JOURNAL OF BACTERIOLOGY, Aug. 2008, p. 5624–5634 Vol. 190, No. 16
0021-9193/08/$08.00+0 doi:10.1128/JB.00594-08

Induction by Cationic Antimicrobial Peptides and Involvement in Intrinsic Polymyxin and Antimicrobial Peptide Resistance, Biofilm Formation, and Swarming Motility of PsrA in *Pseudomonas aeruginosa*▽†

W. James Gooderham, Manjeet Bains, Joseph B. McPhee, Irith Wiegand, and Robert E. W. Hancock*

Center for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada

Received 29 April 2008/Accepted 5 June 2008

Pseudomonas aeruginosa **is an important opportunistic pathogen that causes infections that can be extremely difficult to treat due to its high intrinsic antibiotic resistance and broad repertoire of virulence factors, both of which are highly regulated. It is demonstrated here that the *psrA* gene, encoding a transcriptional regulator, was upregulated in response to subinhibitory concentrations of cationic antimicrobial peptides. Compared to the wild type and the complemented mutant, a *P. aeruginosa* PAO1 *psrA*::Tn*5* mutant displayed intrinsic supersusceptibility to polymyxin B, a last-resort antimicrobial used against multidrug-resistant infections, and the bovine neutrophil antimicrobial peptide indolicidin; this supersusceptibility phenotype correlated with increased outer membrane permeabilization by these agents. The *psrA* mutant was also defective in simple biofilm formation, rapid attachment, and swarming motility, all of which could be complemented by the cloned *psrA* gene. The role of PsrA in global gene regulation was studied by comparing the *psrA* mutant to the wild type by microarray analysis, demonstrating that 178 genes were up- or downregulated ≥2-fold ($P \leq 0.05$). Dysregulated genes included those encoding certain known PsrA targets, those encoding the type III secretion apparatus and effectors, adhesion and motility genes, and a variety of metabolic, energy metabolism, and outer membrane permeability genes. This suggests that PsrA might be a key regulator of antimicrobial peptide resistance and virulence.**

The opportunistic gram-negative bacterium *Pseudomonas aeruginosa* is the most prevalent cause of life-threatening infections in the lungs of cystic fibrosis patients (37) and the third leading cause of severe hospital-acquired infections (24). *P. aeruginosa* can cause substantial morbidity and mortality, due in part to its wide repertoire of virulence factors, and it is extremely difficult to combat due to high intrinsic antibiotic resistance (14). The current treatment of *P. aeruginosa* infections often involves potent β-lactams, aminoglycosides, or fluoroquinolones, or a combination thereof, but resistance can arise nevertheless (14), and there has been a recent emergence of *P. aeruginosa* clinical isolates resistant to virtually all antibiotics. When multidrug resistance occurs, polymyxins have become a drug of last resort (21). Thus, it is important to understand the basis for resistance in this organism and its interrelationship with pathogenesis. For example, there is a well-noted discrepancy between in vitro antibiotic susceptibility and the clinical success of particular antibiotics for *P. aeruginosa* (12, 14). One possible basis for this is the induction of resistance mechanisms due to environmental factors, a process termed adaptive resistance, which is differentiated from acquired or mutational resistance because it reverts upon removal of the antibiotic.

Structurally diverse cationic antimicrobial peptides are part of the innate immune system of complex organisms and can possess direct antimicrobial activity and/or a profound ability to modulate innate immunity (16). Improved synthetic derivatives demonstrate considerable promise against infections by multiply antibiotic-resistant bacteria (13, 28). However, *P. aeruginosa* is able to sense the presence of peptides and to become adaptively resistant, for example, through peptide-mediated regulation of the *arnBCADTEF* (*pmrHFIJKLM*) LPS modification operon, independently of the PmrA-PmrB or PhoP-PhoQ two-component regulatory system (31, 32).

Virulence is similarly complex, representing a series of complex adaptations to growth in a host organism, including biofilm formation, swarming motility, and quorum sensing. For example, in *P. aeruginosa*, motility is important for biofilm formation, virulence, and colonization of different niches (17, 35). There are three basic types of motility. Type IV pili extend and retract to promote twitching motility on solid surfaces, whereas flagella power swimming motility in dilute media. On the other hand, swarming motility appears to be a coordinated and complex adaptation to moderately viscous environments and involves a number of factors that include flagella, type IV pili, quorum sensing, rhamnolipids, etc. (33, 34). There is considerable overlap in the genes utilized in swarming motility and biofilm formation (4, 34, 39), both of which have been proposed to contribute to disease pathogenesis (36) and to lead to increased resistance to several antibiotics (33, 35).

In this study, it was demonstrated that antimicrobial pep-

* Corresponding author. Mailing address: Center for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, 232-2259 Lower Mall Research Station, Vancouver, BC, Canada V6T 1Z4. Phone: (604) 822-2682. Fax: (604) 827-5566. E-mail: bob@cmdr.ubc.ca.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 13 June 2008.

TABLE 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics[a]	Source or reference[b]
Strains		
P. aeruginosa strains		
WT	WT *P. aeruginosa* PAO1; H103	Lab collection
UW WT	UW WT *P. aeruginosa* PAO1	UW (15)
UW-*psrA*	*psrA*::IS*lacZ*/hah-Tc^r; insertion at position 46 (702 bp) in *psrA*; derived from UW WT	UW (15)
psrA mutant	*psrA*::IS*lacZ*/hah-Tc^r; H103 background; Tc^r	This study
psrA (Tn*7*-*psrA*$^+$)	*psrA* mutant with Tn*7*-*psrA*$^+$ integrated; Tc^r Gm^r	This study
PA14	Wild-type *P. aeruginosa* PA14	22
coxA	05_2::A11; derived from PA14	22
etfA	04_4::A12; derived from PA14	22
fhp	09_1::F11; derived from PA14	22
mexC	01_4::H2; derived from PA14	22
pprB	08_3::C3; derived from PA14	22
rhlG	05_3::A8; derived from PA14	22
flp	14_1::F4; derived from PA14	22
rcpA	01_2::B12; derived from PA14	22
tadA	01_2::A7; derived from PA14	22
tadB	04_2::H5; derived from PA14	22
wbpM	03_4::E4; derived from PA14	22
wzz	06_1::F2l; derived from PA14	22
PA1883 (homolog)	12_1::A7; derived from PA14	22
wbpI	*wbpI*::IS*lacZ*/hah-Tc^r; insertion 807 (1,065 bp)	15
wbpL	*wbpL*::IS*lacZ*/hah-Tc^r; insertion 302 (1,020 bp)	15
E. coli strain		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pCR-Blunt II-TOPO	PCR cloning vector; Kan^r	Invitrogen
pCR-*psrA*$^+$	pCR-BluntII-TOPO harboring 1.12-kb *psrA* amplicon	This study
pUC18-mini-Tn*7*T-Gm	Suicide plasmid; Gm^r Amp^r	H. Schweizer (8)
pUC-Tn*7*-*psrA*$^+$	pUC18-mini-Tn*7*T-Gm with 1.12-kb *psrA* fragment from pCR-*psrA*	This study
pTNS2	Transposition helper plasmid; Amp^r	H. Schweizer (8)

[a] Antibiotic resistance phenotypes are indicated as follows: Amp^r, ampicillin resistance for *E. coli* and carbenicillin resistance for *P. aeruginosa*; Gm^r, gentamicin resistance; Kan^r, kanamycin resistance; Tc^r, tetracycline resistance.
[b] UW, University Washington.

tides transcriptionally upregulated the expression of *psrA*, a previously documented *Pseudomonas* regulator of RpoS and the type III secretion system, but one for which the activating signals were unknown (19, 20, 38). Detailed phenotypic studies indicated that PsrA regulated polymyxin and antimicrobial peptide resistance, motility, and biofilm formation. Microarray analysis of the *psrA* mutant provided insight into the basis for these observed phenotypes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Cultures were routinely grown in Luria-Bertani (LB) broth containing 1.8% (wt/vol) Difco agar (Becton Dickinson Co., Oakville, Ontario, Canada), when appropriate. The defined medium used was BM2-glucose minimal medium [62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose] containing 2 mM (high) $MgSO_4$. Antibiotics for selection were used at the following concentrations: tetracycline, 50 to 100 μg/ml for *P. aeruginosa*; ampicillin, 100 μg/ml for *Escherichia coli*; carbenicillin, 500 μg/ml for *P. aeruginosa*; and gentamicin, 30 μg/ml for *P. aeruginosa* and 15 μg/ml for *E. coli*.

Genetic manipulations. Routine molecular biology techniques were performed according to standard protocols (1). Primers were synthesized by Alpha-DNA Inc. (Montreal, Quebec, Canada), and their sequences are available upon request. Plasmid DNA was isolated using QIAprep spin miniprep kits (Qiagen Inc., Mississauga, Ontario, Canada), and agarose gel fragments were purified using a QIAquick gel extraction kit (Qiagen). T4 DNA ligase was from Invitrogen (Burlington, Ontario, Canada), and restriction endonucleases were from New England Biolabs (Mississauga, Ontario, Canada).

Mobilizing the UW-*psrA* transposon mutation into a new PAO1 background. The UW-*psrA* mutation (confirmed to be correct by PCR and sequencing of the junctions of the transposon mutation) was first transferred into our laboratory wild-type (WT) *P. aeruginosa* PAO1 strain H103 as described previously (7). Genomic DNA was isolated from the UW-*psrA* mutant by the hexadecyltrimethyl ammonium bromide method (1). Approximately 1 microgram of this DNA (which contained the tetracycline resistance-encoding transposon IS*lacZ*/*hah*-Tc insertion in *psrA*) was electroporated into WT H103. Cells were allowed to recover for 1 hour at 37°C and then were plated onto LB agar plates containing 100 μg/ml tetracycline. After 18 h of growth at 37°C, tetracycline-resistant transformants were then analyzed by colony PCR, using a transposon-specific primer and a custom gene primer with *Taq* polymerase (Invitrogen), to verify that the transposon was correctly inserted into *psrA*. The new *psrA* mutant allowed better analysis of motility-related phenotypes (H103 is swarm positive under our conditions [see below]) and was therefore used for all experiments reported in this study.

Genetic complementation of *psrA*. Forward and reverse primers for *psrA* were designed from the *P. aeruginosa* PAO1 genome sequence (www.pseudomonas.com), using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), to clone *psrA* together with 347 bp of upstream DNA with the native promoter and 67 bp of downstream DNA. Amplification of *psrA* from *P. aeruginosa* WT H103 genomic DNA was carried out using high-fidelity Platinum *Pfx* DNA polymerase (Invitrogen) with the primers PsrA-L (5′-CGGAGCACAGAGAAAGGAGA-3′) and PsrA-R (5′-GACTTGAAGCCGAGTTCCTG-3′). The resulting PCR product was then cleaned (Qiagen PCR purification kit), and the amplicons were cloned into pCR-Blunt II-TOPO, using a Zero Blunt TOPO PCR cloning kit (Invitrogen), and transformed into One Shot TOP10 cells

(Invitrogen), creating pCR-*psrA*$^+$. An NsiI fragment containing the *psrA* gene was excised from pCR-*psrA*$^+$ and subcloned into pUC18mini-Tn7T-Gm, generating pUC18-mini-Tn7T-Gm-*psrA*$^+$. pUC18mini-Tn7T-Gm-*psrA*$^+$ was coelectroporated with pTNS2 into the *psrA* mutant, using sucrose electroporation (7). As previously described, gentamicin-resistant transformants were analyzed by colony PCR, using primers PglmS-up and PTn7L, to determine the correct transposon integration of mini-Tn*7* into the chromosome (8).

Killing curves. Overnight *P. aeruginosa* cultures were diluted 1/100 in fresh BM2-glucose minimal medium containing 2 mM Mg^{2+}. Upon reaching the mid-logarithmic phase of growth (optical density at 600 nm [OD_{600}], ~0.5), 1 ml of each culture was pelleted, resuspended in 1 ml 1× BM2 salts (buffer), and diluted 1/10 in prewarmed 1× BM2 salts. Killing was then initiated by the addition of 1 μg/ml polymyxin B sulfate (Sigma, St. Louis, MO) or 64 μg/ml indolicidin for analysis of intrinsic resistance. Flasks were shaken at 37°C, and aliquots were withdrawn at the designated times to assay for survivors by plating diluted 100-μl aliquots onto LB agar plates.

Outer membrane permeabilization assays. *P. aeruginosa* outer membrane barrier function and the efficiency of the self-promoted uptake route were determined using the 1-*N*-phenyl naphthylamine (NPN) assay (23). Cultures were grown to mid-logarithmic phase in BM2-glucose minimal medium containing 2 mM $MgSO_4$. The cells were then harvested, washed, and resuspended to an OD_{600} of 0.5 in 5 mM HEPES, pH 7.0, 5 mM glucose, and 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Two milliliters of each bacterial suspension was placed in a quartz cuvette with a magnetic stir bar. NPN (Sigma) was then added to the cuvette at a concentration of 10 μM, and the fluorescence (baseline) was measured using an LS-50B fluorescence spectrophotometer (Perkin-Elmer, Inc., Waltham, MA) (excitation and emission wavelengths of 350 nm and 420 nm, respectively). Indolicidin peptide was then added to initiate the assay, and the increase in fluorescence due to peptide-mediated entry of the hydrophobic fluorophor NPN into the membrane was measured until a stable signal was observed (indicating that additional partitioning of NPN into the membrane had stopped).

Biofilm and attachment assays. Static microtiter biofilm assays were performed as described previously (34). Overnight LB cultures were diluted 1/1,000 in fresh LB broth, and 100 μl was inoculated into wells of a 96-well polystyrene round-bottomed microtiter plate (Becton Dickinson). For PA14 strains, overnight cultures were diluted 1:500 in BM2-glucose medium containing 2 mM $MgSO_4$ and 0.5% Casamino Acids. Plates were then incubated at 37°C without shaking. At the specified time point, medium and planktonic cells were discarded, and the wells were washed three times with distilled H_2O. Surface-attached bacteria were then stained with 0.1% (wt/vol) crystal violet for 20 min, followed by ethanol solubilization of crystal violet-stained cells for quantification of A_{600}.

Rapid attachment was assayed as described previously, with slight modifications (25). Overnight cultures were first diluted 1/100 in fresh LB medium and grown to an OD_{600} of ~0.5, and 100 μl was added to each well of a 96-well polystyrene microtiter plate. Cells were allowed to attach for 30 min at room temperature prior to being stained with crystal violet as described above.

Motility assays. Swimming motility was assayed on BM2-glucose plates containing 0.3% (wt/vol) agar. Swarming was assayed on modified BM2-glucose plates containing 0.5% (wt/vol) agar and with 0.5% (wt/vol) Casamino Acids (or 0.1% for PA14 strains) substituted for 7 mM $(NH_4)_2SO_4$ (34). Swimming and swarming motilities were assayed by inoculating 1 μl of mid-logarithmic-phase liquid culture grown in BM2-glucose containing 2 mM Mg^{2+} onto the motility plate, incubating the plate for 16 to 18 h at 37°C, and measuring motility zone diameters. Twitching motility was assessed by toothpick inoculating cells from agar plates into thin LB-1% agar plates, down to the agar-plastic interface, and measuring the twitch zone diameter after 24 and 48 h of incubation at 37°C.

Growth curves. Overnight cultures were grown in BM2-glucose containing 2 mM Mg^{2+}, and 0.1 ml was diluted into 10 ml fresh medium. Flasks were shaken at 37°C, and aliquots were withdrawn periodically to determine the cell density as the OD_{600}. Similarly, determination of planktonic growth at 37°C in static 96-well polystyrene microtiter plates (simple biofilm conditions) was assayed by monitoring the OD_{600}.

Microarray analysis. Detailed technical descriptions of microarray analyses were provided previously (30). In overview, for each strain, microarray analysis involved five independent cultures. *P. aeruginosa* WT and *psrA* mutant cultures were grown with shaking in BM2-glucose medium plus 2 mM $MgSO_4$ at 37°C for 18 h and then diluted 1 in 100 in fresh medium. Cultures were grown at 37°C with shaking (250 rpm) to the mid-logarithmic phase of growth (OD_{600} = 0.5), and then total RNA was isolated using RNeasy Midi columns (Qiagen). Contaminating genomic DNA was removed by treatment with a DNA-free kit (Ambion Inc., Austin, TX). RNA was stored at −80°C with 0.2 U/μl of SUPERase-In RNase inhibitor (Ambion Inc.). RNA quality was assessed by agarose gel electrophoresis and spectrophotometrically. RNA was converted to cDNA, hybridized, and analyzed as previously described. *P. aeruginosa* PAO1 microarray slides were provided by The Institute for Genomic Research (TIGR) Pathogenic Functional Genomics Resource Center (http://pfgrc.tigr.org/). Images of slides were quantified using ImaGene 6.0, standard edition, software (BioDiscovery, Inc., El Segundo, CA). ArrayPipe, version 1.7, was used for assessment of slide quality, normalization, detection of differential gene expression, and statistical analysis, using available genome annotation from www.pseudomonas.com. Data analysis of DNA microarrays was carried out as previously described (30). The five biological replicates were averaged to obtain overall changes for the *psrA* mutant relative to the WT, and two-sided one-sample Student's *t* test was applied to determine significant changes in gene expression. Changes of ≥2-fold with a Student's *t* test *P* value of ≤0.05 were used as the cutoffs for reporting expression changes.

Real-time qPCR. Total RNA was isolated, using RNeasy Midi columns (Qiagen), from *P. aeruginosa* grown in BM2-glucose minimal medium containing 2 mM Mg^{2+}, with or without 2 μg/ml indolicidin, to the mid-logarithmic phase of growth. DNase treatment of RNA samples, cDNA synthesis, and real-time quantitative PCR (qPCR) were carried out as described previously (30). cDNA was diluted 1/1,000, and 1 μl was used as a template for real-time PCR, using 1× SYBR green PCR master mix (Applied Biosystems, Foster, CA) and an ABI Prism 7000 instrument (Applied Biosystems). Forward and reverse primers were designed internal to *psrA*, using PrimerExpress (Applied Biosystems). All reactions were normalized to the *rpsL* gene, encoding the 30S ribosomal protein S12.

Microarray accession number. The MIAMExpress accession number is E-FPMI-14.

RESULTS

Activation of *psrA* transcription in response to antimicrobial peptides. Preliminary studies indicated that the *psrA* (PA3006) gene was induced 2.5-fold ($P < 0.05$) by a subinhibitory concentration (2 μg/ml; one-eighth the MIC) of the bovine antimicrobial peptide indolicidin (11, 31), based on microarray analyses of cultures grown to mid-logarithmic phase under high-Mg^{2+} (2 mM $MgSO_4$) conditions. Independent real-time qPCR experiments confirmed that the transcription of *psrA* was upregulated 3.0- ± 0.3-fold in the presence of indolicidin, with similar induction by the indolicidin variant peptide CP11CN (data not shown). As a positive control, in agreement with previous studies (31), *arnB* (*pmrF*; PA3552), the first gene of the aminoarabinose lipopolysaccharide (LPS) modification operon (PA3552 to -9), was confirmed here by qPCR to be upregulated 54.3- ± 8.1-fold under these conditions, and microarray data confirmed that the indolicidin-regulated *pmrA-pmrB* operon was also upregulated in the presence of a subinhibitory concentration of indolicidin, but the Mg^{2+}-regulated *oprH-phoP-phoQ* operon was not (data not shown).

Contribution of *psrA* to intrinsic antimicrobial peptide and polymyxin B resistance. The influence of the *psrA* gene on intrinsic resistance to peptides was examined. Intrinsic resistance was assayed by growing cells under high (2 mM)-Mg^{2+} conditions to suppress the possibility of induction by limiting Mg^{2+}. The *psrA* mutant exhibited an increased susceptibility to the polycationic lipopeptide polymyxin B, as shown by kill curves (Fig. 1A). This supersusceptibility phenotype could be complemented to WT polymyxin B susceptibility by introducing a single WT *psrA*$^+$ allele into the chromosome of the mutant, using mini-Tn*7* integration technology (Fig. 1A). Similarly, the *psrA* mutant demonstrated supersusceptibility to the cationic antimicrobial peptide indolicidin, which could be complemented back to WT susceptibility (Fig. 1B). Thus, the *psrA* gene product appeared to be essential for normal intrinsic resistance.

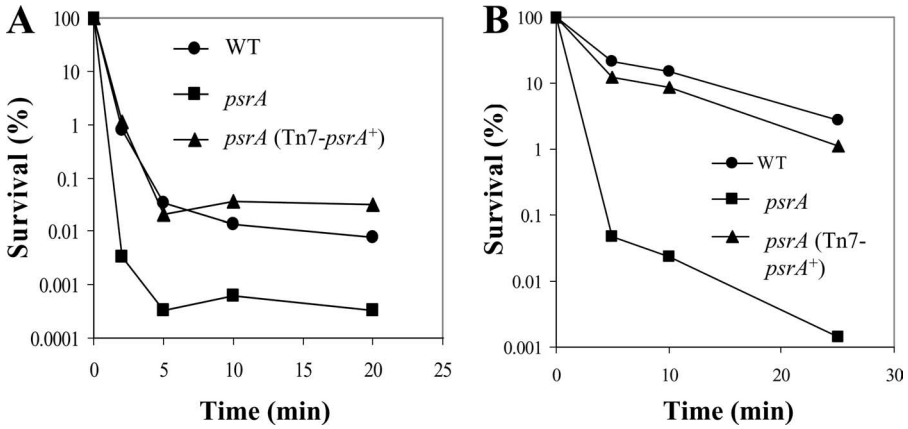

FIG. 1. Intrinsic polymyxin B and antimicrobial peptide supersusceptibility in *psrA* mutants. Intrinsic sensitivity was analyzed by growing cells to mid-log phase in BM2-glucose with 2 mM Mg^{2+}, exposing them to 1 μg/ml polymyxin B (A) or 64 μg/ml indolicidin (B), and plating diluted aliquots to check for survivors. For each condition, one representative experiment is shown of four independent experiments that produced identical trends.

***psrA* mutation affects permeabilization of the outer membrane.** Polycationic molecules such as polymyxin B and antimicrobial peptides pass across the outer membrane by self-promoted uptake. The first stage of self-promoted uptake involves the interaction of the polycation with divalent cation binding sites on surface polyanionic LPS, causing a disruption of the permeability barrier and subsequent uptake of the permeabilizing polycationic antibiotic (16, 23). To address the possibility that altered outer membrane permeability was the basis for peptide supersusceptibility in the *psrA* mutant, NPN was used as a probe for outer membrane permeabilization by indolicidin (Fig. 2). The hydrophobic fluorophor NPN is normally excluded from entering cells due to its inability to penetrate the outer membrane. Upon permeabilization of the outer membrane (as occurs during self-promoted uptake), NPN is taken up and becomes strongly fluorescent in the hydrophobic environment of cell membranes (23). There was no obvious difference in the abilities of the *psrA* mutant and the WT to exclude NPN. However, indolicidin, at concentrations of 3.0 and 1.5 μg/ml, was able to permeabilize the outer membranes of the *psrA* mutant to a greater extent than those of WT cells (Fig. 2A). Thus, the supersusceptibility of the *psrA* mutant to indolicidin correlated with an outer membrane that was more easily permeabilized by this antimicrobial peptide. Similarly, polymyxin B also preferentially permeabilized the *psrA* mutant (Fig. 2B).

Contribution of *psrA* to biofilm formation and attachment. Other genes, such as the PhoQ gene, that regulate antimicrobial peptide resistance also regulate biofilm formation and motility. To assess the ability of the *psrA* mutant to form simple biofilms, static microtiter biofilm assays were employed to demonstrate that the *psrA* mutant displayed significant (>4-fold; $P < 0.05$ by Student's *t* test) impairment in biofilm formation at 18 h (Fig. 3A). Biofilm impairment could be complemented successfully by introducing the WT *psrA* allele into the mutant (Fig. 3A). No observable growth differences were observed when the OD_{600} of planktonic cells was measured as a function of time during the period of growth in the microtiter

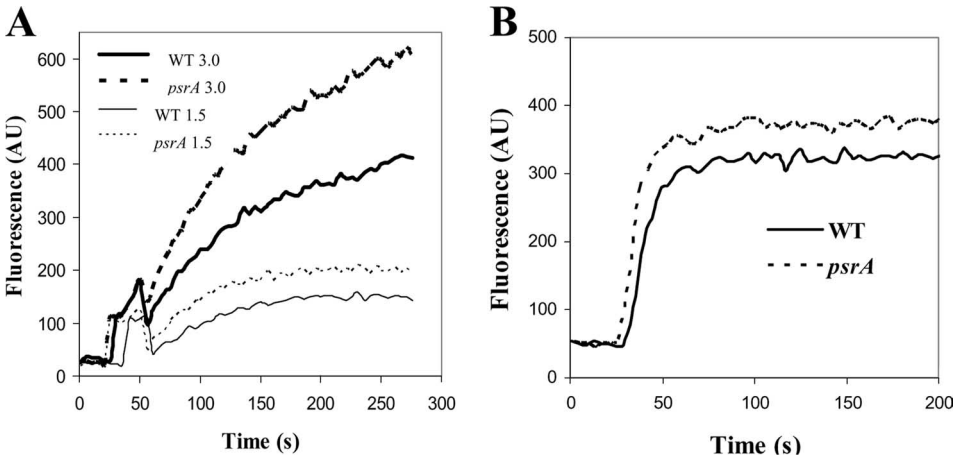

FIG. 2. PsrA mutation effect on outer membrane permeabilization by peptides. Cells from mid-logarithmic-phase cultures of WT and *psrA* mutant strains were exposed to 1.5 or 3 μg/ml of indolicidin (A) or 0.2 μg/ml polymyxin B (B) and the increase in fluorescence due to peptide-stimulated partitioning of NPN into the outer membrane was measured. One representative experiment is shown of three independent experiments, each of which showed reproducible trends. AU, arbitrary units.

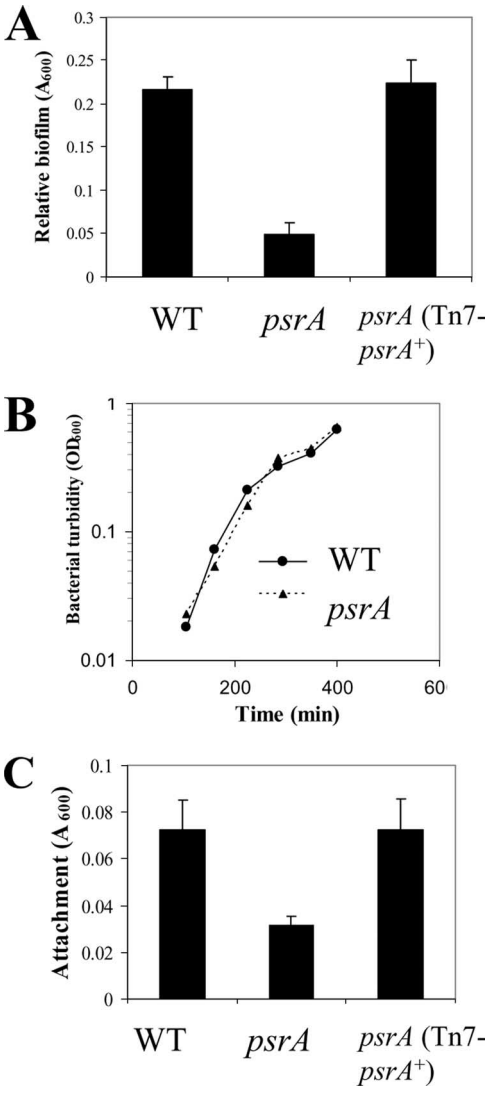

FIG. 3. Defects in biofilm formation and attachment in *psrA* mutants. (A) Requirement for *psrA* in static biofilm formation. Cells were grown at 37°C for 18 h in polystyrene microtiter plates containing LB. Adherent biofilm cells were stained with crystal violet, followed by ethanol solubilization of the crystal violet and quantification (A_{600}) of stained wells. (B) Planktonic growth of the *psrA* mutant under these biofilm conditions was unaffected. Planktonic cells were grown as in biofilm microtiter assays, and turbidity was measured (OD_{600}). (C) Requirement for *psrA* for rapid attachment. Rapid attachment was assayed using mid-log-phase cells for 30 min. Adherent cells were stained with crystal violet, followed by ethanol extraction of the crystal violet for quantification as the A_{600}. Results shown are means with standard deviations for three biological experiments, each with eight technical repeats.

wells (Fig. 3B). Similarly, assessment of growth in defined medium in shaking flasks revealed no differences between the mutant and WT strains (data not shown), indicating no primary growth defect.

To determine whether this biofilm formation phenotype occurred during the initial attachment stage or later during biofilm development, a rapid (30 min) attachment assay was performed. The *psrA* mutant displayed impaired attachment (>2-fold; $P < 0.05$ by Student's *t* test), and this defect could be complemented with the *psrA*$^+$ gene (Fig. 3C).

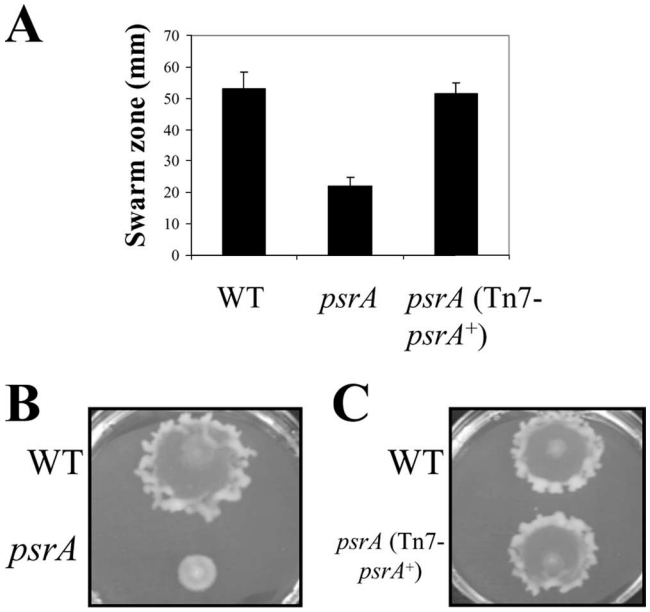

FIG. 4. Swarming motility defect in *psrA* mutants. (A) Swarming motility was evaluated by spot inoculating cells onto BM2 swarm plates containing 0.5% agar, followed by incubation at 37°C for 18 h. Diameters of the characteristic circular PAO1 swarm zones were measured, and means with standard deviations are reported for three biological repeats, each with three technical repeats. (B) Representative WT (top) and *psrA* mutant (bottom) swarming morphologies. (C) Complemented *psrA* mutant swarming morphology. WT (top) and *psrA* (Tn7-*psrA*$^+$) (bottom) morphologies are shown.

Requirement for PsrA for normal swarming. Mutant studies have revealed an intricate relationship between motility and biofilm formation in *P. aeruginosa* (17). Therefore, the *psrA* mutant was assessed for the ability to undergo swimming, twitching, and swarming motility. Neither flagellum-mediated swimming motility nor type IV pilus-mediated twitching motility was significantly affected in the *psrA* mutant. However, the *psrA* mutant demonstrated a severe impairment in swarming motility, with a significant (>2.5-fold; $P < 0.05$ by Student's *t* test) decrease in swarming zone size, and this could be complemented by introducing the WT *psrA* allele (Fig. 4).

Microarray analysis. The above-described complex phenotype indicated that PsrA might control the expression of a substantial regulon. To assess this and to identify candidate genes that might explain the observed *psrA* mutant phenotypes, microarray analysis was performed, comparing the *psrA* mutant to the WT after growth to mid-logarithmic phase in BM2-glucose minimal medium containing 2 mM Mg^{2+}. There were a total of 178 genes that were significantly ($P \leq 0.05$) dysregulated ≥2-fold, among which 70 were upregulated and 108 were downregulated in the mutant relative to the WT (see the table in the supplemental material). A selection of these genes is shown in Tables 2 to 4. Independent quantitative reverse transcription-PCR (qRT-PCR) analysis confirmed the regulation of six of these genes (indicated by asterisks in Tables 2 to 4) and thus provided validation for our *psrA* microarray data. Most previously identified genes with predicted PsrA binding sites in their promoters (18) were observed to be dysregulated in this microarray analysis (Table 2).

TABLE 2. Known PsrA targets (18) significantly dysregulated in *psrA* mutants, determined using a microarray[a]

Gene identifier[b]	Gene name	Change (fold)[c]	Description[a]
PA0506		16.2*	Probable acyl-CoA dehydrogenase
PA2673	*hplV*	−27.9	Probable type II secretion system protein
PA2951	*etfA*	3.3	Electron transfer flavoprotein alpha subunit
PA2952	*etfB*	3.8	Electron transfer flavoprotein beta subunit
PA2953		6.4*	Electron transfer flavoprotein-ubiquinone oxidoreductase
PA3013	*foaB*	15.2*	Fatty acid oxidation complex beta subunit
PA3014	*faoA*	11.6	Fatty acid oxidation complex alpha subunit
PA3622	*rpoS*	−2.3*	Alternative sigma factor RpoS

[a] Only genes showing a ≥2-fold change in the *psrA* mutant are depicted. The *P* value was <0.0001 in all cases.
[b] According to the *P. aeruginosa* genome website (http://www.pseudomonas.com/).
[c] Regulation of genes differentially expressed in the *psrA* mutant relative to the WT. A positive number indicates transcript upregulation in the *psrA* mutant.
*, confirmation of gene regulation by qRT-PCR.

In addition, we observed dysregulation of the entire type III secretion apparatus and its effectors (Table 3), certain adhesion and motility genes, 17 regulators (*rpoS*, *pcrH*, *mdcR*, *toxR*, *arsA*, PA0513, PA1399, PA1976, PA1978, PA2432, PA2469, PA2551, PA3077, PA3409, PA3630, PA4135, and PA4296), and a variety of metabolic and energy metabolism genes (Tables 3 and 4).

Additional mutant phenotypic analyses. The list of genes dysregulated in the *psrA* mutant provided a useful starting

TABLE 3. Type III secretion system, adhesion (*tad*), motility, and type II secretion genes significantly dysregulated in *psrA* mutants, determined using a microarray

Gene identifier[a]	Gene name	Change (fold)[b]	*P* value	Description
Type III secretion genes				
PA0044	*exoT*	4.6	0.0005	Exoenzyme T; type III secretion system effector
PA1695	*pscP*	3.0	<0.0001	Translocation protein in type III secretion
PA1697	*pscN*	2.1	0.004	ATP synthase in type III secretion system
PA1698	*popN*	2.6	0.004	Outer membrane protein PopN
PA1699		2.3	0.003	Conserved hypothetical protein in type III secretion
PA1700		2.0	0.0009	Conserved hypothetical protein in type III secretion
PA1701		4.5	0.0003	Conserved hypothetical protein in type III secretion
PA1702		2.0	0.0002	Conserved hypothetical protein in type III secretion
PA1703	*pcrD*	2.0	0.01	Type III secretory apparatus protein PcrD
PA1705	*pcrG*	3.8	0.002	Regulator in type III secretion
PA1706		2.1	0.001	Type III secretion protein PcrV
PA1707	*pcrH*	2.4	0.0002	Regulatory protein PcrH
PA1708	*popB*	3.1	0.005	Translocator protein PopB
PA1709	*popD*	2.7	0.002	Translocator protein PopD
PA1710	*exsC*	2.1	<0.0001	Exoenzyme S synthesis protein C
PA1712	*exsB*	2.0	0.001	Exoenzyme S synthesis protein B
PA1715	*pscB*	2.5	0.001	Type III export apparatus protein
PA1717	*pscD*	2.8	0.01	Type III export protein PscD
PA1721	*pscH*	2.0	0.01	Type III export protein PscH
PA1723	*pscJ*	2.1	0.01	Type III export protein PscJ
PA2191	*exoY*	2.1	0.0004	Adenylate cyclase ExoY; type III secretion effector
PA3841	*exoS*	2.6	0.001	Exoenzyme S; type III secretion effector
PA3842	*orf1*	3.5	0.001	Chaperone for ExoS secretion
Adhesion and motility genes				
PA0176	*aer2*	−4.2	0.04	Aerotaxis methyl-accepting chemotaxis protein
PA1803	*lon*	−2.0	0.05	ATP-dependent Lon protease
PA4296	*pprB*	−4.9*	0.05	PprB two-component response regulator
PA4300	*tadC*	−2.2	0.04	Flp pilus assembly protein, PilC-like
PA4302	*tadA*	−4.1*	0.01	TadA traffic ATPase in Flp pilus assembly
PA4303	*tadZ*	−2.7	0.02	Flp pilus assembly protein
PA4305	*rcpC*	−2.1	0.05	Flp pilus assembly protein
Type II secretion genes				
PA0683	*hxcY*	−4.4	0.008	Hxc type II secretion system membrane protein
PA1871	*lasA*	10.5	0.002	LasA protease
PA2672	*hplW*	−2.5	0.003	Type II secretion system prepilin peptidase substrate

[a] According to the *P. aeruginosa* genome website (http://www.pseudomonas.com/).
[b] Regulation of genes differentially expressed in the *psrA* mutant relative to the WT. A positive number indicates transcript upregulation in the *psrA* mutant.
*, confirmation of gene regulation by qRT-PCR.

TABLE 4. Other known genes significantly dysregulated in *psrA* mutants, determined using a microarray[a]

Gene identifier[b]	Gene name	Change (fold)[c]	*P* value	Description
PA0106	*coxA*	−3.4	0.01	Cytochrome *c* oxidase, subunit I
PA0217	*mdcR*	−4.8	0.007	Transcriptional regulator
PA0459	*clpC*	−3.5	0.05	ClpA/B protease ATP binding subunit
PA0507		−3.2	0.001	Probable acyl-CoA dehydrogenase
PA0511	*nirJ*	4.8	0.007	Heme d_1 biosynthesis protein
PA0512	*nirH*	2.3	0.04	Conserved hypothetical protein
PA0513	*nirG*	4.5	0.008	Probable transcriptional regulator
PA0517	*nirC*	−2.6	0.001	*c*-type cytochrome
PA0530		−4.3	0.04	Pyridoxal phosphate-dependent aminotransferase
PA0588	*yeaG*	−3.3	0.001	Conserved hypothetical protein
PA0707	*toxR*	−4.2	0.009	ToxR/RegA transcriptional regulator
PA0719		−4.1	0.009	Bacteriophage Pf1 protein
PA0724	*coaA*	2.8	0.02	Coat protein A of bacteriophage Pf1
PA0840		−8.9	0.006	Probable oxidoreductase
PA0852	*cbpD*	2.2	0.04	Chitin-binding protein CbpD
PA1041		−12.9	0.005	OmpA-family outer membrane protein
PA1173	*napB*	−2.6	0.02	Cytochrome *c*-type protein NapB
PA1187	*lcaD*	−2.1	0.04	Acyl-CoA dehydrogenase
PA1399		−2.0	0.004	Probable LysR-family transcriptional regulator
PA1648		−3.0	0.002	Probable oxidoreductase
PA1649		−6.3	0.0003	Probable short-chain dehydrogenase
PA1650		−2.0	0.008	Probable transporter
PA1828		−3.1	0.006	Probable short-chain dehydrogenase
PA1881		−2.2	0.03	Probable oxidoreductase
PA1883		−10.2	0.002	NADH-ubiquinone/plastoquinone oxidoreductase
PA1927	*metE*	2.1	0.007	Methionine synthase
PA1976		−2.2	0.0009	Two-component sensor kinase
PA1978	*agmR*	−2.9	0.003	Two-component response regulator
PA1982	*exaA*	−2.7	0.03	Quinoprotein ethanol dehydrogenase
PA1983	*exaB*	−2.7	0.02	Cytochrome c_{550}
PA1984	*exaC*	−3.6	0.05	Aldehyde dehydrogenase
PA1985	*pqqA*	−4.5	0.002	Pyrroloquinoline quinone biosynthesis protein A
PA2124		3.2	0.01	Probable dehydrogenase
PA2277	*arsR*		0.05	Transcriptional regulator
PA2278	*arsB*		0.02	Ion transport membrane protein
PA2339	*mtlF*	−2.1	0.05	Maltose/mannitol transport protein
PA2350		3.1	0.02	Probable ATP-binding component of ABC transporter
PA2352		2.4	0.03	Probable glycerophosphoryl diester phosphodiesterase
PA2371	*clpV3*	−3.0	0.02	Probable ClpA/B-type protease
PA2396	*pvdF*	2.1	0.05	Pyoverdine synthetase F
PA2398	*fpvA*	2.5	0.03	Ferripyoverdine outer membrane receptor
PA2432		5.8	0.03	Probable transcriptional regulator
PA2469		−4.1	0.01	Probable transcriptional regulator
PA2522	*czcC*	−3.7	0.01	Outer membrane efflux protein
PA2535		−2.3	0.05	Probable oxidoreductase
PA2536	*ynbB*	2.2	0.03	Phosphatidate cytidylyltransferase
PA2550		−3.7	0.001	Probable acyl-CoA dehydrogenase
PA2551		−2.3	0.001	Probable transcriptional regulator
PA2573		−2.9	0.02	Probable chemotaxis transducer
PA2664	*fhp*	−83.1	0.0004	Flavohemoprotein
PA2892		−2.2	0.04	Probable short-chain dehydrogenase
PA2893		−2.4	0.03	Probable very-long-chain acyl-CoA synthetase
PA2939	*pepB*	−4.9	0.007	Secreted aminopeptidase
PA3077		2.5	0.03	Two-component response regulator
PA3145	*wbpL*	−2.5	0.03	WbpL rhamnosyltransferase in LPS biosynthesis
PA3148	*wbpI*	−4.4	0.04	UDP-*N*-acetylglucosamine 2-epimerase WbpI
PA3150	*wbpG*	−2.0	0.05	LPS biosynthesis protein WbpG
PA3152	*hisH2*	−2.3	0.03	Glutamine amidotransferase
PA3277		−4.2	0.0003	Probable short-chain dehydrogenase
PA3327		−2.7	0.02	Probable nonribosomal peptide synthetase
PA3387	*rhlG*	−2.7	0.0002	Beta-ketoacyl reductase
PA3409		−2.1	0.05	Probable transmembrane sensor
PA3418	*ldh*	−3.0	<0.0001	Leucine dehydrogenase
PA3427		−2.6	0.002	Probable short-chain dehydrogenases
PA3454		−2.1	0.002	Probable acyl-CoA thiolase
PA3630		2.2	0.05	Probable transcriptional regulator

Continued on following page

TABLE 4—*Continued*

Gene identifier[b]	Gene name	Change (fold)[c]	*P* value	Description
PA3723	*yqiM*	−3.0	0.005	FMN oxidoreductase
PA3877	*narK1*	−3.8	0.0008	Nitrite extrusion protein 1
PA3957		3.7	0.01	Probable short-chain dehydrogenase
PA4135		−2.9	0.02	Probable transcriptional regulator
PA4497		−4.1	0.01	Binding protein component of ABC transporter
PA4599	*mexC*	−2.0	0.008	RND multidrug efflux membrane fusion protein
PA4654		−5.6	0.02	Major facilitator superfamily transporter
PA4911		−6.6	0.004	Probable permease of ABC amino acid transporter
PA5020		−3.7	0.003	Probable acyl-CoA dehydrogenase
PA5097	*hutT*	2.7	0.02	Amino acid permease
PA5141	*hisA*	2.8	0.02	Histidine biosynthesis protein
PA5187		−3.2	0.0004	Probable acyl-CoA dehydrogenase
PA5188		−2.0	0.0004	Probable 3-hydroxyacyl-CoA dehydrogenase
PA5234		−2.2	0.005	Probable oxidoreductase
PA5302	*dadX*	−3.3	0.02	Catabolic alanine racemase

[a] Dysregulated hypothetical or unclassified open reading frames are not included. Only genes showing a ≥2-fold change in the *psrA* mutant are depicted.
[b] According to the *P. aeruginosa* genome website (http://www.pseudomonas.com/).
[c] Regulation of genes differentially expressed in the *psrA* mutant relative to the WT. A positive number indicates transcript upregulation in the *psrA* mutant.

point toward understanding the basis for the observed *psrA* mutant phenotypes. To understand the phenotypes associated with selected dysregulated genes, transposon mutants from the strain PA14 comprehensive nonredundant library (22) were utilized (Table 1).

The microarray was examined to find genes that might influence peptide susceptibility (since the microarray and time-kill experiments used similar growth conditions). The dysregulation of several genes of the *wbp* gene cluster (Table 4), which is involved in the biosynthesis of B band (serotype O antigen) LPS (3), suggested a possible role for B band LPS in the observed supersusceptibility of the *psrA* mutant. In addition, a small panel of PA14 mutants related to energy metabolism was tested, since our preliminary unpublished observations indi-

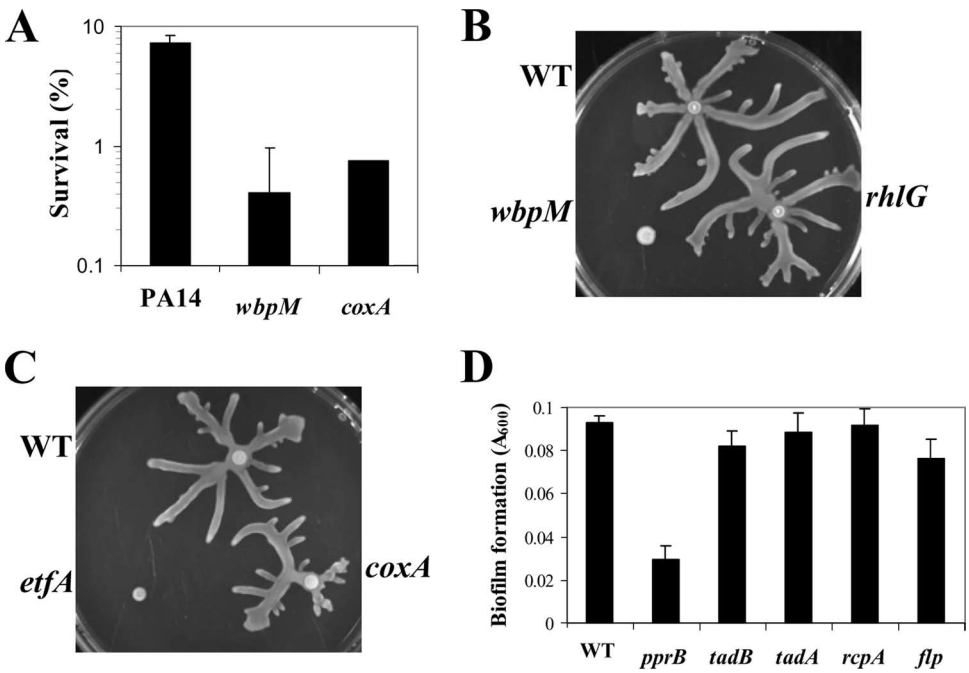

FIG. 5. Peptide susceptibility, swarming, and biofilm analyses of PA14 mutants in selected genes transcriptionally downregulated in *psrA* mutants. (A) Intrinsic indolicidin supersusceptibility time-kill curve analysis of PA14 *coxA* and *wbpM* mutants compared to the WT. Cells were grown to mid-logarithmic phase and exposed to 64 μg/ml indolicidin, and survival was assessed after 25 min. The means plus standard deviations for three independent experiments are shown. (B and C) PA14 mutants in *wbpM* and *etfA* cannot undergo normal swarming motility. Mid-logarithmic-phase cultures were spot inoculated onto PA14-type swarm agar plates and incubated for 18 h at 37°C. Swarming assays were performed with three independent cultures of each strain, and a representative swarm morphology was photographed. (D) Biofilm impairment of the *pprB* mutant. Overnight cultures were diluted 1:500 and then grown for 18 h at 37°C, followed by washing with deionized H_2O and staining with crystal violet. Biofilm formation was repeated three times, each time with six technical replicates, and the data shown are the means with standard deviations for one representative experiment.

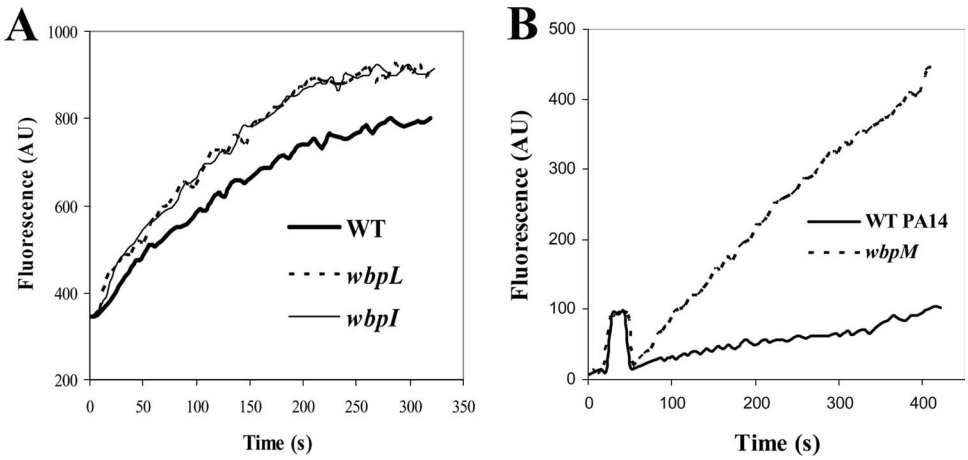

FIG. 6. Mutants in the B band O antigen biosynthetic operon demonstrating altered outer membrane permeabilization by indolicidin. (A) Cells from mid-logarithmic-phase cultures of WT PAO1 and *wbpI* and *wbpL* mutants were exposed to 3.0 μg/ml indolicidin, and the increase in fluorescence due to peptide-promoted partitioning of NPN into the outer membrane was measured. (B) Cultures of WT PA14 and the *wbpM* mutant were exposed to 1.5 μg/ml indolicidin. Data shown are for one representative experiment of at least three independent trials, each of which produced the same trends. AU, arbitrary units.

cated a role for energy metabolism in resistance to antimicrobial peptides.

As shown in Fig. 5A, both *wbpM* (previously shown to lack B band LPS) (3) and *coxA* (cytochrome *c* oxidase subunit 1) mutants showed modest supersusceptibility to indolicidin relative to the WT at 25 min. No differences were seen for mutants in *fhp*, PA1883, *mexC*, and *wzz* (data not shown). Unfortunately, an *etfA* energy metabolism mutant clumped during growth, which made establishing killing curves for this mutant difficult.

No major differences in O antigen chain length expression were seen when LPS was isolated from the WT and the *psrA* mutant and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (data not shown). However, since small changes in LPS that influence functionality, such as substitution by sugars, phosphates, and fatty acids, could be affected by the *psrA* mutation, mutants in *wbpI*, *wbpL* (downregulated in the *psrA* mutant), and *wbpM* were analyzed for possible outer membrane permeability phenotypes. Like the *psrA* mutant, all three of these mutants in LPS B band biosynthetic cluster genes showed increased outer membrane permeabilization by the peptide relative to that of the WT (Fig. 6A and B), although the effect observed with the *wbpM* mutant was more prominent (Fig. 6B).

A panel of PA14 mutants was also analyzed for possible roles in swarming motility and biofilm formation. The *wbpM* mutant showed a major swarming impairment phenotype, as did an *etfA* mutant, although in the latter mutant this might be related to its tendency to clump (Fig. 5B and C). No swarming differences were seen for the *rhlG* (Fig. 5B) *coxA* (Fig. 5C), *mexC*, *wzz*, and PA1883 mutants (data not shown).

The downregulation of certain genes of the type IVb pilus-encoding *tad* cluster led us to analyze mutants in these genes for possible biofilm formation phenotypes. Under simple biofilm growth conditions, none of the *tad* mutants analyzed displayed biofilm impairment (Fig. 5D), confirming previously reported results (9). However, a *pprB* mutant, encoding a two-component response regulator, located adjacent to the *tad* cluster, and substantially downregulated on the arrays (Table 3), demonstrated a significant ($P < 0.001$) biofilm impairment phenotype (threefold) (Fig. 5D).

DISCUSSION

The *psrA* gene of *P. aeruginosa* is an important regulator of both resistance to cationic antimicrobials and virulence features. It is upregulated in response to the cationic antimicrobial peptide indolicidin and mediates cationic peptide resistance and certain virulence-related processes, such as biofilm formation, rapid attachment, and swarming motility. The involvement of PsrA in these phenotypes was supported by studies of a *psrA* mutant and by single-copy complementation of the *psrA* defect.

PsrA was previously characterized by the Venturi group as a positive regulator of transcription of the alternative sigma factor RpoS (19, 20), as also confirmed here. In other *Pseudomonas* spp., PsrA is known to be involved in antifungal metabolite production (6) and in the regulation of quorum sensing (5). However, the direct signals that activate *psrA* were unknown, and the data here now demonstrate that the cationic antimicrobial peptide indolicidin is an activating signal for transcription, consistent with other studies demonstrating that peptides are key regulators of bacterial virulence and resistance (2, 10, 31).

The demonstration that *psrA* contributes to cationic peptide resistance adds another regulator to the increasingly complex regulatory network influencing resistance, which already includes PhoP-PhoQ and PmrA-PmrB (10, 26, 30, 32). However, unlike these two-component regulators, which mediate an increase in resistance to peptides under growth conditions with limiting Mg^{2+}, PsrA mediates intrinsic resistance. Thus, unlike *psrA* mutants, *pmrA* and *phoP* mutants do not demonstrate supersusceptibility under noninducing conditions (26), and there appears to be no obvious regulatory hierarchy, since *psrA* was not apparently transcriptionally regulated by PmrA or PhoP (or vice versa) and there was no substantial overlap in

dysregulated genes (30) (Tables 2 to 4). All three systems, however, appear to mediate resistance by influencing the ability of cationic agents to permeabilize outer membranes (31), and the increase in permeabilization by cationic agents correlated with the supersusceptibility of the *psrA* mutant to polymyxin B and indolicidin (Fig. 2). Microarray experiments were utilized to select candidate genes dysregulated in the *psrA* mutant that might contribute to *psrA* supersusceptibility to peptides. Three genes (*wbpG*, *wbpI*, and *wbpL*) (Table 4) from the LPS B band (serotype O antigen) biosynthesis operon (*wbpGHIJKLM*) were downregulated 2- to 4.4-fold in the *psrA* mutant, indicating that PsrA positively regulates this operon. The link between peptide supersusceptibility and outer membrane permeability of the *psrA* mutant was supported by the observation that mutants in three of these genes (*wbpI*, *wbpL*, and *wbpM*) displayed modest to substantially increased outer membrane permeability (Fig. 6A and B), and the *wbpM* mutant was further shown here to be peptide supersusceptible (Fig. 5A) and swarming deficient (Fig. 5B). This is consistent with observations in *Proteus mirabilis* that LPS O antigen can contribute to both antimicrobial peptide resistance and swarming motility (29). Other possible candidates to explain peptide supersusceptibility would be gene products involved in energy generation and thus, potentially, in interaction of cationic peptides with the cytoplasmic membrane. One of the tested genes, *coxA*, encoding a subunit of cytochrome *c* oxidase, was 3.4-fold downregulated in arrays (Table 4), and a mutant in this gene led to modest supersusceptibility relative to the WT (Fig. 5A).

The substantial swarming impairment displayed by the *psrA* mutant indicated that PsrA is involved in the regulatory mechanisms controlling this complex adaptation (33, 34). Although swarming motility requires both flagella and pili, the *psrA* mutant did not exhibit a defect in either flagellum-mediated swimming or type IV pilus-mediated twitching motility (34), indicating that it did not control a primary motility organelle. PsrA regulation of swarming motility might reflect the downregulation of both Lon protease (Table 3), which is required for normal swarming (27), and the LPS O antigen B band biosynthetic gene cluster, since the *wbpM* mutant was swarming deficient (Fig. 5B).

Biofilm formation in *Pseudomonas* is initiated by attachment of cells to a surface, followed by complex steps leading to development of mature biofilms (35). The *psrA* biofilm impairment phenotype was likely related in part to the early stages of biofilm formation, as the *psrA* mutant displayed a significant impairment in rapid attachment to the polystyrene surface used in the simple biofilm experiments described here (Fig. 3C). Although *psrA* mutants were able to attach and form simple biofilms, they did this at significantly reduced levels compared to the WT. Two possible PsrA-regulated genes that might influence the regulation of biofilm formation by PsrA are *lon* (27) and the *pprB* response regulator gene (Fig. 5D) (found directly adjacent to the *tad* gene cluster), since mutants in both displayed impaired biofilm formation. The finding that the *psrA* mutant displayed both impaired biofilm formation and impaired swarming motility suggests that PsrA is an integral component of the regulatory network that controls these two separate complex adaptations and is consistent with observations that other regulators control both processes (4, 34, 39).

Our results are consistent with previous observations that RpoS is a negative regulator of the type III secretion system, since RpoS is positively regulated by *psrA* (18, 41) (Table 2). PsrA was previously shown to be a positive regulator of the type III secretion system in a mucoid strain of *P. aeruginosa* grown in complex medium (38). In contrast, the data presented here favor negative regulation by PsrA of this secretion system in the nonmucoid *P. aeruginosa* strain PAO1 grown in defined medium (Table 3). We presume that this is because of other underlying regulatory mutations that are known to occur in mucoid isolates of *P. aeruginosa*. Consistent with these observations, the *psrA* mutant presented here had no effect on cytotoxicity toward epithelial cells, which is partially dependent on type III secretion (data not shown).

PA0506, an acyl-coenzyme A (acyl-CoA) dehydrogenase, was highly upregulated in the *psrA* mutant (43-fold, according to qRT-PCR confirmation experiments). This gene was a previously characterized target of PsrA (18), and our microarray analysis confirmed PsrA as a negative regulator of this gene (Table 2). It is noteworthy that PA0506 has previously been shown to be mutated in cystic fibrosis *P. aeruginosa* isolates, consistent with the suggestion that mutation of this gene favors chronic infection and that this gene might be involved in adaptation to the cystic fibrosis lung (40).

Our microarray gene lists uncovered many other interesting genes as part of the PsrA regulon. The downregulation of genes of the *tad* (tight adherence) cluster (Table 3), involved in the assembly of extracellular cell surface Flp pilus appendages (9), was consistent with the attachment defect in *psrA* mutants in the face of normal piliation and twitching motility. However, no differences were seen in biofilm formation by mutants in genes of the *tad* cluster (Fig. 5D). Probable type II secretion system genes (PA0683, PA2672, and PA2673) also showed modest to strong repression (Tables 2 and 3), could encode adhesion-associated products (based on the similarity of pili to the components of the type II secretion system), and thus might contribute to the attachment and biofilm phenotype observed for the *psrA* mutant.

Biofilm formation, attachment, and swarming motility appear to be very important in *P. aeruginosa* colonization and virulence, while it has been strongly suggested that *Pseudomonas* is exposed to cationic antimicrobial peptides during infections and, occasionally, to polymyxin B during therapy. The involvement of PsrA in these processes and its inducibility by a cationic antimicrobial peptide highlight the likely importance of this enzyme in adaptation to the cystic fibrosis lung environment through regulation of virulence and antimicrobial peptide resistance. The results presented here are consistent with the massive complexity of the regulatory network influencing these processes.

ACKNOWLEDGMENTS

Funding from the Canadian Cystic Fibrosis Foundation (CCFF) is gratefully acknowledged. W.J.G. was supported by a CCFF studentship and a Michael Smith Foundation for Health Research-Genome British Columbia junior graduate studentship. R.H. holds a Canada Research Chair. I.W. was supported by the Juergen Manchot Foundation.

We thank Michael Jacobs and Colin Manoil for providing the UW-*psrA* mutant and Joerg Overhage for insightful discussions.

REFERENCES

1. **Ausubel, F. M.** 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York, NY.
2. **Bader, M. W., S. Sanowar, M. E. Daley, A. R. Schneider, U. Cho, W. Xu, R. E. Klevit, H. Le Moual, and S. I. Miller.** 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell **122:**461–472.
3. **Burrows, L. L., R. V. Urbanic, and J. S. Lam.** 2000. Functional conservation of the polysaccharide biosynthetic protein WbpM and its homologues in *Pseudomonas aeruginosa* and other medically significant bacteria. Infect. Immun. **68:**931–936.
4. **Caiazza, N. C., J. H. Merritt, K. M. Brothers, and G. A. O'Toole.** 2007. Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189:**3603–3612.
5. **Chatterjee, A., Y. Cui, H. Hasegawa, and A. K. Chatterjee.** 2007. PsrA, the *Pseudomonas* sigma regulator, controls regulators of epiphytic fitness, quorum-sensing signals, and plant interactions in *Pseudomonas syringae* pv. tomato strain DC3000. Appl. Environ. Microbiol. **73:**3684–3694.
6. **Chin, A. W. T. F., D. van den Broek, B. J. Lugtenberg, and G. V. Bloemberg.** 2005. The *Pseudomonas chlororaphis* PCL1391 sigma regulator *psrA* represses the production of the antifungal metabolite phenazine-1-carboxamide. Mol. Plant-Microbe Interact. **18:**244–253.
7. **Choi, K. H., A. Kumar, and H. P. Schweizer.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64:**391–397.
8. **Choi, K. H., J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat. Methods **2:**443–448.
9. **de Bentzmann, S., M. Aurouze, G. Ball, and A. Filloux.** 2006. FppA, a novel *Pseudomonas aeruginosa* prepilin peptidase involved in assembly of type IVb pili. J. Bacteriol. **188:**4851–4860.
10. **Ernst, R. K., E. C. Yi, L. Guo, K. B. Lim, J. L. Burns, M. Hackett, and S. I. Miller.** 1999. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. Science **286:**1561–1565.
11. **Falla, T. J., D. N. Karunaratne, and R. E. W. Hancock.** 1996. Mode of action of the antimicrobial peptide indolicidin. J. Biol. Chem. **271:**19298–19303.
12. **Flick, M. R., and L. E. Cluff.** 1976. *Pseudomonas* bacteremia. Review of 108 cases. Am. J. Med. **60:**501–508.
13. **Hancock, R. E. W., and H. G. Sahl.** 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. **24:**1551–1557.
14. **Hancock, R. E. W., and D. P. Speert.** 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug Resist. Updat. **3:**247–255.
15. **Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenthner, D. Bovee, M. V. Olson, and C. Manoil.** 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **100:**14339–14344.
16. **Jenssen, H., P. Hamill, and R. E. W. Hancock.** 2006. Peptide antimicrobial agents. Clin. Microbiol. Rev. **19:**491–511.
17. **Klausen, M., A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen.** 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. Mol. Microbiol. **50:**61–68.
18. **Kojic, M., B. Jovcic, A. Vindigni, F. Odreman, and V. Venturi.** 2005. Novel target genes of PsrA transcriptional regulator of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **246:**175–181.
19. **Kojic, M., C. Aguilar, and V. Venturi.** 2002. TetR family member *psrA* directly binds the *Pseudomonas rpoS* and *psrA* promoters. J. Bacteriol. **184:**2324–2330.
20. **Kojic, M., and V. Venturi.** 2001. Regulation of *rpoS* gene expression in *Pseudomonas*: involvement of a TetR family regulator. J. Bacteriol. **183:**3712–3720.
21. **Li, J., R. L. Nation, J. D. Turnidge, R. W. Milne, K. Coulthard, C. R. Rayner, and D. L. Paterson.** 2006. Colistin: the re-emerging antibiotic for multidrug-resistant gram-negative bacterial infections. Lancet Infect. Dis. **6:**589–601.
22. **Liberati, N. T., J. M. Urbach, S. Miyata, D. G. Lee, E. Drenkard, G. Wu, J. Villanueva, T. Wei, and F. M. Ausubel.** 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. USA **103:**2833–2838.
23. **Loh, B., C. Grant, and R. E. W. Hancock.** 1984. Use of the fluorescent probe 1-*N*-phenylnaphthamine to study the interactions of the aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26:**546–551.
24. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. **2:**1051–1060.
25. **Ma, L., K. D. Jackson, R. M. Landry, M. R. Parsek, and D. J. Wozniak.** 2006. Analysis of *Pseudomonas aeruginosa* conditional *psl* variants reveals roles for the *psl* polysaccharide in adhesion and maintaining biofilm structure postattachment. J. Bacteriol. **188:**8213–8221.
26. **Macfarlane, E. L., A. Kwasnicka, M. M. Ochs, and R. E. W. Hancock.** 1999. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol. Microbiol. **34:**305–316.
27. **Marr, A. K., J. Overhage, M. Bains, and R. E. W. Hancock.** 2007. The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. Microbiology **153:**474–482.
28. **Marr, A. K., W. J. Gooderham, and R. E. W. Hancock.** 2006. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. Curr. Opin. Pharmacol. **6:**468–472.
29. **McCoy, A. J., H. Liu, T. J. Falla, and J. S. Gunn.** 2001. Identification of *Proteus mirabilis* mutants with increased sensitivity to antimicrobial peptides. Antimicrob. Agents Chemother. **45:**2030–2037.
30. **McPhee, J. B., M. Bains, G. Winsor, S. Lewenza, A. Kwasnicka, M. D. Brazas, F. S. Brinkman, and R. E. W. Hancock.** 2006. Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg^{2+}-induced gene regulation in *Pseudomonas aeruginosa*. J. Bacteriol. **188:**3995–4006.
31. **McPhee, J. B., S. Lewenza, and R. E. W. Hancock.** 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol. Microbiol. **50:**205–217.
32. **Moskowitz, S. M., R. K. Ernst, and S. I. Miller.** 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J. Bacteriol. **186:**575–579.
33. **Overhage, J., M. Bains, M. D. Brazas, and R. E. W. Hancock.** 2008. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J. Bacteriol. **190:**2671–2679.
34. **Overhage, J., S. Lewenza, A. K. Marr, and R. E. W. Hancock.** 2007. Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn*5-lux* mutant library. J. Bacteriol. **189:**2164–2169.
35. **Parsek, M. R., and E. P. Greenberg.** 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. Trends Microbiol. **13:**27–33.
36. **Parsek, M. R., and P. K. Singh.** 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu. Rev. Microbiol. **57:**677–701.
37. **Rowe, S. M., S. Miller, and E. J. Sorscher.** 2005. Cystic fibrosis. N. Engl. J. Med. **352:**1992–2001.
38. **Shen, D. K., D. Filopon, L. Kuhn, B. Polack, and B. Toussaint.** 2006. PsrA is a positive transcriptional regulator of the type III secretion system in *Pseudomonas aeruginosa*. Infect. Immun. **74:**1121–1129.
39. **Shrout, J. D., D. L. Chopp, C. L. Just, M. Hentzer, M. Givskov, and M. R. Parsek.** 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol. Microbiol. **62:**1264–1277.
40. **Smith, E. E., D. G. Buckley, Z. Wu, C. Saenphimmachak, L. R. Hoffman, D. A. D'Argenio, S. I. Miller, B. W. Ramsey, D. P. Speert, S. M. Moskowitz, J. L. Burns, R. Kaul, and M. V. Olson.** 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. USA **103:**8487–8492.
41. **Yahr, T. L., and M. C. Wolfgang.** 2006. Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. Mol. Microbiol. **62:**631–640.