

## Negative Control of Quorum Sensing by RpoN ( $\sigma^{54}$ ) in *Pseudomonas aeruginosa* PAO1

Karin Heurlier, Valerie Dénervaud, Gabriella Pessi,† Cornelia Reimmann, and Dieter Haas\*

Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne, Switzerland

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In *Pseudomonas aeruginosa* PAO1, the expression of several virulence factors such as elastase, rhamnolipids, and hydrogen cyanide depends on quorum-sensing regulation, which involves the *lasRI* and *rhlRI* systems controlled by *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone, respectively, as signal molecules. In *rpoN* mutants lacking the transcription factor  $\sigma^{54}$ , the expression of the *lasR* and *lasI* genes was elevated at low cell densities, whereas expression of the *rhlR* and *rhlI* genes was markedly enhanced throughout growth by comparison with the wild type and the complemented mutant strains. As a consequence, the *rpoN* mutants had elevated levels of both signal molecules and overexpressed the biosynthetic genes for elastase, rhamnolipids, and hydrogen cyanide. The quorum-sensing regulatory protein QscR was not involved in the negative control exerted by RpoN. By contrast, in an *rpoN* mutant, the expression of the *gacA* global regulatory gene was significantly increased during the entire growth cycle, whereas another global regulatory gene, *vfr*, was downregulated at high cell densities. In conclusion, it appears that GacA levels play an important role, probably indirectly, in the RpoN-dependent modulation of the quorum-sensing machinery of *P. aeruginosa*.

Intercellular communication systems allow bacteria to monitor environmental conditions and to coordinate the expression of several genes, particularly those specifying extracellular products and virulence factors, in a cell density-dependent manner (44). This communication, termed quorum sensing, is based on the production of diffusible signal molecules (autoinducers) which accumulate in the environment during bacterial growth. Upon reaching a threshold concentration at high cellular densities, they can bind to and activate specific transcriptional regulators. In many gram-negative bacteria, quorum-sensing systems consist of pairs of genes in which a *luxI*-type gene encodes an *N*-acylhomoserine lactone (AHL; autoinducer) synthase and a *luxR*-type gene encodes a transcriptional regulator activated by the cognate autoinducer (15, 60, 67).

In the pathogen *Pseudomonas aeruginosa*, which is responsible for nosocomial infections (63) as well as for serious infections in patients suffering from cystic fibrosis, cancer, or burn wounds (6, 62), pathogenicity is due to the production of both cell-associated and extracellular virulence factors, most of which are regulated by quorum sensing (15, 60, 67). *P. aeruginosa* contains two interdependent quorum-sensing systems (26, 32, 45, 70). In the *lasRI* system, the LasI synthase catalyzes the biosynthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL). The LasR-OdDHL complex positively regulates the expression of virulence factors such as elastases (LasB and LasA), exotoxin A, alkaline protease, hydrogen cyanide (HCN), and pyocyanin and, moreover, induces *lasI* itself, form-

ing an autoinduction loop. LasR activated by OdDHL also positively regulates the expression of the *rhlR* gene. RhlR is the transcriptional regulator of the second autoinducer system and functions with *N*-butyryl-L-homoserine lactone (BHL), whose biosynthesis requires the RhlI synthase. The *rhlRI* system enhances the expression of multiple exoproducts such as LasB elastase, HCN, pyocyanin, and rhamnolipids (14). Thus, there is a quorum-sensing hierarchy in which the *las* system is dominant.

Both quorum-sensing systems are positively regulated by the global regulator GacA; in particular, the response regulator GacA has a marked enhancing effect on BHL formation and BHL-dependent virulence factor production (48, 52). In addition, the CRP (cyclic AMP receptor protein) homologue Vfr specifically activates the *lasR* promoter (1, 66). Recently, the QscR protein, a homolog of LasR and RhlR, has been shown to act as a repressor of *lasI* and *rhlI*, leading to a downregulation of quorum-sensing-dependent virulence factors (8). Thus, LasR- and RhlR-controlled quorum-sensing mechanisms are embedded in global regulatory networks. Recent work on sigma factor  $\sigma^{54}$  (RpoN) in *P. aeruginosa* revealed that this alternative sigma factor is important for virulence in several models, e.g., infection of the respiratory epithelium in cystic fibrosis xenografts and of burned mice (9, 21). Reduced virulence of *P. aeruginosa* *rpoN* mutants may be explained, in part, by the fact that *rpoN* function is necessary for the expression of pili and flagella, two important adherence factors (25, 42, 61). However, considering the pleiotropic effects of *rpoN* mutations on metabolic functions in *P. aeruginosa* (5, 27, 40, 61), it has been postulated that the role of RpoN in the regulation of virulence factors could be quite complex, extending beyond positive control of pilin and flagellin synthesis (9). Here we show that RpoN exerts global negative control on the quorum-sensing machinery of *P. aeruginosa*. At least part of this effect appears to be mediated by GacA.

\* Corresponding author. Mailing address: Institut de Microbiologie Fondamentale, Université de Lausanne, CH-1015 Lausanne, Switzerland. Phone: 41 21 692 56 31. Fax: 41 21 692 56 35. E-mail: Dieter.Haas@imf.unil.ch.

† Present address: Department of Genetics and Developmental Biology, Center for Microbial Pathogenesis, University of Connecticut Health Center, Farmington, CT 06030-3710.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Both *Escherichia coli* and *P. aeruginosa* strains were routinely grown in nutrient yeast broth (NYB) or on nutrient agar plates at 37°C (58). L-Glutamine was added at a concentration of 1 mM for growing the *rpoN* mutant strains in NYB or in minimal medium E (64). For  $\beta$ -galactosidase and autoinducer extraction experiments, *P. aeruginosa* strains were grown with aeration in NYB in 50-ml Erlenmeyer flasks. When required, antibiotics were added to media at the following concentrations: tetracycline (Tc), 25  $\mu\text{g ml}^{-1}$  (*E. coli*) or 125  $\mu\text{g ml}^{-1}$  (*P. aeruginosa*); ampicillin (Ap), 100  $\mu\text{g ml}^{-1}$  (*E. coli*); gentamicin (Gm), 10  $\mu\text{g ml}^{-1}$ ; kanamycin (Km), 400  $\mu\text{g ml}^{-1}$ ; and spectinomycin (Sp), 1,500  $\mu\text{g ml}^{-1}$  (*P. aeruginosa*).

To counterselect *E. coli* S17-1 donor cells in matings with *P. aeruginosa* for gene replacement, chloramphenicol (Cm) was used at a concentration of 10  $\mu\text{g ml}^{-1}$ ; mutant enrichment experiments were performed with tetracycline at a final concentration of 20  $\mu\text{g ml}^{-1}$  and carbenicillin at a final concentration of 2,000  $\mu\text{g ml}^{-1}$ . Flagellar swimming was tested as described by Rashid and Kornberg (51) on NYB containing 1 mM L-glutamine solidified with 0.3% (wt/vol) agar. Swarming was tested on plates containing 0.5% (wt/vol) agar, 8 g of nutrient broth per liter (Oxoid), 5 g of glucose per liter, and 1 mM L-glutamine (51). Twitching motility was assayed on 1% (wt/vol) agar supplemented with Luria broth and 1 mM L-glutamine (29).

**DNA manipulation and cloning procedures.** Small- and large-scale preparations of plasmid DNA were made by the cetyltrimethylammonium bromide method (13) and with JetStar columns (Genomed, Basel, Switzerland), respectively. Chromosomal DNA was purified from *P. aeruginosa* as described elsewhere (16). Restriction enzyme digestions, PCRs, ligation, electrophoresis, and electroporation were performed with standard procedures (52) and as described elsewhere (17, 46). Nucleotide sequences of PCR-derived constructs were determined on both strands with a dye terminator kit (Perkin-Elmer product 402080) and an ABI Prism 373 sequencer. Comparison of nucleotide and deduced amino acid sequences was performed with the Genetics Computer Group program GAP.

**Plasmid constructions.** Plasmid pME3829 was obtained by subcloning a 3.1-kb *Bam*HI-*Hind*III fragment from pRP1-*rpoN* (kindly provided by Y. Itoh), containing genes *PA4461* and *rpoN*, into pBBR1MCS (Fig. 1). Plasmid pME3836 was constructed by PCR-amplifying a 744-bp fragment (3 min at 95°C; 25 cycles of 1 min at 95°C, 30 s at 58°C, 1 min at 72°C; and 3 min at 72°C) with PAO1 chromosomal DNA as the template, with the primers qscR-3 (5'-AGGCCAGG ATCTGTTTATTGTCT-3') and qscR-4 (5'-GACAAAATCTGCAGATATCC CTCT-3'). Artificial restriction sites (italic) for *Bam*HI and *Pst*I, respectively, were incorporated into these primers. The resulting 730-bp *Bam*HI-*Pst*I fragment, including a potential Lux box located 486 bp upstream of the ATG and the first eight codons of *qscR*, was fused in-frame with the '*lacZ*' reporter gene from pNM482 in vector pME6010.

To obtain plasmid pME3338, a 2.1-kb *Eco*RI-*Hind*III fragment containing the *rpoN* gene (Fig. 1) was excised from pME3829 and inserted into the *Spe*I site of pME3280b (S. Zuber and D. Haas, unpublished data). To construct plasmid pME3851, the *rhlR* promoter region and first nine *rhlR* codons were amplified by PCR as above with the primers RR1 (5'-CGTTCGTCGAGACCCGGC-3') and RR2 (5'-AAAAGTGCAGCAGCAAAAAGCCTCCGTC-3'), which are *Xho*I and *Pst*I tagged, respectively (italic); plasmid pME3840 was used as the template. The 0.3-kb PCR product was cut with *Xho*I, blunted, cut with *Pst*I, fused in frame with the '*lacZ*' reporter gene of pNM482, and cloned into pME6010. To generate plasmid pME3858, a 0.4-kb *Sma*I-*Pst*I fragment from pME3827, containing the *lasR* promoter region and the first 23 codons of *lasR*, was fused in frame with the '*lacZ*' reporter gene from pNM482 in vector pME6010.

A putative *rpoN* recognition site overlapping the 3' end of the *lasR* (*lux*) box in the *rhlI* promoter was mutated as follows. Primer rhlR-1 (5'-CCGTGGATC CGGCGATCCTC-3'), which anneals around the *Bam*HI site (italic) in the *rhlR* coding sequence, and primer Acl-2 (5'-GATGAACGTTTGGCAACTGCCAG ATCTGGT-3'), which is complementary to part of the *lasR* box in the *rhlI* promoter and carries an artificial *Acl*I site (italic), were used to PCR-amplify a 551-bp fragment from pME3846. A second PCR fragment of 183 bp was amplified from pME3846 with primer Acl-3 (5'-GCCAAACGTTTCATCTCTTTA GTCT-3'), which is tagged with an artificial *Acl*I site (italic) and anneals immediately downstream of the putative *rpoN* box, and primer rhlI-4 (5'-AAAAGTGCAGCGGAAAGCCCTTCCAGCG-3'), which is complementary to codons 8 to 13 of *rhlI* and carries an artificial *Pst*I site (italic). The two PCR fragments were ligated to each other at their *Acl*I sites, fused as a 0.71-kb *Bam*HI-*Pst*I fragment to the '*lacZ*' gene of pNM481, and cloned into pME6010. Sequence analysis of

the resulting construct, pME6186, confirmed that in the putative *rpoN* recognition sequence GGCAGGTTGCCTGC, the last three bases were replaced by AAA, destroying the second half-site of the GG-N<sub>10</sub>-GC motif.

***P. aeruginosa* mutant constructions.** Care was taken to construct all mutants and chromosomal reporter fusions in the same PAO1 background. In PAO6358, a 0.9-kb fragment was deleted in-frame in the *rpoN* gene. The deletion includes the helix-turn-helix motif and the *rpoN* box (Fig. 1) and was obtained as follows. A 604-bp *Kpn*I-*Sac*I fragment from pME3829, including the first 190 codons of *rpoN*, was linked to a 620-bp *Sac*I-*Bam*HI fragment containing the last six codons of *rpoN*, gene *PA4463*, and the beginning of *pstN* (Fig. 1), which had been amplified by PCR as above with primers KH4 (5'-AAAAGAGCTCCGCAAGC GACTGGTGTGA-3') and KH5 (5'-AAAAGGATCCGGCGATGCCATTGCC GAA-3') and cut at the artificial restriction sites for *Sac*I and *Bam*HI (italic). The resulting 1.22-kb fragment was cloned into the suicide plasmid pME3087 digested with *Kpn*I and *Bam*HI, giving plasmid pME3834. In conjugation with PAO1 as the recipient and S17-1/pME3834 as the donor, tetracycline-resistant transconjugants having a chromosomally integrated pME3834 plasmid were selected. After carbenicillin enrichment, glutamine-auxotrophic colonies were obtained and verified by PCR for their 0.9-kb deletion in *rpoN* with primers KH5 and KH10 (5'-TCCAGCAGGAAATCCAGGAAG-3') (Fig. 1).

In strain PAO6359, the *rpoN* gene was interrupted by the insertion of a kanamycin resistance cassette ( $\Omega$ -Km). We obtained this mutant by transducing the mutation from PAO4460 (40) into PAO1 with the temperate phage E79tv-2 (39) as described before (17). Kanamycin-resistant transductants were auxotrophic for glutamine. The double *rpoN gacA* mutant PAO6363 was obtained by transduction of the *gacA* mutation of PAO6281 into PAO6358 with phage E79tv-2.

In strain PAO6366, the *qscR* gene was interrupted by the insertion of a spectinomycin-streptomycin resistance cassette ( $\Omega$ -Sp/Sm). The *qscR* gene was amplified by PCR with PAO1 chromosomal DNA as the template and primers QSCR1 (5'-AAAAGAGCTCATGGAGCGTGGAGAAGAAC-3') and QSCR2 (5'-TAAAGGATCCTATCCGGCCATTCGGTGAAT-3'), which contain artificial restriction sites for *Sac*I and *Bam*HI (italics). The 2-kb  $\Omega$ -Sp/Sm resistance cassette from pHP45 $\Omega$  was inserted as an *Eco*RI fragment into the *Eco*RI site located 30 codons downstream of the *qscR* ATG start codon. The resulting 3.15-kb fragment was cloned into the suicide plasmid pME3087, giving pME3828. This plasmid, carrying *qscR*: $\Omega$ -Sp/Sm, was mobilized into PAO1 and chromosomally integrated, with selection for tetracycline resistance. The chromosomal insertion in a tetracycline-sensitive, spectinomycin-resistant clone was verified by Southern blotting.

**Construction of chromosomal insertions in the Tn7 attachment site.** Complementation by a single copy of the *rpoN*<sup>+</sup> gene was carried out with a Tn7-based system developed for gram-negative bacteria (3, 22). Chromosomal insertion of the mini-Tn7 construct pME3338 carrying *rpoN*<sup>+</sup> (Fig. 1) was obtained via a triparental mating between the recipient PAO6358 (grown overnight at 43°C), *E. coli* SM10 $\lambda$ pir carrying the pUX-BF13 helper plasmid, and *E. coli* S17-1/pME3338, with selection for gentamicin and chloramphenicol resistance. The *rpoN*<sup>+</sup> insertion in the resulting strain, PAO6360, was verified by the loss of the auxotrophy for glutamine and by Southern blotting analysis.

Chromosomal *vfr*'-'*lacZ* strains were constructed as follows. First, the *vfr* gene was amplified by PCR from chromosomal DNA of PAO1 with the *Xho*I (italic)-tagged primer pVFR1 (5'-CATCCTCGAGGAAGGCTTCGC-3') and the *Eco*RI (italic)-tagged primer pVFR2 (5'-GGAATTCATGGGTGCTGTTC A-3'). The resulting 1.15-kb PCR fragment was cleaved with *Eco*RI and *Xho*I and inserted into pBluescriptII-SK to give pME6157. The blunted 0.6-kb *Xho*I-*Pvu*I fragment of pME6157 was fused to '*lacZ*' in the *Sma*I site of pNM482, and the resulting *vfr*'-'*lacZ* fusion on a 3.7-kb *Eco*RI-*Dra*I fragment was ligated to a 0.35-kb *Sph*I (T4 DNA polymerase treated)-*Eco*RI fragment carrying the transcription stop signal of the  $\Omega$ -Sp/Sm cassette, and cloned into the blunted *Hind*III site of the Tn7 delivery vector pME6313. The transcription stop signal upstream of the *vfr*'-'*lacZ* fusion in this construct, named pME6165, prevents potential readthrough from the gentamicin resistance gene. The *vfr*'-'*lacZ* fusion was delivered to the chromosome of PAO1 and PAO6358 by triparental mating with *E. coli* S17-1/pME6165 and *E. coli* SM10 $\lambda$ pir/pUX-BF13 as donors. Gentamicin-resistant transconjugants were checked by Southern analysis.

A translational *gacA*'-'*lacZ* fusion excised from plasmid pME6118 as a 4.5-kb *Eco*RI-*Dra*I fragment was ligated to the *Sph*I (T4 DNA polymerase treated)-*Eco*RI fragment carrying the transcription stop signal of  $\Omega$ -Sp/Sm and inserted into the blunted *Hind*III site of pME6313. The mini-Tn7 *gacA*'-'*lacZ* construct of the resulting plasmid, pME6166, was delivered to the chromosome of PAO1 and PAO6358 as described above. The resulting strains, PAO6320 and PAO6361, were checked by Southern analysis.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype <sup>a</sup>	Reference or origin
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild type	23
PAO4460	<i>rpoN</i> ::Ω-Km	40
PAO6281	PAO1 <i>gacA</i> ::Ω-Sp/Sm	52
PAO6304	PAO1 <i>att</i> Tn7:: <i>vfr</i> '-' <i>lacZ</i> Gm <sup>r</sup>	This study
PAO6320	PAO1 <i>att</i> Tn7:: <i>gacA</i> '-' <i>lacZ</i> Gm <sup>r</sup>	This study
PAO6358	PAO1 Δ <i>rpoN</i>	This study
PAO6359	PAO1 <i>rpoN</i> ::Ω-Km	This study
PAO6360	Δ <i>rpoN att</i> Tn7:: <i>rpoN</i> <sup>+</sup> Gm <sup>r</sup>	This study
PAO6361	Δ <i>rpoN att</i> Tn7:: <i>gacA</i> '-' <i>lacZ</i> Gm <sup>r</sup>	This study
PAO6362	Δ <i>rpoN att</i> Tn7:: <i>vfr</i> '-' <i>lacZ</i> Gm <sup>r</sup>	This study
PAO6363	Δ <i>rpoN gacA</i> ::Ω-Sp/Sm	This study
PAO6366	PAO1 <i>qscR</i> ::Ω-Sp/Sm	This study
PAK-SR	Spontaneous Sm <sup>r</sup> mutant of PAK	25
PAK-N1	<i>rpoN</i> ::Ω-Tc in PAK-SR	25
<i>Escherichia coli</i>		
DH5α	F <sup>-</sup> <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF)U169 deoR λ(φ80dlacZΔM15)</i>	55
S17-1	<i>pro thi hsdR</i> <sup>+</sup> Tp <sup>r</sup> Sm <sup>r</sup> ; chromosome::RP4-2 Tc::Mu-Km::Tn7	57
SM10/λpir	<i>thi-1 thr-1 leuB26 tonA21 lacYI supE44 recA</i> integrated RP4-2 Tc <sup>r</sup> ::Mu Km <sup>r</sup> /λpir	37
Plasmids		
pBBR1MCS	Broad-host-range cloning vector; Cm <sup>r</sup>	30
pBluescript-II KS, SK	Cloning vectors; ColE1 replicon; Ap <sup>r</sup>	Stratagene
pECP60	<i>rhlA</i> '-' <i>lacZ</i> translational fusion on pSW205; Ap <sup>r</sup>	46
pHP45Ω	ColE1 replicon carrying a Ω-Sp/Sm cassette; Sp <sup>r</sup> Sm <sup>r</sup> Ap <sup>r</sup>	50
pME3087	Suicide vector, with <i>EcoRI-SstI-KpnI-BamHI-XbaI-PstI-SphI-HindIII</i> MCS, ColE1 replicon; Tc <sup>r</sup>	65
pME3280b	Mini-Tn7 gene delivery vector based on pUX-BF5 with <i>HindIII-PstI-MluI-SpeI</i> MCS; Gm <sup>r</sup> Ap <sup>r</sup>	S. Zuber and D. Haas, unpublished
pME3338	pME3280b with 2.1-kb <i>EcoRI-HindIII</i> fragment of pME3829 containing <i>rpoN</i> ; Gm <sup>r</sup> Ap <sup>r</sup>	This study
pME3827	<i>lasR</i> Gm <sup>r</sup>	47
pME3828	pME3087 carrying <i>qscR</i> ::Ω-Sp/Sm on 3.15-kb fragment; Tc <sup>r</sup>	This study
pME3829	pBBR1MCS with 3.1-kb <i>BamHI-HindIII</i> insert including genes <i>PA4461</i> and <i>rpoN</i> from pRP1- <i>rpoN</i> ; Cm <sup>r</sup>	This study
pME3834	pME3087 carrying 1.2-kb <i>KpnI-BamHI</i> insert with a deletion in <i>rpoN</i> ; Tc <sup>r</sup>	This study
pME3836	<i>qscR</i> '-' <i>lacZ</i> translational fusion in pME6010, with 700 bp upstream of start codon and 8 codons of <i>qscR</i> fused to ' <i>lacZ</i> ' gene from pNM482; Tc <sup>r</sup>	This study
pME3840	<i>rhlRI</i> ' Cm <sup>r</sup>	47
pME3846	<i>rhlI</i> '-' <i>lacZ</i> translational fusion; Tc <sup>r</sup>	48
pME3850.1	<i>hcnA-lacZ</i> transcriptional fusion; Tc <sup>r</sup>	47
pME3851	<i>rhlR</i> '-' <i>lacZ</i> , translational fusion; Tc <sup>r</sup>	This study
pME3853	<i>lasI</i> '-' <i>lacZ</i> , translational fusion; Tc <sup>r</sup>	48
pME3858	<i>lasR</i> '-' <i>lacZ</i> translational fusion in pME6010, with 0.4-kb <i>SmaI-PstI</i> fragment from pME3827 fused to ' <i>lacZ</i> ' gene from pNM482; Tc <sup>r</sup>	This study
pME6010	Cloning vector; Tc <sup>r</sup>	20
pME6118	ColE1 replicon, with 1.4-kb <i>EcoRI-BamHI</i> fragment containing <i>gacA</i> '-' <i>lacZ</i> translational fusion; Ap <sup>r</sup>	52
pME6157	pBLS-II SK carrying <i>vfr</i> gene with its promoter region, Ap <sup>r</sup>	This study
pME6165	pME6313 with 0.6-kb blunt end <i>XhoI-PvuI</i> fragment from pME6157, containing promoter region and 5' end of <i>vfr</i> fused with ' <i>lacZ</i> ' gene, giving <i>vfr</i> '-' <i>lacZ</i> translational fusion; Gm <sup>r</sup>	This study
pME6166	pME6313, with 1.4-kb <i>EcoRI-BamHI</i> fragment from pME6118 ( <i>gacA</i> '-' <i>lacZ</i> ) preceded by 0.35-kb <i>SphI-EcoRI</i> fragment from pHP45Ω and containing transcription and translation termination signals of Ω-Sp/Sm; Gm <sup>r</sup>	This study
pME6186	<i>rhlI</i> '-' <i>lacZ</i> translational fusion in which <i>rpoN</i> potential box GGCAG-N <sub>5</sub> -CTGCC at positions -43/-31 was replaced with GGCAG-N <sub>5</sub> -CAAAC; Tc <sup>r</sup>	This study
pME6313	Mini-Tn7 gene delivery vector based on pUX-BF5 with <i>HindIII-PstI-SmaI-SpeI</i> MCS; Gm <sup>r</sup> Ap <sup>r</sup>	H. Winteler and D. Haas, unpublished
pNM481, 482	Cloning vectors for translational ' <i>lacZ</i> ' fusions; ColE1 replicon; Ap <sup>r</sup>	38
pRP1- <i>rpoN</i>	pUC119 with <i>Sall-HincII</i> insert of 3.1 kb including genes <i>PA4461</i> and <i>rpoN</i> ; Ap <sup>r</sup>	Y. Itoh, unpublished
pTS400	<i>lasB</i> '-' <i>lacZ</i> translational fusion on pSW205; Ap <sup>r</sup>	44
pUX-BF5	"Carrier" plasmid containing <i>att</i> Tn7::mini-Tn7-Km system; Km <sup>r</sup>	3
pUX-BF13	"Helper" plasmid containing Tn7 transposition functions; R6K replicon; Ap <sup>r</sup>	3

<sup>a</sup> MCS, multiple cloning site; Tp, trimethoprim.

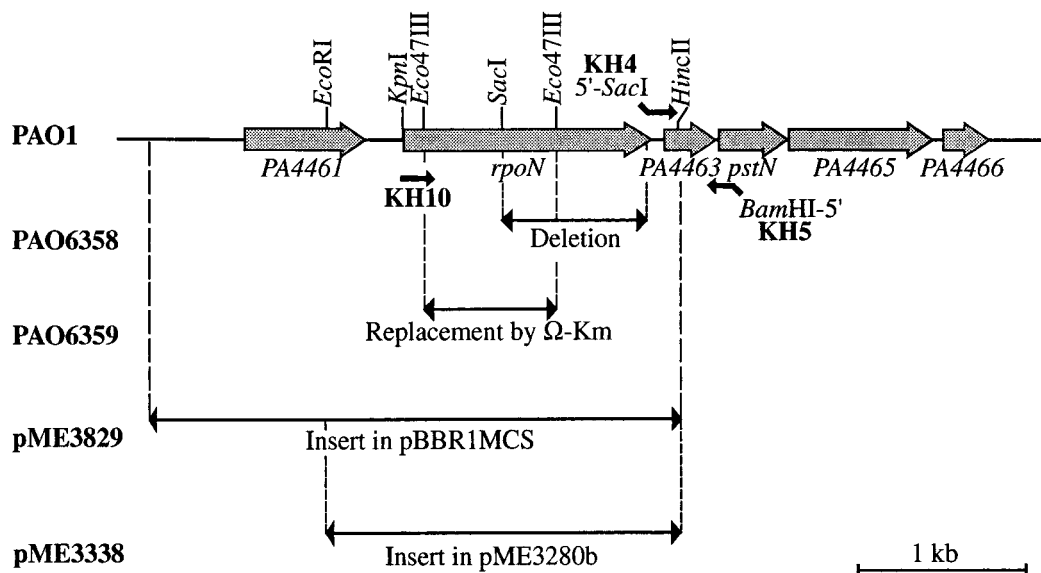


FIG. 1. Chromosomal region of *P. aeruginosa* PAO1 surrounding the *rpoN* gene (59). The construction of strains PAO6358 and PAO6359 and of plasmids pME3338 and pME3829 is described in Materials and Methods. KH4, KH5, and KH10 indicate primers used in PCR. The assignment of *pstN* to open reading frame *PA4464* is based on sequence comparison with the *rpoN* gene cluster in *E. coli* (28).

**Semiquantitative determination of autoinducer concentrations by thin-layer chromatography.** *P. aeruginosa* strains were cultivated with shaking in 20 ml of NYB amended with 1 mM glutamine in 50-ml Erlenmeyer flasks at 37°C to obtain an optical density at 600 nm ( $OD_{600}$ ) of 0.6 or 1.5. Cells were removed by centrifugation, and the pH of supernatants was adjusted to 5.0 prior to extraction with 3 volumes of dichloromethane in a separating funnel. Water was removed from the solvent phase with anhydrous  $Na_2SO_4$ , and dichloromethane was evaporated with a rotary evaporator. The extracts were concentrated 200-fold by dissolving them in aqueous 50% (vol/vol) acetonitrile. The presence of AHLs was tested by  $C_{18}$  reverse-phase (Merck) thin-layer chromatography, developed by elution in methanol-water (60:40, vol/vol), and revealed by overlaying either *Chromobacterium violaceum* CV026 (36), for BHL, or *Agrobacterium tumefaciens* NTL4/pZLR4 (7), for OdDHL. The amounts of BHL and OdDHL were estimated by comparison with standards, i.e., 4, 6, or 8 nmol of BHL and 50, 100, or 150 pmol of OdDHL.

**$\beta$ -Galactosidase assay.** *P. aeruginosa* strains were cultivated with shaking in 20 ml of NYB with 1 mM glutamine in 50-ml Erlenmeyer flasks at 37°C.  $\beta$ -Galactosidase specific activities were determined by the method of Miller (55).

## RESULTS

**Growth characteristics of *P. aeruginosa rpoN* mutants.** As the *rpoN* gene is the first gene in a cluster of five (27, 59), we constructed an in-frame deletion mutation in *rpoN* to avoid potential polar effects on the expression of the downstream genes. The resulting *rpoN* mutant, PAO6358, was complemented by a single *rpoN*<sup>+</sup> copy inserted into the unique chromosomal Tn7 attachment site in strain PAO6360 (Fig. 1). An *rpoN*:: $\Omega$ -Km insertion mutant, PAO6359, was also constructed (Fig. 1). Both *rpoN*-negative strains were auxotrophic for glutamine and unable to swim, swarm, and twitch, in agreement with previous studies showing that *P. aeruginosa rpoN* mutants are defective for flagella and type IV pili (21, 25, 29, 40, 61). In rich medium (NYB) amended with 1 mM L-glutamine, both *rpoN* mutants had a longer doubling time (about 70 min) than the wild-type PAO1 and the complemented mutant PAO6360 (about 40 min), indicating that substrate utilization was somewhat impeded by the loss of RpoN function. It is known that

the utilization of several amino acids as C and N sources depends on RpoN (40, 61). However, both the wild type and the *rpoN* mutant reached stationary phase at similar levels ( $2.5 \times 10^9$  to  $3 \times 10^9$  cells/ml). Under anaerobic conditions in a GasPak jar, the *rpoN* mutant PAO6358 and the wild-type PAO1 grew similarly on nutrient agar amended with 1 mM L-glutamine and either 100 mM  $KNO_3$  or 5 mM  $KNO_2$ , suggesting that RpoN is not essential for denitrification.

**Effects of *rpoN* null mutations on AHL production in *P. aeruginosa*.** To investigate whether RpoN influences the production of the quorum-sensing signals OdDHL and BHL, we quantified AHL levels at cell densities corresponding to early exponential phase ( $OD_{600} = 0.6$ , i.e., about  $6 \times 10^8$  cells/ml) and to late exponential phase ( $OD_{600} = 1.5$ ) (Table 2). The *rpoN* mutants PAO6358 and PAO6359 produced about two times more OdDHL and about five times more BHL than did the parent strain during both early and late exponential phases of growth. As the *rpoN* deletion mutant was phenotypically similar to the insertion mutant, only the former was complemented. In the complemented *rpoN* mutant, PAO6360, autoinducer levels were close to those of the wild type (Table 2). A similar regulation phenomenon was also detected in overnight cultures of an *rpoN* mutant of strain PAK-N1 (PAK-SR *rpoN*:: $\Omega$ -Tc [25]), which produced six to seven times more AHL than did the parental wild type, PAK-SR (data not shown). By comparison with strain PAO1, strain PAK-SR yielded fivefold-lower autoinducer levels (data not shown).

**Effects of *rpoN* null mutations on expression of *lasRI* and *rhIRI* quorum-sensing genes.** To confirm the regulatory effects of RpoN on AHL synthesis, we studied the expression of the *lasR*, *lasI*, *rhIR*, and *rhII* genes with translational *lacZ* fusions in the wild-type PAO1, in both *rpoN* mutants PAO6358 and PAO6359, and in the complemented mutant PAO6360. Null mutation of the *rpoN* gene resulted in an approximately three-

TABLE 2. Negative control of autoinducer production by RpoN in *P. aeruginosa*<sup>a</sup>

Strain	Genotype	OdDHL ( $\mu$ M)		BHL ( $\mu$ M)	
		OD <sub>600</sub> = 0.6	OD <sub>600</sub> = 1.5	OD <sub>600</sub> = 0.6	OD <sub>600</sub> = 1.5
PAO1	Wild type	0.15 $\pm$ 0.03	0.38 $\pm$ 0.02	0.13 $\pm$ 0.03	0.67 $\pm$ 0.34
PAO6358	$\Delta$ rpoN	0.38 $\pm$ 0.03	0.76 $\pm$ 0.04	0.50 $\pm$ 0.10	3.11 $\pm$ 0.38
PAO6359	rpoN:: $\Omega$ -Km	0.28 $\pm$ 0.03	0.82 $\pm$ 0.15	0.55 $\pm$ 0.10	3.33 $\pm$ 0.34
PAO6360	$\Delta$ rpoN attTn7::rpoN <sup>+</sup>	0.05 $\pm$ 0.03	0.41 $\pm$ 0.09	0.06 $\pm$ 0.05	0.95 $\pm$ 0.25

<sup>a</sup> Concentrations of OdDHL and BHL were estimated for *P. aeruginosa* strains grown aerobically in NYB amended with 1 mM glutamine (see Materials and Methods). Data shown are mean values  $\pm$  standard deviations for three independent experiments.

fold derepression of *lasR* on pME3858 and *lasI* on pME3853 at cell densities below an OD<sub>600</sub> of 1.0, but these effects were reversed at high cell densities (Fig. 2A and B). The complemented mutant PAO6360 showed *lasR* and *lasI* expression similar to that in the parent (Fig. 2A and B). RpoN was also found to control the second quorum-sensing circuit in that translational *rhlR*'-'*lacZ* and *rhlI*'-'*lacZ* fusions (on pME3851 and pME3846, respectively) were induced four- to fivefold at low and high cell densities (Fig. 2C and D). We additionally tested transcriptional *lasR-lacZ* and *rhlR-lacZ* fusions in an *rpoN* and in a wild-type background. The derepressing effect of the *rpoN* mutation was similar to that found with the analogous translational fusions (data not shown). In conclusion, the  $\beta$ -galactosidase activities of the *lasRI* and *rhlRI* fusion constructs closely paralleled AHL levels (Table 2).

**Mutation in *rpoN* leads to increased expression of exoproduct genes.** Quorum sensing controls the production of numerous extracellular enzymes and metabolites in *P. aeruginosa*; we chose to measure three of them, elastase, rhamnolipids, and HCN (67), by following the expression of some representative structural genes: *lasB* (for elastase), *rhlA* (the first of two genes for rhamnolipid synthesis), and *hcnA* (the first of three genes encoding HCN synthase). All three genes are most strongly induced by a synergistic action of both the *las* and *rhl* quorum-sensing systems (47, 68). Translational *lacZ* fusions of the *lasB* and *rhlA* genes on plasmids pTS400 and pECP60, respectively, showed a two- to fourfold increase of expression in the *rpoN* mutant PAO6358 compared to the levels in the wild type and in the complemented *rpoN* mutant. An *hcnA-lacZ* transcriptional fusion on pME3850.1 gave a similar result (data not shown). These experiments as well as the autoinducer determinations (Table 2) and the quorum-sensing expression studies (Fig. 2) were all conducted in the same growth medium, NYB amended with 1 mM glutamine. Growth reached a plateau at an OD<sub>600</sub> of 2.5 to 3.0 under these conditions. The results obtained with the *lasB*, *rhlA*, and *hcnA* fusions are consistent with an overall negative effect of RpoN acting transiently on *lasRI* and constantly on *rhlRI* expression.

**Mechanisms of quorum-sensing control by RpoN.** We considered four hypotheses, which are not mutually exclusive, to explain the effects of RpoN on quorum-sensing in strain PAO1. (i) RpoN could directly repress the *lasR*, *lasI*, *rhlR*, and *rhlI* genes at their promoters, similar to the negative effect that  $\sigma^{54}$  can exert on alginate synthesis by binding directly to the *algD* promoter in *P. aeruginosa* (5). (ii) RpoN could repress Vfr, a positive regulator of *lasR* gene expression (1). (iii) RpoN could activate QscR, a negative effector of quorum sensing (8,

33). (iv) RpoN could repress GacA, a positive regulator of the expression of the *lasR*, *rhlR*, and *rhlI* genes (48, 52).

(i) RpoN recognizes a TGGCAC-N<sub>5</sub>-TTGCA consensus sequence in which the TGGC-N<sub>9</sub>-GC motif is most strongly conserved (4, 53). We searched for the presence of such a motif in the *lasR*, *lasI*, *rhlR*, and *rhlI* promoter regions; note that the transcription start sites have been determined experimentally for the *lasR*, *lasI*, and *rhlI* genes (1, 41, 48, 56). We did not find evidence for a conserved RpoN motif in the *lasR*, *lasI*, and *rhlR* promoter regions at positions that would be compatible with a repressive effect of RpoN, nor did we detect such a motif in the promoter of the *rsaL* gene, which exerts negative control on the *lasI* gene (11). By contrast, in the *rhlI* promoter, a potential RpoN recognition sequence, TGGCAG-N<sub>5</sub>-CTGCC, was found at positions -43 to -31 relative to the +1 site. This sequence overlaps a *lasR* (*lux*) box placed at -57 to -38. A 3-bp mutation replacing TGC at -33 to -31 with AAA was constructed in the *rhlI* promoter region. This mutation leaves the *lasR* box intact but would be expected to interfere with recognition of RpoN. However, the expression of an *rhlI*'-'*lacZ* fusion was not influenced by this mutation in the wild type or the *rpoN* mutant PAO6358 (data not shown).

(ii) In order to determine if the influence of RpoN on quorum sensing was mediated by Vfr, we measured the expression of a chromosomal translational *vfr*'-'*lacZ* fusion in a wild-type (PAO6304) and an *rpoN* mutant background (PAO6362). The expression of *vfr* was low throughout growth and significantly reduced in the *rpoN* background at high cell densities (Fig. 3). Inspection of the *vfr* promoter (54), however, did not reveal the presence of an RpoN recognition sequence, suggesting that the positive effect of RpoN on *vfr* expression may be indirect. The consequence of this regulation will be considered in the Discussion.

(iii) A *qscR*:: $\Omega$ -Sp/Sm insertion mutant, PAO6366, was constructed and tested for expression of the *lasR*, *lasI*, *rhlR*, and *rhlI* genes with the *lacZ* fusion constructs pME3858, pME3853, pME3851, and pME3846, respectively. However, each fusion gave a similar expression profile in the wild-type PAO1 and in the *qscR* mutant PAO6366 (data not shown). A translational *qscR*'-'*lacZ* fusion (constructed as detailed in Materials and Methods) was expressed at a low level of 3 to 4 Miller units in the wild-type PAO1 as well as in the *rpoN* mutant PAO6358, at an OD<sub>600</sub> of 2.0. These results suggest that RpoN does not modulate the quorum-sensing machinery via QscR.

(iv) In order to check if the influence of RpoN on quorum sensing was a result of RpoN-mediated control of *gacA* expression, we assayed a chromosomal translational *gacA*'-'*lacZ* fu-

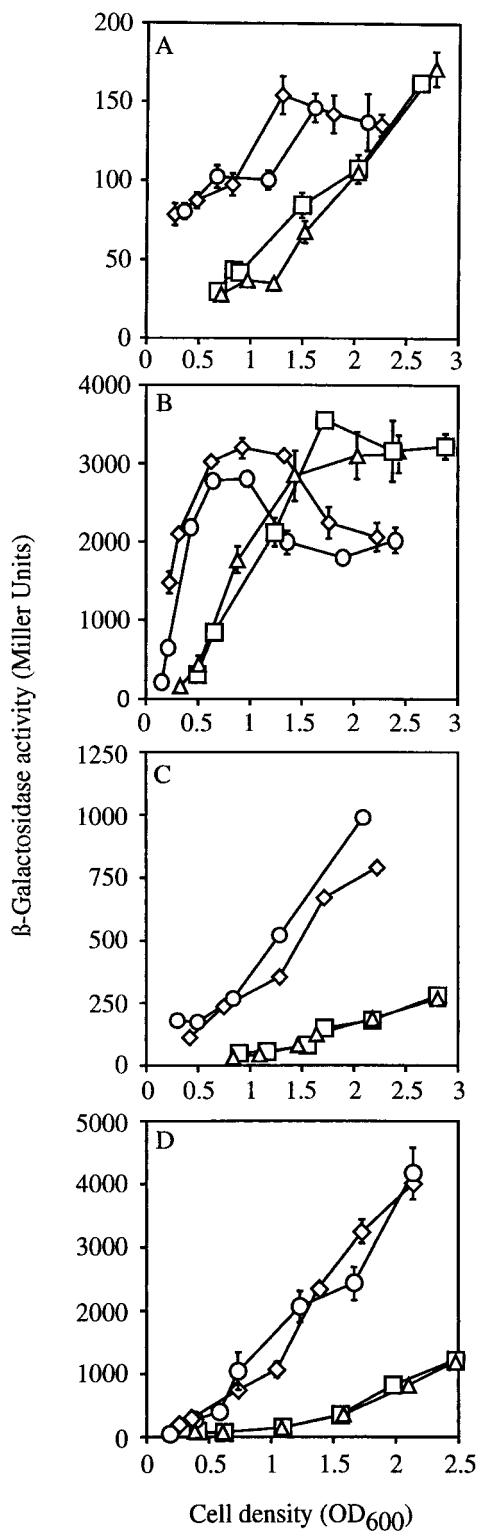


FIG. 2. RpoN control of *lasR*, *lasI*, *rhlR*, and *rhlI* expression.  $\beta$ -Galactosidase activities resulting from the translational fusions *lasR*'-*lacZ* carried by pME3858 (A), *lasI*'-*lacZ* on pME3853 (B), *rhlR*'-*lacZ* on pME3851 (C), and *rhlI*'-*lacZ* on pME3846 (D) were determined in *P. aeruginosa* wild-type PAO1 (□), in the *rpoN* deletion mutant PAO6358 (◇), in the *rpoN* insertion mutant PAO6359 (○), and in the *rpoN* deletion mutant complemented with monocopy *rpoN*<sup>+</sup> PAO6360 (△), grown in NYB with 1 mM glutamine. Each point is the mean  $\pm$  standard deviation for three cultures.

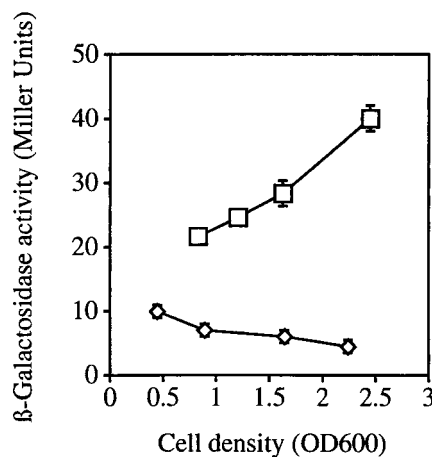


FIG. 3. Influence of RpoN on *vfr* expression.  $\beta$ -Galactosidase activities from the chromosomally located translational fusion *vfr*'-*lacZ* were determined in the wild-type PAO6304 (□) and in the *rpoN* mutant background strain PAO6362 (◇). Each result is the mean  $\pm$  standard deviation for three cultures. Bacteria were grown in NYB with 1 mM glutamine.

sion in the wild type (PAO6320) and the *rpoN* deletion mutant (PAO6361). A marked negative effect of RpoN on *gacA* expression occurred throughout growth (Fig. 4). In the wild type as well as in the complemented *rpoN* mutant, *gacA* expression was about three times lower than in the *rpoN* mutant (Fig. 4). In agreement with previous data (52), the expression levels of the *lasB*'-*lacZ*, *rhlA*'-*lacZ*, and *hcnA*-*lacZ* fusions were very low in a *gacA* mutant compared with those in the wild type and the *rpoN* mutant (Fig. 5A, B, and C).

If the repressive effects of RpoN on the quorum-sensing machinery were essentially a consequence of repression of GacA, then we might expect that a *gacA rpoN* double mutant

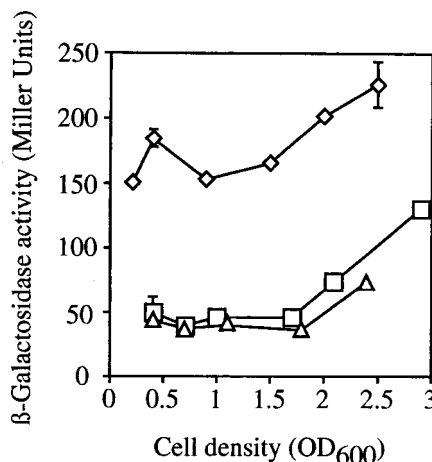


FIG. 4. Influence of RpoN on *gacA* expression.  $\beta$ -Galactosidase activities from the chromosomally located translational fusion *gacA*'-*lacZ* were determined in the wild-type PAO6320 (□), in the *rpoN* mutant PAO6361 (◇), and in the *rpoN* mutant PAO6361 complemented with pME3829 (△). Each result is the mean  $\pm$  standard deviation for three cultures. Bacteria were grown in NYB with 1 mM glutamine.

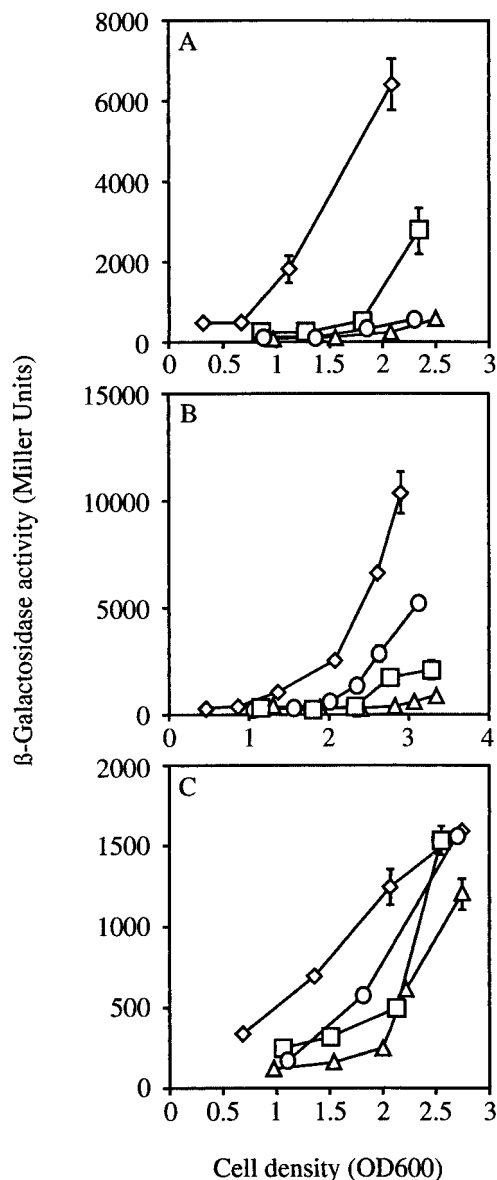


FIG. 5. Influence of RpoN and GacA on *lasB*, *rhlA*, and *hcnA* expression.  $\beta$ -Galactosidase activities from the translational fusions *lasB'*-*lacZ* on pTS400 (A) and *rhlA'*-*lacZ* on pECP60 (B) and from the transcriptional fusion *hcnA*-*lacZ* on pME3850.1 (C) were determined in the wild-type PAO1 ( $\square$ ), the *rpoN* mutant PAO6358 ( $\diamond$ ), the *rpoN gacA* double mutant PAO6363 ( $\circ$ ), and the *gacA* mutant PAO6281 ( $\triangle$ ). Each result is the mean  $\pm$  standard deviation for three cultures. Bacteria were grown in NYB with 1 mM glutamine.

would not differ substantially from a *gacA* mutant in terms of quorum-sensing-dependent expression. In the case of the *lasB'*-*lacZ* fusion, this was indeed observed (Fig. 5A). However, in the case of the *rhlA'*-*lacZ* and *hcnA*-*lacZ* fusions, the *gacA rpoN* mutant gave intermediate expression levels (Fig. 5B and C), indicating that a simple linear model ( $\text{RpoN} \rightarrow \text{LasRI/RhlRI} \rightarrow \text{RhlA/HcnA}$ ) would incompletely describe the situation and that RpoN may act on the expression of the *rhlA* and *hcnA* genes via GacA-independent pathways. The *hcnA*-*lacZ* fusion of pME3850.1 used is controlled tightly and

probably exclusively by LasR and RhlR (47). It is likely that RpoN also acts on quorum sensing via regulators other than GacA, but these remain to be identified.

## DISCUSSION

In *P. aeruginosa*, the alternative sigma factor RpoN has several roles. As in enteric bacteria, it is a vital component of nitrogen assimilation. *rpoN* mutants of strains PAO1 and PAK but not strain PA14 are auxotrophic for glutamine (21, 40, 61). In strain PAO, RpoN cooperates with the two-component systems NtrB-NtrC and CbrA-CbrB in the utilization of various carbon and nitrogen sources, e.g., arginine, histidine, and polyamines (40, 61). Moreover, PAO and PAK mutants that are defective for *rpoN* do not produce flagella and pili and therefore do not show swimming and twitching mobility (25, 61; this study). For the transcription of flagellar and pilus genes,  $\sigma^{54}$  needs the transcriptional regulators FleQ, FleR, and PilR (2, 35). Remarkably, the genomic sequence of strain PAO predicts >20 transcriptional regulators activating  $\sigma^{54}$ ; however, the function of most of these has not yet been discovered (59). Whether any of these is involved in the modulation of quorum-sensing activity reported here is also unknown. Although a direct repressive effect of RpoN on some quorum-sensing gene promoter has not been rigorously excluded, it seems more likely that RpoN, together with some transcriptional regulator(s), would transcribe one or several regulatory elements that control quorum sensing.

Certain transcriptional regulators that interact with  $\sigma^{54}$ , e.g., NtrC and CbrB, are response regulators of two-component systems in *P. aeruginosa*. Since the cognate sensor kinases NtrB and CbrA are involved in the utilization of various N sources (40), we considered the possibility that the production of AHLs by *P. aeruginosa* might be influenced by the N source. However, we did not observe any significant differences in AHL levels produced aerobically by *P. aeruginosa* PAO1 cells when the growth medium contained either ammonium (a good N source) or nitrate (a poor N source) (data not shown).

One role of the postulated RpoN-dependent control element(s) is to downregulate the expression of the global regulator GacA. This downregulation occurs at both low and high cell densities (Fig. 4). By contrast, RpoN has a positive effect on the expression of another quorum-sensing regulator, Vfr, especially during late growth phases (Fig. 3). Vfr positively controls the expression of the *lasR* regulator (1). It therefore appears that the derepressing effect of an *rpoN* mutation on *lasR* expression (Fig. 2A and B) could be the result of the enhanced expression of GacA during early growth phases and at low cell densities. During later stages of growth and at higher cell densities, the downregulation of *vfr* may compensate for the upregulation of *gacA* in an *rpoN* mutant. This would explain the observation that *lasR* and, indirectly, *lasI* are not overexpressed in an *rpoN* mutant background at an OD<sub>600</sub> of  $\geq 1.5$  (Fig. 2A and B).

The signal transduction pathways by which GacA acts on the expression of target genes, many of which are involved in the synthesis of exoproducts and biofilm formation (43), are incompletely understood at present (19). In *P. aeruginosa*, a detailed analysis of *hcnABC* expression revealed the existence of two GacA-dependent pathways (47, 48, 52). In the first

pathway, GacA exerts a positive effect on the expression of the *lasR* and *rhIRI* genes and, as a consequence, also on the transcription of LasR- and RhIR-dependent genes, including *hcnABC*. In the second, AHL-independent pathway, GacA exerts a positive effect on target gene expression at a posttranscriptional level; in the case of the *hcnABC* genes, this effect requires a sequence surrounding the *hcnA* ribosome-binding site (48) and the RNA-binding protein RsmA (49). In both pathways, the DNA sequences directly recognized by the GacA protein, presumably in its phosphorylated form, have remained elusive. We considered the possibility that RpoN might regulate the expression of RsmA. However, we found no evidence for such an effect in experiments with an *rsmA'*-*lacZ* fusion and Western blotting (our unpublished data).

It is interesting that another alternative sigma factor, the stress and stationary-phase sigma factor RpoS ( $\sigma^{38}$ ), is also involved in quorum-sensing regulation in *P. aeruginosa*. In an *rpoS* null mutant, BHL levels are elevated throughout growth, essentially due to derepression of *rhIRI* expression, by comparison with the wild-type strain PAO1. As a consequence, expression of exoproduct genes, e.g., *hcnB*, is increased in an *rpoS* mutant (69). There is also evidence that the *rhIRI* system can positively control the RpoS level (31). Environmental conditions greatly influence the relative amounts of sigma factors in bacterial cells (24). These considerations led us to propose an empirical model in which GacA and some sigma factors (such as RpoN and RpoS) globally exert opposite effects on the quorum-sensing machinery in *P. aeruginosa*.

Interactions between quorum sensing and RpoN have also been proposed in *E. coli*. Addition of a signal molecule termed autoinducer 2 (a furanone compound) to *E. coli* cultures induces, among a large number of genes, the *ybhH* gene (corresponding to the gene *PA4463* lying downstream of *rpoN*; Fig. 1) as well as the *ygeV* gene, encoding a  $\sigma^{54}$ -dependent regulator homologous to LuxO, which is a component of the quorum-sensing cascade regulating bioluminescence in *Vibrio harveyi* (12, 34).

Considering the fact that inactivation of *rpoN* leads to the loss of two important adherence factors (pili and flagella) in *P. aeruginosa* on the one hand (21, 25, 61) and to an overexpression of several quorum-sensing-regulated virulence genes (*lasB*, *rhLAB*, and *hcnABC*) on the other hand, it would have been difficult to predict the virulence properties of a *P. aeruginosa rpoN* mutant in an animal model. The work of Hendrickson et al. (21) showed that an *rpoN* mutant of strain PA14 can manifest pathogenicity differently depending on the host. For nematodes and burnt mice, the *rpoN* mutant was less virulent than the wild type, whereas both strains were equally able to kill wax moth larvae (21).

RpoN is required in *P. aeruginosa* strain CHA for type III secretion of exotoxin S and exotoxin T, and thus RpoN makes a contribution to cytotoxicity in this strain (10). Moreover, since many pathogenicity models use relatively high infectious doses, the adherence properties of *P. aeruginosa* in these models may be less important than in most clinical situations. To some extent, the decreased virulence of *P. aeruginosa rpoN* mutants in some models might then be a consequence of a decreased ability to utilize a large number of organic substrates, not just N sources (40), and to produce secreted toxins (10).

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