carotovora*, the global regulator protein RsmA (repressor of secondary metabolism) is a posttranscriptional negative regulator of extracellular enzyme and *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) synthesis (6).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference or origin
P. aeruginosa		
PAO1	Wild type	Holloway Collection
PAZH13	*rsmA* deletion mutant, derivative of PAO1	This study
Plasmids		
pBluescript II KS	Cloning vector; ColE1 replicon; Ap^r	Stratagene
pLPL105	pBluescript II KS containing *lasRI* genes on a 2.4-kb *Bam*HI/*Xho*I fragment	28
pME6001	Cloning vector derived from pBRR1MCS, Gm^r	8
pME6010	pACYC177-pVS1 shuttle vector, Tc^r	8
pME3826	pME6010 with a 0.78-kb *hcnA* upstream fragment and a translational *hcnA′*::*′lacZ* fusion containing the first nine *hcnA* codons (*Eco*RV site)	22
pME3843	pME6010 containing a 1.09-kb *Bgl*II/*Pst*I p*tac-hcn* fragment and a translational *hcnA′*::*′lacZ* fusion containing the first 9 *hcnA* codons (*Eco*RV site)	2
pME3846	pME6010 with a 666-bp *rhlI* upstream fragment and a translational *rhlI′*::*′lacZ* fusion containing the first 14 *rhlI* codons	This study
pME3849	pME6001 with *rsmA* gene on a 311-bp *Bam*HI/*Sma*I fragment	This study
pME3853	pME6010 with 174-bp *lasI* upstream fragment and translational *lasI′*::*′lacZ* fusion containing first 13 *lasI* codons	This study
pME3859	pME6010 with 194-bp *rsmA* upstream fragment and translational *rsmA′*::*′lacZ* fusion containing first 7 *rsmA* codons	This study
pME3860	pME3843 derivative containing 3-bp insertion in RBS	This study
pMP21	pMMB190 carrying 2-kb *Pst*I fragment containing *rhlRI* gene from *P. aeruginosa* PAO1, Ap^r	12
pNM481	*′lacZ* fusion vector, Ap^r	18a
pNM482	*′lacZ* fusion vector, Ap^r	18a

RsmA is considered to function as an RNA-binding protein that reduces the levels of *hslI* (also called *carI* and *expI*; the gene coding for the 3-oxo-C6-HSL synthase) transcripts, thus influencing AHL production (6). RsmA is a homologue of CsrA (carbon storage regulator), a protein that was initially identified in *Escherichia coli* as a global regulator affecting cell size, cell surface properties, and the regulation of carbon metabolism (25, 26). CsrA is a 61-amino-acid protein containing an RNA-binding motif that is also found in eukaryotic RNA-binding proteins. By controlling access to the ribosome-binding site (RBS) and by altering mRNA stability, CsrA is considered to function as a posttranscriptional regulator (16, 25). In *E. coli*, the regulatory activity of CsrA is modulated by CsrB, an untranslated RNA which binds to about 20 CsrA molecules, titrating the available concentration of free CsrA (25). A regulatory RNA related to CsrB has also been identified in *E. carotovora* (RsmB) (17) and in *P. fluorescens* F113 (PrrB) (1).

In several different *Pseudomonas* species, the GacS/GacA two-component global regulatory system positively regulates production of secondary metabolites (e.g., HCN and antibiotics) and exoenzymes (5, 11, 14, 24). Overexpression of *rsmA* in *P. fluorescens* CHA0 mimics a GacA defect (2), while overproduction of PrrB in a *P. fluorescens* F113 *gacA* or *gacS* mutant restores HCN and 2,4-diacetylphloroglucinol synthesis (1). In *P. fluorescens* CHA0, a strain that does not have an AHL-dependent quorum-sensing system, the RsmA protein appears to act in the vicinity of the RBS of a target gene(s), e.g., the HCN biosynthesis cluster *hcnABC* (2, 25). In *P. aeruginosa*, the *hcnABC* cluster is regulated not only by GacA, but also via the *las* and *rhl* quorum-sensing circuitry (22, 23). Furthermore, GacA exerts a positive effect on the transcription of *lasR*, *rhlR*, and *rhlI*; consequently, the production of C4-HSL is both delayed and reduced in a *P. aeruginosa gacA* mutant (24). In the present study, we sought to determine whether, and at what level, RsmA is involved in regulating the production of quorum-sensing-dependent secondary metabolites and virulence determinants in *P. aeruginosa.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *P. aeruginosa* was routinely grown in nutrient yeast broth (NYB) or on nutrient agar (NA) plates (24). When required, gentamicin and tetracycline were added at 10 μg ml^{-1} and 125 μg ml^{-1}, respectively.

DNA manipulation. Plasmid DNA preparations, restriction enzyme digestions, ligations, and agarose gel electrophoresis were performed using standard procedures (27). Restriction fragments were purified from agarose gels using the Geneclean II Kit (BIO 101). Transformation of *P. aeruginosa* was carried out by electroporation (22). Nucleotide sequences were determined by automated sequencing, and the data were analyzed with Blast, Gap, and Bestfit using the Genetics Computer Group package.

Cloning, overexpression, and mutation of *P. aeruginosa rsmA* gene. To amplify the *P. aeruginosa rsmA* gene sequence, we used degenerate PCR primers based on the *E. coli* and *E. carotovora csrA* and *rsmA* sequences, respectively (rsm-F: 5′-ATG CTI RTY YTR WCI CGI MRA RT-3′; rsm-R1: 5′-GGT ART CTK TTY ISK RTG RAY-3′). The PCR product obtained was sequenced and used to screen a cosmid bank for the *P. aeruginosa rsmA* gene, which was located on a 1.5-kb *Pst*I fragment and introduced into pBluescript II KS. For *rsmA* overexpression studies, the *P. aeruginosa rsmA* gene was generated by PCR using primers rsmA1 (5′-CTGGCCAAGGAAAGCATCAAC-3′) and rsmA2 (5′-CT CCGCAA*CCCGGG* GCGCATG-3′). The PCR fragment was trimmed to 311 bp with *Bam*HI and *Sma*I (site shown in italics) and placed under the control of the *lac* promoter in vector pME6001 (2) to give pME3849 (Table 1). The *P. aeruginosa rsmA* mutant PAZH13 was constructed by gene replacement using methods described before (33) and contains an internal deletion of 54 codons in the *rsmA* open reading frame (ORF) and has a translational stop codon after amino acid position 3.

Construction of translational fusions. Primers RA1 (5′-GCTC*GAATTC*GTG AGTGACGCTGACAGG-3′; *Eco*RI site in italics) and RA2 (5′-GCTC*CTGC AG*CCGACGAGTCAGAATCAG-3′; *Pst*I site in italics) and plasmid pME3849 (Table 1), used as a PCR template, served to clone the upstream region and the first seven codons of the *rsmA* gene, which was fused in-frame with the *′lacZ* gene from pNM482 in vector pME6010, to give the *rsmA′-′lacZ* reporter pME3859 (Table 1).

The *lasI′-′lacZ* translational fusion was constructed as follows. The *lasI* gene region contained in pLPL105 (28) was amplified by PCR using primers L1 (5′-AAAA*CTGCAG*TTTATCGAACTCTT-3′) and L2 (5′-GCTC*GGATCC*GA

CGTTTCTTCGAG-3′). The 174-bp PCR product, containing 98 bp of the promoter region, was trimmed with *Pst*I and *Bam*HI (sites in italics) and fused in frame to *′lacZ* from pNM482 and introduced into pME6010, giving pME3853 (Table 1). Plasmid pME3846 (Table 1) was constructed by fusing in frame the *rhlI* gene on a 704-bp *Bam*HI/*Pst*I fragment with the *′lacZ* gene from pNM481 in pME6010 (8). A 751-bp-long PCR product was obtained by amplification of the *rhlI* region contained in plasmid pMP21 (12) using primers R1 (5′-GCTGGAG CGATACCAGATGCA-3′), which anneals 702 bp upstream of the *rhlI* translational start site, and R2 (5′-AAAA*CTGCAG*CGGAAAGCCCTTCCAGCG-3′), which anneals at position +22 and contains an artificial *Pst*I site (italic).

The construction of plasmid pME3826 with a translational *hcnA′-′lacZ* fusion of *P. aeruginosa* strain PAO1 has been reported previously (22). In plasmid pME3843, the *hcnA* promoter of plasmid pME3826 was replaced by the constitutively expressed *tac* promoter as described (2). A derivative of pME3843 which contains a 3-bp insertion (italic) in the RBS (CAC*ACA*GG) was constructed using the oligonucleotide-annealing technique (7). The 76-bp-long *Kpn*I/*Pst*I region of pME3843 containing the *hcn* 5′ untranslated region and the first nine codons of the *hcnA* coding region was replaced with a *Kpn*I/*Pst*I linker carrying a mutated Shine-Dalgarno sequence, resulting in plasmid pME3860.

β-Galactosidase assay. For β-galactosidase measurements by the Miller method (27), *P. aeruginosa* cells were grown in NYB with shaking at 37°C.

Detection of RsmA and PA-IL lectin by Western blotting. *P. aeruginosa* PAO1, PAO1(pME3849), and PAZH13 were grown in NYB at 37°C with shaking. Bacterial cells were harvested at intervals and lysed by sonication. After standardizing for total protein content, each sample was heated in lithium dodecyl sulfate buffer (Novex), subjected to electrophoresis on 10% Bis-Tris NuPage gels (Novex), and electroblotted onto nitrocellulose. RsmA and PA-IL were detected using polyclonal antibodies raised against the purified *Yersinia enterocolitica* RsmA and the purified lectin, respectively, as described before (33).

Exoproduct assays. Unless otherwise stated, *P. aeruginosa* strains were grown in NYB at 37°C with shaking to an optical density at 600 nm (OD_{600}) of approximately 2.5. Cell-free culture supernatants were assayed for casein-hydrolyzing proteolytic activity (3), elastolytic activity (3), staphylolytic activity (10), and HCN production (22). For pyocyanin, *P. aeruginosa* was grown for 24 h at 37°C with shaking in glycerol-alanine medium and assayed as described before (24).

AHL production. *P. aeruginosa* cultures were grown in NYB at 37°C with shaking and sampled at OD_{600}s of 0.6 and 2.5, which correspond to late exponential and stationary phase, respectively. Cell-free supernatants (10 ml) adjusted to pH 5.0 were extracted with dichloromethane twice with 10 ml each, and the presence of AHLs in 1 to 5 μl of extract was assayed using C_{18} reverse-phase thin-layer chromatography (TLC) overlaid with the indicator organisms *Chromobacterium violaceum* CV026 (18) for the detection of C4-HSL and *Agrobacterium tumefaciens* (*traG-lacZ*) (29) for the detection of 3-oxo-C12-HSL. The levels of AHLs were determined by comparison with known amounts of C4-HSL and 3-oxo-C12-HSL standards synthesized as described before (4).

Nucleotide sequence accession number. The sequence reported here has been assigned accession number AF061757 in the GenBank database.

RESULTS

Cloning and expression of *rsmA* from *P. aeruginosa* PAO1. The *P. aeruginosa rsmA* gene consists of a 186-bp ORF which encodes a 6.9-kDa polypeptide of 61 amino acid residues and is flanked by ORFs with homology to *E. coli* genes encoding aspartate kinase (*lysC*) and a serine tRNA (*serV*) (www.pseudomonas.com). Blast searches revealed that RsmA of *P. aeruginosa* is closely related to other members of this family of RNA-binding proteins, having 93% identity to *Serratia liquefaciens* CsrA (AF074437), 92% to *E. coli* CsrA (L07596), 89% to *E. carotovora* RsmA (L40173), 85% to *Y. enterocolitica* CsrA (25), and 84% to *P. fluorescens* RsmA (AF136151). As with all CsrA and RsmA orthologs, the *P. aeruginosa* RsmA protein contains a putative RNA-binding motif (25) (KH motif). The *P. aeruginosa* RsmA is functionally homologous to CsrA of *E. coli*, since introduction of a plasmid-borne *rsmA* copy complemented an *E. coli csrA* mutant by restoring glycogen synthesis back to wild-type levels (data not shown).

To determine the relationship between growth phase and *rsmA* expression in *P. aeruginosa*, we constructed a translational *rsmA′-′lacZ* fusion. Initially, *rsmA* expression was low, but then it increased approximately threefold over the growth curve (Fig. 1A). This finding was confirmed by Western blotting, which revealed the presence of a single protein of approximately 7 kDa, the level of which increased throughout growth (Fig. 1B and C). A *gacA* mutation had no significant effect on *rsmA′-′lacZ* expression in the exponential phase but caused a

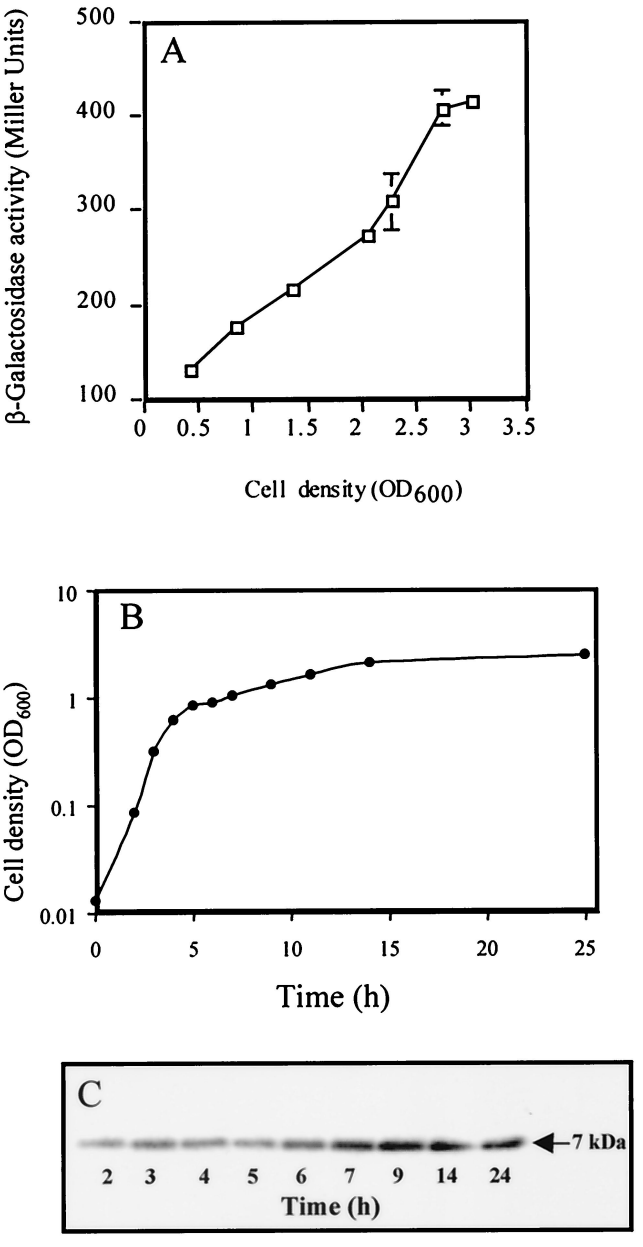

FIG. 1. Expression of *rsmA* in *P. aeruginosa.* (A) Cell density-dependent β-galactosidase expression from a translational *rsmA′-′lacZ* fusion carried by pME3859 in the *P. aeruginosa* PAO1 wild-type strain. Each point is the mean of three measurements ± standard deviation. (B) PAO1 growth curve. (C) Production of RsmA protein by *P. aeruginosa* PAO1. Samples for immunoblot analysis were taken throughout the growth curve at hourly intervals from 2 to 7 h and then at 9, 14, and 25 h.

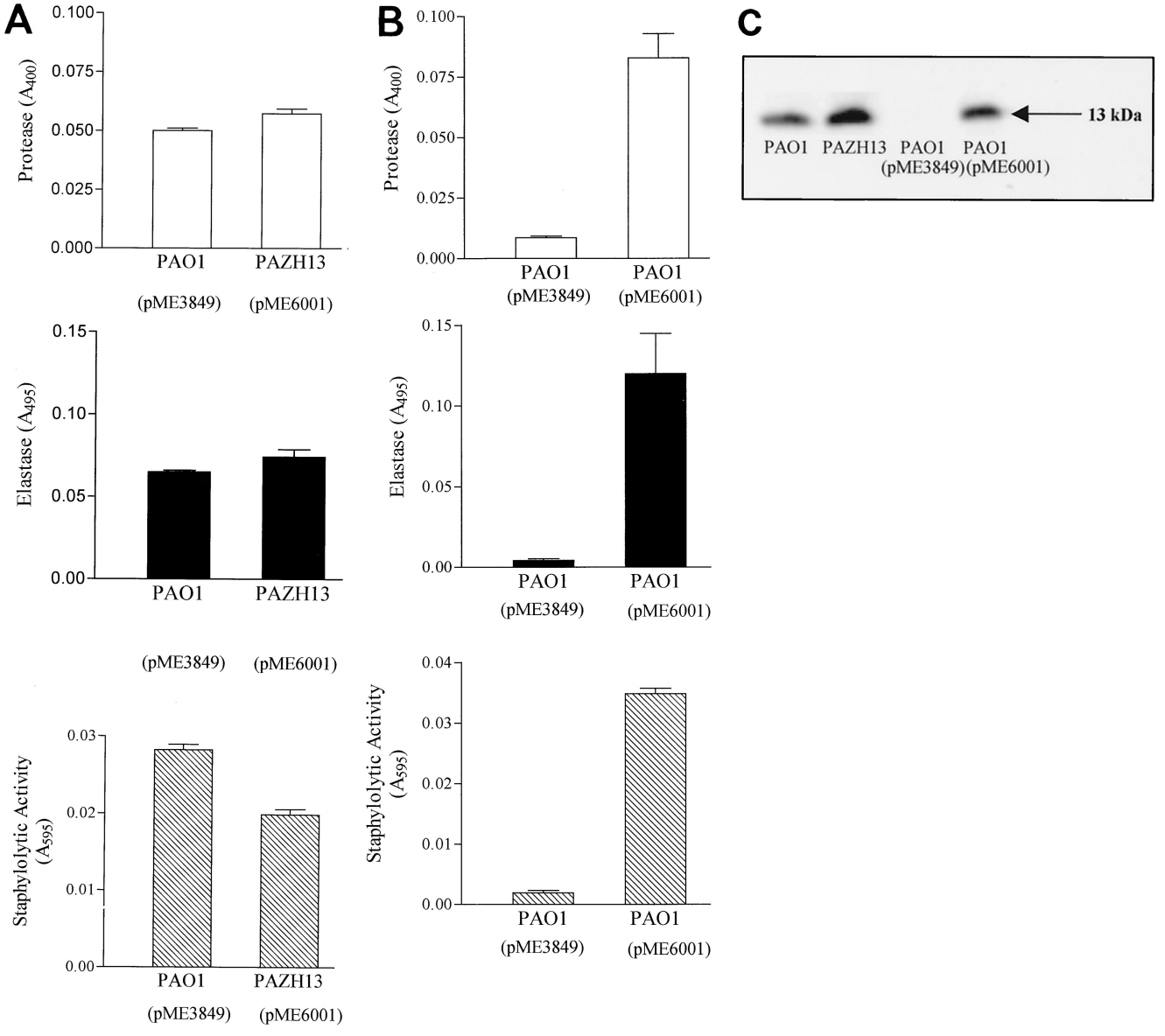

FIG. 2. Influence of RsmA on exoenzyme and PA-IL lectin production. Total protease, elastase, and staphylolytic activities for (A) PAO1 and PAZH13 (*rsmA*) and (B) PAO1(pME3849) (*rsmA*$^{++}$) and PAO1(pME6001) (vector control) were determined spectrophometrically as described in Materials and Methods in cultures grown at 37°C with shaking to an OD_{600} of 2.5. (C) Western blot showing the production of the PA-IL lectin in PAO1, PAZH13 (*rsmA* mutant), PAO1(pME3849) (*rsmA*$^{++}$), and PAO1(pME6001) (vector control).

slight (30%) reduction in early stationary phase (data not shown).

Phenotypic consequences of overexpression or deletion of *rsmA*. To investigate the role of *P. aeruginosa* RsmA in the regulation of virulence determinants and secondary metabolites, we examined the effects of *rsmA* overexpression (*rsmA*$^{++}$) or deletion. For overexpression, *rsmA* from *P. aeruginosa* PAO1 was cloned into the multicopy vector pME6001 under the control of the *lac* promoter (pME3849) and introduced into strain PAO1. In addition, an *rsmA* chromosomal deletion mutant was constructed in PAO1 by allelic exchange as described in Materials and Methods. When grown with good aeration in NYB at 37°C, the *rsmA* mutant grew slightly more slowly (doubling time of 36 min) than the parent strain (doubling time of 31 min; data not shown).

Cell-free supernatants of PAO1, the *rsmA* mutant PAZH13, and the *rsmA*-overexpressing strain PAO1(pME3849) grown to an OD_{600} of 2.5 were assayed for proteolytic, elastolytic, and staphylolytic activities. Protease and elastase activities in the *rsmA* mutant PAZH13 were similar to those in the wild-type, while staphylolytic (LasA protease) activity was reduced by approximately 30% (Fig. 2A). In contrast, overexpression of *rsmA* substantially reduced the three different exoenzyme activities by >80% (Fig. 2B). Furthermore, while production of the cytotoxic "internal lectin" PA-IL (33) was abolished in the *rsmA*$^{++}$ strain, it was produced in the *rsmA* mutant (Fig. 2C).

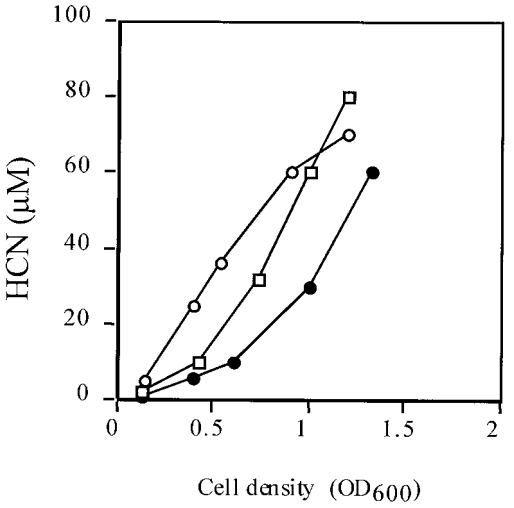

FIG. 3. Cell density-dependent HCN production in *P. aeruginosa*. HCN production was measured in wild-type PAO1 (squares), the *rsmA* mutant PAZH13 (open circles), and the *rsmA*-overexpressing strain PAO1(pME3849) (solid circles). Each point is the mean of three independent experiments.

On *Pseudomonas* isolation agar, which selects for pyocyanin production, the level of the blue pigment produced by the *rsmA* mutant was much higher than that produced by the wild-type strain (data not shown). In parallel, the *rsmA* mutant grown to late exponential phase (OD_{600} of 2.5 to 3.0) in glycerol-alanine liquid medium produced five times more pyocyanin than did the wild-type PAO1 (56 ± 4 versus 10 ± 0 μg ml^{-1}), and the *rsmA*$^{++}$ strain produced 10 times less pyocyanin than the wild type (1 ± 0 versus 10 ± 0 μg ml^{-1}).

Influence of *rsmA* on cyanogenesis. To determine whether, in *P. aeruginosa,* RsmA controls HCN production, we followed HCN production throughout the growth curve in wild-type PAO1, the *rsmA* mutant, and the *rsmA*$^{++}$ strain. The production of HCN was greater in the *rsmA* mutant than in the wild-type PAO1 during the early stages of growth (Fig. 3). As the cell density increased, the difference in the levels of HCN produced by the two strains decreased, until the wild-type culture contained a higher concentration of HCN than the mutant. By comparison, the *rsmA*$^{++}$ strain produced less HCN than either the wild-type or the mutant (Fig. 3).

Effect of *rsmA* on AHL production. In *P. aeruginosa*, the production of numerous exoproducts, including exoenzymes, pyocyanin, and HCN, is under AHL-dependent quorum-sensing control (31, 34). To determine whether RsmA influences the production of 3-oxo-C12-HSL and C4-HSL, we quantified AHL levels both early and late in the exponential phase of growth (Table 2). Although the *rsmA* mutant produced more 3-oxo-C12-HSL than did the parent strain in the early exponential phase, this effect was reversed by the time that the cultures reached late log phase. C4-HSL production followed a similar pattern except that the mutant produced about twice the parental level of C4-HSL by late log phase. The *rsmA*$^{++}$ strain, however, produced significantly lower levels of both AHLs (Table 2).

RsmA-dependent regulation of *lasI* and *rhlI*. To confirm the modulatory effect of RsmA on AHL synthesis as a function of growth, we examined the RsmA-dependent expression of the *P. aeruginosa* genes encoding the AHL synthases, *lasI* and *rhlI*. In an *rsmA* mutant background, expression of a translational *lasI'-'lacZ* fusion was induced prematurely to a level some 10-fold higher than the wild-type level during the exponential phase (Fig. 4A). When RsmA was overproduced, expression of the *lasI'-'lacZ* fusion was delayed until the bacterial cells reached an OD_{600} of around 1.0. As noted above with HCN production, mutation of *rsmA* resulted in elevated expression levels during the early stages of growth. As the bacterial cell population reached the late exponential phase ($OD_{600} > 1.5$), expression of the *lasI'-'lacZ* fusion in the wild-type background surpassed that of the mutant.

The second *P. aeruginosa* quorum-sensing circuit, *rhl*, was also influenced by RsmA. Compared with the wild type and *rsmA* mutant, expression of *rhlI* in the *rsmA*-overexpressing strain was drastically reduced throughout the growth curve (Fig. 4B). In the *rsmA* mutant, *rhlI* expression was slightly advanced compared with the parent strain and reached a lower final level in stationary phase. Overall, the negative effects of RsmA on the quorum-sensing machinery paralleled those on the exoproducts.

RsmA-mediated control of *hcn* expression also occurs at a posttranscriptional level. Expression of the *hcn* operon in the AHL-negative *P. fluorescens* strain CHA0 is regulated by RsmA under the control of the two-component regulatory system GacS/GacA and involves the RBS region of *hcnA* (2). To investigate whether a similar posttranscriptional control of *hcn* expression is conserved in *P. aeruginosa*, the native anaerobic and quorum-sensing-dependent *hcn* promoter (22) was replaced by the constitutively expressed *tac* promoter in pME3843. Figure 5 reveals that posttranscriptional control of *P. aeruginosa hcn* expression was also exerted at the level of the RBS region; insertion of three nucleotides (ACA) into the *hcn* RBS of pME3860 caused a loss of RsmA control (Fig. 5).

TABLE 2. RsmA-dependent production of *P. aeruginosa* autoinducers 3-oxo-C12-HSL and C4-HSL[a]

Strain (genotype)	Concn (μM) at indicated OD_{600}			
	3-oxo-C12-HSL		C4-HSL	
	0.6	2.5	0.6	2.5
PAO1 (*rsmA*$^{+}$)	0.18 ± 0.02	0.53 ± 0.09	0.25 ± 0.1	6.0 ± 1.0
PAZH13 (*rsmA*)	0.45 ± 0.01	0.47 ± 0.05	0.97 ± 0.14	13.0 ± 1.0
PAO1/pME3849 (*rsmA*$^{++}$)	0.08 ± 0.06	0.13 ± 0.03	0.06 ± 0.02	2.0 ± 0

[a] Concentrations of 3-oxo-C12-HSL and C4-HSL were estimated for *P. aeruginosa* strains grown in NYB by TLC analysis (see Materials and Methods) when cells reached an OD_{600} of 0.6 and 2.5. The experiment was performed in triplicate. Values are means ± standard deviations.

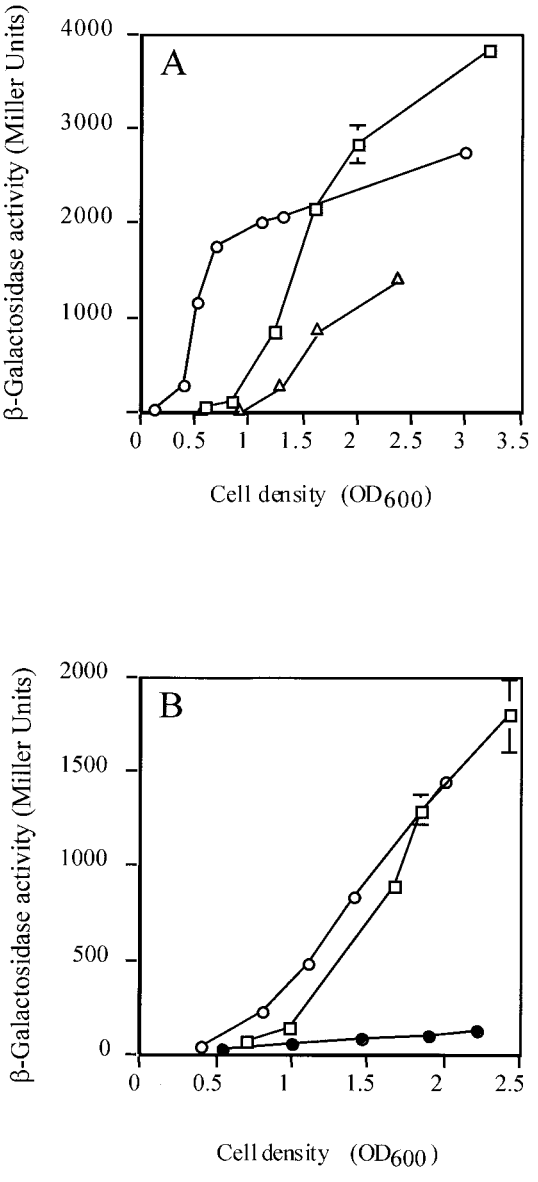

FIG. 4. RsmA control of the *lasI* and *rhlI* genes encoding the AHL synthase genes. Expression of translational (A) *lasI'-'lacZ* and (B) *rhlI'-'lacZ* fusions in wild-type *P. aeruginosa* (open squares), in an *rsmA* mutant (open circles), and in an *rsmA*-overexpressing strain (open triangles in panel A; closed circles in panel B). Each point is the mean of three measurements ± standard deviation.

DISCUSSION

The widespread occurrence of conserved *rsmA* and *csrA* genes in many different gram-positive and gram-negative bacteria, including a number of human and plant pathogens, suggests that CsrA and RsmA proteins may be functionally equivalent (25). Indeed, the *P. aeruginosa rsmA* gene can complement an *E. coli csrA* mutant with respect to repression of glycogen biosynthesis, and the RsmA protein is recognized by antibodies raised against the *Y. enterocolitica* counterpart.

In *P. aeruginosa*, we have shown that overexpression of *rsmA* results in severe downregulation of protease, elastase, and staphylolytic activities as well as reduced production of lectin, HCN, and pyocyanin. Since each of these phenotypes is regulated via the *las/rhl* quorum-sensing cascade (13, 21), it is conceivable that this repression is a consequence of a reduction in the levels of 3-oxo-C12-HSL and C4-HSL. The levels of both AHLs as well as expression of the AHL synthase genes *lasI* and *rhlI* were reduced in the *rsmA*$^{++}$ strain, a finding which is consistent with this hypothesis. However, the addition of exogenous 3-oxo-C12-HSL and C4-HSL to the *P. aeruginosa rsmA*$^{++}$ strain did not restore exoenzyme production to wild-type levels (F. Williams, unpublished data). This suggests that the negative effect of RsmA overproduction on the corresponding exoproduct genes is not only a direct consequence of the repression of AHL synthesis but also involves an effect downstream of the quorum-sensing circuitry, possibly at the level of the structural genes.

Interestingly, while overexpression of *rsmA* had a greater effect on *rhlI* than *lasI* expression, the *rsmA* mutant produced approximately twofold more C4-HSL in stationary phase than the parent. This finding does not correlate directly with the data obtained for the translational *rhlI'-'lacZ* fusion, which exhibited a lower level of activity in stationary phase than the wild type. The reason(s) for this observation is not clear but may reflect higher levels of substrate availability for RhlI (*S*-adenosylmethionine and either butanoyl-coenzyme A or butanoyl-acyl carrier protein [9]) in the mutant compared with the wild type.

Mutation of *rsmA* in the plant pathogen *E. carotovora* results in elevated production of extracellular enzyme virulence determinants and a hypervirulent phenotype (6). In *P. aeruginosa*, the corresponding mutation had only minor effects on exoenzyme and lectin synthesis. In contrast, the production of HCN in the *P. aeruginosa rsmA* mutant was induced prematurely and enhanced during the exponential stage of growth. Beyond an OD_{600} of 1.5, however, the wild-type strain produced more HCN than the mutant, presumably because the negative effect of RsmA is overcome by other regulators during the onset of stationary phase. The *rsmA* mutant also synthesized high levels of pyocyanin, indicating that RsmA functions as a negative regulator of secondary metabolites. Whether this is sufficient to confer a hypervirulent phenotype on *P. aeruginosa* is not yet known, although an *rpoS* mutant which also overproduces HCN and pyocyanin (while retaining wild-type levels of elastase and staphylolytic activity) was reported to be more virulent than the parent strain in a rat chronic lung infection model (30). A similar observation has been made for *E. carotovora* (19) in that an *rpoS*-negative mutant is a more virulent plant pathogen than the parent. In *Erwinia*, this appears to be a consequence of the modulation of *rsmA* expression, which is positively controlled by RpoS (19). Whether this is also the case for *P. aeruginosa* remains to be established; the *lacZ* fusion and Western blot data presented in this paper show that *rsmA* is expressed throughout growth with an approximately threefold enhancement in stationary phase. This finding is consistent with a possible role for RpoS in modulating *rsmA* expression in *P. aeruginosa*.

Previous work on *P. fluorescens* CHA0, which does not produce AHLs (2), has indicated that RsmA lies downstream of the GacA regulatory network and that the posttranscriptional control by the GacA/RsmA system in this organism involves a

Plasmids	ß-Galactosidase activity (Miller units)		
	wt	*rsmA*	*rsmA*$^{++}$
pME3826	206 ± 23	408 ± 31	122 ± 3
pME3843	7396 ± 428	22502 ± 428	3658 ± 361
pME3860	13192 ± 428	17470 ± 2600	n.d.

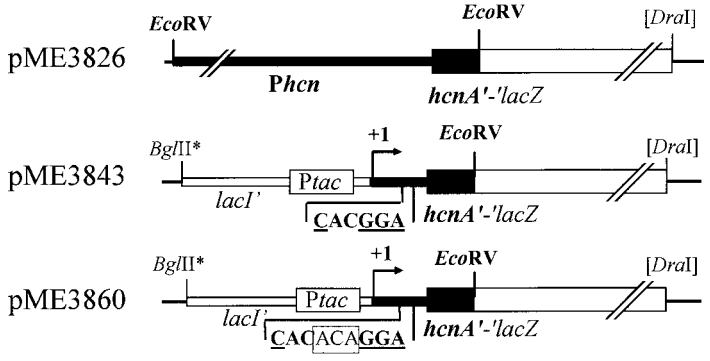

FIG. 5. Posttranscriptional RsmA-dependent regulation of *P. aeruginosa* translational *hcn'-'lacZ* fusions. Each reporter was constructed in the vector pME6010 as described in Materials and Methods. The *hcn* region is indicated in boldface; the 3-bp insertion in the *hcn* RBS of pME3860 is boxed; artificially introduced restriction sites are marked with *. β-Galactosidase activities were determined in *P. aeruginosa* PAO1 wild-type (wt), PAZH13 *(rsmA* mutant), and PAO1(pME3849) (*rsmA*$^{++}$), grown in NYB to an OD_{600} of 1.0 to 1.2. Activities are the means ± standard deviation from three measurements. n.d., not determined.

specific recognition sequence in the region of the RBS of *hcnA* (2). By replacing the natural quorum-sensing-dependent *hcn* promoter of *P. aeruginosa* (22) with the constitutively expressed *tac* promoter, we have shown that expression of a translational *hcnA'-'lacZ* fusion is regulated posttranscriptionally by RsmA. An insertion of three nucleotides (ACA) into the *hcn* Shine-Dalgarno sequence resulted in a loss of RsmA control, demonstrating the importance of the RBS region for the negative control exerted by RsmA. Thus, it would appear that in *P. aeruginosa* RsmA modulates quorum-sensing-dependent phenotypes at multiple levels.

Although the loss by mutation of *gacA* in *P. aeruginosa* does not affect the production of secondary metabolites and exoenzymes as strongly as in *P. fluorescens* (12, 19, 24), it is nevertheless clear that GacA and RsmA exert overall opposing effects on HCN, pyocyanin, and exoenzyme production in both organisms. In *P. aeruginosa*, the *las* and *rhl* systems constitute an additional regulatory layer which is also modulated by GacA and RsmA in a growth phase-dependent manner. However, the direct targets of GacA and RsmA in the quorum-sensing system have not yet been identified. In conclusion, the global control exerted by GacA and RsmA in *P. aeruginosa* does not appear to operate via a linear signal transduction pathway but via both transcriptional modulation of the quorum-sensing circuitry and AHL-independent translational modulation at the RBS of target structural genes.

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