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Reverse Engineering Antibiotic Sensitivity in a Multidrug-Resistant *Pseudomonas aeruginosa* Isolate

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Antibiotic resistance is a pervasive and growing clinical problem. We describe an evaluation of a reverse engineering approach for identifying cellular mechanisms and genes that could be manipulated to increase antibiotic sensitivity in a resistant *Pseudomonas aeruginosa* isolate. We began by chemically mutating a broadly resistant isolate of *P. aeruginosa* and screening for mutants with increased sensitivity to the aminoglycoside amikacin, followed by performing whole-genome transcriptional profiling of the mutant and wild-type strains to characterize the global changes occurring as a result of the mutations. We then performed a series of assays to characterize the mechanisms involved in the increased sensitivity of the mutant strains. We report four primary results: (i) mutations that increase sensitivity occur at a high frequency (10^{-2}) relative to the frequency of those that increase resistance (10^{-5} to 10^{-10}) and occur at a frequency 10^4 higher than the frequency of a single point mutation; (ii) transcriptional profiles were altered in sensitive mutants, resulting in overall expression patterns more similar to those of the sensitive laboratory strain PAO1 than those of the parental resistant strain; (iii) genes found from transcriptional profiling had the more dramatic changes in expression-encoded functions related to cellular membrane permeability and aminoglycoside modification, both of which are known aminoglycoside resistance mechanisms; and finally, (iv) even though we did not identify the specific sites of mutation, several different follow-up MIC assays suggested that the mutations responsible for increased sensitivity differed between sensitive mutants.

Antibiotic resistance develops rapidly after the introduction of a new antibiotic and now exists, to some extent, for all antibiotics. Resistance has even evolved for drugs specifically designed to prevent selection for resistance (10, 23, 27, 30, 43, 52, 74, 85, 86). These factors underline the importance of understanding the genetic and phenotypic bases underlying antibiotic resistance and developing new strategies to combat the proliferation of resistant organisms. One approach has been to combine new and conventional antibiotics to simultaneously increase the sensitivities of resistant organisms and target essential genes (40, 50, 51, 73). The success of such an approach is dependent upon the identification of genes and/or mechanisms that might be targeted to increase sensitivity. We report here on our efforts to evaluate a reverse engineering approach for identifying such genes and mechanisms. Specifically, we have identified aminoglycoside-sensitive mutants of a multiple-drug-resistant *Pseudomonas aeruginosa* isolate and characterized the global changes in gene expression associated with mutations that restored sensitivity in two of these mutants as well as the resistant parental strain and the sensitive laboratory strain PAO1.

We chose to study a clinical isolate, named B1, that showed high levels of resistance to five aminoglycosides tested: amikacin, gentamicin, kanamycin, streptomycin, and tobramycin. B1 was found to be of serotype O12, a serotype that has been associated with multiresistance to a number of antibiotic classes, including aminoglycosides and beta-lactams (4, 15, 46, 65, 66). B1 was chemically mutagenized and screened to identify mutants that exhibited increased levels of susceptibility to amikacin. Based on the fact that resistance can arise due to genetic mutation (2, 13, 14, 29), we expected that it would also be possible to restore sensitivity to an already resistant strain through mutation. We hypothesized that the frequency of finding mutations that increase sensitivity would be higher than the frequency of isolating mutants with increased resistance. This is based on the premise that, with only a limited number of point mutations providing a selective advantage in the presence of an antibiotic, a larger percentage of mutations may either have no impact or decrease the level of antibiotic tolerance. Although the frequency of finding sensitivity mutations is a key consideration in assessing the potential of combination therapies, the ability to identify sensitivity-restoring genes/mechanisms is the critical parameter in the design of such therapies.

We hypothesized that whole-genome transcriptional profiling with Affymetrix PAO1 GeneChips could be used for such a purpose. To test this hypothesis, we compared the expression patterns among strain B1, two of its susceptible mutants (named M5 and M31), and laboratory strain PAO1 (81). Moreover, we assessed the resistance mechanisms suggested by transcriptional profiling by MIC assays performed under a variety of conditions (in the presence of verapamil, carbonyl cyanide *m*-chlorophenylhydrazone [CCCP], and polymyxin B and with spheroplasts) as well as PCR-based gene identification approaches.

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MATERIALS AND METHODS

Strains and culturing conditions. All isolates of *P. aeruginosa* were obtained from Mike Vasil at the University of Colorado Health Sciences Center, Denver. All strains were cultured in Luria-Bertani (LB) broth at 37°C with constant

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TABLE 1. MICs of PAO1, B1, and the sensitive mutants characterized

Strain	MIC (μg/ml)[a]							
	AMK (*n*)[b]	AMK + CP (*n*)	AMK + VP (*n*)	POLYB (*n*)	GEN (*n*)	KAN (*n*)	STR (*n*)	TOB (*n*)
PAO1	4 (25)	4 (8)	4 (8)	2 (8)	4 (4)	200 (4)	64 (4)	4 (4)
B1	25 (21)	25 (8)	25 (8)	2 (8)	200 (4)	800 (4)	>4,000 (4)	200 (4)
M5	8 (13)	8 (8)	8 (8)	2 (8)	200 (4)	800 (4)	>4,000 (4)	200 (4)
M31	4 (25)	4 (8)	4 (8)	2 (8)	64 (4)	400 (4)	2,000 (4)	64 (4)
M50	10 (6)	10 (8)	10 (8)	2 (8)				
M52	8 (6)	8 (8)	8 (8)	2 (8)				

[a] AMK, amikacin; CP, carbonyl cyanide *m*-chlorophenylhydrazone (250 μM); VP, verapamil (100 μg/ml); POLYB, polymyxin B; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; TOB, tobramycin.
[b] *n*, number of replicates.

shaking at 225 rpm. Serotyping was performed by Adriana Vasil at the University of Colorado Health Sciences Center.

Disk susceptibility and MIC determination. Susceptibility to aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, tobramycin) was determined by methods similar to the disk diffusion method described previously (58) with LB agar plates. LB agar plates were inoculated by swabbing with a turbid culture. Immediately following inoculation, BBL Sensi-Discs (Fisher Scientific, Pittsburgh, PA) were placed with sterile forceps onto the plate. The plates were incubated overnight, and the diameters around the disks were measured.

MICs were determined by a standard broth dilution method in LB medium (1). The lowest concentration at which no growth was noted after 18 h was deemed the MIC. Cultures at an optical density at 600 nm of 0.6 were used for MIC studies For preparation of the medium to be used in studies of MICs determined in the presence of CCCP or verapamil, either CCCP was added to LB medium to achieve a 250 μM solution (87) or verapamil was added to LB medium to achieve a final concentration of 100 μg/ml. This medium was then used in the standard broth dilution MIC assay, with the appropriate levels of amikacin added.

For MIC assays performed with spheroplasts, spheroplasts of *P. aeruginosa* were formed by treating the cells with EDTA and lysozyme (77). Spheroplast formation was checked by osmotic shock (5, 59).

Random mutagenesis and sensitivity screening. Random mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; TCI America, Portland, OR) was carried out as described previously (53). Cells were exposed to MNNG at a concentration of 50 μg/ml for 8 min. This level of mutagenesis was sufficient to result in the survival of 50% of the exposed cells. The mutated cells were then plated onto LB agar plates. The contents of the master plates were then replica plated (44) onto LB agar plates containing increasing levels of amikacin. MIC studies were carried out with the colonies that grew on the master plate but not on the imprints to confirm sensitive phenotypes.

For studies conducted to examine the frequencies of sensitivity-restoring mutations, mutated cells were plated onto LB agar plates and incubated overnight. The cells were then patched into 384-well plates containing LB medium. These plates were then patched by using a 384-pin blot replicator into 384-well plates containing increasing amounts of amikacin. After 18 h of incubation, growth in the 384-well plates was confirmed by once again replica plating the cells from the amikacin-containing plates into plates containing LB medium with no amikacin. These plates were then incubated, and growth was determined visually after 18 h.

Detection of plasmids. Strain B1 was examined for the presence of plasmids. No plasmids were found from plasmid extractions with a QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA) or the hot alkaline method of Kado and Liu (36) which has been shown to be effective for the isolation of plasmids in the size range of 2.6 to 350 MDa.

LPS gels. Lipopolysaccharide (LPS) was isolated by first lysing the cells and treating the lysate with proteinase K (26). LPS was run on 16.5% acrylamide Tris-Tricine gels (Bio-Rad, Hercules, CA) and silver stained (20).

Transcriptional profiling. Cultures were grown in medium containing amikacin at a concentration of 50% of the respective MIC and were incubated until logarithmic phase. Volumes of the culture (10 ml) were collected and immediately immersed in liquid nitrogen for 10 s. The samples were then centrifuged (5,000 × *g*, 10 min, 4°C) and the supernatant was removed. The cell pellets were then once again immersed in liquid nitrogen for 15 s and then stored at −80°C for later RNA extraction.

RNA was extracted from strain B1 and its mutants by using a TRIzol Max bacterial RNA isolation kit (Invitrogen Life Technologies, Carlsbad, CA), followed by further purification with an RNeasy Mini kit (QIAGEN), according to the manufacturer's specifications. RNA from strain PAO1 was extracted by using just the RNeasy Mini kit, but the samples were further purified by using an additional RNeasy mini spin column (63).

Microarray probes were prepared according to the Affymetrix (Santa Clara, CA) *P. aeruginosa* GeneChip expression analysis protocol, with the slight modification that 2× PCR Enhancer Solution (Invitrogen Life Technologies) was added during cDNA synthesis. Target hybridization, washing, staining, and scanning were performed by the University of Colorado DNA Microarray Facility, according to the manufacturer's specifications, by using a GeneChip hybridization oven, a GeneChip fluidics station, a GeneArray scanner, and GeneChip operating software (v1.1) (Affymetrix).

Microarray data analysis. Microarray data were analyzed with ArrayAssist (Stratagene, La Jolla, CA). The data were analyzed by first performing robust multichip averaging (6, 32, 33), followed by principal component analysis (72), hierarchical clustering (18), and *t* tests. Data for a third replicate of strain B1 were removed from the analysis due to the large discrepancies in the results of principal component analysis and hierarchical clustering between it and the two other replicates. Additionally, the third replicate of B1 was generated by using a microarray from a different batch of Affymetrix GeneChips. Log fold differences are of base 2.

RESULTS

Screening of mutants for increased amikacin sensitivity. Our efforts here were directed at reverse engineering of sensitivity into a resistant isolate of *P. aeruginosa* and then determining the extent to which the transcriptional profiles could be used to elucidate sensitivity genes and/or mechanisms. First, we determined how much we could increase the sensitivity of a resistant isolate and how frequently we would identify mutants with increased sensitivity. To do so, multidrug-resistant isolate B1 was subjected to random chemical mutagenesis with MNNG and screened to identify mutants with increased levels of sensitivity to amikacin. We chose chemical mutagenesis over other forms of mutatgenesis, such as random or targeted insertion-based mutagenesis, because the B1 isolate studied was found to be resistant to many antibiotics for which there are available selective markers, including chloramphenicol, tetracycline, zeocin, blasticidin, carbenicillin, mercury, trimethoprim, and nalidixic acid, and because chemical mutagenesis can result in mutations that affect not only the expression of a gene but also the function of the encoded gene product.

Initially, 850 mutants were screened by using replica plating for increased susceptibility to amikacin. Of the 850 mutants, 4 were confirmed to have MICs lower than that of B1 (amikacin MIC, 25 μg/ml) (Table 1). Three mutants (M5, M50, and M52) had MICs at intermediate levels of 8, 10, and 8 μg/ml, respectively, while mutant M31 had an MIC equal to that of laboratory strain PAO1 (4 μg/ml). It is of note that we performed the

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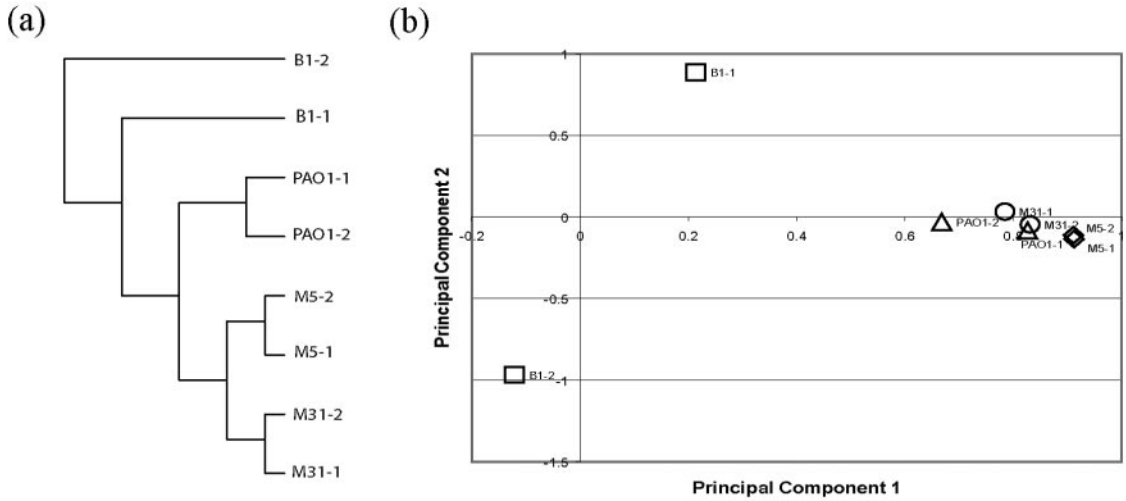

FIG. 1. (a) Hierarchical clustering (b) and principal component analysis of transcriptional profiles of strain B1, strain PAO1, and sensitive mutants M5 and M31. Replicates are labeled 1 and 2.

MIC assays with changes in concentrations less than the standard twofold change, with significance determined by *t* tests.

Our initial round of reverse engineering suggested that the frequency of sensitivity-conferring mutations was surprisingly high (4/850). To more thoroughly investigate the frequency of sensitivity-conferring mutations, we screened an additional 1,500 mutants produced from a single round of random mutagenesis. The frequency of mutations leading to an 80% decrease in the MIC was found to be about $4.8 \times 10^{-2} \pm 0.01$.

To gain an idea of the number of places on the genome that could be mutated to increase susceptibility to amikacin, we compared the frequencies for which we found sensitive mutants to the frequency of finding a mutant resistant to rifampin, which is known to be due to a single point mutation. The frequency of finding B1 mutants resistant to rifampin when they were subjected to the same level of exposure to MNNG was found to be 3.4×10^{-6}, over 4 orders of magnitude less than the frequency of finding a sensitivity-increasing mutation.

Finally, we wanted to assess in greater detail whether or not sensitivity could be further increased through recursive mutagenesis. To do so, we subjected the most sensitive mutant resulting from a single round of mutagenesis, mutant M31, to a second round of random mutagenesis with MNNG. From this additional round, a total of close to 3,000 mutants were screened for increased susceptibility to amikacin. Interestingly, none were found to have further increased sensitivity relative to that of the M31 parental strain.

Transcriptional profiling. Transcriptional profiles were obtained for strain B1, the sensitive mutants M31 and M5, and strain PAO1 grown in the presence of half of their respective amikacin MICs. Amikacin was included in the medium so that the profiles of the strains would reflect the transcription of the strains under similar levels of antibiotic stress. In all cases, cells were cultured identically and samples were obtained in early exponential phase. To determine the extent to which sensitivity mutations altered the overall transcriptional profiles, we performed hierarchical clustering and principal component analysis with the gene expression data corresponding to each strain evaluated. Hierarchical clustering results (Fig. 1a) revealed that the sensitive mutants had expression patterns more similar to that of the laboratory strain PAO1 than to that of parental isolate B1. To further assess this unexpected result, we used principal component analysis (Fig. 1b) to reduce the large amount of data generated from the microarray experiments to a few key variables, called principal components, that account for much of the variation among samples (72). Again, the principal component analysis values corresponding to replicate microarrays of the sensitive mutants and PAO1 strain clustered tightly together, while those of strain B1 were separated from the other samples along the first and second principal components (which accounted for 54% and 23% of the overall variation, respectively). This indicated that, with respect to these principal components, PAO1 and the sensitive mutants were more similar to each other than to B1. The discrepancies observed between the B1 replicates could, in part, be explained by the difficulty of working with the mRNA of this isolate. This, however, does not detract from the finding that the sensitive mutants exhibited expression patterns more similar to that of PAO1 than to those of either of the B1 replicates. This result suggests that the major variation in the overall gene expression data is the result of differences between the B1 profiles and all other profiles.

Our results indicated that significant changes in gene expression had occurred among the resistant and the sensitive strains. We wanted to determine if the changes in gene expression associated with increased sensitivity were coordinated between the two different sensitive mutants, which, if this were the case, would suggest that both mutants had converged upon similar overall gene expression phenotypes, or if the mutants had altered expression of entirely different sets of gene. In order to assess this possibility, *t* tests were performed to identify the number of genes with significantly altered expression among each strain evaluated. Between mutant M5 and strain B1, the difference in the expression of 694 genes both was statistically significant ($P \leq 0.05$) and showed at least a 1 log fold (base 2) difference. A twofold change in expression (equivalent to 1 log

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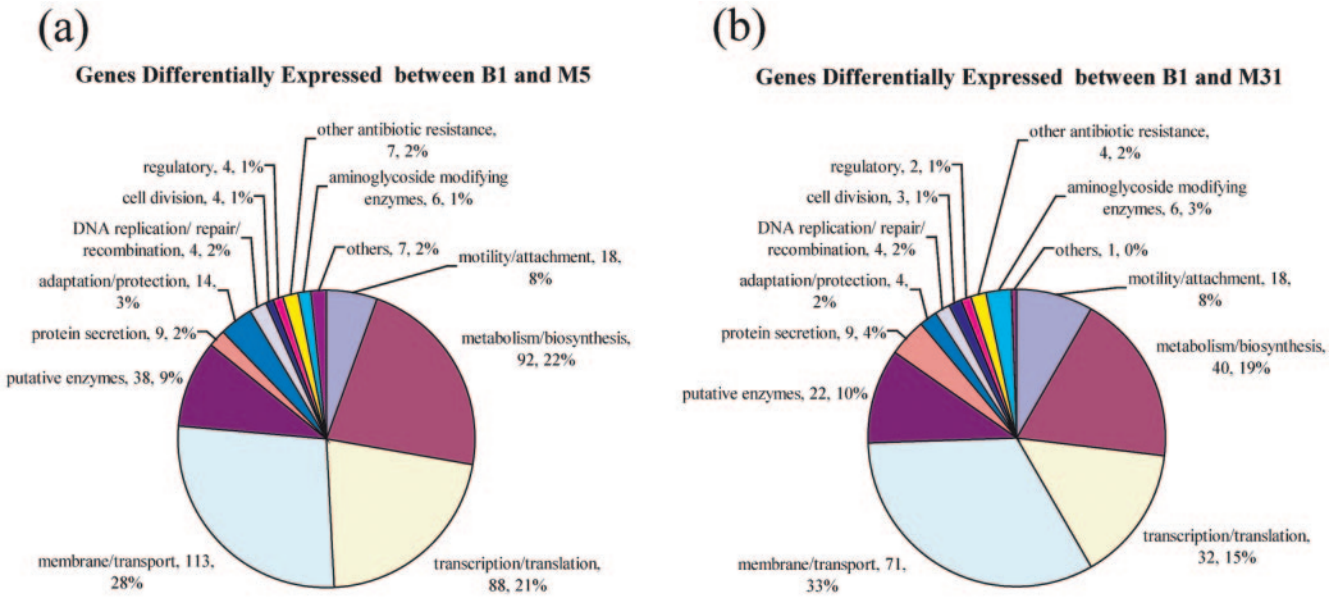

FIG. 2. Differentially expressed genes (log fold difference [base 2] ≥ 1; $P \leq 0.05$) between sensitive mutants and strain B1. The pie charts show the distribution of the functional categories of differentially expressed genes between (a) B1 and M5 and (b) B1 and M31. Following the name of each functional category are the number of genes in that category and the percentage of differentially expressed genes for which those genes account.

fold, base 2) has previously been found to be sufficient for the detection of nearly 95% of differentially expressed genes by the use of Affymetrix GeneChips (11). The number of genes differentially expressed between M31 and B1 was also high (350 genes). Of these sets of genes, only 101 genes were found to be differentially expressed in both mutants. It should be noted that the changes in gene expression not only might be due directly to a mutation in that gene or a regulator of that gene but also might be due to the response of the overall genetic regulatory network to such mutations.

Of the genes that were differentially expressed in mutant M5 or M31 compared with their expression in B1, a vast majority were genes that were grouped into three functional categories or that had no assigned function. The largest proportion of these genes were categorized as unclassified or as having only hypothetical functions (281 genes for M5 and 134 genes for M31). The largest functional category of genes (Fig. 2) comprised genes involved in cell permeability, LPS synthesis, efflux, and the transport of small molecules. There were changes in the expression levels in 113 of these genes for M5 and 71 for M31. The second and third categories contained genes involved in transcription or translation (88 for M5 and 32 for M31) and genes involved in metabolism, catabolism, and biosynthesis (92 for M5 and 40 for M31). Of particular interest, six genes encoding for aminoglycoside-modifying enzymes (AMEs) were found in both M5 and M31 to exhibit decreased expression relative to that in B1, suggesting that both strains would be similarly altered in aminoglycoside-modifying activity. Table 2 provides a detailed summary of the selected genes belonging to the AME or permeability functional class that had significant changes in expression.

PCR of AME genes. In effort to confirm the presence of a number of genes encoding AMEs, we used primers specific for particular AME genes and attempted to amplify these genes by PCR (80, 82, 84). PCR of *aadB*, *ant(4′)-IIa*, and *aac(3)-Ib* based on this method gave no bands when they were run on agarose gels. PCR of *aadA6*, *aac(3)-IIIb*, and *aac(3)-IIIc* gave several bands when they were run on agarose gels; but none was of the appropriate size. These bands were not observed by using the same conditions with PAO1 genomic DNA. Finally, we were able to amplify by PCR an *aac(6)-Ib* gene from strain B1 as well as its sensitive mutants.

Assays and MIC studies to further characterize sensitive mutants. Several hypotheses were developed on the basis of our transcriptional profile studies. First, the observed increase in the sensitivity of M5 and M31 could be due to the decreased expression of several AME genes relative to their levels of expression in strain B1. Second, the sensitivities of M5 and M31 could be the result of mutations affecting amikacin permeability. To test these hypotheses, we assessed changes in the MICs for the mutants strains, B1, and PAO1 (i) to multiple aminoglycosides to assess AME contributions, (ii) to amikacin in the presence of compounds that effect afflux pump activity, (iii) to the polycationic antibiotic polymyxin B to assess altered LPS mechanisms, and, finally, (iv) with spheroplasts of each strain to delineate between the mechanisms present within the plasma membrane and those due to the outer membrane and LPS structures (Table 1).

To examine changes in mutants M5 and M31 that may have been due to AMEs, MIC studies with four additional aminoglycosides were carried out (49). Only the MIC of amikacin for M5 was affected. Since genes affecting the accumulation of amikacin within the cell are less likely to be amikacin specific, the increased sensitivity of M5 is likely due to the decreased activity of an AME that has the capacity to modify amikacin. In contrast, M31 had decreased resistance not only to amikacin

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TABLE 2. Genes differentially expressed between B1 and sensitive mutants M5 and M31

Function and gene ID	Gene name	Log fold difference (base 2)		Description
		B1/M5	B1/M31	
AME				
Pae AF133699cds4[a]	*aadB*	3.1	3.1	Aminoglycoside adenylyltransferase
Pae AF140629cds1[a]	*aadA6*	3.2	3.0	Aminoglycoside adenylyltransferase
Pae L06157cds2[a]	*aac(3)-Ib*	2.1	1.9	Aminoglycoside 3′-*N*-acetyltransferase
Pae L06160cds[a]	*aac(3)-IIIc*	3.0	2.7	Aminoglycoside 3′-*N*-acetyltransferase
Pae L06161cds[a]	*aac(3)-IIIb*	2.2	2.2	Aminoglycoside 3′-*N*-acetyltransferase
Pae M98270cds3[a]	*ant(4′)-IIa*	3.7	3.8	Aminoglycoside-4′-adenyltransferase
Permeability/membrane				
PA0013		1.55	1.05	Conserved hypothetical membrane protein
PA0203		3.22	3.10	Probable binding protein component of ABC transporter
PA0450		1.79	1.09	Probable phosphate transporter
PA0786		1.81	1.28	Probable transporter
PA0885		2.16	1.50	Probable C4-dicarboxylate transporter
PA1308		−2.50	−1.95	Hypothetical membrane protein
PA1361		1.54	1.23	Probable transporter
PA1386		3.21	3.11	Probable ATP-binding component of ABC transporter
PA1735		1.21	1.08	Hypothetical membrane protein
PA2042		−1.51	−1.32	Probable transporter (membrane subunit)
PA2070		1.38	1.12	Hypothetical membrane protein
PA2219	*opdE*	2.98	2.90	Membrane protein
PA2397	*pvdE*	2.44	2.29	Pyoverdine biosynthesis protein
PA2398	*fpvA*	2.19	2.18	Ferripyoverdine receptor
PA2549		1.66	1.52	Conserved hypothetical membrane protein
PA2853	*oprI*	−4.36	−4.11	Outer membrane lipoprotein OprI precursor
PA3141	*wbpM*	1.97	2.29	Nucleotide sugar epimerase/dehydratase
PA3145	*wbpL*	2.60	2.66	O-antigen initiating glycosyltransferase
PA3146	*wbpK*	3.22	3.08	Probable NAD-dependent epimerase/dehydratase
PA3148	*wbpI*	2.08	2.15	Probable UDP-*N*-acetylglucosamine 2-epimerase
PA3149	*wbpH*	3.55	3.50	Probable glycosyltransferase
PA3153	*wzx*	1.63	1.68	Putative O-antigen translocase
PA3157		3.79	3.79	Probable acetyltransferase
PA3158	*wbpB*	2.40	2.34	Probable oxidoreductase
PA3159	*wbpA*	3.34	3.35	Probable UDP-glucose/GDP-mannose dehydrogenase
PA3160	*wzz*	3.23	3.12	O-antigen chain length regulator
PA3176	*ghS*	1.90	1.83	Sodium/glutamate symporter GhS
PA3337	*rfaD*	−2.70	−1.92	ADP-L-glycero-D-mannoheptose 6-epimerase
PA3514		4.09	3.91	Probable ATP-binding component of ABC transporter
PA3748		−2.73	−2.81	Conserved hypothetical membrane protein
PA3753		−1.39	−1.42	Probable ferripyochelin binding protein
PA4020	*mpl*	−1.22	−1.15	Murein tripeptide ligase
PA4501		1.78	1.37	Probable porin
PA4503		1.08	1.25	Probable permease of ABC transporter
PA4514		2.47	2.62	Probable outer membrane receptor for iron transport
PA4996	*rfaE*	−1.64	−1.61	LPS biosynthesis protein
PA5265		2.93	2.75	Hypothetical membrane protein
Pae AF035937cds10[a]	*wbpT*[b]	2.69	2.72	Putative glycosyltransferase
Pae AF035937cds11[a]	*wbpU*[b]	3.50	3.50	Putative glycosyltransferase
Pae AF035937cds12[a]	*wbpV*[b]	2.74	2.63	Hypothetical protein involved in O-antigen biosynthesis
Pae AF035937cds13[a]	*wbpL*[b]	1.34	1.28	Probable O-antigen initiating glycosyltransferase
Pae AF035937cds3[a]	*wzz*[b]	4.16	3.99	O-antigen chain length regulator
Pae AF035937cds6[a]	*wbpQ*[b]	2.61	2.73	Probable B-band O-antigen polymerase
Pae AF035937cds7[a]	*wzx*[b]	2.96	2.95	Putative O-antigen translocase
Pae AF035937cds9[a]	*wbpS*[b]	3.76	3.70	Hypothetical protein
Pae AF147795cds11[a]	*wbpL*[c]	2.39	2.39	O-antigen initiating glycosyltransferase
Pae AF147795cds3[a]	*wzx*[c]	3.23	3.13	Putative O-antigen translocase
Pae AF147795cds8[a]	*wbiD*[c]	3.02	3.02	Putative UDP-*N*-acetylglucosamine2-epimerase

[a] Affymetrix Transcript identification (ID).
[b] Genes from *P. aeruginosa* serogroup O6 O-antigen locus.
[c] Genes from *P. aeruginosa* serogroup O11 O-antigen locus.

but also to tobramycin, gentamicin, kanamycin, and streptomycin. This would indicate that M31 has mutations that confer either increased accumulation of amikacin or, possibly, decreased AME activity.

To further examine the possibility of altered amikacin accumulation, the MIC of amikacin was determined in the presence of CCCP and verapamil. CCCP is an uncoupling proton ionophore that can carry protons across the cytoplasmic membrane

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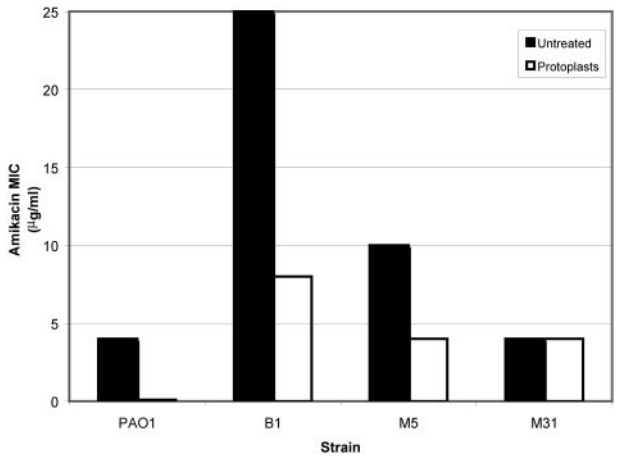

FIG. 3. Amikacin MICs for untreated cells and spheroplasts of strain PAO1, strain B1, and mutants M5 and M31. In all cases, the standard deviation was 0 ($n = 4$).

and that can thus inhibit efflux pumps and other transporters that are dependent upon the proton motive force as a source of energy (47, 48, 69, 87). Verapamil, a calcium channel blocker, has been noted to inhibit efflux pumps, including ATP-binding cassette (ABC) efflux pumps (12, 35, 45). CCCP (250 μM) and verapamil (100 μg/ml) had no impact upon the MIC of amikacin for PAO1, M5, M31, or B1, indicating that efflux pumps are not substantially contributing to the changes in resistance levels.

Since efflux did not appear to contribute to resistance, we next sought to determine if altered uptake was affected. Polymyxin B is a polycationic antibiotic that is thought to exhibit self-promoted uptake mechanisms for entry into the cell membrane similar to those of aminoglycosides by first interacting with LPS molecules (62). Cross-resistance to polymyxin B and aminoglycosides has been noted for a number of systems (55, 70), possibly due to overall changes in the charge of the outer membrane (83) or the presence of outer membrane proteins that may be blocking antibiotic binding to the membrane (60, 61). The MICs for B1, M5, and M31 were the same as that for PAO1, indicating that changes in sensitivity levels were likely not due to significant changes that would hinder polycationic antibiotic binding to the LPS.

Finally, to delineate between resistance conferred by the outer membrane and resistance mechanisms within the cytoplasmic membrane, spheroplasts of B1, M31, M5, and PAO1 were made; and the MIC of amikacin was then determined. Interestingly, the MICs for spheroplasts of B1, M5, and PAO1 all decreased, while the MIC for the spheroplast for M31 was the same as that for unaltered M31 (Fig. 3). It should be noted that slight changes in MICs may not be clinically relevant but may be biologically relevant. Based upon replicates of this experiment, our results have been shown to be repeatable and statistically significant. The MIC for B1 spheroplasts decreased significantly, but not to the level of those for the M5 and M31 spheroplasts. The M5 spheroplasts had the same MIC as M31 and the M31 spheroplasts. This suggests that the resistance of B1 is due in large part to altered outer membrane permeability and, to a lesser extent, internal mechanisms or mechanisms related to the cytoplasmic membrane. M5 has an increased level of amikacin resistance compared with that for M31, and this increase is likely due to a mutation in permeability. Once the permeability barrier has been removed, the MIC drops to that for M31. The unchanged MICs for M31 and the M31 spheroplasts indicate that the susceptibility of M31 is due to mutations that affect cell permeability. The fact that the MICs for M5 and M31 spheroplasts did not drop to the levels for the PAO1 spheroplasts indicates that there are still mechanisms aside from the permeability of the outer membrane that are contributing to resistance. Furthermore, the internal resistance mechanisms of M5 and M31 are attenuated compared with those of B1.

DISCUSSION

From our screening for sensitive mutants of B1, we found that mutations leading to sensitivity occurred at a relatively high frequency, $4.8 \times 10^{-2} \pm 0.01$, leading to an 80% decrease in the MIC, whereas the frequencies of mutations leading to increased resistance are reported to be 10^{-5} to 10^{-10} (16, 21, 28, 39, 42). The range of MICs found suggested that a number of different mutations or combination of mutations that led to increased sensitivity could have occurred. Since mutagenesis with MNNG has been shown to mutate hot spots, resulting in a number of mutations concentrated within a few minutes on the genome (24), particularly at the DNA replication fork (9), these results suggested that more than a single point mutation and likely more than one gene are responsible for the identified increased sensitivity phenotypes. Our inability to find mutants, created after a second round of mutagenesis, that had increased sensitivity levels compared with those of the parent mutant implies that there may be a lower limit to which sensitivity can be restored through chemical mutagenesis. This may be because *P. aeruginosa* has a large number of intrinsic resistance traits that would have to be altered in combination to further increase susceptibility to amikacin.

A notable detriment to the use of chemical mutagenesis is the lack of an efficient way to determine the mutations that occur. One of the promises of transcriptional profiling is an improved ability to decipher the genetic basis of relevant phenotypes through comparisons of gene expression profiles. Thus, we next sought to determine if transcriptional profiling could be used to identify relevant genetic alterations in two sensitive mutants of the B1 isolate. We reasoned that if only minor changes were required for the restoration of sensitivity, then the transcriptional profiles of sensitive mutants should strongly correlate with the profile of the parental resistant strain and not with the profile of the laboratory PAO1 strain. We hypothesized that the profiles of sensitive mutants would differ in the expression of a limited number of genes, which might allow prediction of the sensitivity-restoring mechanism or mechanisms.

From hierarchical clustering and principal component analysis of gene expression data obtained from strain B1, mutants M5 and M31, and strain PAO1, we found that the sensitive mutants had significant changes in gene expression and that their gene expression patterns were more similar to that of PAO1 than to that of their parent isolate, B1. Upon examination of the genes that were differentially expressed in the sen-

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sitive mutants compared with their expression in B1, we found that (i) a large number of genes had altered expression in both mutants and (ii) the overall changes in gene expression were similarly distributed among different functional categories for both M5 and M31.

According to GeneChip analysis, several AMEs exhibited decreased expression in both M5 and M31. Among them were the genes for aminoglycoside 3′-*N*-acetyltransferases [*aac(3)-Ib*, *aac(3)-IIIb*, and *aac(3)-IIIc*], aminoglycoside adenyltransferases from integron cassettes (*aadA6* and *aadB*), as well as an aminoglycoside-4′-adenyltransferase (*ant(4′)-IIa*). Amikacin is believed to be protected by steric hindrance or folding from modification by aminoglycoside 3′-*N*-acetyltransferases (54). The *aadA6* gene has been shown to render resistance to streptomycin and spectinomycin (57, 79), while *aadB* has been linked to gentamicin, kanamycin, and tobramycin resistance (56, 79). The AME gene with the largest difference in expression between B1 and the sensitive mutants was *ant(4′)-IIa*, which encodes for an AME that has been shown to confer resistance to both tobramycin and amikacin (79). However, the level of amikacin resistance displayed by B1 is an order of magnitude lower than the MICs previously reported for other *P. aeruginosa* strains expressing this gene (34, 75, 78). Aminoglycoside-resistant isolates of serotype O12 have been reported to react with probes for the genes *ant(3′)* and *aac(6′)-I* and *-II* (46).

When attempting to confirm the presence of genes encoding for AMEs by PCR with AME-specific primers, we were able to amplify by PCR an *aac(6)-Ib* gene from strain B1 and mutants M5 and M31. This gene has been associated with amikacin resistance, with the reported levels of amikacin resistance of strains carrying this gene being similar to the levels found in strain B1 and the sensitive mutants (22). It is feasible that a decrease in expression due to a mutation in a regulator of this gene could cause a decrease in tolerance to amikacin or that a mutation in this gene could also affect its specificity for amikacin (41). Probes for this gene are not available on the Affymetrix *P. aeruginosa* GeneChip, which explains its absence from our transcriptional profiling studies. Based on this analysis and the high level of resistance to several aminoglycosides displayed by B1, we suspect that B1 does contain a set of AMEs that is partially responsible for its increased overall aminoglycoside tolerance but that these enzymes are not effective at modifying amikacin or are poorly expressed.

Transcriptional profiling also suggested that genes related to the cellular membrane or transport of small molecules were of interest. Of particular note was the change in expression of genes involved in O-antigen synthesis and assembly. The Affymetrix PAO1 GeneChip has probes for O-antigen genes from serotypes O6 (3) and O11 (17), in addition to PAO1 serotype O5 (8). Although strain B1 and its sensitive mutants are of serotype O12, genes involved in O-antigen biosynthesis (*wbp* genes) and assembly (*wzx* and *wzz*) from the O5, O6, and O11 serogroups showed 2- to 4-log-fold decreases in expression levels in sensitive mutants M5 and M31 compared with that in B1. The changes noted in genes from several serotypes may be explained by the fact that hybridization occurs for sequences with greater than approximately 70% identity. Notably, genes related to the B-band O antigen had significant changes in expression, suggesting that altered B-band synthesis could be responsible for the increased sensitivities of the M5 and M31 mutants. There are mixed reports of the impact that the loss of O antigens has upon resistance. The loss of the B-band O antigen has been shown to result in an increase in resistance levels to aminoglycosides in *P. aeruginosa* (7, 38), possibly because the B band is highly anionic and may thus attract and attach to the highly cationic aminoglycosides. On the other hand, the loss of O-specific antigen, and possibly other parts of the core region, has also been shown to decrease resistance to gentamicin, with the explanation that negatively charged sites of the lipid A of LPS may be involved in aminoglycoside uptake (76). Despite the changes in gene expression noted, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of LPS showed that all strains had smooth LPS, with no detectable differences in the LPS of B1 and the sensitive mutants.

Other genes that were identified as having significant changes in expression included *oprF*, *oprG*, and *oprI*, which encode outer membrane protein or lipoprotein precursors. These genes were all shown to have higher levels of expression in mutants M5 and M31 than in strain B1. OprF is a major porin of *P. aeruginosa* (25), and a decrease in expression of this porin has been observed in antibiotic-resistant strains of *P. aeruginosa* (37, 68) (the strains were not tested for aminoglycoside resistance). Additionally, M5 and M31 also have decreased expression of probable ABC-type transporters. Certain ABC transporters have been found to serve as efflux pumps for antibiotics and have been shown to increase the levels of resistance to a number of antibiotics (19, 31, 64, 67). Recently, inactivation of the *glmR* gene, which is believed to be involved in amino sugar metabolism, has been shown to increase sensitivity to aminoglycosides (71). This gene was found to have no significant change in expression between B1, M5, and M31.

Transcriptional profiling results suggested that the increased sensitivity of M5 and M31 could be due in part to modified AME activity or changes in amikacin permeation. We found that M5 had a change in its MIC for amikacin but not for other aminoglycosides, while M31 had a decreased tolerance for other aminoglycosides, in addition to amikacin. Additionally, spheroplasts of M5 had decreased tolerance to amikacin, while spheroplasts of M31 had no changes in amikacin resistance. We rationalized that mutations affecting AMEs would likely be more specific for different aminoglycosides, while mutations affecting permeability would likely be less specific and would possibly be indicated by changes in MICs between untreated cells and spheroplasts of cells. Thus, our results imply that M5 has mutations that affected the ability of AMEs to modify amikacin and that M31 has mutations that affect amikacin permeability and possibly mutations that affect AME activity.

Conclusion. This work describes an assessment of a reverse engineering approach for the identification and characterization of sensitive mutants of a resistant *P. aeruginosa* isolate. Our results indicate that the frequency of identification of sensitive mutants was unexpectedly high and was several orders of magnitude higher than the reported frequencies of finding resistant mutants. From the frequencies of detection of mutations, along with the various levels of amikacin resistance among the mutants that were identified, it was shown that there are multiple ways in which sensitivity could be increased.

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This result was bolstered by the dramatic and different changes in gene expression observed for each sensitive mutant, as well as the findings of detailed MIC assays performed with the two mutants examined in greater detail.

A key issue in our evaluation was whether or not whole-genome transcriptional profiling could be used to gain insight into sensitivity-restoring genes/pathways, which is the critical information required for the development of new therapies. Unexpectedly, we found that the changes leading to sensitivity resulted in dramatic but only partially coordinated alterations in gene expression between the two sensitive mutants. Furthermore, the transcriptional profiles of the sensitive mutants more closely resembled that of sensitive laboratory strain PAO1 than that of the resistant parental isolate. Analysis of such transcriptional profiles indicated that changes in amikacin permeability and/or modification by AMEs was the most likely source of the increased sensitivity in mutants M5 and M31, which was later confirmed by several additional assays. Although the transcriptional profiling results provided interesting general insights into sensitivity-restoring mechanisms, the surprisingly large number of genes displaying significant changes in expression prohibited the identification of any single target gene or mutation. Furthermore, this study was hindered by the lack of an array containing probes for all of the genes found within the resistant isolate but not found within the PAO1 genome. Future applications might benefit from the use of a combination of this approach with the use of conventional genetic strategies involving screening of a knockout library to study the impact that gene disruption may have upon sensitivity or screening of an overexpression library in which the effects of increased copy numbers of genes could be examined.

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