High-Level Pacidamycin Resistance in *Pseudomonas aeruginosa* Is Mediated by an Opp Oligopeptide Permease Encoded by the *opp-fabI* Operon

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Pacidamycins (or uridyl peptide antibiotics) possess selective *in vivo* activity against *Pseudomonas aeruginosa*. An important limitation for the therapeutic use of pacidamycins with *P. aeruginosa* is the high frequency (10^{-6} to 10^{-7}) at which resistant mutants emerge. To elucidate the mechanism(s) of this resistance, pacidamycin-resistant *P. aeruginosa* mutants were isolated. Two types of mutants were obtained. Type 1, or high-level resistance mutants with a pacidamycin MIC of 512 μg/ml, were more abundant, with a frequency of $\sim 2 \times 10^{-6}$, and did not show cross-resistance with other antibiotics. Type 2, low-level resistance mutants, were isolated with a frequency of $\sim 10^{-8}$ and had a pacidamycin MIC of 64 μg/ml (the MIC for the wild-type strain was 4 to 16 μg/ml). These mutants were cross-resistant to levofloxacin, tetracycline, and erythromycin and were shown to overexpress either the MexAB-OprM or MexCD-OprJ multidrug resistance efflux pumps. High-level resistant mutants were isolated by transposon mutagenesis and one insertion was localized to *oppB*, one of two periplasmic binding protein components of an oligopeptide transport system which is encoded by the *opp-fabI* operon. The Opp system is required for uptake of pacidamycin across the inner membrane, since various *opp*, but not *fabI*, mutants were resistant to high levels of pacidamycin. Both of the two putative Opp periplasmic binding proteins, OppA and OppB, were required for pacidamycin uptake. Although both impaired uptake into and efflux from the cell can cause pacidamycin resistance in *P. aeruginosa*, our data suggest that impaired uptake is the primary reason for the high-frequency and high-level pacidamycin resistance.

Pacidamycins are uridyl peptide antibiotics that were first discovered in 1989 (1–3) (pacidamycin 4, used in this study, is shown in Fig. 1). Since that time, at least 10 related compounds have been reported. Despite their discovery over 2 decades ago, the biosynthetic gene clusters for pacidamycin biosynthesis were only recently identified and characterized in *Streptomyces coeruleorubidus* (4, 5). The pacidamycins have an unusual spectrum of antibacterial activity. While they possess *in vivo* activity against *Pseudomonas aeruginosa*, one of the organisms most refractory to antibacterial therapy, they are not active against many other bacteria, for example, *Escherichia coli* and *Staphylococcus aureus* (2). Pacidamycins target MraY, or translocase I, an essential enzyme in peptidoglycan biosynthesis in most Gram-positive and Gram-negative bacteria (6). Located on the cytoplasmic face of the inner membrane, MraY catalyzes the formation of the first lipid intermediate, undecaprenylpyrophosphate-*N*-acetylmuramyl-pentapeptide (or lipid I), in cell wall synthesis. Although *E. coli* and *S. aureus* are intrinsically resistant to pacidamycin, MraY purified from either bacterium can be efficiently inhibited *in vitro* by pacidamycin (6). High-level intrinsic pacidamycin resistance in these bacteria could be explained by lack of uptake of the peptide antibiotic, efficient extrusion via efflux pumps, or a combination of these mechanisms. The intrinsic resistance of *E. coli* to mureidomycins was previously attributed to efflux by the AcrAB-TolC pump (7, 8). A significant limitation for the therapeutic use of pacidamycins with *P. aeruginosa* is the high frequency (10^{-6} to 10^{-7}) at which resistant mutants emerge. In this paper, we report that both impaired uptake into and efflux from the cell are pacidamycin resistance determinants in this bacterium. However, the primary reason for high-frequency and high-level resistance is loss of uptake into the cell due to mutations in the *opp* operon encoding an oligopeptide transport system.

(Portions of this work were previously presented in part as a poster at the 101st General Meeting of the American Society for Microbiology, Orlando, FL, 20 to 24 May 2001 [9].)

MATERIALS AND METHODS

Bacterial strains and media. *Pseudomonas aeruginosa* strains used in this study are listed in Table 1. *E. coli* strains used for cloning were DH5α (15) and HPS1 (16). Bacteria were generally cultivated in Lennox LB broth base or agar (Life Technologies, Grand Island, NY). Other growth media used in this study were super optimal broth (SOB) (17), peptone tryptic soy broth (PTSB) (18), and M9 minimal medium (28) supplemented with 1% Difco Casitone (Becton, Dickinson and Company, Franklin Lakes, NJ). For plasmid maintenance, media were supplemented with 100 μg/ml of ampicillin or 15 μg/ml gentamicin for *E. coli* and 200 μg/ml carbenicillin or 30 μg/ml gentamicin for *P. aeruginosa*, unless noted otherwise.

MIC determinations. Antimicrobial susceptibilities were assessed by determining MICs using the 2-fold broth microdilution method by following Clinical and Laboratory Standards Institute guidelines (20).
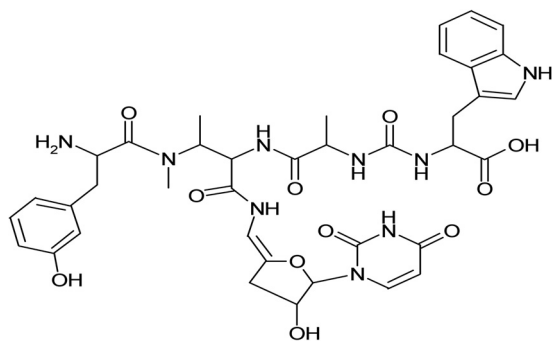
FIG 1 Structure of the uridylpeptide antibiotic pacidamycin 4 employed in this study.

Selection of pacidamycin-resistant mutants. Spontaneous pacidamycin-resistant mutants of wild-type strain PAO1 (PAM1020) were selected on LB agar plates containing pacidamycin at either 50 μg/ml (4× MIC) or 200 μg/ml (16× MIC) using overnight cultures grown in LB. The potential efflux-mediated multidrug resistance phenotype was assessed by replica plating on LB plates containing levofloxacin at 0.5 μg/ml (4× MIC). Levofloxacin was used since it is a substrate of major Mex pumps from *P. aeruginosa* (12). Pacidamycin-resistant mutants that were also resistant to levofloxacin were selected on plates contacting pacidamycin at 50 μg/ml. Such mutants were much less (50- to 100-fold) frequent than mutants resistant to pacidamycin alone. In order to carefully estimate the frequency of emergence of pacidamycin-resistant mutants that exhibited a multidrug resistance phenotype, such mutants were also selected directly on plates containing both pacidamycin (at 50 μg/ml) and levofloxacin (at 0.5 μg/ml). Frequency of resistance emergence was calculated as a ratio of viable colonies from antibiotic-containing and antibiotic-free plates.

Transposon mutagenesis and transduction. Transposon mutagenesis of PAM1020 to identify pacidamycin resistance mutants was performed with the mini-D3112 bacteriophage transposable element mini-D171 (encoding tetracycline resistance) (21). Mutants were selected on LB agar plates containing 100 μg/ml tetracycline and 200 μg/ml pacidamycin. Transductions into *P. aeruginosa* recipient strains were performed with phage F116L (22).

Inverse PCR. Inverse PCR was used to localize mini-D171 insertions resulting in pacidamycin resistance. Chromosomal DNA was isolated from the mutant PAM1194 (PAM1020 *pac*::TET-16), digested with HindIII, ligated, and used as a template for PCR with primer 1 (primer sequences are shown in Table 2), which anneals close to the left end of mini-D171), and primer 2, annealing upstream of the HindIII site in mini-D171. The resulting ~600-bp PCR fragment was cloned into pCR2.1 (Life Technologies) and sequenced using M13 reverse and M13 forward (−40) primers.

PAβN uptake assay. Opp transport activity was assessed utilizing Phe-Arg-β-naphthylamide (PAβN) as the substrate. PAβN is nonfluorescent in solution but is cleaved enzymatically inside cells to produce highly fluorescent β-naphthylamine (23). Since PAβN is effluxed via the MexAB-OprM system, mutants defective in this system were used for the uptake assays. Cultures of *P. aeruginosa* were grown in LB broth to an optical density (at 600 nm) of ~1, washed, and resuspended in 50 mM K_2HPO_4 (pH 7.0), 1 mM $MgSO_4$, 0.4% glucose. Assays were performed in 96-well plates in a final volume of 200 μl and were initiated by addition of 250 μg/ml PAβN (Sigma, St. Louis, MO) to suspensions of intact cells. Fluorescence was measured using excitation at 320 nm and emission at 460 nm.

Construction of chromosomal *opp* mutants by allelic exchange. Using standard methods for PCR amplification of GC-rich templates (including 5% [vol/vol] dimethyl sulfoxide [DMSO]) and *Taq* DNA poly-

merase (Life Technologies), a 1,745-bp *oppE* gene fragment was PCR amplified from ~100 ng of PAO1 genomic DNA using primers *oppE*#1 and *oppE*#2, which introduced SacI sites flanking the *oppE* gene fragment. Cycle conditions were an initial 5 min of denaturation at 96°C, followed by 35 cycles of 96°C for 45 s, 60°C for 45 s, 72°C for 1 min, and a final extension step for 10 min at 72°C. The resulting 1,745-bp fragment was cloned into the TA cloning vector pCR2.1 to form pOPPE1. A plasmid-borne *oppE* insertional mutant was generated by ligation of a 853-bp gentamicin resistance (GENʳ) cassette from pPS856 (14) into the single SmaI site located within the *oppE* gene fragment contained on pOPPE1. This step generated pOPPE2, whose *oppE*::GENʳ fragment was then cloned into the gene replacement vector pEX18Ap (14) to yield pOPPE3. For transfer to the *P. aeruginosa* chromosome, pOPPE3 was transformed into *E. coli* mobilizer strain SM10 (24) and conjugally transferred to PAO1, and gentamicin-resistant merodiploids were selected on VBMM plates containing 30 μg/ml gentamicin (14). Merodiploids were resolved by sucrose counterselection, and the gentamicin resistance marker was excised from the chromosome using Flp recombinase as previously described (14) to yield the unmarked *oppE* mutant strain PAO315. Inser-

TABLE 1 *Pseudomonas aeruginosa* strains and plasmids used in this study

Strain/plasmid	Characteristic(s)	Source or reference
Strains		
PAO1	Prototype strain (PAM1020); obtained from N. Gotoh	10
PAO1-UW	Sequenced prototype PAO1 strain	11
PAM1032	*nalB* (MexAB-OprM up)	12
PAM1033	*nfxB* (MexCD-OprJ up)	12
PAM1034	*nfxC* (MexEF-OprN up)	12
PAM1043	*nalB*-type (MexAB-OprM up)	This study
PAM1044	*nfxB*-type (MexCD-OprJ up)	This study
PAM1135	PAO1 *pac-1*	This study
PAM1154	PAO1 *oprM*::Hgʳᵃ	This study
PAM1194	PAO1 *pac*::mini-D171ᵇ	This study
PAM1316	PAM1194 *oprM*::Hgʳ	This study
PAM1348	PAM1135 *oprM*::Hgʳ	This study
PAM2386	PAO1 *pac*::mini-D171ᵇ,ᶜ	This study
PAO235	PAO1 *fabI*::FRT	This study
PAO315	PAO1 *oppE*::FRT	This study
PAO449	PAO1 *oppA*::FRT	This study
PAO306	PAO1 with chromosomally integrated *fabI'*-*lacZ*	This study
PW4183	PAO1-UW *oppA*::IS*lacZ*/hah	13
PW4184	PAO1-UW *oppA*::IS*lacZ*/hah	13
PAO1284	PAO1 Δ*oppB*::FRT	This study
Plasmids		
pCR2.1	TA cloning vector for PCR fragments; Ampʳ Kanʳ	Life Technologies
pEX18Ap	Gene replacement vector; Ampʳ	14
pPS856	Source for GENʳ cassette	14
pOPPA1	pCR2.1 with 1,852-bp *oppA* fragment	This study
pOPPA2	pOPPA1 with GENʳ cassette from pPS856	This study
pOPPA3	pEX18Ap with *oppB*::GENʳ fragment from pOPPA2	This study
pOPPB1	pCR2.1 with 834-bp *oppB* fragment	This study
pOPPB2	pOPPB1 with GENʳ cassette from pPS856	This study
pOPPB3	pEX18Ap with *oppB*::GENʳ fragment from pOPPB2	This study
pOPPE1	pCR2.1 with 1,745-bp *oppE* fragment	This study
pOPPE2	pOPPE1 with GENʳ cassette from pPS856	This study
pOPPE3	pEX18Ap with *oppE*::GENʳ fragment from pOPPE2	This study

ᵃ Hgʳ, mercury resistance determinant from Tn*501*.
ᵇ Insertion mapping revealed that in this strain, *pac* is identical to *oppB*.
ᶜ The mini-D171 element was transferred from PAM1194 to PAO1 by F116L-mediated transduction selecting tetracycline resistance encoded by mini-D171.

TABLE 2 Oligonucleotides used in this study

Name	Sequence[a]
Primer 1	5′-GCAGCTCTTGCTCAATCC
Primer 2	5′-CAAGCGACTTATGGGAGG
oppE#1	5′-CTGCTGGAGCTCATCGGCGAAGC
oppE#2	5′-ATGGAGAGCTCGCTGGCGACTCC
oppB-up	5′-GGAGCTCGACAAGGTGGTCGC
oppB-down	5′-GGAGCTCAGTGGCTGGCCGTC
oppA-up	5′-CAAGCTTGGCCAGAACCCCGG
oppA-down	5′-AGGTACCAGTTGGGAATGCTGTAG
FabI-2	5′-CGCGGGTGGTCACCGCGG
OppE-1	5′-ATGGCCCCGGCAAGCGCG
P244	5′-CTGCGGCAACTGGAGGGCAAG
P245	5′-CCGGGGCCCTGAGCTTGTCGTT
lacZ-148	5′-GGGTAACGCCAGGGTTTTCC
P$_{ser-down}$	5′-AGTTCGGCCTGGTGGAACAACTCG

[a] Newly introduced SacI (*oppE*#1 and *oppE*#2), HindIII (*oppA*-up), KpnI (*oppA*-down), and ApaI (P245) restriction sites are underlined.

tional mutagenesis of *oppE* was verified by genomic Southern analysis using the biotin-labeled 1,745-bp *oppE* DNA fragment as the probe by following previously described procedures (14).

Using similar procedures and primers *oppB*-up and *oppB*-down, a 834-bp partial *oppB* fragment was amplified from PAO1 genomic DNA and cloned into pCR2.1 to form pOPPB1. pOPPB2 then was obtained by replacing a 204-bp NruI fragment from within the partial *oppB* coding sequence with a 1,077-bp gentamicin resistance marker-containing SmaI fragment from pPS856. Finally, a 1,811-bp HindIII-XhoI fragment of pOPPB2 was cloned between the HindIII-SalI sites of pEX18Ap to form pOPPB3. After transformation into SM10, pOPPB3 was conjugally transferred into PAO1 and the unmarked Δ*oppB* mutant PAO1284 was derived by utilizing the procedures described above for *oppE*. The presence of the correct *oppB* mutation was verified by PCR and DNA sequence analysis.

For isolation of an *oppA* mutant, this gene was amplified from PAO1 using primers *oppA*-down and *oppA*-up, and a 1,852-bp *oppA* fragment was cloned into pCR2.1 to form pOPPA1. pOPPA2 next was obtained by inserting a blunt-ended 1,054-bp GEN^r SacI fragment from pPS856 into an NruI site located within *oppA* (although we intended to delete three NruI fragments encompassing 1,044 bp of *oppA*, we inadvertently ended up isolating a GEN^r insertion in the third, most distal NruI site). Finally, a 2,902-bp HindIII-KpnI fragment of pOPPA2 was cloned between the same sites of pEX18Ap to form pOPPA3. The unmarked *oppA*::*FRT* mutant PAO449 was derived by utilizing the procedures described above for *oppE*, and PCR analysis and DNA sequencing were used to verify the presence of the *oppA* insertion.

***P. aeruginosa oppA* transposon mutants.** The two *oppA* transposon mutants PW4183 and PW4184 were obtained from the sequence-verified two-allele *P. aeruginosa* PAO1 transposon mutant library (13). The two mutants contain transposon IS*lacZ*/hah inserted either early (PA4184; nucleotide 85 of 1830) or late (PA4183; nucleotide 1528 of 1830) in *oppA*. In both mutants the transposon is oriented in the same orientation as *oppA* (or reverse with respect to the annotation of the chromosome), such that the outward-facing neomycin resistance gene promoter engineered into the transposon (25) should direct transcription of downstream genes, minimizing or eliminating polar effects on genes in the same transcriptional unit, in this instance *oppB* (Fig. 2). Transposon locations were verified by PCR amplification with the recommended primers, followed by sequencing of the PCR products.

Gene fusion constructs. A *fabI′*-*lacZ* transcriptional fusion was constructed by PCR amplifying a 377-bp DNA fragment from PAO1 chromosomal DNA using the primer pair P244 and P245. The *fabI* fragment contained the last 68 codons of *oppE*, the 21-bp *oppE*-*fabI* intergenic region, and the first 45 codons of *fabI*. This fragment was then subcloned into the integrative *lacZ* fusion vector mini-CTX-*lacZ* (26) between the

EcoRI and SmaI sites and integrated at the φCTX attachment site on the PAO1 chromosome using established procedures (27). Strain PAO306 (*fabI′*-*lacZ*) was obtained after removal of the tetracycline resistance selection marker using Flp recombinase (27). The presence of the correct fusion was verified by amplifying PCR fragments containing the 5′ *lacZ* sequences, the cloned chromosomal DNA segment, and portions of the mini-CTX-*lacZ* vector sequences with primers lacZ-148 and P$_{ser-down}$. The resulting PCR fragment was sequenced with primer lacZ-148. The strains harboring the *fabI′*-*lacZ* fusion were grown in various media to an optical density at 600 nm of ~1, and β-galactosidase activities were measured (28).

RT-PCR analysis. For RNA isolation, cells of PAO1 were grown overnight at 37°C in 1% proteose peptone broth 3 (Difco). The cells were diluted 1:100 into the same medium and incubated at 37°C until the cell density reached an optical density (540 nm) of 0.5 to 0.7. Total RNA was isolated from these cells using the RNeasy kit (Qiagen, Valencia, CA). The reverse transcriptase (RT) reaction mixture contained 1 μg of total RNA, 10 pmol of primer FabI-2, and 5% DMSO. This mixture was heated at 70°C for 10 min before addition of RNasin, RT buffer, dithiothreitol (DTT), and deoxynucleoside triphosphates (dNTPs). After 2 min at 48°C, 10 U reverse AMV transcriptase was added, and the mixture was incubated at 48°C for 50 min before the RT was inactivated by heating to 70°C for 15 min. Five-μl aliquots of the PCR mixes were used as the templates in PCR mixtures containing 0.2 mM dNTPs, 10 pmol of FabI-2, 10 pmol of OppE-1, 5% DMSO, 1× PCR buffer, and 5 U *Taq* polymerase. Cycle conditions were 35 cycles of 95°C for 45 s, 65°C for 45 s, 72°C for 1 min, and a final extension step for 10 min at 72°C. The sizes of the amplicons were analyzed by electrophoresis on a 2% agarose gel in Tris-acetate-EDTA (TAE) buffer (19), and their nucleotide sequences were determined.

RESULTS AND DISCUSSION

Identification of spontaneous pacidamycin-resistant mutants.
Exposure of PAO1 (MIC, 4 to 16 μg/ml) resulted in ready emergence of pacidamycin-resistant mutants of two types (Table 3). Type 1 mutants were obtained with a frequency of 2×10^{-6}. These
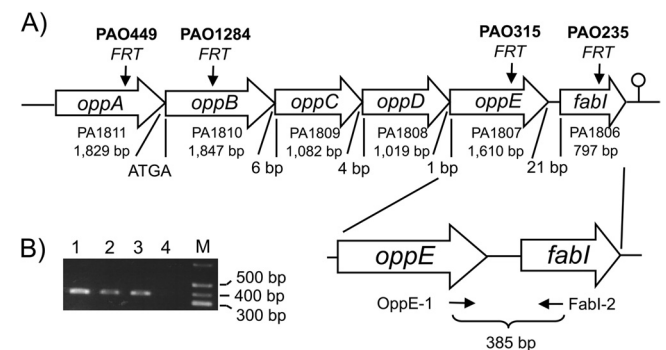

FIG 2 Gene organization in the *P. aeruginosa opp-fabI* operon. (A) The genes present in the *opp-fabI* operon are indicated by gene name, PAO1 gene annotation number, and size. Distances between genes are also shown. ATGA indicates overlap of the *oppB* ATG initiation codon with the *oppA* TGA stop codon. Arrows mark the relative positions of *FRT* insertions in site-specific mutants with mutant names shown above the arrows. The lollipop symbol indicates a putative transcriptional terminator. *oppA* and *oppB* encode periplasmic binding proteins; *oppC* and *oppD* encode membrane transport proteins; *oppE* encodes a traffic ATPase; *fabI* encodes an enoyl-acyl carrier protein reductase. (B) RT-PCR analysis of *oppE* and *fabI* coexpression. Amplification was achieved with primers OppE1 and FabI-2, which yielded the expected indicated 384-bp product. PCR fragments were analyzed by agarose gel electrophoresis. Lanes: 1, 2, and 3, PCR products from three separate RNA and cDNA preparations; 4, blank (same as lane 1 but without reverse transcriptase addition); M, Hi-Lo DNA size ladder from Minnesota Molecular (Minneapolis, MN).

TABLE 3 Properties of spontaneous pacidamycin-resistant *P. aeruginosa* mutants

Strain	Description	MIC (μg/ml)				
		Pacidamycin	Levofloxacin	Carbenicillin	Erythromycin	Imipenem
PAO1	Wild type	4–16	0.25	32	128	1
Type 1	High-level resistance					
PAM1135	*pac-1*	>512	0.25	32	128	1
Type 2	Low-level resistance					
PAM1043	MexAB-OprM up	64	1	256	256	1
PAM1044	MexCD-OprJ up	64	2	16	>512	0.125
Controls						
PAM1032	*nalB* (MexAB-OprM up)	64	1	256	256	1
PAM1033	*nfxB* (MexCD-OprJ up)	64	2	16	>512	0.25
PAM1034	*nfxC* (MexEF-OprN up)	16	4	16	128	4

so-called *pac-1* mutants, exemplified by PAM1135, were highly resistant to pacidamycin (MIC, >500 μg/ml) but not cross-resistant to other antibiotics. Type 2 mutants emerged less frequently (10^{-7} to 10^{-8}), showed intermediate resistance to pacidamycin (64 μg/ml), and exhibited cross-resistance with other antibiotics. Antibiotic resistance profiles of two type 2 mutants analyzed in more detail, PAM1043 and PAM1044, were similar to MexAB-OprM- and MexCD-OprJ-expressing control strains (Table 3). RT-PCR analysis confirmed overexpression of *mexB* and *mexD* in PAM1043 and PAM1044, respectively (data not shown). This indicated that the intermediate pacidamycin resistance levels observed in PAM1043 and PAM1044 most likely were due to up-regulation of MexAB-OprM and MexCD-OprJ, respectively. Pacidamycin was not a substrate for MexEF-OprN, since the MICs of PAO1 and a MexEF-OprN-overexpressing strain were the same.

Because it has been well established that MexAB-OprM plays a major role in the intrinsic antibacterial resistance of *P. aeruginosa* (29–31), we sought to further characterize the impact of this efflux pump on pacidamycin susceptibility (Table 4). Cells expressing an inactive MexAB-OprM system (PAM1154) were more susceptible to pacidamycin than wild-type PAO1, indicating that this efflux pump plays a role in intrinsic pacidamycin resistance. However, inactivation of MexAB-OprM or the presence of the efflux pump inhibitor PAβN (32 and data not shown) did not reduce resistance to pacidamycin in type 1 mutants, corroborating the notion that high-level resistance was not affected by this efflux system. As expected, in control experiments, inactivation of MexAB-OprM or the presence of PAβN did reduce the levofloxacin MIC for PAM1135 from 0.25 to 0.015 μg/ml (data not shown). It should be

TABLE 4 Impact of MexAB-OprM on *P. aeruginosa* susceptibility to pacidamycin

Strain	Description	MIC (μg/ml)		
		Pacidamycin	Levofloxacin	Carbenicillin
PAO1	Wild type	16	0.25	32
PAM1154	PAO1 (PAM1020) *oprM*::Hg^r	4	0.015	0.5
PAM1135	PAO1 (PAM1020) *pac-1*	>512	0.25	32
PAM1348	PAM1135 *oprM*::Hg^r	>512	0.015	0.5

noted that inactivation of OprM also impairs the function of the MexXY efflux pump, resulting in hypersusceptibility to antimicrobials not extruded by MexAB-OprM, e.g., aminoglycosides, but the role of MexXY in pacidamycin resistance, if any, remains to be assessed.

A transposon mutant exhibiting high-level pacidamycin resistance maps to an oligopeptide permease operon. To assess the molecular mechanism(s) underlying high-level pacidamycin resistance in type 1 mutants, wild-type PAO1 was subjected to mini-D171 transposon mutagenesis, and mutants exhibiting high-level (>200 μg/ml) pacidamycin resistance were selected. One of the resulting mutants, PAM1194 (*pac*::mini-D171), was further characterized (Table 5). The resistance profile of this mutant was the same as that of the spontaneous *pac-1* mutant PAM1135, and inactivation of MexAB-OprM had no effect on the pacidamycin resistance. Transduction of mini-D171 from PAM1194 into the wild-type PAO1 resulted in high-level resistance (PAM2386), demonstrating that mini-D171 insertion was linked to a pacidamycin resistance locus.

By inverse PCR, determination of the DNA sequence adjacent to mini-D171, and BLAST searches of the published *P. aeruginosa* chromosomal sequences, including PAO1 (11), the transposon insertion in PAM1194 was localized to the *oppB* gene, encoding a protein with 63% similarity to a periplasmic binding protein encoded by the *oppA* gene of *Helicobacter pylori* (33).

The *P. aeruginosa* PAO1 *opp* operon (Fig. 2) contains five genes that encode proteins of a probable oligopeptide transport system. The first two genes, *oppA* and *oppB*, whose coding sequences overlap, contain type I signal peptides; thus, they are probably periplasmic binding proteins. The next three genes of the operon, *oppC*, *oppD*, and *oppE*, are separated from *oppB* by 6 bp of untranslated sequence. Based on similarity with amino acid motifs found in proteins from other binding protein-dependent systems, *oppC* and *oppD* encode membrane transporter components and *oppE* encodes an ATPase component. Interestingly, there is no transcriptional terminator downstream of *oppE*. Rather, this gene is separated from another gene, *fabI*, by only 21 bp. A transcriptional terminator is found downstream of *fabI* (34). The previously identified *fabI* gene encodes the triclosan-sensitive enoyl-acyl carrier protein (ACP) reductase gene of the *de novo* fatty acid biosynthetic pathway (35).

To determine whether the *opp* genes and *fabI* are located in the

TABLE 5 Characterization of *P. aeruginosa* transposon and defined *opp* mutants exhibiting high-level pacidamycin resistance

Strain	Description	MIC (µg/ml)			
		Pacidamycin	Levofloxacin	Carbenicillin	Tetracycline
PAO1	Wild type	16	0.25	32	4
PAM1194	PAO1 *pac*::mini-D171[b]	>512	0.25	32	512[a]
PAM1135	PAO1 *pac-1*	>512	0.25	32	ND
PAM1316	PAO1194 *oprM*::Hg[r]	>512	0.015	0.5	32
PAM2386	PAO1 *pac*::mini-D171[b,c]	>512	1	256	>512[a]
PAO235	PAO1 *fabI*::FRT	32	0.25	ND	ND
PAO315	PAO1 *oppE*::FRT	>512	0.25	ND	ND
PAO1284	PAO1 Δ*oppB*::FRT	>512	0.25	ND	ND
PAO449	PAO1 *oppA*::FRT	256	0.25	ND	ND
PW4183	PAO1 *oppA*::IS*lacZ*/hah	>512	ND	ND	ND
PW4184	PAO1 *oppA*::IS*lacZ*/hah	>512	ND	ND	ND

[a] The mini-D171 element contains a tetracycline resistance marker.
[b] Insertion mapping revealed that in this strain, *pac* is identical to *oppB*.
[c] The tetracycline resistance marker was transferred from PAM1194 to PAO1 by F116L-mediated transduction.

same operon, reverse transcription-PCR (RT-PCR) was performed. Preliminary analyses using a *fabI′-lacZ* transcriptional fusion had shown that the *opp* operon was transcribed at low but detectable levels in most rich (e.g., LB, PTSB, SOB, or proteose peptone broth) and minimal (e.g., M9 plus 1% Casitone) media. The β-galactosidase activity levels in PAO1 containing *fabI′-lacZ* integrated at the mini-CTX attachment site on the chromosome (26, 27) ranged between 24 and 171 Miller units (28) in cells grown in either LB, PTSB, SOB, proteose peptone broth, or M9 plus 1% Casitone. Therefore, RNA was isolated from cells grown in proteose peptone broth and reverse transcribed using a primer (FabI-2) that anneals to sequences early in *fabI* (Fig. 2A depicts approximate locations of primer binding sites), and the resulting cDNA was PCR amplified using primers OppE-1 and FabI-2. RT-PCR yielded a single amplicon of the expected size (Fig. 2B), and its identity was verified by nucleotide sequencing. This finding demonstrates that the *opp* genes and *fabI* are cotranscribed as part of the same operon. The RT-PCR product was also produced in *P. aeruginosa* cells grown under various growth conditions; for example, no significant differences were observed in rich or defined minimal media (data not shown).

Opp proteins but not FabI are responsible for high-level pacidamycin resistance. To determine the requirement of various *opp* operon genes and their products for pacidamycin transport, several defined insertion or deletion mutants were isolated and characterized. Inactivation of *oppA*, *oppB*, and *oppE* resulted in high-level pacidamycin resistance (256 to >512 µg/ml), but the susceptibility of the *fabI* mutant PAO235 was similar to that of wild-type PAO1 (Table 5). These results are consistent with the notion that the Opp system is required for uptake of pacidamycin across the inner membrane and that the activity of this transport system is responsible for the observed pacidamycin susceptibility of wild-type *P. aeruginosa*. This mutant analysis also indicated that both OppA and OppB periplasmic binding proteins were required for pacidamycin uptake and that they cannot compensate for each other. OppA (609 amino acids) and OppB (615 amino acids) are closely related (42.5% identity in CLUSTALW alignments), but neither *P. aeruginosa* OppA nor OppB shows significant homology to the 543-amino-acid *E. coli* OppA (identity, 16.4 and 15.7%, respectively). Despite expression of OppB in the nonpolar deletion mutant PAO449 or the nonpolar transposon mutants

PW4182 and PW4184 (downstream genes are transcribed from the strong neomycin resistance gene promoter engineered into the transposon; see Materials and Methods), these three mutants still exhibit high-level pacidamycin resistance. Similarly, despite expression of OppA, the Δ*oppB* mutant PAO1284 still exhibits high-level resistance.

Pacidamycin-resistant mutants are affected in peptide transport. As mentioned above, using a single-copy, chromosomally integrated *fabI′-lacZ* transcriptional fusion, we demonstrated that the *opp* operon was expressed at low but detectable levels in cells grown in various media, and no significant differences were observed in rich or defined minimal media. Therefore, transport activity was assessed in LB broth-grown cells, utilizing PAβN as the substrate. Decreased uptake of PAβN was observed in the spontaneous or transposon-induced pacidamycin-resistant mutants (Fig. 3). The residual uptake of this dipeptide is probably due to the expression of a separate dipeptide transport system.

Concluding remarks. In summary, we have identified the mechanisms associated with *P. aeruginosa*'s resistance to the uridyl peptide antibiotic pacidamycin. High-level resistance emerges with high frequency through mutations in the Opp transporter, a binding protein-dependent ABC transporter used for oligopeptide import. Mutations in the *oppA*, *oppB*, and *oppE* genes, encoding two periplasmic binding proteins and an ATPase component, respectively, resulted in high-level pacidamycin resistance. These did not display cross-resistance to other antibiotics, consistent with high-level resistance being due to decreased uptake of pacidamycin rather than upregulation of multisubstrate efflux pumps. The Opp permease contains two putative periplasmic binding proteins, OppA and OppB. We observed that lack of OppA cannot be compensated for by OppB and vice versa, indicating that neither binding protein alone can mediate pacidamycin transport, which is indicative of an oligomeric binding protein requiring both OppA and OppB subunits. The Opp system was shown to be a bona fide oligopeptide transport system by demonstrating that transport of PAβN is impaired in *opp* mutants. Oligopeptide permease systems are often involved in uptake of toxic peptide compounds. For instance, the *E. coli* and *Salmonella enterica* serovar Typhimurium Opp permease is required for uptake of the phaseolotoxin ([N$^\delta$,N′-sulfodiaminophosphinyl] L-ornithyl-alanyl-homoarginine) from *Pseudomonas syringae* pv.
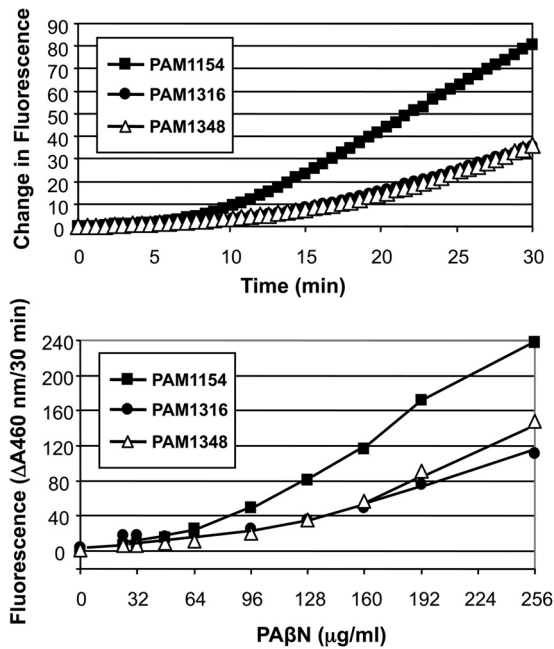
FIG 3 *P. aeruginosa opp* operon encodes a functional peptide transport system. Cells of the indicated strains were grown in LB broth to an optical density (at 600 nm) of ~1, washed, and resuspended in 50 mM K_2HPO_4 (pH 7.0), 1 mM $MgSO_4$, and 0.4% glucose. Assays were initiated by addition of PAβN to suspensions of intact cells, and fluorescence was measured. Strains assayed were PAM1154 (PAO1 *oprM*::Hg[r]), PAM1316 (*oppB*::mini-D171 *oprM*::Hg[r]), and PAM1348 (*pac-1 oprM*::Hg[r]).

phaseolica, and *opp* mutants were resistant to phaseolotoxin (36). Spontaneous *Listeria monocytogenes* resistance to the tripeptide herbicide bialaphos (consisting of L-phosphinotricin, an analog of L-glutamate, and two L-alanine residues) occurs primarily via mutations affecting oligopeptide permease-mediated uptake (37). Disk diffusion susceptibility assays showed that in contrast to PAO1, which is susceptible to bialaphos, Δ*oppB* and *oppE* insertion mutants are bialaphos resistant, indicating that the *opp* system is also required for import of this tripeptide herbicide (data not shown). Isolates with low-level resistance of *P. aeruginosa* to pacidamycin were found at a lower frequency, and this resistance was mediated by the MexAB-OprM and MexCD-OprJ efflux pumps. Consistent with multidrug efflux pump expression, these isolates were cross-resistant to other antibiotics known to be pump substrates. In summary, development of pacidamycins as anti-*Pseudomonas* drugs faces a dual threat of resistance and reduced uptake and efflux.

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REFERENCES

1. **Chen RH, Buko AM, Whittern DN, McAlpine JB.** 1989. Pacidamycins, a novel series of antibiotics with anti-*Pseudomonas aeruginosa* activity. II. Isolation and structural elucidation. J. Antibiot. **42**:512–520.
2. **Fernandes PB, Swanson RN, Hardy DJ, Hanson CW, Coen L, Rasmussen RR, Chen RH.** 1989. Pacidamycins, a novel series of antibiotics with anti-*Pseudomonas aeruginosa* activity. III. Microbiologic profile. J. Antibiot. **42**:521–526.
3. **Karwowski JP, Jackson M, Theriault RJ, Chen RH, Barlow GJ, Maus ML.** 1989. Pacidamycins, a novel series of antibiotics with anti-*Pseudomonas aeruginosa* activity. I. Taxonomy of the producing organism and fermentation. J. Antibiot. **42**:506–511.
4. **Zhang W, Ostash B, Walsh CT.** 2010. Identification of the biosynthetic gene cluster for the pacidamycin group of peptidyl nucleoside antibiotics. Proc. Natl. Acad. Sci. U. S. A. **107**:16828–16833.
5. **Rackham EJ, Gruschow S, Goss RJ.** 2011. Revealing the first uridyl peptide antibiotic biosynthetic gene cluster and probing pacidamycin biosynthesis. Bioeng. Bugs **2**:218–221.
6. **Brandish PE, Kimura KI, Inukai M, Southgate R, Lonsdale JT, Bugg TD.** 1996. Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-*N*-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. Antimicrob. Agents Chemother. **40**:1640–1644.
7. **Mao W, Warren M, Malouin F, Galazzo J, Lee A, Lomovskaya O.** 2001. AcrAB-TolC efflux pump contributes to the intrinsic resistance to pacidomycin in *Escherichia coli*, poster A-35. Abstr. 101st Gen. Meet. Am. Soc. Microbiol.. American Society for Microbiology, Washington, DC.
8. **Gotoh N, Murata T, Ozaki T, Kimura T, Kondo A, Nishino T.** 2003. Intrinsic resistance of *Escherichia coli* to mureidomycin A and C due to expression of the multidrug efflux system AcrAB-TolC: comparison with the efflux systems of mureidomycin-susceptible *Pseudomonas aeruginosa*. J. Infect. Chemother. **9**:101–103.
9. **Lomovskaya O, Mistry A, Warren M, Lee A.** 2001. Characterization of pacidomycin resistant mutants in *Pseudomonas aeruginosa*, poster A-34. Abstr. Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC.
10. **Watson JM, Holloway BW.** 1978. Chromosome mapping in *Pseudomonas aeruginosa*. J. Bacteriol. **133**:1113–1125.
11. **Stover CK, Pham X-Q, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Spencer D, WongGK-S, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV.** 2000. Complete genome sequence of *Pseudomonas aeruginosa*, an opportunistic pathogen. Nature **406**:959–964.
12. **Lomovskaya O, Lee A, Hoshino K, Ishida H, Mistry A, Warren MS, Boyer E, Chamberland S, Lee VJ.** 1999. Use of a genetic approach to evaluate the consequence of inactivation of efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43**:1340–1346.
13. **Held K, Ramage E, Jacobs M, Gallagher L, Manoil C.** 2012. Sequence-verified two-allele transposon mutant library for *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **194**:6387–6389.
14. **Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212**:77–86.
15. **Liss L.** 1987. New M13 host: DH5αF′ competent cells. Focus **9**:13.
16. **Schweizer HP.** 1994. A method for construction of bacterial hosts for *lac*-based cloning and expression vectors: α complementation and regulated expression. Biotechniques **17**:452–456.
17. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
18. **Ohman DE, Cryz SJ, Iglewski BH.** 1980. Isolation and characterization of a *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. J. Bacteriol. **142**:836–842.
19. **Sambrook J, Russell DW.** 2001. Molecular cloning, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
20. **Clinical and Laboratory Standards Institute.** 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; M07-A9. Approved standard, 9th ed. CLSI, Wayne, PA.
21. **Darzins A, Casadaban MJ.** 1989. Mini-D3112 bacteriophage transposable elements for genetic analysis of *Pseudomonas aeruginosa*. J. Bacteriol. **171**:3909–3916.
22. **Krishnapillai V.** 1972. A novel transducing phage. Mol. Gen. Genet. **114**:134–143.
23. **Goldbarg JA, Rutenburg AM.** 1958. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. Cancer **11**:283–291.
24. **de Lorenzo V, Timmis KN.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived mini-transposons. Methods Enzymol. **235**:386–405.

25. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, Manoil C. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **100:**14339–14344.

26. Becher A, Schweizer HP. 2000. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single copy chromosomal *lacZ* and *lux* gene fusions. Biotechniques **29:**948–954.

27. Hoang TT, Kutchma AJ, Becher A, Schweizer HP. 2000. Integration proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid **43:**59–72.

28. Miller JH. 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

29. Poole K, Krebes K, McNally C, Neshat S. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.

30. Li XZ, Nikaido H, Poole K. 1995. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:** 1948–1953.

31. Poole K, Srikumar R. 2001. Multidrug efflux in Pseudomonas aeruginosa: components, mechanisms and clinical significance. Curr. Topics Med. Chem. **1:**59–71.

32. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshino K, Ishida H, Lee VJ. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Chemother. **45:**105–116.

33. Weinberg MV, Maier RJ. 2007. Peptide transport in *Helicobacter pylori*: Rokles of Dpp and Opp systems and evidence for additional peptide transporters. J. Bacteriol. **189:**3392–3402.

34. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS. 2011. Pseudomonas Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res. **39:**D596–D600.

35. Hoang TT, Schweizer HP. 1999. Characterization of the *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase: a target for triclosan and its role in acylated homoserine lactone synthesis. J. Bacteriol. **181:**5489–5497.

36. Staskawicz B, Panopoulos N. 1980. Phaseolotoxin transport in *Escherichia coli* and *Salmonella typhimurium* via the oligopeptide permease. J. Bacteriol. **142:**474–479.

37. Borezee E, Pellegrini E, Berche P. 2000. OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. Infect. Immun. **68:**7069–7077.