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Adaptive Resistance to the "Last Hope" Antibiotics Polymyxin B and Colistin in *Pseudomonas aeruginosa* Is Mediated by the Novel Two-Component Regulatory System ParR-ParS[▽][†]

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As multidrug resistance increases alarmingly, polymyxin B and colistin are increasingly being used in the clinic to treat serious *Pseudomonas aeruginosa* infections. In this opportunistic pathogen, subinhibitory levels of polymyxins and certain antimicrobial peptides induce resistance toward higher, otherwise lethal, levels of these antimicrobial agents. It is known that the modification of lipid A of lipopolysaccharide (LPS) is a key component of this adaptive peptide resistance, but to date, the regulatory mechanism underlying peptide regulation in *P. aeruginosa* has remained elusive. The PhoP-PhoQ and PmrA-PmrB two-component systems, which control this modification under low-Mg^{2+} conditions, do not appear to play a major role in peptide-mediated adaptive resistance, unlike in *Salmonella* where PhoQ is a peptide sensor. Here we describe the identification and characterization of a novel *P. aeruginosa* two-component regulator affecting polymyxin-adaptive resistance, ParR-ParS (PA1799-PA1798). This system was required for activation of the *arnBCADTEF* LPS modification operon in the presence of subinhibitory concentrations of polymyxin, colistin, or the bovine peptide indolicidin, leading to increased resistance to various polycationic antibiotics, including aminoglycosides. This study highlights the complexity of the regulatory network controlling resistance to cationic antibiotics and host peptides in *P. aeruginosa*, which has major relevance in the development and deployment of cationic antimicrobials.

Pseudomonas aeruginosa is a Gram-negative ubiquitous environmental bacterium that is capable of infecting a wide range of plant and animal hosts (40). In humans, it is responsible for eventually fatal chronic lung infections in patients with cystic fibrosis (CF), as well as serious acute infections in immunocompromised and injured individuals, where it is a serious problem in ventilator-associated pneumonia and septic burn wounds (32, 43). Indeed, *P. aeruginosa* is the third leading cause of nosocomial infections (2).

The ability of this microorganism to adapt to different environments is, to a large extent, due to intricate regulatory networks. *P. aeruginosa* PAO1 has one of the largest collections of regulatory proteins found in bacteria, representing just less than 10% of all open reading frames (ORFs) (47), and a major subset of these ORFs corresponds to two-component regulatory systems. These systems classically consist of a histidine kinase sensor protein, located in the cytoplasmic membrane, and a cytoplasmic response regulator. When the periplasmic domain of the sensor kinase detects and interacts with an effector molecule, the protein autophosphorylates at a conserved cytoplasmic histidine residue. This phosphate is subsequently transferred to an aspartate residue of the response regulator, and the phosphorylated regulator will then activate and/or repress transcription of its target genes (41). The *P. aeruginosa* genome encodes 64 response regulators and 63 histidine kinases, as well as 16 atypical kinases (41). Although some of these regulatory systems have been shown to control key aspects of *P. aeruginosa* antibiotic resistance and virulence (19), the function of many of them remains undetermined.

Infections caused by *P. aeruginosa* are very difficult to treat because of its high level of intrinsic resistance to drugs (24). In addition, *P. aeruginosa* can also exhibit adaptive resistance, a phenomenon by which certain environmental cues, including subinhibitory concentrations of antibiotics, can transiently induce resistance to otherwise lethal doses of antimicrobial agents (33, 3). This is one of the reasons why *in vitro* analysis of antibiotic resistance does not necessarily reflect the clinical effectiveness of a specific drug. In *P. aeruginosa*, adaptive resistance to polymyxins and cationic antimicrobial peptides is known to occur in response to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} cations (33, 36). This adaptation is controlled by the two-component regulators PhoP-PhoQ and PmrA-PmrB which upregulate the expression of the *arnBCADTEF* lipopolysaccharide (LPS) modification operon (36). The products of these *arn* genes participate in the addition of 4-aminoarabinose to lipid A of LPS, thereby reducing the net negative charge of LPS and limiting the interaction and self-promoted uptake of polycationic antibiotics, such as polymyxins, host defense peptides, and aminoglycosides, with the outer membrane. Nevertheless, this adaptation to low divalent cations is unlikely to be clinically meaningful, as

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain[a] or plasmid	Relevant genotype or characteristics[b]	Reference(s) or source
Bacterial strains		
P. aeruginosa		
WT	Wild-type *P. aeruginosa* PAO1; strain H103	3, 47
UW-WT	UW wild-type *P. aeruginosa* PAO1	26
UW-PA1799	PA1799::IS*lacZ*/hah-Tc^r; insertion at 492 (708) bp in PA1799; derived from strain UW-WT	26
UW-PA1798	PA1798::IS*lacZ*/hah-Tc^r; insertion at 307 (1,287) bp in PA1798; derived from UW-WT	26
parR mutant	PA1799::IS*lacZ*/hah-Tc^r, H103 background; Tet^r	This study
parS mutant	PA1798::IS*lacZ*/hah-Tc^r, H103 background; Tet^r	This study
parR (Tn7-*parRS*$^+$) complemented strain	PA1799 mutant with Tn7-PA1798-1799$^+$ integrated; Tc^r Gm^r	This study
UW-PA1797	PA1797::IS*lacZ*/hah-Tc^r; insertion at 423 (1,833) bp in PA1797; derived from UW-WT	26
UW-PA1559	PA1559::IS*lacZ*/hah-Tc^r; insertion at 406 (732) bp in PA1559; derived from UW-WT	26
UW-*pagL*	*pagL*::IS*phoA*/hah-Tc^r; insertion at 196 (522) bp in *pagL*; derived from UW-WT	26
UW-PA4782	PA4782::IS*phoA*/hah-Tc^r; insertion at 147 (246) bp in PA4782; derived from UW-WT	26
UW-*pmrB*	*pmrB*::IS*phoA*/hah-Tc^r; insertion at 67 (1,434) bp in *pmrB*; derived from UW-WT	26
UW-*arnB*	*arnB*::IS*phoA*/hah-Tc^r; insertion at 327 (1,149) bp in *arnB*; derived from UW-WT	26
UW-*arnC*	*arnC*::IS*lacZ*/hah-Tc^r; insertion at 408 (1,020) bp in *arnC*; derived from UW-WT	26
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*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-PA1798-1799$^+$	pCR-Blunt II-TOPO harboring 2.3-kb PA1798-PA1799 amplicon	This study
pUC18-mini-Tn7T-Gm	Suicide plasmid; Gm^r Amp^r	5
pUC-Tn7-PA1798-1799$^+$	pUC18-mini-Tn7T-Gm with 2.3-kb PA1798-PA1799 fragment from pCR-PA1798-PA1799	This study
pTNS2	Transposition helper plasmid; Amp^r	5
pUCP*lux*-p$_{PA3552}$	pUCP23 containing a transcriptional fusion between the *arnB* promoter and *luxCDABE*	36

[a] UW, University of Washington Genome Science Center.
[b] Antibiotic resistance phenotypes: 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. An "insertion at 492 (708) bp" means that the insertion is at nucleotide 492 within a 708-bp-long gene. "hah" is a 63-codon insertion which encodes an influenza hemagglutinin epitope together with a hexahistidine metal affinity purification tag.

the human body contains 1 to 2 mM divalent cations. More alarmingly, peptides themselves are also able to induce adaptive resistance via LPS modification (36). Subinhibitory concentrations of peptides can induce the expression of the *pmrAB* and *arnBCADTEF* operons in *P. aeruginosa*. However, neither PhoP-PhoQ nor PmrA-PmrB seems to be essential for this upregulation (36). Thus, an unidentified regulatory system has been proposed to respond to peptides and promote the activation of the *arnBCADTEF* and *pmrAB* operons independently of PhoP-PhoQ and PmrA-PmrB, consequently increasing resistance to antimicrobial peptides (36, 19).

The polymyxins, a group of cationic antimicrobial lipopeptides synthesized by bacteria, are being used increasingly to treat infections by multiresistant *P. aeruginosa* strains (31). Despite the fact that polymyxins have been used for decades, the pharmacokinetics and pharmacodynamics of polymyxins are still poorly understood. Consequently, there is the possibility that bacteria are exposed to sublethal concentrations during treatment.

Cationic antimicrobial peptides are widely present in nature, being produced by bacteria and complex organisms, such as plants and animals. In the latter, peptides constitute key parts of their host defense mechanisms. As such, *P. aeruginosa* comes into contact with the human cationic host defense (antimicrobial) peptides produced by epithelial surfaces (e.g., β-defensins and cathelicidins), during infection. The combination of antimicrobial and immunomodulatory activity has made host defense peptides very attractive for the design of new

drugs to combat bacterial infections (23). Therefore, it is desirable to develop peptides with a poor ability to induce an adaptive bacterial response while maintaining good activity. All these factors make it important to attain a better understanding of the molecular mechanisms behind adaptive resistance to cationic antimicrobial peptides in *P. aeruginosa*.

The aim of this study was the identification of the major sensor/regulator controlling polymyxin- and cationic-peptide-mediated adaptive resistance in *P. aeruginosa*. We demonstrate that the novel two-component regulator ParR-ParS is a key component in the cascade that leads to the induction of the LPS modification operon in response to subinhibitory concentrations of peptides. Additionally, analysis of the transcriptome of a *parR* mutant in the presence of subinhibitory indolicidin (a bovine host defense peptide) provided new insights into the genes involved in the regulatory pathway activated by this peptide.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antimicrobial agents. The bacterial strains and plasmids used in this study are described in Table 1. Cultures were routinely grown in Luria-Bertani (LB) broth containing 2.0% (wt/vol) Difco agar (Becton-Dickinson Co.) when appropriate. The defined minimal medium used was BM2-glucose [62 mM potassium phosphate buffer (pH 7), 7 mM (NH$_4$)$_2$SO$_4$, 10 μM FeSO$_4$, 0.4% (wt/vol) glucose] containing 2 mM MgSO$_4$ (high Mg^{2+}) or 20 μM MgSO$_4$ (low Mg^{2+}). The antibiotics and concentrations used for selection in *P. aeruginosa* were as follows: tetracycline, 50 μg/ml; carbenicillin, 500 μg/ml; and gentamicin, 30 μg/ml. The antibiotics and concentrations used for selection in *Escherichia coli* were as follows: ampicillin, 100 μg/ml; and kanamycin, 50

μg/ml. All antibiotics were purchased from Sigma. Peptides were synthesized by 9-fluorenylmethoxy carbonyl (Fmoc) methods and were 95% pure by high-performance liquid chromatography (HPLC) and mass spectrometry (MS).

Transferring the UW-PA1798 and UW-PA1799 transposon mutations into a different *P. aeruginosa* PAO1 background. The UW-PA1798 and UW-PA1799 transposon mutations (Table 1) were first confirmed to be correct by colony PCR as recommended by the University of Washington Genome Science Center (UW) (26). These mutations were then transferred into our laboratory wild-type (WT) *P. aeruginosa* PAO1 strain (H103) as described previously (6, 17). Briefly, genomic DNA was isolated from each of the UW mutants, and 1 μg was electroporated into competent cells of strain H103. After cells recovered for 1 h at 37°C, aliquots were plated onto LB agar plates containing 50 μg/ml tetracycline. The following day, tetracycline-resistant colonies were analyzed by PCR to verify the correct site of transposon insertion.

Genetic complementation. In order to complement the mutation in *parR* (PA1799), the operon *parRS* (PA1798-1799$^+$) was cloned together with 185 bp of upstream DNA (native promoter) and 50 bp of downstream DNA. This fragment was amplified from *P. aeruginosa* WT PAO1 genomic DNA using Platinum *Pfx* DNA polymerase (Invitrogen), and the PCR product was purified with the Qiagen PCR purification kit. Forward and reverse primers for PA1798-PA1799 were designed from the *P. aeruginosa* PAO1 genome sequence (*Pseudomonas* Genome Database v2 at www.pseudomonas.com) using Primer3 (44). These primers were as follows: PA1799-F (F for forward) (5′-GAATCAAGGGCATG CATTCTA-3′) and PA1798-R (R for reverse) (5′-GTCCTTACGGCTTTGTC AGC-3′). The generated amplicon was then cloned into pCR-Blunt II-TOPO, using the Zero Blunt TOPO PCR cloning kit (Invitrogen), and transformed into One Shot TOP10 *E. coli* cells (Invitrogen), creating pCR-PA1798-1799$^+$. An NsiI fragment containing PA1798-PA1799$^+$ was excised from pCR-PA1798-1799 and subcloned into pUC18-mini-Tn7T-Gm, generating pUC-Tn7-PA1798-1799$^+$. Next, pUC-Tn7-PA1798-PA1799$^+$ was coelectroporated with pTNS2 (transposition helper plasmid) into electrocompetent cells of the *parR* mutant prepared by the sucrose method (6). Gentamicin-resistant transformants were analyzed by colony PCR using primers P*glmS*-up and PTn7L to determine the correct transposon integration of mini-Tn7 into the chromosome as previously described (5).

Luminescence gene expression assays. Induction of the *luxCDABE* fusion under different conditions was carried out by the method of McPhee et al. (36). Different *P. aeruginosa* strains carrying plasmid pUCP*lux*-p$_{PA3552}$ were grown overnight in LB broth containing carbenicillin. The following day, the cells were pelleted and resuspended in BM2-glucose liquid medium containing either high or low Mg^{2+} concentration. These cell suspensions were then diluted 1:20 into the same medium. When necessary, different peptides or aminoglycosides were added to the medium. The production of luminescence at different time points was measured using a SPECTRAFluorPlus luminometer (Tecan, San Jose, CA). Luminescence was corrected for growth by measuring the optical density at 620 nm (OD$_{620}$).

Killing curves. *P. aeruginosa* cultures were grown overnight in BM2-glucose (high Mg^{2+}) and diluted 1:50 the next morning into fresh BM2-glucose (high Mg^{2+}) with or without subinhibitory indolicidin (4 μg/ml). Upon reaching the mid-logarithmic phase of growth (OD$_{600} \sim 0.5$), 1 ml of culture was harvested, centrifuged, and resuspended in 1 ml of 1× BM2 salts. This cell suspension was diluted 1:10 into 1× BM2 salts, and killing was then initiated by the addition of 10.0 μg/ml or 1.0 μg/ml polymyxin B sulfate (Sigma) for the analysis of adaptive and intrinsic resistance, respectively. The tubes were continuously shaken at room temperature using a benchtop nutator (BD Clay Adams nutator, model 1105). To assay for survivors, 50-μl aliquots were withdrawn at the designated times and serially diluted 10-fold, and 100-μl portions from serial dilutions of these aliquots were plated onto LB agar plates.

MIC determination. MICs were assessed according to the standard broth microdilution method (7) as described by CLSI/NCCLS guidelines (M7-A7 [7] and M100-S17 [7a]) using BM2-glucose minimal medium containing 2 mM or 20 μM Mg^{2+}. The MIC was determined following 24 h of growth at 37°C and represented the minimal concentration at which no growth could be visually observed with the naked eye. For MICs using cationic antimicrobial peptides, polypropylene microtiter plates were used to prevent peptide binding to the plate surface and artificially high MICs (50). The amino acid sequences of the peptides used in this study are included in Table 2. To determine the adaptive MICs to peptides and aminoglycosides, cells were grown before and during MIC determination in medium supplemented with 4 μg/ml indolicidin.

Assessment of antimicrobial interactions between peptides. To determine whether there was synergistic action between certain peptides, the checkerboard method was used (8). This technique is a modification of the broth microdilution method, in which two drugs are diluted in a two-dimensional fashion. As a result

TABLE 2. Antimicrobial peptides used in this study

Peptide	Sequence[a]	Reference
Polymyxin B	fa-B^L-T^L-B(B^L-B^L-FL^L-B^L-B^L-T^L-)	49
Colistin	fa-B^L-T^L-B(B^L-B^L-LL^L-B^L-B^L-T^L-)	49
CP11N	ILKKWPWWPWRRK-NH$_2$	15
Indolicidin	ILPWKWPWWPWRR-NH$_2$	46
CP26	KWKSFIKKLTSAAKKVVTTAKPLISS	16
CP28	KWKLFKKIGIGAVLKVLTTGLPAL KLTK	39
Bac2A	RLARIVVIRVAR-NH$_2$	50
Bactenecin	RLCRIVVIRVCR	42
LL-37	LLGDFFRKSKEKIFKEFKRIVQRIK DFLRNLVPRTES	20
Polyphemusin	RRWC$_1$FRVC$_2$YRGFC$_2$YRKC$_1$R	51

^{a} Sequences are shown in the one-letter amino acid code. In the first two sequences, the sequences for polymyxin B and colistin, fa indicates a 6-methyloctanoyl or 6-methylheptanoyl fatty acid chain, B indicates α,γ-diaminobutirate, and a superscripted L indicates that the amino acid is the L-enantiomer. For the remaining sequences, -NH$_2$ indicates amidation of the carboxyl terminus and numbered cysteines represent residues joined by disulfide bonds.

of this, each well in the microtiter plate contains a unique combination of concentrations of the two drugs. The fractional inhibitory concentration (FIC) for each drug was determined by dividing the concentration necessary to inhibit growth in a specific row or column by the MIC of the bacterium for that drug by itself (10). The FIC index (FICi), calculated as the sum of the FICs, was interpreted as follows: a FICi of ≤0.5 represents synergy, a FICi of ≥4.0 represents antagonism, and a FICi of > 0.5 to 4 represents no interaction. As in the MIC determinations, the medium used was BM2-glucose containing 2 mM Mg^{2+}, and polypropylene microtiter plates were used. Results were read after 24 h of incubation at 37°C.

Microarray analysis. Microarray analysis was carried out as previously described (35). In overview, four independent cultures of each *P. aeruginosa* strain, WT and the *parR* mutant, were grown with shaking in BM2-glucose medium containing 2 mM MgSO$_4$ at 37°C for 18 h and then diluted 1:100 into fresh medium supplemented with 4 μg/ml indolicidin. Cultures were grown to the mid-logarithmic phase of growth (OD$_{600} \sim 0.5$) at 37°C with shaking (250 rpm), and then total RNA was isolated using the RNeasy Midi RNA isolation kit (Qiagen) following the manufacturer's instructions. Contaminating genomic DNA was removed by treatment with the DNA-free kit (Ambion Inc., Austin, TX). RNA was stored at −80°C with 0.2 U μl^{-1} of SUPERase-In RNase inhibitor (Ambion Inc.). The quality of the RNA was assessed spectrophotometrically and by agarose gel electrophoresis. RNA was converted to cDNA, hybridized, and analyzed as previously described (35). *P. aeruginosa* PAO1 microarray slides were provided by the J. Craig Venter Institute (JCVI) (formerly The Institute for Genomic Research [TIGR]) Pathogenic Functional Genomics Resource Center (http://pfgrc.jcvi.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 the *Pseudomonas* Genome Database v2 at www .pseudomonas.com. The four biological replicates were averaged to obtain overall changes for the *parR* 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 or greater with a Student *t* test *P* value of ≤0.05 were used as the cutoffs for reporting expression changes.

Reverse transcription-quantitative PCR (RT-qPCR). Three independent cultures of the *P. aeruginosa* WT strain and the *parR* mutant were grown to the mid-logarithmic phase in BM2-glucose minimal medium containing 2 mM Mg^{2+} with or without 4 μg/ml indolicidin. Total RNA was then isolated, using RNeasy minicolumns (Qiagen). DNase treatment of RNA samples, cDNA synthesis, and real-time PCR (qPCR) were carried out as described previously (35). cDNA was diluted 1:100, and 2.5 μl was used as a template for each reaction, using 1× SYBR green PCR master mix (Applied Biosystems, Foster, CA) and an ABI Prism 7000 instrument (Applied Biosystems). Internal forward and reverse primers for each gene were designed using PrimerExpress (Applied Biosystems). Experiments were repeated with three independent cultures, each tested in duplicate. Comparison to the *rpsL* gene, encoding the 30S ribosomal protein S12, allowed calculation of the fold change by the threshold cycle (C_T) method (31a).
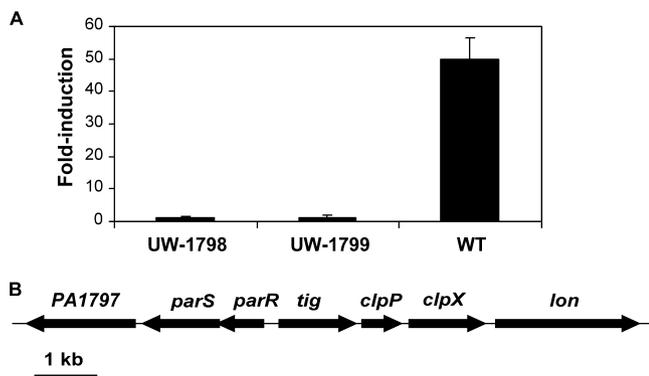
A

B

FIG. 1. Identification of the *parRS* operon. (A) Fold induction of pPA3552::*lux* fusion in BM2-glucose minimal medium supplemented with 2 mM Mg^{2+} in the presence of 4 μg/ml indolicidin compared to induction in the absence of indolicidin in the WT (H103) and mutant strains UW-PA1798 (UW-1798) and UW-PA1799 (UW-1799). The pPA3552::*lux* fusion contained a promoterless *lux* cassette fused to the promoter of the *arnB* gene and reported on the transcription of the eight-gene operon encoding the *arn* LPS modification operon. The results provided correspond to one representative experiment of at least three independent assays which displayed the same trends. (B) Schematic representation showing the genomic context of the *parRS* operon. The black arrows represent ORFs.

RESULTS

Initial identification of ParR-ParS. Indolicidin, a bovine host defense peptide (Table 2), is known to be a strong inducer of the *arnBCADTEF* LPS modification operon. Examination of the promoter regions of genes that were dysregulated by indolicidin indicated a motif similar to that of PmrA and PhoQ (35). We sought to identify regulators involved in this induction. To do so, we utilized plasmid pUCP*lux*-P$_{PA3552}$, which carries a transcriptional fusion of the *arnB* (PA3552) promoter with a promoterless *luxCDABE* cassette (36). This reporter construct allows quantification of the expression from the *arnB* promoter by measuring luminescence. Plasmid pUCP*lux*-P$_{PA3552}$ was electroporated into a panel of UW mutants (UW for University of Washington Genome Science Center) (26) that harbored transposon insertions in different response regulator genes that were most similar to PmrA and/or PhoP, namely, PA0756, PA1799, PA2479, PA2523, PA2657, PA2809, PA3204, PA3205, and PA4885. These strains were grown with and without 4 μg/ml indolicidin, and luminescence production was determined. This permitted the identification of an intragenic transposon mutation, located within the response regulator-encoding gene PA1799 (Table 1), which rendered the cells unresponsive to *arnB* induction by indolicidin (Fig. 1A); no other mutants showed any effect on peptide-mediated induction. The same unresponsive phenotype could be observed in the cognate sensor kinase-encoding mutant UW-PA1798. The transposon mutations were then transferred into a new *P. aeruginosa* PAO1 background corresponding to our routine laboratory wild-type (WT) strain H103 (Table 1), resulting in new PA1798 and PA1799 mutant strains that were used for all subsequent experiments in this paper. These genes will hereafter be referred to as *parR* (PA1799) and *parS* (PA1798) for peptide adaptive resistance regulator and sensor, respectively.

The *parR* and *parS* genes are predicted to be cotranscribed according to the database of prokaryotic operons (34) and their sequential location in the *P. aeruginosa* PAO1 genome (Fig. 1B). Analysis of the amino acid sequence of ParS with program SOSUI 1.11 (25) revealed the presence of two putative transmembrane domains (residues 8 to 30 and 132 to 154) flanking a periplasmic loop (residues 28 to 134), which probably corresponds to the sensor domain of the protein. The charge of this periplasmic loop at pH 7.0 was estimated to be −6.6, using the program Protein Calculator v3.3 (http://www.scripps.edu/~cdputnam/protcalc.html), a result consistent with a putative function in polycation binding.

Given that the best-characterized adaptive peptide resistance mechanism thus far is that of *Salmonella*, the sequence of *parR* was compared with the *Salmonella enterica* serovar Typhimurium genome, using the tool BLAST Microbial Genomes from the National Center for Biotechnology Information website. The results showed that, among the *P. aeruginosa* response regulators, ParR had the highest similarity to *Salmonella* PhoP (51%); however, the highest homologue of ParR was actually the response regulator RstA (62% similarity). Likewise, protein ParS is 58% similar to the *S. enterica* sensor kinase RstB. This resemblance only concerned the intracellular domain, whereas no significant similarity could be found between the sensor domain of ParS and any protein in the *Salmonella* genome.

Mutations in *parR* and *parS* affect peptide-mediated expression of *arnB*, but not induction in low Mg^{2+}. The plasmid pUCP*lux*-P$_{PA3552}$ was transformed into the *parR* and *parS* mutants, and the production of luminescence under different conditions was assayed. The induction of this operon by indolicidin and low Mg^2 was tested in the *parR* and *parS* mutants, the WT, and a complemented *parR* mutant [*parR*-Tn7 (*parRS*⁺)]. Unlike the WT, neither mutant showed a significant induction of reporter luminescence in the presence of indolicidin (Fig. 2A). This phenotype could be complemented by the introduction of a single copy of the WT *parRS*⁺ operon into the chromosome of the *parR* mutant by means of mini-Tn7 integration (6) (Fig. 2A).

However, there was no significant difference in luminescence production among the four strains when incubated under inducing low-Mg^{2+} or noninducing high-Mg^{2+} conditions (Fig. 2A). This indicated that the upregulation of the *arn* LPS modification operon mediated by *parRS* was a specific response to the presence of the antimicrobial peptide.

Next, the ability of different peptides to induce the LPS modification operon in the WT and the *parR* mutant strain was determined. The peptides tested were indolicidin, polymyxin B, colistin, CP28, polyphemusin, and LL-37 (see Table 2 for peptide sequences). The aminoglycoside tobramycin was also assayed as a negative control, as it is known that it does not upregulate the *arn* operon, even though LPS modification increases resistance to aminoglycosides (36). First, the concentration that elicited the highest increase in luminescence production relative to the nonantibiotic control was determined. Of note, the upregulation caused by doses around normally inhibitory concentrations could be tested because the inoculum size was much higher than that used for routine MIC deter-
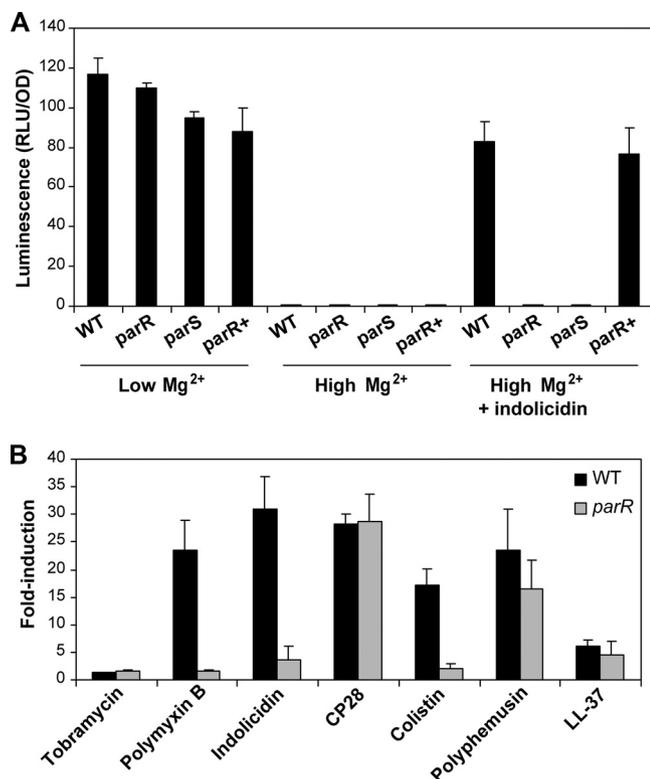
FIG. 2. ParR-ParS mediates the upregulation of the LPS modification operon in response to certain antimicrobial peptides. (A) Induction of pPA3552::*lux* fusion in bacteria grown in BM2-glucose minimal medium supplemented with 20 μM Mg^{2+}, 2 mM Mg^{2+}, or 2 mM Mg^{2+} and 4 μg/ml indolicidin. The WT (H103), *parR* and *parS* mutants, and the complemented *parR* mutant were studied. The pPA3552::*lux* fusion contained a promoterless *lux* cassette fused to the promoter of the *arnB* gene and reported on the transcription of the eight-gene operon encoding the *arn* LPS modification operon. Luminescence is shown in relative light units per optical density (RLU/OD). The results provided correspond to one representative experiment of at least four independent assays which displayed the same trends. (B) Fold induction of pPA3552::*lux* fusion in the WT (H103) and the *parR* mutant grown in BM2-glucose minimal medium supplemented with 2 mM Mg^{2+} and different antimicrobial compounds. The values shown here correspond to the means plus standard deviations (error bars) of four independent experiments at the concentration of each antibiotic leading to the highest induction level. These antibiotics and concentrations are as follows: tobramycin, 0.5 μg/ml; polymyxin B, 1 μg/ml; indolicidin, 4 μg/ml; colistin, 1 μg/ml; CP28, 3 μg/ml; polyphemusin, 2 μg/ml; and LL-37, 32 μg/ml.

mination. The effect of the selected concentrations on the WT and the mutant was then assessed (Fig. 2B). As previously indicated by McPhee et al. (36), not all peptides were able to induce upregulation of the *arn* genes involved in the adaptive response to the same degree (Fig. 2B). The strongest inducer of luminescence in the WT was indolicidin at a concentration of 4 μg/ml, leading to a 31-fold increase. Conversely, only a 4-fold increase could be observed in the *parR* mutant. The peptides of bacterial origin, polymyxin B and colistin, showed patterns similar to that of indolicidin, although the degree of induction in the WT was marginally lower. The presence of 1 μg/ml of polymyxin B or colistin induced the expression of the *arnB* promoter in the WT by 23- and 17-fold, respectively, whereas the increase in the mutant was negligible. Intriguingly,

the results obtained with the α-helical peptide CP28 seem to indicate that there could be distinct regulatory pathways for different peptides, since both the WT and the mutant experienced an upregulation of about 28-fold when 3 μg/ml CP28 was added to the medium. In a similar manner, a concentration of 2 μg/ml polyphemusin induced 24- and 17-fold in the WT and the *parR* mutant, respectively. According to this, ParR-ParS was not necessary to detect and respond to these two peptides. The human peptide LL-37 did not trigger a major response in either strain even at a concentration of 32 μg/ml, with only 6- and 4-fold increases in expression of the *arn* operon in the WT and the *parR* mutant, respectively (Fig. 2B). Statistical comparison of the data corresponding to the WT and the mutant with Student's *t* test confirmed the existence of differential induction in the *parR* mutant by indolicidin, polymyxin B, and colistin (*P* values < 0.05), whereas the relative induction values found for CP28, polyphemusin, LL-37, and tobramycin were not significantly different between the *parR* mutant and its parent strain (*P* values > 0.05).

The *parR* and *parS* mutants display reduced adaptive resistance to polymyxin B. Killing curves were carried out in order to investigate the adaptive and intrinsic polymyxin B resistance of the *parR* mutant. To prevent the stimulatory effect of a low Mg^{2+} concentration (20 μM) on peptide resistance, the cells were grown in BM2-glucose medium with a high Mg^{2+} concentration (2 mM) before they were killed with polymyxin B. Additionally, the medium was supplemented with subinhibitory indolicidin (4.0 μg/ml) to test adaptive resistance. Under noninducing conditions (high Mg^{2+} alone), the *parR* mutant and the WT exhibited similar levels of killing with 1 μg/ml polymyxin B, approximately 3 log units in 10 min (Fig. 3A). The results obtained in the adaptive resistance assay were, however, quite different for the two strains. After preincubation in the presence of indolicidin, the WT cells showed only 1 log unit of killing within 10 min when challenged with 10 μg/ml polymyxin B, which indicates that they had become adaptively resistant (Fig. 3B). In contrast, the *parR* mutant was supersusceptible to polymyxin B under the same conditions, with 4-log-unit reduction in survivors after 10 min of killing (Fig. 3B). Adaptive peptide killing assays performed with the complemented strain, the *parR* (Tn7-*parRS*$^+$) strain, showed levels of adaptive resistance equivalent to those of the WT (Fig. 3B). This indicates that the *parRS* genes are required for the adaptive peptide resistance response in *P. aeruginosa*.

Susceptibility of *parRS* mutants to different classes of antibiotics. MICs were determined to establish whether the *parR* and *parS* mutants presented increased susceptibility or resistance to different classes of drugs. As can be seen in Table 3, both mutant strains displayed supersusceptibility (4-fold) toward polymyxin B and colistin compared to the WT in BM2-glucose medium with high Mg^{2+}. No difference could be observed under these conditions with any of the other antibiotics tested, including antimicrobial peptides (indolicidin, CP11CN, CP26, CP28, Bac2A, bactenecin, LL-37, and polyphemusin), aminoglycosides (tobramycin and gentamicin), quinolones (ciprofloxacin), and β-lactams (ceftazidime) (Table 3).

To explain these data, we hypothesized that the presence of certain concentrations of polymyxin B in MIC assay wells can elicit an adaptation response in the WT, but not in the mutants. To test this, WT cells were grown in different concen-
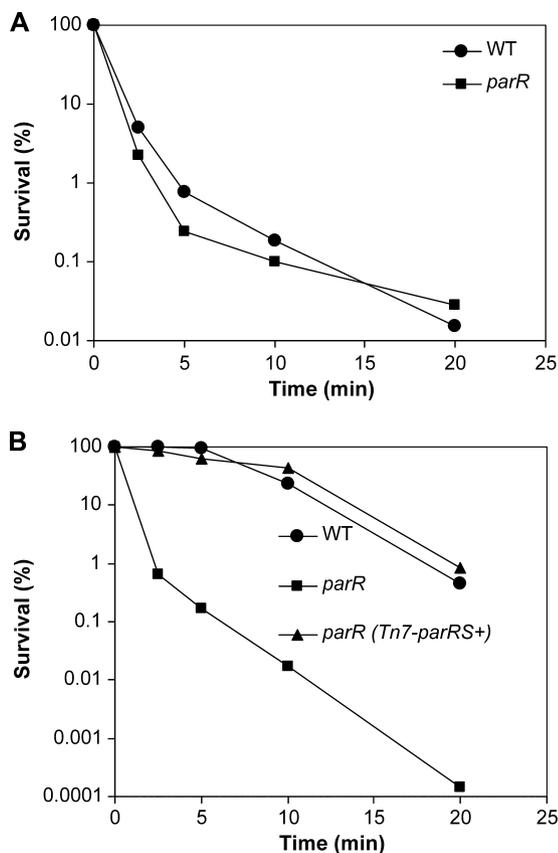
FIG. 3. Comparison of intrinsic (A) and adaptive (B) polymyxin B resistance in the WT and *parR* mutant. Sensitivity was analyzed by means of killing curves. Cells from the different strains were grown to mid-log phase in BM2-glucose minimal medium with 2 mM Mg^{2+} (A) or the same medium supplemented with 4 μg/ml indolicidin (B) and exposed to 1 μg/ml (A) or 10 μg/ml (B) of polymyxin B. Diluted aliquots were then plated to assay for survivors. The results presented correspond to one representative experiment for each condition out of four independent experiments which produced identical trends.

trations of polymyxin B (0.125, 0.25, and 0.5 μg/ml) and then challenged with a lethal dose (1 μg/ml) of the same antibiotic. The results obtained showed that these concentrations of polymyxin B made the cells more resistant to the killing effect of the same compound in a dose-dependent manner (data not shown). This hypothesis also agrees with the data obtained in the luminescence assay (Fig. 2B). Also, when the MICs were determined for cells grown in low-Mg^{2+} conditions, both mutant strains underwent an increase in resistance to polymyxin B equivalent to that of the WT, while remaining 4-fold more susceptible (Table 3), indicating that this difference in MIC was insensitive to low-Mg^{2+} regulation.

Since the ParR-ParS system seemed to be specifically required for peptide-induced adaptive resistance, the MICs in the presence of subinhibitory indolicidin (4.0 μg/ml) (adaptive MICs) were also determined. The same concentration of indolicidin was added to the medium during growth of the culture used as inoculum for the MIC test. The difference in MICs between the WT or *parR* mutant complemented with a single copy of the *parRS* operon and the *parR* or *parS* mutant in-

creased from 4-fold in the absence of indolicidin to 64-fold, a result that reflected in part peptide-induced adaptive resistance. Interestingly, while indolicidin increased by 8-fold the polymyxin B resistance of the WT and complemented mutant, as expected due to induction of adaptive resistance, the *parR* and *parS* mutants showed a slight 2-fold increase in susceptibility (Table 3). This was likely due to interactions between indolicidin and polymyxin B, as these antibiotics demonstrated an average fractional inhibitory concentration index (FICi) of 0.127 ± 0.033 over four independent experiments. Presumably, in the WT and complemented strains, the induction of polymyxin B resistance by subinhibitory indolicidin masked any synergy between the two peptides. Adaptive MICs to colistin followed trends identical to those observed for polymyxin B (Table 3).

The adaptive MICs of other peptides were also determined with various results (Table 3). For example, the activity of CP28 was not altered by the presence of indolicidin or by the

TABLE 3. MICs of different antibiotics toward *P. aeruginosa* WT, *parR* and *parS* mutants, and complemented *parR*/Tn7-*parRS*⁺ strain[a]

Medium and antibiotic	MIC (μg/ml) (mode) in:			
	WT	*parR* mutant	*parS* mutant	*parR*/Tn7-*parRS*⁺ strain
Minimal medium with high Mg^{2+} (2 mM)				
Polymyxin B	1	0.25	0.25	1
Colistin	1	0.25	0.25	1
Indolicidin	64	64	64	64
CP11CN	64	64	64	64
CP26	32	32	32	32
CP28	3	3	3	3
Bac2A	128	128	128	NT[b]
Bactenecin	128	128	128	NT
LL-37	16	16	16	16
Polyphemusin	2	2	2	2
Tobramycin	2	2	2	2
Gentamicin	2	2	2	NT
Ceftazidime	1	1	1	1
Ciprofloxacin	0.1	0.1	0.1	0.1
Minimal medium with high Mg^{2+} (2 mM) and supplemented with 4 μg/ml indolicidin[c]				
Polymyxin B[c]	8	0.125	0.125	8
Colistin[c]	8	0.125	0.125	8
CP28[c]	3	3	3	3
LL-37[c]	64	16	16	64
Polyphemusin[c]	0.5	0.25	0.25	0.5
Tobramycin[c]	4	2	2	4
Gentamicin[c]	16	2	2	NT
Minimal medium with low Mg^{2+} (20 μM)				
Polymyxin B	16	4	4	16

[a] MICs were determined by serial 2-fold dilutions in BM2-glucose minimal medium with 2 mM Mg^{2+}, unless indicated otherwise. The MIC represents the concentration at which no growth was visually observed after 24 h of incubation at 37°C. The values shown are the modes of 4 to 8 independent experiments.
[b] NT, not tested.
[c] The cultures used to inoculate the MIC plates were also grown in the presence of indolicidin.

TABLE 4. Selected genes from the microarray analysis of genes expressed differently in the presence of 4 μg/ml indolicidin in the *parR* mutant compared to the WT[a]

GeneID	Gene name	Fold change[b]	*P* value	Description
PA0282	*cysT*	−2.3	0.03	Sulfate transport protein CysT
PA0517	*nirC*	2.7	0.04	Probable *c*-type cytochrome precursor
PA0523	*norC*	6.0	0.01	Nitric oxide reductase subunit C
PA0524	*norB*	10.7	0.009	Nitric oxide reductase subunit B
PA1559		−4.2	<0.001	Hypothetical protein
PA1560		−2.9	<0.001	Hypothetical protein
PA1797		−3.3	<0.001	Hypothetical protein
PA3392	*nosZ*	2.1	0.04	Nitrous oxide reductase precurser
PA3396	*nosL*	2.0	0.04	NosL protein
PA3446		−5.8	0.0007	Hypothetical protein
PA3552	*arnB*	−1.6	0.0009	Hypothetical protein
PA3553	*arnC*	−2.0	<0.001	Probable glycosyltransferase
PA3554	*arnA*	−2.5	<0.001	Hypothetical protein
PA3555	*arnD*	−2.9	<0.001	Hypothetical protein
PA3556	*arnT*	−2.1	0.003	Inner membrane L-Ara4N transferase ArnT
PA3559	*pmrE*	−3.3	<0.001	Probable nucleotide sugar dehydrogenase
PA3920		2.3	0.0059	Membrane proteins/transport of small molecules
PA4661	*pagL*	−2.5	<0.001	Lipid A 3-*O*-deacylase
PA4773		−5.1	<0.001	Hypothetical protein
PA4774		−7.4	<0.001	Hypothetical protein
PA4775		−3.3	<0.001	Hypothetical protein
PA4776	*pmrA*	−2.1	0.003	Two-component system response regulator
PA4777	*pmrB*	−1.7	0.002	Two-component system signal sensor kinase
PA4782		−6.7	<0.001	Hypothetical protein

[a] The full list of dysregulated genes is given in Table S1 in the supplemental material.
[b] Fold change in gene expression in the presence of 4 μg/ml indolicidin in the *parR* mutant compared to gene expression in the presence of 4 μg/ml indolicidin in the WT.

mutation of *parRS*, as the MIC values were the same for all strains under inducing and noninducing conditions. For the human cathelicidin LL-37 as well as the aminoglycosides tobramycin and gentamicin, the WT cells became 2- to 8-fold more resistant when indolicidin was present in the medium (Table 3), and this response was abolished in the *parR* and *parS* mutants (Table 3). The presence of indolicidin led to 4- and 8-fold-increased polyphemusin susceptibility in the WT or complemented strain and the *parR* and *parS* mutants, respectively (Table 3). This could be explained by interaction assays showing synergy between polyphemusin and indolicidin with an average FIC of 0.057 ± 0.009 over four independent experiments.

Transcriptional analysis. Microarrays were performed in order to compare the transcriptional responses of the *parR* mutant and the WT in the presence of a subinhibitory concentration of indolicidin (4 μg/ml). Under these conditions, the mutant exhibited an altered expression (≥2-fold) of 114 genes, of which 66 were upregulated and 48 were downregulated. A list of selected genes from the microarray can be seen in Table 4 (for the full list, see Table S1 in the supplemental material). The microarray data confirmed that the expression of the LPS modification operon in the presence of indolicidin was lower in the mutant than in the WT, consistent with the luminescence assay results. In addition to induction of the LPS modification operon, which is also induced under low-Mg^{2+} conditions, further overlap with the Mg^{2+} regulon could be observed (35). Thus, the following genes known to be dysregulated under Mg^{2+} limitation, PA0282, PA1559-1560, PA3446, PA3920, PA4773-4775-*pmrAB*-PA4778, and PA4782 (35), were found here to be downregulated in the *parR* mutant. Furthermore, all

but PA3920 are positively regulated by PmrA-PmrB (35). The operon containing *pmrAB* is known to be modulated by both Mg^{2+} and the presence of peptides (36), and PmrA-PmrB is able to induce the expression of the LPS modification operon when Mg^{2+} is limiting in the medium, leading to increased resistance to peptides. However, peptide regulation of the *arn* and PA4774-*pmrAB* operons occurs, at least in part, independently of PmrA-PmrB (or PhoP-PhoQ) (36). Therefore, the overlaps between the PmrA-PmrB and ParR-ParS regulons might reflect similar DNA binding site motifs as well as action through peptide regulation of PmrA-PmrB.

Other genes of note included downregulated *pagL*, which encodes a lipid A 3-*O*-deacylase situated in the outer membrane. This finding is particularly relevant, as the presence of a specific LPS structure, deacylated at position 3, was previously demonstrated in isolates from infants with cystic fibrosis (CF) (11). The PA1797 gene, located just downstream of the *parRS* operon (but not in the *parRS* operon itself), was transcribed at a lower level in the *parR* mutant than in the WT. The product of the PA1797 gene is predicted to be a lipoprotein (30) of unknown function, which shares similarity with β-lactamases according to PFAM prediction. It is also worth mentioning that several genes involved in the denitrification route, *nirC*, *norC*, *norB*, *nosZ*, and *nosL*, were upregulated in the *parR* mutant compared to the WT, as some of these genes are known to be involved in aminoglycoside susceptibility (45).

Microarray data often underestimate the extent of dysregulation due to the very low concentrations of nucleic acids used for hybridization. To validate the data obtained in the microarray, the involvement of the Par system and indolicidin in the regulation of several genes (PA1559, PA1797, *arnB*, *pagL*,

TABLE 5. RT-qPCR analysis of the expression of selected genes in the *parR* mutant and WT cells grown in BM2-glucose minimal medium with or without indolicidin peptide

Gene	Fold change (mean ± SD) in gene expression[a]			
	WT/*parR* mutant		WT with indolicidin/ WT without indolicidin	*parR* mutant with indolicidin/ *parR* mutant without indolicidin
	With indolicidin	Without indolicidin		
PA1559	16.46 ± 6.13	NT[b]	NT	NT
PA1797	114.8 ± 50.01	1.56 ± 0.70	25.3 ± 5.8	0.38 ± 0.26
PA1799 (*parR*)	0.66 ± 0.08	NT	1.05 ± 0.015	NT
PA3552 (*arnB*)	16.34 ± 7.32	NT	NT	NT
PA4661 (*pagL*)	5.26 ± 0.53	1.33 ± 0.44	4.06 ± 0.64	1.00 ± 0.20
PA4774	23.34 ± 14.66	NT	NT	NT
PA4777 (*pmrB*)	4.88 ± 1.86	1.13 ± 0.50	2.27 ± 0.39	0.54 ± 0.24
PA4782	8.73 ± 1.92	NT	NT	NT

[a] Fold change in gene expression comparing gene expression in the WT to gene expression in the *parR* mutant (WT/*parR* mutant) in the absence and presence of 4 μg/ml indolicidin or comparing gene expression in the presence and absence of 4 μg/ml indolicidin in the WT or *parR* mutant.
[b] NT, not tested.

PA4774, *pmrB*, and PA4782) was confirmed using the more accurate method of reverse transcription-quantitative PCR (RT-qPCR) (Table 5). These genes were found to be suppressed between 5- and 100-fold in the *parR* mutant, indicating that this regulator and indolicidin were involved in the activation of gene expression; indeed, when the indolicidin peptide was omitted, there was no effect of *parR* on the expression of the three genes tested. Additionally, RT-qPCR results showed that *pmrB*, PA1797, and *pagL* were upregulated in the presence of indolicidin in the WT, but not in the *parR* mutant (Table 5). In contrast to these results, *parR* was not autoregulatory, as the expression of the *parRS* operon did not change in the presence of subinhibitory indolicidin in either the mutant or the WT (Table 5).

Polymyxin B MICs of selected *P. aeruginosa* mutants. After determining the genes that were differentially expressed in the *parR* mutant, we attempted to investigate which ones might be involved in the adaptive response to peptides. With that objective, polymyxin B intrinsic and adaptive MIC tests were performed with mutants carrying transposon insertions in selected genes from the microarray. As shown in Table 6, the PA1559 and *pagL* mutants were as resistant to polymyxin B as the WT was. Some differences could be seen, however, in the

PA1797, *pmrB*, and PA4782 mutants. The MICs of these mutants were similar to that of the WT under noninducing conditions, but the adaptive MICs of these strains were 4-fold lower for the PA1797 and *pmrB* mutants and 2-fold lower for the PA4782 mutant (Table 6), consistent with the possibility that these genes modulate the development of adaptive resistance. Strains with mutations in the *arn* LPS modification operon, specifically in the *arnB* and *arnC* genes, exhibited phenotypes similar to those of the *parR* and *parS* mutants (Table 6). This is consistent with the conclusion that the major effect of the *parRS* mutations was through the lack of induction of the LPS modification operon. Of note, none of the mutations in mutant strains with phenotypes different from that of the WT are predicted to have polar effects due to the transposon insertion.

DISCUSSION

A better understanding of the mechanisms involved in modulating antibiotic susceptibility inside the host is essential to achieve greater success in the battle against bacterial infections. This paper reports the identification of a two-component regulator, ParR-ParS, that plays a decisive role in the acquisition of peptide-induced adaptive resistance to antimicrobial peptides and aminoglycosides in *P. aeruginosa*.

Previous studies have demonstrated the involvement of PhoP-PhoQ and PmrA-PmrB in cationic peptide resistance in *P. aeruginosa* under Mg^{2+}-deficient growth conditions (36). In contrast, neither of these two-component regulators has a major influence when antimicrobial peptides are the activating signal (36). We showed here that while ParR-ParS is necessary for the induction of the LPS modification *arn* operon by polymyxins and the antimicrobial peptide indolicidin, it is not required for the activation of this operon under Mg^{2+}-limiting conditions. Hence, this regulator seems to be specific to adaptive resistance mediated by certain peptides. Our results show analogies but also striking differences with the situation in *S. enterica* serovar Typhimurium. In this organism, PhoP-PhoQ is able to sense both low Mg^{2+} and antimicrobial peptides and, through the upregulation of PmrA-PmrB, induces the expression of genes involved in the addition of aminoarabinose to

TABLE 6. Polymyxin B MICs of selected *P. aeruginosa* mutants[a]

Strain	MIC (μg/ml) (mode)	
	Without indolicidin	With indolicidin
WT	1	8
parR mutant	0.25	0.125
UW-PA1559	1	8
UW-PA1797	0.5–1[b]	1–2[b]
UW-*pagL*	1	8
UW-*pmrB*	0.5–1[b]	2
UW-PA4782	1	4
UW-*arnB*	0.25	0.25
UW-*arnC*	0.25	0.125

[a] MICs were determined by serial 2-fold dilutions in BM2-glucose minimal medium with 2 mM Mg^{2+} without indolicidin or with 4 μg/ml indolicidin. The MIC represents the concentration at which no growth was visually observed after 24 h of incubation at 37°C. Results are the mode of 5 or 6 independent experiments.
[b] The MICs varied over the 5 or 6 different experiments.

LPS. This, in turn, leads to an increase in polymyxin B resistance (12, 21). Thus, peptides interact with and activate the *Salmonella* sensor PhoQ by displacing the Mg^{2+} cations from their binding site in the sensor domain. Conversely, the sensor domain of *P. aeruginosa* PhoQ cannot substitute for that of *Salmonella* and does not bind peptides (1).

In *Pseudomonas*, it is clear that there are two Mg^{2+} sensory systems, PhoP-PhoQ and PmrA-PmrB, and at least one additional peptide sensor, ParS. Although the specific mechanism by which ParS senses peptides is still to be determined, it seems possible that the mechanism might be analogous to that of *Salmonella* PhoQ, since both proteins have a negatively charged sensor domain; however, the basis for the selectivity observed here remains to be elucidated.

The closest homologues of ParR and ParS found in the *Salmonella* genome correspond to RstB and RstA, respectively. Nevertheless, the periplasmic sensor domain of ParR does not share any significant similarity with that of RstA or with any other protein from *S. enterica*. This emphasizes the differences between the specific mechanisms by which these two microorganisms sense peptides, even though the triggered responses are analogous. In *Salmonella*, RstB-RstA is activated by PhoP-PhoQ in low Mg^{2+} and at acidic pH, and it upregulates the expression of iron acquisition genes like *feoAB* (4). A similar regulatory hierarchy between PhoP-PhoQ and RstB-RstA was found in *Escherichia coli* (9). In contrast, expression of the *parRS* operon is not under the control of the *P. aeruginosa* PhoP-PhoQ counterparts, as indicated by previous microarray analysis (35).

The abilities of *P. aeruginosa* to sense and respond to the clinically important antibiotics polymyxin B and colistin are dependent on ParR-ParS. This finding is relevant in a clinical setting, since colistin is one of the antibiotics used in the treatment of CF patients (27). The fact that *Pseudomonas* exposure to polymyxins can induce increased resistance to selected antimicrobial peptides and aminoglycosides is of particular concern. The ability of polymyxin B to upregulate the *pmrAB* operon was first demonstrated by McPhee et al. (36). More recently, it was shown that colistin can upregulate the *arn* operon in a subpopulation of biofilm cells and induce tolerance (22, 38). The human cathelicidin LL-37 was, however, a weak inducer of the LPS modification operon. In fact, the transcriptional responses that occurred in the presence of this host defense peptide do not include the *arn* operon or any of the PmrA-dependent genes upregulated by indolicidin (37). Surprisingly, not all antimicrobial peptides seem to require the participation of ParR-ParS in order to induce the LPS modification operon. A clear example is the upregulation of the *arnB* promoter by CP28 and polphemusin. According to this, it appears that different peptides and/or different concentrations of a peptide may activate distinct sensing mechanisms corresponding to convergent regulatory pathways. The nature of the alternative sensor/regulator is yet to be identified, although the partial involvement of PhoQ and/or PmrB cannot be discarded.

The data gathered in this study indicate that ParR-ParS contributes exclusively to adaptive resistance to peptides and aminoglycosides, but not to intrinsic resistance. Among peptides, polymyxins are affected the most by this mechanism of adaptive resistance. Some host defense peptides, like LL-37

and polphemusin, show some modest effects, whereas the action of the α-helical peptide CP28 is independent of this bacterial adaptive response. We also found evidence for very strong synergistic interactions between some antimicrobial peptides, particularly polphemusin and indolicidin. This suggests that it might be possible to counteract the effects of adaptive resistance by designing therapeutic programs based on combinations of peptides with synergistic action. Moreover, it would be of particular interest to develop peptides that exhibit such an interaction with human host defense peptides. The presence of a sublethal concentration of indolicidin increased the resistance to the tobramycin and gentamicin aminoglycosides in a ParR-ParS-dependent manner. This effect is probably due to a reduced ability of aminoglycosides, which are cationic molecules, to interact with the more positively charged cell surface when LPS modification is present. The fact that sublethal doses of certain peptides can induce *Pseudomonas* cells to become more resistant not only to other peptides but also to aminoglycosides is quite alarming, as both types of antibiotics are used in the treatment of CF patients. On the other hand, awareness of this phenomenon will be advantageous so that more efficient therapeutic programs can be implemented.

The transcriptome of the *parR* mutant in the presence of a subinhibitory concentration of indolicidin was substantially different from that of the WT under the same conditions. Although ParR-ParS does not influence the response to limiting Mg^{2+}, a significant number of genes that can be upregulated at low Mg^{2+} concentrations were actually downregulated in the *parR* mutant compared to the WT (35). These genes include the *arn* LPS modification operon (PA3552-3559), the operon containing *pmrAB* (PA4773-4777), PA0282, PA1559-1560, PA3446, PA3920, and PA4782. Most of these overlapping genes are under the control of PmrA-PmrB and have typical PmrA recognition sites in their promoters (35). McPhee et al. (35) also demonstrated that many of these genes are induced in the presence of subinhibitory CP11N, a peptide related to indolicidin. Lipid A modifications, including aminoarabinose addition, have been observed in isolates from cystic fibrosis patients (14, 13). These modifications can be induced *in vitro* under low Mg^{2+} conditions (36), but to date, all the information available points toward other host factors, such as peptides, as being responsible for the appearance of these modifications during infection. The results in this study suggest that ParS could be one protein capable of sensing the presence of antimicrobial peptides during infection.

The *pagL* gene encodes a deacylase involved in LPS modification. Lipid A 3-O deacylation and palmitoylation reduce the ability of lipid A to activate the host Toll-like receptor 4, suggesting that these modifications help pathogens to avoid innate immune recognition (29). The expression of *pagL* in *S. enterica* is activated directly by PhoP-PhoQ (48). It has been shown recently that in *S. enterica pmrA* and *pmrE* mutants, the protein PagL is released from a latency state and compensates for the loss of inducible polymyxin B resistance (28). In *P. aeruginosa*, *pagL* is not part of the PhoP-PhoQ regulon (35), and it is upregulated by ParR-ParS in the presence of subinhibitory concentrations of peptides. The *pagL* gene is constitutively expressed in isolates from CF patients and in laboratory-adapted strains, but not in isolates from environmental

samples or from patients with acute infections (11). Our current data, however, indicate that *pagL* is not necessary for peptide-induced resistance.

Despite the importance of ParR-ParS in modulating transcriptional changes that occur in the presence of subinhibitory indolicidin, the *parRS* operon itself is not regulated by this peptide. Nor is its expression altered by the lack of a functional ParR product. On the other hand, the PA1797 gene, located downstream of but transcribed independently of the *parRS* operon, is significantly induced by indolicidin in a ParR-dependent manner. Moreover, a PA1797 mutant seems to have a reduced ability to acquire adaptive resistance compared to the WT. It is tempting to speculate that the product of PA1797 might play an intermediary role in the cascade initiated by ParR-ParS or that its product has some direct function in resistance. However, the specific function of the PA1797 product still needs to be elucidated. Even though PA1797 is not dysregulated during growth under low-Mg^{2+} conditions or in a PhoP mutant (35), its expression is induced 4-fold in a *phoQ* mutant (18). It would be interesting to determine whether this reflects the existence of some kind of interaction between PhoP-PhoQ and ParR-ParS.

In conclusion, the data presented here constitute a substantial step forward toward understanding adaptive resistance to polymyxin B and cationic antimicrobial peptides in *P. aeruginosa*. A greater insight into the molecular mechanisms involved will be essential for the optimum design of new peptides for therapeutic purposes. In that sense, it will be important to evaluate the ability of specific peptides to induce adaptive resistance in order to select those that do not significantly trigger this response. Neglect to do so would lead to decreased susceptibility to subsequent treatment with peptides or aminoglycosides. In addition, our results indicate that adaptive resistance can be counteracted in some cases by taking advantage of synergistic interactions between peptides. Thus, the identification of *parRS* is of great significance within this context.

ACKNOWLEDGMENTS

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