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*  

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*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* PA01 *psrA*::Tn5 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* < 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 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-
tides transcriptionally upregulated the expression of \( psrA \), a previously documented \textit{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 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 in this study are described in Table 1.

#### 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).

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**Table 1.** \textit{P. aeruginosa} strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristics$^a$</th>
<th>Source or reference$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains \textit{P. aeruginosa} strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>WT \textit{P. aeruginosa} PA01; H103</td>
<td>Lab collection</td>
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<tr>
<td>UW WT</td>
<td>UW WT \textit{P. aeruginosa} PA01</td>
<td>UW (15)</td>
</tr>
<tr>
<td>UW-psrA</td>
<td>\textit{psrA}:\textit{Islacz}/hah-Tc$^e$; insertion at position 46 (702 bp) in \textit{psrA}; derived from UW WT</td>
<td>UW (15)</td>
</tr>
<tr>
<td>\textit{psrA} mutant</td>
<td>\textit{psrA}:\textit{Islacz}/hah-Tc$^e$; H103 background; Te$^e$</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{psrA} (Tn7-psrA$^+$)</td>
<td>\textit{psrA} mutant with Tn7-psrA$^+$ integrated; Te$^e$ Gm$^e$</td>
<td>This study</td>
</tr>
<tr>
<td>PA14</td>
<td>Wild-type \textit{P. aeruginosa} PA14</td>
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</tr>
<tr>
<td>ccoA</td>
<td>05_2::A11; derived from PA14</td>
<td>22</td>
</tr>
<tr>
<td>etfA</td>
<td>04_4::A12; derived from PA14</td>
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<tr>
<td>flp</td>
<td>09_1::F11; derived from PA14</td>
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<td>mexC</td>
<td>01_4::H2; derived from PA14</td>
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<tr>
<td>pprB</td>
<td>08_3::C3; derived from PA14</td>
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<td>rhIG</td>
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<td>01_2::A7; derived from PA14</td>
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<td>taaB</td>
<td>04_2::H5; derived from PA14</td>
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<tr>
<td>\textit{wbpM}</td>
<td>03_4::E4; derived from PA14</td>
<td>22</td>
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<tr>
<td>\textit{wzz}</td>
<td>06_1::F2l; derived from PA14</td>
<td>22</td>
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<tr>
<td>PA1883 (homolog)</td>
<td>12_1::A7; derived from PA14</td>
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</tr>
<tr>
<td>\textit{wbpl}</td>
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<td>15</td>
</tr>
<tr>
<td>\textit{wbpl}</td>
<td>\textit{wbpl}:\textit{Islacz}/hah-Tc$^e$; insertion 302 (1,020 bp)</td>
<td>15</td>
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<tr>
<td>\textit{E. coli} strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10</td>
<td>\textit{F}–\textit{mcrA} $\Delta$(\textit{mrr-bsdRMs-merBC}) $\beta$80lacZ$\Delta$M15 $\Delta$lacX74 recA1 ara$\Delta$139 $\Delta$(ara-leu)7697 galU galK rpsL (Str$^+$) endA1 napG</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

$^a$ Antimicrobial resistance phenotypes are indicated as follows: Amp$^r$, ampicillin resistance for \textit{E. coli} and carbenicillin resistance for \textit{P. aeruginosa}; Gm$^e$, gentamicin resistance; Kan$^{\text{R}'}$, kanamycin resistance; Te$^e$, tetracycline resistance.

$^b$ UW, University Washington.
transposon integration of mini-Tn7

As previously described, gentamicin-resistant transformants were analyzed by psrA was excised from pCR-carded, and the wells were washed three times with distilled H2O. Surface-shaking. At the specified time point, medium and planktonic cells were dis-

Shaking (250 rpm) to the mid-logarithmic phase of growth (OD600 = 0.05) were used as the cutoffs for reporting expression changes. Real-time qPCR. Total RNA was isolated, using RNAesy Midi columns (Qiagen), from P. aeruginosa grown in BM2-glucose minimal medium containing 2 mM Mg2+, with or without 2 µg/ml indolicidin, to the mid-logarithmic phase of growth. DNase treatment of RNA samples, cDNA synthesis, and real-time quan-
titative 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 1/10 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 ppsrA, using PrimerExpress (Applied Biosystems). All reac-
tions were normalized to the rpsL gene, encoding the 30S ribosomal protein S12. Microarray accession number. The MIAMEExpress accession number is E-

Preliminary studies indicated that the ppsrA (PA3006) gene was induced 2.5-fold (P < 0.05) by a subinhibitory concentra-
tion (2 µg/ml; one-eighth the MIC) of the bovine anti-
microbial peptide polymyxin B, as shown by kill curves. The influence of the

psrA mutant to WT polymyxin B susceptibility by introduc-
ing a single WT ppsrA+ allele into the chromosome of the mutant, using mini-Tn7 integration technology (Fig. 1A). Similarly, the ppsrA mutant demonstrated supersusceptibility to the cationic antimicrobial peptide polymyxin B, which could be com-
plemented back to WT susceptibility (Fig. 1B). Thus, the ppsrA gene product appeared to be essential for normal intrinsic resistance.
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
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).

**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 ($P < 0.05$) dysregulated 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).

**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.

**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).
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 point for further studies. A variety of metabolic and energy metabolism genes (Tables 3 and 4) were upregulated in the psrA mutant, indicating potential changes in the metabolic landscape of the bacteria.

#### Table 2. Known PsrA targets (18) significantly dysregulated in psrA mutants, determined using a microarray

<table>
<thead>
<tr>
<th>Gene identifier</th>
<th>Gene name</th>
<th>Change (fold)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0506</td>
<td>hpiV</td>
<td>16.2*</td>
<td>Probable acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>PA2673</td>
<td>etfA</td>
<td>-27.9</td>
<td>Probable type II secretion system protein</td>
</tr>
<tr>
<td>PA2951</td>
<td>etfB</td>
<td>3.3</td>
<td>Electron transfer flavoprotein alpha subunit</td>
</tr>
<tr>
<td>PA2952</td>
<td></td>
<td>3.8</td>
<td>Electron transfer flavoprotein beta subunit</td>
</tr>
<tr>
<td>PA2953</td>
<td></td>
<td>6.4*</td>
<td>Electron transfer flavoprotein-ubiquinone oxidoreductase</td>
</tr>
<tr>
<td>PA3013</td>
<td>foaB</td>
<td>15.2*</td>
<td>Fatty acid oxidation complex beta subunit</td>
</tr>
<tr>
<td>PA3014</td>
<td>foaA</td>
<td>11.6</td>
<td>Fatty acid oxidation complex alpha subunit</td>
</tr>
<tr>
<td>PA3622</td>
<td>rpoS</td>
<td>-2.3*</td>
<td>Alternative sigma factor RpoS</td>
</tr>
</tbody>
</table>

* 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).

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

<table>
<thead>
<tr>
<th>Gene identifier</th>
<th>Gene name</th>
<th>Change (fold)</th>
<th>P value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0044</td>
<td>exoT</td>
<td>4.6</td>
<td>0.0005</td>
<td>Exoenzyme T; type III secretion system effector</td>
</tr>
<tr>
<td>PA1695</td>
<td>pscP</td>
<td>3.0</td>
<td>&lt;0.0001</td>
<td>Translocation protein in type III secretion</td>
</tr>
<tr>
<td>PA1697</td>
<td>pscN</td>
<td>2.1</td>
<td>0.004</td>
<td>ATP synthase in type III secretion system</td>
</tr>
<tr>
<td>PA1698</td>
<td>popN</td>
<td>2.6</td>
<td>0.004</td>
<td>Outer membrane protein PopN</td>
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<tr>
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<td></td>
<td>2.3</td>
<td>0.003</td>
<td>Conserved hypothetical protein in type III secretion</td>
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<td>2.0</td>
<td>0.0009</td>
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<tr>
<td>PA1701</td>
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<td>0.0002</td>
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<tr>
<td>PA1703</td>
<td>pcrD</td>
<td>2.0</td>
<td>0.01</td>
<td>Type III secretory apparatus protein PcrD</td>
</tr>
<tr>
<td>PA1705</td>
<td>pcrG</td>
<td>3.8</td>
<td>0.002</td>
<td>Regulator in type III secretion</td>
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<td>Type III secretion protein PcrV</td>
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<td>PA1708</td>
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<td>0.005</td>
<td>Translocator protein PopB</td>
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<td>PA1709</td>
<td>popD</td>
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<td>PA1710</td>
<td>exsC</td>
<td>2.1</td>
<td>&lt;0.0001</td>
<td>Exoenzyme S synthesis protein C</td>
</tr>
<tr>
<td>PA1712</td>
<td>exsB</td>
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<td>0.001</td>
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<td>0.001</td>
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<td>pscD</td>
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<td>0.01</td>
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<tr>
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<td>0.0004</td>
<td>Adenylate cyclase ExoY; type III secretion effector</td>
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<tr>
<td>PA3841</td>
<td>exoS</td>
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<td>0.001</td>
<td>Exoenzyme S; type III secretion effector</td>
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<tr>
<td>PA3842</td>
<td>orfI</td>
<td>3.5</td>
<td>0.001</td>
<td>Chaperone for ExoS secretion</td>
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Adhesion and motility genes

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<th>Gene name</th>
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<tr>
<td>PA0176</td>
<td>aer2</td>
<td>-4.2</td>
<td>0.04</td>
<td>Aerotaxis methyl-accepting chemotaxis protein</td>
</tr>
<tr>
<td>PA1803</td>
<td>lon</td>
<td>-2.0</td>
<td>0.05</td>
<td>ATP-dependent Lon protease</td>
</tr>
<tr>
<td>PA4296</td>
<td>pprB</td>
<td>-4.9*</td>
<td>0.05</td>
<td>PprB two-component response regulator</td>
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<tr>
<td>PA4300</td>
<td>tadC</td>
<td>-2.2</td>
<td>0.04</td>
<td>FliP pilus assembly protein, PillC-like</td>
</tr>
<tr>
<td>PA4302</td>
<td>tadA</td>
<td>-4.1*</td>
<td>0.01</td>
<td>TadA traffic ATPase in FliP pilus assembly</td>
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<td>0.02</td>
<td>FliP pilus assembly protein</td>
</tr>
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<td>PA4305</td>
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<td>0.05</td>
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Type II secretion genes

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>PA0683</td>
<td>hcxY</td>
<td>-4.4</td>
<td>0.008</td>
<td>Hcx type II secretion system membrane protein</td>
</tr>
<tr>
<td>PA1871</td>
<td>laxA</td>
<td>10.5</td>
<td>0.002</td>
<td>LasA protease</td>
</tr>
<tr>
<td>PA2672</td>
<td>hpiW</td>
<td>-2.5</td>
<td>0.003</td>
<td>Type II secretion system prepilin peptidase substrate</td>
</tr>
</tbody>
</table>

* 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 pscA mutants, determined using a microarray

<table>
<thead>
<tr>
<th>Gene identifier</th>
<th>Gene name</th>
<th>Change (fold)</th>
<th>P value</th>
<th>Description</th>
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<tbody>
<tr>
<td>PA0106</td>
<td>coxA</td>
<td>−3.4</td>
<td>0.01</td>
<td>Cytochrome c oxidase, subunit I</td>
</tr>
<tr>
<td>PA0217</td>
<td>mdcR</td>
<td>−4.8</td>
<td>0.007</td>
<td>Transcriptional regulator</td>
</tr>
<tr>
<td>PA0459</td>
<td>clpC</td>
<td>−3.5</td>
<td>0.05</td>
<td>ClpA/B protease ATP binding subunit</td>
</tr>
<tr>
<td>PA0507</td>
<td></td>
<td>−3.2</td>
<td>0.001</td>
<td>Probable acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>PA0511</td>
<td>nirJ</td>
<td>4.8</td>
<td>0.007</td>
<td>Heme d1 biosynthesis protein</td>
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<td>nirH</td>
<td>2.3</td>
<td>0.04</td>
<td>Conserved hypothetical protein</td>
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<td></td>
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<td>0.008</td>
<td>Probable transcriptional regulator</td>
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<td>nirC</td>
<td>−2.6</td>
<td>0.001</td>
<td>c-type cytochrome</td>
</tr>
<tr>
<td>PA0530</td>
<td></td>
<td>−4.3</td>
<td>0.04</td>
<td>Pyridoxal phosphate-dependent aminotransferase</td>
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<td>yeaG</td>
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<td>0.01</td>
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<td>0.009</td>
<td>ToxR/RegA transcriptional regulator</td>
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<td>0.009</td>
<td>Bacteriophage Pf1 protein</td>
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<td>0.02</td>
<td>Coat protein A of bacteriophage Pf1</td>
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<td></td>
<td>−8.9</td>
<td>0.006</td>
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<td>Chitin-binding protein</td>
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<td>OmpA-family outer membrane protein</td>
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<td>Cytochrome c-type protein</td>
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<td>Acyl-CoA dehydrogenase</td>
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<td>Probable LysR-family transcriptional regulator</td>
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<td>0.008</td>
<td>Probable transporter</td>
</tr>
<tr>
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<td>0.006</td>
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<td>−10.2</td>
<td>0.002</td>
<td>NADH-ubiquinone/plastoquinone oxidoreductase</td>
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<td>PA1927</td>
<td>metE</td>
<td>2.1</td>
<td>0.007</td>
<td>Methionine synthase</td>
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<tr>
<td>PA1976</td>
<td></td>
<td>−2.2</td>
<td>0.009</td>
<td>Two-component sensor kinase</td>
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<tr>
<td>PA1978</td>
<td>agrR</td>
<td>−2.9</td>
<td>0.03</td>
<td>Two-component response regulator</td>
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<td>PA1982</td>
<td>exaA</td>
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<td>Quinoprotein ethanol dehydrogenase</td>
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<td>0.02</td>
<td>Cytochrome c150l</td>
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<tr>
<td>PA1984</td>
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<td>0.05</td>
<td>Aldehyde dehydrogenase</td>
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<td>PA1985</td>
<td>pqqA</td>
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<td>PA2124</td>
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<td>3.2</td>
<td>0.01</td>
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<tr>
<td>PA2277</td>
<td>arsR</td>
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<td>0.02</td>
<td></td>
<td>Ion transport membrane protein</td>
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<tr>
<td>PA2339</td>
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<td>Maltose/mannitol transport protein</td>
</tr>
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<td>0.02</td>
<td>Probable ATP-binding component of ABC transporter</td>
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<td>PA2352</td>
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<td>0.03</td>
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<td>PA2371</td>
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<td>Ferriproverdine outer membrane receptor</td>
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<td>0.01</td>
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</tr>
<tr>
<td>PA2522</td>
<td>czcC</td>
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<td>0.01</td>
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<td>Probable oxidoreductase</td>
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<td>0.001</td>
<td>Probable acyl-CoA dehydrogenase</td>
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<td>0.001</td>
<td>Probable transcriptional regulator</td>
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<td>−2.9</td>
<td>0.02</td>
<td>Probable chemotaxis transducer</td>
</tr>
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<td>PA2664</td>
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<td>0.0004</td>
<td>Flavohemoprotein</td>
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<td>PA2892</td>
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<td>0.04</td>
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<tr>
<td>PA2893</td>
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<td>Probable very-long-chain acyl-CoA synthetase</td>
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<tr>
<td>PA2939</td>
<td>pepB</td>
<td>−4.9</td>
<td>0.007</td>
<td>Secreted aminopeptidase</td>
</tr>
<tr>
<td>PA3077</td>
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<td>2.5</td>
<td>0.03</td>
<td>Two-component response regulator</td>
</tr>
<tr>
<td>PA3145</td>
<td>wbpL</td>
<td>−2.5</td>
<td>0.03</td>
<td>WbpL thymosyltransferase in LPS biosynthesis</td>
</tr>
<tr>
<td>PA3148</td>
<td>wbpI</td>
<td>−4.4</td>
<td>0.04</td>
<td>UDP-N-acetylglucosamine 2-epimerase WbpI</td>
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<td>PA3150</td>
<td>wbpG</td>
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<td>LPS biosynthesis protein WbpG</td>
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<td>Glutamine amidotransfer</td>
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<td>0.02</td>
<td>Probable nonribosomal peptide synthetase</td>
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<td>0.0002</td>
<td>Beta-ketoacyl reductase</td>
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<td>0.05</td>
<td>Probable transmembrane sensor</td>
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<td>&lt;0.0001</td>
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<td>−2.6</td>
<td>0.002</td>
<td>Probable short-chain dehydrogenases</td>
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<td>0.002</td>
<td>Probable acyl-CoA thiolase</td>
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<td>PA3630</td>
<td></td>
<td>2.2</td>
<td>0.05</td>
<td>Probable transcriptional regulator</td>
</tr>
</tbody>
</table>

Continued on following page
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 H2O 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.]

<table>
<thead>
<tr>
<th>Gene identifier</th>
<th>Gene name</th>
<th>Change (fold)</th>
<th>P value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
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<td>PA0723</td>
<td>yqiM</td>
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<td>0.005</td>
<td>FMN oxidoreductase</td>
</tr>
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<td>narK1</td>
<td>-3.8</td>
<td>0.0008</td>
<td>Nitrite extrusion protein 1</td>
</tr>
<tr>
<td>PA0397</td>
<td></td>
<td>3.7</td>
<td>0.01</td>
<td>Probable short-chain dehydrogenase</td>
</tr>
<tr>
<td>PA0415</td>
<td></td>
<td>-2.9</td>
<td>0.02</td>
<td>Probable transcriptional regulator</td>
</tr>
<tr>
<td>PA0449</td>
<td></td>
<td>-4.1</td>
<td>0.01</td>
<td>Binding protein component of ABC transporter</td>
</tr>
<tr>
<td>PA0459</td>
<td>mexC</td>
<td>-2.0</td>
<td>0.008</td>
<td>RND multidrug efflux membrane fusion protein</td>
</tr>
<tr>
<td>PA04654</td>
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<td>-5.6</td>
<td>0.002</td>
<td>Major facilitator superfamily transporter</td>
</tr>
<tr>
<td>PA04911</td>
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<td>0.004</td>
<td>Probable permease of ABC amino acid transporter</td>
</tr>
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<td>0.003</td>
<td>Probable acyl-CoA dehydrogenase</td>
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<td>0.02</td>
<td>Amino acid permease</td>
</tr>
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<td>Histidine biosynthesis protein</td>
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</tr>
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<td>PA05302</td>
<td>dadX</td>
<td>-3.3</td>
<td>0.02</td>
<td>Catabolic alanine racemase</td>
</tr>
</tbody>
</table>

* Dysregulated hypothetical or unclassified open reading frames are not included. Only genes showing a ≥2-fold change in the psrA mutant are depicted.

* According to the *P. aeruginosa* genome website (http://www.pseudomonas.com/).

* Regulation of genes differentially expressed in the psrA mutant relative to the WT. A positive number indicates transcript upregulation in the psrA mutant.
cated a role for energy metabolism in resistance to antimicrobial peptides.

As shown in Fig. 5A, both \textit{wbpM} (previously shown to lack B band LPS) \((3)\) and \textit{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 \textit{rhlG}, \textit{psrA}, \textit{mexC}, and \textit{wzz} (data not shown). Unfortunately, an \textit{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 \textit{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 \textit{psrA} mutation, mutants in \textit{wbpI}, \textit{wbpL} (downregulated in the \textit{psrA} mutant), and \textit{wbpM} were analyzed for possible outer membrane permeability phenotypes. Like the \textit{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 \textit{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 \textit{wbpM} mutant showed a major swarming impairment phenotype, as did an \textit{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 \textit{rhlG} (Fig. 5B) \textit{coxA} (Fig. 5C), \textit{mexC}, \textit{wzz}, and PA1883 mutants (data not shown).

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

**DISCUSSION**

The \textit{psrA} gene of \textit{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 \textit{psrA} mutant and by single-copy complementation of the \textit{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 \textit{Pseudomonas} \textit{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 \textit{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 \textit{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 \textit{psrA} mutants, \textit{pmrA} and \textit{phoP} mutants do not demonstrate supersusceptibility under noninducing conditions (26), and there appears to be no obvious regulatory hierarchy, since \textit{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 (wpbG, wpbI, and wpbL) (Table 4) from the LPS B band (serotype O antigen) biosynthesis operon (wpbGHIJKLM) 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 (wpbI, wpbL, and wpbM) displayed modest to substantially increased outer membrane permeability (Fig. 6A and B), and the wpbM 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 wpbM 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 pil 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.

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