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Determinants of Intrinsic Aminoglycoside Resistance in *Pseudomonas aeruginosa*

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Screening of a transposon insertion mutant library of *Pseudomonas aeruginosa* for increased susceptibility to paromomycin identified a number of genes whose disruption enhanced susceptibility of this organism to multiple aminoglycosides, including tobramycin, amikacin, and gentamicin. These included genes associated with lipid biosynthesis or metabolism (*lptA*, *faoA*), phosphate uptake (*pstB*), and two-component regulators (*amgRS*, PA2797-PA2798) and a gene of unknown function (PA0392). Deletion mutants lacking these showed enhanced panaminoglycoside susceptibility that was reversed by the cloned genes, confirming their contribution to intrinsic panaminoglycoside resistance. None of these mutants showed increased aminoglycoside permeation of the cell envelope, indicating that increased susceptibility was not related to enhanced aminoglycoside uptake owing to a reduced envelope barrier function. Several mutants (*pstB*, *faoA*, PA0392, *amgR*) did, however, show increased cytoplasmic membrane depolarization relative to wild type following gentamicin exposure, consistent with the membranes of these mutants being more prone to perturbation, likely by gentamicin-generated mistranslated polypeptides. Mutants lacking any two of these resistance genes in various combinations invariably showed increased aminoglycoside susceptibility relative to single-deletion mutants, confirming their independent contribution to resistance and highlighting the complexity of the intrinsic aminoglycoside resistome in *P. aeruginosa*. Deletion of these genes also compromised the high-level panaminoglycoside resistance of clinical isolates, emphasizing their important contribution to acquired resistance.

Pseudomonas aeruginosa is a common nosocomial pathogen (36, 86) that causes a variety of infections, with one of the most frequent sites of *P. aeruginosa* infection being the lungs of cystic fibrosis (CF) patients (27). Aminoglycosides are commonly used to treat such infections, with aerosolized tobramycin and/or intravenous (i.v.) tobramycin or amikacin used to treat prechronic infections of *P. aeruginosa* in CF patients as well as acute exacerbations that occur following the onset of chronicity (10, 64, 82). Aminoglycoside use in treating pulmonary infections in CF patients is, however, associated with resistance development (9, 30, 58, 61), which complicates antipseudomonal chemotherapy.

Aminoglycoside antibiotics bind the 16S rRNA component of the 30S ribosomal subunit, disrupting translation and causing the accumulation of truncated/aberrant polypeptides (15). These polypeptides insert into the cytoplasmic membrane and compromise membrane integrity (16), ultimately permitting accumulation of additional drug and total inhibition of all cellular ribosomes (43) as well as activating stress responses that culminate in the production of reactive oxygen species (ROS), which appear to be responsible for the lethal effect of aminoglycosides (51). Intriguingly, initial uptake of these antimicrobials into bacterial cells is an active (i.e., energy-requiring) process, although the details are as yet unclear (81).

Bacterial resistance to aminoglycosides typically results from enzymatic modification of the drug, drug efflux, and target modification (43), with target modification involving mutation of genes for 16S rRNA and ribosomal proteins (43) or methylation of 16S rRNA by transposon-encoded methyltransferases (28). 16S rRNA methyltransferase-mediated aminoglycoside resistance is rarely seen in *P. aeruginosa* (19, 84), where the most common mechanism of aminoglycoside resistance involves aminoglycoside-modifying enzymes encoded by transmissible genes that are acquired by horizontal gene transfer (22, 49, 68). This is not, however, the case for CF patient isolates of *P. aeruginosa*, where these mechanisms are almost unknown (35, 68, 73). The predominant identified mechanism of aminoglycoside resistance in this case is efflux by the MexXY-OprM multidrug efflux system (39, 40), which is also responsible for earlier reports of so-called impermeability-type panaminoglycoside resistance that was frequently seen in CF patient *P. aeruginosa* isolates (68). Aminoglycoside resistance linked to lipopolysaccharide (LPS) changes in the *P. aeruginosa* outer membrane (OM; the site of initial binding of aminoglycosides during entry into bacterial cells [31, 46]) has also been reported (6, 25) and may, in some instances, be linked to an LPS modification locus (*arn*) (65) previously implicated in resistance to polycationic antimicrobials (e.g., polymyxins) (60) and regulated by the PhoPQ two-component system (TCS) (56). PhoPQ has, in fact, been implicated in aminoglycoside (including amikacin) resistance (56). Less commonly, resistance owing to ill-defined defects in aminoglycoside uptake (4, 7, 79), including defects in respiration/electron transport (5, 8, 25), and mutations in ribosomal components (25, 52) have been reported in *P. aeruginosa*. A recent study of *P. aeruginosa* transposon (Tn) insertion mutants screened for decreased susceptibility to aminoglycosides (tobramycin) identified a number of genes apparently linked to low-level aminoglycoside resistance, including a number involved in LPS biosynthesis and energy metabolism (72). An independent screen of a *P. aeruginosa* Tn mutant library for decreased resis-

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tance to several antimicrobial classes also identified genes linked to energy metabolism/respiration whose inactivation enhanced tobramycin susceptibility (21). Here we report a number of novel genes whose inactivation enhances susceptibility specifically to aminoglycosides. Intriguingly, all these appear to contribute independently to resistance, in some instances via an apparent impact on cytoplasmic membrane stability, highlighting the complexity of the *P. aeruginosa* intrinsic aminoglycoside resistome. Most importantly, these genes also contribute to the panaminoglycoside resistance of clinical (CF patient) isolates.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were cultured at 37°C in Luria (L) broth or on L agar, unless otherwise indicated, with antimicrobials added as necessary. The pUT::mini-Tn*5-tet* plasmid was maintained in *Escherichia coli* with 100 μg/ml ampicillin or 10 μg/ml tetracycline. The pUC19 and pBluescript II SK (+) plasmids and their derivatives were maintained in *E. coli* with 100 μg/ml ampicillin. The pDSK519 plasmid and its derivatives were maintained in *E. coli* with 50 μg/ml kanamycin. Plasmids pEX18Tc and pRK415 and their derivatives were maintained in *E. coli* with 5 to 10 μg/ml tetracycline and in *P. aeruginosa* with 50 to 75 μg/ml tetracycline.

DNA methods. Standard protocols were used for restriction endonuclease digestions, ligations, transformations, and agarose gel electrophoresis, as previously described (71). Plasmid DNA was extracted from *E. coli* using a Fermentas GeneJET plasmid miniprep kit or a Qiagen plasmid midikit according to protocols provided by the manufacturers. Chromosomal DNA was extracted from *P. aeruginosa* using a Qiagen DNeasy blood and tissue kit according to a protocol provided by the manufacturer. PCR products and restriction endonuclease digest products requiring purification were purified using a Promega Wizard SV Gel and PCR cleanup system according to a protocol provided by the manufacturer. $CaCl_2$-competent *E. coli* (71) and electrocompetent *P. aeruginosa* (11) cells were prepared as previously described. Oligonucleotide synthesis was performed by Integrated DNA Technologies (Coralville, IA), and nucleotide sequencing was performed by ACGT Corporation (Toronto, ON, Canada).

Transposon mutagenesis. Aminoglycoside-resistant *P. aeruginosa* strain K2153 or K2413-T3 was mutagenized with mini-Tn*5-tet* following mobilization of mini-Tn5-*tet*-carrying plasmid pUT from *E. coli* SM10 (λ*pir*) as described previously (78), except that K2413-derived mutants were selected on 50 μg/ml tetracycline and 5 μg/ml chloramphenicol (to counterselect donor *E. coli* cells) following a 6-hour incubation of the mating mixture on L-agar plates. Mini-Tn*5-tet* insertion mutants showing increased susceptibility to aminoglycosides were subsequently identified by first screening on plates containing paromomycin (512 μg/ml for K2153-derived mutants, 64 μg/ml for K2413-derived mutants), and the mutants that were not able to grow were subsequently examined for increased susceptibility to additional aminoglycosides (as well as paromomycin). The mini-Tn*5-tet*-disrupted genes from panaminoglycoside-susceptible mutants were recovered following the cloning of mini-Tn*5-tet*-containing PstI-derived genomic DNA fragments into pBluescript II SK (+) (selected in *E. coli* DH5α on 10 μg/ml tetracycline) and sequencing of the transposon-flanking chromosomal DNA as described previously (78).

Deletion strain construction. Derivatives of *P. aeruginosa* strains with deletions of various genes were generated by constructing deletions in plasmid pEX18Tc and mobilizing them into these strains from *E. coli* S17-1 as before (63). *P. aeruginosa* transconjugants harboring chromosomal inserts of the deletion vectors were selected on L-agar plates containing tetracycline (50 μg/ml for all K767 derivatives except Δ*lptA*, where 75 μg/ml was used; 100 μg/ml for derivatives of K2160 and K2162) and chloramphenicol (5 μg/ml; to counterselect *E. coli* S17-1). These were subsequently streaked onto L agar containing sucrose (10% [wt/vol]), with sucrose-resistant colonies screened for the appropriate deletion using colony PCR with 2.5 U *Taq* polymerase in 10% (vol/vol) dimethyl sulfoxide (DMSO) (74). Colony PCR was carried out using the respective Up-F and Down-R primers for each deletion, except for Δ*mexXY* (mexXY-F, 5′-CTTGACCAGGGCCTCGTAG-3′; mexXY-R, 5′AAGGC CGAACTGGAGCAG-3′), with samples heated for 3 min at 95°C, followed by 34 cycles of 45 s at 95°C, 45 s at 65°C, and 3 min at 72°C, before finishing with 5 min at 75°C (except for Δ*lptA*, where samples were heated for 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 65°C, and 3 min at 72°C, before finishing with 7 min at 75°C).

Gene deletions were constructed by amplifying, via PCR, 1-kb fragments upstream and downstream of the sequences being deleted and cloning these individually into plasmid pUC19 or pBluescript for sequencing (to ensure that no mutations had been introduced during PCR) and then together into pEX18Tc. PCR fragments were gel purified and digested with restriction enzymes (sites incorporated into PCR primers) prior to cloning into appropriately digested plasmids.

For ΔPA2798, the upstream and downstream fragments were amplified using primers 2798UP-F (5′-GAC TGA ATT CCC GTA CGT GAT GCT GCC GTT-3′; EcoRI site underlined) and 2798UP-R (5′-GAC TTC TAG AGA AGT TGC TGT CTT CCA AGT-3′; XbaI site underlined) and primers 2798Down-F (5′-GAC TTC TAG AGC CGG AGG CAC CCT CGA CGG-3′; XbaI site underlined) and 2798Down-R (5′-GAC TAA GCT TGG TCT CGC GCA TCT ATC GCT-3′; HindIII site underlined). The 50-μl PCR mixtures contained 1 μg of *P. aeruginosa* K767 chromosomal DNA as the template, 0.2 μM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1× *Pfu* (with Mg) buffer, and 1.25 U *Pfu* DNA polymerase (Promega). Following an initial denaturation step at 95°C for 3 min, the mixture was subjected to 30 cycles of heating at 95°C for 45 s, 63.1°C (annealing temperature) for 45 s, and 72°C for 1 min, before finishing with a 5-min incubation at 72°C.

For ΔPA2797, the upstream and downstream fragments were amplified using primers 2797Up-F (5′-GTA CGA ATT CAC GCC GAT CAT CGT ACT TTC-3′; EcoRI site underlined) and 2797Up-R (5′-GTA CTC TAG AACTCA TGC AAG GTT CCT GCT C-3; XbaI site underlined) and primers 2797Down-F (5′-GTA CTC TAG ACATTA ACC GGT CGATCC CTT-3′; XbaI site underlined) and 2797Down-R (5′-GTA CAA GCT TCC ATG ACT TCG AAG CTG GAA C-3′; HindIII site underlined). The reaction mixture and parameters were as described above for ΔPA2798, except that 1 U Vent DNA polymerase in 1× Thermopol buffer (New England BioLabs) was used and 10% (vol/vol) DMSO was included.

For Δ*pstB*, the upstream and downstream fragments were amplified using primers pstBUp-F (5′-GAC TGA ATT CCA AGG TCC TGG AAG AGC AGC-3′; EcoRI site underlined) and pstBUp-R (5′-GAC TTC TAG ACA TGG GAA GCT CCC TCA ATC-3′; XbaI site underlined) and primers pstBDown-F (5′-GAC TTC TAG ATA CAT CAC CGG CCG CTA CGG-3; XbaI site underlined) and pstBDown-R (5′-GAC TAA GCT TCG TCA TCC ACC ACT AGC GCG-3′; HindIII site underlined). The reaction mixture and parameters were as described above for ΔPA2798, except that an annealing temperature of 58.6°C was used.

For ΔPA0392, the upstream and downstream fragments were amplified using primers 0392Up-F (5′-GAC TGA ATT CCG AAC TGC TGC TGG ACC TGA-3′; EcoRI site underlined) and 0392Up-R (5′-GAC TTC TAG ATC TGG AGG ATA TAG ATA GCA-3′; XbaI site underlined) and primers 0392Down-F (5′-GAC TTC TAG ACG GCA TGC CCC AGC AGT TGA-3′; XbaI site underlined) and 0392Down-R (5′-GAC TAA GCT TCG AAG TTG ACC AGG GCG TGG-3′; HindIII site underlined). The reaction mixture and parameters were as described above for ΔPA2798, except that annealing temperatures of 49°C (upstream arm) and 63.1°C (downstream arm) were used and 10% (vol/vol) DMSO was included. In the process of cloning the downstream fragment into pUC19, a Dam methylation site overlapping the XbaI site was created. In order to excise this fragment from pUC and clone it into pEX18Tc carrying the upstream fragment, it was necessary to prepare plasmid DNA from the *E. coli dam* mutant strain GM2163.

For Δ*proC*, upstream and downstream fragments were amplified us-

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
E. coli		
DH5α	ϕ80d*lacZ*ΔM15 *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA gyrA96 relA1* F^- Δ(*lacZYA-argF*)*U169*	1
SM10(λ*pir*)	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r λ*pir*	62
S17-1	*thi pro hsdR recA* tra^+	76
GM2163	F^- dam-13::Tn9 (Cm^r) dcm-6 hsdR2 (r_k^- m_k^+) leuB6 hisG4 thi-1 araC14 lacY1 galK2 galT22 xylA5 mtl-1 rpsL136 (Str^r) fhuA31 tsx-78 glnV44 mcrA mcrB1	NEB
P. aeruginosa		
K767	PAO1 wild type	59
K2153	Panaminoglycoside-resistant clinical (CF patient) isolate	77
K2363	K2153 *amgR*::mini-Tn*5 tet*	This study
K3104	K2153 *pstB*::mini-Tn*5 tet*	This study
K2358	K2153:*proC*::mini-Tn*5 tet*	This study
K2359	K2153 *faoA*::mini-Tn*5 tet*	This study
K2365	K2153 PA2798::mini-Tn*5 tet*	This study
K2361	K2153 PA0392::mini-Tn*5 tet*	This study
K2413	K767 ΔPA5471	63
K2413-T3	MexXY-expressing panaminoglycoside-resistant derivative of K2413	S. Fraud, unpublished data
K3158	K2413-T3 *lptA*::mini-Tn*5 tet*	This study
K1525	K767 Δ*mexXY*	17
K3159	K767 Δ*amgR*	This study
K3160	K767 Δ*pstB*	This study
K3161	K767 Δ*faoA*	This study
K3162	K767 ΔPA2798	This study
K3163	K767 ΔPA2797	This study
K3164	K767 ΔPA0392	This study
K3165	K767 Δ*lptA*	This study
K3166	K767 Δ*mexXY* Δ*amgR*	This study
K3167	K767 Δ*mexXY* Δ*pstB*	This study
K3168	K767 Δ*mexXY* Δ*faoA*	This study
K3169	K767 Δ*mexXY* ΔPA2798	This study
K3170	K767 Δ*mexXY* ΔPA2797	This study
K3171	K767 Δ*mexXY* ΔPA0392	This study
K3172	K767 Δ*mexXY* Δ*lptA*	This study
K3173	K767 Δ*amgR* Δ*pstB*	This study
K3174	K767 Δ*amgR* Δ*faoA*	This study
K3175	K767 Δ*amgR* ΔPA2798	This study
K3176	K767 Δ*amgR* ΔPA2797	This study
K3177	K767 Δ*amgR* ΔPA0392	This study
K3178	K767 Δ*amgR* Δ*lptA*	This study
K3179	K767 Δ*pstB* Δ*lptA*	This study
K3180	K767 Δ*pstB* Δ*faoA*	This study
K3181	K767 Δ*pstB* ΔPA0392	This study
K3182	K767 Δ*pstB* ΔPA2798	This study
K3183	K767 Δ*lptA* ΔPA0392	This study
K3184	K767 Δ*faoA* ΔPA0392	This study
K3185	K767 Δ*lptA* Δ*faoA*	This study
K2160	Panaminoglycoside-resistant clinical (CF patient) isolate	77
K3192	K2160 Δ*amgR*	This study
K3193	K2160 Δ*pstB*	This study
K3194	K2160 Δ*faoA*	This study
K3195	K2160 ΔPA2798	This study
K3196	K2160 ΔPA0392	This study
K3197	K2160 Δ*lptA*	This study
K2162	Panaminoglycoside-resistant clinical (CF patient) isolate	77
K3198	K2162 Δ*amgR*	This study
K3199	K2162 Δ*pstB*	This study
K3200	K2162 Δ*faoA*	This study
K3201	K2162 ΔPA2798	This study
K3202	K2162 ΔPA0392	This study
K3203	K2162 Δ*lptA*	This study

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Relevant characteristics[a]	Reference or source
Plasmids		Reference
pUT::mini-Tn*5-tet*	Delivery vector for mini-Tn*5 tet* Ap^r Tc^r	18
pUC19	Cloning vector; Ap^r	85
pDSK519	Broad-host-range cloning vector; Km^r	48
pBluescript II SK (+)	Cloning vector; Ap^r	Stratagene
pEX18Tc	Broad-host-range gene replacement vector; *sacB* Tc^r	38
pRK415	Broad-host-range cloning vector; P_{lac}-MCS Tc^r	48
pCSV05-01	pEX18Tc::Δ*mexXY*	17
pCG005	pEX18Tc::Δ*amgR*	This study
pCG006	pEX18Tc::Δ*pstB*	This study
pCG007	pEX18Tc::Δ*faoA*	This study
pCG008	pEX18Tc::ΔPA2797	This study
pCG009	pEX18Tc::ΔPA2798	This study
pCG010	pEX18Tc::ΔPA0392	This study
pJDT1	pEX18Tc::Δ*lptA*	This study
pCG017	pEX18Tc::Δ*proC*	This study
pCG011	pRK415::*amgR*	This study
pCG012	pRK415::*pstB*	This study
pCG013	pRK415::*faoA*	This study
pCG014	pRK415::PA2797	This study
pCG015	pRK415::PA2798	This study
pCG016	pRK415::PA0392	This study
pJDT3	pRK415::*lptA*	This study

[a] Cm^r, chloramphenicol resistance; Str^r, streptomycin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; P_{lac}-MCS, *lac* promoter upstream of multicloning site.

ing primers proCUp-F (5′-CGA TGA ATT CGA GAG GTG CAG GTC CGA AG-3′; EcoRI site underlined) and proCUp-R (5′-CG ATT CTA GAG GGT GTG CTC ATA GGG GAT T-3′; XbaI site underlined) and primers proCDown-F (5′-CGA TTC TAG ACA GCT TGG CCA ATA AGG AGT-3′; XbaL site underlined) and proCDown-R (5′-CGA TAA GCT TGT CTT TCC ATG CTC ATC CAG −3′; HindIII site underlined). The reaction mixture and parameters were as described above for ΔPA2798, except that Exact DNA polymerase (5 Prime, Inc., Gaithersburg, MD) was employed in 1× Exact buffer and 1× 5P solution, the annealing temperature was 65°C, and 40 cycles of amplification were used.

For Δ*faoA*, the upstream and downstream fragments were amplified using primers faoAUp-F (5′-TGA CGA ATT CTG ACT CTA GAA GTC CAG AGC AAT GTC CTG C-3′; EcoRI site underlined) and faoAUp-R (5′-TGA CGG ATC CCA AGA GGC TTA ACC GTG ATG-3′; BamHI site underlined) and primers faoADown-F (5′-TGA CGG ATC CGT TAA TCG CGG AAC GAC TAG-3′; BamHI site underlined) and faoADown-R (5′-GAT CTC TAG ACT TGA GGT CCT TCA GCA CA-3′; XbaI site underlined). The reaction mixture was formulated as described above for ΔPA2798, except that 1 U of Phusion High-Fidelity DNA polymerase in 1× Phusion GC buffer (Fermentas) was employed, primers were present at 0.5 μM, and 5% (vol/vol) DMSO was included. Amplification of the upstream and downstream fragments was achieved using an initial denaturation step of 98°C for 30 s, followed by 30 cycles of 98°C for 30 s, 65°C for 30 s, and 72°C for 1 min, before finishing with 72°C for 5 min.

For Δ*lptA*, the upstream and downstream fragments were amplified using primers lptAUp-F (5′-GAC TGA ATT CCT GTA GAA GTC CTG GCG GT-3′; EcoRI site underlined) and lptAUp-R (5′-GAC TTC TAG ATG GCC TGC ACT GTC GAC AT 3′; BamHI site underlined) and primers lptADown-F (5′-GAC TTC TAG AGC CGT CGG TGG TCT CGT GA-3′; BamHI site underlined) and lptADown-R (GAC TCT GCA GCG GCG CTG GAG AAA CTG GT; PstI site underlined). The reaction mixture and parameters were as described above for Δ*faoA*, except that primers were used at 0.6 μM and the 30 cycles of amplification involved 10 s at 98°C, 30 s at 65°C, and 25 s at 72°C, before finishing with 7 min at 72°C.

For Δ*amgR*, the upstream and downstream fragments were amplified using primers amgRUp-F (5′-GAC TGA ATT CCT GTA GAA GTC CTG GCG GT-3′; EcoRI site underlined) and amgRUp-R (5′-GAC TTC TAG ATG GCC TGC ACT GTC GAC AT 3′; XbaI site underlined) and primers amgRDown-F (5′-GAC TTC TAG AGC CGT CGG TGG TCT CGT GA-3′; XbaI site underlined) and AmgRDown-R (5′-GAC TCT GCA GCG GCG CTG GAG AAA CTG GT-3′; PstI site underlined). The reaction mixture and parameters were as described above for amplification of the ΔPA2798 deletion fragments.

For ΔPA0006, the upstream and downstream fragments were amplified using primers 0006UP-F (5′-GAC TGA ATT CCC TTG ATG TTG TCG GG-3′; EcoRI site underlined) and 0006UP-R (GAC TTC TAG AAT CGT CGG AGT CGA GG-3′; XbaI site underlined) and primers 0006Down-F (5′-GAC TTC TAG AAG TCG CCA GCG CAT TA-3′; XbaI site underlined) and 0006Down-R (5′-GAC TCT GCA GTC GGT TCG GTC GAT CG-3′; PstI site underlined). The reaction mixture and amplification parameters were as described above for Δ*faoA*, except that the extension time at 72°C was 30 s.

For construction of Δ*mexXY* mutants of *P. aeruginosa*, plasmid pCSV05-01 (pEX18Tc:: Δ*mexXY*) was employed as described previously (77).

Gene cloning. Various genes were amplified from *P. aeruginosa* K767 chromosomal DNA using primers tagged with restriction sites to facilitate their cloning into plasmid pRK415, which was selected in $CaCl_2$-competent *E. coli* DH5α. Following sequencing of the PCR-amplified and cloned genes to ensure that no errors had been introduced during PCR, plasmids were electroporated into *P. aeruginosa*. The PA2798 gene was amplified using primers 2798-F (5′-GCA TAA GCT TTA GGA CCC ACT GTT TCC GG-3′; HindIII site underlined) and 2798-R (5′-GAT CGA ATT CGA CCT CAC CGA CGA ATT TCA-3′; EcoRI site underlined) in a reaction mixture formulated as described above for amplification of the PA2798 deletion fragments, with the exception that 10% (vol/vol) DMSO was included. PCR parameters were also as described above for the PA2798 deletion fragments, except that an annealing temperature of 60°C was used and extension at 72°C was for 2 min 45 s rather than 1 min. The PA2797 gene was amplified using primers 2797-F (5′-GAT CAA GCT TCG GAG ATG CCG GAT GAT AT-3′; HindIII site underlined) and

2797-R (5′-GAT CGG ATC CCA GCG GAG GAT TCA GCA G-3′; BamHI site underlined) using Exact DNA polymerase (2.5 U) in a reaction mixture containing 1 μg of *P. aeruginosa* K767 chromosomal DNA as the template, 1 μM each primer, and 0.3 mM each dNTP in 1× Exact buffer. Following an initial denaturation step at 95°C for 5 min, the mixture was subjected to 30 cycles of heating at 94°C for 45 s, 45.5°C for 45 s, and 72°C for 1 min, before finishing with a 5-min incubation at 72°C. The *pstB* and *amgR* genes were amplified using primers pstB-F (5′-CTA GAA GCT TCT GCG CGA GAA GTA CAA GGC-3′; HindIII site underlined) and pstB-R (5′-CTA GGA ATT CAA CGA GCT ACG GAG AGC GC-3′; EcoRI site underlined) and primers amgR-F (5′-CTA GAA GCT TGC CGC TAC CTG GGC GAT AA-3′; HindIII site underlined) and amgR-R (5′-CTA GGA ATT CCC TGT TGC GGG TAA GAC GAC-3′; EcoRI site underlined), respectively, in reaction mixtures formulated as described above for amplification of the PA2797 deletion fragments using the parameters described for amplification of the PA2798 deletion fragments. The PA0392 gene was amplified using primers 0392-F (5′-CTA GAA GCT TGT GGA GCA GGC CCT GAA C-3′; HindIII site underlined) and 0392-R (5′-CTA GGA ATT CGT GGC AAT CGA GGA GCA G-3′; EcoRI site underlined) in a reaction mixture formulated as described above for amplification of the *faoA* deletion fragments. PCR parameters were as described above for the Δ*faoA* fragments, except that the extension time at 72°C was 30 s as opposed to 1 min. The *faoA* gene was amplified using primers faoA-F (5′-GAC TGG ATC CGG CGT CCG ATG TGT AAG TTC-3′; BamHI site underlined) and faoA-R (5′-GAT CGA ATT CGT CTC TCG GAT TCA GGC TC-3′; EcoRI site underlined) in a reaction mixture formulated as described above for amplification of the *faoA* deletion fragments. PCR parameters were also as described above for the Δ*faoA* fragments, except that an annealing temperature of 63.5°C was used and annealing was carried out for 30 s. The *lptA* gene was amplified using primers lptA-F (5′-GACTAAGCTTTCGACGATCTGGCGGCAG T-3′; HindIII site underlined) and lptA-R (5′-GACTGAATTCCGAATC GGCGCACAGTACG-3′; EcoRI site underlined) in a reaction mixture formulated as described above for amplification of the *lptA* deletion fragments. PCR parameters were also as described above for amplification of the *lptA* deletion fragments.

Antimicrobial susceptibility testing. The susceptibility of bacterial strains to various antimicrobials was assessed using the 2-fold serial dilution technique in 96-well microtiter plates as previously described (44).

NPN assay. Aminoglycoside (gentamicin) promotion of 1-*N*-phenylnaphthylamine (NPN) uptake into *P. aeruginosa*, as a measure of aminoglycoside interaction with and permeation of membranes, was assessed as described previously (55), except that cells were treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 5 μM) rather than KCN.

Membrane depolarization assay. The assay for measuring cytoplasmic membrane (CM) depolarization promoted by gentamicin treatment of *P. aeruginosa* was loosely based on a previously described assay (51). Overnight L-broth cultures of *P. aeruginosa* were diluted 1:99 in L broth (100 ml) and grown until early logarithmic phase (optical density at 600 nm [OD_{600}] = 0.3 to 0.5), at which time the culture was divided in two and gentamicin (final concentration, 5 μg/ml) was added to one of the cultures. Samples (5 ml) of the gentamicin-treated and untreated control cultures were taken immediately and then hourly over 3 h and exposed to the membrane potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DIBAC_4(3)$; Invitrogen] at 37°C for 5 min in the dark at a final concentration of 10 μg/ml. Bacteria were then pelleted by centrifugation (10 min at 3,000 rpm in a microcentrifuge) and resuspended in phosphate-buffered saline to a final OD_{600} of 0.1. Membrane depolarization-dependent fluorescence emitted by cells was then measured using a Varian Cary Eclipse fluorescent spectrophotometer with excitation and emission wavelengths of 490 and 518, respectively.

Gentamicin killing assay. *P. aeruginosa* cultures were grown as described above for the membrane depolarization assay, samples were taken from the gentamicin-containing cultures at the indicated time points, and dilutions were plated on L agar and incubated overnight at 37°C in order to determine viable cell numbers over time following gentamicin exposure.

RESULTS

Identification of genes contributing to intrinsic aminoglycoside resistance in *P. aeruginosa*. Random transposon mutagenesis of panaminoglycoside-resistant *P. aeruginosa* strains K2153 and K2413-T3 was undertaken to identify a gene(s) contributing to aminoglycoside resistance. Plate screening for mutants showing increased susceptibility first to paromomycin and then to other aminoglycosides identified several showing a panaminoglycoside susceptibility phenotype (data not shown). Cloning and sequencing of the transposon-flanking chromosomal DNA identified several genes whose disruption correlated with increased aminoglycoside susceptibility, including *faoA*, *proC*, *pstB*, *amgR*, *lptA*, PA0392, and PA2798. *proC* occurs upstream of PA0392 in a probable 3-gene operon, while PA2798 occurs upstream of PA2797 in a probable 2-gene operon, such that Tn insertions in these genes might impact aminoglycoside susceptibility as a result of polar effects on the downstream genes and not as a result of the gene disruptions themselves. Individual in-frame deletions of each these genes were engineered into the chromosome of the *P. aeruginosa* prototroph strain K767 to assess their link to panaminoglycoside susceptibility. With the exception of the mutant with the *proC* deletion (data not shown), all of the resultant mutant strains were panaminoglycoside susceptible, and this was reversed by cloned wild-type copies of the corresponding genes (Table 2). Of note, none of the deletion mutants showed any change in susceptibility to nonaminoglycosides, including carbenicillin, norfloxacin, nalidixic acid, chloramphenicol, and tetracycline (data not shown), while spectinomycin MICs were reduced ≤2-fold. These genes thus contribute to intrinsic aminoglycoside resistance in *P. aeruginosa*.

Transposon insertion mutants showing a panaminoglycoside-susceptible phenotype and disrupted in *pstB* (encoding a component of a high-affinity phosphate transport system [66]), *amgR* (encoding the response regulator component of the *amgRS* TCS homologue of the *Escherichia coli* OmpR-EnvZ envelope stress-response regulators [54]), *faoAB* (encoding a multienzyme complex involved in degradative fatty acid [FA] β-oxidation [47]), and PA0392 (encoding a conserved hypothetical product of unknown function [29, 54]) have been reported previously. Of the novel intrinsic aminoglycoside resistance genes, *lptA* encodes a homologue of the *E. coli* PlsC lysophosphatidic acid acyltransferase (LPA), responsible for adding the second FA to glycerol-3-phosphate in the synthesis of phospholipids (PLs) (13), while PA2798 is part of a 2-gene operon that includes PA2797, where PA2797 is annotated as an anti-anti-sigma factor whose activity is dictated by its phosphorylation state (inactive upon phosphorylation), and PA2798 encodes a probable sensor phosphatase that acts on the PA2797 gene product, thus activating it.

Intrinsic aminoglycoside resistance genes contribute to aminoglycoside resistance independently of AmgR(S) and MexXY. A previous study revealed that *mexXY* is not regulated by the AmgRS two-component putative stress-response regulatory protein pair and that these genes likely contribute independently to intrinsic aminoglycoside resistance (54). Results presented here confirm the additive effects of eliminating *amgR* and *mexXY*. A mutant lacking both, K3166, was more susceptible to aminoglycosides than either single-knockout strain (Table 3). Given the previously shown link of AmgRS to membrane stress, the possi-

TABLE 2 Panaminoglycoside susceptibility[a] of *P. aeruginosa* mutants

Strain	Plasmid	Relevant property	MIC (μg/ml)[b]						
			GEN	PAR	SPC	KAN	TOB	AMI	STR
K767		Wild type	2	256	512	64	1	2	32
K3163		PA2797$^-$	0.25 (8)	16 (16)	128 (4)	16 (4)	0.125 (8)	0.25 (8)	8 (4)
K3162		PA2798$^-$	0.5 (4)	32 (8)	256 (2)	8 (8)	0.25 (4)	0.5 (4)	8 (4)
K3162	pRK415	PA2798$^-$	0.25	16	128	16	ND	ND	ND
K3162	pCG014	PA2798$^+$	2	128	512	64	ND	ND	ND
K3160		PstB$^-$	0.5 (4)	32 8)	256 (2)	8 (8)	0.5 (2)	0.5 (4)	8 (4)
K3160	pRK415	PstB$^-$	0.5	32	128	16	ND	ND	ND
K3160	pCG012	PstB$^+$	1	64	512	32	ND	ND	ND
K3164		PA0392$^-$	0.5 (4)	32 (8)	256 (2)	16 (4)	0.5 (2)	1 (2)	8 (4)
K3164	pRK415	PA0392$^-$	0.5	32	256	16	ND	ND	ND
K3164	pCG015	PA0392$^+$	2	128	512	64	ND	ND	ND
K3159		AmgR$^-$	1 (2)	64 (4)	512 (1)	16 (4)	0.5 (2)	0.5 (4)	8 (4)
K3159	pRK415	AmgR$^-$	1	64	512	16	ND	ND	ND
K3159	pCG011	AmgR$^+$	4	512	1024	128	ND	ND	ND
K3161		FaoA$^-$	0.5 (4)	16 (16)	512 (1)	16 (4)	0.5 (2)	1 (2)	8 (4)
K3161	pRK415	FaoA$^-$	0.5	16	512	16	ND	ND	ND
K3161	pCG013	FaoA$^+$	1	128	512	32	ND	ND	ND
K3165		LptA$^-$	0.5 (4)	64 (4)	512 (1)	16 (4)	0.5 (2)	0.5 (4)	8 (4)
K3165	pRK415	LptA$^-$	0.5	64	512	16	0.5	0.5	8
K3165	pJDT03	LptA$^+$	1	256	512	64	ND	ND	ND
K1525		MexXY$^-$	0.5 (4)	16 (16)	64 (8)	32 (2)	0.5 (2)	0.5 (4)	2 (16)

[a] Aminoglycoside susceptibility of *P. aeruginosa* mutants with deletions of genes linked to intrinsic aminoglycoside resistance in a transposon mutagenesis screen. The impact of the corresponding cloned genes and vector controls on susceptibility was also assessed.
[b] Fold changes in MIC for the mutants relative to that for wild type are indicated in parentheses. GEN, gentamicin; PAR, paromomycin; SPC, spectinomycin; KAN, kanamycin; TOB, tobramycin; AMI, amikacin; STR, streptomycin.

bility that AmgRS contributed to aminoglycoside resistance as a result of protection against aberrant polypeptide accumulation in aminoglycoside-treated cells and their disruption of membranes (54), and the identification of a number of lipid/membrane-related genes as determinants of aminoglycoside resistance (i.e., *lptA*, *faoAB*), it was reasoned that AmgRS and MexXY might represent two distinct mechanisms of aminoglycoside resistance, with one (involving AmgRS and lipid/membrane-related genes) based on limiting aberrant polypeptide-mediated membrane damage. To assess this, deletions in each of *pstB*, *faoA*, *lptA*, PA0392, and PA2798 were coupled with deletions in *mexXY* and *amgR*. Interestingly, all double knockouts showed increased panaminoglycoside susceptibility relative to single *mexXY* and *amgR* knockouts (Table 3), indicating that all worked additively or synergistically and, apparently, independently in contributing to intrinsic aminoglycoside resistance. Still, *amgR* double knockouts were generally more aminoglycoside susceptible than the *mexXY* double knockouts; i.e., loss of *amgR*(*S*) rendered cells more sensitive to aminogly-

TABLE 3 Impact on aminoglycoside susceptibility of intrinsic aminoglycoside resistance gene knockouts in combination with Δ*mexXY* and Δ*armR*

Strain	Relevant property	MIC (μg/ml)[a]						
		GEN	PAR	SPC	KAN	TOB	AMI	STR
K767	Wild type	2	256	512	64	1	2	32
K1525	MexXY$^-$	0.5 (4)	16 (16)	64 (8)	32 (2)	0.5 (2)	0.5 (4)	2 (16)
K3166	MexXY$^-$, AmgR$^-$	0.125 (16, 4)	4 (64, 4)	32 (16, 2)	8 (8, 4)	0.125 (8, 4)	0.125 (16, 4)	0.5 (64, 4)
K3169	MexXY$^-$, PA2798$^-$	0.0625 (32, 8)	4 (64, 4)	32 (16, 2)	8 (8, 4)	0.125 (8, 4)	0.125 (16, 4)	1 (32, 2)
K3167	MexXY$^-$, PstB$^-$	0.25 (8, 2)	4 (64, 4)	64 (8, 1)	8 (8, 4)	0.25 (4, 2)	0.25 (8, 2)	1 (32, 2)
K3171	MexXY$^-$, PA0392$^-$	0.25 (8, 2)	4 (64, 4)	32 (16, 2)	8 (8, 2)	0.25 (4, 2)	0.25 (8, 2)	1 (32, 2)
K3168	MexXY$^-$, FaoA$^-$	0.25 (8, 2)	4 (64, 4)	64 (8, 1)	8 (8, 4)	0.25 (4, 2)	0.25 (8, 2)	1 (32, 2)
K3172	MexXY$^-$, LptA$^-$	0.125 (16, 4)	16 (16, 1)	64 (8, 1)	16 (4, 2)	0.125 (8, 4)	0.125 (16, 4)	1 (32, 2)
K3159	AmgR$^-$	0.5 (4)	32 (8)	256 (2)	16 (4)	0.25 (4)	0.25 (8)	8 (4)
K3175	AmgR$^-$, PA2798$^-$	0.03125 (64, 16)	2 (128, 16)	32 (16, 8)	2 (32, 8)	0.03125 (32, 8)	0.03125 (64, 8)	0.5 (64, 16)
K3173	AmgR$^-$, PstB$^-$	0.03125 (64, 16)	4 (64, 8)	64 (8, 4)	4 (16, 4)	0.0625 (16, 4)	0.0625 (32, 4)	0.5 (64, 16)
K3177	AmgR$^-$, PA0392$^-$	0.0625 (32, 8)	4 (64, 8)	64 (8, 4)	4 (16, 4)	0.0625 (16, 4)	0.0625 (32, 4)	0.5 (64, 16)
K3174	AmgR$^-$, FaoA$^-$	0.125 (16, 4)	8 (32, 4)	128 (4, 2)	8 (8, 2)	0.0625 (16, 4)	0.0625 (32, 4)	1 (32, 8)
K3178	AmgR$^-$, LptA$^-$	0.25 (8, 2)	16 (16, 2)	256 (2, 1)	16 (4, 1)	0.25 (4, 1)	0.125 (16, 2)	2 (16, 4)

[a] Fold changes in MIC for the mutants relative to that for wild type are indicated in parentheses. Where two numbers are indicated, the first is the fold change in MIC for the indicated mutant relative to that for wild type, and the second is the fold change in MIC for the indicated double mutant relative to that for its parent MexXY$^-$ or AmgR$^-$ single mutant. GEN, gentamicin; PAR, paromomycin; SPC, spectinomycin; KAN, kanamycin; TOB, tobramycin; AMI, amikacin; STR, streptomycin.

TABLE 4 Impact of loss of multiple intrinsic aminoglycoside resistance genes on aminoglycoside susceptibility in *P. aeruginosa*

Strain	Relevant property	MIC (μg/ml)[a]						
		GEN	PAR	SPC	KAN	TOB	AMI	STR
K767	Wild type	2	256	512	64	1	2	32
K3160	PstB$^-$	0.5 (4)	32 (8)	256 (2)	16 (4)	0.5 (2)	0.5 (4)	8 (4)
K3179	PstB$^-$, LptA$^-$	0.25 (8)	16 (16)	256 (2)	16 (4)	0.25 (4)	0.25 (8)	2 (16)
K3180	PstB$^-$, FaoA$^-$	0.25 (8)	16 (16)	256 (2)	8 (8)	0.125 (8)	0.25 (8)	2 (16)
K3181	PstB$^-$, PA0392$^-$	0.25 (8)	8 (32)	128 (4)	8 (8)	0.125 (8)	0.25 (8)	2 (16)
K3182	PstB$^-$, PA2798$^-$	0.125 (16)	4 (64)	64 (8)	4 (16)	0.0625 (16)	0.125 (16)	2 (16)
K3161	FaoA$^-$	0.5 (4)	16 (16)	512 (1)	16 (4)	0.5 (2)	1 (2)	8 (4)
K3165	LptA$^-$	0.5 (4)	64 (4)	512 (1)	16 (4)	0.5 (2)	0.5 (4)	8 (4)
K3164	PA0392$^-$	0.5 (4)	32 (8)	256 (2)	16 (4)	0.5 (2)	1 (2)	8 (4)
K3185	FaoA$^-$, LptA$^-$	0.125 (16)	8 (32)	256 (2)	8 (8)	0.0625 (16)	0.25 (8)	2 (16)
K3184	FaoA$^-$, PA0392$^-$	0.25 (8)	16 (16)	128 (4)	8 (8)	0.125 (8)	0.25 (8)	4 (8)
K3183	LptA$^-$, PA0392$^-$	0.25 (8)	16 (16)	256 (2)	16 (4)	0.125 (8)	0.25 (8)	4 (8)

[a] Fold changes in MIC for the mutants relative to that for wild type are indicated in parentheses. GEN, gentamicin; PAR, paromomycin; SPC, spectinomycin; KAN, kanamycin; TOB, tobramycin; AMI, amikacin; STR, streptomycin.

cosides upon loss of a second intrinsic aminoglycoside resistance determinant. Of note, while most double knockouts were markedly more susceptible to aminoglycosides than the Δ*amgR* single-knockout strain (at least 4-fold), the impact of *lptA* loss in this background was minimal (≤2-fold), suggesting that LptA might be in some way linked to the AmgRS-regulated stress-response pathway.

Cumulative effects of intrinsic aminoglycoside resistance genes. Since the various resistance genes operated nominally independently of *amgRS* and *mexXY*, it was of interest to assess whether they also operate independently of one another. To assess this, various combinations of knockouts were engineered in individual strains, and the impact on resistance was determined. Again, all double knockouts examined showed enhanced aminoglycoside susceptibility compared with their counterpart single knockouts (Table 4), indicating that they have independent and cumulative effects on resistance.

Contribution of intrinsic aminoglycoside resistance genes to the panaminoglycoside resistance of clinical isolates. To determine whether genes linked to intrinsic aminoglycoside resistance in a prototroph strain were significant contributors to the panaminoglycoside resistance of clinical (i.e., CF patient) isolates, deletions of these genes were individually engineered into two representative panaminoglycoside-resistant CF patient isolates, K2160 and K2162 (Table 5). The mechanism(s) of aminoglycoside resistance in these isolates is not known, although a contribution of MexXY-OprM has been reported (77). Elimination of any of these genes increased susceptibility to multiple aminoglycosides, particularly to those most commonly used to treat *P. aeruginosa* infections (e.g., gentamicin, up to 8- to 32-fold; amikacin, up to 8- to 16-fold; tobramycin, up to 8-fold). Moreover, the impact of these gene deletions on the susceptibility of the clinical isolates to amikacin, gentamicin, and tobramycin was generally greater than

TABLE 5 Impact of loss of intrinsic aminoglycoside resistance genes on aminoglycoside susceptibility of clinical *P. aeruginosa* isolates

Strain	Relevant property	MIC (μg/ml)[a]					
		GEN	PAR	SPC	KAN	TOB	AMI
K2160	Panaminoglycoside resistant	32	1,024	512	256	8	32
K3192	AmgR$^-$	8 (4)	512 (2)	256 (2)	64 (4)	1 (8)	4 (8)
K3193	PstB$^-$	8 (4)	512 (2)	256 (2)	64 (4)	2 (4)	16 (2)
K3194	FaoA$^-$	4 (8)	256 (4)	256 (2)	64 (4)	2 (4)	8 (4)
K3195	PA2798$^-$	4 (8)	256 (4)	256 (2)	64 (4)	1 (8)	4 (8)
K3196	PA0392$^-$	4 (8)	256 (4)	256 (2)	64 (4)	2 (4)	8 (8)
K3197	LptA$^-$	8 (4)	512 (2)	512 (1)	128 (2)	2 (4)	8 (8)
K2168	MexXY$^-$	8 (4)	128 (8)	64 (8)	128 (2)	4 (2)	8 (8)
K2162	Panaminoglycoside resistant	256	>4,096	2,048	1,024	32	128
K3198	AmgR$^-$	64 (4)	4,096 (2)[b]	1,024 (2)	512 (2)	8 (4)	32 (4)
K3199	PstB$^-$	16 (16)	2,048 (4)[b]	1,024 (2)	256 (4)	16 (2)	16 (8)
K3200	FaoA$^-$	64 (4)	1,024 (8)[b]	2,048 (1)	512 (2)	16 (2)	32 (4)
K3201	PA2798$^-$	8 (32)	1,024 (8)[b]	1,024 (2)	512 (2)	4 (8)	8 (16)
K3202	PA0392$^-$	16 (16)	1,024 (8)[b]	1,024 (2)	256 (4)	8 (4)	16 (8)
K3203	LptA$^-$	64 (4)	4,096 (2)[b]	2,048 (1)	512 (2)	8 (4)	32 (4)
K2170	MexXY$^-$	16 (16)	256 (32)[b]	128 (16)	256 (4)	8 (4)	16 (8)

[a] Fold changes in MIC for the mutants relative to that for the corresponding wild-type clinical parent strain are indicated in parentheses. GEN, gentamicin; PAR, paromomycin; SPC, spectinomycin; KAN, kanamycin; TOB, tobramycin; AMI, amikacin.

[b] Because a precise paromomycin MIC was not determined for the K2162 parent of these mutants, the fold change reported in parentheses is a minimum.

that of the same deletions in wild-type *P. aeruginosa* strain K767 (compare Tables 5 and 3), arguing that they not only contribute to intrinsic resistance but also are important for the acquired aminoglycoside resistance of these isolates. In many cases, the effect of the gene deletions on the susceptibility of the clinical isolates to these aminoglycosides was equal to or greater than that seen when the *mexXY* efflux genes were deleted, indicating that *amgR*, *pstB*, PA2798, *faoA*, *lptA*, and PA0392 are as important as this well-known efflux contributor to aminoglycoside resistance in clinical strains (68). The impact of these deletions was less noteworthy for paromomycin and, intriguingly, spectinomycin in the clinical strains, where only the *mexXY* knockout had any appreciable impact on resistance (Table 5).

Impact of intrinsic aminoglycoside resistance gene deletions on aminoglycoside permeation of membranes. Aminoglycosides enter bacterial cells, including those of *P. aeruginosa*, via a so-called self-promoted uptake route (33) that involves their permeation of the OM (55). One possibility for the enhanced panaminoglycoside susceptibility of the aforementioned mutants is increased aminoglycoside interaction with/permeation of membranes and subsequent uptake into *P. aeruginosa*. Aminoglycoside interaction with and promotion of permeation of membranes were assessed in wild-type (strain K767) and mutant cells using enhanced NPN fluorescence following exposure to gentamicin. In the absence of gentamicin, no increase in NPN fluorescence was observed in K767, while at low (5 μg/ml) and high (20 μg/ml) concentrations of this aminoglycoside, NPN fluorescence increased over time (Fig. 1), indicative of gentamicin association with membranes and promotion of membrane permeation. Gentamicin-promoted membrane permeation was also observed for the mutant strains at both gentamicin concentrations, and this was indistinguishable from that seen for K767 (Fig. 1). Thus, the enhanced aminoglycoside susceptibility of these mutants is not explained by their increased membrane association/permeation and, so, uptake into the cell.

Impact of intrinsic aminoglycoside resistance gene deletions on aminoglycoside-promoted cytoplasmic membrane depolarization. Aminoglycosides are known to promote mistranslation, with mistranslated polypeptides inserting into and damaging cytoplasmic membranes (16). In *E. coli*, this aminoglycoside-dependent membrane perturbation initiates a cascade of events, including activation of the CpxRA envelope stress-response pathway, which leads to the production of ROS and, ultimately, cell death (51). Disruption of the intrinsic aminoglycoside resistance genes in *P. aeruginosa* might, therefore, increase susceptibility to aminoglycosides by increasing the susceptibility of membranes to perturbation by aminoglycoside-generated mistranslated polypeptides. One measure of cytoplasmic membrane perturbation is membrane depolarization, and, indeed, aminoglycoside-promoted membrane depolarization has been demonstrated in *E. coli* and correlates well with aminoglycoside-promoted cell killing (51). Moreover, *E. coli* mutants showing enhanced aminoglycoside susceptibility showed higher levels of gentamicin-promoted membrane depolarization than wild type (51), consistent with the membranes of these mutants being more susceptible to perturbation by aminoglycoside-generated mistranslated polypeptides. As seen in Fig. 2, exposure of wild-type *P. aeruginosa* strain K767 to a supra-MIC of gentamicin (5 μg/ml) promoted membrane depolarization, which increased over the 3-hour exposure period, in contrast to unexposed K767, where no increase was seen. Gentamicin-promoted membrane depolarization that increased over time was also seen for the mutant strains, with the Δ*pstB*, Δ*faoA*, and ΔPA0392 mutants and, to a lesser extent, the Δ*amgR* mutant showing an increase in gentamicin-promoted membrane depolarization relative to strain K767 after 1 h of exposure to the drug (Fig. 2). This correlated with the period of maximal killing of the cells by gentamicin (≥99%; Fig. 3), suggesting that the enhanced susceptibility of these mutants to aminoglycosides results from enhanced aminoglycoside-promoted membrane perturbation. The ΔPA2798 (at all time points) and Δ*lptA* (at the 1-hour time point) mutants, in contrast, appeared to be less susceptible to gentamicin-promoted membrane depolarization than the wild type (Fig. 2). As such, the enhanced aminoglycoside susceptibility of these mutants did not result from enhanced susceptibility of their membranes to aminoglycoside-promoted perturbation.

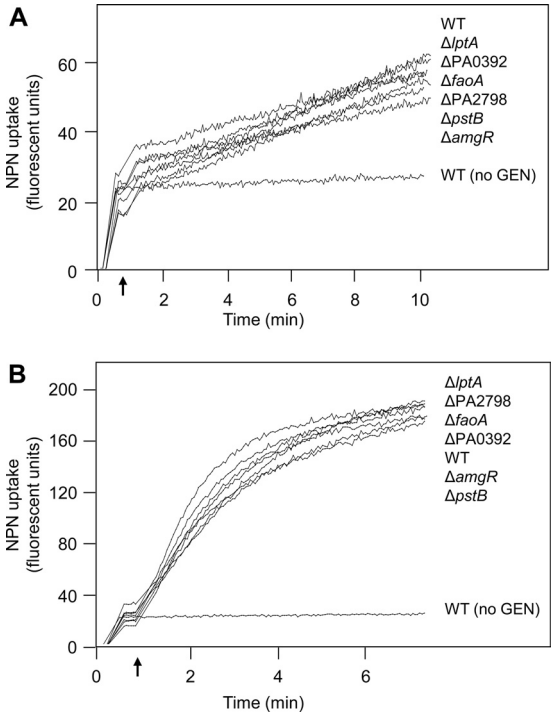

FIG 1 Impact of intrinsic aminoglycoside resistance gene knockouts on gentamicin-promoted outer membrane permeability. Membrane permeability, as assessed by NPN fluorescence, was measured over time following exposure of wild-type (WT; strain K767) and mutant *P. aeruginosa* strains to 5 μg/ml (A) and 20 μg/ml (B) of gentamicin (added at the time indicated by the arrow). The strains listed at the right (top to bottom) correspond to the traces at the left (top to bottom, respectively) at the final time points. Data for wild-type strain K767 not treated with gentamicin (no GEN) are also shown. The data are representative of at least 2 independent experiments for each strain.

DISCUSSION

Aminoglycoside uptake into and action on bacterial cells are complex processes that involve LPS binding/OM permeation (31, 32, 46), CM traversal using the membrane potential (8, 32), and ribosome disruption, leading to production of membrane-damaging mistranslated polypeptides (16). Polypeptide disruption of the CM triggers a cell envelope stress response (51) that ultimately leads to the generation of ROS (i.e., hydroxyl radicals), whose oxidation of key macromolecules appears to be responsible for the lethal effects of these agents (24, 51). As such, mutations leading to

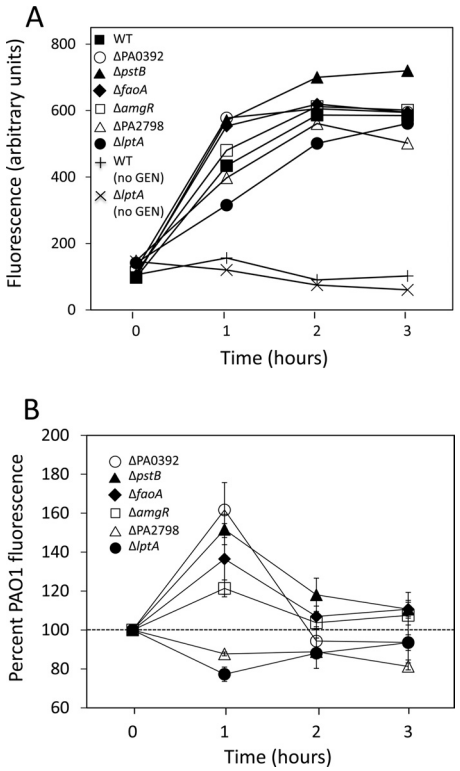

FIG 2 Impact of intrinsic aminoglycoside resistance gene knockouts on gentamicin-promoted cytoplasmic membrane depolarization. Cytoplasmic membrane depolarization, as assessed by $DIBAC_4(3)$ fluorescence, was measured over time following exposure of wild-type (WT; strain K767) and mutant *P. aeruginosa* strains to 5 μg/ml of gentamicin (added at time zero). (A) Results of a representative experiment showing increased gentamicin-promoted fluorescence over time. Background fluorescence in the absence of gentamicin is shown for wild-type strain K767 but is representative of all strains, with the exception of the indicated Δ*lptA* strain. (B) Gentamicin-promoted fluorescence over time for the indicated mutants as a percentage of gentamicin-promoted fluorescence for wild-type strain K767. The data are the mean ± standard deviation of 3 to 4 independent experiments where the fluorescence data were adjusted for the background fluorescence of gentamicin-unexposed cells prior to calculating percentages.

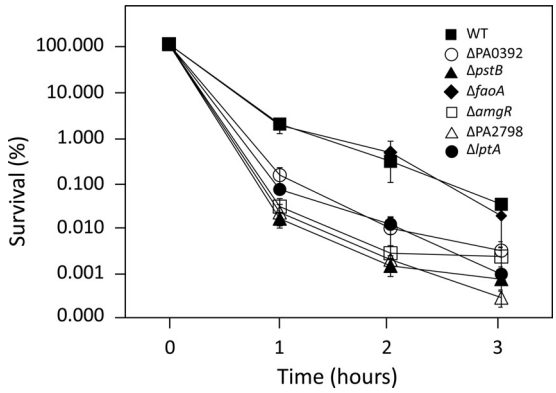

FIG 3 Gentamicin-promoted killing of *P. aeruginosa*. The impact of gentamicin (5 μg/ml) on the viability of wild-type (WT; strain K767) and mutant *P. aeruginosa* was assessed over time by determining viable cell counts at the indicated time points. Results are presented as percent survival and are the mean ± standard deviation of 3 independent experiments.

alterations in LPS binding/OM permeation (6), CM potential (67), envelope stress responses (51, 54), and ROS production (50) can influence aminoglycoside susceptibility. The current study identifies several apparently unrelated genes whose disruption enhances aminoglycoside susceptibility in *P. aeruginosa*, confirming them to be determinants of intrinsic aminoglycoside resistance in this organism. The *amgRS* genes, encoding a TCS that apparently regulates an envelope stress response, have previously been identified to be determinants of intrinsic aminoglycoside resistance (54), as have the *faoA*, *pstB*, and PA0392 genes (29, 54). While the *amgRS* link to resistance is explained, at least in part, by their control of protease genes whose products may well function to turn over aminoglycoside-generated mistranslated polypeptides, thereby ameliorating their perturbation of the CM (37), the nature of the link of these other genes was undefined. This work highlights the probable contribution of several of these genes to maintenance of CM integrity and/or stability, with their mutational loss compromising this and so rendering the CM more susceptible to aminoglycoside-promoted perturbation, likely by aminoglycoside-generated mistranslated polypeptides. In agreement with this, susceptibility to spectinomycin, a ribosome-targeting agent related to the aminoglycosides but known not to generate mistranslated protein products (14), was only minimally enhanced (≤2-fold increase) in the *amgR*, *faoA*, *pstB*, and PA0392 deletion strains. Indeed, only the *mexXY* deletion substantially increased susceptibility to spectinomycin, suggesting that the increased aminoglycoside susceptibility of a *mexXY* knockout is not explained by an increased sensitivity to CM perturbation by mistranslated polypeptides and, thus, that MexXY-OprM contributes to aminoglycoside resistance via some mechanism other than alleviation of mistranslated polypeptide-mediated CM stress. While antimicrobial export is the simplest explanation for its contribution to resistance, a *mexZ* mutant expressing MexXY and showing elevated aminoglycoside MICs did not exhibit any change in aminoglycoside (tobramycin) accumulation relative to its $MexZ^+$ parent strain in a recent study (57).

Intriguingly, the enhanced susceptibility to aminoglycoside-promoted CM perturbation in the *amgR*, *faoA*, *pstB*, and PA0392 deletion strains typically occurs 1 hour after initial exposure to an aminoglycoside only, during the period of maximal killing (≥2-log-unit decline in viable cell numbers), suggesting that the two are linked. In *E. coli* there is also a spike of membrane depolarization (and hydroxyl radical formation) 1 h after initial aminoglycoside exposure, and this, too, parallels the period of maximal cell killing (51). Moreover, aminoglycoside-sensitive *E. coli hflKC* mutants show enhanced gentamicin-promoted membrane depolarization at 1 h postexposure (51), reminiscent of what we saw here with the *pstB*, *faoA*, and PA0392 mutants. HflKC regulates a protease, FtsH, which is responsible, in part, for turning over aberrant/misfolded membrane-associated polypeptides (41), suggesting that the increased susceptibility and membrane depolarization of the mutants result from increases in the levels of aminoglycoside-generated mistranslated polypeptides and, so, membrane perturbation. Consistent with this and with enhanced aminoglycoside-promoted membrane depolarization in *P. aeruginosa* being an indicator of enhanced CM perturbation by aminoglycoside-generated mistranslated polypeptides, an *ftsH* mutant of *P. aeruginosa* also exhibits enhanced susceptibility to aminoglycosides (37).

FaoAB/FadBA-mediated FA oxidation is typically associated

with growth on/metabolism of exogenously provided FAs (3), although the observation that its mutational loss provides for enhanced aminoglycoside-promoted membrane perturbation and aminoglycoside susceptibility suggests that it may also play a role in membrane FA turnover in response to envelope stress. In the face of membrane perturbation by aminoglycoside-generated mistranslated polypeptides, for example, *P. aeruginosa* might normally replace shorter and/or unsaturated FAs with longer and/or saturated FAs so as to enhance membrane stability and, possibly, limit CM perturbation by mistranslated polypeptides. Certainly, there are many examples of bacteria modulating the FA composition of their membranes in response to membrane perturbants (e.g., organic solvents) to stabilize these structures and, so, limit membrane perturbation (23, 69, 83).

A link between *pstB* function and the CM is obvious, given the importance of phosphate for synthesis of a key CM component, PLs. Loss of the Pst inorganic phosphate transporter effectively renders cells phosphate limited, even in rich media, and it is well-known that phosphate limitation has a negative impact on PL incorporation into bacterial membranes. In *Pseudomonas fluorescens*, for example, increased incorporation of the non-PL ornithine amide lipid occurs in membranes of cells grown under phosphate-limiting conditions, at the expense of PLs (20), although it is not clear whether this would impact membrane stability. *pst* mutations have also been reported in *E. coli*, where they are linked to membrane FA alterations, with a tendency toward an increase in unsaturated FAs (53). This would tend to make the CM more fluid and, possibly, more susceptible to perturbation by mistranslated polypeptides. In light of the information presented above, it is likely that a *pstB* mutation impacts the lipid composition of *P. aeruginosa* so as to enhance CM perturbation by aminoglycoside-generated mistranslated polypeptides. PA0392 has no known function at present, though its homologue in *E. coli*, YggT (36.7% amino acid identity), appears to play a role in osmotic stress tolerance (42). Given the impact of a PA0392 deletion on aminoglycoside-dependent CM perturbation and reports that membrane PL and FA changes occur in response to osmotic stress in several bacteria and may contribute to osmotic stress tolerance (70), PA0392 may well influence the CM lipid composition in ways that stabilize this structure, contributing to aminoglycoside resistance and, perhaps, tolerance to osmotic stress. Of particular note is the greater increase in aminoglycoside susceptibility seen upon loss of *faoA*, *pstB*, or PA0392 in a mutant already lacking *amgR* relative to that seen upon loss of these genes in an otherwise wild-type strain. This presumably reflects the heightened impact of the absence of an envelope stress response in mutants with compromised and, so, more stress-sensitive CMs.

The link between *lptA* and the CM is readily apparent, yet mutation of *lptA* does not provide for any obvious increase in aminoglycoside-promoted CM perturbation. A homologue of *E. coli plsC*, *lptA* is capable of complementing a *plsC*(Ts) mutant (2), consistent with LptA functioning as an LPA. Its closest homologue, however, is *Neisseria meningitidis* NlaB (36% identity), a second LPA in this organism (the first is encoded by *nlaA* [80]). Like *N. meningitidis* (and unlike *E. coli*), *P. aeruginosa* apparently expresses two LPAs (PA4351 is a close homologue of NlaA; 54% similarity and 33% identity), which likely explains the ability to derive an *lptA* knockout in this organism when knockouts of *E. coli plsC* are lethal (12). A previously reported *lptA* mutant of *P. aeruginosa* PAO1 showed alterations in membrane FAs (decrease in $C_{16:0}$, increase in $C_{18:0}$, and loss of $C_{17:0\ cyclo}$) and evidence for reduced membrane fluidity (2). This is consistent with the reduced aminoglycoside-promoted membrane perturbation seen in this mutant and, as such, is unlikely to explain its enhanced aminoglycoside susceptibility. Possibly, given that elimination of *lptA* in a strain already lacking *amgR* has a minimal additional impact on aminoglycoside susceptibility, loss of *lptA* somehow compromises activation of the AmgRS TCS by aminoglycoside-generated mistranslated polypeptides. In light of the reduced membrane fluidity and reduced aminoglycoside-promoted membrane perturbation in the *lptA* mutant, one possibility is that this results from a reduced mistranslated polypeptide perturbation of the *lptA* mutant membranes (i.e., reduced membrane stress). If true, however, there would then be less need for the AmgRS-regulated envelope stress response, a major component of which are proteases that likely turn over aberrant polypeptides. If these aberrant polypeptides are less able to perturb the membranes of an *lptA* mutant, there would be less need to turn them over. Still, it may be that aminoglycoside-generated mistranslated polypeptides have additional deleterious effects on the cell, independent of CM perturbation, such that their turnover by AmgRS-controlled proteases would still be beneficial and their loss (in the absence of AmgRS activation) would compromise aminoglycoside resistance.

Interestingly, *N. meningitidis* NlaB is encoded as part of an operon with *gmhX*, whose product is required for incorporation of L-glycero-D-manno-heptose into lipooligosaccharide (75), and intriguingly, the closest homologue to *gmhX* in *P. aeruginosa* is the PA0006 gene (51% identical, 67% overall similarity), which occurs as part of a probable 2-gene operon with *lptA*. A homologue of *gmhX* and PA0006 in *E. coli*, YaeD (also known as GmhB), is also linked to heptose synthesis, though as part of LPS biosynthesis. Given this possible link to LPS biosynthesis, the operon organization of PA0006 and *lptA*, and the known connection between LPS and aminoglycoside uptake and resistance, it was of interest to assess the impact of a PA0006 knockout on aminoglycoside susceptibly. Unfortunately, and despite repeated attempts, a deletion mutant lacking PA0006 could not be recovered, suggesting that this gene may well be essential. While the link to a probable LPS biosynthetic gene suggests that *lptA* may well play a role (nonessential) in the synthesis of this macromolecule and that its loss produces aminoglycoside resistance as a result of some defect in LPS, deletion of *lptA* did not adversely impact LPS binding/permeation of the OM. Thus, it may also operate independently of PA0006, at least as regards its contribution to aminoglycoside resistance. Despite the probably operonic link between *proC* and PA0392, for example, only a Δ*proC* mutant displays proline auxotrophy (data not shown), while only the PA0392 deletion impacts aminoglycoside resistance.

PA2797 and PA2798 are the respective homologues of the *Bacillus* SpoIIAA and SpoIIE proteins that regulate the SpoIIAB anti-sigma factor of the sporulation sigma factor σ^F (26) and the *Bacillus* RbsV and RbsU proteins that regulate the RbsW anti-sigma factor of the stress-response sigma factor σ^B (34). One possibility, then, is that these proteins ultimately regulate a, possibly, stress-response sigma factor in *P. aeruginosa* whose lack (in the PA27987 and PA2798 mutants, which would be defective in acting on/sequestering their presumed anti-sigma factor target to promote sigma factor activation) enhances susceptibility to aminoglycoside-promoted stress. This would appear to be independent of aminoglycoside-promoted membrane perturbation, which is not

enhanced and is, in fact, reduced in the PA2798 mutant. Strikingly, loss of PA2798 in an *amgR* background has a marked and greater impact on aminoglycoside susceptibility than loss of this gene in an otherwise wild-type background. As such, these two systems may regulate overlapping stress responses such that the full impact of a stress-response defect vis-à-vis aminoglycoside susceptibility is not felt until both are absent. While the probable sigma factor controlled by PA2797-PA2798 is unknown, disruption of this locus has recently been linked to increased colistin tolerance (45), although a mechanism was not elucidated.

Significantly, the intrinsic aminoglycoside resistance genes identified here were also important contributors to the panaminoglycoside resistance of clinical strains, including strains where MexXY-OprM has been implicated in resistance (77). Given their apparent link to membrane stability and the not unexpected role of membrane perturbability in aminoglycoside susceptibility, this is not surprising. These results do suggest, however, that undermining CM integrity and/or compromising adaptive responses to CM perturbation can enhance the susceptibility of *P. aeruginosa* to aminoglycosides, and thus, targeting components of CM biosynthesis or these adaptive responses might be useful in promoting aminoglycoside susceptibility.

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