ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2008, p. 4213–4219 Vol. 52, No. 12
0066-4804/08/$08.00+0 doi:10.1128/AAC.00507-08

Novel Genetic Determinants of Low-Level Aminoglycoside Resistance in *Pseudomonas aeruginosa*▽

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Received 17 April 2008/Returned for modification 23 May 2008/Accepted 17 September 2008

Pseudomonas aeruginosa **strains isolated from patients with persistent lung infections and cystic fibrosis have been found to gradually develop aminoglycoside resistance over time. The aim of this study was to identify potential contributors to low-level aminoglycoside resistance, which may cause such graduated increases in resistance. The Harvard *P. aeruginosa* PA14 nonredundant library, consisting of approximately 5,800 mutants, was screened for twofold or greater increases in tobramycin resistance. Mutants carrying mutations in a total of 135 unique genes were identified and confirmed to have reduced susceptibility to tobramycin. Many of these genes were involved predominantly in energy metabolism; however, most of these mutants did not exhibit growth defects under the conditions tested, although some exhibited the small-colony phenotype and/or defects in growth under anaerobic conditions. Lipopolysaccharide mutants were also identified, and it was found that tobramycin had a reduced ability to permeabilize the outer membranes of these mutants. The results of this study emphasize the complexity of the interactions that tobramycin may have within the bacterial cell and introduce a large number of novel genes which may play a role in tobramycin resistance.**

The majority of deaths of cystic fibrosis patients can be attributed to the progressive deterioration of lung function resulting from chronic infection by pathogens such as *Pseudomonas aeruginosa* (25). Antibiotic treatment of such chronic infections may temporarily suppress symptoms; however, it does not eradicate the pathogen. To overcome the inability of orally and parenterally administered antibiotics to adequately penetrate lung tissue and secretions, the aerosolized aminoglycoside tobramycin was formulated to directly target the site of infection. Clinical trials of aerosolized tobramycin revealed that long-term use of the agent against *P. aeruginosa* results in small, graduated increases in tobramycin MICs upon repeated isolation of the organism (21, 22), although the mechanisms contributing to this resistance have yet to be described.

Tobramycin is a bactericidal agent that targets the 30S ribosome and interferes with protein synthesis. Uptake occurs in three phases: an initial, reversible ionic-binding phase, a phase of slow energized uptake, and finally a phase of very rapid energy-dependent uptake (2, 4, 9). Despite the traditionally held view that antibiotic action can be simplified to interaction with a single target, it is evident that aminoglycosides exert pleiotropic effects on the cell (8, 9), as these effects may be antagonized by a variety of compounds known to affect cellular metabolic processes. Furthermore, the bactericidal nature of aminoglycosides such as tobramycin cannot be accounted for simply by protein synthesis inhibition or misreadings during translation because other protein synthesis inhibitors, such as chloramphenicol, and agents that promote misreading, including modified amino acids, are bacteriostatic. A limited number of cytochrome mutations leading to aminoglycoside resistance have been previously identified by selecting mutagenized *P. aeruginosa* strains on aminoglycoside-containing media (1, 3). As aminoglycosides exert a number of effects on the bacterial cell, we believe the potential sites for development of resistance are numerous. The purpose of this study was to identify the extent of the tobramycin "resistome," i.e., potential genetic contributors to the graduated development of aminoglycoside resistance in *P. aeruginosa*, using a comprehensive mutant library for a tobramycin resistance screen.

MATERIALS AND METHODS

Bacterial strains. The *P. aeruginosa* PA14NR set described by Liberati et al. (15) was used for screening genes related to tobramycin resistance. This mutant library comprises approximately 5,800 mutants representing around 4,600 genes. It was constructed using a mariner-based transposon, MAR2xT7, containing the resistance cassette *aacC1*, which confers resistance to the aminoglycosides gentamicin, astromicin, and sisomicin, but not to tobramycin. In addition, two PAO1 mutant libraries, the mini-Tn*5-luxCDABE* mutant library described by Lewenza et al. (14) and the University of Washington transposon mutant library (11), were used for verification and cross-referencing purposes.

Tobramycin resistance screening. *P. aeruginosa* PA14 mutants were inoculated into 100 μl of Mueller-Hinton broth (MHB) in 96-well plates and incubated at 37°C overnight. Overnight cultures were diluted 1:100 into MHB and were replica plated onto MH agar containing 0.5 μg/ml tobramycin (the MIC of the wild-type strain under these conditions). Growth was assessed at 24 and 48 h. Resistance was defined as a twofold or greater increase in the MIC compared to that for the parent strain, PA14. MICs at 24 and 48 h were determined at least in triplicate, using broth microdilution in cation-adjusted MHB (CAMHB), according to CLSI guidelines (6).

Tobramycin kill curves. Kill curves were performed in triplicate, in 96-well plates to simulate the MIC conditions, using selected mutants representing the major gene class functions identified in the screen. Cultures were grown in CAMHB at 37°C to an optical density at 600 nm (OD_{600}) of 0.4, and 1.25 ml was harvested by centrifugation. Pellets were resuspended in 1 ml CAMHB and then diluted 1:50. Fifty microliters of each sample was inoculated into the wells of 96-well plates, each containing 50 μl of a 4-μg/ml tobramycin solution, yielding a starting inoculum containing approximately 3×10^6 to 4×10^6 CFU/ml in 2 μg/ml tobramycin. Separate 96-well plates were inoculated for each time point,

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▽ Published ahead of print on 29 September 2008.

and aliquots were plated at 0, 10, 20, 30, 40, 50, and 60 min. Plates were incubated at 37°C overnight, and colony counts were performed to obtain numbers of CFU/ml at each time point.

Determination of growth rate. Growth rates were determined for all tobramycin-resistant mutants related to energy metabolism. These mutants were inoculated and grown overnight in 3 ml CAMHB at 37°C. Two microliters of each overnight culture was inoculated in 200 μl CAMHB, and growth at 37°C was monitored using a Tecan Spectrafluor Plus by measuring the OD_{620} every 20 min.

Assessment of aerobic and anaerobic growth defects. Cell suspensions were prepared to a 0.5 McFarland standard in LB broth and diluted 1:20 in 200 μl each of LB broth (aerobic) and LB broth containing 15 mM KNO_3 (anaerobic) in 96-well microtiter plates. Anaerobic plates were placed in an anaerobic jar containing a GasPak Plus hydrogen and carbon dioxide generator envelope. All plates and jars were incubated at 37°C for 24 h. The OD_{600} of the mutants was measured in the 96-well plates and compared to the OD_{600} of PA14.

Tobramycin outer membrane interaction studies. The hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN) was used as described by Loh et al. to study the interaction of tobramycin with the outer membranes of the tobramycin-resistant lipopolysaccharide (LPS) mutants compared to its interaction with that of PA14 (16). Briefly, a 50-ml sample of mid-log-phase cells was harvested by centrifugation at 3,000 × *g* for 10 min and resuspended in 5 mM sodium HEPES buffer (pH 7.2), with 5 μM carbonyl cyanide *m*-chlorophenylhydrazone, at an OD_{600} of 0.5. NPN was added to a final concentration of 10 μM, and baseline fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a Perkin Elmer LS 50B fluorescent spectrophotometer. Tobramycin was added to final concentrations of 1, 5, 10, 20, 40, and 60 μg/ml, and increases in fluorescence were recorded.

Cloning of the PA5300 strain. To complement the PAO1 PA5300::*lux* mutant strain and the PA14 PA5300 mutant strain, a PCR amplicon was generated using Phusion high-fidelity DNA polymerase (Finnzymes) from PAO1 genomic DNA using forward primer PA5300EcoRI and reverse primer PA5300BamHI. The amplicon was then ligated into the broad-host-range plasmid vector pUCP18 and transformed into *Escherichia coli* strain XL1-BlueMRF′. Plasmids were isolated (Qiagen) and transformed into both mutants as well as wild types PAO1 and PA14 by electroporation (5).

Cloning of *radA*. In order to complement the PA14 *radA* mutant, the genomic DNA of *P. aeruginosa* PA14 was used as a template to amplify the whole *radA* gene using Phusion high-fidelity DNA polymerase (Finnzymes). The PCR fragment was subcloned using the Zero Blunt Topo kit (Invitrogen). The vector was cut with XbaI and HindIII (Invitrogen), and the *radA* fragment was cloned into the low-copy-number vector pBBR1MCS-1 (13) and transformed into *E. coli* Top10 (Invitrogen). Plasmids were isolated (Qiagen) and electroporated into the PA14 *radA* mutant as well as into wild-type PA14 (5).

RESULTS AND DISCUSSION

Tobramycin resistance screening and confirmation. Screening of a preexisting transposon library allowed for the identification of a large number of potential genetic contributors to tobramycin resistance that have not been identified using the traditional method of using randomly mutagenized *P. aeruginosa* strains selected on antibiotic media. This tobramycin resistance screen identified a total of 348 mutants with reduced susceptibility compared to the PA14 parent strain. MICs determined using broth microdilution confirmed that 186 of these mutants have an MIC at least twofold greater than the MIC of the PA14 parent strain. These 186 mutants represented a total of 135 genes, as depicted in Fig. 1. A major benefit of this type of screen is that even small changes in resistance may be detected. Here the highest MIC found in the screen was an eightfold increase compared to that of PA14 for the *mutL* mutant (MIC of 4 μg/ml versus 0.5 μg/ml for the wild type). Additionally, 21 of the mutants identified had a fourfold increase in the MIC, while the remaining 165 mutants had a twofold increase in the MIC. Although twofold changes in MICs are typically considered within the acceptable range of error, we were able to confirm the increased tobramycin MIC in nearly all of these mutants in at least duplicate experiments, either by the redundancy of the PA14 mutant library or by the availability of corresponding mutants in either the mini-Tn*5-luxCDABE* library or the University of Washington PAO1 library. Of the 135 genes found in the PA14 screen, 44 genes had redundant mutants within the PA14 library, 31 genes were available in corresponding mutants in the PAO1 mini-Tn*5-luxCDABE* library, and 4 genes had corresponding mutants in the University of Washington PAO1 library. In total, 70 genes were confirmed to have increased tobramycin resistance in at least two independent mutants. An additional 60 genes that were not represented by more than one mutant belonged to operons with which other mutants had confirmed resistance. Only six genes neither were confirmed in duplicate experiments nor belonged to an operon. In addition, we were able to successfully restore wild-type tobramycin susceptibility in both the PAO1 and PA14 PA5300 mutants.

Classification of tobramycin-resistant mutants using pseudoCAP. Although twofold changes in MICs are typically considered within the acceptable range of error for this assay, the study of El'Garch et al. not only confirmed the reliability of twofold changes but indicated that independent mutants could act in a combinatorial fashion (7). Furthermore, slight increases in the MIC not only may tip the MIC above the clinical breakpoint but also may be indicative of a genotype with enhanced adaptability to stress conditions, such as exposure to antibiotics.

This enhanced adaptability has been observed for cystic fibrosis infections, for which the isolation of antibiotic-resistant strains with a hypermutator phenotype due to the presence of mutations in DNA mismatch repair systems, particularly in *mutL*, *mutS*, or *uvrD*, has previously been reported (18, 19). A number of isolates with other unknown genotypes have also been found to exhibit this hypermutator phenotype. Similarly, mutations in regulatory genes are known to lead to a cascade of effects ultimately causing adaptive resistance (20). When the mutants exhibiting reduced susceptibility to tobramycin were categorized according to their PseudoCAP functional class, according to the *Pseudomonas* Genome Database (www.pseudomonas.com) (Fig. 2), seven regulatory genes were identified along with eight distinct mutations known to be involved in DNA replication and repair processes, including mutations in *mutL*, *mutS*, *mutY*, *micA*, *mfd*, *nth*, *uvrA*, and *radA*.

The most notable observation from the classification of the tobramycin-resistant mutants was, however, the large proportion of energy metabolism mutants. To rule out decreased growth as the reason for reduced tobramycin susceptibility, all 186 mutants were assessed for major growth deficiencies using an endpoint OD_{600} at 24 h as a measure of yield of growth. Of 186 mutants assayed, mutations in only 25 genes, comprising 10 individual genes and three operons (Table 1), were found to cause a significant reduction in growth yield under either aerobic or anaerobic conditions or both. Additionally, growth rates were determined for all energy metabolism mutants. With the exception of mutants belonging to the PA4429-PA4431 operon, for which mutants had a doubling time of approximately 80 min, all of the energy metabolism mutants exhibited normal growth in CAMHB compared to PA14, with doubling times of approximately 50 min. Finally, growth on solid media was assessed for the 57 energy metabolism mutants

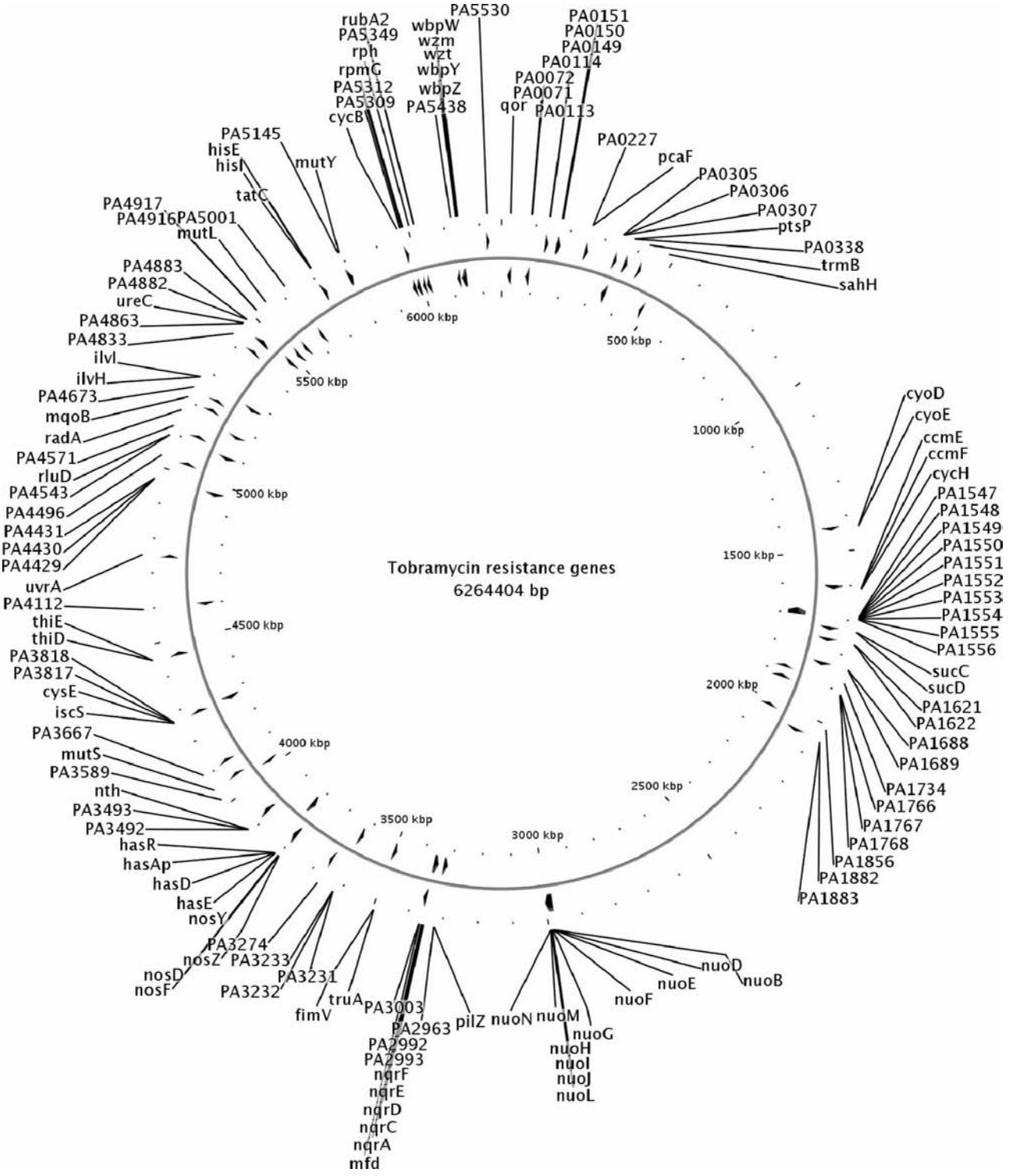

FIG. 1. Distribution of genes identified in the tobramycin resistance screen around the PAO1 genome. This genome image was generated by using CGView.

by observing colony morphology. Only 10 appeared as small-colony variants (of which 3 also had an observed growth defect in liquid media); all 10 of these had mutations in genes encoding cytochromes. Clinical isolates exhibiting this poorly understood small-colony phenotype have been shown to have increased antibiotic resistance compared to revertant colonies, and recovery of these isolates is strongly associated with daily inhalation of tobramycin or colistin (10).

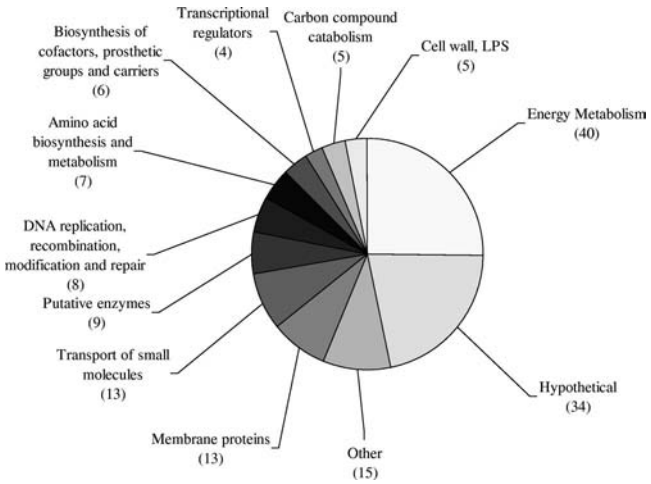

FIG. 2. Distribution of the 135 genes found to be involved in tobramycin resistance, grouped by PseudoCAP functional class. NB: the numbers of genes represented do not add up to 135, as many genes are represented in more than one functional group.

Several aminoglycoside-resistant cytochrome mutants were previously isolated by selective passaging on antibiotic media (1, 3); however, this study emphasizes the vast number of cytochrome genes which may impact aminoglycoside susceptibility. The definitive role that cytochromes play in aminoglycoside activity in *P. aeruginosa* is unknown. It is, however, known that the mechanism by which aminoglycosides cross the cytoplasmic membrane is an energy-dependent process which relies directly on the electron transport chain. Indeed, it has been suggested that a threshold membrane potential is required to support aminoglycoside uptake (17). Thus, for these energy metabolism mutants, we propose that resistance may result as a function of decreased uptake due to altered membrane potential. Nonetheless, cytochrome mutations were not the only energy metabolism mutations identified in the tobramycin screen. Of particular note is the identification of a number of genes related to NADH reduction, including the *nuo* and *nqr* operons, as well as the putative NADH:ubiquinone oxidoreductase encoded by PA3493. These results, along with the identification of several mutations involved in the tricarboxylic acid cycle and the cytochrome–electron transport chain mutations, are consistent with recent views suggesting that NADH depletion triggers the production of free radicals which ultimately contribute to antibiotic killing (12).

Tobramycin kill curves. Tobramycin kill curves mimicking MIC conditions were performed for selected mutants of various functional classes, including six energy metabolism mutants (PA1320, PA1480, PA1551, PA2638, PA2644, and PA5300), two transcriptional regulator mutants (PA0149 and PA5438), one DNA replication and repair mutant (PA4609), two LPS mutants (PA5447 and PA5450), two small molecule transport mutants (PA3408 and PA5070), two membrane protein mutants (PA1767 and PA3115), and two hypothetical protein mutants (PA0338 and PA1588) (Fig. 3). All selected mutants showed a significant reduction ($P < 0.05$ by one-tailed Student's t test) in the extent of killing at 60 min by 2 μg/ml tobramycin compared to the wild type, with the exception of the PA3115 mutant ($P = 0.068$) (Fig. 4). As a number of mutants found in the tobramycin screen were impaired in

TABLE 1. Tobramycin resistance mutations causing defective growth in LB broth under aerobic and/or anaerobic conditions and small-colony variants

Defect or variant type	PAO1 ortholog(s) of mutant gene	Gene name	Gene description
General growth defect	PA0432	*sahH*	*S*-Adenosyl-L-homocysteine hydrolase
	PA1547		Putative membrane protein
	PA1548		Putative cytochrome oxidase maturation protein, cbb_3 type
	PA1588	*sucC*	Succcinyl-CoA synthetase beta subunit
	PA4429		Putative cytochrome c_1 precursor
	PA4430		Putative cytochrome *c* reductase, iron-sulfur subunit
	PA5070	*tatC*	Sec-independent protein translocase TatC
Aerobic growth defect	PA3818		Inositol-1-monophosphatase
	PA3975		Possible phosphomethylpyrimidine kinase
Anaerobic growth defect	PA1480	*ccmF*	Cytochrome *c*-type biogenesis protein CcmF
	PA2638-PA2648	*nuo*	NADH dehydrogenase I
	PA2960	*pilZ*	Type IV fimbrial biogenesis protein PilZ
	PA3233		Putative signal transduction protein
	PA4673		Putative GTP-binding protein
	PA4916		Putative ADP-ribose pyrophosphatase
Small-colony variants (energy metabolism mutations)	PA1480	*ccmF*	Cytochrome *c*-type biogenesis protein CcmF
	PA1483	*cycH*	Cytochrome *c*-type biogenesis protein
	PA1552		Putative cytochrome *c* oxidase, cbb_3 type, subunit III
	PA1553		Putative cytochrome *c* oxidase, cbb_3 type, subunit II
	PA1554	*ccoN*	Cytochrome oxidase subunit, cbb_3 type
	PA4429		Putative cytochrome c_1 precursor
	PA4430		Putative cytochrome *b*
	PA4431		Putative cytochrome *c* reductase, iron-sulfur subunit
	PA5300	*cycB*	Cytochrome c_5

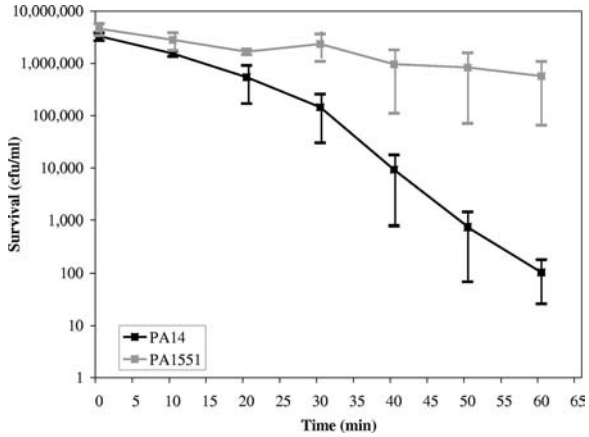

FIG. 3. Impact of PA1551 gene mutation on tobramycin killing of strain PA14. Error bars represent the standard deviations calculated from at least three separate experiments.

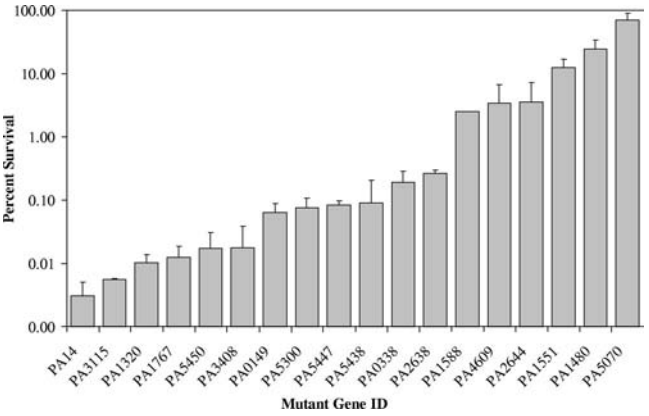

FIG. 4. Influence of tobramycin resistance mutations on killing by tobramycin. Represented is the percent a survival after a 60-min exposure to 2 μg/ml tobramycin for selected tobramycin-resistant mutants compared to wild-type PA14. Error bars represent the standard deviations calculated from at least three separate experiments.

DNA mismatch repair systems and many of these mutants are characterized by a hypermutator phenotype, it may be assumed that resistance in these mismatch repair mutants is due to a highly probable second-site mutation. We recently demonstrated that the *radA* mutant from the PA14 library shows an elevated mutation rate compared to PA14 and that the complemented strain has a mutation rate similar to that of the wild type (26). The kill curves performed here for the same *radA* mutant show that the tobramycin resistance phenotype is stable under the conditions tested. As these kill curves are performed over a short period of time, only 60 min, this indicates that a second-site mutation is not developing upon treatment. Tobramycin MICs were determined for the complemented *radA* strain to determine whether resistance in this mutant was due primarily to the *radA* mutation or to a preexisting second-site mutation. Tobramycin susceptibility was not restored in the complemented strain, indicating that resistance was due to a preexisting secondary mutation. In contrast, complementation of the PA5300 mutant, which does not exhibit a hypermutator phenotype, restored susceptibility to wild-type MIC levels.

Interaction of tobramycin with the outer membranes of LPS mutants. *P. aeruginosa* is capable of expressing two distinct forms of LPS, known as the A band and the B band (O antigen), which differ with respect to their O polysaccharides. The O polysaccharide of A-band LPS is a conserved polyrhamnose molecule, and proteins involved in the assembly of the A-band LPS are encoded by the PA5447-PA5454 operon (23, 24). Several genes of this operon, namely, *wbpZ*, *wbpY*, *wzt*, *wzm*, and *wbpW*, were identified in the tobramycin resistance screen. Tobramycin crosses the outer membrane of the bacterial cell via the process of self-promoted uptake (9). It competitively binds LPS, displacing divalent cations and disrupting the integrity of the outer membrane, thus causing increased membrane permeability. NPN is a fluorescent probe that fluoresces weakly in aqueous solution but strongly in a nonpolar or hydrophobic environment. Under normal conditions, NPN is excluded from the membrane and does not fluoresce. Upon disruption of the outer membrane, e.g., by aminoglycosides (16), NPN partitions into the outer membrane interior and an increase in fluorescence can be observed. The initial rate of increase in fluorescence varies with the concentration of aminoglycoside and defines the extent of outer membrane permeabilization, which in turn relates to the extent of self-promoted uptake. The NPN assay was used to determine if the mutations in A-band LPS genes were in fact contributing to resistance, resulting in a reduced ability of tobramycin to interact with and permeabilize the outer membranes of these mutants. A maximum increase in fluorescence intensity was achieved using 40 μg/ml tobramycin for PA14 and 60 μg/ml for the six LPS mutants, indicating that higher concentrations of tobramycin were required to attain a similar level of disruption to the outer membrane. Differences in permeabilization were observed for all LPS mutants compared to the wild type at all concentrations assayed above 1 μg/ml, at which concentration no permeabilization was observed for any of the mutants or the wild type during the relatively short period that could be assessed in this assay. These findings reflect the substantially delayed killing (~1 h) observed when using concentrations near the MIC. While a concentration of 5 μg/ml resulted in slight permeabilization of the wild-type outer membrane, this was not observed for any of the LPS mutants during the time frame observed. At 10 μg/ml tobramycin, the initial rate of fluorescence increase was approximately twofold higher in the PA14 parent strain than in any of the LPS mutants tested (Fig. 5), suggesting that the reduced ability of tobramycin to permeabilize the outer membranes of these mutants explains their reduced susceptibility.

Concluding remarks. The results presented here indicate that a wide variety of genetic determinants may affect aminoglycoside resistance. This finding is in contrast to the previously held belief, supported by direct in vitro selection studies of randomly mutagenized *P. aeruginosa* strains, that mutations leading to aminoglycoside resistance are relatively rare. In previous studies, aminoglycoside-resistant mutants were selected on media containing >12 μg/ml gentamicin from *P. aeruginosa* isolates subjected to ethyl methane sulfonate mutagenesis, while at a much lower selection concentration of 3.2 μg/ml gentamicin, nonmutatgenized cultures did not produce

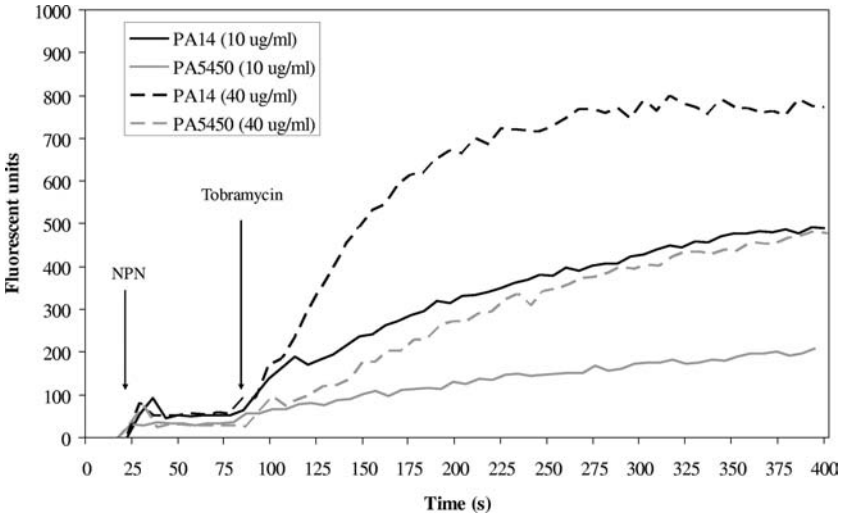

FIG. 5. Interaction of tobramycin with the outer membrane of the tobramycin-resistant *wbpZ* mutant (PA5450) compared to its interaction with wild-type PA14, using 10 μg/ml and 40 μg/ml tobramycin.

any aminoglycoside-resistant mutants (1, 3). The contrasting results demonstrated here are almost certainly because the ability to screen existing comprehensive libraries permitted us to identify many mutants with only twofold changes in resistance, which would be difficult to obtain through direct selection. Despite the large number of genes identified here, it is of importance to note that this type of screen has the limitation of identifying only nonessential genes and so may in fact underestimate the size of the tobramycin "resistome." Nonetheless, we believe that this screen is a particularly sensitive method of detecting nonessential genes involved in resistance, as we have not only successfully detected mutations in two genetic backgrounds but have also independently identified other known resistance genes, such as the *nuo* (7) and cytochrome (1, 3) genes. Although the changes in the MICs of the mutants are modest, such modest changes have been shown to be real, and when double, triple, and quadruple mutants were generated for genes that when independently mutated lead to only twofold increases in the MICs, the mutations were shown to be capable of acting cumulatively to result in higher levels of resistance (7). By including such modest changes here, we have provided strong evidence for a very extensive aminoglycoside "resistome." We have also related several phenotypes often associated with clinical aminoglycoside resistance to genes identified in this screen. Although the clinical relevance of the individual mutations found in this screen has yet to be elucidated, these results provide a large framework for future studies investigating the gradual development of aminoglycoside resistance due to the potential cumulative effects of genetic mutations. As well, they may provide insights into the actual mechanisms by which aminoglycosides act upon the cell.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health Research. K.N.S. holds a Natural Sciences and Engineering Council of Canada postgraduate scholarship and a Michael Smith Foundation for Health Research senior graduate studentship. R.E.W.H. holds a Canada Research Chair.

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