AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

An Amino Acid Substitution in Elongation Factor EF-G1A Alters the Antibiotic Susceptibility of *Pseudomonas aeruginosa* LasR-Null Mutants

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ABSTRACT The opportunistic bacterium *Pseudomonas aeruginosa* uses the LasR-I quorum-sensing system to increase resistance to the aminoglycoside antibiotic tobramycin. Paradoxically, *lasR*-null mutants are commonly isolated from chronic human infections treated with tobramycin, suggesting there may be a mechanism that permits the emergence of *lasR*-null mutants under tobramycin selection. We hypothesized that some other genetic mutations that emerge in these isolates might modulate the effects of *lasR*-null mutations on antibiotic resistance. To test this hypothesis, we inactivated *lasR* in several highly tobramycin-resistant isolates from long-term evolution experiments. In some of these isolates, inactivating *lasR* further increased resistance, compared with decreasing resistance of the wild-type ancestor. These strain-dependent effects were due to a G61A nucleotide polymorphism in the *fusA1* gene encoding amino acid substitution A21T in the translation elongation factor EF-G1A. The EF-G1A mutational effects required the MexXY efflux pump and the MexXY regulator ArmZ. The *fusA1* mutation also modulated Δ*lasR* mutant resistance to two other antibiotics, ciprofloxacin and ceftazidime. Our results identify a gene mutation that can reverse the direction of the antibiotic selection of *lasR* mutants, a phenomenon known as sign epistasis, and provide a possible explanation for the emergence of *lasR*-null mutants in clinical isolates.

IMPORTANCE One of the most common mutations in *Pseudomonas aeruginosa* clinical isolates is in the quorum sensing *lasR* gene. In laboratory strains, *lasR* disruption decreases resistance to the clinical antibiotic tobramycin. To understand how *lasR* mutations emerge in tobramycin-treated patients, we mutated *lasR* in highly tobramycin-resistant laboratory strains and determined the effects on resistance. Disrupting *lasR* enhanced the resistance of some strains. These strains had a single amino acid substitution in the translation factor EF-G1A. The EF-G1A mutation reversed the selective effects of tobramycin on *lasR* mutants. These results illustrate how adaptive mutations can lead to the emergence of new traits in a population and are relevant to understanding how genetic diversity contributes to the progression of disease during chronic infections.

KEYWORDS LasR, antibiotic resistance, evolution, *Pseudomonas aeruginosa*, quorum sensing, tobramycin

Pseudomonas aeruginosa is an opportunistic multidrug-resistant pathogen that regulates about 10% of its genes using quorum sensing, a type of bacterial communication that is activated in a population density-dependent manner (1–4). One type of quorum sensing involves a LuxR-family signal receptor and a LuxI-family signal synthase (2, 5, 6). In *P. aeruginosa*, there are two complete LuxR-I-type quorum-sensing systems; the LasR-I

Editor Conrad W. Mullineaux, Queen Mary University of London

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The authors declare no conflict of interest.

Received 18 April 2023
Accepted 22 April 2023
Published 16 May 2023

system, which produces and responds to the signal *N*-3-oxododecanoyl-homoserine lactone (3OC12-HSL) (2–4, 7), and the RhlR-I system, which produces and responds to the signal *N*-butanoyl-L-homoserine lactone (C4-HSL) (8). In laboratory strains, the LasR-I system regulates the expression of the *rhlR* and *rhlI* genes, resulting in a hierarchy in quorum sensing, wherein LasR is the master regulator (5, 7, 9). The LasR-I system is essential for pathogenesis in several acute infection animal models (10–12).

We, and others, have shown that the LasR-I system increases *P. aeruginosa* resistance to tobramycin, a clinically relevant antibiotic that is commonly used to treat acute and chronic *P. aeruginosa* infections (13–17). Quorum-sensing systems in other bacteria can also increase antibiotic resistance (18), suggesting that the control of antibiotic resistance by quorum sensing might provide some advantages that are conserved across several bacterial species and environments. The *P. aeruginosa* LasR-I system increases tobramycin resistance in planktonic cultures (17) and also in biofilm conditions (13–16). Tobramycin can also suppress the proliferation of *lasR* mutants that emerge *de novo* during the propagation of populations in certain conditions in the laboratory (17). These results suggest tobramycin treatment could have a strong selective pressure on quorum sensing in a clinical setting.

Clinical isolates of *P. aeruginosa* are remarkably genetically diverse in part due to adaptations to the cystic fibrosis lung environment (19–21). One of the most commonly observed adaptations in clinical infections is a *lasR* mutation, resulting in the loss of quorum-sensing function (21–25). Although LasR contributes to pathogenesis in acute infection models with laboratory strains (26–28), the emergence of LasR mutants in a clinical setting correlates with worse outcomes in infection (22, 29–32). It is counterintuitive that *lasR*-null mutations emerge in infections of patients treated with tobramycin when these mutations increase sensitivity to tobramycin in laboratory strains. There are several potential explanations for this puzzling finding. Certain nutritional conditions can select for *lasR* mutants (23, 33–35), which could possibly overcome tobramycin effects. Alternatively, some conditions have been shown to alter the physiology of *lasR* mutants to make them more tobramycin resistant (36, 37). Whether the conditions of the infection environment contribute to the selection of *lasR* mutations in infections of tobramycin-treated patients remains unknown.

Here, we sought to explore another possibility for *lasR* mutant emergence in infections of tobramycin-treated patients: the possibility that the effects of tobramycin on *lasR*-null mutants might be dependent on genetic background. In particular, we hypothesized that the tobramycin susceptibility of Δ*lasR* mutants might be modified by epistatic gene interactions (38). We identify one such mutation, a point mutation in the gene encoding the translation accessory factor EF-G1A (*fusA1*). We show that *fusA1* G61A (EF-G1A^{A21T}) facilitates the emergence of Δ*lasR* mutations in populations under tobramycin selection. Our results show that antibiotic susceptibility of *lasR* mutants can be genotype dependent and support the idea that genetic interactions could contribute to the emergence of *lasR*-null mutations in a clinical setting.

RESULTS

Δ*lasR* has strain-dependent effects on tobramycin resistance. We previously characterized six tobramycin-resistant genetic isolates of *P. aeruginosa* PA14 with mutations in unique genes (e.g., *fusA1* and *ptsP*, see Table S1 in the supplemental material) (17). We used these isolates to test the hypothesis that antibiotic adaptations could alter the effect of Δ*lasR* mutations on antibiotic resistance. We used allelic exchange to delete *lasR* from each of the six isolates from our prior study (termed T1 to T6). We compared the MIC of the Δ*lasR* isolates with their parent (Fig. 1A). Similar to our prior results (17), deleting *lasR* caused a small decrease in tobramycin resistance of PA14, although in this study the difference was not significant due to comparisons with a wider range of MICs in our statistical analyses. Deleting *lasR* in the T1 isolate increased tobramycin resistance ~2-fold (Fig. 1A). Deleting *lasR* in T3 had a smaller but similar effect (Fig. 1A). In pairwise comparisons with the PA14 wild-type strain, the effects of Δ*lasR* on the tobramycin MIC were significantly different in T1 and T3 ($P < 0.05$, Fig. 1A). We did not observe significantly different effects of deleting *lasR* on the MIC for any of the other four isolates (Fig. S1).

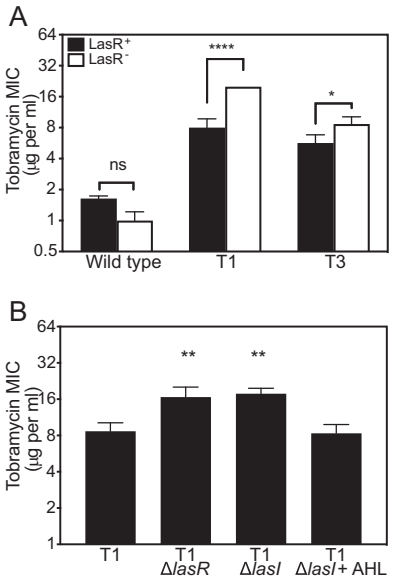

FIG 1 Tobramycin resistance of Δ*lasR* mutants is strain dependent. (A) The MIC of tobramycin was determined for each strain carrying intact *lasR* (LasR$^+$, black bars) or Δ*lasR* (LasR$^-$, white bars). The significance of the effect of strain (wild type, T1, or T3) and *lasR* allele (LasR$^+$ or LasR$^-$) and the interaction of the two on MIC was determined by two-way ANOVA by performing pairwise comparisons of each LasR$^{+/-}$ strain pair with that of PA14. The interaction was significant for T1 ($F_{1,8}$ = 156.8 and $P < 0.0001$) and T3 ($F_{1,8}$ = 10.67 and $P < 0.05$). Pairwise comparisons of LasR$^+$ and LasR$^-$ of each strain were determined using Sidak's *post hoc* test with *P*-values adjusted for multiple comparisons; *, $P < 0.05$; ****, $P < 0.0001$; ns, not significant. (B) Tobramycin MIC of T1 and T1-mutated strains. Where indicated, AHL (3OC12-HSL) was added before inoculating culture tubes at the 10-μM final concentration. Statistical analysis was done by one-way ANOVA and Dunett's multiple comparisons with T1; **, $P < 0.01$. For panels A and B, results are the average of three independent experiments and the vertical bars indicate standard deviation.

To further confirm the effects of the LasR-I system on tobramycin resistance in the T1 isolate, we deleted the *lasI* signal synthase gene (Fig. 1B). Because LasI synthesizes the LasR signal, we anticipated that deleting *lasI* would cause changes in tobramycin MIC similar to that of a *lasR* deletion. Consistent with this expectation, deleting *lasI* significantly increased tobramycin resistance in T1, and adding synthetic 3OC12-HSL to the Δ*lasI* mutant culture restored resistance levels to that of T1 (Fig. 1B). These results offer further support that the effects of disrupting the LasR-I circuit on tobramycin resistance are strain dependent.

***fusA1* (G61A) modulates the effects of Δ*lasR* on tobramycin resistance.** We hypothesized that one or more genetic mutations in T1 and T3 altered the susceptibility of Δ*lasR* mutants to tobramycin. In our prior study, we determined that the T1 and T3 isolates have only two common mutations, *fusA1* G61A (EF-G1A^{A21T}) and *ptsP* 1547T (a frameshift mutation that is predicted to result in an inactivated protein) (Table S1) (17). We individually introduced each of these mutations to PA14 and PA14 Δ*lasR* using allelic exchange and compared the MIC of the mutated strains to that of the PA14 parent strains (Fig. 2A). Introducing the *fusA1* G61A mutation on its own did not change the MIC of PA14. However, deleting *lasR* in PA14 *fusA1* G61A increased the MIC of the *fusA1* G61A mutant ~2-fold, which was a significantly different effect from deleting *lasR* in PA14 ($P < 0.0001$). In contrast, deleting *lasR* in the *ptsP* 1547T mutant was not significantly different from that of PA14 ($P = 0.9865$). These results suggested that the *fusA1* G61A mutation is responsible for increasing the tobramycin resistance of Δ*lasR* mutants.

Next, we asked if ectopic expression of the wild-type *fusA1* could restore the MIC to that of wild type. To do so, we fused the wild-type *fusA1* gene to a rhamnose-inducible promoter (P*rha*) and introduced this cassette to the neutral *attB* site in the genome of T1 and the PA14 *fusA1* G61A mutant (Fig. 2B and Fig. S2). In wild-type *fusA1*-expressing strains, the effects of deleting *lasR* on the tobramycin MIC were indistinguishable from that of the parent (T1 or PA14 *fusA1* G61A carrying P*rha-fusA1* compared with Δ*lasR* of each strain). The results show that the defects caused by the G61A mutation can be restored by expression of the wild-type

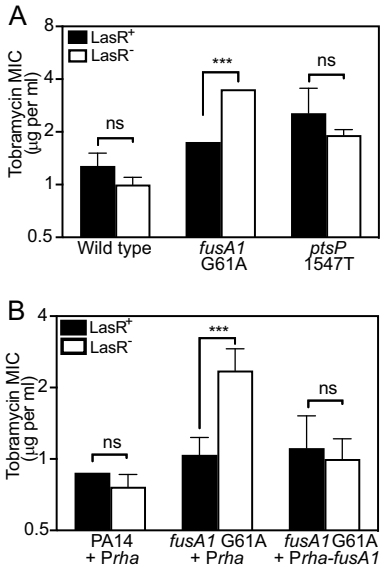

FIG 2 Effects of *fusA1* G61A and Δ*lasR* on tobramycin resistance. The MIC of tobramycin was determined for each strain carrying intact *lasR* (LasR$^+$, black bars) or Δ*lasR* (LasR$^-$, white bars). The significance of the effect of strain (indicated along the *x* axis) and *lasR* allele (LasR$^+$ or LasR$^-$) and the interaction of the two on MIC was determined by 2-way ANOVA by performing pairwise comparisons of each LasR$^{+/-}$ strain pair with that of PA14 (A) or PA14 P*rha* (B). Pair-wise comparisons of LasR$^+$ and LasR$^-$ of each strain were determined using Sidak's *post hoc* test with *P* values adjusted for multiple comparisons; ***, $P < 0.001$; ns = not significant. (A) There was a statistically significant interaction between the effects of strain and *lasR* for *fusA1* G61A ($F_{1,8} = 156.8$, $P < 0.0001$) but not for *ptsP* 1547T ($F_{1,8} = 0.3642$, $P = 0.5629$). An additional Sidak's *post hoc* test comparing PA14 and *fusA1* G61A of the LasR$^+$ strains revealed no significance ($P > 0.4$). (B) Strains had either the empty CTX-1 P*rha* or the CTX-1 P*rha-fusA1* cassette inserted into the neutral *attB* site in the genome. Rhamnose was added to all cultures at 0.1% final concentration. There was a statistically significant interaction between the effects of strain and *lasR* for *fusA1* G61A P*rha* ($F_{1,8} = 17.41$, $P < 0.005$) but not for *fusA1* G61A + P*rha-fusA1* ($F_{1,8} = 1.7 \times 10^{-7}$, $P = 0.997$). For panels A and B, results are the average of three independent experiments and the vertical bars indicate standard deviation.

fusA1 and that this mutation is recessive. These results also validate the conclusion that the *fusA1* G61A mutation causes the observed effects on *lasR*-dependent tobramycin resistance.

Strain-dependent effects of tobramycin on the proliferation of Δ*lasR* mutants in cocultures. We sought to assess the selective effects of tobramycin on Δ*lasR* mutants in different strain backgrounds using coculture competition experiments. When PA14 is coinoculated with PA14 Δ*lasR*, the Δ*lasR* mutant rapidly proliferates to a higher frequency because it has a fitness advantage over PA14; however, Δ*lasR* mutants are suppressed in identical cocultures grown with subinhibitory tobramycin (17). We hypothesized that tobramycin would enhance rather than suppress the proliferation of Δ*lasR* mutants in strains carrying the *fusA1* G61A mutation. To test this hypothesis, we inoculated cocultures with either PA14 or PA14 *fusA1* G61A and a 1% initial population of each respective Δ*lasR* mutant and grew the cocultures with or without tobramycin. Cocultures were transferred daily to fresh medium, and the proportion of Δ*lasR* mutants in the final population was assessed after two daily transfers. The results (Fig. 3) showed that tobramycin had significantly different effects on Δ*lasR* mutant proliferation in each of the two strain backgrounds ($P < 0.0001$). At the end of the PA14 experiment, the Δ*lasR* mutants were ~30% of the population in cocultures grown with no tobramycin but reached only ~0.5% of the tobramycin-treated population (Fig. 3, left), consistent with prior results (17). In cocultures with strains carrying *fusA1* G61A, the Δ*lasR* mutants similarly reached ~30% of the population in the absence of tobramycin but further increased to ~60% of the population in the presence of tobramycin (Fig. 3, right). These results show that tobramycin selection of Δ*lasR* mutations is reversed in the *fusA1* G61A mutant compared with the wild-type parent and support the idea that *fusA1* mutations could permit the emergence of *lasR* mutations in the population.

Effects of other *fusA1* mutations on tobramycin MIC of Δ*lasR* mutants. The *fusA1* gene encodes elongation factor G1A (EF-G1A), a GTPase protein that hydrolyzes GTP to drive the elongation and recycling steps of protein synthesis (39, 40). EF-G1A is an essential

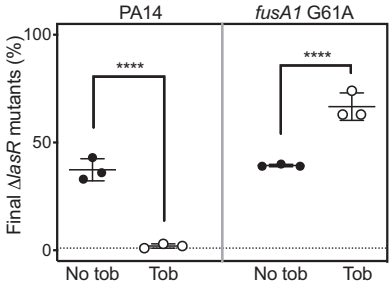

FIG 3 Tobramycin effects on Δ*lasR* proliferation in cocultures. Cocultures of wild-type PA14 and PA14 Δ*lasR* (left) or of *fusA1* G61A and *fusA1* G61A Δ*lasR* (right) were grown with no tobramycin (No tob, closed circles) or with tobramycin (Tob, open circles) at the highest concentration that permits growth (0.3 μg/mL for PA14 or 2 μg/mL for *fusA1* G61A). In both cases, the Δ*lasR* mutant was started at 1% of the total coculture population. After being combined, cocultures were inoculated into casein medium and subsequently transferred to fresh medium daily for 2 days. Final population densities ranged from 1 to 5 $\times$ 10^9 cells per mL. Each data point represents a single experiment and vertical lines represent standard deviation. Statistical analysis by two-way ANOVA was used to determine the significance of the effect of the tobramycin and the *lasR* allele and interaction between the two on the relative fitness of the Δ*lasR* mutant, and the interaction was significant ($P < 0.0001$ and $F_{1,8} = 41.26$). Pair-wise comparisons of $LasR^+$ and $LasR^-$ for each condition (+/− tob) were performed using Sidak's *post hoc* analysis with *P* values adjusted for multiple comparisons; ****, $P < 0.0001$.

component in translation, and *fusA1*-null mutations are not viable (39–41). Nevertheless, *fusA1* has been reported to be among the most frequently mutated genes in clinical isolates (42–45), and in at least some cases, *fusA1* mutations increase tobramycin resistance (41, 42, 46). EF-G1A has 5 domains (labeled I to V in Fig. 4A). The $EF\text{-}G1A^{A21T}$ substitution is within a motif in domain 1 called the Walker-A P-loop, which is responsible for binding phosphoryl groups and catalyzing phosphoryl transfer of NTPs (47).

To determine the prevalence of the *fusA1* G61A mutation in natural isolates, we surveyed 4,312 sequenced strains in the *Pseudomonas* Genome Database (48). Only four strains were

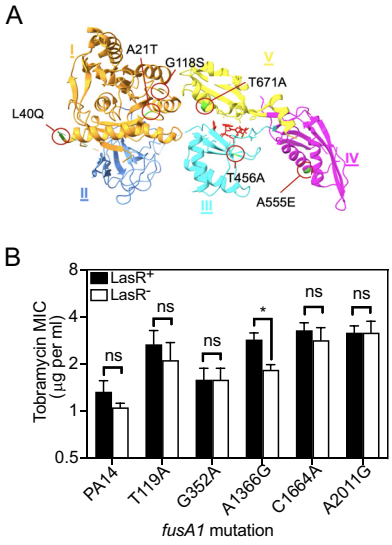

FIG 4 Other *fusA1* mutations and their effects on tobramycin resistance of Δ*lasR* mutants. (A) Ribbon diagram of the *fusA1*-encoded protein elongation factor-G (EF-G1A) (PDB ID: 4FN5). The protein was crystallized bound to argyrin, which is shown in red. Each one of the amino acid substitutions is indicated with green coloration in the ribbon diagram and a red circle and was identified using the UCSF ChimeraX (82) (B) The MIC of tobramycin was determined for each strain carrying the wild-type *fusA1* allele (PA14) or PA14 with the indicated amino acid substitution in the encoded EF-G1A. Each strain had either an intact *lasR* ($LasR^+$, black bars) or Δ*lasR* ($LasR^-$, white bars). Results are the average of three independent experiments and the vertical bars indicate standard deviation. The significance of the effect of the *fusA1* allele and the *lasR* allele and the interaction of the two on MIC was determined by 2-way ANOVA by performing pairwise comparisons of each $LasR^{+/-}$ strain pair with that of PA14. Only A1366G showed a significant interaction ($P < 0.05$, $F_{1,8} = 10.25$). Pairwise comparisons of $LasR^+$ and $LasR^-$ of each strain were determined using Sidak's *post hoc* test with *P* values adjusted for multiple comparisons. *, $P < 0.05$; ns, not significant.

TABLE 1 Antibiotic susceptibility of *lasR* and *fusA1* G61A mutant strains

	Antibiotic susceptibility[a,b]				
Strain	**Cipro**	**Imi**	**Tet**	**Pip**	**Ceft**
PA14	0.09 (0.01)	0.7 (0.1)	5 (2)	50	49 (2)
PA14 Δ*lasR*	0.08 (0.01)	0.8 (0.1)	5 (2)	50	11 (5)****
fusA1 G61A	0.06	0.8 (0.1)	6 (2)	50	13
fusA1 G61A Δ*lasR*	0.12 (0.03)**[c]	0.8 (0.2)	4 (2)	50	10 (2)

[a]Antibiotic susceptibility was determined by MIC as described in Materials and Methods. Cipro, ciprofloxacin; Imi, imipenem; Tet, tetracycline; Pip, piperacillin; Ceft, ceftazidime. The values represent the average of three independent MIC experiments with the standard deviation in parentheses. Standard deviation was zero where not indicated.

[b]The significance of the effects of *fusA1* (wild-type *fusA1* in PA14 or G61A) and *lasR* allele (intact *lasR* or Δ*lasR*) and the interaction of the two on MIC was determined by two-way ANOVA. The interaction was significant for ciprofloxacin ($P < 0.01$ and $F_{1,8} = 13.98$) and for ceftazidime ($P < 0.0001$ and $F_{1,8} = 91.16$). There was no significance of interaction ($P > 0.05$) for imipenem, tetracycline, and piperacillin.

[c]Superscripted asterisks indicate the significance of comparing $LasR^+$ and $LasR^-$ of each strain (PA14 or *fusA1* G61A) using Sidak's *post hoc* analysis with *P* values adjusted for multiple comparisons of all strains. There was no significance ($P > 0.05$) unless indicated. **, $P < 0.01$; ****, $P < 0.0001$.

identified with any substitution in amino acid 21, all of them an A to T substitution at amino acid 21 caused by a G61A nucleotide polymorphism (Table S2). Interestingly, none of these four EF-G1A^{A21T} strains were *lasR*-mutated. However, the environmental constraints particular to these strains are unknown, making the interpretation of this finding difficult. Our results indicate *fusA1* G61A mutations do occur naturally but are relatively rare.

fusA1 is a hot spot for single-nucleotide mutations in clinical isolates in *P. aeruginosa* (41, 42, 45, 49, 50). To test whether other *fusA1* mutations can enhance or alter the tobramycin resistance of Δ*lasR* mutants, we introduced five *fusA1* mutations to the genome of PA14 or PA14 Δ*lasR* by allelic exchange and determined the tobramycin MIC of each strain (Fig. 4). In all but one of the *fusA1*-mutated strains (A1366G, corresponding with an amino acid substitution T456A), the effect of LasR on antibiotic resistance was not significantly altered compared with that of PA14. In the *fusA1* A1366G strain, Δ*lasR* decreased tobramycin resistance, which is the opposite of the effect observed with the G61A mutation. There were no effects of deleting *lasR* for the other *fusA1* mutations tested (T119A, G352A, C1664A, and A2011G, corresponding with amino acid substitutions L40Q, G118S, A555E, and T671A). These results show that not all *fusA1* mutations modulate the tobramycin resistance of Δ*lasR* mutants in the same way.

***fusA1* G61A modulates Δ*lasR* resistance to other antibiotics.** We next asked whether the *fusA1* G61A mutation can also alter the susceptibility of Δ*lasR* mutants to antibiotics other than tobramycin. We tested the antibiotics ciprofloxacin (DNA gyrase inhibitor), ceftazidime (cell wall biosynthesis inhibitor), piperacillin (cell wall biosynthesis inhibitor), and tetracycline (protein synthesis inhibitor) against PA14 and our *fusA1* and *lasR* single and double mutant strains (Table 1). We observed significant strain-dependent effects of the Δ*lasR* mutation on the MIC values for two of these antibiotics: ciprofloxacin ($P < 0.01$) and ceftazidime ($P < 0.0001$). With ciprofloxacin, Δ*lasR* increased the resistance of the *fusA1* G61A mutant, but this mutation caused no changes in the PA14 MIC. With ceftazidime, we observed different effects: the Δ*lasR* mutation and *fusA1* G61A mutations decreased PA14 resistance to the same degree as single mutations and in combination. These results show that *fusA1* G61A mutations modulate the susceptibility of Δ*lasR* mutants to several different classes of antibiotics and demonstrate the broad interaction effect of these genes.

***fusA1* G61A and Δ*lasR* increase tobramycin resistance by activating the MexXY efflux pump.** Some other *P. aeruginosa fusA1* mutations can enhance aminoglycoside resistance through the multidrug efflux pump MexXY (41, 51). Thus, we hypothesized that MexXY is important for increasing the tobramycin resistance of *fusA1* G61A and Δ*lasR* double mutants. As an initial test of this hypothesis, we measured the expression of the *mexX* gene, which is immediately upstream of and in the same operon as *mexY* (Fig. 5A). We quantified *mexX* transcripts in logarithmically growing cells of PA14 *fusA1* G61A and Δ*lasR* single and double mutants. Consistent with our hypothesis, *mexX* transcripts were the highest in *fusA1* G61A and Δ*lasR* double mutants. We also deleted the

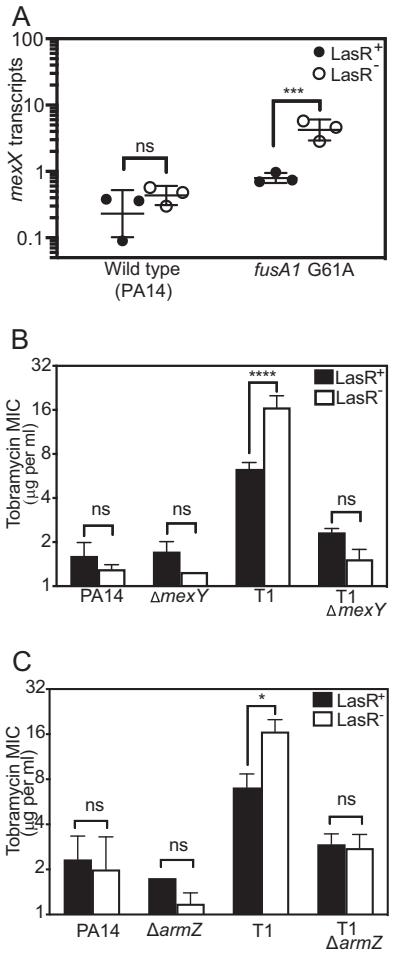

FIG 5 Role of MexXY on tobramycin resistance of Δ*lasR* and *fusA1* G61A mutants. (A) *mexX* transcript levels were determined by droplet digital PCR and normalized to the housekeeping control gene *proC*. Strains were wild-type PA14 or PA14 with the *fusA1* G61A substitution. Each strain had either an intact *lasR* (LasR$^+$, filled circles) or Δ*lasR* (LasR$^-$, open circles). Each point represents in independent experiment; horizontal lines represent the geometric mean, and the vertical lines represent the geometric standard deviation. Statistical analysis by two-way ANOVA showed a significant interaction between the effects of strain and *lasR* allele on *mexX* transcripts ($P < 0.005$, $F_{1,8} = 15.67$). An additional Sidak's *post hoc* test comparing PA14 and *fusA1* G61A of the LasR$^+$ strains revealed no significance ($P > 0.6$). (B and C) The MIC of tobramycin was determined for each strain carrying intact *lasR* (LasR$^+$, black bars) or Δ*lasR* (LasR$^-$, white bars). Results are the average of three independent experiments and the vertical bars show standard deviation. In pairwise comparisons with the LasR$^{+/-}$ PA14 strains, there was a significant interaction between the effects of strain and *lasR* allele for T1 ($P < 0.01$ and $F_{(1,8)} = 29.01$ for panel B and $P < 0.005$ and $F_{(1,8)} = 17.99$ for panel C) but not for the other strains. In panels A to C, pairwise comparisons of LasR$^+$ and LasR$^-$ of each strain were determined using Sidak's *post hoc* test with *P*-values adjusted for multiple comparisons. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

mexY gene that is essential for pump activity. Our attempts to delete *mexY* in *fusA1* G61A were unsuccessful; however, we were able to delete this gene in T1 and T1 Δ*lasR* and also PA14 and PA14 Δ*lasR*. Deleting Δ*mexY* in T1 abolished the LasR-dependent changes in MIC observed in this strain (Fig. 5B), supporting that MexY is important for *fusA1* G61A to increase the tobramycin resistance of Δ*lasR* mutants.

One mechanism of MexXY induction is through the ArmZ regulator (52, 53), which is activated by a mechanism of transcription attenuation in response to perturbations that cause the ribosome to stall (53–55). ArmZ derepresses the MexXY regulator MexZ; thus, activation of ArmZ ultimately leads to increased MexXY activity (52, 53, 56). To test the hypothesis that ArmZ is required for MexXY activation in *fusA1* G61A and Δ*lasR* double mutants, we deleted *armZ* in T1 and the T1 Δ*lasR* mutants and determined the MIC of these strains. Similar to the result of deleting *mexY*, we found that deleting *armZ* abolished

TABLE 2 Growth rates in MOPS minimal medium

Strain/isolate	Doubling time (min)[ab]	Strain	*lasR*	Interaction
PA14	62.5 (2.0)			
PA14 Δ*lasR*	61.4 (2.9)			
fusA1 G61A	66.3 (2.9)	$P < 0.0005$	$P < 0.01$	$P < 0.005$
fusA1 G61A Δ*lasR*	78.2 (2.2)***[c]	$F = 49.91$	$F = 13.81$	$F = 19.73$
T1	67.7 (4.0)	$P < 0.0005$	$P < 0.02$	$P < 0.005$
T1 Δ*lasR*	79.7 (2.5)***	$F = 47.61$	$F = 10.41$	$F = 14.81$
T3	74.9 (3.4)	$P < 0.0001$	ns	$P < 0.04$
T3 Δ*lasR*	83.0 (3.5)*	$F = 96.27$	$F = 4.084$	$F = 6.930$

[a]The values represent the average doubling time during logarithmic growth determined from three independent experiments, with the standard deviation indicated in parentheses. Standard deviation was zero where not indicated.

[b]The significance of the effects of strain (PA14, *fusA1* G61A, T1, or T3) and *lasR* allele (intact *lasR* or Δ*lasR*) and the interaction of the two on MIC was determined by two-way ANOVA by performing pairwise comparisons of each $LasR^{+/-}$ strain pair with that of PA14. The interaction was significant in all cases. For *fusA1* G61A, $P < 0.005$ and $F = 19.73$; for T1, $P < 0.005$ and $F = 14.81$; for T3, $P < 0.04$ and $F = 6.930$.

[c]Asterisks indicate the significance of comparing $LasR^{+}$ and $LasR^{-}$ of each strain using Sidak's *post hoc* analysis with *P* values adjusted for multiple comparisons of all strains. There was no significance ($P > 0.05$) unless indicated. *, $P < 0.05$; ***, $P < 0.001$.

LasR-dependent changes in MIC observed in T1 (Fig. 5C). Together, the results support that MexXY and the ArmZ regulator are both required for *fusA1* G61A to increase the tobramycin resistance of Δ*lasR* mutants.

fusA1 mutations are associated with slower translation rates (41, 51, 57). Thus, we hypothesized that the *fusA1* G61A mutation might cause ribosome stalling effects that are enhanced by the Δ*lasR* mutation, to subsequently activate ArmZ. To test this hypothesis, we measured the doubling times of our *fusA1* G61A and Δ*lasR* mutant strains during logarithmic growth in minimal media, as growth rates can indirectly indicate the effects of *fusA1* mutations on the translation rate (41, 58). We found that *fusA1* G61A alone had no significant effect on growth; however, the growth of this mutant was slowed when combined with Δ*lasR* (Table 2). Although there are other possible explanations for why growth effects might be observed in these mutants, for example, pleiotropic effects of the *fusA1* mutation on gene regulation (51), these results support the idea that the Δ*lasR* and *fusA1* G61A mutants increase *mexXY* expression by increasing ribosome stalling.

DISCUSSION

In this study, we demonstrate that *lasR*-null mutations have strain-dependent effects on antibiotic resistance in *P. aeruginosa*. These effects are caused by mutations in the translation accessory *fusA1* gene (G61A), a commonly mutated gene in clinical isolates. These results add to the growing body of work showing that adaptive mutations can modulate quorum sensing (17, 38, 59–62). Our results show that adaptation can have important consequences on the emergence of *lasR* mutations, which could significantly change the evolutionary landscape of the community. Thus, adaptive mutations are important to the evolution of quorum sensing and may play a critical role in shaping quorum-sensing populations in communities under strong selective pressure, such as those found in infections.

The effects of *fusA1* G61A mutations on Δ*lasR* selection by tobramycin are consistent with a type of epistasis called sign epistasis. Epistasis describes a phenomenon in which the phenotypic effects of one gene mutation are modified by mutations in other genes (38), and sign epistasis is a type of interaction that reverses mutational effects on phenotype (63–65). Sign epistatic effects change the direction of selection; thus, they do not require large changes in phenotype or MIC to cause significant effects on evolution (66, 67). For example, sign epistatic interactions permit the emergence of new traits in a population, as illustrated by our study. Some prior studies have demonstrated sign epistatic effects in bacteria, for example, in the development of cefotaxime resistance in *Escherichia coli* (68) and the evolution of antibiotic resistance in *Mycobacterium tuberculosis* (69) and also in *P. aeruginosa* in the context of rifampicin resistance (70). In the *P. aeruginosa* study, epistatic and sign epistatic interactions were surprisingly common, with ~50% of those interactions

tested showing epistatic effects and 84% of epistatic interactions showing sign epistatic effects. These studies suggest that sign epistatic interactions are prevalent in *P. aeruginosa*. The studies also highlight the potential importance of adaptive mutations that have small antibiotic resistance effects for the evolution of new traits in a population.

Our study provides new information on the effects of *fusA1* mutations on antibiotic resistance. The *fusA1*-encoded protein EF-G1A is important for recycling ribosomes during translation (71). Mutations in EF-G1A are thought to cause ribosomes to get stuck on the mRNA and back up other ribosomes behind them (72). These translation disruptions could cause ribosome stress and subsequently activate the MexXY regulator ArmZ through translation attenuation (53–55). In support of this idea, at least some of the other *fusA1* mutants increase transcription of the *mexXY* genes, and MexXY is important for increased resistance of these strains (41, 51). The *fusA1* G61A mutation seems to cause similar effects but only in combination with Δ*lasR* (Fig. 2 and 5A). These results suggest LasR can somehow ameliorate the effects of the G61A mutation possibly by protecting against ribosome stress. LasR may do this by activating specific effectors or in coordination with other stress responses, for example, through the stress-responsive sigma factor RpoS (73). We find it interesting that LasR does not have interactive effects on tobramycin resistance with any other *fusA1* mutations (Fig. 4B), which suggests either that LasR cannot sufficiently overcome the effects of those other mutations or there are unknown mechanistic differences in the mutational effects.

Our results suggest there may be a variety of epistatic gene interactions with *fusA1* that have previously unappreciated contributions to antibiotic resistance and gene evolution. For example, the *fusA1* G61A mutation increased resistance in the T1 variant by ~4-fold, which was not observed in wild type (Fig. S2). This result suggested other mutations in the T1 isolate enhance the effects of the *fusA1* G61A mutation on tobramycin resistance. The particular mutations in T1 that have interaction effects on *fusA1* G61A remain to be discovered. One other *fusA1* mutation (A1366G) also had interesting effects in our studies. This mutation further enhanced the sensitivity of Δ*lasR* to tobramycin (Fig. 4B). Such genetic interactions that enhance antibiotic sensitivity are particularly relevant to therapeutics because they could pave a path toward developing novel disease interventions that increase the efficacy of existing antibiotics.

MATERIALS AND METHODS

Culture conditions and reagents. Routine growth was in Luria-Bertani broth (LB) for *Escherichia coli* or in LB buffered to pH 7 with 50 mM 3-(morpholino)-propanesulfonic acid (LB-MOPS) for *Pseudomonas aeruginosa* or on LB agar (LBA; 1.5% wt/vol Bacto Agar). Liquid growth media for specific experiments were M9-caseinate (casein broth: 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl, pH 7.4, and 1% sodium caseinate) (17) or a MOPS minimal medium [25 mM D-glucose, freshly prepared 5 μM $FeSO_4$, 15 mM NH_4Cl, and 2 mM K_2HPO_4 added to a 1× MOPS base buffer consisting of 50 mM MOPS, 4 mM Tricine, 50 mM NaCl, 1 mM K_2SO_4, 50 μM $MgCl_2$, 10 μM $CaCl_2$, 0.3 μM $(NH_4)_6Mo_7O_{24}$, 40 μM H_3BO_3, 3 μM cobalt(II) acetate, 1 μM $CuSO_4$, 8 μM $MnSO_4$, and 1 μM $ZnSO_4$] (17, 74). Four percent skim milk agar (SMA) was used for identifying Δ*lasR* mutants in competition experiments. All growth was at 37°C, and liquid cultures were grown with shaking at 250 rpm in 18-mm culture tubes (2-mL cultures), 125-mL baffled flasks (10-mL cultures), or 250-mL baffled flasks (60-mL cultures). For strain construction, we used 100 μg mL^{-1} carbenicillin, 15 μg mL^{-1} gentamicin, and 2.5–10 μg mL^{-1} tetracycline for *E. coli* and 150 to 300 μg mL^{-1} carbenicillin, 50 to 200 μg mL^{-1} gentamicin, and 15 to 100 μg mL^{-1} tetracycline for *P. aeruginosa*. 3OC12-HSL was purchased from Cayman Chemicals (MI, USA), dissolved in acidified ethyl acetate with glacial acetic acid (0.1 mL l^{-1}), and added to an empty sterile conical tube and dried by evaporation before adding liquid media.

Genomic or plasmid DNA was extracted using Qiagen Puregene Core A kit (Hilden, Germany) or IBI Scientific plasmid purification mini-prep kit (IA, USA) while PCR products were purified using IBI Scientific PCR cleanup/gel extraction kits, according to the manufacturer's protocol. Antibiotics were purchased from GoldBio (MO, USA) except for tetracycline, which is from Fisher Scientific (PA, USA).

Bacterial strains and strain construction. All bacterial strains, plasmids, and primers used in this study are provided in the supplemental material. *P. aeruginosa* strain UCBPP-PA14 ("PA14") and PA14 derivatives were used for these studies. Markerless deletions in specific loci of *P. aeruginosa* PA14 were generated using allelic exchange as described previously (75). To generate plasmids for allelic exchange, DNA fragments with the mutated or deleted gene allele plus 500-bp flanking DNA were synthesized (Genscript, NJ) or generated by PCR using primer-incorporated restriction enzyme sites. The synthesized or PCR products were moved to plasmid pEXG2 (76) using restriction enzyme digestion and ligation or isothermal assembly. The subsequent plasmids were transformed into the appropriate *P. aeruginosa* strain using described methods (77). The plasmids for Δ*lasR* (17), Δ*mexY* (78), and Δ*armZ* (56) are described elsewhere. Merodiploids were selected on

Pseudomonas isolation agar (PIA)-carbenicillin (150 to 300 μg mL^{-1}) for Δ*lasR*; PIA-gentamicin (50 to 200 μg mL^{-1}) for *fusA1* G61A, Δ*lasI*, and *ptsP* 1547T; and PIA-tetracycline (15 to 100 μg mL^{-1}) for Δ*mexY* and Δ*armZ*. Deletion mutants were counterselected using NaCl-free 15% sucrose. Putative mutants were verified through antibiotic sensitivity tests and gene-targeted Sanger sequencing. It was of note that in some cases we had difficulty with genetic manipulations involving *fusA1* mutations. In most cases, we found success by optimizing the concentration of antibiotics used for selecting plasmid integrants. For introducing the *fusA1* G61A mutation to the genome of *P. aeruginosa* by allelic exchange, we found success only when we introduced the full-length *fusA1* rather than shorter fragments, possibly because this approach helped to avoid creating disruptions that were not viable. To make the P*rha-fusA1* expression cassette, we PCR-amplified the wild-type *fusA1* gene from PA14 using primer-encoded restriction enzyme sites. The PCR product was digested and ligated to pJM253 (miniCTX1-rhaSR-PrhaBAD) (79). This plasmid was moved into *P. aeruginosa* by conjugation as described previously (79). Transformants were selected on LBA with 200 μg mL^{-1} tetracycline, and the insertion of the cassette in the *attB* site was verified by PCR.

Antimicrobial susceptibility assays. Antibiotic susceptibility was determined by MIC using a modified method from the 2022 guidelines of the Clinical and Laboratory Standards Institute (CSLI) (80), similar to that previously described (17). Briefly, two antibiotic dilution series were made from staggered starting antibiotic concentrations to cover a broader range of concentrations. These were as follows (in μg mL^{-1}): tobramycin, 20 and 7; ceftazidime, 100 and 75; ciprofloxacin 1 and 0.375; piperacillin 100 and 75; imipenem 10 and 3.5; and tetracycline 20 and 7. These were successively diluted in MOPS minimal medium 2-fold in a 200-μL volume in 2-mL tubes. The starter cultures were prepared by growing *P. aeruginosa* in LB-MOPS to an optical density at 600 nm (OD_{600}) of 4. The starter cultures were subsequently diluted 1:40 into each tube containing tobramycin to start the MIC experiment. After 20 h of incubation with shaking, turbidity was measured using a Biotek Synergy 2 plate reader. The MIC was defined as the lowest concentration of antibiotic (μg mL^{-1}) in which bacterial growth was not measurable.

Coculture assays. Overnight (18 h) pure cultures were grown in LB-MOPS, diluted to an OD_{600} of 0.025 for LasR$^-$ or 0.05 to 0.15 for LasR$^+$ into LB-MOPS, and grown to an OD_{600} of ~3.5 before combining at a 99:1 (LasR$^+$:LasR$^-$) ratio and used to start the coculture by diluting 1:40 into casein broth in 18-mm test tubes. In some cases as indicated, tobramycin was added to the casein broth coculture. At 24 h, cocultures were diluted 1:40 into fresh casein broth in a new test tube and the experiment was ended at 48 h. The initial and final total population counts (CFU mL^{-1}) were determined by dilution plating and viable plate counts. The percent Δ*lasR* mutant was determined by patching 200 colonies on SMA where Δ*lasR* mutants form distinct colony phenotypes (17, 22, 23, 81).

Droplet digital PCR. Overnight (18 h) pure cultures were grown in LB-MOPS, subcultured in LB-MOPS, and grown to an OD_{600} of ~4. Stationary-phase *P. aeruginosa* (OD_{600} of 4) was diluted to OD_{600} 0.1 in MOPS minimal medium and grown 2.5 to 3 h (OD_{600} ~0.20 to 0.45). RNA was harvested using the RNeasy minikit (Qiagen), following the manufacturer's instructions. Droplet digital PCR (ddPCR) was performed on a Bio-Rad QX200 ddPCR system using Eva Green supermix. Each reaction mixture contained 1.8 ng μL^{-1} cDNA template, 0.8 μM each primer, and 10 μL Eva Green supermix in a 20-μL final volume. After generating 40 μL of oil droplets, 40 rounds of PCR were conducted using the following cycling conditions: 95°C for 30s, 62°C for 30s, and 68°C for 30s. Absolute transcript levels were determined using Bio-Rad QuantaSoft software. In all cases, a no-template control was run with no detectable transcripts. The reference gene used was the proline biosynthetic gene (*proC*), and the results are reported as the calculated transcript amount of a given gene per calculated *proC* transcript.

Growth curve. Overnight (18 h) pure cultures were grown in LB-MOPS, subcultured in LB-MOPS, and grown to an OD_{600} of ~4. Stationary-phase *P. aeruginosa* (OD_{600} of 4) was diluted to OD_{600} 0.1 in MOPS minimal medium, and OD_{600} was measured in a Jenway spectrophotometer every hour for 8 h. An exponential-fit trendline was fit to the data used to calculate the doubling time.

Statistical analysis. All statistical analyses were carried out using GraphPad Prism version 9.4.0 (GraphPad Software, San Diego, CA). Unless otherwise noted, antibiotic MICs, cocultures, *mexX* transcripts, and growth rates were analyzed using two-way analysis of variance (ANOVA). The significance of LasR$^-$ or antibiotic-dependent effects among strains was determined by finding the interaction term with alpha = 0.05 in pairwise comparisons of each strain/isolate with PA14. The significance of differences between LasR$^+$ and LasR$^-$ within each strain was determined using Sidak's multiple-comparison test in a *post hoc* analysis. Statistically significant differences are defined in the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS

This work was supported by the NIH through grants R35GM133572, CMADP COBRE (P20 GM103638), and K-INBRE (P20 GM103418) and by Inez Jay Fund to J.R.C. V.D.C. was supported by an Undergraduate Research Award from the KU Center for Undergraduate Research and a K-INBRE fellowship (P20 GM103418). K.A.T. was supported by KU Center for Undergraduate Research Emerging Scholars Program, U.S. Department of Education McNair Scholars Program, and Maximizing Access to Research Careers (MARC) (T34GM136453-01). R.G.A.-D. was supported by the Fulbright Foreign Student Program (15160174). B.M.M. was

supported by the NIH Chemical Biology Training Program (T32 GM132061). A.J.H. was supported by the NIH Bridges to Baccalaureate Program (R25 GM060182).

The authors also acknowledge Keith Poole (Queen's University) and Katy Jeannot (Université de Franche-Comté) for providing plasmids; Nicole E. Smalley and Ajai A. Dandekar (University of Washington), and Robert Unckless (University of Kansas) for the insightful suggestions; and Rishita Yadali, Isabelle Parisi, Emma Norris, and Benjamin Smith for their technical support. Molecular graphics and analyses were performed with the University of California, San Francisco (UCSF) ChimeraX, developed by the Resource for Biocomputing, Visualization, and Informatics at UCSF, with support from National Institutes of Health grant R01-GM129325 and the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases.

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