Amplification of Aminoglycoside Resistance Gene *aphA1* in *Acinetobacter baumannii* Results in Tobramycin Therapy Failure

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ABSTRACT Gene amplification is believed to play an important role in antibiotic resistance but has been rarely documented in clinical settings because of its unstable nature. We report a rise in MICs from 0.5 to 16 µg/ml in successive *Acinetobacter baumannii* isolated over 4 days from a patient being treated with tobramycin for an infection by multidrug-resistant *A. baumannii*, resulting in therapeutic failure. Isolates were characterized by whole-genome sequencing, real-time and reverse transcriptase PCR, and growth assays to determine the mechanism of tobramycin resistance and its fitness cost. Tobramycin resistance was associated with two amplification events of different chromosomal fragments containing the *aphA1* aminoglycoside resistance gene part of transposon Tn*6020*. The first amplification event involved low amplification (6 to 10 copies) of a large DNA fragment that was unstable and conferred tobramycin MICs of ≤8 µg/ml. The second event involved moderate (10 to 30 copies) or high (40 to 110 copies) amplification of Tn*6020*. High copy numbers were associated with tobramycin MICs of 16 µg/ml, impaired fitness, and genetic instability, whereas lower copy numbers resulted in tobramycin MICs of ≤8 µg/ml and no fitness cost and were stably maintained *in vitro*. Exposure *in vitro* to tobramycin of the initial susceptible isolate and of the *A. baumannii* AB0057 reference strain led to similar *aphA1* amplifications and elevated tobramycin MICs. To the best of our knowledge, this is the first report of *in vivo* development of antibiotic resistance secondary to gene amplifications resulting in therapy failure.

IMPORTANCE A combination of whole-genome sequencing and mapping were used to detect an antibiotic resistance mechanism, gene amplification, which has been presumed for a long time to be of major importance but has rarely been reported in clinical settings because of its unstable nature. Two gene amplification events in a patient with an *Acinetobacter baumannii* infection treated with tobramycin were identified. One gene amplification event led to high levels of resistance and was rapidly reversible, while the second event led to low and more stable resistance since it incurred low fitness cost on the host. Gene amplification, with an associated rise in tobramycin MICs, could be readily reproduced *in vitro* from initially susceptible strains exposed to increasing concentrations of tobramycin, suggesting that gene amplification in *A. baumannii* may be a more common mechanism than currently believed. This report underscores the importance of rapid molecular techniques for surveillance of drug resistance.

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*A*cinetobacter baumannii has a remarkable ability to develop resistance to antibiotics by horizontal acquisition of foreign genetic information (1) or by mutation (2). These mechanisms have made *A. baumannii* a highly successful human nosocomial pathogen (3). In the clinical setting, point mutations in two-component regulatory systems can result in multiple antibiotic resistance in a single step by overexpression of efflux pumps (4) or resistance to colistin, the last resort antibiotic against this species (5).

Despite moderate side effects and increasing resistance, the aminoglycosides continue to be of therapeutic value. Several mechanisms of aminoglycoside resistance have been observed in

Acinetobacter spp., the most frequent involving drug inactivation by *N*-acetylation, *O*-adenylylation, or *O*-phosphorylation (6).

Resistance genes in *A. baumannii* are generally part of integrons or resistance islands (AbaR) (7–9). The first island described, AbaR1, includes a large array of laterally transferred antimicrobial resistance genes intermixed with a diverse set of transposons and integrons (10). Since then, truncated derivatives of AbaR3, the ancestral resistance island, all integrated at the same site in the c*omM* ATPase gene, have been reported. They are the result of 5′-ward deletions mediated by IS*26* (11, 12).

Gene duplication and amplification (GDA) are believed to play an important role in antibiotic resistance (13). However, because

TABLE 1 Phenotypic characteristics of strains

MRSN[a]	Isolation		Tobramycin MIC (μg/ml)[c]
	Day[b]	Site	
3361	3	Left hip	0.5
3363	3	Right hip	0.5
3364	8	Left hip	0.5
56	11	Left hip	0.5
57	12	Right hip	8
58	13	Left hip	16

[a] De-identified number used to track isolates throughout the Multidrug-resistant Organism Repository and Surveillance Network (MRSN).

[b] Day after initial injury.

[c] MICs were determined in quintuplicate. *A. baumannii* CLSI breakpoints (μg/ml) for tobramycin are as follows: susceptible, $\leq$4; intermediate, 8; resistant, $\geq$16.

of its unstable nature, its relevance in the clinical setting is difficult to assess. Nevertheless, duplication of a 36.4-kb region encompassing bla_{SHV-11} in a clinical isolate of *Klebsiella pneumoniae* linked to a 16-fold increase in amoxicillin resistance has been reported (14). Similarly, resistance to sulfonamide and trimethoprim was associated in a clinical isolate of *Streptococcus agalactiae* with the unstable amplification of a 13.5-kb region carrying five genes involved in dihydrofolate synthesis (15). Duplication of the plasmid-borne bla_{OXA-58} gene has been shown to be responsible for reduced susceptibility to carbapenems in *A. baumannii* (16) and *Acinetobacter genospecies 3* (17).

We report the *in vivo* occurrence of tobramycin resistance in *A. baumannii* from a patient undergoing tobramycin therapy via gene amplification. Tobramycin resistance emerged by two distinct amplification events. The first event involved low amplification of an approximately 15-kb region containing the aminoglycoside resistance gene *aphA1*, which is part of transposon Tn*6020*.

The second event was moderate- or high-level amplification of Tn*6020*. In both cases, tobramycin MICs increased with gene copy number up to a plateau at 16 μg/ml. Very high gene copy numbers (>40) resulted in reduced fitness of the host and were transient in the absence of selection, as opposed to low copy numbers which could be maintained *in vitro* for at least 1 month.

RESULTS

Six *A. baumannii* strains were isolated from the same patient over 10 days and were tested for susceptibility to three clinically important aminoglycosides (Table 1). All were resistant to gentamicin and susceptible to amikacin. Initial isolates (MRSN 3361, MRSN 3363, MRSN 3364, and MRSN 56) were susceptible to tobramycin, but MRSN 57 and MRSN 58 were categorized as intermediate (MIC = 8 μg/ml) and resistant (MIC = 16 μg/ml), respectively. Multilocus sequence typing (MLST) assigned them to sequence type 1 (ST1) (Pasteur scheme) of clonal complex CCI, one of three major *A. baumannii* clonal groups strongly associated with multidrug resistance and hospital outbreaks in many countries (18).

Detection of gene amplification by whole-genome mapping. Whole-genome mapping showed that all isolates were closely related but that MRSN 57 and MRSN 58 had diverged from the main cluster (Fig. 1). This difference was attributable to a localized and unique restriction pattern that was not present in the other four strains. In MRSN 57, this pattern consisted of six identical and tandem restriction fragments approximately 15 kb in size and in MRSN 58 of a single approximately 270-kb region with no NcoI restriction sites. When these fragments were artificially removed, both isolates shared >99% identity with the remaining strains.

Whole-genome sequencing of clinical isolates. Comparative analysis of the genomes of MRSN 56, MRSN 57, and MRSN 58 clinical isolates and reference strain AB0057 identified AbaR28
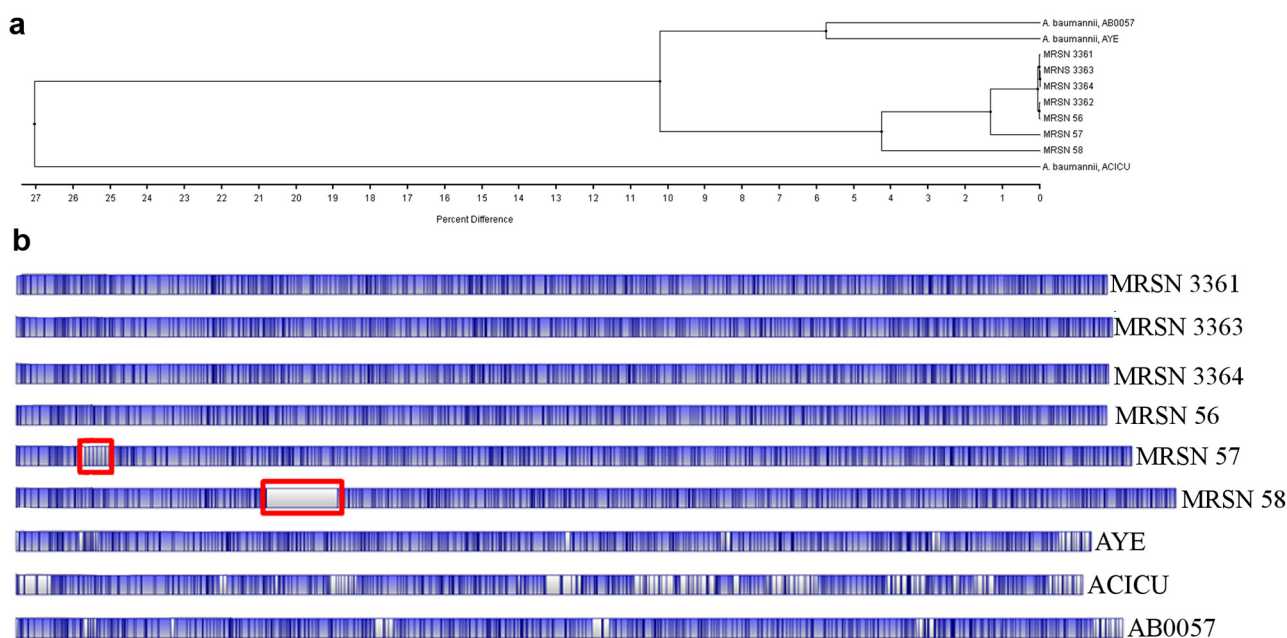

FIG 1 Dendrogram (a) and optical genome map (b) of clinical isolates of *A. baumannii* and reference strains. Strain relatedness was calculated using the unweighted-pair group method using average linkages (UPGMA) (5). Optical maps were compared with *in silico* optical maps of *A. baumannii* reference strains AB0057, AYE, and ACICU generated from GenBank sequences. Blue areas represent regions of alignment compared to isolate MRSN 3361, and white areas represent unaligned regions. Vertical black bars indicate NcoI restriction sites. The areas corresponding to the amplified regions are enclosed in red rectangles.
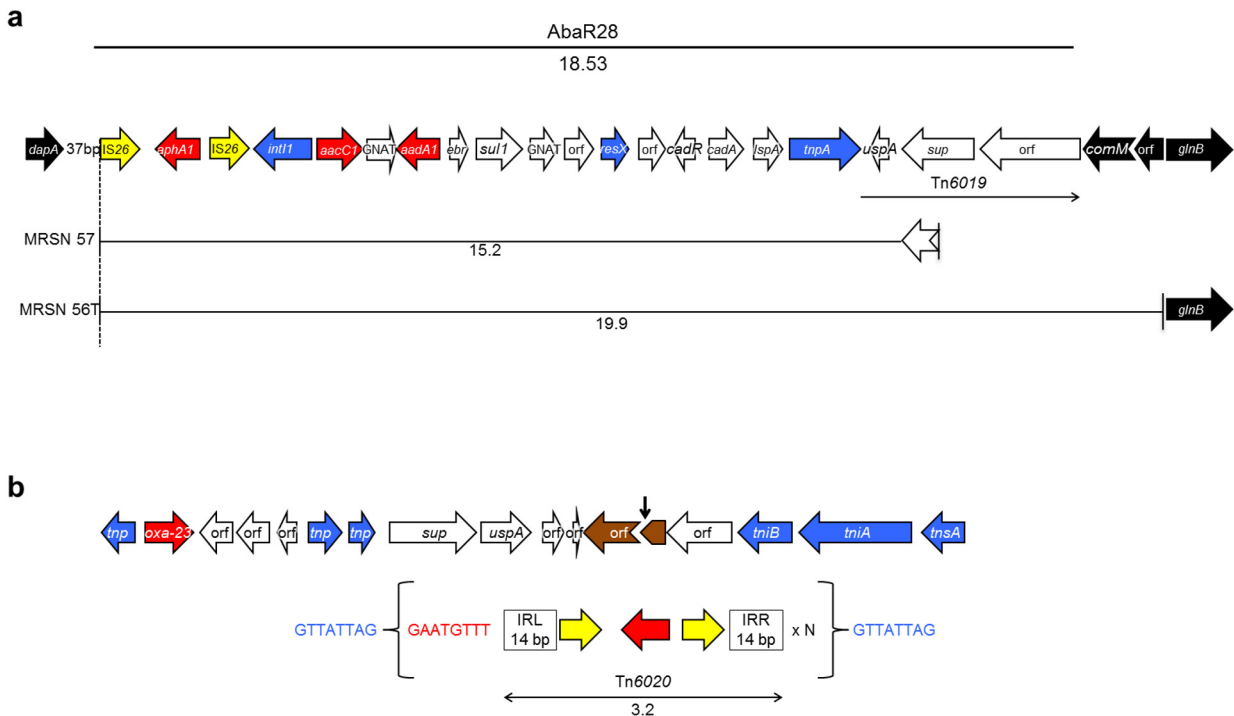
FIG 2 Schematic representation of AbaR28 and amplified units (a) and the genetic environment of Tn6020 (top) and the amplified unit (bottom) in *A. baumannii* isolate MRSN 58 (b). (a) Resistance island AbaR28 is indicated by a thick black line at the top of the panel. Amplified units are indicated by thin black lines. The sizes of the resistance island or amplified units are in kilobases. The open arrows represent coding sequences (yellow arrows, IS26; red arrows, aminoglycoside resistance genes; blue arrows, genes associated with DNA mobility) and indicate the direction of transcription. The notched arrows represent the truncated *comM* and *sup* genes. The chromosomal genes flanking AbaR28 are indicated in black. AbaR28 is a truncated version of AbaR3 generated by a 5′-ward deletion mediated by the IS26 copy located at the left end of Tn6020 which is now the extremity of the island. This element is known to create deletions in adjacent regions (20, 37). The deletion removed the 5′ end of Tn6019 and the 5′ portion of the *comM* target gene. The right end of AbaR28 corresponds to the 26-bp terminal right inverted repeat (IRR) of Tn6019, known to be associated with AbaRs (arrow) (11), and flanked by a 5-bp (ACCGC) duplication of target DNA (5 bp), the characteristic footprint of AbaR3 insertions (20). (b) The vertical black arrow indicates the insertion site of Tn6020 in isolate MRSN 58. The duplicated target sequence is in blue lettering and the sequence carried over 8 bp is in red. Tn6020 is indicated by a double-head arrow, and its size is shown in kilobases. Gene nomenclature was assigned based on the closest BLAST match from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed February 2014) using reference strain AB0057.

(Fig. 2a), a novel deletion derivative of AbaR3 (11, 19), in the three clinical isolates. AbaR28 consisted of 20 open reading frames (ORFs) identical to the 3′ end of AbaR3 in *A. baumannii* AB0057 (19). A truncated *comM*, a gene encoding an ATPase which is a hot spot for insertion of resistance islands in *A. baumannii* (9, 11), was present at the 3′ end of the island alongside the characteristic 5-bp duplication (AACGC) generated upon AbaR3 insertion (20). However, unlike other derivatives of AbaR3 (11), AbaR28 was missing the 5′ remnant of *comM*, most likely following a 5′-ward IS26-mediated deletion that extended 37 bp downstream from *dapA*, a chromosomal gene for a dihydrodipicolinate synthase (Fig. 2a).

Analysis of average genome read coverage identified one area in isolate MRSN 57 and one in MRSN 58 with significantly higher coverage than in MRSN 56. In MRSN 57, this area consisted of a 15.21-kb region, encompassing 19 of the 20 ORFs of AbaR28 (Fig. 2a), that had a mean depth coverage approximately 6 times higher than that of the corresponding region in MRSN 56 and MRSN 58 (see Fig. S1 in the supplemental material). IS26 formed the 5′ end, and the amplified unit terminated 332 bp into the sulfate permease gene (Fig. 2a). In isolate MRSN 58, amplification occurred in a smaller 3.9-kb region, comprising Tn6020 of AbaR28, with mean depth coverage approximately 65 times

higher than that of MRSN 56 and MRSN 57 (Fig. S1). Tn6020 is composed of *aphA1* flanked by two directly repeated copies of IS26 (Fig. 2b). In isolate MRSN 58, a single copy of the transposon was found at the original position, but approximately 65 tandem copies of Tn6020 were identified 281 kb downstream, inserted in a gene for a hypothetical protein (Fig. 2b). The presence of the characteristic 8-bp direct repeat of target DNA confirmed that IS26 mediated transposition at this new locus. Surprisingly, rather than direct tandem copies of Tn6020, each copy of the transposon was connected to the next by 8 bp identical to those upstream from Tn6020 at the original location (Fig. 2b). This short sequence (GAATGTTT) was not part of the unit amplified in MRSN 57. The genomes of MRSN 56, MRSN 57, and MRSN 58 were identical outside the amplified regions.

Confirmation of gene amplification and expression by qPCR. When normalized to the single-copy genes *secE* and *rpiN* (21) by quantitative PCR (qPCR), isolate MRSN 57 had an average of 10 (±2) copies of *aphA1*, *aacC1*, and *uspA* (encoding a universal stress protein at the 3′ end of the amplified unit) and MRSN 58 had an average of 75 (±14) copies of *aphA1* but a single copy of *aacC1* and *uspA* (see Fig. S3 in the supplemental material). Whole-genome mapping and qPCR demonstrated that *aphA1* copy numbers in 3 colonies of MRSN 58 from the same overnight culture
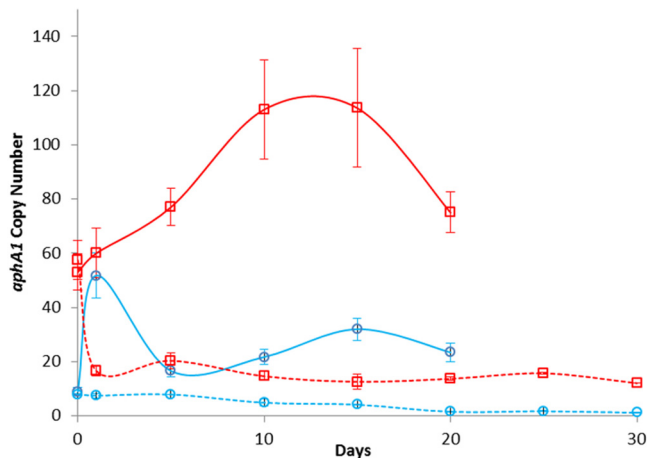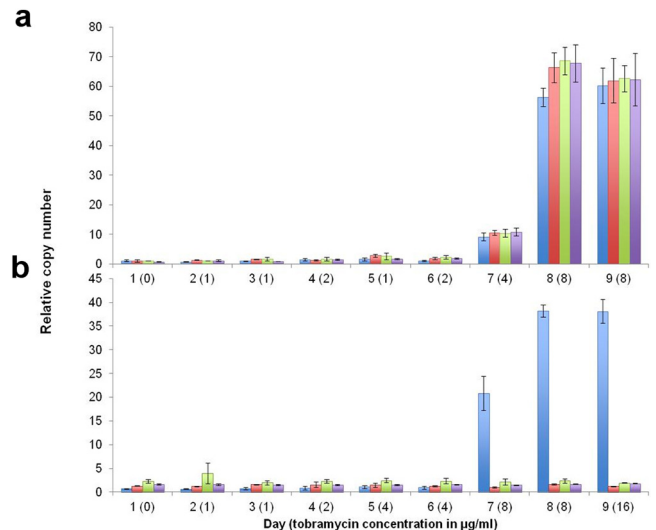
FIG 3 Relative gene copy numbers. Number of copies of *aphA1* in isolates MRSN 57 (blue line) and MRSN 58 (red line) as determined by qPCR during growth with (solid line) and without (dashed line) tobramycin (4 μg/ml). Data are the averages from three independent experiments; error bars represent 1 standard deviation.


FIG 4 In vitro induction of gene amplification. (a and b) Number of copies of *aphA1* (blue), *aacC1* (red), *uspA* (green), and *comM* (purple) genes as determined by qPCR in isolate MRSN 56 (a) and strain AB0057 (b) following growth on increasing concentrations of tobramycin. Data are the averages of three independent experiments; error bars represent 1 standard deviation. No growth was observed for MRSN 56 on 16 μg/ml of tobramycin.

were 60, 69, and 71, corresponding to approximate sizes of 259 kb, 278 kb, and 286 kb, respectively (Fig. S2).

Stability of gene amplification. When the bacteria were grown on antibiotic-free solid medium over 30 days, *aphA1*, *aacC1*, and *uspA* copy number (Fig. 3) and expression (see Fig. S3A in the supplemental material) displayed a gradual decrease from 10 copies in isolate MRSN 57. By day 17, a single copy of the genes was present and thereafter oscillated between 1 and 2, indicating that the 15.2-kb sequence was amplified or lost en bloc. When cultured with tobramycin (4 μg/ml), the number of amplification units and gene expression increased rapidly during the first 2 days from approximately 9 to 60, followed by a gradual decrease through day 6 to approximately 15 copies before rising to 30 copies by day 15 and stayed above 20 for the remainder of the exposure (Fig. 3 and Fig. S3A).

The *aphA1* copy number in strain MRSN 58 declined rapidly from 57 to 17 after the first overnight subculture on antibiotic-free medium and remained moderate with a daily average of 16 (±4) copies over the ensuing 28 days (Fig. 3). When grown in the presence of tobramycin (4 μg/ml), copy number (Fig. 3) and expression (see Fig. S3B in the supplemental material) increased rapidly, reaching 120 (±19) copies on day 10, before gradually declining to 78 (±9) copies by day 20. In all instances, mRNA synthesis was proportional to gene copy number.

Gene amplification can be induced *in vitro*. Tobramycin-susceptible *A. baumannii* MRSN 56 and AB0057 were subcultured daily in the presence of increasing concentrations of tobramycin. Isolate MRSN 56 displayed robust growth on 8 μg/ml tobramycin on days 8 and 9 but failed to grow on 16 μg/ml. This evolving phenotype correlated with a change in *aphA1*, *aacC1*, and *uspA* copy number (Fig. 4) and expression (data not shown), which increased moderately from 1 to 10 copies when grown on 4 μg/ml to approximately 70 copies on 8 μg/ml of tobramycin. However, unlike MRSN 57, the fragment downstream from *comM* was also amplified. Whole-genome sequencing of tobramycin-resistant MRSN 56 on day 8, named MRSN 56T, showed a 19.9-kb region with 30-fold-higher coverage than the remainder of the genome.

This DNA portion had the same 5′ end as that in MRSN 57 but extended 4 kb further in the 3′ direction encompassing all of AbaR28, truncated *comM*, a gene for a conserved hypothetical protein, and terminated 55 bp upstream from *glnB* encoding the nitrogen regulatory protein P-II (Fig. 2A).

Strain AB0057, which carries AbaR3 including Tn*6020*, eventually grew on 8 μg/ml after 7 days and moderately on day 9 on 16 μg/ml. Similarly to MRSN 58, increased resistance correlated with changes in *aphA1* copy number only, from 1 copy on day 6 to >70 copies on day 9 (Fig. 4). Whole-genome sequencing of the tobramycin-resistant AB0057 derivative on day 9, termed AB0057T, identified a 6.4-kb region with 50-fold-higher coverage than the rest of the genome, ca. twice the size of the unit in MRSN 58. This fragment encompassed Tn*6020* but extended 3 kb beyond the 5′ IS*26*, terminating in the *tnpA* gene (see Fig. S4 in the supplemental material). This gene is present in AbaR3 but has been deleted in AbaR28. Amplification occurred *in situ*, but we did not detect repeat sequences at the joint points.

High-level *aphA1* amplification results in reduced fitness. Whether an increase in gene copy number affects fitness can be evaluated by growth rate (GR) determination (22, 23). Initial data indicated that there were no significant differences between the growth rate of tobramycin-susceptible strain MRSN 56 and those of MRSN 57 and MRSN 58 when measured in the absence of antibiotic (the GR of MRSN 57 and MRSN 58 relative to that of MRSN 56 was 1.00 ± 0.02). However, individual colonies from the initial culture of MRSN 58 displayed variable *aphA1* amplification: among 18 colonies studied, 12 had an *aphA1* copy number between 8 and 13, and five had a copy number between 39 and 72. The GRs of the eighteen colonies were determined, and the same suspension of each colony was used to (i) determine the tobramycin MIC, (ii) prepare DNA to quantify *aphA1* copy number by qPCR, and (iii) purify RNA for quantification of *aphA1* expression by quantitative reverse transcriptase PCR (qRT-PCR). The
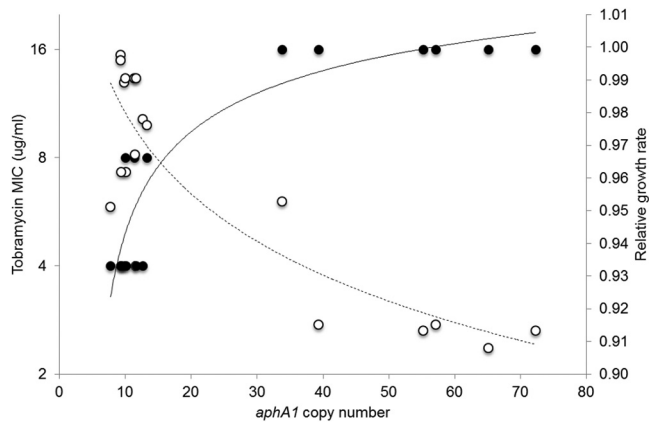
FIG 5 Biological cost and resistance associated with gene amplification. Tobramycin MICs (filled circles) and relative growth rates (open circles) of individual colonies of isolate MRSN 58 containing various copy numbers of the *aphA1* gene. Eighteen individual colonies were grown at 37°C up to an OD_{600} of 0.9 in BHI broth, and the growth rates and tobramycin MICs by microdilution were determined. The same bacterial suspension was used to purify DNA for *aphA1* gene copy number determination by qPCR. Experiments were performed at least three times independently. The logarithmic tendency curves have R^2 values of 0.9 (solid line) and 0.8 (dashed line).

level of mRNA production was proportional to gene copy number (see Fig. S3 in the supplemental material).

The group of 12 colonies with low *aphA1* copy numbers (from 8 to 13) had tobramycin MICs of 4 to 8 µg/ml and a relative GR of ≥0.95. The five colonies with high *aphA1* copy numbers (39 to 72) had tobramycin MICs of 16 µg/ml and a significantly diminished relative GR of ≤0.92 ($P < 0.001$). Interestingly, a colony with an intermediate *aphA1* copy number ($n = 34$) presented with an intermediate loss of fitness (relative GR of 0.95) and a tobramycin MIC of 16 µg/ml (Fig. 5).

Altogether, there was a significant decrease in the growth rate of cells with high copy numbers of *aphA1*, whereas there was no detectable burden for the cells with an average of 10 copies of the gene, which was the predominant group in strain MRSN 58. The latter genotype was stable over a period of 30 days even in the absence of selection and incurred little or no fitness cost.

DISCUSSION

Collection of serial *A. baumannii* isolates from the same patient provided the opportunity to investigate antibiotic resistance by two independent but related events involving amplification of the aminoglycoside phosphotransferase gene *aphA1*. The first event was moderate amplification of a 15.2-kb region, including transposon Tn*6020*, composed of *aphA1* flanked by two directly repeated copies of IS*26*, and the second event was replicative transposition and subsequent amplification of Tn*6020* (Fig. 2). Tobramycin MICs (Table 1) and gene transcription (see Fig. S3 in the supplemental material) were crudely related to the *aphA1* gene copy number but reached a plateau at 16 µg/ml above 30 copies of the gene, and high gene copy number incurred a fitness cost on the host bacterium (Fig. 5).

All clinical isolates contained antibiotic resistance island AbaR28 (Fig. 2), and the gene amplification events in these strains involved portions of AbaR28.

In isolate MRSN 57, a 15.2-kb region containing 19 of the 20 ORFs in AbaR28 (Fig. 2) was amplified *in situ* from 2 to 61 times

(Fig. 3). The 5′ end was formed by IS*26*, and part of the *supA* gene for a sulfate permease constituted the 3′ end of the amplified fragment. A similar event was observed in MRSN 56T, following *in vitro* amplification by passage of the initial susceptible isolate MRSN 56 on medium with increasing concentrations of tobramycin. The two events could be accounted for by IS*26*-mediated amplification via formation of deletion circles and reinsertion (24).

In strain MRSN 58, a smaller 3.2-kb unit composed of Tn*6020* underwent replicative transposition at another site in the chromosome followed by amplification at the new locus through nonequal homologous recombination between directly repeated copies of IS*26* (25). We previously reported tandem amplification of *aphA1* by similar homologous recombination between two flanking IS*15* direct copies in Tn*1525* from *Salmonella enterica* serovar Panama when challenged by kanamycin (26).

In strain AB0057, which carries AbaR3, a larger 6.4-kb region was amplified *in situ* after growth in the presence of tobramycin (see Fig. S4 in the supplemental material) by a mechanism likely similar to that in MRSN 57 and MRSN 56T.

Measurement of the fitness cost associated with gene amplification has shown that the growth rate of the host might be indistinguishable from that of the single-copy counterpart or it may be greatly reduced under nonselective conditions depending on the size and location of the amplification (13). In agreement with these observations, we found no biological cost associated with amplification when the growth rate was measured by the usual method (22). However, when individual colonies from the same plate were examined, we observed a diminished growth rate and higher tobramycin MIC (16 µg/ml) for the rare colonies that harbored high copy numbers (>35) of the *aphA1* gene. Hence, greater amplification results in higher levels of resistance to tobramycin but exerts a toll on the host that results in a significant fitness cost.

The mechanism by which Aph(3′)-I confers tobramycin resistance is of interest, as tobramycin lacks the 3′ hydroxyl group which is the target of this phosphotransferase (27). Tobramycin resistance in *Escherichia coli* by overproduction of Aph(3′)-I has been reported (28). In that study, high-level expression of *aphA1* was due to the presence of a strong promoter upstream of the structural gene for the enzyme, and resistance resulted from the formation of a complex between the protein and tobramycin in a stoichiometric manner (28). Thus, trapping of tobramycin by the enzyme could account for the reproducible correlation between increasing tobramycin MICs and higher *aphA1* copy number (Fig. 5). As in *E. coli*, the resistance achieved, although of clinical relevance, was of intermediate level and reached a plateau at 16 µg/ml (Fig. 5). The saturable intracellular sequestration of the drug suggests that the binding between tobramycin and Aph(3′)-I is not very tight. Our observations are compatible with the notion that a tobramycin concentration of 4 µg/ml represents a crude threshold: at higher concentrations, the *aphA1* copy number appeared to coalesce between ca. 30 and 55 in isolate MRSN 57 and between 60 to 120 copies in MRSN 58. At lower drug concentrations, the copy number was maintained at approximately 8 and 15 copies in MRSN 57 and MRSN 58, respectively (Fig. 3).

Detection of gene amplification is difficult due to its transient nature, and thus, its role in antibiotic resistance is thought to be considerably underestimated (13). Typically, amplification is rapidly lost from the bacterial population in the absence of selective pressure (29) primarily by homologous recombination between

repeated sequences mediated through *recA* (30, 31). The decrease in copy number was evident in both isolates MRSN 57 and MRSN 58, eventually leading to a single copy in MRSN 57 (Fig. 3). Consistent with this mechanism, analysis of *recA* in the clinical isolates did not reveal any mutation in the gene or in its upstream regulatory elements. In MRSN 58, the Tn*6020* copy number decreased rapidly from 60 to 17 after a single passage on antibiotic-free medium, but amplification was maintained at an average of 16 (± 4) copies over the ensuing 28 days (Fig. 3). As both the size and location play a role in determining the stability and fitness cost of amplification (29), we speculate that these factors may explain the contrasting evolution of *aphA1* copy numbers in MRSN 57 and MRSN 58. The growth rate of clones of MRSN 58 carrying less than 30 copies of the transposon was indistinguishable from that of the progenitor, susceptible MRSN 56, confirming that the fitness cost of the additional genes was minimal.

We provide the first description of *in vivo* gene amplifications that resulted in the development of antibiotic resistance with subsequent treatment failure. Resistance emerged through two IS*26*-mediated events involving the amplification of a large or small chromosomal region, both containing the *aphA1* resistance gene. As a result of gene dosage effect, production of the aminoglycoside phosphotransferase increased over 50-fold, resulting in the intracellular sequestration of the drug, as demonstrated subsequently for a truncated aminoglycoside acetyltransferase (32). Detection and analysis of gene amplification in clinical isolates were achievable only through a combination of active surveillance with translational research, a strategy that has been alluded to for the detection of these transient events (13). Finally, our data demonstrate that GDA are very dynamic processes that could be readily reproduced *in vitro* and may play a heretofore underappreciated role in clinical antibiotic resistance.

MATERIALS AND METHODS

Case report. All isolates (Table 1) were cultured from a 20-year-old male polytrauma victim sustained in combat operations in Afghanistan (day 1). Cultures obtained from day 3 grew *A. baumannii* (isolates MRSN 3361 and MRSN 3363) susceptible to tobramycin (MIC = 0.5 μg/ml). Tobramycin-susceptible MRSN 3364 was cultured on day 8 from a tissue sample collected from the left hip injury. Upon stabilization of the patient, he was transferred to the United States for definitive care, arriving on day 9, where antibiotics were changed to intravenous tobramycin, meropenem, and vancomycin based on culture results. *A. baumannii* MRSN 56 was cultured from the left hip wound on day 11 and was susceptible to tobramycin (MIC = 0.5 μg/ml). Throughout this escalation of care, tobramycin was continuously administered. On day 12, MRSN 57 was cultured from a tissue sample collected from the right hip but had an MIC of tobramycin of 8 μg/ml. The following day, MRSN 58 was cultured from a tissue sample collected from the left hip. MRSN 58 had a tobramycin MIC of = 16 μg/ml, which denotes resistance by CLSI guidelines (33). Consequently, tobramycin therapy was discontinued, and the patient was prescribed colistin while continuing meropenem.

Whole-genome mapping. Whole-genome mapping (WGM) with NcoI using the Argus system (OpGen Inc., MD, USA) was performed as described previously (5).

Determination of gene copy number by real-time PCR (qPCR). DNA was collected using a modified rapid extraction method (34). Briefly, a 10-μl loopful of bacterial cells was suspended in 300 μl of sterile water, and 20 μl was added to 40 μl of lysis buffer (1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl [pH 8.0], and 1 mM EDTA). The resulting solution was heated to 95°C for 12 min, and 2 μl was used for qPCR.

Primers (see Table S1 in the supplemental material) targeting seven loci within or flanking the amplified unit in *A. baumannii* MRSN 57 were designed using Primer Express 2.0 (Life Technologies, NY) and tested for sensitivity and specificity using MIQE (*m*inimum *i*nformation for *p*ublication of *q*uantitative real-time PCR *e*xperiments) guidelines (35). Standard curves were generated using serial dilutions of DNA from 10^7 to 10^1 genome copies of *A. baumannii* AB0057 as described previously (21). The gene copy number was normalized to the single-copy genes *secE* and *rpiN* to calculate relative copy number per genome (21). Experiments were performed from a minimum of three biological replicates with two technical replicates per run.

RNA extraction and qRT-PCR. Total RNA extraction and subsequent quantitative reverse transcriptase PCR (qRT-PCR) were performed as described previously (5).

Whole-genome sequencing. Whole-genome sequencing was performed on isolates MRSN 56, MRSN 57, and MRSN 58 using a MiSeq benchtop sequencer (Illumina Inc., CA). Libraries were generated from 550-bp fragments of high-quality DNA using the TruSeq DNA PCR-free LT sample preparation kit (Illumina Inc.). Paired-end sequencing (250 bp) was performed using the MiSeq reagent kit v2 (500-cycle) kit. Newbler version 2.7 (454 Life Sciences, CT) was used to assemble MiSeq sequencing reads into *de novo* contigs and sequencing reads against reference DNA sequences. *A. baumannii* AB0057 and AYE were used for comparative genomic analyses. Reference and *de novo* contigs were combined using Geneious (Biomatters, Auckland, New Zealand) to construct a scaffold genome assembly and verified using a whole-genome map (36).

Stability of gene copy number. A single colony of isolates MRSN 57 and MRSN 58 was plated in triplicate onto Mueller-Hinton (MH) agar with and without tobramycin (4 μg/ml). After growth overnight at 37°C, half of the cells of two individual colonies was inoculated separately onto fresh MH agar plates and the other half was used to isolate DNA for qPCR as described above. This process was repeated daily for 20 and 30 days in the presence or absence of tobramycin, respectively. MRSN 56, the progenitor susceptible strain, was cultured in parallel on antibiotic-free medium, and 15 colonies were recovered on days 0, 6, 12, and 20 for mRNA extraction and gene expression studies.

***In vitro* induction of gene amplification.** *A. baumannii* MRSN 56 and AB0057 were cultured on MH agar supplemented with tobramycin (1 μg/ml) in triplicate. Strain AB0057 was selected, as it is of the same sequence type (ST1) as the clinical isolates and carries the AbaR3 resistance island with an intact copy of Tn*6020* (19). Single colonies were transferred daily onto plates with increasing concentrations of tobramycin from 1 to 16 μg/ml. Bacteria were harvested for RNA and DNA extraction daily.

Growth rates. Growth rates were determined in microplates coupled to a Multiskan spectrophotometer (Thermo Fisher Scientific, MA). Strains were grown overnight at 37°C, the cultures were diluted in 5 ml of LB to an optical density at 600 nm (OD$_{600}$) of 0.15 and grown at 37°C with shaking. At the beginning of the stationary phase, i.e., at an OD$_{600}$ of 0.9, the cultures were diluted 10^5-fold to inoculate approximately 1×10^4 bacteria into 200 μl of LB in a 96-well microplate that was incubated at 37°C with shaking. Absorbance was measured at 600 nm every 3 min. Each culture was replicated three times in the same microplate. Growth rates, performed in two independent experiments, were determined at the beginning of the exponential phase, and relative growth rates were calculated as the ratio of the growth rate of the strains versus that of susceptible MRSN 56.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00915-14/-/DCSupplemental.

Figure S1, JPG file, 0.1 MB.
Figure S2, JPG file, 0.2 MB.
Figure S3, JPG file, 0.2 MB.
Figure S4, JPG file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.

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