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Characterization of a *Pseudomonas aeruginosa* Efflux Pump Contributing to Aminoglycoside Impermeability

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Pseudomonas aeruginosa **can employ many distinct mechanisms of resistance to aminoglycoside antibiotics; however, in cystic fibrosis patients, more than 90% of aminoglycoside-resistant *P. aeruginosa* isolates are of the impermeability phenotype. The precise molecular mechanisms that produce aminoglycoside impermeability-type resistance are yet to be elucidated. A subtractive hybridization technique was used to reveal gene expression differences between PAO1 and isogenic, spontaneous aminoglycoside-resistant mutants of the impermeability phenotype. Among the many genes found to be up-regulated in these laboratory mutants were the *amrAB* genes encoding a recently discovered efflux system. The *amrAB* genes appear to be the same as the recently described *mexXY* genes; however, the resistance profile that we see in *P. aeruginosa* is very different from that described for *Escherichia coli* with *mexXY*. Direct evidence for AmrAB involvement in aminoglycoside resistance was provided by the deletion of *amrB* in the PAO1-derived laboratory mutant, which resulted in the restoration of aminoglycoside sensitivity to a level nearly identical to that of the parent strain. Furthermore, transcription of the *amrAB* genes was shown to be up-regulated in *P. aeruginosa* clinical isolates displaying the impermeability phenotype compared to a genotypically matched sensitive clinical isolate from the same patient. This suggests the possibility that AmrAB-mediated efflux is a clinically relevant mechanism of aminoglycoside resistance. Although it is unlikely that hyperexpression of AmrAB is the sole mechanism conferring the impermeability phenotype, we believe that the Amr efflux system can contribute to a complex interaction of molecular events resulting in the aminoglycoside impermeability-type resistance phenotype.**

Resistance to aminoglycosides in *Pseudomonas aeruginosa* is usually mediated either by specific enzymatic modification of the drug or by an undefined mechanism that has commonly been referred to as impermeability resistance. Aminoglycoside impermeability-type resistance (AGIR) was originally described for clinical isolates of *P. aeruginosa* with strains exhibiting diminished uptake of gentamicin in energy-dependent phases I and II and no detectable acetylation or adenylylation activity and having ribosomes that were sensitive to the inhibitory effect of aminoglycosides (3). AGIR strains are now commonly characterized as panaminoglycoside resistant in the absence of modifying enzymes, and the characterization of strains exhibiting this phenotype can be inferred from the pattern of sensitivity to aminoglycosides by a disk diffusion-based assay referred to as the aminoglycoside resistance profile (AGRP) (43). Extensive surveys of aminoglycoside resistance in clinical isolates have established the prevalence of the AGIR phenotype in *Pseudomonas* isolates (24, 25, 34, 38, 44). In general, these studies found that among clinical strains of *P. aeruginosa*, impermeability-type resistance was the single most common mechanism even though it was identified less frequently than that caused by modifying enzymes as a whole. In contrast, impermeability resistance predominates (>90%) among *P. aeruginosa* isolates from patients with cystic fibrosis (CF) (23).

While much has been learned since the original characterization of AGIR strains, the specific mechanism(s) involved in this type of resistance remains unclear. Impermeability-type resistance has been circumstantially linked with changes in outer membrane composition of *P. aeruginosa*, including alterations in the structure of lipopolysaccharide (6, 15, 49), overexpression of outer membrane protein (OMP) OprH (30, 50), and changes in the electron transport chain (4, 5). In this study we employed a PCR-based, subtractive hybridization technique, representational difference analysis (RDA) (20), to examine differential gene expression associated with the AGIR phenotype in *P. aeruginosa*. These analyses led to the identification of many genes that may be differentially expressed in AGIR strains. One such genomic region, the *amrAB* locus, was observed to be up-regulated in AGIR strains, including clinical isolates. These genes encode a *P. aeruginosa* transporter belonging to the resistance, nodulation, and cell division (RND) family of efflux systems (39) and appear to be the same as the recently described *mexXY* genes from *P. aeruginosa*. However, our data shows that the effects of this efflux system in *P. aeruginosa* are quite different from those described when these genes were expressed in *Escherichia coli* (26). A knockout of this putative efflux system in an AGIR strain restored sensitivity to aminoglycosides in this mutant. Although these data provide a direct line of evidence supporting a role for AmrAB in the AGIR phenotype, the large number of other genes identified in the RDA analyses supports the hypothesis that impermeability-type resistance to aminoglycosides in *P. aeruginosa* is more complex. It is likely that mutations in multiple loci are necessary to achieve high-level resistance while maintaining strain viability. In addition, regulatory mutations that affect the up-regulation of the *amrAB* locus may also result in expression level changes of other *P. aeruginosa* loci that contribute to the AGIR phenotype.

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Description	Source or reference
Strains		
P. aeruginosa		
PAO1	Wild type	P. Phibbs, S. Lory, University of Washington
PAO200	PAO1 with unmarked Δ(*mexAB-oprM*)	H. Schweitzer (41)
2547	Spontaneous tobramycin-resistant derivative of PAO1	This study
2548	Spontaneous tobramycin-resistant derivative of 2547	This study
3579	PAO1 with unmarked Δ(*amrR*)	This study
3580	2547 with unmarked Δ(*amrB*)	This study
3582	PAO1(pAMR-1)	This study
3583	PAO1(pUCP20) plasmid control strain	This study
3737	3579(pXZL34)	This study
913	CF clinical isolate	CHMC
1030	CF clinical isolate	CHMC
1085	CF clinical isolate	CHMC
1104	CF clinical isolate	CHMC
1151	CF clinical isolate	CHMC
1168	CF clinical isolate	CHMC
1200	CF clinical isolate	CHMC
1249	CF clinical isolate	CHMC
1250	CF clinical isolate	CHMC
1365	CF clinical isolate	CHMC
1452	CF clinical isolate	CHMC
1457	CF clinical isolate	CHMC
1520	CF clinical isolate	CHMC
E. coli		
DH5α	F$^-$ f80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Gibco-BRL
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ψ 80d*lacZ*Δ M15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara*, *leu*)7697 *galU* λ$^-$ *rpsL nupG*	Gibco-BRL
S17.1λpir	*thi pro hsdR recA* Tra$^+$	P. Phibbs, S. Lory, University of Washington
SM10	Km^r; mobilizer strain (*thi-1 thr leu tonA lacY supE recA*::RP4-2Tc::Mu)	H. Schweizer, Colorado State University (42)
Plasmids		
pGEM-T	Ap^r; T-tailed cloning vector	Promega Corp.
pUCP20	Ap^r Cb^r, *ori1600*$^+$; broad-host-range cloning vector (GenBank no. U07165)	H. Schweizer (47)
pAMR-1	pUCP20 derivative carrying the *amrAB* genes on a 4.9-kb *Eco*RV-*Eco*RI fragment	This study
pXZL34	pVLT31 derivative carrying the *oprM* gene under the control of an IPTG-inducible promoter	R. Hancock, University of British Columbia (48)
pEX18T	Ap^r Cb^r; *sacB*$^+$ *oriT*$^+$; gene replacement vector (GenBank no. AF004910)	H. Schweizer, Colorado State University (42)
pPS858	Ap^r Gm^r; source of Gm^r-GFP fragment flanked by FRT sites	H. Schweizer (17)
pFLP2	Ap^r Cb^r; source of yeast Flp recombinase (GenBank no. AF048702)	H. Schweizer (17)
pEX-AMRR	pEX18T derivative used for the deletion of the *amrR* gene	This study
pEX-AMR3	pEX18T derivative used for the deletion of the *amrAB* genes	This study

[a] Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; CHMC, Children's Hospital and Medical Center, Seattle, Washington; GFP, green fluorescent protein structural gene; FRT, yeast Flp site-specific recombinase recognition sequence.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *P. aeruginosa* PAO1 was used to generate the spontaneous mutant 2547 by plating 10^8 cells from an overnight culture onto Mueller-Hinton agar (BBL) containing 4 μg of tobramycin per ml. Colonies that appeared after 48 h at 37°C were subsequently characterized for susceptibility to other aminoglycosides. Mutant 2548 was generated in a similar manner by plating strain 2547 onto media containing 32 μg of tobramycin per ml. Biochemical profiles were determined by the API-20NE strip test (Biomerieux), and strains were tested for isogenicity by pulsed-field gel electrophoresis (PFGE) (12). Strain 3579 was constructed by utilizing the pEX-AMRR plasmid to generate a 540-bp deletion within the open reading frame (ORF) of *amrR* (Table 1; Fig. 1). Strain 3580 was constructed in a similar manner utilizing the pEX-AMR3 plasmid for deletion of a 3.5-kb fragment containing the 3′ terminus of the *amrA* gene and a majority of the *amrB* gene (Table 1; Fig. 1). Construction of recombinant plasmids and generation of unmarked deletions are described in detail below. Plasmid pAMR-1 (Fig. 1) was constructed by subcloning a 4.9-kb *Eco*RV-*Eco*RI fragment, carrying the *amrAB* genes, from a cosmid library into pUCP20. The plasmid construction was carried out in *Escherichia coli* DH5α prior to transformation into *P. aeruginosa* by electroporation (9).

Growth media and susceptibility testing. *E. coli* strains were cultivated in Lennox L broth or agar (Gibco-BRL). *P. aeruginosa* strains were maintained on blood agar (Remel) or L agar and propagated in cation-adjusted Mueller-Hinton broth (BBL) unless otherwise noted. Growth curves were determined by dilution (1:50) of overnight cultures in fresh Mueller-Hinton broth and growth at 37°C in a shaking incubator (250 rpm). Vogel-Bonner (VB) medium (46) was used for selective isolation of *P. aeruginosa* and supplemented with 5% sucrose for negative selection of strains carrying the *sacB* gene. Strains containing the pXZL34 plasmid were maintained on L agar containing tetracycline and 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (48). Antibiotics at various concentrations were used for selection, as follows: for *E. coli*, ampicillin (100 μg/ml), gentamicin (10 μg/ml), and tetracycline (10 μg/ml); and for *P. aeruginosa*, carbenicillin (500 μg/ml), gentamicin (200 μg/ml unless otherwise indicated), and tetracycline (100 μg/ml). All antibiotics were supplied by Sigma Chemical Co. (St. Louis, Mo.). MICs were determined by microbroth dilution according to National Committee for Clinical Laboratory Standards guidelines (29). Disk susceptibility determina-

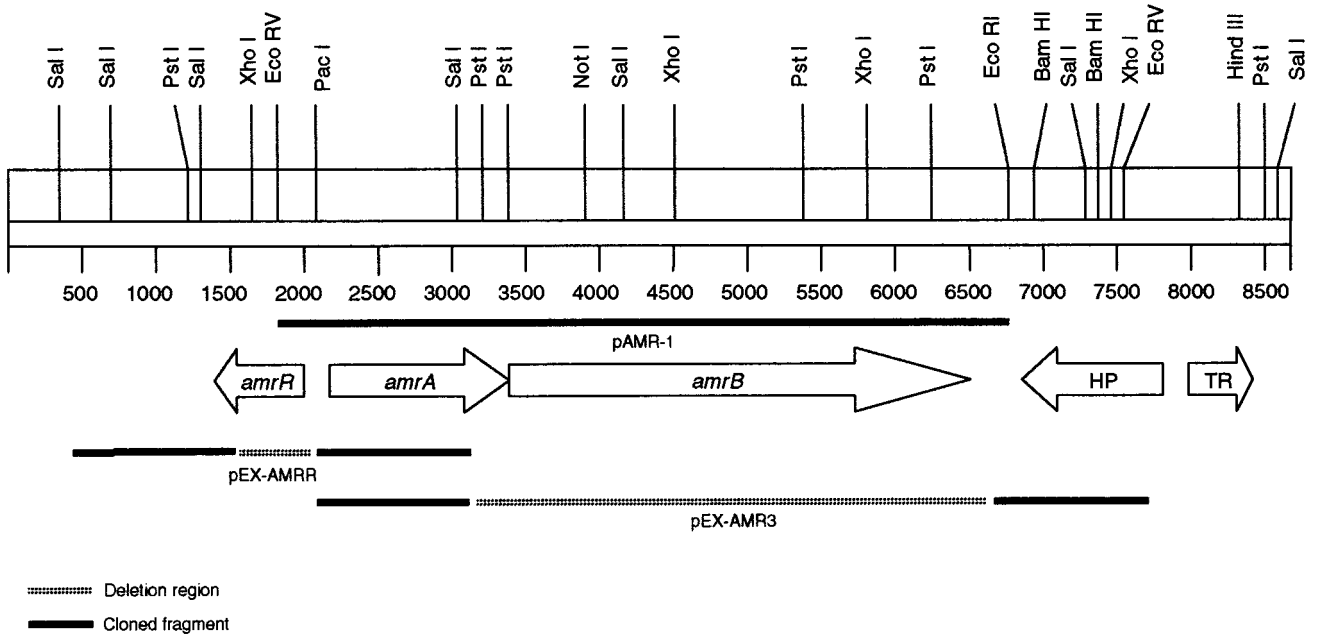

FIG. 1. Physical map of the *amr* locus in *P. aeruginosa*. Arrows designate complete ORF. The region downstream of *amrB* contains two ORFs, one (HP) with homology (54% similar) to *yjiK*, encoding a hypothetical protein from *E. coli*, and the other (TR) possessing homology (74% similar to *merR* from *Archaeoglobus fulgidus*) to the MerR family of bacterial response regulators. See Materials and Methods for a detailed description of the representative plasmids.

tions were also done according to National Committee for Clinical Laboratory Standards guidelines (28). AGRP assay results were interpreted as described by Shaw et al. (43).

RDA. The protocol described by Lisitsyn et al. (20) was modified for use with bacterial cDNA as a means of analyzing gene expression. Total bacterial RNA (3 μg), isolated from mid-log-phase cultures, was DNase treated and converted to double-stranded cDNA. As a positive control, MS2 bacteriophage RNA was spiked into a background of PAO1 RNA at 100 copies per cell equivalent and used as a tester against PAO1. The first strand of cDNA was synthesized in a 20-μl random-primed reverse transcription reaction mixture containing 1× first-strand buffer (50 mM Tris-HCl [pH 8.3], 40 mM KCl, 6 mM $MgCl_2$; Gibco-BRL), 100 ng of primer [2:1:1 mix of N_6 $(SN)_3$, and $(NS)_3$, where S = G or C] 0.5 mM deoxynucleoside triphosphates (dNTPs), 10 mM dithiothreitol, 5% dimethyl sulfoxide (DMSO), and 200 U of reverse transcriptase (SuperScript II; Gibco-BRL). The second strand was synthesized in a 150-μl polymerization reaction mixture containing 1× second-strand buffer [20 mM Tris-HCl (pH 6.9), 4.6 mM $MgCl_2$, 90 mM KCl, 0.15 mM β-NAD^+, 10 mM $(NH_4)_2SO_4$], 0.2 mM dNTPs, 40 U of *E. coli* DNA polymerase I, 10 U of *E. coli* DNA ligase, 2 U of ribonuclease H (all enzymes obtained from Gibco-BRL). All adapter sequences and PCR conditions were as described by Lisitsyn et al. (20), with exceptions as noted. Purified cDNA was digested with *Dpn*II and ligated with oligonucleotide (R-Bgl) adapters. For all ligation reactions, 100 ng of cDNA and 0.5 nmol of each adapter were combined in a T4 DNA ligase reaction (Boehringer Mannheim) at 16°C for 4 h. PCR amplification primed off the terminal adapter sequences was used to produce driver and tester amplicons for subsequent hybridization and selective amplification. Amplicons were purified by extraction with equal volumes of phenol (pH 8.0) and phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and precipitated with isopropanol by using tRNA as the carrier. All subsequent PCRs and digests were purified using QiaQuick PCR purification columns (Qiagen) as per the manufacturer's instructions. R-Bgl adapters were removed by digestion with *Dpn*II, and only tester amplicons were ligated with new (J-Bgl) adapters. A 100-ng amount of the adapterless driver amplicon and 0.1 ng of the newly ligated tester amplicon were denatured and hybridized in a 50-μl phenol emulsion containing the following: 1.5 M sodium thiocyanate, 120 mM phosphate buffer (equimolar mono- and dibasic phosphate), 10 mM EDTA, and 12% (vol/vol) unbuffered phenol. Initial denaturation at 100°C for 10 min was followed by three cycles of hybridization (15 min at 25°C) and denaturation (2 min at 65°C) and a final hybridization (15 min at 25°C). A driver-only reaction was included as a negative control for each hybridization and selective amplification. Hybridizations were extracted with 100 μl of chloroform and concentrated to 20 μl over a QiaQuick column. Selective amplifications of the tester-tester hybrid DNA were performed by two rounds of PCR, with priming from the adapter sequence and an intervening mung bean nuclease digest to remove any remaining single-stranded DNA. Primary amplification of the purified hybridization reaction was done in a 50-μl volume as follows: 1× PCR buffer [67 mM Tris-HCl (pH 8.8), 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 100 μg of bovine serum albumin per ml], 0.5 mM dNTPs, 0.2 nmol of primer (same 24-bp oligonucleotide adapter to which the tester was ligated), 5% DMSO, and 2 U of *Taq* polymerase. Reactions were hot-started in the absence of primer and incubated for 10 min at 72°C in order to fill in target adapter ends. Primer was added, and reactions were amplified for 15 cycles of 1 min at 95°C and 3 min at 72°C and then finished with 10 min at 72°C. Purified reaction products were digested with mung bean nuclease (New England Biolabs) for 30 min at 30°C and purified again. Secondary amplification, scaled to a 100-μl reaction volume, was as described for the primary PCR, but the fill-in incubation prior to primer addition was omitted. The resulting difference products were subjected to two more rounds of hybridization and selective amplification, with N-Bgl adapters used for the second round and J-Bgl adapters used for the third round, as described above. Third-round difference products were cloned into pGEM-T and transformed into competent *E. coli* DH10B cells. Colony PCR was used to amplify the inserted difference product with primers directed to the flanking vector sequence. PCR products were sequenced and analyzed by Basic Local Alignment Search Tool (BLAST) (2) queries of nonredundant public databases, including nucleotide databases (GenBank, EMBL, DDBJ, and PDB) and protein databases (GenBank CDS translations, PDB, SwissProt, PIR, and PRF), and queries at the National Center for Biotechnology Information (29a) as well as by comparison to *P. aeruginosa* sequences from the Pseudomonas Genome Project.

RT-PCR. A 1-μg sample of DNase-treated RNA was converted to single-stranded cDNA by reverse transcription with the reverse primers specific to the gene of interest. Specific primer pairs were as follows: for *amrA*, amrA-F1 (5′-CATCAGCGAACGCGAGTACACCGAAGCG-3′) and amrA-R1 (5′-CAC GTAGATCGGATCGATCTGCTCGACGC-3′); for *amrB*, amrB-F1 (5′-CTGG GTGATCTCCCTGCTGATCGTGCTC-3′) and amrB-R2 (5′-ACTCGACGAT CTTCAGGCGGTTCTGCAC-3′); and for *rps*L, rpsL-For (5′-GCAACTATCA ACCAGCTGGTG-3′) and rpsL-Rev (5′-GCTGTGCTCTTGCAGGTTGTG-3′). Primers specific to the constitutively expressed gene *rpsL* were used as a positive control. Reverse transcription reaction conditions were as follows: 1× first-strand buffer (50 mM Tris-HCl [pH 8.3], 40 mM KCl, 6 mM $MgCl_2$; Gibco-BRL), 10 mM ditheothreitol, 2 pmol of reverse primer per target transcript, 0.5 mM dNTPs, 5% DMSO, 22 U of anti-RNase (Ambion), and 200 U of reverse transcriptase (Gibco-BRL). The same reaction omitting the reverse transcriptase (RT) was used as a negative control. RT reaction mixtures were serially diluted 1:3 in 10 mM Tris-HCl (pH 8.0), and 5 μl of each dilution was used as the template for PCR amplification with the following: 1× PCR buffer [67 mM Tris-HCl (pH 8.8), 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 100 μg of bovine serum albumin per ml], 0.2 mM dNTPs, 10 pmol of each primer (forward and reverse), 5% DMSO, and 2.5 U of *Taq* polymerase (Boehringer Mannheim). Reaction mixtures were incubated for 90 s at 95°C and amplified for 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C and finished with 10 min at 72°C. A 10-μl volume of each reaction product was examined by 1.5% (wt/vol) agarose gel electrophoresis.

TABLE 2. Aminoglycoside resistance phenotypes

Strain	Patient and/or relevant genotype[b]	AGRP zone diameter (mm)[a]											Disc AGRP Phenotype
		Apra	Forta	21562	21561	Gent	Tobra	Amk	Isep	Net	22591	Kan	Neo	
PAO1	Wild type	20	20	17	16	16	21	21	20	18	18	8	14	Sensitive
2547	Isogenic PAO1 mutant	10	6	7	7	6	10	6	7	8	9	6	6	Permeability
2548	Isogenic 2547 mutant	6	6	6	6	6	6	6	6	6	6	6	6	Permeability
1168	40-03, 22-7	20	19	16	16	16	20	17	18	16	17	6	12	Sensitive
1250	40-03, 22-7	19	8	6	6	10	19	12	13	11	16	6	6	Permeability
1249	40-03, 22-7	6	6	6	6	6	6	6	6	6	6	6	6	Permeability
1151	59-01, 11-1	27	22	20	20	21	27	23	22	22	23	6	6	Sensitive
1030	59-01, 10-1	8	6	6	9	6	18	6	10	11	12	6	6	Permeability
1200	59-01, 10-1	6	6	6	6	6	6	6	6	6	6	6	6	Permeability
913	48-06, 7-1	29	29	28	28	25	29	30	30	30	25	10	18	Sensitive
1085	48-07, 7-1	27	28	25	26	22	26	24	24	24	22	9	16	Sensitive
1365	47-17, 4-1	30	21	22	22	23	31	28	27	27	26	6	16	Sensitive
1457	01-09, ND	26	20	21	21	21	27	24	25	22	22	11	16	Sensitive
1104	50-03, 24-4	6	6	6	6	6	6	6	6	6	6	6	6	Permeability
1452	67-03, ND	6	6	6	6	6	6	6	6	6	6	6	6	Permeability
1520	53-11, ND	6	6	6	6	6	6	6	6	6	6	6	6	Permeability

[a] Disk diameter is 6 mm. A value of 6 mm indicates no zone of inhibition. Apra, apramycin; Forta, fortamicin; Gent, gentamicin; Tobra, tobramycin; Amk, amikacin; Isep, isepamicin; Net, netilmicin; Kan, kanamycin; Neo, neomycin.

[b] Genotypes for the clinical isolates were determined by randomly amplified polymorphic DNA fingerprinting as described by Bukanov et al. (7). ND, not determined.

Generation of unmarked deletions. Unmarked deletions of the *amrR* gene in PAO1 and the *amrB* gene in strain 2547 were generated according to the method described by Schweizer (41). DNA homologous to sequence flanking the region of deletion was amplified by PCR. Primers 5′ for-amrR (5′-CGTCGCGGGTT TTCTGGGATCCCTCTTTGG-3′) and 5′ rev-amrR (5′-GCAGGAATTCGCG ATGCGGATTGCGGAAC-3′) generated a 1-kb fragment with *Bam*HI and *Eco*RI restriction sites introduced on the respective ends and flanking the 5′ end of *amrR*. Similarly, primers 3′ for-amrR (5′-GGAAAGCTTGGTGGCGAGGA AGGCATTGG-3′) and 3′ rev-amrR (5′-GCCTCTAGAGCCTGCGCAGTTCT CCCTAC-3′) generated a 1.1-kb fragment with *Hin*dIII and *Xba*I restriction sites introduced on the respective ends and flanking the 3′ end of *amrR*. The 5′ and 3′ ends flanking the *amrAB* deletion region were generated with the same respective restriction sites by using primers 5′ for-amr3 (5′-CGTCGCGGGTT TTCTGGAATTCCTCTTTGG-3′), 5′ rev-amr3 (5′-AGCAATTCGGGATCCG GATTGCGGAACAG-3′), 3′ for-amr3 (5′-CAAGCCTGATGCCTCTAGAGA AACTCTCGC-3′), and 3′ rev-amr3 (5′-GAATCTGGTTCAAGCTTGAGCAG CGCTACG-3′). PCR fragments were amplified with 3 cycles at an annealing temperature of 55°C and 31 cycles at an annealing temperature of 60°C. Fragments were digested with the appropriate enzymes and 5′ and 3′ fragments were cloned sequentially into plasmid pEX18T. The Gm^r-GFP cassette from pPS858 was isolated as a *Bam*HI fragment and cloned directly into pEX18T containing flanking ends for *amrR*. Due to *Bam*HI restriction sites in the *amrAB* 3′ fragment, the Gm^r-GFP cassette and *Xba*I-digested pEX18T containing flanking ends for *amrAB* were treated with Klenow fragment and blunt-end ligated. All plasmids were constructed and propagated in *E. coli* DH5α. The final constructs were introduced into the *E. coli* donor strain, S17.1λpir, by chemical transformation and conjugally transferred to *P. aeruginosa*. Transconjugates were selected and propagated on L agar containing gentamicin. Secondary selection on L agar with carbenicillin was used to identify double-crossover events of the Gm^r and Cb^s phenotypes. In the case of pEX-AMR3, primary selection yielded only colonies which were Gm^r and Cb^r, or single-crossover events. One of these strains was grown overnight in L broth containing gentamicin. Culture dilutions (10^{-2} and 10^{-4}; volume, 100 μl) were plated on VB plus 5% sucrose agar containing gentamicin in order to select for the loss of the *sacB* gene. Colonies arising after 48 h were screened for carbenicillin resistance and propagated on L agar containing gentamicin (100 μg/ml). *E. coli* SM10 was used to mobilize the plasmid pFLP2, which contains the yeast Flp recombinase, into Gm^r and Cb^s strains of *P. aeruginosa* by conjugal transfer. Primary selection for *P. aeruginosa* carrying the pFLP2 plasmid was done on VB agar containing carbenicillin. Plasmids were cured by streaking colonies onto VB agar plus 5% sucrose and subsequently were confirmed to be Gm^s and Cb^s. Both strains 3579 and 3580 were confirmed to contain the proper deletions by comparing them to appropriate parent and single-crossover and double-crossover event and strains by Southern blot analyses (45).

Outer membrane SDS-PAGE gels. OMPs of *P. aeruginosa* were prepared by the method of Poxton et al. (37). Bacterial cells were grown to mid- to late-log phase. Cell pellets were resuspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4). Cells were broken by ultrasonic disruption, and unbroken cells were removed by low-speed centrifugation (10,000 × *g* for 10 min). Membranes were subsequently pelleted by high-speed centrifugation (100,000 × *g* for 30 min), resuspended in 10 mM HEPES with 2% (wt/vol) sodium *N*-lauroyl sarcosine, and incubated at room temperature for 1 h. Insoluble membrane proteins were pelleted by high-speed centrifugation (100,000 × *g* for 30 min) and resuspended in distilled water. Samples were assayed for total protein using the *DC* Protein Assay (Bio-Rad) as per the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% acrylamide/bis-acrylamide (37.5:1) SDS slab gel and analyzed by staining with GELCODE Blue Stain Reagent (Pierce).

Southern blot hybridizations. *Sal*I-digested genomic DNA (1 μg) was run on a 0.7% agarose gel. DNA was blotted onto positively charged nylon membranes overnight (Nytran Plus; Schleicher & Schuell) by standard alkaline transfer (40). Dry membranes were cross-linked with UV light and probed with random-primed [α-^{32}P]dCTP-labeled DNA. Mutants were confirmed by probing blots with the PCR fragments that were cloned into the recombination vectors. The deletion mutants were confirmed to be unmarked by probing with the Gm^r-GFP cassette.

Nucleotide sequence accession numbers. The sequences of the *amrR* and *amrAB* genes have been deposited with GenBank under the accession no. AF147719. Other relevant genes can be referenced with the following GenBank accession no.: AF073776 (*mexGH* [*P. aeruginosa*]), AB015853 (*mexXY* [*P. aeruginosa*]), and AF072887 (*amrAB* [*Burkholderia pseudomallei*]).

RESULTS

Characterization of spontaneous tobramycin-resistant mutants. Two spontaneous AGIR mutants were isolated in a stepwise fashion from strain PAO1. The mutants, designated 2547 and 2548, are isogenic to PAO1 as determined by PFGE (data not shown), and they displayed a panaminoglycoside-resistant phenotype, which is characteristic of AGIR strains, in the AGRP assay (Table 2). Strain 2547 showed an intermediate-level aminoglycoside impermeability phenotype, whereas 2548, with no zone of inhibition to any of the tested aminoglycosides, showed a high-level impermeability phenotype. In addition to enhanced aminoglycoside resistance the mutants exhibited other altered phenotypic characteristics compared to PAO1. Neither of the mutants were able to hydrolyze urea, and both displayed impaired growth in culture with rich media (Fig. 2).

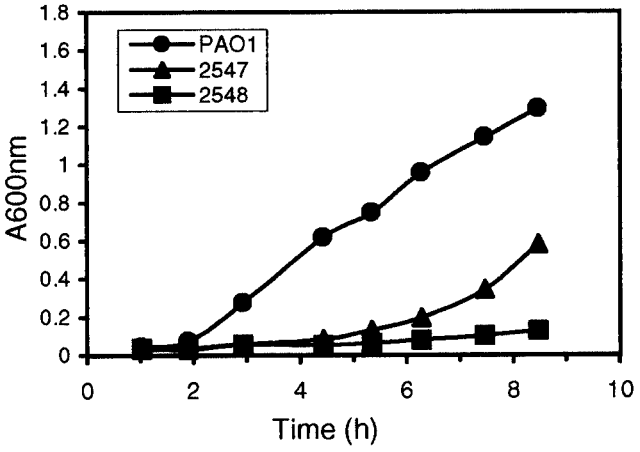

FIG. 2. Comparison of the growth rates of PAO1 and spontaneous tobramycin-resistant mutants.

Identification of the *amr* locus. RDA subtractions were designed to identify genes that are up-regulated in the mutants 2547 and 2548 in comparison to strain PAO1. Three subtractions were done with an excess of PAO1-derived cDNA as the driver. Tester cDNA was derived from in vitro cultures of strains 2547, 2548, and 2548 grown in the presence of 16 μg of tobramycin per ml. An average of 200 difference products per subtraction were sequenced, and approximately 30 open reading frames (ORFs) were identified from each subtraction. One of these ORFs which was identified in all three experiments was designated *amrB* based on strong homology (79% similarity of amino acid sequences) to a previously identified efflux pump component that affects aminoglycoside and macrolide resistance in *B. pseudomallei* (27). BLAST analysis of the *Pseudomonas* genome revealed that *amrB* was located downstream of another gene, designated *amrA*, which has homology to the membrane fusion proteins of the RND-type transporters. Homology of the predicted amino acid sequence of AmrB and genomic organization of the *amrAB* genes (Fig. 1) indicate that AmrAB belongs to the RND family of transporters; however, unlike the previously characterized Mex pumps, no OMP was identified downstream of *amrB*. More recently, the sequence of the *amrAB* genes was found to be highly similar to two other GenBank sequences from PAO1, *mexGH* (1) and *mexXY* (26). While the deduced amino acid sequences of *amrAB* and *mexXY* are only 84 to 89% similar, respectively, the restriction pattern published by Mine et al. (26) is the same as that deduced for the *amrAB* genes. In addition, BLAST analysis of the PAO1 genome (presently more than 99% complete) revealed no other matches for the *mexXY* or *mexGH* genes that were perfect or better than *amrAB*. Therefore, it seems likely that *amrAB*, *mexXY*, and presumably *mexGH* are the same genes.

AmrR transcriptional regulation of *amrAB*. The region located upstream of *amrA* contains an ORF, designated *amr*R, belonging to the TetR family of bacterial response regulators (Fig. 1). To determine whether this putative regulator was involved in the transcription of *amrAB*, an unmarked deletion of *amrR* was constructed in strain PAO1. The deletion was generated using a gentamicin resistance, green fluorescent protein selectable marker cassette that is flanked by yeast Flp recombinase target sites (17). Generation of an unmarked deletion within a gene by a Flp-based system has been demonstrated in other organisms and shown to cause no polar effects on downstream transcription (8, 21). The resulting strain, 3579, was shown by RT-PCR to transcribe the *amrA* gene at least 15-fold and the *amrB* gene at least 12-fold compared to the parental strain PAO1 (Fig. 3). The transcription levels in strain 3579 were approximately equivalent to those in a strain carrying *amrAB* on a multicopy plasmid, strain 3582, but are still approximately sixfold less than those of the spontaneous mutants 2547 and 2548 (Fig. 3). It has been shown previously that other *P. aeruginosa* strains that overexpress Mex pumps have mutations in their respective transcriptional regulatory genes (18, 35, 36). However, sequencing of the *amrR* gene in both 2547 and 2548 revealed no mutations in either the putative 5′ promoter region or the *amrR* coding sequence compared to wild-type PAO1. Therefore, although it is apparent that *amrR* can negatively regulate transcription of *amrAB*, loss of this regulatory function does not seem to be the cause of the up-regulation observed in mutants 2547 and 2548. However, we recently identified an *rplA* mutation in strains 2547 and 2548 that theoretically would result in a truncated form of ribosomal protein L1. Complementation of mutant 2547 with a wild-type copy of the *rplA* gene restored aminoglycoside sensitivity, growth rate, and *amrAB* transcription levels back to PAO1 levels but did not complement the urease defect (16).

Deletion of *amrAB* from strain 2547 results in a reduction in MICs of aminoglycosides. To determine whether the increased transcription of *amrAB* contributes to the AGIR phenotype of the laboratory mutants, an unmarked deletion of *amrB* was constructed in strain 2547 by the same system described above. Strain 3580 (2547Δ*amrB*) shows MIC values returned to within a fourfold difference of wild-type (PAO1) levels for four of the six aminoglycosides tested (Table 3). Both kanamycin and tobramycin also showed reduced MIC values compared to strain 2547, but in these cases the changes were less than or equal to fourfold. Strains 2547 and 3580 did not show differences in the MICs of any of the other nonaminoglycoside drugs tested, and in addition, the deletion of *amrB* in strain 3580 did not complement the other phenotypic characteristics, slow growth and lack of urease activity, seen in strain 2547 compared to PAO1.

Up-regulation of *amrAB* alone does not alter MICs of aminoglycosides for strain PAO1. Despite the overexpression of *amrAB* in strains 3579 and 3582, the MICs for these strains did not change significantly relative to those of PAO1 (Table 3). Since MexXY has been shown to associate with OprM in *E. coli* (26), we investigated the possibility that OprM might contribute to an increase of aminoglycoside resistance in strain 3579, which overexpresses *amrAB* (*mexXY*) as a result of an unmarked deletion of the *amrR* gene. Transformation of strain 3579 with the plasmid pXZL34, which has been shown previously to express OprM under the control of an IPTG-inducible promoter (48), resulted in strain 3737. Growth of strain 3737 in

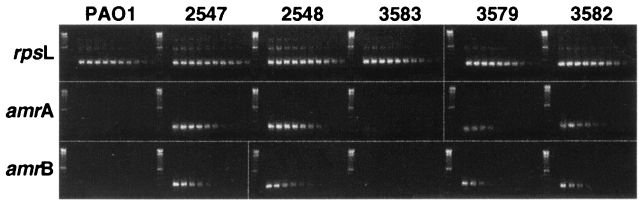

FIG. 3. 1.5% agarose gels of RT-PCR products showing relative expression of the genes *rpsL* (positive control), *amrA*, and *amrB*. For each set the two left-most lanes show a 100-bp DNA ladder and an RT-negative template (negative control), respectively, and the other lanes show an RT-positive template (serial 1:3 dilutions).

TABLE 3. Susceptibilities of *P. aeruginosa* strains to various antibiotics[a]

Strain	Relevant genotype	MIC (μg/ml)													
		Apra	Gent	Amk	Kan	Neo	Tobra	Nor	Oflox	Cipro	Lom	Erm	Clar	Clin	Tet
PAO1	Wild type	16	2	4	128	16	0.25	2	2	0.5	4	>32	>8	>32	32
3583	PAO1(pUCP20)	16	4	8	128	16	0.5	2	2	0.25	4	>32	>8	>32	32
3582	PAO1(pAMR-1)	16	2	8	256	64	1	4	4	1	8	>32	>8	>32	32
3579	PAO1Δ(*amrR*)	32	4	8	128	32	0.5	2	4	0.5	8	>32	>8	>32	32
PAO200	PAO1Δ(*mexAB-oprM*)	2	<1	2	32	2	0.25	<0.06	<0.06	<0.015	0.13	32	2	>32	0.5
2547	PAO1 mutant	256	32	128	>512	256	16	4	4	1	8	>32	>8	>32	32
3580	2547Δ(*amrB*)	16	2	16	256	16	4	2	1	0.5	2	>32	>8	>32	16
2548	2547 mutant	>512	128	256	>512	>512	64	4	4	1	8	>32	>8	>32	32

[a] Apra, apramycin; Gent, gentamicin; Amk, amikacin; Kan, kanamycin; Neo, neomycin; Tobra, tobramycin; Nor, norfloxacin; Oflox, ofloxacin; Cipro, ciprofloxacin; Lom, lomefloxacin; Erm, erythromycin; Clar, clarithromycin; Clin, clindamycin; Tet, tetracycline.

0.05 mM IPTG did not impact susceptibility to any of the antibiotics tested (data not shown). In addition, we analyzed outer membrane preparations made from strains PAO1, 2547, and 2548 by SDS-PAGE to look for differences in the levels of the OprM. These gels revealed a dramatic decrease in the amount of OprM present in strains 2547 and 2548 compared to PAO1 (Fig. 4), indicating that OprM is unlikely to be the outer membrane component associated with this efflux system.

***amrB* transcription is increased in CF clinical isolates that display impermeability-type aminoglycoside resistance.** In order to address the possible clinical relevance of the Amr system, we used RT-PCR to examine *amrB* gene expression level differences among clinical isolates with varying AGRP phenotypes. Transcription of *amrB* can be detected in clinical isolates of *P. aeruginosa* that display an impermeability phenotype at levels that are at least 6- to 15-fold greater than that of strain PAO1 (Fig. 5), and gene expression is generally higher in the AGIR strains (Fig. 5A) compared to those clinical isolates with a sensitive phenotype (Fig. 5B). In particular, the isogenic clinical isolates 1168, 1250, and 1249 have AGRP phenotypes (Table 2) and *amrB* RT-PCR results that mimic directly those of PAO1 and the mutants 2547 and 2548. A trend towards increased *amrB* transcription was not observed in strains that produce aminoglycoside-modifying enzymes or have multiple AGRP phenotypes (data not shown).

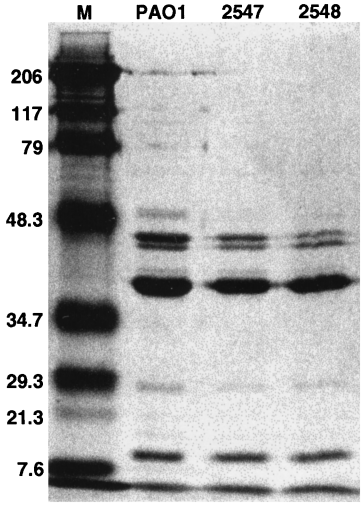

FIG. 4. SDS-PAGE analysis of OMPs. The OprM protein, known to be expressed in wild-type *P. aeruginosa*, runs in the 50-kDa monomeric form with the inclusion of β-mercaptoethanol in the sample buffer (48). The molecular weight standards (M) are listed to the left in kilodaltons.

DISCUSSION

Drug efflux is a prevalent mechanism of antibiotic resistance in *P. aeruginosa*. Until recently this mechanism of resistance had been seemingly limited to quinolones, β-lactams, tetracycline and chloramphenicol (14, 31). However, new evidence for *B. pseudomallei* now indicates an even broader potential for efflux-mediated acquired and intrinsic antibiotic resistance. The recently described *B. pseudomallei* genes, *amrAB*, have

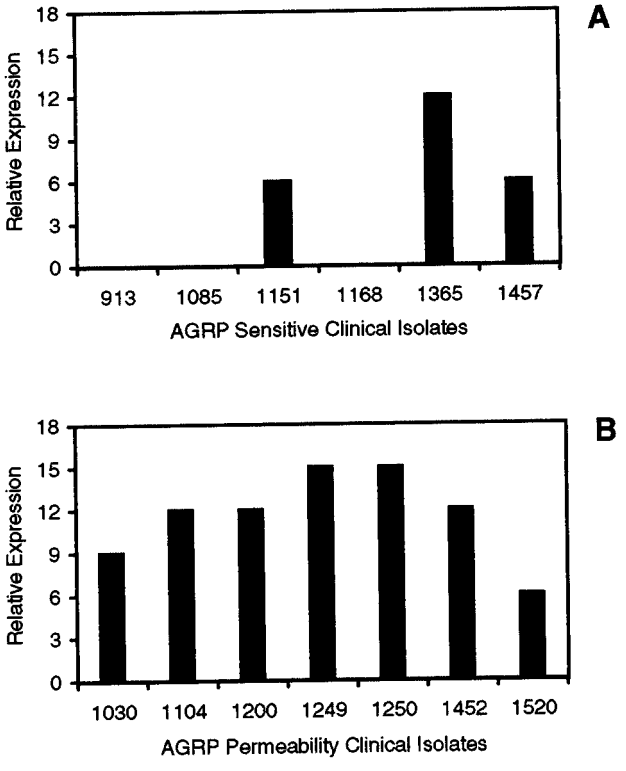

FIG. 5. Graphs representing expression of *amrB* in *P. aeruginosa* clinical isolates having the sensitivity (A) or permeability (B) phenotype from patients with CF relative to that in strain PAO1 as determined by RT-PCR. Relative expression levels are plotted as the number of 1:3 dilutions showing amplification on a 1.5% agarose gel multiplied by three. PAO1 had no detectable amplification, so the relative expression value represents at least that fold increase in expression of *amrB* by a particular strain compared to PAO1.

TABLE 4. Kirby-Bauer disk diffusion assay for selected quinolones

Strain	Relevant genotype	Zone diameter (mm)[a]				
		Nor	Oflox	Cipro	Lom	Nal
PAO1	Wild type	28	20	31	23	7
2547	PAO1 mutant	20	12	27	13	7
2548	2547 mutant	19	10	26	11	7
3580	2547Δ(*amrB*)	28	18	36	24	7

[a] Disk diameter is 6 mm. Disk content (in μg/ml) and resistance and sensitivity ranges (R and S, respectively; also in μg/ml) for the drugs tested were as follows: for norfloxacin (Nor), disk content = 10, R ≤ 12, and S ≥ 17; for ofloxacin (Oflox), disk content = 5, R ≤ 12, and S ≥ 16; for ciprofloxacin (Cipro), disk content = 5, R ≤ 15, and S ≥ 21; for lomefloxacin (Lom), disk content = 10, R ≤ 18, and S ≥ 22; and for nalidixic acid (Nal), disk content = 30, R ≤ 13, and S ≥ 19.

been shown to contribute to the intrinsic resistance of this organism to both macrolides and aminoglycosides (27).

Aminoglycoside impermeability-type resistance is poorly understood despite the fact that it is the predominant manifestation of aminoglycoside resistance in *P. aeruginosa* isolates infecting the lungs of CF patients (23). In this study we show that spontaneous mutants of PAO1 displaying the AGIR phenotype also display up-regulation of the *amrAB* genes. The deletion of the *amrB* gene resulted in the reversal of the panaminoglycoside resistance phenotype, and in contrast to the recent work describing expression of the *mexXY* genes in *E. coli* (26), our data did not show any change in the MICs of a number of quinolones and macrolides for strain 3580 compared to those for strain 2547. It was shown recently that the frequency of emergence of fluoroquinolone resistance in a triple deletion strain of PAO1 (Δ*mexAB-oprM*::Cm Δ*mexEF-oprN*::ΩHg Δ*mexCD-oprJ*::Gm) was essentially undetectable (22). These data would argue against the role of an additional efflux system actively contributing to quinolone resistance in *P. aeruginosa*. Using disk susceptibility assays we did detect small differences in the zones of inhibition for lomefloxacin, norfloxacin, and ofloxacin for strains PAO1, 2547 and 3580 (Table 4). However, the comparison of these subtle differences with the changes seen in the MICs of quinolones for strain PAO200 (Δ*mex*AB-*opr*M) (Table 3) leads us to believe that the observed quinolone effect is more likely to be the result of regulatory cross talk with other efflux systems rather than an affinity of AmrAB for quinolones. Recent data demonstrating coordinated regulation of expression for the MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux pumps (19) supports the possibility that altering the expression of a single pump may have downstream effects on any number of other efflux systems. In fact the apparent decrease seen for OprM in the mutants 2547 and 2548 compared to PAO1 would be consistent with the quinolone disk resistance data noted above and support a hypothesis of coordinated regulation between MexAB-OprM and the AmrAB efflux systems.

Although it is clear from our studies that *amrAB* can play a role in aminoglycoside resistance, up-regulation of the *amrAB* genes alone in wild-type *P. aeruginosa* (PAO1) did not affect susceptibility to aminoglycosides or any of the other antibiotics tested, including quinolones and macrolides. This is not unexpected since RND-type systems are known to require three proteins for active efflux of drugs from the cytoplasm, an inner membrane RND-transport protein, a periplasmic membrane fusion protein, and an OMP (11, 31–33). It is likely that the lack of enhanced resistance displayed by the above strains is due to the absence of corresponding expression of an appropriate OMP. Recent observations by Mine et al. suggest that OprM could be the proper accessory protein for AmrAB (26). Nonetheless, SDS-PAGE gels of outer membrane preparations revealed that very little OprM is even present in the mutant 2547. Our results suggest that the OMP that acts in conjunction with AmrAB in affecting aminoglycoside resistance in *P. aeruginosa* is unlikely to be OprM but instead some other yet to be identified protein. There are many potential choices for an OMP that will function with the AmrAB pump. Analysis of the newly sequenced PAO1 genome has identified ten potential RND-type drug efflux loci. Four of these loci, including the Amr locus, lack downstream OMPs. Additional phylogenetic analyses have identified three large families of OMPs, each consisting of 18 to 27 members, and numerous smaller families in the genome (13). One large family is most closely related to those proteins previously characterized as having a role in antimicrobial efflux and contains a subfamily that is most related to OprM, OprN, and OprJ. Comparisons of trees generated using these OMPs and the corresponding proposed cytoplasmic membrane and pump components suggests that in some cases there has been a shuffling of the OMP genes relative to the periplasmic and cytoplasmic membrane components of the efflux machinery (13). This is reminiscent of a previous report on other efflux OMPs that suggests that the OMP components evolve independently of the other components (33). The genome analysis reveals a complex set of efflux systems and corresponding OMPs and highlights the need for additional understanding of how these systems may be interrelated and regulated.

Despite the fact that AGIR has been observed in the clinic for decades, the molecular mechanisms of this phenotype are still not well understood. The RDA experiments presented in this study identified a large number of genes that are potentially involved in this type of aminoglycoside resistance and suggest that the AGIR phenotype is multifaceted. While we have shown here that the AmrAB efflux system can contribute to aminoglycoside resistance, it seems that efflux may be only one of the factors that can contribute to this phenotype in spontaneously occurring mutants of *P. aeruginosa*. The ability to generate stepwise mutants resistant to increasing levels of aminoglycosides (i.e., strain 2548) and the up-regulation of the *amrAB* genes in a one-step, low-level resistant mutant, strain 2547, in addition to other phenotypic differences, such as reduced growth rate and lack of urease activity, also support the theory that AGIR is a cumulative, multifactorial process. While we have shown here that the *amrR* gene can negatively regulate transcription of *amrAB*, the mutation in 2547 resulting in up-regulation at the *amr* locus does not seem to be related to the *amrR* gene. Alternate regulatory pathways for the currently characterized Mex pumps are just starting to surface. For instance, a recent publication has demonstrated growth-phase regulation for the *mexAB-oprM* system and shown that this regulation is not dependent on MexR (10). The *rplA* mutation we recently identified in strains 2547 and 2548 and its link to aminoglycoside resistance require more study. However, this mutation may affect some global regulatory function that results in deregulation and hyperexpression of *amrAB* as well as many other genes. In any case, it seems that the functional regulation of this pump is quite complex and the identification of other regulatory systems affecting the expression and function of the *amr* locus could shed light on the pleiotropic effects noted above.

The association between the AGIR phenotype and hyperexpression of the AmrAB efflux system was initially observed in a laboratory-generated mutant of PAO1. However, the potential relevance of this system to the AGIR observed in clinical isolates is also supported by RT-PCR data demonstrating sub-

stantially increased transcription of *amrAB* in clinical strains displaying the AGIR phenotype when compared to a genotypically matched sensitive isolate (e.g., 1168, 1250, and 1249). Because *amrAB* up-regulation alone does not appear to be sufficient to affect aminoglycoside resistance and because *amrAB* hyperexpression may not be universally necessary for AGIR one would not expect a direct correlation between *amr*B expression and resistance phenotype in all strains. However, a general trend of higher-level *amrB* expression was observed in clinical isolates displaying the AGIR phenotype compared to sensitive isolates. These studies support the hypothesis that the AGIR phenotype is the result of a complex interaction of molecular events and suggest the possibility that multiple molecular scenarios may result in similar AGIR phenotypes. The latter point may contribute to the difficulty in understanding the exact mechanism of this type of resistance. Ultimately, understanding the complicated and potentially diverse manifestations of impermeability resistance may require looking beyond aminoglycoside resistance to other phenotypic differences among this population and perhaps in the process redefine the nature of impermeability resistance. In conclusion, we believe efflux of aminoglycosides by the AmrAB system to be one of the contributing factors in the multifactorial AGIR phenotype, but further study will be necessary to understand the intricate aspects of this type of resistance in *P. aeruginosa*.

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