Aminoglycoside Heteroresistance in *Acinetobacter baumannii* AB5075

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ABSTRACT Heteroresistance is a phenomenon where a subpopulation of cells exhibits higher levels of antibiotic resistance than the general population. Analysis of tobramycin resistance in *Acinetobacter baumannii* AB5075 using Etest strips demonstrated that colonies with increased resistance arose at high frequency within the zone of growth inhibition. The presence of a resistant subpopulation was confirmed by population analysis profiling (PAP). The tobramycin-resistant subpopulation was cross resistant to gentamicin but not amikacin. The increased tobramycin resistance phenotype was highly unstable, and cells reverted to a less resistant population at frequencies of 60 to 90% after growth on nonselective media. Furthermore, the frequency of the resistant subpopulation was not increased by preincubation with subinhibitory concentrations of tobramycin. The tobramycin-resistant subpopulation was shown to replicate during the course of antibiotic treatment, demonstrating that these were not persister cells. In *A. baumannii* AB5, a large plasmid (p1AB5075) carries *aadB*, a 2″-nucleotidyltransferase that confers resistance to both tobramycin and gentamicin but not amikacin. The *aadB* gene is part of an integron and is carried adjacent to four additional resistance genes that are all flanked by copies of an integrase gene. In isolates with increased resistance, this region was highly amplified in a RecA-dependent manner. However, in a *recA* mutant, colonies with unstable tobramycin resistance arose by a mechanism that did not involve amplification of this region. These data indicate that tobramycin heteroresistance occurs by at least two mechanisms in *A. baumannii*, and future studies to determine its effect on patient outcomes are warranted.

IMPORTANCE *Acinetobacter baumannii* has become an important pathogen in hospitals worldwide, where the incidence of these infections has been increasing. *A. baumannii* infections have become exceedingly difficult to treat due to a rapid increase in the frequency of multidrug- and pan-resistant isolates. This has prompted the World Health Organization to list *A. baumannii* as the top priority for the research and development of new antibiotics. This study reports for the first time a detailed analysis of aminoglycoside heteroresistance in *A. baumannii*. We define the mechanistic basis for heteroresistance, where the *aadB(ant2″)Ia* gene encoding an aminoglycoside adenylyltransferase becomes highly amplified in a RecA-dependent manner. Remarkably, this amplification of 20 to 40 copies occurs stochastically in 1/200 cells in the absence of antibiotic selection. In addition, we provide evidence for a second RecA-independent mechanism for aminoglycoside heteroresistance. This study reveals that aminoglycoside resistance in *A. baumannii* is far more complex than previously realized and has important implications for the use of aminoglycosides in treating *A. baumannii* infections.

Antibiotic heteroresistance occurs when subpopulations of an isogenic bacterial strain exhibit decreased susceptibility to a particular antibiotic (1). Although the clinical significance of heteroresistance has been the subject of debate, resistant subpopulations of otherwise susceptible strains have been demonstrated to mediate treatment failure in animal models (2) and have been associated with treatment failure in human patients (3–6). Heteroresistance has been reported to a wide variety of antibiotics, including β-lactams, glycopeptides, and antimicrobial peptides (1, 7–9). However, reports of aminoglycoside heteroresistance are uncommon. In 1947, it was reported that populations of type b *Haemophilus influenzae* contained rare cells with increased streptomycin resistance, although the mechanism responsible for the formation of these cells was not investigated (10). More recently, decreased expression of the porin gene *ompC* was associated with nonmutational kanamycin-resistant subpopulations in *Salmonella enterica* (11). However, to our knowledge, heteroresistance to tobramycin or gentamicin has not been previously reported.

Acinetobacter baumannii is a Gram-negative, nosocomial, opportunistic pathogen (12–14). Widespread antibiotic resistance in this species recently led the World Health Organization to name carbapenem-resistant *A. baumannii* as its most critical priority pathogen for research and development of new interventions (15). Aminoglycoside resistance in *A. baumannii* has been associated with the acquisition, increased expression, and/or gene amplification of aminoglycoside-modifying enzymes and efflux pumps (13, 16, 17). The multidrug-resistant isolate AB5075 carries a number of antibiotic resistance genes, many of which are carried on the large plasmid p1AB5075. This plasmid includes an integron-like structure encoding four aminoglycoside-modifying enzymes, including the tobramycin resistance gene *aadB*, and a chloramphenicol resistance transporter (18). The plasmid also carries an additional tobramycin-modifying enzyme gene, *aacA4* (18, 19). A recent study of loci required for tobramycin resistance in AB5075 showed that in addition to *aadB* and *aacA4*, 34 chromosomal genes also contribute to resistance to this drug (19).

AB5075 is resistant to tobramycin and gentamicin, meaning that its MIC values for these drugs are above the CLSI breakpoints (19, 20). Here, we report that AB5075 also exhibits tobramycin and gentamicin heteroresistance, as it produces subpopulations of cells that grow at concentrations of these drugs that are higher than the MIC for the general population. The subpopulations with increased resistance were shown to be unstable. We demonstrated that the integron-like structure of five adjacent antibiotic resistance genes, including *aadB*, becomes amplified to 20 to 40 copies in this resistant subpopulation. While this amplification was RecA dependent, colonies with increased tobramycin resistance could also be selected in a *recA*::*Tc* mutant. These resistant isolates did not contain amplifications of the region containing *aadB*, indicating that tobramycin heteroresistance can occur by at least two distinct mechanisms.

RESULTS

An AB5075 subpopulation exhibits increased tobramycin and gentamicin resistance. When performing tobramycin Etest assays with the *A. baumannii* strain AB5075, we observed colonies arising at a high frequency within the zone of inhibition, consistent with a phenomenon termed heteroresistance (Fig. 1A). Heteroresistance was not observed during Etest assays with colistin, rifampin, or tetracycline (data not shown). In order to characterize the population with increased tobramycin resistance, colonies representative of this subpopulation were isolated by plating AB5075 on agar plates with various inhibitory concentrations of tobramycin. In general, colonies representing the resistant subpopulation were heterogenous in size on tobramycin plates but exhibited normal size on medium without drug. The colony size differences in the presence of tobramycin likely reflect differences in the levels of resistance. The frequency of resistant colonies decreased with increasing drug concentrations, so we chose a concentration twice the baseline MIC to determine the frequency. In three
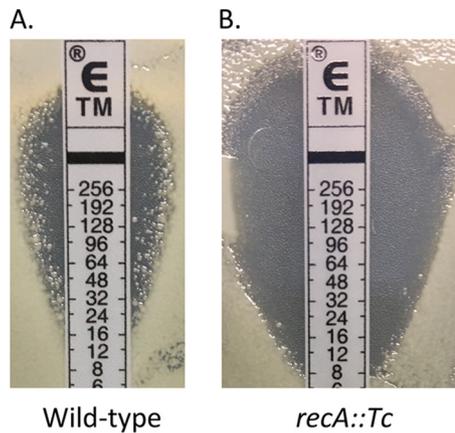
FIG 1 AB5075 produces a subpopulation of cells with increased resistance to tobramycin. Virulent opaque (VIR-O) wild-type (A) or *recA::Tc* (B) cells of AB5075 were grown to 1×10^7 CFU/ml, and 100 μl was plated on LB agar to obtain a lawn of growth. After plating, a tobramycin Etest strip was added, and the plate was incubated for 16 h at 37°C.

independent experiments, colonies with increased resistance arose at an average frequency of 0.52% ± 0.24%. Among the resistant subpopulation, both virulent opaque (VIR-O) and avirulent translucent (AV-T) colony variants were observed, as described previously (21, 22). Because VIR-O and AV-T variants were previously reported to exhibit subtle differences in tobramycin resistance (21, 23), both VIR-O and AV-T tobramycin-resistant colonies were selected for further characterization. These resistant isolates were designated hetR-O2, hetR-O3, hetR-T1, and hetR-T4.

The subpopulation with increased tobramycin resistance was analyzed for cross-resistance to other antimicrobials. Etest assays were used to compare antimicrobial susceptibilities of the tobramycin-resistant isolates to those of wild-type VIR-O and AV-T variants with baseline levels of resistance. Etest assays for a variety of antimicrobials were conducted, revealing that hetR-O2, hetR-O3, hetR-T1, and hetR-T4 all exhibited cross-resistance to gentamicin but not amikacin (Table 1). In addition, cross-resistance to non-aminoglycosides was not observed (Table 1).

TABLE 1 The tobramycin-resistant subpopulation exhibits increased cross-resistance to gentamicin but not other antimicrobials

| Direct comparison of strains	MIC (μg/ml) of drug[a]:						
	TOB	CST	RIF	TET	AMK	CHL	GEN
Comparison 1							
VIR-O	48	1	4	3	192	256	96
hetR-O2	128	1	4	3	192	>256	192
Comparison 2							
VIR-O	64	1	4	3	96	96	32
hetR-O3	192	1	4	3	96	128	128
Comparison 3							
AV-T	48	1	4	2	128	256	96
hetR-T1	256	1	4	2	128	192	>256
Comparison 4							
AV-T	32	1	4	3	64	96	32
hetR-T4	384	1	4	3	64	96	128

[a]Abbreviations: TOB, tobramycin; CST, colistin; RIF, rifampin; TET, tetracycline; AMK, amikacin; CHL, chloramphenicol; GEN, gentamicin. MICs were measured after 16 (TOB, CST, RIF, and TET) or 5 (AMK, CHL, and GEN) h of growth at 37°C. MICs were determined using Etest strips. Direct comparisons between isolates were performed by inoculating two cultures at an optical density A_{600} of 0.1 along the same Etest strip. Two independent experiments were performed for each comparison to assess reproducibility; data from a single representative experiment are shown.

The tobramycin-resistant subpopulation is not induced by tobramycin and is not composed of persister cells. Further phenotypic characterization of the tobramycin-resistant subpopulation was performed. The ability of subinhibitory concentrations of tobramycin to induce the formation of the tobramycin-resistant subpopulation was first investigated. Population analysis profile (PAP) results for treated and untreated cultures of AB5075 were identical (Fig. 2A), suggesting that the formation of the tobramycin-resistant subpopulation was not increased by the presence of tobramycin. To confirm that the tobramycin-resistant subpopulation is capable of growing in the presence of antibiotic, a killing assay with AB5075 was conducted. This assay clearly demonstrated that the tobramycin-resistant subpopulation was capable of growing in high tobramycin concentrations, as the CFU counts increased during treatment (Fig. 2B). These data show that AB5075 forms a subpopulation with increased resistance to tobramycin that is distinct from persister cells, which are antibiotic tolerant at the expense of metabolic activity and active growth (24). Although exposure to a subinhibitory concentration of tobramycin does not increase the frequency of the resistant subpopulation, selection with a high level of drug kills off the majority of susceptible cells, allowing the resistant subpopulation to dominate the culture.

Increased tobramycin resistance is an unstable phenotype. The stability of the increased tobramycin resistance phenotype was first tested in liquid culture. Cultures containing a mix of VIR-O and AV-T cells were grown, treated with tobramycin, and then subcultured without drug. Serial dilutions of cultures at each step indicated that following subculture without drug, the population reverted from being almost entirely tobramycin resistant to containing only about 10% resistant cells (Fig. 2C). To determine whether VIR-O and AV-T representatives of the tobramycin-resistant subpopulation behave similarly in terms of their resistance stability, the hetR-O2 and hetR-T1 stocks were examined for loss of resistance on agar plates. Colonies of hetR-O2 and hetR-T1 were passaged from plates containing tobramycin onto plates with and without drug. After 24 h and 48 h of growth, individual colonies were resuspended and serially diluted onto plates with and without tobramycin to determine the percent resistance within individual colonies (Fig. 2D and E). Interestingly, hetR-O2 and hetR-T1 exhibited differing levels of resistance stability. At 24 h, the percent resistance values of hetR-T1 were similar for colonies grown with and without tobramycin, with about half of the cells from each condition exhibiting resistance. In contrast, hetR-O2 colonies grown without tobramycin for 24 h lost their increased resistance, whereas the majority of cells taken from colonies grown with tobramycin remained resistant (Fig. 2D). In colonies at 48 h, the frequency of resistant cells for both hetR-O2 and hetR-T1 continued to drop (Fig. 2E). Taken together, these results show that increased tobramycin resistance in AB5075 is an unstable phenotype, although the level of instability varies within the tobramycin-resistant subpopulation.

Tobramycin-resistant subpopulations exhibit increased expression of *aadB*. The mechanism of tobramycin heteroresistance in AB5075 was investigated by performing quantitative reverse transcriptase PCR (qRT-PCR) on hetR-O2 and hetR-T1, as well as the parental AB5075 VIR-O and AV-T variants not exposed to tobramycin. Two genes, *aadB* and *aacA4*, known to confer tobramycin resistance were selected for gene expression analysis (19). Differences in *aadB* or *aacA4* expression between the wild-type VIR-O and AV-T variants were not observed (Fig. 3A and B). However, hetR-O2 and hetR-T1 exhibited 5- and 15-fold-increased expression of *aadB*, respectively, compared to VIR-O (Fig. 3A). No differences in expression were observed for *aacA4* (Fig. 3B). These results are consistent with the aminoglycoside resistance profiles of hetR-O2 and hetR-T1, as *aadB* is associated with resistance to both tobramycin and gentamicin but not amikacin (25).

Tobramycin-resistant subpopulations contain a highly amplified region that includes *aadB*. To determine whether the preexisting tobramycin-resistant subpopulation in AB5075 could be due to gene amplification, we measured gene copy number of the region surrounding *aadB*. The *aadB* gene is found on the large plasmid p1AB5075 and is carried adjacent to four other resistance genes that are all flanked by two copies of an integrase (*intI*) gene in the same orientation (Fig. 4). We hypothesized
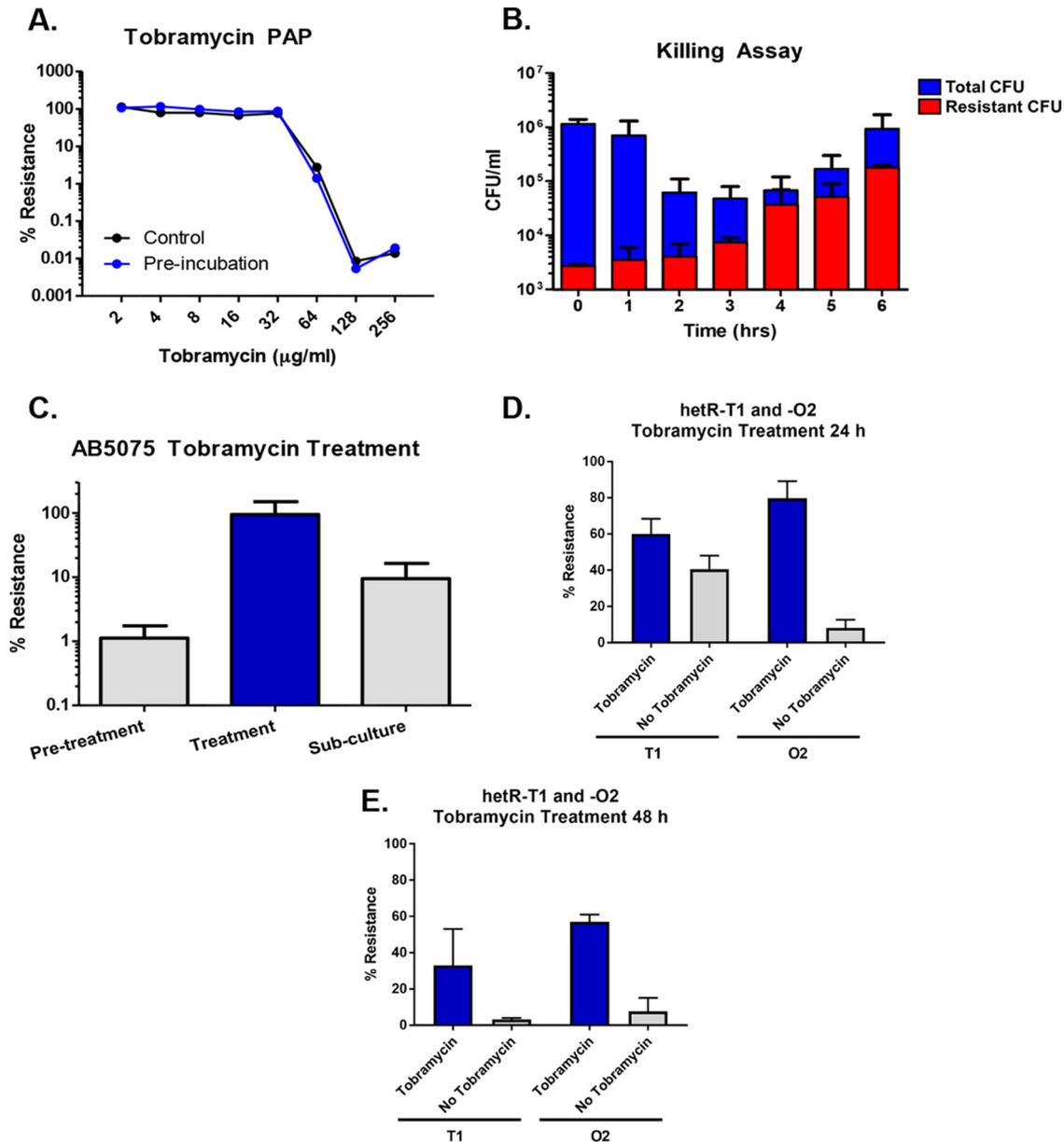
FIG 2 Characterization of tobramycin heteroresistance in AB5075. (A) Preincubation of AB5075 with a subinhibitory concentration of tobramycin had no effect on the population analysis profile (PAP) for this strain, indicating that increased resistance is not induced by exposure to this drug. (B) Incubation of AB5075 in liquid culture with 64 μg/ml of tobramycin resulted in killing of the majority of the population, while the CFU of the tobramycin-resistant subpopulation continued to increase. This indicates that the tobramycin-resistant subpopulation is distinct from persisters, which would be unable to replicate in the presence of drug. (C) Increased tobramycin resistance is unstable in liquid cultures. Following exposure of AB5075 to 64 μg/ml tobramycin, the majority of the surviving population exhibited increased tobramycin resistance. However, when tobramycin selection was removed, most of the cells in the population lost their increased resistance. (D) Twenty-four-hour stability of the tobramycin resistance phenotype in colonies was examined using stocks of hetR-O2 and hetR-T1. Colonies were plated from stock onto plates supplemented with tobramycin. Individual colonies were picked and passaged onto plates with 40 μg/ml tobramycin or with no tobramycin. After 24 h of growth, individual colonies were resuspended and the percentages of resistant cells within each colony were determined. Data represent the means and standard errors of means for four (hetR-T1) or three (hetR-O2) independent replicates. (E) Forty-eight-hour stability of the tobramycin resistance phenotype was assessed for stocks of hetR-O2 and hetR-T1. The experiment was performed as described for panel D but with colonies incubated on plates with and without 40 μg/ml of tobramycin for 48 h. Data represent the means and standard errors of means for two independent replicates.

that if an amplification event were occurring, it would be within the interval flanked by the *intI* genes, possibly facilitated by recombination between the *intI* genes on adjacent plasmids during DNA replication. Gene copy number was measured by quantitative PCR (qPCR) using genomic DNA (gDNA) from the hetR-O2 and hetR-T1 isolates, as well

A.

B.
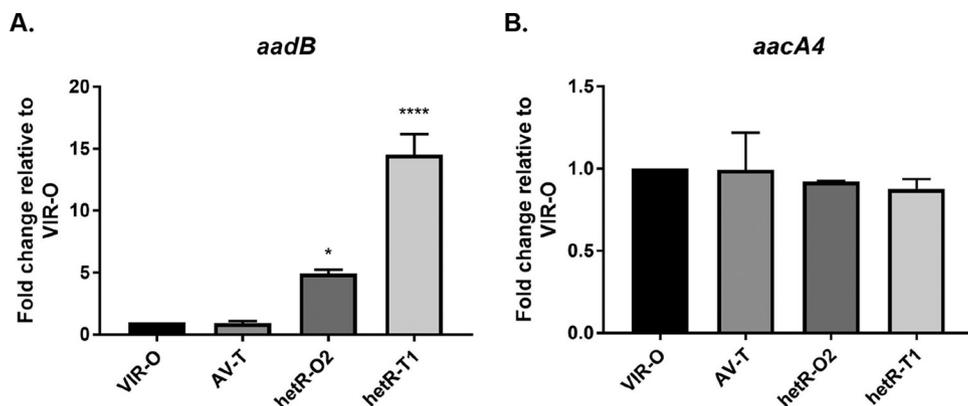
FIG 3 Increased expression of *aadB* in cells with increased tobramycin resistance. Expression of aminoglycoside resistance genes *aadB* (A) and *aacA4* (B) was quantified by qRT-PCR. Data are presented as the averages and standard errors of means of three independent biological replicates (*, $P < 0.05$, and ****, $P < 0.0001$, relative to AB5075 VIR-O by one-way analysis of variance with Dunnett's posttest).

as gDNA generated from VIR-O cells not exposed to tobramycin. The relative copy numbers of the *strB* and *aadB* genes (located immediately inside the interval flanked by *intI*) and the *ABUW_4052* and *ABUW_RS19335* genes (located immediately outside the *intI* region) were normalized to levels of *aacA4*, which is carried outside this region and served as a control for changes in plasmid copy number. In both the hetR-O2 and hetR-T1 isolates, the *strB* and *aadB* genes were highly amplified, whereas the *ABUW_4052* and *ABUW_RS19335* genes were not amplified (Table 2). As presented previously in Fig. 2D and E, cells lost tobramycin resistance when grown in the absence of antibiotic. Consistent with this loss of resistance, the levels of *aadB* amplification were reduced in colonies at 24 h in the absence of selection (Table 3). These results strongly suggest that tobramycin heteroresistance in AB5075 is due to the amplification of *aadB*.

To confirm the role of *aadB* in heteroresistance, we obtained three independent *aadB*::T26 transposon insertion mutants from the University of Washington library. However, PCR analysis of the mutants revealed that all three had two copies of *aadB*, a wild-type copy and a T26-disrupted copy (data not shown). Similarly, in our wild-type VIR-O AB5075 parent strain, qPCR analysis indicated that there were two copies of *strB* and *aadB* relative to the *aacA4* gene (Table 2), which is in contrast to the published genome sequence, where a single copy of each gene is present (18). Southern blot analysis confirmed that the region between the *intI* genes was duplicated in our AB5075 parental strain (data not shown). Due to the duplication of *aadB* in our parental strain and the presence of a wild-type copy of *aadB* in the University of Washington library mutants, we have been unable to construct and test a defined *aadB* mutant.

Tobramycin heteroresistance can occur by RecA-dependent and -independent mechanisms. To determine if the above amplification event between duplicated copies of the *intI* gene required homologous recombination, tobramycin heteroresistance was examined in a *recA*::*Tc* mutant. The *recA*::*Tc* mutant exhibited intrinsic levels of tobramycin resistance that were lower than the wild-type parent (Fig. 1B and Table 4). Although this strain still appeared heteroresistant, the frequency of tobramycin-resistant
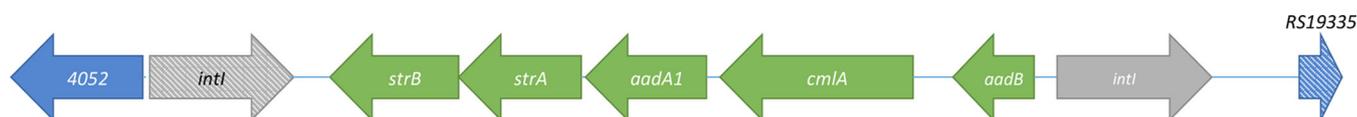
FIG 4 Amplified region in p1AB5075 that includes *aadB*. The genes surrounding *aadB* on p1AB5075 are shown. Gene annotations are based on the most recent sequence annotation of p1AB5075 available on NCBI (accession NZ_CP008707.1); some small annotated open reading frames are not shown. Based on qPCR results, open reading frames annotated in green were amplified in the tobramycin-resistant subpopulation, whereas open reading frames annotated in blue were not changed. The two integrase genes annotated in gray were not examined by qPCR.

TABLE 2 hetR-O2 and hetR-T1 exhibit gene amplifications that include *aadB*

Strain	Relative copy no. (mean ± SD) of gene[a]:			
	ABUW_4052	strB	aadB	ABUW_RS19335
VIR-O	1.31 ± 0.09	2.94 ± 0.07	2.45 ± 0.06	0.72 ± 0.01
hetR-O2	1.80 ± 0.53	20.26 ± 6.18*	17.23 ± 6.80*	0.83 ± 0.31
hetR-T1	1.44 ± 0.04	55.62 ± 9.27*	44.07 ± 8.26*	0.64 ± 0.03

[a]Relative copy number of genes surrounding *aadB* measured in three biological replicates. Relative copy numbers were measured by qPCR using normalization to *aacA4*, a presumed single-copy gene located on the same plasmid. Copy numbers of *aadB* and *strB* were significantly increased in hetR-O2 and hetR-T1 relative to VIR-O not exposed to tobramycin (*, $P < 0.001$ relative to VIR-O by two-way analysis of variance with Dunnett's posttest).

colonies arising in the zone of clearing was lower than in wild-type cells (Fig. 1B). Introduction of the wild-type *recA* gene into the *recA*::Tc mutant partially restored heteroresistance (see Fig. S1 in the supplemental material). Interestingly, when six tobramycin-resistant isolates from the *recA*::Tc mutant were tested for amplification of *aadB*, the copy number was similar to the *recA*::Tc parent strain (Table 4). This indicates that increased resistance arose in these isolates by a mechanism that did not involve *aadB* amplification. When three of these resistant isolates, 1-2, 1-4, and 1-10, were cultured for approximately 30 generations in the absence of tobramycin, the frequency of cells retaining tobramycin resistance was 11%, 23%, and 35%, respectively, demonstrating that these isolates are not stable mutants. The resistant subpopulation in the *recA*::Tc mutant consisted of both VIR-O and AV-T cells (data not shown).

To confirm that the tobramycin-resistant subpopulation in the *recA*::Tc mutant was capable of growing in the presence of antibiotic, a tobramycin killing assay was conducted (Fig. S2A). This indicated that the tobramycin-resistant subpopulation was capable of growing in the presence of drug and was not composed of persister cells. In addition, PAP analysis demonstrated that pretreatment with tobramycin did not alter the frequency of cells that became resistant to tobramycin in the *recA*::Tc background (Fig. S2B).

DISCUSSION

This study demonstrates that subpopulations of *A. baumannii* AB5075 exhibit heterogeneous levels of resistance to aminoglycosides. The subpopulation with increased tobramycin resistance can be visualized using both an Etest MIC assay and PAP analysis. Application of selective concentrations of tobramycin resulted in the outgrowth of the tobramycin-resistant subpopulation, allowing it to dominate the surviving culture. However, once selective pressure was removed, the majority of the subpopulation returned to baseline levels of resistance. Independent isolates from the resistant subpopulation were found to exhibit increased expression and amplification of the 2″-aminoglycoside nucleotidyltransferase gene *aadB*. This gene is likely involved in heteroresistance as the substrate profile of this enzyme matches the resistance profile observed, i.e., resistance to tobramycin and gentamicin but not amikacin.

The role of phenotypic heterogeneity in the formation of subpopulations that are able to survive antibiotic treatment is well appreciated. This has been best studied in

TABLE 3 *aadB* copy number in the presence and absence of antibiotic selection

Strain	Copy no. (mean ± SD) of gene[a]:	
	aadB	ABUW_RS19335
hetR-O2 + Tob	40.35 ± 0.62	0.72 ± 0.16
hetR-O2 − Tob	16.75 ± 0.16*	0.71 ± 0.03
hetR-T1 + Tob	55.12 ± 4.35	0.72 ± 0.05
hetR-T1 − Tob	40.38 ± 10.71	0.77 ± 0.08

[a]Copy numbers of *aadB* and *ABUW_RS19335* relative to *aacA4* in hetR-O2 and hetR-T1 colonies grown with (+) or without (−) tobramycin for 24 h. The means and standard deviations of two biological replicates are shown. The copy number of *aadB* was significantly decreased in hetR-O2 grown without tobramycin compared to this strain grown in the presence of drug (*, $P < 0.05$ by paired two-tailed t test).

TABLE 4 Relative copy number of *aadB* in *recA*::Tc isolates with increased tobramycin resistance

Strain	*aadB* relative copy no.[b]	MIC (μg/ml)
recA::Tc	2.49[a]	8
hetR-*recA*-1-1	3.34	16
hetR-*recA*-1-2	3.15	64
hetR-*recA*-1-3	2.85	16
hetR-*recA*-1-4	2.64	32
hetR-*recA*-1-5	2.63	16
hetR-*recA*-1-10	2.72	96

[a]The *recA*::Tc control sample was used as a control in three independent experiments; the value shown represents the average relative copy number across the three experiments.
[b]Relative copy number of *aadB* normalized to *aacA4* was measured using gDNA isolated from a *recA* transposon mutant and 6 independently isolated derivatives with increased tobramycin resistance. Data shown are from a single sample for each strain. None of the tobramycin-resistant *recA*::Tc isolates exhibit increased copy number of *aadB*, suggesting that gene amplification of *aadB* in wild-type cells is RecA dependent.

the case of dormant persister cells and slow-growing small-colony variants (SCVs), both of which exhibit increased tolerance to antibiotics, including aminoglycosides (26–29). Both persistence and SCV formation confer antibiotic tolerance at the expense of normal growth. In contrast, heteroresistance involves the formation of a subpopulation of cells with increased antibiotic resistance, which maintain the ability to actively grow during antibiotic exposure (1). Although heteroresistance has been reported in many species, including *A. baumannii* (30, 31), reports of aminoglycoside heteroresistance are exceedingly rare. This study is the first report of aminoglycoside heteroresistance in *A. baumannii* and to our knowledge only the second definitive report of this phenomenon in any species. In *Salmonella enterica*, aminoglycoside heteroresistance has been shown to occur when a subpopulation of cells expresses decreased levels of the porin gene *ompC*. This limits uptake of kanamycin into the cells, causing increased resistance (11).

This study demonstrates that tobramycin heteroresistance can arise by the extensive amplification (20 to 40 copies) of a region carried on the large p1AB5075 plasmid that includes five resistance genes in tandem, including *aadB*, flanked by copies of an integrase (*intI*) gene. The mechanism by which the *aadB*-containing region gets extensively amplified is unclear, but our work has established that RecA is required. Duplications can occur by nonequal recombination between directly repeated regions on adjacent replicons (32). Gene amplification of the aminoglycoside-modifying enzyme gene *aphA1* has previously been reported to cause unstable tobramycin resistance in *A. baumannii* AB0057 and a clinical isolate (16). However, in that study, amplifications were dependent on prior exposure to tobramycin and were likely selected for by the antibiotic. Heteroresistance resulting from gene amplification of a chromosomal locus has also been reported in *Salmonella enterica*, where amplification of a region containing *pmrD* conferred colistin heteroresistance (33). Our data suggest that extensive amplifications preexist in 1/200 cells (i.e., the frequency of the resistant subpopulation) in the absence of any selective tobramycin pressure. As extensive gene amplifications typically require growth in the presence of selective pressures, this suggests that additional mechanisms, such as the rolling-circle-dependent generation of tandem arrays, may contribute to amplification of this region in *A. baumannii* (32). In a *recA*::Tc mutant, cells with increased resistance still arose, but none of the isolates examined contained duplications of the *aadB* gene (Table 4). Therefore, heteroresistance can occur by at least one additional mechanism. A recent study by Gallagher et al. demonstrated that at least 32 chromosomally carried genes in AB5075 function to maintain intrinsic tobramycin resistance (19). In principle, amplification of any of these genes could potentially lead to increased tobramycin resistance and may account for the resistant subpopulation that does not contain amplification of *aadB*. However, if this amplification is occurring, it does not appear to require RecA.

When the stability of the tobramycin-resistant subpopulation was examined, con-

trasting results were found for the virulent opaque (VIR-O) and avirulent translucent (AV-T) isolates (Fig. 2D). As long as selection was maintained, the majority of hetR-O2 cells remained tobramycin resistant; however, resistance was lost in approximately 90% of the population in colonies grown without drug for 24 h. In contrast, cells of hetR-T1 lost their increased resistance at roughly the same rate regardless of whether selection was maintained, with approximately 50% of the population in a 24-h colony maintaining the increased resistance state. However, in both isolates the loss of heteroresistance at high rates is consistent with the unstable nature of extensive duplications (32). The increased stability of heteroresistance in hetR-T1 in the absence of selection may be due to the larger number of tandem repeats that includes *aadB* (Table 2).

The clinical relevance of the aminoglycoside heteroresistance phenomenon described here remains to be determined. The plasmid-borne *aadB* gene is common in *A. baumannii*, and strains carrying this gene should exhibit clinically relevant resistance to tobramycin and gentamicin (34–37). Further research is needed to determine whether *A. baumannii* strains identified as being aminoglycoside sensitive also exhibit heteroresistance, which could pose problems for appropriately treating these infections. It is possible that strains lacking *aadB* could still exhibit heteroresistance by an *aadB*-independent mechanism, similarly to the *recA::Tc* mutant discussed in this work. The data presented here illustrate that the full picture of antibiotic resistance in *A. baumannii* is more complicated than has been traditionally recognized, with both the acquisition of resistance determinants and phenotypic heterogeneity contributing to resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *A. baumannii* were maintained at −80°C in 15% glycerol. Pure stocks of opaque and translucent variants were prepared as previously described (23). Liquid cultures were prepared in sterile LB broth, supplemented as needed with tobramycin (Sigma-Aldrich, St. Louis, MO) at the concentrations indicated. Resistance stability experiments and experiments to select isolates with increased resistance were performed using 0.5× LB supplemented with 0.8% agar. All other experiments were performed using regular LB supplemented with 1.5% agar. Plates were supplemented with tobramycin as indicated.

A T26 insertion mutant in *recA* was obtained from the AB5075 transposon mutant library maintained at the University of Washington (18). A culture of this strain was grown overnight at 37°C with shaking and used to prepared genomic DNA (gDNA) as outlined below. A culture of VIR-O AB5075 was grown at 37°C with shaking to late log phase and used to prepare electrocompetent cells by washing three times with 10% glycerol. The *recA::T26* mutant DNA was electroporated into these cells, and transformants were selected on LB supplemented with 10 μg/ml tetracycline. A single colony was isolated and designated the *recA::Tc* strain. The presence of the *recA* mutation was confirmed by PCR.

Isolation of tobramycin-resistant subpopulations was conducted by plating serial dilutions of an early-log-phase culture of the wild-type or *recA::Tc* VIR-O variant on 0.5× LB and 0.5× LB containing tobramycin (Sigma-Aldrich, St. Louis, MO) at 2.5, 5, 10, 15, 20, 25, and 30 μg/ml. Colonies exhibiting increased resistance were apparent at 15 μg/ml tobramycin for wild type and at 5 μg/ml for the *recA::Tc* mutant. Resistant colonies were restreaked on 0.5× LB with tobramycin and examined under a stereomicroscope with oblique lighting to determine whether they were opaque or translucent variants.

Oligonucleotides. All oligonucleotides used in this study are listed in Table S1 in the supplemental material.

Complementation of the *recA* mutant. The wild-type *recA* gene was amplified by PCR using the primers oSA77 (5′-GCTCATCGTTTCGTTTGAAC-3′) and oSA78 (5′-GAATAAAAACGTCGAGTTGTG-3′) (Table S1). This fragment was then cloned into the SmaI site of pQF1266Blue, a derivative of pQF50 (38) where a hygromycin resistance gene has been cloned into the *bla* gene encoding β-lactamase. In addition, this plasmid contains an origin of replication from pWH1266 (39) cloned into the NcoI site. The resulting plasmid was designated p*recA*.

MIC assays. MICs of different antibiotics were measured using Etest strips (bioMérieux, Marcy-l'Etoile, France). For the tobramycin MICs used to visualize heteroresistance, AB5075 wild-type or *recA::Tc* VIR-O cells were inoculated into LB broth and grown at 37°C with shaking to a concentration of 1.1×10^7 to 1.3×10^7 CFU/ml. Lawns were inoculated by spreading 100 μl of culture onto an LB plate, followed by application of the Etest strip. The plate was photographed following incubation for 16 h at 37°C.

For MIC experiments reported in Table 1, strains were inoculated into LB broth, grown overnight statically at room temperature, grown at 37°C with shaking to an optical density at 600 nm (OD_{600}) of 0.1, and stored at 4°C for use later in the day. Etest strips were placed on LB plates, and strains were inoculated next to each strip by spotting 10 μl of culture next to the bottom of the strip, tilting the plate so that the culture spread up the side of the strip, and removing excess culture at the top of the strip by pipetting. Two strains were inoculated on each side of each strip to facilitate a direct comparison of

susceptibility. MIC values were recorded after incubation for 5 h or 16 h at 37°C, as noted. MIC experiments were performed two independent times to confirm the reproducibility of trends.

For the recA::Tc mutant, colonies that were growing in the zone of tobramycin inhibition were placed into a small vial of 20% glycerol and stored at −80°C. MIC experiments were performed by growing cells from the −80°C glycerol stock for several hours in LB and performing Etest assays as outlined above for the data presented in Table 1.

PAP. Population analysis profile (PAP) analysis was performed by growing bacteria overnight to stationary phase and then plating serial dilutions on LB agar with or without various concentrations of tobramycin (Spectrum, New Brunswick, NJ). Plates were then incubated at 37°C, and CFU were enumerated after 24 h. Percent tobramycin resistance was calculated as the number of bacterial colonies that grew on tobramycin plates divided by the number of bacteria that grew on LB alone without drug.

Tobramycin killing assays. Briefly, AB5075 was grown overnight to stationary phase in LB medium and serially diluted to 1×10^6 CFU/ml. Tobramycin (Spectrum) was added at a concentration of 64 μg/ml. One-hundred-microliter aliquots were taken at desired time points, serially diluted, and plated on LB medium alone (to quantify total CFU) or LB plates containing 64 μg/ml tobramycin (to quantify resistant CFU).

Stability measurements of the resistant subpopulation. For experiments conducted with broth cultures, AB5075 was grown overnight to stationary phase in LB medium. The bacteria were then serially diluted and plated on LB agar plates with and without 64 μg/ml tobramycin (Spectrum) to enumerate total and resistant CFU for the pretreatment group (day 1). A subculture (1:1,000) was then grown overnight in LB supplemented with 64 μg/ml tobramycin, serially diluted, and plated on LB agar with or without 64 μg/ml tobramycin to enumerate total and resistant CFU for the treatment group (day 2). This process was repeated in LB broth without antibiotics (day 3), and dilutions were plated on agar plates with and without 64 μg/ml tobramycin to enumerate total and resistant CFU.

For experiments with hetR-O2 and hetR-T1 colonies, cells from the two subpopulations were struck from freezer stocks onto 0.5× LB supplemented with 20 μg/ml tobramycin (Sigma-Aldrich). Plates were incubated overnight at 37°C, and single colonies were resuspended in 1 ml LB and struck onto 0.5× LB agar with and without 40 μg/ml tobramycin. After 24 h or 48 h of incubation at 37°C, individual colonies were resuspended in 1 ml LB and serial dilutions were plated in duplicate on 0.5× LB with and without 40 μg/ml tobramycin. Plates were incubated for up to 48 h at 37°C, and colonies were enumerated to determine the percent tobramycin resistance by comparing the CFU on plates with and without tobramycin.

RNA isolation. Cultures of different A. baumannii AB5075 subpopulations were grown in LB medium at 37°C with shaking to an OD_{600} of 0.5. Cells were pelleted by centrifugation, and RNA was isolated using the MasterPure RNA purification kit according to the manufacturer's protocol (Epicentre, Madison, WI). Contaminating DNA was degraded by two treatments with Turbo DNA-free according to the manufacturer's protocol (Invitrogen, Waltham, MA). DNA contamination was evaluated by PCR with purified RNA as the template, and RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer.

qRT-PCR. Total RNA (1 μg) was used to prepare cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) with random primers and either iScript or SuperScript III (Invitrogen, Waltham, MA) reverse transcriptase. Reactions with mixtures lacking reverse transcriptase were also performed as a control for the presence of contaminating DNA. Incubation conditions for cDNA synthesis were 25°C for 5 min, 42°C for 45 min, and 85°C for 5 min. cDNA reaction mixtures and controls were then diluted 1:10 with sterile water and used as a template for reverse transcriptase quantitative PCR (qRT-PCR). Oligonucleotide primer pairs for qRT-PCR were designed to amplify approximately 150-bp fragments from each gene of interest and were generated using the Primer-BLAST program available at https://www.ncbi.nlm.nih.gov/tools/primer-blast. qRT-PCR was performed using iQ Sybr green Supermix (Bio-Rad, Hercules, CA) on a Bio-Rad CFX Connect cycler. Cycle parameters were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 20 s. Melt curve data were then collected to confirm the specificity of the oligonucleotide primer pairs. Data were generated using cDNA prepared from three independent RNA isolations, and qRT-PCRs were performed in technical triplicate to ensure accuracy. Fold changes in gene expression relative to the control strain (VIR-O) and a control gene (clpX) were determined using the threshold cycle ($2^{-\Delta\Delta C_T}$) method (40).

Genomic DNA isolation. For experiments presented in Table 2, cultures of different AB5075 subpopulations were grown in LB medium with shaking at 37°C to an OD_{600} of 0.5. For experiments presented in Table 3, colonies were grown for 24 h on 0.5× LB with or without 40 μg/ml tobramycin; individual colonies were then resuspended in 1 ml of LB. In both cases, cells were pelleted by centrifugation and resuspended in Tris-EDTA (TE). Cells were lysed by incubation with 0.5% SDS and 400 μg/ml proteinase K for 1 h at 37°C. Following lysis, NaCl was added to a final concentration of 0.7 M, and DNA was extracted twice with equal volumes of phenol-chloroform-isoamyl alcohol. DNA was precipitated by mixing with 1.5 volumes of 95% ethanol until a precipitate formed. DNA pellets were collected by centrifugation, washed twice with 75% ethanol, dried, and resuspended in molecular-grade water.

qPCR. Concentrations of gDNA samples were determined using a NanoDrop ND-100 spectrophotometer. Samples were diluted to a concentration of 15 μg/ml, and qPCR was performed as outlined above for cDNA samples. Standard curves using 10-fold serial dilutions of wild-type gDNA were used to ensure that primers exhibited similar efficiencies. Relative gene copy numbers normalized to aacA4 were determined using the equation $2^{-\Delta C_T}$ (40).

Statistical analyses. Statistical analyses were performed with Prism 7 (GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00271-18.

FIG S1, PDF file, 0.5 MB.

FIG S2, PDF file, 0.1 MB.

TABLE S1, PDF file, 0.1 MB.

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