Targeting a bacterial stress response to enhance antibiotic action

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This report describes the identification and analysis of a 2-component regulator of *Pseudomonas aeruginosa* that is a potential aminoglycoside antibiotic combination therapy target. The regulator, AmgRS, was identified in a screen of a comprehensive, defined transposon mutant library for functions whose inactivation increased tobramycin sensitivity. AmgRS mutations enhanced aminoglycoside action against bacteria grown planktonically and in antibiotic tolerant biofilms, against genetically resistant clinical isolates, and in lethal infections of mice. Drugs targeting AmgRS would thus be expected to enhance the clinical efficacy of aminoglycosides. Unexpectedly, the loss of AmgRS reduced virulence in the absence of antibiotics, indicating that its inactivation could protect against infection directly as well as by enhancing aminoglycoside action. Transcription profiling and phenotypic analysis suggested that AmgRS controls an adaptive response to membrane stress, which can be caused by aminoglycoside-induced translational misreading. These results help validate AmgRS as a potential antibiotic combination target for *P. aeruginosa* and indicate that fundamental stress responses may be a valuable general source of such targets.

aminoglycoside | AmgRS | *Pseudomonas aeruginosa* | 2-component regulator | CpxRA

The need for new antibiotics has become critical for a number of reasons. First, heavy antibiotic use and person-to-person spread of bacteria have accelerated the emergence of resistant strains, and this problem is continually worsening (1). The bacterium *Pseudomonas aeruginosa* is a prime example: 30% of clinical isolates from critically ill patients are now resistant to three or more drugs. This resistance leads to treatment failure and increased mortality rates (2). Second, existing antimicrobials work poorly in chronic infections, even when the bacteria are susceptible when tested ex vivo. A key factor limiting therapeutic efficacy in such infections is that the organisms live in biofilms, bacterial clusters which are antibiotic tolerant (3, 4). Finally, antibiotic treatment often fails in overwhelming infections such as severe Gram-negative sepsis (5), and little progress has been made in improving outcomes. Thus, new agents are needed to treat infections caused by resistant organisms and biofilms and for acute infections like severe sepsis.

For more than a decade, genomic approaches have been used to identify potential antibiotic targets which are essential for viability and conserved across species (6, 7). Unfortunately, the results of pursuing such functions as single-agent targets have been disappointing (8–10). One explanation for this may be that the best targets were favored in the evolution of naturally-occurring antibiotics and are already exploited by existing drugs (6, 9, 11).

An alternative to essential functions as drug targets is the set of gene products whose inactivation enhances the activity of existing antibiotics (8, 12). Such functions are potential combination therapy targets. Although dedicated resistance functions like inactivating enzymes and efflux pumps have received the greatest attention as combination targets (12, 13), it appears likely that many more such targets exist (14–16). In the work reported here, we have identified potential drug targets for aminoglycoside combination therapy in *P. aeruginosa*. We carried out proof-of-principle experiments to help validate the clinical relevance of one potential target, a 2-component regulator that appears to control a conserved membrane stress response.

We focused on enhancing aminoglycoside activity against *P. aeruginosa* for several reasons. *P. aeruginosa* is a major cause of both chronic and acute infections. Although aminoglycosides are probably the most valuable treatment available for such infections, rates of bacterial resistance are steadily increasing (17). Furthermore, the high aminoglycoside dosages and sustained treatments needed to fight *P. aeruginosa* infections can lead to serious side effects, such as hearing loss and kidney damage (18). Enhancing aminoglycoside efficacy through inhibition of combination targets could reduce antibiotic failure in life-threatening infections and decrease adverse effects by reducing dosages required for treatment.

Results

Identification of Aminoglycoside Antibiotic Combination Targets. We sought to identify potential combination targets for aminoglycosides using a comprehensive method. To achieve this, we screened a library of arrayed, sequence-defined *P. aeruginosa* PAO1 transposon mutants (19) for strains exhibiting increased tobramycin sensitivity (see *Materials and Methods*). The transposons used to create the library can generate alkaline phosphatase or beta-galactosidase translational gene fusions and encode tetracycline resistance. Since the mutant library includes mutations in nearly all nonessential genes, this screening strategy should identify the majority of functions whose inactivation enhances tobramycin activity. The results of screens for mutants exhibiting two "test" phenotypes (altered motility or auxotrophic growth) support this assumption (19). The screen of 42,240 *P. aeruginosa* strains identified tobramycin-sensitive insertion mutations in 61 genes and 10 intergenic regions (Table S1). The genomic locations of all 71 insertions were verified by resequencing. In addition, the phenotypic assignments of about half of the genes were confirmed by the identification of multiple alleles and additional genetic linkage analysis (see *Materials and Methods*). The group is enriched in genes encoding membrane and small molecule transport proteins, and represents about a 5-fold increase in the total number of functions associated with tobramycin sensitivity (Table S2).

Although most of the mutations led to 2-fold decreases in tobramycin minimal growth inhibitory concentrations (MIC) (Table S1), 16 had larger effects (Table 1). The assignments in this strong group were verified by the identification of multiple alleles with increased sensitivity, linkage analysis, and complementation (Table 1 and Fig. S1). The set included mutations inactivating several functions previously associated with aminoglycoside intrin-

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Table 1. Antibiotic sensitivities of strong tobramycin hypersensitive mutants

Mutant ORF*	Gene	Function*	Alleles identified‡	Reference allele§	Wild-type complementation¶	MIC (μg/ml)								
						Tob	Gen	Kan	Par	Str	Spec	Ery	Nal	Carb
None (PAO1)	–	–	–	–		1	2	128	256	16	1024	256	512	16
PA3303 (Control)	–	–	–	–		1	2	128	256	16	1024	256	512	16
PA0016	*trkA*	Potassium transport	3	C3$^+$		0.25	1	32	64	8	1024	256	512	8
PA0427	*oprM*	Multidrug efflux	3	O1		0.25	0.25	32	32	1	256	16	512	1
PA2018	*mexY*	Multidrug efflux	3	B19$^+$		0.25	0.25	32	32	2	256	64	512	16
PA2019	*mexX*	Multidrug efflux	1	B16		0.25	0.25	32	32	2	128	32	512	8
PA2494†	*mexF*	Multidrug efflux	6	L18$^+$	–	0.125	0.5	32	64	2	256	512	64	32
PA3013	*foaB*	Fatty acid oxidation	2	A23		0.25	1	32	128	8	1024	256	512	16
PA3016	–	Hypothetical	2	F22$^+$	+	0.125	0.5	32	64	8	1024	256	512	16
PA3686	*adk*	Adenylate kinase	1	L21$^+$		0.25	1	64	128	8	512	256	512	16
PA4398	–	Two-component sensor	1	F11$^+$		0.25	1	32	128	8	512	64	512	8
PA4942	*hflK*	Protease accessory factor	1	I15$^+$	+	0.25	1	32	128	8	1024	256	512	16
PA4961	–	Hypothetical	2	E8		0.25	1	64	128	8	256	64	512	2
PA5199	*amgS*	Two-component sensor	3	A3$^+$	+	0.125	0.25	32	64	2	1024	128	256	8
PA5200	*amgR*	Two-component regulator	2	M7$^+$	+	0.063	0.125	32	64	1	512	128	256	4
PA5366	*pstB*	Phosphate transport	2	H12		0.25	0.5	32	64	4	256	64	512	8
PA5471	–	Regulator	1	M5$^+$		0.25	0.5	32	16	1	256	64	512	16
PA5528	–	Hypothetical	2	G8$^+$	+	0.125	0.25	32	64	4	1024	256	512	16

The minimal inhibitory concentrations for growth on LB-agar are presented. All genes with at least one mutant allele leading to 4-fold or greater decrease in tobramycin MIC are shown with the exceptions of mutations in *mexB,* expected to be polar on *oprM*. PA5199 and PA5200 were originally annotated as *envZ* and *ompR*, respectively, based on homology to the *E. coli* regulator. These were renamed *amgS* and *amgR* ("aminoglycoside resistance") because their mutant phenotypes differed significantly from those of *E. coli envZ-ompR* mutants. Tob, tobramycin; Gen, gentamicin; Kan, kanamycin; Par, paromomycin; Str, streptomycin; Spec, spectinomycin; Ery, erythromycin; Nal, nalidixic acid; Carb, carbenicillin.

*http://v2.pseudomonas.com/index.jsp. The "control" strain carries a transposon insertion in PA3303 which does not alter tobramycin sensitivity.

†Aminoglycoside sensitivity phenotype due to apparent gain-of-function alleles (see *Text*).

‡Number of independent tobramycin hypersensitive alleles identified.

§Genetic linkage (+) of the transposon insertion and tobramycin hypersensitivity was confirmed by recombination (by transformation) of the reference allele into wild-type (strain MPAO1), followed by retesting of tobramycin sensitivity.

¶Complementation was carried out by introducing plasmids carrying wild-type versions of the mutated genes into strains carrying the reference mutant alleles indicated and examining the tobramycin MIC of the resulting strains.

sic resistance, including an efflux pump (MexXY-OprM) and its regulator (PA5471) (20, 21), a membrane protease accessory factor (HflK) (14, 22), and a hypothetical protein (PA5528) (16). Newly identified functions whose inactivation significantly increased sensitivity included a 2-component regulator [PA5200/5199, annotated as "OmpR/EnvZ" (www.Pseudomonas.com) but here named "AmgRS" ("*am*inoglycoside resistance")], potassium and phosphate transporters (Trk and Pst systems) and two unassigned gene products (PA3016 and PA4961) (Table 1). Insertions in one gene (*mexF*) were unusual in that they generated gain-of-function changes (see *Mechanism of Action of AmgRS Inactivation*).

Mutations inactivating the AmgRS 2-component regulator produced the greatest increases in tobramycin sensitivity observed, reducing the MIC 8- to 16-fold (Table 1). Inactivation of this regulator also increased susceptibility to all other aminoglycosides tested but had modest effects on the action of spectinomycin, erythromycin, nalidixic acid, and carbenicillin. Inactivation of *amgRS* did not lead to a significant decrease in growth rate on antibiotic-free media (see below). To examine the function of AmgRS in more detail, an unmarked *amgRS* deletion mutant was constructed (see *Materials and Methods*). This isolate was also highly tobramycin sensitive, and the phenotype was complemented by a plasmid carrying wild-type *amgRS* (Fig. S1). The marked sensitivity of the *amgRS* mutant raised the possibility that the AmgRS regulator could be targeted to enhance tobramycin activity in clinical situations where antibiotics often fail. We performed proof-of-principle studies to test this idea.

Proof-of-Principle Tests of AmgRS as a Combination Target. An important cause of antibiotic failure in many chronic infections is the infecting organism's growth in biofilms. Biofilm growth produces marked phenotypic resistance even when bacteria are inherently antibiotic-sensitive (4). We investigated the effect of inactivating *amgRS* on biofilm-mediated resistance using two systems that measure antibiotic action in different ways. Inactivation of *amgRS* clearly increased tobramycin killing of biofilms grown using continuous media flow as measured by live-dead staining (23) (Fig. 1*A*). Using a model in which biofilms are grown on permeable filters (24), we also found that tobramycin treatment killed approximately 100-fold more bacteria when *amgRS* was inactivated as compared to wild type (Fig. 1*B*).

Antibiotic resistance due to mutation can also lead to treatment failure and an increased risk of death during infection. To investigate whether AmgRS could be targeted to sensitize resistant strains, we inactivated it in 23 *P. aeruginosa* isolates from chronically infected cystic fibrosis patients, including 11 strains which were highly tobramycin resistant (MIC $\geq$ 8 μg/ml) and 2 mucoid strains (25). The resistance of these strains results from intensive and repeated antibiotic treatment (25). For all strains, including those with extremely high resistance, tobramycin sensitivity was markedly increased by inactivation of *amgR* (Fig. 1*C*). Loss of AmgRS function also increased the sensitivity of a highly resistant PAO1 strain expressing a 16S rRNA methylase (26), although the resistance of the mutant strain remained relatively high (Fig. S2). These results suggest that an agent inhibiting AmgRS could increase aminoglycoside sensitivity in isolates with different levels of resistance and employing diverse resistance mechanisms.

Another circumstance in which current drugs are unsatisfactory is severe sepsis, in which treatment failure can occur even if organisms are intrinsically antibiotic sensitive. To examine whether

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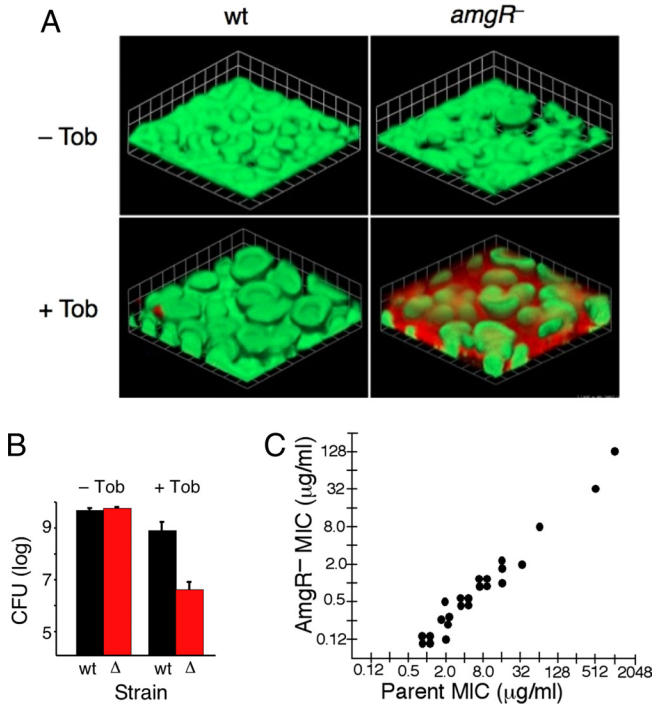

Fig. 1. Mutations in *amgRS* increase tobramycin sensitivity in biofilms and resistant clinical isolates. (*A*) Wild-type and mutant bacterial biofilms produced in flow cells exposed or unexposed to tobramycin were stained for live (green) and dead (red) cells. (*B*) Biofilm bacteria grown on filters over nutrient agar were exposed to tobramycin and assayed for surviving colony forming units. (*C*) The tobramycin minimal inhibitory concentrations of 23 cystic fibrosis isolates (and MPAO1) carrying an *amgR*$^{-}$ insertion are compared to their *amgRS*$^{+}$ parents. Two mucoid strains represented in the set exhibited MIC decreases of 4- and 8-fold when *amgR* was inactive. WT, strain MPAO1; *amgR*$^{-}$, *amgR*::IS*phoA*/hah M7; Δ, Δ*amgRS*.

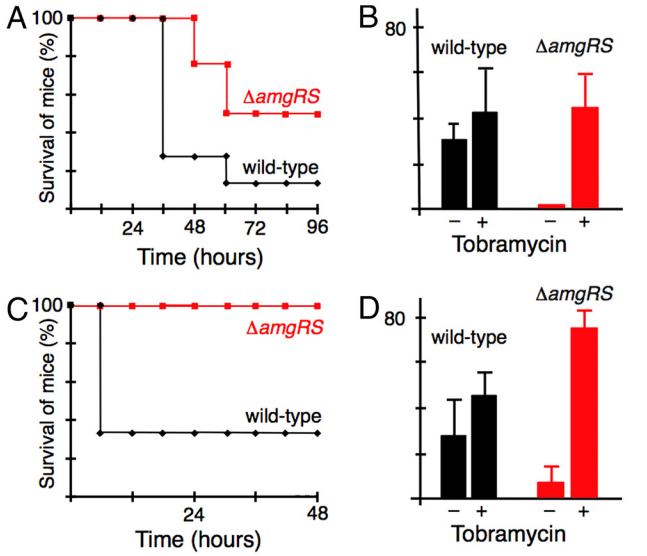

Fig. 2. Behavior of *amgRS* mutants in acute infection. The survival of mice following intratracheal (*A* and *B*) and intraperitoneal (*C* and *D*) infection are shown. (*A* and *C*) Mice were administered equal numbers of wild-type and Δ*amgRS* mutant bacteria. (*B* and *D*) Effect of tobramycin treatment on survival. To compensate for the virulence defect of the Δ*amgRS* mutant, we administered twice the number of mutant as wild-type bacteria in intra-tracheal infections (*B*), and 4 times the number of mutant as wild-type bacteria in intraperitoneal infections (*D*). Survival after 4 (*B*) or 2 (*D*) days is shown. *A* and *C* represent 3 separate experiments, and *B* and *D* represent averages of 3 independent experiments, with $n = 12$–33 animals per data point. The difference in outcome with tobramycin treatment in both *B* and *D* was statistically significant for the mutant ($P < 0.05$) but not for the wild-type strain ($P = 0.21$–0.31).

targeting AmgRS could increase aminoglycoside efficacy in lethal sepsis, we tested the mutant in intra-tracheal and intra-peritoneal infections (Fig. 2). Unexpectedly, we found that the *amgRS* mutation reduced mortality even in the absence of tobramycin treatment (Fig. 2 *A* and *C*). Thus, loss of AmgRS directly compromises bacterial virulence. To determine whether targeting AmgRS could increase treatment efficacy apart from this virulence defect, we increased the inoculum of the mutant to amplify the severity of the infection. As shown in Fig. 2 *B* and *D*, infections produced using a high inoculum of the *amgRS* mutant were more tobramycin susceptible than those caused by lower numbers of wild-type bacteria, even though the high mutant inoculum produced a more severe infection. Taken together, these data indicate that targeting AmgRS could reduce bacterial virulence directly, as well as increase the efficacy of aminoglycosides against biofilms, antibiotic resistant strains, and in acute lethal infections.

Mechanism of Action of AmgRS Inactivation. To explore how inactivation of AmgRS increases aminoglycoside sensitivity, we used microarray analysis to identify the genes whose expression is controlled by this regulator in tobramycin-exposed cells. AmgRS strongly regulated the expression of a number of genes involved in proteolysis and membrane transport (Table 2 and Table S3). The genes were induced in wild-type by tobramycin exposure, implying that AmgRS is activated by such exposure. Expression of the aminoglycoside-specific efflux pump (MexXY-OprM) was not significantly affected by the absence of AmgRS, although the expression of three other transporters, two membrane proteases, and a protease-associated function was strongly AmgRS-dependent. Although the AmgRS protein sequences are highly similar to those of *E. coli* OmpR-EnvZ, none of the *E. coli* homologues of the AmgRS-dependent genes require OmpR-EnvZ for expression (27). Furthermore, none of the major *P. aeruginosa* outer membrane porin genes were significantly regulated by AmgRS (Table S3). However, several of the *E. coli* homologues of the AmgRS-regulated genes are controlled by the 2-component regulator CpxRA (28–30). In *E. coli*, CpxRA is activated by misfolded membrane proteins and mediates an envelope stress response (30–34).

There are phenotypic similarities between the *amgRS* and *cpxRA* mutants as well. *E. coli cpxRA* loss-of-function mutations increase tobramycin sensitivity (35), although the effect is smaller than that seen for the *P. aeruginosa* mutations. Mutations in both regulators also compromise growth at alkaline pH (31) (Fig. S3) and modulate sensitivity to exported hybrid proteins (see below) (33).

Although the AmgRS and CpxRA regulators exhibit functional similarities, there are differences as well. The AmgRS regulator appears to lack an auxiliary factor homologous to a protein (CpxP) which functions with CpxRA (31). In addition, the homologues of several of the genes regulated most strongly by CpxRA in *E. coli*, including *degP* and *dsbA*, were not controlled by AmgRS under the conditions we examined (Table S3) (36).

If *amgRS* mutations increase aminoglycoside sensitivity due to a defect in responding to membrane stress, other mutations that increase membrane stress might also sensitize bacteria to the antibiotics. Candidates for such mutations were the unusual class of *mexF* insertions which increases tobramycin sensitivity (Table 1). We were originally surprised to find *mexF* mutations represented in the tobramycin sensitive group, since previous studies had not associated the MexEF-OprN efflux pump with aminoglycoside resistance, although it is associated with nalidixic acid resistance (37). To examine the unexpected association in more detail, we retrieved all 14 *mexF* insertion mutants in the mutant library,

Table 2. Genes whose transcriptional induction by tobramycin requires AmgRS

Gene*	*E. coli* homologue	AmgRS$^+$ /AmgRS$^-$		+Tob/–Tob		Function
		+ Tob	– Tob	AmgRS$^+$	AmgRS$^-$	
PA2830	*htpX*†	7.6	2.9	3.4	1.3	Membrane protease
PA1331	*yegH*	7.4	2.6	3.9	1.3	Transporter
PA2549	*ygjT*†	5.4	1.7	5.8	1.8	Transporter
PA3712	*yebE*†	4.9	3.2	2.6	1.7	Hypothetical
PA5528	None	4.7	3.1	3.4	2.2	Hypothetical
PA3575	*yceJ*	3.7	2.3	2.6	1.6	Cytochrome
PA1882	*sugE*	3.7	1.6	2.6	1.2	Transporter
PA3787	*nlpD*	2.8	1.1	2.7	1.1	Membrane protease
PA2604	*yccA*†	2.5	1.8	2.5	1.8	Membrane protease modulator

The expression ratios of wild-type relative to Δ*amgRS*, and of tobramycin-treated relative to -untreated cultures is presented for the genes whose expression showed the greatest dependence on AmgRS. The genes carry a conserved upstream sequence (consensus: GAAANANNNNGNAACA) which may correspond to an AmgR interaction site. The complete set of differentially expressed genes is listed in Table S3. Tob, tobramycin.

*All genes whose expression after tobramycin exposure required AmgRS (≥2.5-fold expression in wild-type relative to the Δ*amgRS* strain) and whose expression was induced in wild-type by tobramycin (≥2.5-fold) are shown. Cultures were treated with 1 μg/ml tobramycin for fifteen minutes prior to harvest

†*E. coli* homologue is regulated by CpxRA.

resequenced them to confirm their transposon locations, and assayed their sensitivities to nalidixic acid and tobramycin (Table S4). All 14 of the mutations increased sensitivity to nalidixic acid, implying that they inactivated the MexEF-OprN efflux pump. However, only 6 of the *mexF* insertions conferred increased sensitivity to tobramycin. All 6 encode hybrid proteins consisting of MexF attached to alkaline phosphatase and are predicted to be targeted to the cytoplasmic membrane, the subcellular location of MexF (Table S4). The increased aminoglycoside sensitivity of the mutants could thus result from inner membrane stress caused by the aberrant hybrid proteins. For example, the hybrids could overload the putative AmgRS stress response pathway, compromising its capacity to defend cells against aminoglycoside-induced mistranslated proteins (38). Previous studies have shown that membrane protein-alkaline phosphatase hybrid proteins are often toxic (39). Two additional findings support this interpretation. First, as expected from the model, the aminoglycoside sensitivity due to production of the *mexF-phoA* hybrids was genetically dominant in partial diploid cells expressing wild-type *mexF* (Table S4), although the loss-of-function phenotype (nalidixic acid sensitivity) was, as expected, recessive. Second, the hybrid proteins whose expression increased tobramycin sensitivity were highly toxic to *amgRS* mutants in the absence of antibiotics at neutral pH (Table S4). The results indicate that the toxic effects of the *mexF-phoA* hybrids are ameliorated through AmgRS action. The results suggest two ways in which the hybrid proteins may increase tobramycin sensitivity: by compromising the membrane and facilitating aminoglycoside uptake and by overloading the AmgRS response so that it is less able to respond to aminoglycoside-induced mistranslation.

If the mechanism we have proposed for MexF-PhoA action is correct, the production of other membrane-disruptive proteins should also enhance aminoglycoside sensitivity. Recent studies have identified a novel class of small hydrophobic proteins from *E. coli* whose expression compromises the inner membrane barrier and is toxic (40). To test whether production of the *E. coli* peptides in *P. aeruginosa* increased tobramycin sensitivity, we expressed one of them (IbsC) at a sublethal level and examined tobramycin sensitivity (Fig. S4). Indeed, like production of MexF-PhoA hybrids, expression of IbsC greatly enhanced tobramycin sensitivity. Intriguingly, loss of AmgRS only modestly increased the toxicity of the *E. coli* protein in the absence of antibiotic. The results indicate that weakening the inner membrane through IbsC production enhances aminoglycoside sensitivity, but that, unlike for the MexF-PhoA proteins, the AmgRS response did not significantly protect cells from the *E. coli* protein. It is also possible that sublethal IbsC synergizes with tobramycin through a mechanism independent of membrane damage, and this accounts for the absence of a larger AmgRS mutant effect.

Discussion

The goal of the project described here was to identify functions whose inhibition could increase the effectiveness of aminoglycoside antibiotics in treating *P. aeruginosa* infections. Pursuing such combination therapy targets rather than sole-agent targets is attractive in that the approach builds on the clinical success of established antibiotics rather than attempting to find comparable substitutes. The steps we followed in the study were: (*i*) to screen a comprehensive, defined transposon mutant library for potential targets whose inactivation increased sensitivity to tobramycin; (*ii*) to prioritize the targets based on the level of tobramycin sensitivity; (*iii*) to help validate the clinical relevance of one promising target, a 2-component regulator called AmgRS; and (*iv*) to help define the mechanism by which loss of AmgRS increases aminoglycoside sensitivity.

The screen for functions whose inactivation increased aminoglycoside sensitivity used a transposon mutant library with an average of about 5 insertions per gene (19). The redundancy was important both for helping avoid missed assignments and for eliminating false positives by providing confirmatory hits. The screen identified mutations in 61 genes, with insertions in 16 leading to strong phenotypes. The mutant associations were confirmed by the identification of multiple independent alleles increasing sensitivity, genetic linkage tests, and complementation analysis. The group included the principle functions previously associated with intrinsic aminoglycoside resistance, indicating that the screen was relatively comprehensive. As expected, there was no overlap of our group with a large set of transposon mutants exhibiting elevated rather than reduced aminoglycoside resistance (41).

The mutations leading to the greatest increase in tobramycin sensitivity inactivated a 2-component regulator, AmgRS. To help examine whether AmgRS could be a favorable drug target, we examined the consequence of inactivating it in several clinically relevant contexts. Mutations inactivating AmgRS increased tobramycin killing of drug-tolerant biofilms, sensitized clinical isolates exhibiting high-level aminoglycoside resistance, and improved outcomes in lethal murine infections. The results indicate that inhibiting AmgRS has the potential to increase aminoglycoside efficacy in 3 clinical situations in which antibiotic treatment often fails and help validate it as a potential combination drug target.

Unexpectedly, the *amgRS* mutant was considerably less virulent than its parent in acute murine infections in the absence of antibiotic. The deficiency is not due to a general growth defect,

since the mutant grew as well as its parent under laboratory conditions (e.g., see Fig. S3). We assume that loss of AmgRS sensitizes organisms to adverse conditions imposed by the host. Inactivating AmgRS could thus compromise infection directly as well as sensitize bacteria to aminoglycoside treatment.

Mechanistic studies suggest that AmgRS controls a response that protects bacteria from envelope damage. The conclusion is based on results implying that the AmgRS regulator is functionally similar to an *E. coli* regulator (CpxRA) which controls a well-characterized envelope stress response (34, 36). Nearly half of the genes strongly regulated by AmgRS have *E. coli* homologues regulated by CpxRA, and mutants defective in the two regulators shared several fundamental phenotypes. Remarkably, there was no such functional overlap detected with OmpR-EnvZ, the closest *E. coli* sequence homologue of AmgRS. Although AmgRS and CpxRA appear functionally related, there are significant differences in the genes controlled and mutant phenotypes corresponding to the two regulators. Overall, the AmgRS response appears to be more focused on protecting the cytoplasmic membrane and less on the periplasm than the CpxRA response.

A model for how AmgRS protects *P. aeruginosa* from aminoglycosides builds on the mechanism by which streptomycin is thought to kill *E. coli* (42, 43). At low concentrations, aminoglycoside antibiotics cause translational misreading, and the resulting mistranslation products damage the inner membrane. The damage allows ions and small molecules to leak from the cytoplasm, promotes increased uptake of antibiotic, and leads ultimately to bacterial death. We hypothesize that AmgRS mutations increase aminoglycoside sensitivity by sensitizing cells to the membrane-damaging polypeptides resulting from translational misreading. The functions induced through activation of AmgRS by aminoglycoside treatment include two membrane proteases (HtpX and NlpD) and a protease-associated factor (YccA), which should help eliminate misfolded proteins (30, 44). Their reduced expression in AmgRS mutants should thus reduce the capacity of cells to tolerate antibiotic exposure.

This model can account for a number of other observations. First, the production of several aberrant cytoplasmic membrane proteins (MexF-PhoA hybrids and the membrane disruptive IbsC protein) increased aminoglycoside sensitivity. These proteins may mimic the membrane-damaging effects of mistranslation products and thus enhance aminoglycoside uptake. Other mutations enhancing tobramycin sensitivity could also act by destabilizing the inner membrane, which may help account for the high representation of changes affecting membrane proteins in the mutant set (Table S2). Second, the hybrid proteins that increased aminoglycoside sensitivity were toxic to *amgRS* mutants in the absence of antibiotic. The result supports the assumption that the AmgRS response protects cells from aberrant membrane proteins. Third, mutations affecting 4 proteases not significantly regulated by AmgRS increase tobramycin sensitivity (Table S1). The strongest inactivated a factor (HflK) (Table 1) associated with a membrane protease (FtsH) which may be particularly important (14, 45). Fourth, mutations inactivating the Trk potassium transport system increased tobramycin sensitivity (Table 1). Inactivating this system may decrease cells' capacity to tolerate membrane damage-induced potassium leakage, an early consequence of aminoglycoside action (46).

Our model differs from one proposed recently for *E. coli* in which activation of the Cpx stress response contributes to aminoglycoside lethality by pushing the cell into a state which provokes oxidative stress (22, 47). The *E. coli* model was based in part on findings of reduced sensitivity of *cpx* deletion mutants to gentamicin, a result which contrasts with our observations of enhanced aminoglycoside sensitivity of AmgRS mutants of *P. aeruginosa*.

It may be possible to identify compounds which interfere with the AmgRS response either by inhibiting the regulator directly or overwhelming the pathway it controls with excess substrate (e.g., an unfolded membrane protein). Two-component regulators have been targeted successfully in a number of small molecule screens, and several chemical classes of inhibitors defined (48, 49). Since the AmgRS response is required for full virulence, its inhibition is expected to provide the dual benefit of compromising infection directly as well as enhancing antibiotic sensitivity. Our analysis used regulator null mutants, and we have not yet defined the level of pharmacological inhibition required to achieve significant effects.

Expression and mutant profiling studies indicate that, in general, sublethal doses of antibiotics activate fundamental physiological responses which help bacteria to tolerate the drugs (14, 50, 51). These stress responses, including the regulators which control them, may provide a rich source of novel, conserved antibiotic combination therapy targets.

Materials and Methods

Additional information is available in the *SI Text*.

Strains. The reference strain (MPAO1; this laboratory's version of PAO1) and its defined transposon (IS*phoA*/hah and IS*lacZ*/hah) mutant library have been described (19), as have the cystic fibrosis clinical isolates (25). The unmarked Δ*amgRS* mutation (deleted from bp + 14 of *amgR* to 6 bp downstream of the *amgS* termination codon) was generated by recombination in MPAO1 using a pEX18Gm derivative carrying the deletion allele (52). Similarly, the *amgR*::IS*phoA*/hah M7 allele was transferred into clinical isolates by recombination using a pEX18Gm derivative carrying a chromosome-derived 7.7 kb Acc65I-SacI fragment which includes the insertion and flanking sequences. *MexF* insertions from the *P. aeruginosa* MPAO1 transposon mutant library were transferred into MPAO1 Δ*amgRS* through chromosomal DNA transformation and *red* recombination with selection for tetracycline resistance (53), with strains cured of the *red* plasmid before analysis.

Identification of Tobramycin Hypersensitive Mutants. The 42,240 member transposon mutant library of *P. aeruginosa* MPAO1 was screened in several steps for strains showing enhanced sensitivity to tobramycin. In the first step, ca. 1 μl aliquots of cells from freezer plates (384-well format) were diluted into 60 μl LB and incubated 2 days at 37 °C, followed by spotting (*ca.* 1 μl aliquots containing 10^5–10^6 cells) on LB agar supplemented with tobramycin (1.0 μg/ml) and LB agar alone. This level of tobramycin partially inhibited the growth of spots of wild-type cells and was intended to provide a sensitive detection of mutants exhibiting increased sensitivity. The plates were incubated 2 days at 37 °C and scored visually for spots showing reduced growth on the medium containing tobramycin. This step identified 924 candidate mutants affecting 708 genes and intergenic regions. The scoring was carried out with a low threshold for inclusion (i.e., one expected to lead to a high false discovery rate) since this approach provided the most complete identification of mutants in previous ''test'' screens (19). A subset of 227 of these candidates judged to be most promising based on the magnitude of tobramycin sensitivity and the recovery of multiple independent mutants affected in the same gene (≥20% of the representatives in the library) was rearrayed in 96-well format for additional tests. The efficiency of plating on LB containing 0.5 μg/ml tobramycin was then determined by serial dilutions and spotting. A set of 149 mutants exhibiting <20% plating efficiency (the MPAO1 parent plates at an efficiency of approximately 1.0) were single colony-purified and arrayed again. (In 12 cases, 2 distinct colony morphologies were observed during purification and both were rearrayed.) The purified mutants were resequenced to verify transposon locations. In several cases, the newly-determined sequences did not correspond to the original assignments, presumably due to mutant cross-contamination introduced during replication of the library. In these cases, additional single colony purification, sequencing and tobramycin sensitivity tests were carried out to correct the discrepancies. In addition, 14 mutants were eliminated because of inconsistent sensitivity assay results or lack of genetic linkage of the transposon insertion and tobramycin sensitivity trait. To test genetic linkage, selected insertion mutations had been transferred into the MPAO1 parent using the Red recombinase method (53), followed by assay of tobramycin sensitivity (Table S1). Four strains with weaker alleles of genes already represented by strong alleles were also added to the collection. The final set of hypersensitive strains consisted of 112 unique mutants corresponding to 61 genes and 10 intergenic regions (Table S1).

Biofilm Assays. In Fig. 1*A*, biofilms were grown under continuous medium flow for 3 days in the absence of antibiotics (23) and then treated with 10 μg/ml tobramycin for 4 h. To measure killing after treatment, cells were stained with 30 μM propidium iodide which labels nonviable cells red (23). Colony biofilms (Fig.

1*B*) were grown on polycarbonate membranes (0.2 μm, 25 mm dia.) for 48 h as previously described (24), and transferred to LB agar lacking NaCl containing 10 μg/ml tobramycin. To measure the susceptibility of planktonic cells under the same conditions, suspensions from overnight cultures were inoculated directly on membranes on the surface of plates. After 24 h the biofilm was homogenized to produce a cell suspension, and viable cell counts were determined by plating on LB agar.

Animal Infections. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Washington. Eight-to-ten week old C57BL/6 mice (Jackson Laboratory) were housed in a pathogen-free environment and received food and water ad libitum. *P. aeruginosa* was grown to mid-log phase in LB lacking NaCl and diluted in 10 mM K_2PO_4, pH 7.0 to the desired cell density. For intra-tracheal infections, mice were anesthetized, intubated, and bacteria (5×10^6 cells) deposited in the lungs as previously described (23). For intra-peritoneal infections, mice were given 0.5 ml injections of bacterial suspensions (1×10^7 cells total). Animals were treated with either 0.7 mg/kg tobramycin in water, or PBS alone by the intra-peritoneal route. Animals were euthanized if they became moribund.

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