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Regulation of *rpoS* Gene Expression in *Pseudomonas*: Involvement of a TetR Family Regulator

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The *rpoS* gene encodes the sigma factor which was identified in several gram-negative bacteria as a central regulator during stationary phase. *rpoS* gene regulation is known to respond to cell density, showing higher expression in stationary phase. For *Pseudomonas aeruginosa,* it has been demonstrated that the cell-density-dependent regulation response known as quorum sensing interacts with this regulatory response. Using the *rpoS* promoter of *P. putida,* we identified a genomic Tn*5* insertion mutant of *P. putida* which showed a 90% decrease in *rpoS* promoter activity, resulting in less RpoS being present in a cell at stationary phase. Molecular analysis revealed that this mutant carried a Tn*5* insertion in a gene, designated *psrA* (*Pseudomonas* sigma regulator), which codes for a protein (PsrA) of 26.3 kDa. PsrA contains a helix-turn-helix motif typical of DNA binding proteins and belongs to the TetR family of bacterial regulators. The homolog of the *psrA* gene was identified in *P. aeruginosa*; the protein showed 90% identity to PsrA of *P. putida*. A *psrA*::Tn*5* insertion mutant of *P. aeruginosa* was constructed. In both *Pseudomonas* species, *psrA* was genetically linked to the SOS *lexA* repressor gene. Similar to what was observed for *P. putida*, a *psrA* null mutant of *P. aeruginosa* also showed a 90% reduction in *rpoS* promoter activity; both mutants could be complemented for *rpoS* promoter activity when the *psrA* gene was provided in *trans*. *psrA* mutants of both *Pseudomonas* species lost the ability to induce *rpoS* expression at stationary phase, but they retained the ability to produce quorum-sensing autoinducer molecules. PsrA was demonstrated to negatively regulate *psrA* gene expression in *Pseudomonas* and in *Escherichia coli* as well as to be capable of activating the *rpoS* promoter in *E. coli*. Our data suggest that PsrA is an important regulatory protein of *Pseudomonas* spp. involved in the regulatory cascade controlling *rpoS* gene regulation in response to cell density.

The *rpoS* gene codes for sigma factor RpoS (also called σ^s and σ^{38}) (12, 18), which was identified as a central regulator during stationary phase in *Escherichia coli*; this factor is involved in the survival of famine conditions and in cross-protection against osmotic, acidic, oxidative, and heat stresses (19, 22). Since then, it has been identified in various gram-negative bacteria, including several species belonging to the fluorescent pseudomonads; these findings demonstrate that in these bacteria as well this factor has an important regulatory role, including adaptation to nutrient-limiting conditions, survival in the presence of several environmental stresses, and the production of virulence factors (16, 17, 34, 37, 42, 43).

RpoS is an alternative sigma factor, resulting in the alteration of RNA polymerase core specificity and thereby switching gene expression at stationary phase. The levels of RpoS within a bacterial cell are carefully controlled, since perturbations in the relative amounts can have severe consequences. Thus, the regulation of RpoS levels is of crucial importance (8). In *E. coli*, the levels of RpoS are extremely low in exponential growth phase but increase markedly upon entry into stationary phase (15). The regulation of RpoS in *E. coli* remains a subject of extensive investigation, since regulation occurs at the transcriptional, posttranscriptional, and protein levels (6, 13, 20). At the transcriptional level, it has been observed that in rich media there is a considerable increase in transcription at the transition to stationary phase and that the cyclic AMP-cyclic AMP receptor protein complex is involved either directly or indirectly in this regulation. However, RpoS levels in *E. coli* appear to be regulated mainly at the posttranscriptional level through susceptibility to proteolysis. RpoS is rapidly degraded by the ClpXP protease (29, 38); this degradation absolutely requires the phosphorylated form of a two-component response regulator called SprE or RssB (33), which in turn is modulated by a LysR regulatory family protein called LrhA (13). It is still unclear which signals and effector molecules trigger the regulators responsible for the regulation of this RpoS proteolysis.

The regulation of *rpoS* in *Pseudomonas* has also been addressed recently. The cell-cell communication device, called quorum sensing (10), used by gram-negative bacteria to regulate several physiological processes has been demonstrated to be involved in the control of *rpoS* transcription in *Pseudomonas aeruginosa* (21). There are at least two chemically and genetically independent quorum-sensing systems in *P. aeruginosa*, designated the LasR-LasI and the RhlR-RhlI systems, each having a cognate *N*-acylhomoserine lactone (AHL) (21); these systems are involved in the regulation of a large number of exoproducts in response to cell density. It has been reported that the LasR-LasI elements regulate the activation at the transcriptional level of the RhlR-RhlI system, which then directs the regulation of *rpoS* transcription, resulting in RpoS accumulation in response to cell density (21). A recent study, however, reports that RpoS regulates *rhlI* transcription (45); thus, RpoS and the quorum-sensing system in *P. aeruginosa* are part of the same regulatory network. Future work is required

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TABLE 1. Plasmids used

Plasmid	Relevant characteristics[a]	Reference or source
pUC18	Ap^r; ColE1 replicon	47
pBluescript KS	Ap^r; ColE1 replicon	Stratagene
pBluescript SK	Ap^r; ColE1 replicon	Stratagene
pQE30	Ap^r; ColE1 replicon; His_6 expression vector	Qiagen
pQE31	Ap^r; ColE1 replicon; His_6 expression vector	Qiagen
pREP-4	*lacI*; Km^r; p15A replicon	Qiagen
pRK2013	Km^r Tra^+ Mob^+; ColE1 replicon	9
pPH1JI	IncP1; Gm^r	1
pMP220	Promoter probe vector; IncP1; Tc^r	39
pMP77	Promoter probe vector; IncQ; Cm^r	39
pLAFR3	Broad-host-range cloning vector; IncP1; Tc^r	41
pH3.5	*rpoS* gene of strain WCS358 in pBluescript	17
pSB1075	Ap^r; ColE1 replicon; AHL biosensor	46
pMK962	*rpoS* promoter cloned in pCU18	This study
pRPO77	*rpoS* promoter cloned in pMP77	This study
pRPO220A	*rpoS* incomplete promoter cloned in pMP220	This study
pRPO220B	*rpoS* promoter cloned in pMP220	This study
pPPSR18	*psrA* promoter cloned in pUC18	This study
pPPSR220	*psrA* promoter cloned in pMP220	This study
pCOS17	pLAFR3 containing WCS358 DNA	This study
pCOS18	pLAFR3 containing WCS358 DNA	This study
pAPC17	pUC18 containing 680 bp of *P. putida* MT17 DNA bordering Tn5	This study
pBSH10	pBluescript containing 10-kb *Hin*dIII fragment from pCOS17	This study
pBS25	pBluescript containing 2.5-kb *Pst*I fragment from pCOS17	This study
pLM17E	pLAFR3 containing 5-kb *Eco*RI fragment from pCOS17	This study
pLM17P	pLAFR3 containing 2.5-kb *Pst*I fragment from pCOS17	This study
pMKP25	pMP77 containing 2.5-kb *Pst*I fragment from pCOS17	This study
pPSRPAO1	2.2-kb PCR *Eco*RI fragment from PAO1 genome harboring *psrA* in pBluescript	This study
pPSRPAO1::Tn5	pPSRPAO1 with Tn5 insertion in *psrA*	This study
pSRE8	pLAFR3 harboring 8-kb *Eco*RI fragment from pPSRPAO1::Tn5	This study
pBS25::Tn5R	pBS25 with Tn5 insertion in *psrA*	This study
pCOS17::Tn5R	pCOS17 with Tn5 insertion in *psrA*	This study
pQEPSRA	*psrA* cloned in pQE30	This study
pQERPOS	*rpoS* cloned in pQE31	This study

[a] Ap^r, Km^r, Sm^r, Tc^r, Gm^r, and Cm^r, resistant to ampicillin, kanamycin, streptomycin, tetracycline, gentamicin, and chloramphenicol, respectively.

to precisely define the molecular events leading to *rpoS* regulation. In *P. fluorescens* Pf-5, the GacS-GacA two-component regulatory system positively influences *rpoS* expression (44). This two-component system is well conserved in *Pseudomonas* spp. and regulates the production of several secondary metabolites.

The *rpoS* gene of *P. putida* WCS358 has been identified (17). In this study, we used *rpoS*-reporter gene transcriptional fusions to identify a Tn*5* mutant of strain WCS358 that had considerably reduced *rpoS* expression. This mutant had a Tn*5* insertion in a regulatory gene (designated *psrA*) coding for a protein (designated PsrA) belonging to the TetR regulatory family. A homolog of this gene in *P. aeruginosa* encodes a protein having 90% identity with PsrA of *P. putida*. This gene was inactivated by transposon mutagenesis and homologous recombination; the resulting mutant also showed a 90% reduction in *rpoS* promoter activity. Our data suggest that in *Pseudomonas* spp., *psrA* plays an important role in *rpoS* expression.

MATERIALS AND METHODS

Strains, plasmids, media, and chemicals. The strains used in this study included *E. coli* HB101 (35), DH5α (14), and XL1-Blue (4); *P. putida* WCS358, a plant-growth-promoting strain isolated from the rhizosphere of potato roots (11); *P. aeruginosa* PAO1 (Holloway collection); and *Chromobacterium violaceum* CVO26, a double mini-Tn*5* mutant derived from ATCC 31532. This mutant is nonpigmented, and production of the purple pigment can be induced by providing exogenous AHL inducer molecules (27). *E. coli* and *P. aeruginosa* PAO1 were grown in LB medium (28) at 37°C, whereas *P. putida* WCS358 was cultured in LB medium or in M9 minimal medium (35) at 30°C. The following antibiotic concentrations were used: tetracycline, 10 μg/ml (*E. coli*), 40 μg/ml (strain WCS358), and 300 μg/ml (PAO1); kanamycin, 100 and 300 μg/ml (PAO1); ampicillin, 100 μg/ml; nalidixic acid, 25 μg/ml; chloramphenicol, 25 μg/ml (*E. coli*), 250 μg/ml (strain WCS358), and 500 μg/ml (PAO1); and gentamicin, 10 μg/ml (*E. coli*), 40 μg/ml (strain WCS358), and 60 μg/ml (PAO1). The plasmids used in this study are listed in Table 1.

The *rpoS* promoter transcriptional fusions were constructed as follows. First, a 135-bp fragment consisting of the −9 to −144 DNA region, where position 0 is the ATG codon of the *rpoS* gene of *P. putida* WCS358 (17), was cloned into promoter probe vector pMP220 by making use of two synthetic oligonucleotides (5′-CCTTTGCTGCAGTTCGAACTCAGA-3′ and 5-′CCCGTGGATCCACTC CAGTTCCTG-3′). One oligonucleotide had a *Pst*I restriction site inserted and the other had a *Bam*HI site, and they were cloned into the *Bgl*II and *Pst*I sites in pMP220 to yield pRPO220A. Second, a *Hin*dIII-*Aat*II DNA fragment of 962 bp obtained from plasmid pH3.5 and containing 920 bp of DNA upstream of the ATG of the *rpoS* gene of strain WCS358 (17) was end filled and cloned into the *Sma*I site of pUC18 to yield pMK962. pMK962 was then digested with *Bam*HI and *Kpn*I, and the 974-bp fragment was cloned into pMP220 and pMP77, both digested with *Bgl*II and *Kpn*I, to yield pRPO220B and pRPO77, respectively.

Plasmid pPSRPAO1 harbors a 2.2-kb fragment of PAO1 genomic DNA. It was constructed by amplifying by PCR, using the PAO1 genome as a template and two oligonucleotides (5′-ACCTTGCTGAATTCGCGCTTGAAGCG-3′ and 5′-CGCCACATGGGAATTCGGCCTCGGCC-3′), a 2.2-kb fragment located at positions 3368363 to 3370563 in the PAO1 genome (www.pseudomonas.com). This fragment was cloned as an *Eco*RI (the two oligonucleotides used harbored *Eco*RI restriction sites) fragment of pBluescript to yield pPSRPAO1.

The *psrA* promoter was cloned into promoter probe vector pMP220 as follows. An *Xmn*I-*Sac*II fragment of 435 bp (Fig. 1) containing the *psrA* promoter was blunted by end filling and cloned into the *Sma*I site of pUC18 to generate

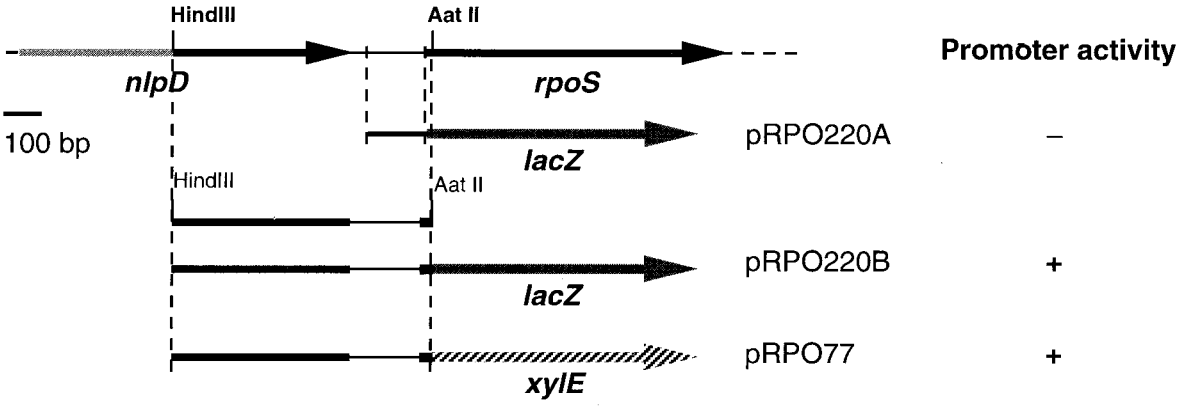


FIG. 1. Strategy for construction of *rpoS-lacZ* and *rpoS-xylE* transcriptional fusions. The genetic map shows the location of *rpoS* in the *P. putida* WCS358 genome, as described by Kojic et al. (17). Plasmid constructs pRPO220A and pRPO220B are derivatives of promoter probe vector pMP220. pRPO77 is derived from pMP77 (see the text for details). Also shown is whether the transcriptional fusion had promoter activity, as detected using the reporter gene. Plasmid construct pRPO220A contains the *rpoS-nlpD* intergenic region cloned upstream of a promoterless *lacZ* gene. Plasmid constructs pRPO220B and pRPO77 contain the indicated 962-bp fragment cloned upstream of promoterless *lacZ* and *xylE* genes, respectively (see the text for details).

pPPSR18. The promoter was then removed as a *Bam*HI-*Kpn*I fragment and cloned into *Bgl*II-*Kpn*I-digested pMP220 to yield pPPSR220. The *psrA* gene was cloned into expression vector pQE30 as follows. The gene was amplified by PCR using two oligonucleotides (*psrA-Bam*HI-start, 5′-GGAATAATCGGATCCCA ATCGGAAACCG-3′, and *psrA-Hin*dIII-end, 5′-GCGCAAGCTTAGCCGAA GCGCCCTGCCCC-3′) and cloned as a *Bam*HI-*Hin*dIII fragment into the corresponding sites of pQE30, yielding pQEPSRA and resulting in *psrA* being in frame with the six histidines. The *P. putida* WCS358 *rpoS* gene was cloned into expression vector pQE31 as follows. The gene was amplified by PCR using two oligonucleotides (*rpoS-Bam*HI-start, 5′-CTATAACAATGGATCCCAATAAA GAAGCGCC-3′ and *rpoS-Hin*dIII-end, 5′-GTCTTAAGCTTGCGAACAGCG TATTACTGG-3′) and cloned as a *Bam*HI-*Hin*dIII fragment into the corresponding sites of pQE31, yielding pQERPOS.

Recombinant DNA techniques. Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with the Klenow fragment of DNA polymerase, Southern hybridization, transformation of *E. coli,* and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis were performed as described by Sambrook et al. (35). Analytical amounts of plasmids were isolated as described by Birnboim (3), whereas preparative amounts were purified with Qiagen columns. Total DNA from *Pseudomonas* was isolated by Sarkosyl-pronase lysis as described by Better et al. (2). Triparental matings from *E. coli* to *Pseudomonas* were performed with an *E. coli*(pRK2013) helper strain (9). The DNA sequence flanking transposon mutant *P. putida* MT17 was determined using arbitrary PCR. In this procedure, the DNA flanking the Tn*5* insertion site was enriched in two rounds of amplification using primers specific to the ends of the Tn*5* element and primers of random sequence which annealed to chromosomal sequences flanking the transposon as described by O' Toole and Kolter (31).

Protein expression, analysis, and purification and antibodies against PsrA and RpoS. Expression and purification of His_6-PsrA (pQEPSRA) and His_6-RpoS (pQERPOS) were carried out with *E. coli* M15(pREP-4) according to the instructions of the supplier (Qiagen, Hilden, Germany). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corp.) using a tank system according to the manufacturer's instructions. The membrane was subjected to Western blot analysis using polyclonal antibodies against either RpoS or PsrA. After incubation with the secondary horseradish peroxidase-labeled antibody, the proteins were detected with 3-3′-diaminobenzidine tetrahydrochloride tablets (Sigma, St. Louis, Mo.). Antibodies against PsrA and RpoS were produced in rabbits by injecting the purified protein. No significant cross-reaction was observed in this study.

Reporter gene fusion assays; purification, detection, and visualization of autoinducer molecules; pyocyanin quantification; and stress response assays. β-Galactosidase activity was determined essentially as described by Miller (28) with the modifications of Stachel et al. (40). The purification, detection, and visualization of AHL inducer molecules from culture supernatants were performed essentially as described by McClean et al. (27) and Kojic et al. (17). *C. violaceum* CVO26 and *E. coli*(pSB1075) were used as the indicator strains for C_4-homoserine lactone (HSL) and 3-oxo-C_{12}-HSL, respectively, on thin-layer chromatography plates to detect the presence of AHL molecules (27, 46). As controls, chemically synthesized C_4-HSL and 3-oxo-C_{12}-HSL (kindly donated by P. Williams) were used. Pyocyanin production was measured as described by Essar et al. (7) The measurement of cell viability to survive heat stress and osmotic stress and sensitivity to hydrogen peroxide were determined as described by Kojic et al. (17).

Identification of *rpoS* regulatory mutants and their complementation with a WCS358 gene bank. About 200 cells per plate of a Tn*5* mutant bank of strain WCS358 (26), harboring promoter fusion pRPO77, were spread on LB plates containing kanamycin (100 μg/ml) and chloramphenicol (250 μg/ml). These plates were incubated for 2 days at 30°C before being sprayed with a 0.1 M catechol solution. Colonies with a decrease in yellow color were purified and further studied. Tn*5* mutants which had reduced promoter activity were complemented for promoter activity as follows. About 4×10^9 cells each of *E. coli* HB101 harboring the *P. putida* WCS358 gene bank in pLAFR3 and *E. coli* (pRK2013) and 2×10^8 cells of mutant MT17(pRPO77) were mixed, and the suspension was applied to a 0.45-μm-pore-size membrane filter (Millipore) on an LB plate. The plate was incubated overnight at 30°C before the cells were resuspended and plated on LB plates containing nalidixic acid (25 μg/ml), ampicillin (100 μg/ml), kanamycin (100 μg/ml), chloramphenicol (250 μg/ml), and tetracycline (40 μg/ml). These plates were incubated for 2 days at 30°C, and the cells were resuspended and pooled. About 100 cells were spread on LB plates containing chloramphenicol (250 μg/ml), kanamycin (100 μg/ml), and tetracycline (40 μg/ml). These plates were incubated at 30°C for 2 days before being sprayed with a 0.1 M catechol solution. Transconjugants that turned yellow were purified and further assayed.

Construction of a *psrA* mutant of *P. putida* WCS358. Transposon Tn*5* insertions within recombinant plasmid pBS25 were obtained as described by Magazin et al. (25) with *E. coli* HB101::Tn*5* as the source of the transposon. *E. coli* HB101 cells containing Tn*5* insertions within plasmid pBS25 were identified by purifying plasmid DNA from HB101::Tn*5*(pBS25), using it to transform *E. coli* DH5α, and selecting for plasmids having ampicillin and kanamycin resistance. Transposon insertions in the *psrA* gene of *P. putida* were then mapped by restriction and Southern analyses and DNA sequencing using a Tn*5*-based primer (5′-GAACG TTACCATGTTAGGAGGTC-3′). One Tn*5* insertion in the *psrA* gene in pBS25 was identified (the plasmid was designated pBS25::Tn*5*R) and was located 428 bp downstream of the putative ATG codon of the *P. putida psrA* gene (see Fig. 3A). This Tn*5* insertion in pBS25 was transferred to the *psrA* gene harbored in pCOS17 by homologous recombination in the following way. pBS25::Tn*5* was used to transform HB101(pCOS17), and the resulting construct, HB101 (pCOS17)(pBS25::Tn*5*), was grown overnight. The culture was used for a triparental conjugation into *P. putida* WCS358 with *E. coli*(pRK2013) as a helper. After appropriate selection, pCOS17::Tn*5*R was selected. It was verified that Tn*5* had been transferred by double-crossover homologous recombination from pBS25::Tn*5* to pCOS17. Plasmid pCOS17::Tn*5* was then used in a marker exchange technique (5) in order to introduce insertion mutations site specifically

with the *psrA* gene of *P. putida*; in this experiment, pPH1JI was used as the incoming incompatible plasmid as previously described (1). The fidelity of the marker exchange event in the *P. putida psrA*::Tn*5* mutant was confirmed by Southern analysis (data not shown). This mutant was designated *P. putida* M17R.

Construction of a *psrA*::Tn*5* mutant of *P. aeruginosa* PAO1. Transposon Tn*5* insertions within recombinant plasmid pPSRPAO1 were obtained as described by Magazin et al. (25) with *E. coli* HB101::Tn*5* as the source of the transposon as described above. One Tn*5* insertion in the *psrA* gene in pPSRPAO1 was identified (the plasmid was designated pPSRPAO::Tn*5*) and was located 478 bp downstream of the putative ATG codon of the PAO1 *psrA* gene. Plasmid pPSRPAO1::Tn*5* was digested with *Eco*RI, and the 8-kb fragment harboring the 2.2-kb *Eco*RI fragment of PAO1 DNA with a Tn*5* insertion was cloned into the corresponding site of pLAFR3 to yield pPSRE8. This plasmid was conjugated into *P. aeruginosa* PAO1 and used in a marker exchange technique (5) in order to introduce insertion mutations site specifically with the *psrA* gene of *P. aeruginosa* PAO1 (1). The fidelity of the marker exchange event in the PAO1 *psrA*::Tn*5* mutant was confirmed by Southern analysis (data not shown).

DNA sequence determination and analysis. DNA segments of various sizes were created from plasmid pBS25 using a nested deletion kit (Amersham-Pharmacia). These constructs were either encapsidated as single-stranded DNA upon infection with helper phage VCSM13 (Stratagene Co.) or used directly for DNA sequencing. Nucleotide sequences were determined by the dideoxy chain termination method (36) using [α-^{35}S]dATP for labeling and 7-deaza-dGTP (Amersham-Pharmacia) instead of dGTP. The DNA fragments were separated with a Bio-Rad electrophoresis kit. The nucleotide sequences presented here were determined in both orientations and across all restriction sites.

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AJ293485.

RESULTS

Isolation and characterization of *P. putida* WCS358 regulatory mutants affected in *rpoS* expression. Two transcriptional fusions were constructed using the *P. putida* WCS358 *rpoS* promoter and a promoterless *lacZ* gene as described in Materials and Methods (Fig. 1). The first fusion (pRPO220A) contained a 135-bp fragment of the *rpoS* promoter starting from 9 bp upstream of the ATG and reaching 17 bp away from the stop codon of the *nlpD* gene, which is located upstream and transcribed in the same orientation as the *rpoS* gene (Fig. 1). The second fusion (pRPO220B) contained 920 bp upstream of the ATG codon, as depicted in Fig. 1. The first fusion did not show any β-galactosidase activity; thus, there was no promoter element in the *nlpD-rpoS* intergenic region. However, the second, larger construct displayed significant promoter activity, demonstrating that the *rpoS* promoter was contained in this fragment within the *nlpD* gene (Fig. 1). This same fragment was cloned in promoter probe vector pMP77, yielding pRPO77 (Fig. 1), which contained a promoterless *xylE* gene. This procedure provided a convenient plate assay for detecting promoter activity compared to the more sensitive detection of β-galactosidase activity.

A transposon Tn*5* insertion mutant bank of *P. putida* strain WCS358 harboring promoter fusion pRPO77 was assayed for promoter activity. With this construct, promoter activity can be conveniently detected on plates by assaying for the *xylE* gene product, catechol 2,3-dioxygenase (XylE), which converts catechol to a yellow product. Bacterial colonies exhibiting promoter activity become yellow when sprayed with a catechol solution. Ten thousand mutants were screened, and 1 mutant, designated MT17, showed very little yellow coloration after being sprayed with a catechol solution. In order to confirm the reduced *rpoS* promoter activity, plasmid pRPO77 was cured from mutant MT17 by growth for several generations in the absence of antibiotics and then selection for a chloramphenicol-sensitive colony. pRPO77 was then conjugated into the mutants, and it was reconfirmed that MT17(pRPO77) displayed less yellow color than wild-type WCS358(pRPO77) when sprayed with a catechol solution.

The activity of the *rpoS* promoter was quantified in mutant MT17 after the introduction of plasmid pRPO220B. This plasmid contains the same promoter as pRPO77 but cloned upstream of a promoterless *lacZ* gene, providing a more convenient way to quantify promoter activity through assaying for β-galactosidase activity. The *rpoS* promoter showed strong activity in parent strain WCS358, whereas in mutant strain MT17 it displayed only 10% the activity shown in parent strain WCS358 (Fig. 2). It was concluded that mutant MT17 had a Tn*5* insertion in a locus affecting *rpoS* gene expression.

Complementation of *P. putida* MT17 Tn*5* regulatory mutants. A gene bank containing partially digested *Hin*dIII chromosomal DNA fragments of strain WCS358 cloned in the corresponding site in cosmid vector pLAFR3 was introduced into the regulatory mutants. The transconjugants were complemented for the restoration of the promoter activity of plasmid pRPO77 as described in Materials and Methods. Two cosmids, pCOS17 and pCOS18, were isolated that complemented mutant MT17 for promoter activity. Restriction analysis revealed that pCOS17 and pCOS18 shared a 10-kb *Hin*dIII fragment.

A portion of the DNA sequence flanking Tn*5* was cloned using an arbitrary PCR method as described in Materials and Methods. This allowed the cloning into pUC18 of a 680-bp fragment (pAPC17) flanking Tn*5* in mutant MT17. This fragment was sequenced and used as a probe in Southern analysis, which allowed the localization of the complementing region within the 10-kb *Hin*dIII fragment of pCOS17 and pCOS18. This 10-kb *Hin*dIII fragment was cloned into the corresponding site in pBluescript to yield pBSH10. Further mapping using the 680-bp PCR fragment from pAPC17 as a probe revealed that within the 10-kb *Hin*dIII fragment, a 5-kb *Eco*RI fragment and a 2.5-kb *Pst*I fragment contained the DNA flanking the Tn*5* transposon in mutant MT17. Both of these fragments were cloned into the corresponding site in vector pLAFR3, yielding pLM17E and pLM17P, respectively, and were conjugated into *P. putida* MT17(pRPO77). Both of these subclones restored *rpoS* promoter activity (data not shown). The 2.5-kb *Pst*I fragment was also cloned into the IncQ plasmid pMP77 to yield pMKP25, which was transferred to *P. putida* MT17 (pRPO220B); this procedure restored wild-type levels of *rpoS* promoter activity, as detected by β-galactosidase activity (Fig. 2). It was concluded that the 2.5-kb *Pst*I fragment could complement the mutant and contained all the necessary information to restore *rpoS* promoter activity in mutant MT17.

Characterization of the complementing DNA. The 2.5-kb *Pst*I fragment from pCOS17 and pCOS18 (i.e., construct pBS25) was sequenced in both directions (GenBank accession number AJ293485). The genetic map of the region is presented in Fig. 3A. Tn*5* in mutant MT17 was inserted within an open reading frame (ORF) of 714 bp and coding for a putative protein of 237 amino acids and having a predicted molecular mass of 26,359 Da (Fig. 3B). The precise position of the Tn*5* insertion in *P. putida* MT17 was 178 bp downstream of the putative ATG of this ORF. The gene representing this ORF was designated *psrA* (*Pseudomonas* sigma regulator). Western

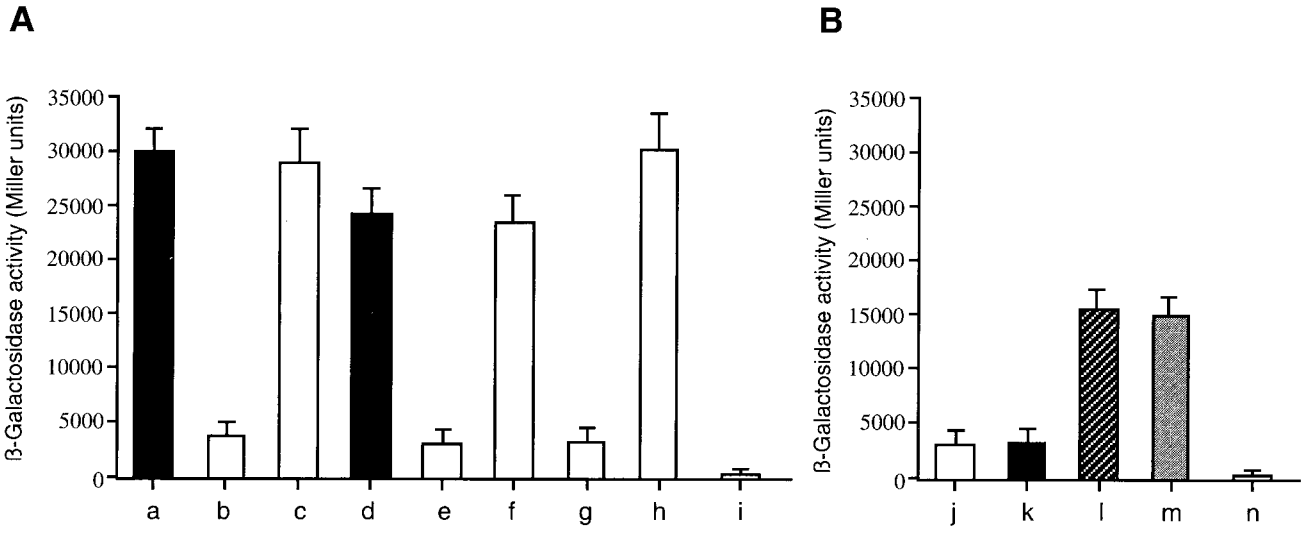


FIG. 2. β-Galactosidase activities. (A) β-Galactosidase activities driven by the *rpoS-lacZ* fusion pRPO220B. All measurements were done in triplicate; the mean and standard error are shown. Cells harboring plasmids were grown for 16 h in LB medium in the presence of appropriate antibiotics. *P. putida* MT17 is a *psrA* null mutant of parent strain WCS358, *P. putida* MT17R is also a *psrA* null mutant, and *P. aeruginosa* MKV8 is a *psrA* null mutant of parent strain PAO1. Plasmid pRPO220B (IncP; Tc^r) is an *rpoS-pacZ* transcriptional fusion, whereas pMKP25 (IncQ; Cm^r) harbors a 2.5-kb *Pst*I insert of *P. putida* WCS358 DNA carrying the complete *psrA* gene. (B) β-Galactosidase activities driven by the *psrA-lacZ* fusion pPPSR220. Cells harboring plasmids were grown for 16 h in LB medium in the presence of appropriate antibiotics. See the text for details. Columns: a, *P. putida* WCS358(pRPO220B); b, *P. putida* MT17(pRPO220B); c, *P. putida* MT17(pRPO220B)(pMKP25); d, *P. aeruginosa* PAO1(pRPO220B); e, *P. aeruginosa* MKV8(pRPO220B); f, *P. aeruginosa* MKV8(pRPO220B)(pMKP25); g, *P. putida* MT17R(pRPO220B); h, *P. putida* MT17R(pRPO220B)(pMKP25); i, *P. putida* WCS358(pMP220); j, *P. putida* WCS358(pPPSR220); k, *P. putida rpoS*::Tn*5* WCS358 (pPPSR220); l, *P. putida* MT17(pPPSR220); m, *P. putida* MT17R(pPPSR220); n, *P. putida* WCS358(pMP220).

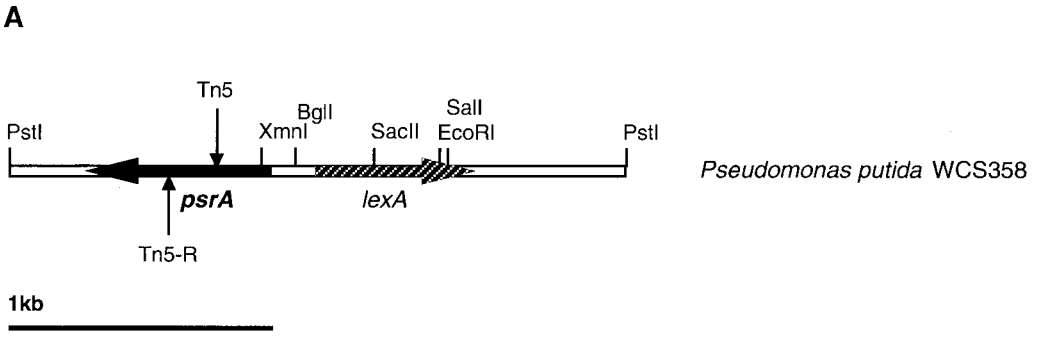
analysis using anti-PsrA antibodies revealed that mutant MT17 no longer produced PsrA (Fig. 4). This ORF was preceded by a potential Shine-Dalgarno sequence and was located 216 bp upstream of and in the orientation opposite that of the first codon of the *lexA* gene (GenBank accession number AJ293485). The LexA repressor protein identified here displayed a high level of identity to the characterized LexA proteins of other gram-negative bacteria (data not shown).

Regeneration of the *psrA*::Tn*5* mutant of *P. putida* WCS358. In order to unequivocally confirm that the *psrA* gene alone was responsible for the phenotype in mutant MT17, this gene was insertionally inactivated in *P. putida* WCS358 as described in Materials and Methods. In the regenerated *psrA* null mutant, *rpoS* promoter activity was reduced by 90%, just as in *P. putida* MT17, as identified by genetic selection. It was concluded that the ORF coding for the PsrA protein was responsible for the phenotype of reduced *rpoS* gene expression.

PsrA belongs to the TetR family of bacterial regulators. A computer-assisted homology search between PsrA and proteins deposited in data banks revealed significant homology to bacterial regulators belonging to the TetR family (Prosite accession number PS01081). Many of the proteins of this family appear to be repressors, and their targets are often genes encoding proteins involved in cell envelope permeability. They have similar molecular masses (from 21 to 27 kDa). As a signature pattern, there is a conserved region that starts four residues before the helix-turn-helix motif and ends six residues after it and that is located near the N terminus. For PsrA, the signature is between amino acids 10 and 56, as depicted in Fig. 3B. The two proteins which displayed the highest level of identity to PsrA were a 175-amino-acid TetR regulator of *Vibrio parahaemolyticus* (30), showing 40% identity, and a regulator called IfeR (207 amino acids) of *Agrobacterium tumefaciens* (32), showing 34% identity. Both of these regulators are genetically linked to operons encoding efflux pumps. An alignment of the first 60 amino acids of PsrA with these two proteins is depicted in Fig. 3B. Apart from these proteins, PsrA displayed significant identity in the first 60 amino acids (approximately 30%) to 18 putative TetR family regulators (data not shown). We have found a similar gene upstream of the *lexA* gene in *P. aeruginosa* (www.pseudomonas.com); the genetic organization is like that in *P. putida*. This putative gene codes for a protein which is 90% identical to PsrA (Fig. 3B); it has not been characterized but has been identified through sequencing of the *P. aeruginosa* genome (see below).

Growth phase dependence of *rpoS* expression in *P. putida*. The growth phase dependence of *rpoS* expression was assessed using the pRPO220B *rpoS-lacZ* fusion (Fig. 4). Expression was relatively constant during early and exponential growth and then increased approximately fivefold in late exponential and early stationary phases. In *psrA*::Tn*5* mutant MT17, *rpoS* expression was low throughout growth. The induction observed in the parent strain was not detected; *rpoS* expression was approximately 10% that seen in strain WCS358 (Fig. 4). *P. putida* MT17 harboring *psrA* in plasmid pMKP25 regained promoter activity and inducibility in response to cell density.

The *psrA* gene of *P. aeruginosa* is also involved in *rpoS* gene expression. To investigate the role of the putative *psrA* gene of *P. aeruginosa* PAO1, we insertionally inactivated this locus in *P. aeruginosa*. A *psrA* null mutant was constructed and designated *P. aeruginosa* MKV8. Similar to what was observed for *P. putida* WCC358, a 90% reduction in *rpoS* promoter activity was



B

(i)

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PsrA        ----MAQSETVERILDAAEQLFAERGFAETSLRLITSKAGVNLAAVNYHFGSKKALIQA 55
V.para      ---MAPRSTTKEKILDVAEGLFAEYGFNDTSLRTITGKANVNLASVNYHFGDKKTLVRA 56
IfeR       MRRTKEQAAETGRQILQAAETLFLDKGYDNVSLEEIAALSGVTRGAIHWHFKNKHGLLLA 60
                  : * .:**:.** ** : *: :.**. *:. :.*. .::::** .*: *: *
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(ii)

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PsrA       MAQSETVERILDAAEQLFAER GFAETSLRLITSKAGVNLAAVNYHFGSKKAL IQAVFSRF 60
PsrA*      MAQSETVERILDAAEQLFAEK GFAETSLRLITSKAGVNLAAVNYHFGSKKAL IQAVFSRF 60
           ********************: ********************************** ********

PsrA       LGPFCASLERELERRQARPE-QKPSLEELLEMLVEQALAVQPRSNNDLSIFMRLLGLAFS 120
PsrA*      LGPFCASLEKELDRRQAKPEAQHATLEDLLHLLVSQAMAVKPRSGNDLSIFMRLLGLAFS 120
           *********:**:****:** *:.:**:**.:**.**:**:***.***************

PsrA       QSQGHLRRYLEDMYGKVFRRYMLLVNEAAPRVPPLELFWRVHFMLGAAAFSMSGIKALRA 180
PsrA*      QSQGHLRKYLEEVYGKVFRRYMLLVNEAAPKLPPIELFWRVHFMLGAAAFSMSGIKALRA 180
           *******:***::*****************::**:************************

PsrA       IAETDFGINTSIEQVMRLMVPFLAAGMRADSGVTDEAMAAAQLRPRSKTSTSATTAKA 237
PsrA*      MAETDFGVNTSTEQVMHLMVPFFAAGMRAESGIDDPLLAGAQLRPRNKTP-----AKA 233
           :******:*** ****:*****:******:**: *  :*.******.**.     ***
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FIG. 3. Gene map and protein sequence alignments. (A) Genetic organization of the region (*Pst*I fragment; see text for details) containing the *psrA* regulatory gene in *P. putida* WCS358. Shown is the Tn*5* insertion position located in mutant MT17. Also shown is the Tn*5* insertion (shown as Tn*5*-R) located in the regenerated *psrA* mutant, designated MT17R. The DNA sequence of this 2.5-kb fragment can be found in GenBank accession number AJ293485. (B) (i) Protein sequence alignment, using the one-letter code, of the first 60 amino acids of PsrA, a TetR regulator of *V. parahaemolyticus* (V.para) (GenBank accession number Q56726), and IfeR of *A. tumefaciens* (GenBank accession number O68442). (ii) Protein sequence alignment, using the one-letter code, of the PsrA proteins from *P. putida* (PsrA) and *P. aeruginosa* (PsrA*) (this study). In bold are shown the amino acids which constitute the TetR family signature pattern (Prosite accession number PS01081). A star indicates conservation of identical amino acids, a colon indicates a conserved substitution, and a period indicates a semiconserved substitution.

observed in the mutant strain relative to the wild-type strain (Fig. 2). This reduction in promoter activity was restored when the *P. putida psrA* gene harbored in pMKP25 was introduced in *trans* into *P. aeruginosa* MKV8(pRPO220B) (Fig. 2). It was concluded that this gene also plays a role in *rpoS* gene expression in *P. aeruginosa*.

Phenotype of the *Pseudomonas psrA*::Tn*5* mutants. RpoS is known to confer cross-protection against heat and osmotic shock as well as hydrogen peroxide. It was reported that *P. putida* WCS358 containing *rpoS*::Tn*5* was more sensitive to these three environmental stresses (17). When stationary-phase cultures of mutant MT17 were exposed to a sudden shift in temperature from 30 to 50°C, it was found to be twofold more sensitive at 50°C than the parent strain (data not shown). Similarly, when mutant MT17 was subjected to an increase in osmotic pressure caused by the addition of a high concentration of salt, the mutant was almost twofold more sensitive than strain WCS358 (data not shown). We also tested protection against hydrogen peroxide. In exponentially growing cells, there was no difference in sensitivity between the *psrA* mutant and the parent strain; however, in stationary-phase cells, the zone of inhibition was approximately 10% larger (data not shown), most probably due to a decrease in catalase activity (42).

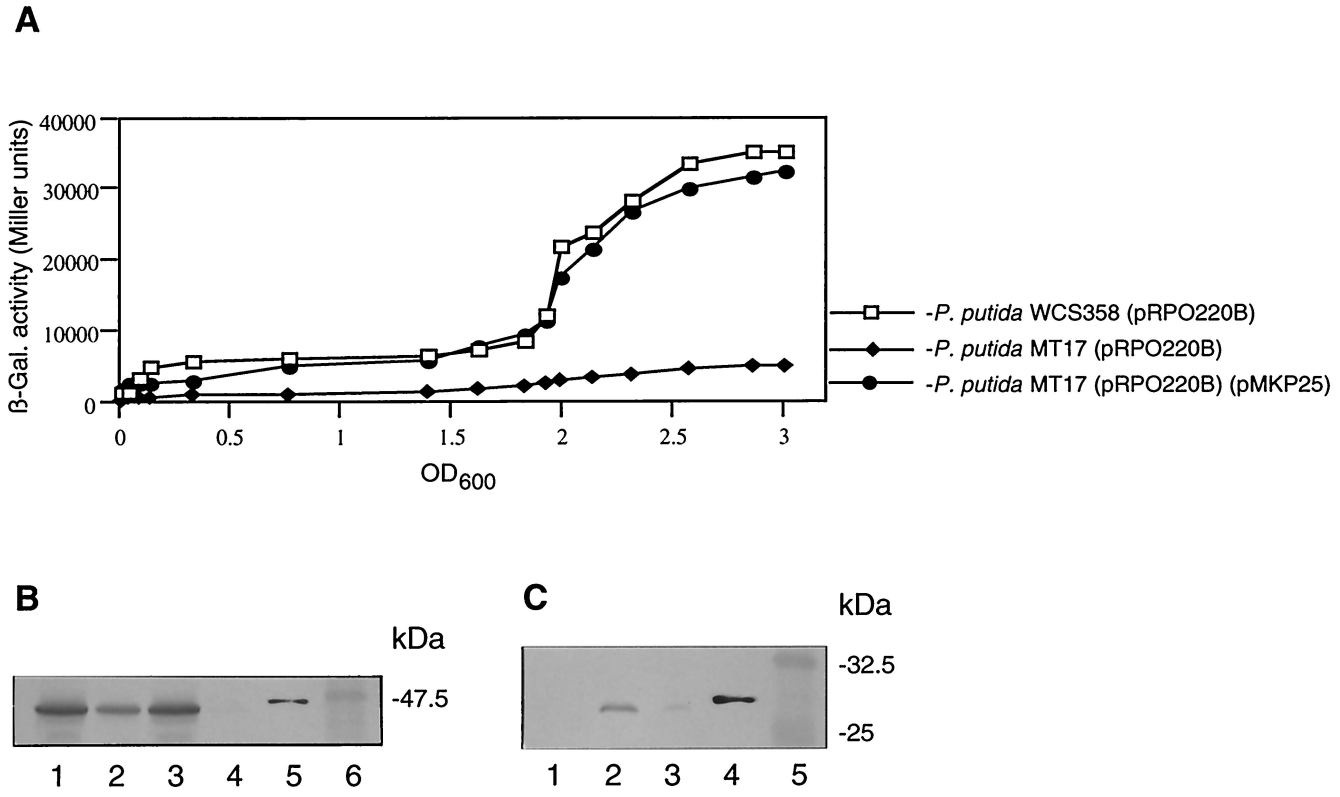

FIG. 4. β-Galactosidase activities and Western analyses. (A) Growth and β-galactosidase (β-Gal.) activities of *P. putida* WCS358(pRPO220B), *psrA* null mutant MT17(pRPO220B), and MT17(pRPO220B)(pMKP25). The values were determined with LB medium, and the means of triplicate experiments are shown. OD_{600}, OD at 600 nm. (B) Cellular RpoS levels of overnight cultures (OD, 2.3) of *P. putida* WCS358 (lane 1), *P. putida* MT17 (lane 2), *P. putida* MT17(pMKP25) (lane 3), *rpoS*:: Tn*5* *P. putida* (lane 4), purified His_6-RpoS (lane 5), and prestained molecular mass markers (lane 6). Immunoblot analysis was performed with 30 μg of total cellular protein per lane, 20 ng of purified His_6-RpoS (lane 5), and anti-RpoS antibodies. (C) Cellular PsrA levels of overnight cultures (OD, 2.4) of *P. putida* MT17 (lane 1), *P. putida* MT17(pMKP25) (lane 2), *P. putida* WCS358 (lane 3), and purified His_6-PsrA (lane 4). Immunoblot analysis was performed with 30 μg of total cellular protein per lane, 20 ng of purified His_6-PsrA (lane 4), prestained molecular mass markers (lane 5), and anti-PsrA antibodies.

It was reported that in *P. aeruginosa,* RpoS negatively regulates the production and secretion of pyocyanin pigment (42), the *rpoS* mutants showing a clear dark-blue coloration compared to the wild type. The *psrA* mutants of *P. aeruginosa* PAO1 also displayed a dark-blue coloration compared to the wild type. Therefore, we determined pyocyanin production in *P. aeruginosa* MKV8 and in PAO1 and found that the *psrA* mutant produces 2.5-fold more pyocyanin than the wild type (data not shown).

Finally, we examined levels of RpoS proteins in 24-h-old stationary-phase cultures of *P. putida* WCS358 and the *psrA* knockout *P. putida* MT17 using anti-RpoS antibodies. As depicted in Fig. 4, RpoS levels in *P. putida* MT17 were significantly lower than those in the wild type and the complemented mutant. Using a densitometer (Pharmacia LKB Ultroscan), we determined that RpoS levels in overnight cultures of the *psrA* mutant were approximately 50% those in parent strain WCS358 or the complemented mutant (Fig. 4).

***Pseudomonas psrA* mutants produce autoinducer molecules.** In *P. aeruginosa,* quorum sensing was implicated in *rpoS* gene expression and/or vice versa (21, 45). We tested the production of autoinducer (AHL) molecules in the *psrA* null mutant of *P. aeruginosa*. *P. aeruginosa* PAO1 synthesized the two identified and characterized AHLs, namely, *N*-3-oxododecanoyl-L-homoserine lactone, encoded by the *lasI* gene, and *N*-butanoyl-L-homoserine lactone, encoded by the *rhlI* gene, as detected by violacein production with the biosensor *C. violaceum* CVO26 and by light production with *E. coli*(pSB1075), respectively. AHL molecules were purified in a volume corresponding to 5×10^8 CFU at optical densities (ODs) of 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2, and 2.5 from culture supernatants of *P. aeruginosa* PAO1 and the *psrA* mutant *P. aeruginosa* MKV8 grown on LB plates. The purified extracts were placed on thin-layer chromatography plates and overlaid with the bacterial sensor. No significant differences were observed for the *psrA* mutant *P. aeruginosa* MKV8 (data not shown). It was previously reported that *P. putida* WCS358 produces at least three different autoinducer molecules, as detected using the biosensor CVO26 (17). Purifying autoinducer molecules from spent supernatants of *P. putida* WCS358 and *P. putida* MT17R revealed that in the *psrA* null mutant, there was no difference in the production of these molecules. It was concluded that PsrA of *P. aeruginosa* and *P. putida* does not play a major role in the regulation of autoinducer biosynthesis.

***psrA* is autoregulated.** In order to establish whether PsrA regulated its own synthesis, the promoter of *psrA* fused to a promoterless β-galactosidase gene in construct pPPSR220 was introduced into wild-type strain WCS358, *psrA* mutant MT17,

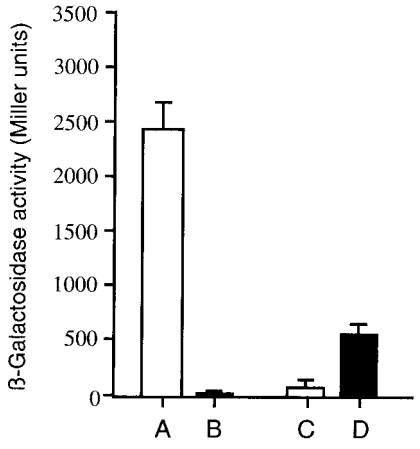

FIG. 5. β-Galactosidase activities in *E. coli* DH5α driven by the *rpoS-lacZ* (pRPO220B) and *psrA-lacZ* (pPPSRA220) fusions. Plasmid pQEPSRA contains the *psrA* gene and expresses the PsrA protein, whereas plasmid pQE30 is the vector (see text for details). All measurements were done in triplicate; the mean and standard error are shown. Cells harboring plasmids were grown for 16 h in LB medium in the presence of appropriate antibiotics. Columns: A, *E. coli*(pQE30)(pPPSR220); B, *E. coli*(pQEPSRA)(pPPSR220); C, *E. coli*(pQE30)(pRPO220B); D, *E. coli*(pQEPSRA)(pRPO220B).

and WCS358 containing *rpoS*::Tn*5* (*rpoS* mutant) (17). As depicted in Fig. 2, in mutant MT17 *psrA* gene expression was considerably increased (approximately fourfold), indicating that PsrA had a negative effect on its own synthesis. No difference was observed between the *rpoS* mutant and the wild type.

Effect of PsrA on *rpoS* and *psrA* promoters in *E. coli*. We determined whether PsrA had an effect on *psrA* and *rpoS* expression in a heterologous background (*E. coli*). The *psrA* gene was cloned into expression vector pQE30 (construct designated pQEPSRA), resulting in His_6-PsrA production in *E. coli,* as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). For *E. coli* (pQEPSRA), the *psrA-lacZ* fusion (pPPSR220) and the *rpoS-lacZ* fusion (pRPO220B) were introduced in independent experiments, and β-galactosidase activity was determined. As shown in Fig. 5, the *psrA* promoter in *E. coli* was active, giving approximately 2,445 Miller units; this activity was repressed in the presence of pQEPSRA. On the other hand, the *rpoS* promoter in *E. coli* produced only 60 Miller units; this activity was increased by approximately 10-fold to 550 Miller units in the presence of pQEPSRA. As expected from the results obtained with *Pseudomonas*, PsrA appeared to act as a repressor of *psrA* expression and as an activator of *rpoS* expression.

DISCUSSION

The regulation of *rpoS* expression in *P. putida* WCS358 was investigated. Using an *rpoS* transcriptional fusion, we identified a Tn*5* genomic mutant, MT17, which showed a 90% decrease in *rpoS* promoter activity. This mutant had a Tn*5* insertion in an ORF of 714 bp and coding for a protein (PsrA) of 26.3 kDa. The PsrA protein contains, near its N terminus, a helix-turn-helix motif and belongs to the TetR family of bacterial regulators (Fig. 3B). Many of the regulators belonging to the TetR regulatory family act as repressors of transcription, their targets very often being genes which encode proteins involved in membrane permeability (23, 24). PsrA likely acts as a positive regulator of *rpoS* gene expression, since a null mutant resulted in a considerable decrease in *rpoS* gene expression and in less RpoS protein being present in the cell (Fig. 4). It is not known whether this regulatory action is direct or indirect, through another regulatory component(s). PsrA acts as a repressor of its own synthesis, since *psrA* was expressed at a much higher level in *P. putida* MT17 than in wild-type WCS358, indicating that PsrA regulates its own synthesis in a negative way. The observation that PsrA represses *psrA* and activates *rpoS* in *E. coli* is an indication that it might do so directly. The repression was strong and clear, whereas the activation of *rpoS* was rather weak compared to promoter activities in *Pseudomonas*. Thus, it is likely that some other factors are missing in *E. coli*.

To our knowledge, this study represents the first genetic screen of *Pseudomonas* spp. using an *rpoS* transcriptional fusion and selecting for down-regulated mutants. Our selection led to the identification of only one mutant having Tn*5* in the *psrA* gene. The screen made use of the *xylE* reporter gene, which is less sensitive than other reporter systems (e.g., *lacZ*). However, mutants not showing a strong decrease in promoter activity cannot be detected in plate assays. The fact that the *P. putida rpoS* promoter exhibited strong promoter activity made the selection even more difficult. It is therefore possible that other mutants which showed lower levels of *rpoS* expression were not detected. The fact that a mutant exhibiting no *rpoS* promoter activity was not isolated could indicate that the regulation of RpoS occurs at different levels, as in *E. coli*, or that more transcriptional activators are involved. Our data showed that in *Pseudomonas,* there is an important regulator controlling *rpoS* transcription. However, additional control could take place in response to various stresses or to the stringent response. At present we do not know to which effector molecule(s) or environmental stimuli PsrA responds.

Previous studies have indicated that two other regulatory systems are involved in the regulation of *rpoS* expression in *P. aeruginosa*, namely, the LasR-LasI and RhlI-RhlR quorum-sensing systems and the GacA-GacS two-component regulatory system (see above) (21, 45). Surprisingly, no mutations in either of these systems were isolated in the genetic selection. We have demonstrated that the *rpoS* regulatory gene *psrA* is also present in *P. aeruginosa* PAO1 and that, as in *P. putida,* it plays a role in *rpoS* expression, since a *psrA* null mutant of PAO1 displayed a 90% reduction in *rpoS* promoter activity. By analysis of the production of quorum-sensing autoinducer molecules, it was verified that *psrA* mutants of both *P. putida* and *P. aeruginosa* still produced autoinducer molecules. Thus, it does not appear that PsrA influences *rpoS* gene expression through the biosynthesis of these molecules. Similarly, a reduction of *rpoS* expression in the *psrA* mutants did not result in any alteration in the production of autoinducer molecules. Following the contradictory reports of Latifi et al. (21) and Whiteley et al. (45), in which RpoS was linked to autoinducer production, we did not conclude in this study that PsrA plays a major role. However, it must be stressed that small quantities of RpoS were still made in *psrA* mutants.

Experiments thus far have indicated that in *Pseudomonas,* unlike in *E. coli*, *rpoS* appears to be extensively regulated at the

transcriptional level through the use of various regulators. More work is needed to show whether the regulation of *rpoS* expression by PsrA occurs directly or indirectly or through quorum sensing and/or other regulatory components and to determine which effector molecules act on which regulators.

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