Tobramycin adaptation alters the antibiotic susceptibility of *Pseudomonas aeruginosa* quorum sensing-null mutants

Authors: Rhea G. Abisado-Duque[a, b, §], Kade A. Townsend[a, §], Brielle M. Mckee[a, §], Kathryn Woods[a], Pratik Koirala[a], Alexandra J. Holder[a], Vaughn D. Craddock[a], Matthew Cabeen[c], and Josephine R. Chandler[a, #]

[§]Contributed equally

[a]Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045

[b]Present address: Rhea G. Abisado-Duque, Department of Biology, Ateneo de Manila University, Quezon City, Philippines

[c]Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK, USA

[#]Address correspondence to Josephine R. Chandler, jrchandler@ku.edu.

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ABSTRACT (250 words)

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 allowing the *lasR*-null mutants to persist under tobramycin selection. We hypothesized that the effects of inactivating *lasR* on tobramycin resistance might be dependent on the presence or absence of other gene mutations in that strain, a phenomenon known as epistasis. To test this hypothesis, we inactivated *lasR* in several highly tobramycin-resistant isolates from long-term evolution experiments. We show that the effects of Δ*lasR* on tobramycin resistance are strain dependent, which is due to a single mutation in the *fusA1* gene encoding the translation elongation factor EF-G1A (G61A nucleotide substitution). The *fusA1* G61A mutation confers a strong selective advantage to Δ*lasR* mutants under tobramycin treatment. The effects of *fusA1* G61A on Δ*lasR*-dependent tobramycin resistance are dependent on the MexXY efflux pump and the MexXY regulator ArmZ. The *fusA1* mutation also modulates Δ*lasR* mutant resistance to two other antibiotics, ciprofloxacin and ceftazidime. Our results provide a possible explanation for the emergence of *lasR*-null mutants in clinical isolates and illustrate the importance of epistatic gene interactions in the evolution of quorum sensing.

50 **IMPORTANCE**

51 *Pseudomonas aeruginosa* causes chronic infections in the airways of patients with the genetic
52 disease cystic fibrosis. In these clinical isolates the quorum-sensing regulator LasR frequently
53 becomes mutated. The emergence of LasR-null mutants can have important consequences to
54 clinical outcomes. We performed studies of tobramycin-resistant isolates from laboratory
55 evolution experiments to understand the factors that contribute to the emergence of LasR
56 mutants. Although mutating *lasR* was deleterious in our laboratory strain, we observed that this
57 same mutation was beneficial in some of the isolates we tested. This difference was due to a
58 single mutation in the *fusA1* gene encoding the translation elongation factor EF-G1A (nucleotide
59 G61A). We show that *fusA1* G61A confers an advantage to *lasR* mutants under tobramycin
60 selection. Thus in populations undergoing adaptation to new environments, some mutations
61 could arise that permit the emergence of *lasR*-null mutations and ultimately alter the course of
62 infection.

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75 **INTRODUCTION**

76 *Pseudomonas aeruginosa* is an opportunistic multidrug resistant pathogen that regulates
77 about 10% of its genes using quorum sensing, a type of bacterial communication that is activated
78 in a population density-dependent manner (1-4). One type of quorum sensing involves a LuxR-
79 family signal receptor and a LuxI-family signal synthase (2, 5, 6). In *P. aeruginosa,* there are two
80 complete LuxI-R-type quorum-sensing systems; the LasI-R system, which produces and
81 responds to the signal *N*-3-oxododecanoyl-homoserine lactone (3OC12-HSL) (2-4, 7) and the
82 RhlI-R system, which produces and responds to the signal *N*-butanoyl-L-homoserine lactone
83 (C4-HSL) (8). In laboratory strains, the LasR-I system regulates expression of the *rhlR* and *rhlI*
84 genes, resulting in a hierarchy in quorum sensing, wherein LasR is the master regulator (5, 7, 9).
85 The LasI-R system is essential for pathogenesis in several acute infection animal models (10-12).

86 We, and others, have shown that the LasR-I system increases *P. aeruginosa* resistance to
87 tobramycin, a clinically relevant antibiotic that is commonly used to treat acute and chronic *P.*
88 *aeruginosa* infections (13-17). Quorum-sensing systems in other bacteria can also increase
89 antibiotic resistance (18), suggesting that the control of antibiotic resistance by quorum sensing
90 might provide some advantages that are conserved across several bacterial species and
91 environments. The *P. aeruginosa* LasR-I system increases tobramycin resistance in planktonic
92 cultures (17) and also in biofilm conditions (13-16). Tobramycin can also suppress the
93 proliferation of *lasR* mutants that emerge de novo during propagation of populations in certain

94 conditions in the laboratory (17). These results suggest tobramycin treatment could have a strong
95 selective pressure on quorum sensing in a clinical setting.

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

109 Here, we sought to explore another possible explanation for *lasR* mutant emergence in
110 infections of tobramycin-treated patients; that the effects of tobramycin on *lasR*-null mutants
111 might be dependent on genetic background. In particular, we hypothesized that tobramycin
112 susceptibility of Δ*lasR* mutants might be modified by epistatic gene interactions (38). We
113 identify one such mutation; a point mutation in the gene encoding the translation accessory factor
114 EF-G1A (*fusA1*). We show that the *fusA1* G61A ($FusA1^{A21T}$) mutation facilitates the emergence
115 of Δ*lasR* mutations in populations under tobramycin selection. Our results show that antibiotic

116 susceptibility of *lasR* mutants can be genotype dependent and support the idea that epistatic

117 interactions could contribute to the emergence of LasR-null mutations in a clinical setting.

118

119 **RESULTS**

120 **Δ*lasR* has strain-dependent effects on tobramycin resistance**

121 We previously characterized six tobramycin-resistant genetic isolates of *P. aeruginosa*
122 PA14 with mutations in unique genes (e.g. *fusA1* and *ptsP*, see Table S1)(17). We used these
123 isolates to test the hypothesis that antibiotic adaptations could alter the effect of Δ*lasR* mutations
124 on antibiotic resistance. To test this hypothesis, we used allelic exchange to delete *lasR* from
125 each of the six isolates from our prior study (termed T1-T6). We compared the minimum
126 inhibitory concentration (MIC) of the Δ*lasR* isolates with their parent (Fig. 1A). Similar to our
127 prior results (17), deleting *lasR* caused a small decrease in tobramycin resistance of PA14,
128 although in this study the difference was not significant due to comparisons with a wider range
129 of MICs in our statistical analyses. Deleting *lasR* in the T1 isolate increased tobramycin
130 resistance ~2-fold (Fig. 1A). Deleting *lasR* in T3 had a smaller, but similar effect (Fig. 1A). In
131 pairwise comparisons with the PA14 wild-type strain, the effects of Δ*lasR* on tobramycin MIC
132 were significantly different in T1 and T3 ($p<0.05$, Fig. 1A). We did not observe significantly
133 different effects of deleting *lasR* on the MIC for any of the other 4 isolates (Fig. S1).

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

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142 ***fusA1* (G61A) modulates the effects of Δ*lasR* on tobramycin resistance**

143 We hypothesized that one or more genetic mutations in T1 and T3 altered the
144 susceptibility of Δ*lasR* mutants to tobramycin. We previously described that the T1 and T3
145 isolates have only two common mutations, *fusA1* G61A ($FusA1^{A21T}$) and *ptsP* 1547T (a
146 frameshift mutation that is predicted to result in an inactivated protein) (Table S1)(17). We
147 individually introduced each of these mutations to PA14 and PA14 Δ*lasR* using allelic exchange
148 and compared the MIC of the mutated strains to that of the PA14 parent strains (Fig. 2A). We
149 found that deleting *lasR* in PA14 *fusA1* G61A increased the MIC of the *fusA1* G61A mutant ~2-
150 fold, which was a significantly different effect than deleting *lasR* in PA14 ($p < 0.0001$). In
151 contrast, deleting *lasR* in the *ptsP* 1547T mutant was not significantly different from that of
152 PA14 ($p=0.9865$). These results suggested that the *fusA1* G61A mutation somehow increases
153 tobramycin resistance of Δ*lasR* mutants. We next asked if ectopic expression of the wildtype
154 *fusA1* would restore the MIC to that of wild type. To do so, we fused the wild-type *fusA1* gene to
155 a rhamnose-inducible promoter (P*rha*) and introduced this cassette to the neutral *attB* site in the
156 genome of T1 and the PA14 *fusA1* G61A mutant (Fig. 2B and Fig. S2). In wild-type *fusA1*
157 expressing strains, the effects of deleting *lasR* on the tobramycin MIC were indistinguishable
158 from that of the parent (T1 or PA14 *fusA1* G61A carrying P*rha-fusA1* compared with Δ*lasR* of
159 each strain). Thus, the effects of the *fusA1* G61A mutation on *lasR*-dependent tobramycin
160 resistance can be restored by complementation with wild type *fusA1*.

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162 **Tobramycin has strain-dependent effects on the proliferation of Δ*lasR* mutants in**
163 **cocultures**

164 We sought to assess the selective effects of tobramycin on Δ*lasR* mutants in different
165 strain backgrounds using coculture competition experiments. When PA14 is co-inoculated with
166 PA14 Δ*lasR*, the Δ*lasR* mutant rapidly proliferates to higher frequency because it has a fitness
167 advantage over PA14; however, Δ*lasR* mutants are suppressed in identical cocultures grown with
168 subinhibitory tobramycin (17). We hypothesized that tobramycin would enhance rather than
169 suppress the proliferation of Δ*lasR* mutants in strains carrying the *fusA1* G61A mutation. To test
170 this hypothesis, we inoculated cocultures with either PA14 or PA14 *fusA1* G61A and a 1% initial
171 population of each respective Δ*lasR* mutant and grew the cocultures with or without tobramycin.
172 Cocultures were transferred daily to fresh medium and the proportion of Δ*lasR* in the final
173 population was assessed after 2 daily transfers. The results (Fig. 3) showed that tobramycin had
174 significantly different effects on Δ*lasR* mutant proliferation in each of the two strain
175 backgrounds ($p<0.0001$). At the end of the PA14 experiment the Δ*lasR* mutants were ~30% of
176 the population in cocultures grown with no tobramycin but reached only ~0.5% of the
177 tobramycin-treated population (Fig. 3, left), consistent with prior results (17). In cocultures with
178 strains carrying *fusA1* G61A, the Δ*lasR* mutants similarly reached ~30% of the population in the
179 absence of tobramycin but further increased to ~60% of the population in the presence of
180 tobramycin (Fig. 3, right). These results support the idea that under tobramycin selection, the
181 *fusA1* G61A mutation causes Δ*lasR* mutations to become beneficial, rather than deleterious, to
182 bacterial fitness.
183

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

188 G1A is an essential component in translation, and *fusA1*-null mutations are not viable (39-41).
189 Nevertheless, *fusA1* has been reported to be among the most frequently mutated genes in clinical
190 isolates (42-45), and in at least some cases *fusA1* mutations increase tobramycin resistance (41,
191 42, 46). EF-G1A has 5 domains (labeled I-V in Fig. 4A). The FusA1 A21T substitution is within
192 a motif in domain 1 called the Walker-A P-loop, which is responsible for binding phosphoryl
193 groups and catalyzing phosphoryl transfer of NTPs (47). $FusA1^{A21T}$ has not been reported in the
194 literature to our knowledge, however, a survey of 4,312 sequenced strains identified 4 strains
195 with this mutation (Table S1), indicating *fusA1* G61A mutations do occur naturally but are
196 relatively rare.

197 Several other mutations in *fusA1* have been characterized (Fig. 4A)(41). To test whether
198 these mutations enhance or alter tobramycin resistance of Δ*lasR* mutants, we introduced five
199 *fusA1* mutations to the genome of PA14 or PA14 Δ*lasR* by allelic exchange and determined the
200 tobramycin MIC of each strain (Fig. 4B). In all but one (A1366G) of the *fusA1*-mutated strains,
201 the effect of LasR on antibiotic resistance was not significantly altered compared with that of
202 PA14. In *fusA1* A1366G, the Δ*lasR* mutation decreased tobramycin resistance compared with the
203 $LasR^{+}$ strain, which is the opposite of the effect observed with the G61A mutation. These results
204 show that not all *fusA1* mutations modulate tobramycin resistance of Δ*lasR* mutants and that
205 different mutations have independent effects.

206

207 **_fusA1_ G61A modulates Δ_lasR_ resistance to other antibiotics**

208 We next asked whether the *fusA1* G61A mutation can also alter the susceptibility of Δ*lasR*
209 mutants to antibiotics other than tobramycin. We tested the antibiotics ciprofloxacin (DNA

210 gyrase inhibitor), ceftazidime (cell wall biosynthesis inhibitor) piperacillin (cell wall
211 biosynthesis inhibitor), and tetracycline (ribosome inhibitor) against PA14 and our *fusA1* and
212 *lasR* single and double mutant strains (Table 1). We observed significant strain-dependent effects
213 of the Δ*lasR* mutation on the MIC values for two of these antibiotics: ciprofloxacin ($p < 0.01$)
214 and ceftazidime ($p < 0.0001$). With ciprofloxacin, Δ*lasR* increased resistance of the *fusA1* G61A
215 mutant but this mutation caused no changes in the PA14 MIC. With ceftazidime we observed
216 different effects: the Δ*lasR* mutation decreased resistance in PA14 but not in the *fusA1* G61A
217 mutant. These results show that *fusA1* G61A mutations modulate susceptibility of Δ*lasR* mutants
218 to several different classes of antibiotics.

219

220 ***fusA1* G61A and Δ*lasR* increase tobramycin resistance by activating the MexXY efflux**
221 **pump**

222 Some other *P. aeruginosa fusA1* mutations can enhance aminoglycoside resistance
223 through the multidrug efflux pump MexXY (41, 48). Thus, we hypothesized that MexXY is
224 important for increasing the tobramycin resistance of *fusA1* G61A and Δ*lasR* double mutants. As
225 an initial test of this hypothesis, we measured expression of the *mexX* gene, which is
226 immediately upstream of and in the same operon as *mexY* (Fig. 5A). We quantified *mexX*
227 transcripts in logarithmically growing cells of PA14 *fusA1* G61A and Δ*lasR* single and double
228 mutants. Consistent with our hypothesis, *mexX* transcripts were the highest in *fusA1* G61A and
229 Δ*lasR* double mutants. We also deleted the *mexY* gene that is essential for pump activity. Our
230 attempts to delete *mexY* in *fusA1* G61A were unsuccessful; however, we were able to delete this
231 gene in T1 and T1 Δ*lasR*, and also PA14 and PA14 Δ*lasR*. Deleting Δ*mexY* in T1 abolished the

232 LasR-dependent changes in MIC observed in this strain (Fig. 5B), supporting that MexY is
233 important for *fusA1* G61A to increase tobramycin resistance of Δ*lasR* mutants.

234 One mechanism of MexXY induction is through the ArmZ regulator (49, 50), which is
235 activated by a mechanism of transcription attenuation in response to perturbations that cause the
236 ribosome to stall (50-52). ArmZ de-represses the MexXY regulator MexZ; thus activation of
237 ArmZ ultimately leads to increased MexXY activity (49, 50, 53). To test the hypothesis that
238 ArmZ is required for MexXY activation in *fusA1* G61A and Δ*lasR* double mutants, we deleted
239 *armZ* in T1 and the T1 Δ*lasR* mutants and determined the MIC of these strains. Similar to the
240 result of deleting *mexY,* we found that deleting *armZ* abolished LasR-dependent changes in MIC
241 observed in T1 (Fig. 5C). Together, the results support that MexXY and the ArmZ regulator are
242 both required for *fusA1* G61A to increase the tobramycin resistance of Δ*lasR* mutants.

243 The ArmZ regulator is activated by ribosome stalling, and *fusA1* mutations are associated
244 with slower translation rates (41, 48, 54). Thus, we hypothesized that the *fusA1* G61A mutation
245 might cause ribosome stalling effects that are enhanced in combination with Δ*lasR*. To test this
246 hypothesis, we measured the doubling times of our *fusA1* G61A and Δ*lasR* mutant strains during
247 logarithmic growth in minimal media, as growth rates can be an indirect indication of translation
248 rate. We found that *fusA1* G61A alone had no significant effect on growth, however, in
249 combination with Δ*lasR*, we observed slowed growth (Table 2). These results support the idea
250 that MexXY activation in Δ*lasR* and *fusA1* G61A double mutants might be caused by increased
251 ribosome stalling.

252

253

254 **DISCUSSION**

255 In this study, we demonstrate that *lasR*-null mutations have strain-dependent effects on
256 resistance to certain antibiotics, which are caused by specific mutations in the translation
257 accessory *fusA1* gene (G61A). These results add to the growing body of work supporting that
258 adaptive mutations can cause unexpected effects on quorum-sensing phenotypes. For example, in
259 a prior study we showed that *ptsP* mutations can influence LasR-dependent cooperation and
260 cheating dynamics (17). In addition, inactivation of the efflux pump regulator MexT enables
261 activation of the RhlR-I system as well as the non-AHL Pseudomonas quinolone signal (PQS) in
262 *lasR*-null mutants (55-57). These are all examples of epistatic gene interactions, where the effect
263 of one gene mutation is modified by mutations in other genes (38). Epistatic interactions have
264 consequences for evolutionary outcomes because they can place constraints on adaptation; for
265 example, some mutations will become favorable only after other mutations have accumulated
266 (58). These studies highlight how epistatic gene interactions can contribute to the evolution of
267 quorum sensing during adaptation to changing environments.

268 Our finding that *fusA1* G61A mutations modulate the effects of Δ*lasR* on tobramycin
269 resistance are consistent with a type of epistasis called sign epistasis. Sign epistasis is a
270 phenomenon where a trait is beneficial in certain genetic backgrounds and harmful in others (58-
271 60). Under tobramycin selection, Δ*lasR* mutations were deleterious in the laboratory strain PA14
272 but beneficial in *fusA1* G61A mutant strains (Fig. 3). Sign epistasis has particularly dramatic
273 effects on evolution because it can reverse the effects of selection (61, 62). Sign epistasis has
274 been demonstrated in *P. aeruginosa* in the context of rifampicin resistance (63), where
275 combinations of mutations causing rifampicin resistance were shown to have epistatic effects on
276 fitness in the absence of any antibiotic. About half of the interactions studied showed epistatic

277 effects on fitness, and a surprisingly high number of these (84%) were sign epistatic. Sign
278 epistasis has also been demonstrated in the development of cefotaxime resistance in *Escherichia*
279 *coli* (64) and the evolution of antibiotic resistance in *Mycobacterium tuberculosis* (65). Such
280 studies illustrate that sign epistatic interactions are strikingly common across a variety of gene
281 mutations and bacterial species. Our findings provide a proof-of-principle that sign epistasis can
282 cause dramatic effects on the evolution of quorum sensing by reversing the effects of antibiotic
283 selection of *lasR* mutants. It will be interesting to determine whether mutations other than those
284 characterized in our study can have similar effects.

285 The *fusA1* G61A mutation had relatively mild effects in a wild type strain, but more
286 severe growth defects in combination with the *lasR* mutation (Table 2). These results suggest
287 that the deleterious effects of the *fusA1* G61A mutation may be compensated by some unknown
288 function of LasR. In the case of other *fusA1* mutations, it is thought that the mutations in the
289 encoded EF-G1A protein cause the ribosome to remain "stuck" on the mRNA, which might
290 cause stalling when other ribosomes get backed up behind it (66). We posit that LasR activates
291 some function that somehow protects from ribosome stalling. Consistent with FusA1^{A21T} having
292 a defect that is exacerbated by loss of LasR, expression of wild-type, fully functional *fusA1*
293 abrogated the effects of *lasR* deletion on tobramycin resistance in a *fusA1* G61A background
294 (Fig. 2B). Future studies are aimed at understanding how LasR might compensate for the
295 deleterious effects of *fusA1* G61A mutations.

296 We found it interesting that one other *fusA1* mutation (A1366G) had the opposite effect
297 as the G61A mutation. The *fusA1* A1366G mutation decreased tobramycin resistance of Δ*lasR*
298 mutants (Fig. 4B), whereas the *fusA1* G61A mutation increased resistance. From these results,
299 we posit that LasR may enhance the deleterious effects of the A1366G *fusA1* mutation rather

300 than providing protection as we propose for G61A *fusA1*. Most other *fusA1* mutations did not
301 have interactive effects with *lasR.* These results show that LasR and *fusA1* gene interactions can
302 be neutral, beneficial or deleterious depending on the type of *fusA1* mutation. Our results support
303 the idea that different *fusA1* mutations increase antibiotic resistance through different pathways.
304 Our result that some mutations increase antibiotic sensitivity of Δ*lasR* mutants opens the door to
305 the intriguing possibility of combination therapies that rely on epistatic gene interactions. For
306 example, combining a LasR inhibitor with a therapeutic that enhances antibiotic sensitivity of a
307 *lasR* mutant could be an effective novel approach to therapeutic design.

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309

310 **MATERIALS AND METHODS**

311 **Culture conditions and reagents**

312 Routine growth was in Luria-Bertani broth (LB) for *Escherichia coli* or in LB buffered to
313 pH 7 with 50 mM 3-(morpholino)-propanesulfonic acid (LB-MOPS) for *Pseudomonas*
314 *aeruginosa* or on LB agar (LBA; 1.5% wt/vol Bacto Agar). Liquid growth media for specific
315 experiments were M9-caseinate (casein broth); [6 g liter^{-1} Na_2HPO_4, 3 g liter^{-1} KH_2PO_4, 0.5 g
316 liter^{-1} NaCl, 1 g liter^{-1}NH_4Cl, pH 7.4, 1% sodium caseinate)](17), or a MOPS minimal medium
317 [25 mM d-glucose, freshly prepared 5 μM $FeSO_4$, 15 mM NH_4Cl, and 2 mM K_2HPO_4 added to a
318 1× MOPS base buffer consisting of 50 mM MOPS, 4 mM Tricine, 50 mM NaCl, 1 mM K_2SO_4,
319 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,
320 1 μM $CuSO_4$, 8 μM $MnSO_4$, and 1 μM $ZnSO_4$] (17, 67, 68). 4% skim milk agar (SMA) was used
321 for identifying Δ*lasR* mutants in competition experiments. All growth was at 37ºC and liquid

322 cultures were grown with shaking at 250 rpm in 18 mm culture tubes (2-ml cultures), 125 ml
323 baffled flasks (10-ml cultures) or 250-ml baffled flasks (60-ml cultures). For strain construction,
324 we used 100 μg ml^{-1} carbenicillin, 15 μg ml^{-1} gentamicin, and 2.5–10 μg ml^{-1} tetracycline for *E.*
325 *coli* and 150–300 μg ml^{-1} carbenicillin, 50–200 μg ml^{-1} gentamicin, and 15–100 μg ml^{-1}
326 tetracycline for *P. aeruginosa.* 3OC12-HSL was purchased from Cayman Chemicals (MI, USA),
327 dissolved in acidified ethyl acetate with glacial acetic acid (0.1 ml l^{-1}) and added to an empty
328 sterile conical tube and dried by evaporation prior to adding liquid media.

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

334

335 **Bacterial strains and strain construction**

336 All bacterial strains, plasmids, and primers used in this study are provided in the
337 Supplementary Material. *P. aeruginosa* strain UCBPP-PA14 ('PA14') and PA14 derivatives
338 were used for these studies. Markerless deletions in specific loci of *P. aeruginosa* PA14 were
339 generated using allelic exchange as described previously (69). To generate plasmids for allelic
340 exchange, DNA fragments with the mutated or deleted gene allele plus 500 bp flanking DNA
341 were synthesized (Genscript, New Jersey USA) or generated by PCR using primer-incorporated
342 restriction enzyme sites. The synthesized or PCR product were moved to plasmid pEXG2 (70)
343 using restriction enzyme digestion and ligation or isothermal assembly. The subsequent plasmids

344 were transformed into the appropriate *P. aeruginosa* strain using described methods (71). The
345 plasmids for Δ*lasR* (68), Δ*mexY* (72), and Δ*armZ* (53) were described elsewhere. Merodiploids
346 were selected on Pseudomonas Isolation Agar (PIA)-carbenicillin (150–300 μg ml^{-1}) for Δ*lasR*;
347 PIA-gentamicin (50–200 μg ml^{-1}) for *fusA1* G61A, Δ*lasI*, and *ptsP* 1547T; and PIA-tetracycline
348 (15–100 μg ml^{-1}) for Δ*mexY* and Δ*armZ*. Deletion mutants were counterselected using NaCl-free
349 15% sucrose. Putative mutants were verified through antibiotic sensitivity tests and gene-targeted
350 Sanger sequencing. To make the P*rha-fusA1* expression cassette, we PCR-amplified the wild-
351 type *fusA1* gene from PA14 using primer-encoded restriction enzyme sites. The PCR product
352 was digested and ligated to pJM253 (miniCTX1-rhaSR-PrhaBAD)(73). This plasmid was moved
353 into *P. aeruginosa* by conjugation as described previously (73). Transformants were selected on
354 LBA with 200 μg ml^{-1} tetracycline and the insertion of the cassette in the *attB* site was verified
355 by PCR.

356

357 **Antimicrobial susceptibility assays**

358 Antibiotic susceptibility was determined by MIC using a modified method from the 2022
359 guidelines of the Clinical and Laboratory Standards Institute (CSLI) (74), similar to that
360 previously described (17). Briefly, two antibiotic dilution series were made from staggered
361 starting antibiotic concentrations to cover a broader range of concentrations in MOPS minimal
362 medium, and successively diluted 2-fold in a 200 μl volume in 2 ml tubes. The starter cultures
363 were prepared by growing *P. aeruginosa* in LB-MOPS to an optical density at 600 nm (OD_{600})
364 of 4. The starter cultures were subsequently diluted 1:40 into each tube containing tobramycin to
365 start the MIC experiment. After 20 h of incubation with shaking, turbidity was measured using a

366 Biotek Synergy 2 plate reader. The MIC was defined as the lowest concentration of tobramycin
367 (μg ml^{-1}) in which bacterial growth was not measurable.

368 **Coculture assays**

369 Overnight (18 h) pure cultures were grown in LB-MOPS, diluted to an OD_{600} of 0.025 for
370 $LasR^-$ or 0.05–0.15 for $LasR^+$ into LB-MOPS, and grown to an OD_{600} of ~3.5 before combining
371 at a 99:1 ($LasR^+$: $LasR^-$) ratio and used to start the coculture by diluting 1:40 into casein broth in
372 18 mm test tubes. In some cases as indicated, tobramycin was added to the casein broth
373 coculture. At 24 h, cocultures were diluted 1:40 into fresh casein broth in a new test tube and the
374 experiment was ended at 48 h. The initial and final total population counts (CFU ml^{-1}) were
375 determined by dilution plating and viable plate counts. The % Δ*lasR* mutant was determined by
376 patching 200 colonies on SMA where Δ*lasR* mutants form distinct colony phenotypes (17, 22,
377 23, 75).

378

379 **Droplet digital PCR**

380 Overnight (18 h) pure cultures were grown in LB-MOPS, subcultured in LB-MOPS, and
381 grown to an OD_{600} of ~4. Stationary-phase *P. aeruginosa* (OD_{600} of 4) was diluted to OD_{600} 0.1
382 in MOPS minimal medium and grown 2.5-3 hours (OD_{600} ~0.20-0.45). RNA was harvested using
383 the RNeasy minikit (Qiagen), following the manufacturer's instructions. Droplet digital PCR
384 (ddPCR) was performed on a Bio-Rad QX200 ddPCR system using Eva Green supermix. Each
385 reaction mixture contained 1.8 ng μl^{-1} cDNA template, 0.8 μM of each primer, and 10 μl Eva
386 Green supermix in a 20-μl final volume. After generating 40 μl of oil droplets, 40 rounds of PCR
387 were conducted using the following cycling conditions: 95°C for 30s, 62°C for 30s, and 68°C for

388 30s. Absolute transcript levels were determined using Bio-Rad QuantaSoft software. In all cases,
389 a no-template control was run with no detectable transcripts. The reference gene used was
390 proline biosynthetic gene (*proC*), and the results are reported as the calculated transcript amount
391 of a given gene per calculated *proC* transcript.

392

393 **Growth Curve**

394

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

399

400 **Statistical analysis**

401 All statistical analyses were carried out using GraphPad Prism version 9.4.0 (GraphPad Software,
402 San Diego, CA). Unless otherwise noted, antibiotic MICs, cocultures, *mexX* transcripts and growth
403 rates were analyzed using 2-way analysis of variance (ANOVA). Significance of LasR- or
404 antibiotic-dependent effects among strains was determined by finding the interaction term with
405 alpha = 0.05 in pair-wise comparisons of each strain/isolate with PA14. Significance of
406 differences between $LasR^+$ and $LasR^-$ within each strain was determined using Sidak's multiple
407 comparisons test in a posthoc analysis. Statistically significant differences are defined in the figure
408 legends.

409

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427

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641

642

643 **TABLES**

644 **Table 1.** Antibiotic susceptibility of *lasR* and *fusA1* G61A mutant strains.

645

	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 the 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 2-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 significance of comparing $LasR^+$ and $LasR^-$ of each strain (PA14 or *fusA1* G61A) using Sidak's posthoc analysis with p-values adjusted for multiple comparisons of all strains. No significance ($p>0.05$) unless indicated. **, $p<0.01$, **** $p<0.0001$.

668 **Table 2.** Growth rates in MOPS minimal medium[a].

669

670 671 672

Strain/Isolate	Doubling time (min)[a, b]	strain	*lasR*	interaction
673 PA14	62.5 (2.0)			
674 PA14 Δ*lasR*	61.4 (2.9)			
675 *fusA1* G61A	66.3 (2.9)	p<0.0005	p<0.01	p<0.005
676 *fusA1* G61A Δ*lasR*	78.2 (2.2)[***, c]	F = 49.91	F = 13.81	F = 19.73
677 T1	67.7 (4.0)	p<0.0005	p<0.02	p<0.005
678 T1 Δ*lasR*	79.7 (2.5)[***]	F = 47.61	F = 10.41	F = 14.81
679 T3	74.9 (3.4)	p<0.0001	ns	p<0.04
680 T3 Δ*lasR*	83.0 (3.5)[*]	F = 96.27	F = 4.084	F = 6.930

681

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

685 [b] The significance of the effects of strain (PA14, *fusA1* G61A, T1 or T3) and *lasR* allele (intact
686 *lasR* or Δ*lasR*) and the interaction of the two on MIC was determined by 2-way ANOVA by
687 performing pairwise comparisons of each $LasR^{+/-}$ strain pair with that of PA14. The interaction
688 was significant in all cases. For *fusA1* G61A p<0.005 and F = 19.73; for T1 p<0.005 and F =
689 14.81; for T3 p<0.04 and F = 6.930.

690 [c] Superscripted asterisks indicate significance of comparing $LasR^+$ and $LasR^-$ of each strain using

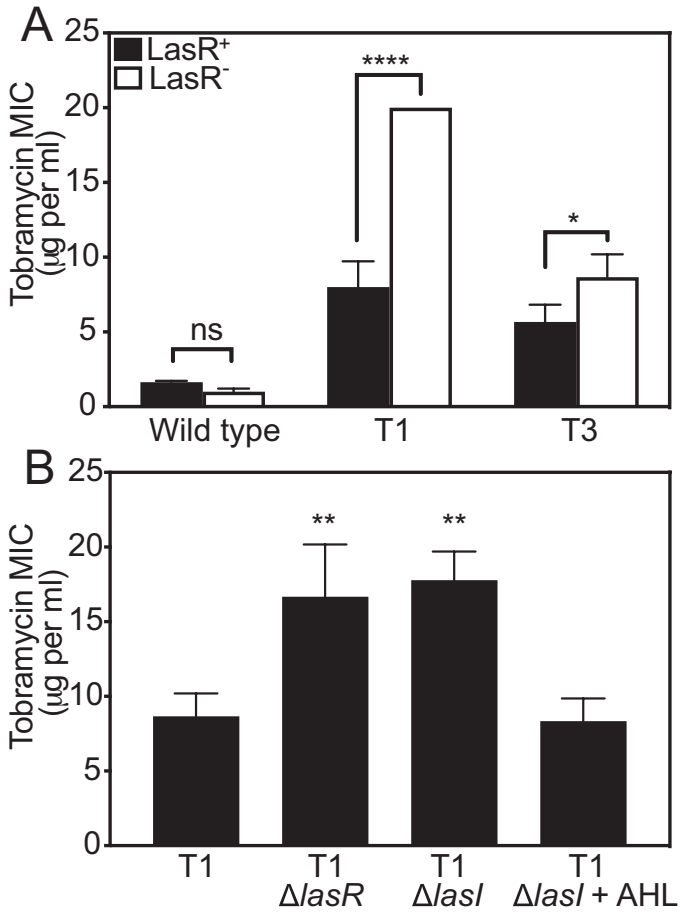
691 Sidak's posthoc analysis with p-values adjusted for multiple comparisons of all strains. No

692 significance ($p>0.05$) unless indicated. * $p<0.05$, *** $p<0.001$.

693

694 **FIGURES**

695



696

697 **Fig. 1. Tobramycin resistance of Δ*lasR* mutants is strain dependent. A.** The minimum
698 inhibitory concentration (MIC) of tobramycin was determined for each strain carrying intact *lasR*
699 ($LasR^+$, black bars) or Δ*lasR* ($LasR^-$, white bars). The significance of the effect of strain (wild
700 type, T1 or T3) and *lasR* allele ($LasR^+$ or $LasR^-$) and the interaction of the two on MIC was
701 determined by 2-way ANOVA by performing pair-wise comparisons of each $LasR^{+/-}$ strain pair
702 with that of PA14. The interaction was significant for T1 ($F_{1,8}$ = 156.8 and $p<0.0001$) and T3
703 ($F_{1,8}$ = 10.67 and $p < 0.05$). Pairwise comparisons of $LasR^+$ and $LasR^-$ of each strain were
704 determined using Sidak's post-hoc test with p-values adjusted for multiple comparisons; *,
705 $p<0.05$; ****, $p < 0.0001$; ns = not significant. **B.** Tobramycin MIC of T1 and T1-mutated

706 strains. Where indicated, AHL (3OC12-HSL) was added prior to inoculating culture tubes at 10
707 μM final concentration. Statistical analysis was by one-way ANOVA and Dunett's multiple
708 comparisons with T1; **, $p<0.01$. For both panels, results are the average of three independent
709 experiments and the vertical bars indicate standard deviation.

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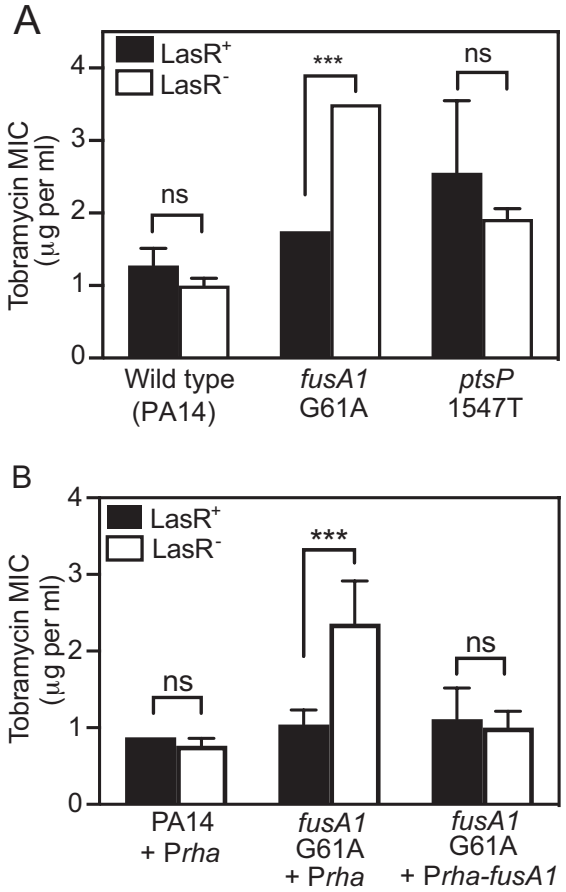

712

713 **Fig. 2. Effects of *fusA1* G61A and Δ*lasR* on tobramycin resistance.** The minimum inhibitory
714 concentration (MIC) of tobramycin was determined for each strain carrying intact *lasR* ($LasR^+$,
715 black bars) or Δ*lasR* ($LasR^-$, white bars). The significance of the effect of strain (indicated along
716 the X axis) and *lasR* allele ($LasR^+$ or $LasR^-$) and the interaction of the two on MIC was determined
717 by 2-way ANOVA by performing pair-wise comparisons of each $LasR^{+/-}$ strain pair with that of
718 PA14 (A) or PA14 P*rha* (B). **A.** There was a statistically significant interaction between the effects
719 of strain and *lasR* for *fusA1* G61A ($F_{1,8} = 156.8$, $p < 0.0001$) but not for *ptsP* 1547T ($F_{1,8} = 0.3642$,
720 $p = 0.5629$). **B.** Strains had either the empty CTX-1 P*rha* or the CTX-1 P*rha-fusA1* cassette
721 inserted into the neutral *attB* site in the genome. Rhamnose was added to all cultures at 0.4% final
722 concentration. There was a statistically significant interaction between the effects of strain and

723 *lasR* for *fusA1* G61A P*rha* ($F_{1,8}$ = 17.41, $p < 0.005$) but not for *fusA1* G61A + P*rha-fusA1* ($F_{1,8}$ =
724 1.7×10^{-7}, $p = 0.997$). For both panels, results are the average of three independent experiments and
725 the vertical bars indicate standard deviation. Pair-wise comparisons of $LasR^+$ and $LasR^-$ of each
726 strain were determined using Sidak's post-hoc test with p-values adjusted for multiple
727 comparisons; ***, $p<0.001$; ns = not significant.

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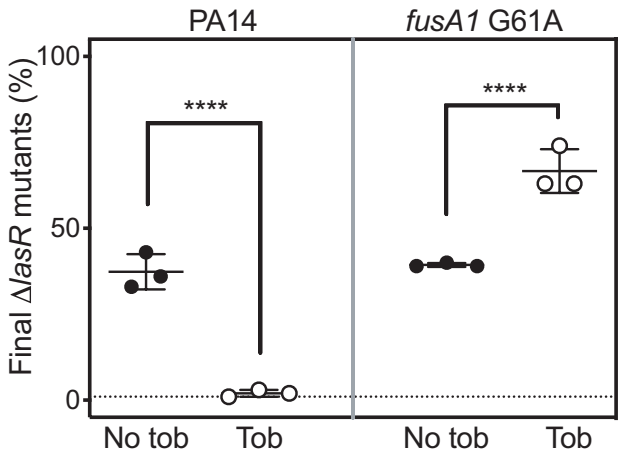

729

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

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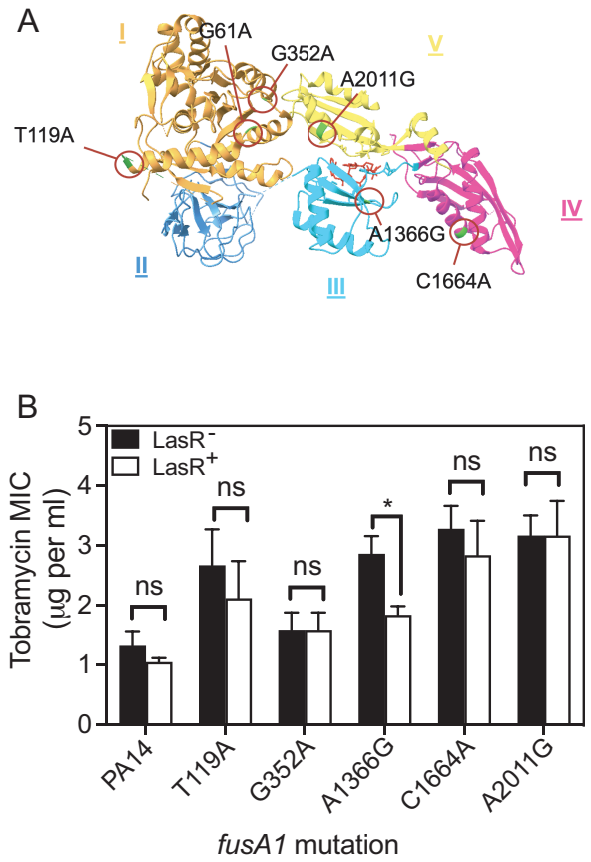

745

746 **Fig. 4. Other *fusA1* mutations and their effects on tobramycin resistance of Δ*lasR* mutants.**
747 **A.** Ribbon diagram of the *fusA1*-encoded protein Elongation Factor-G (EF-G) (PDB ID: 4FN5).
748 The protein was crystallized bound to argyrin, which is shown in red. Each of the mutations
749 assessed in panel B are indicated with green coloration in the ribbon diagram and a red circle and
750 were identified using the UCSF ChimeraX (76) **B.** The minimum inhibitory concentration (MIC)
751 of tobramycin was determined for each strain carrying the wild type *fusA1* allele (PA14) or PA14
752 with the indicated *fusA1* nucleotide mutation. Each strain had either an intact *lasR* ($LasR^+$, black
753 bars) or Δ*lasR* ($LasR^-$, white bars). Results are the average of three independent experiments and
754 the vertical bars indicate standard deviation. The significance of the effect of the *fusA1* allele and
755 the *lasR* allele and the interaction of the two on MIC was determined by 2-way ANOVA by
756 performing pair-wise comparisons of each $LasR^{+/-}$ strain pair with that of PA14. Only A1366G

757 showed a significant interaction ($p<0.05$, $F_{1,8} = 10.25$). Pairwise comparisons of $LasR^+$ and $LasR^-$
758 of each strain was determined using Sidak's post-hoc test with p-values adjusted for multiple
759 comparisons. *, $p<0.05$.

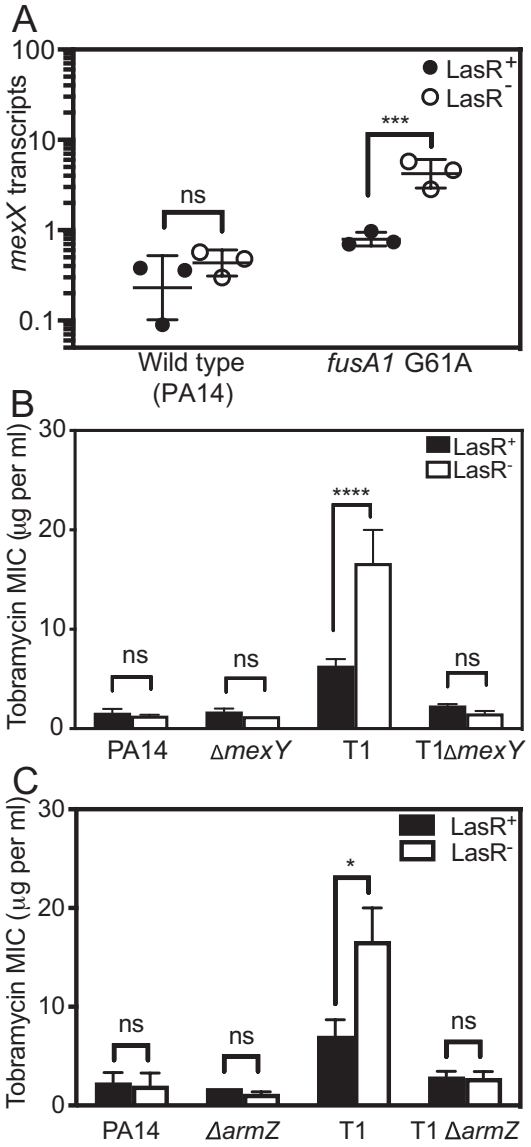

Fig. 5. Role of MexXY on tobramycin resistance of Δ*lasR* and *fusA1* G61A mutants. A. *mexX* transcript levels were determined by droplet digital PCR and normalized to the housekeeping control gene *proC.* Strains were wild type PA14 or PA14 with the *fusA1* G61A substitution. Each strain had either 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 2-way ANOVA showed a significant interaction between the effects of strain and *lasR* allele on *mexX* transcripts

768 ($p<0.005$, $F_{(1, 8)} = 15.67$). **B and C.** The minimum inhibitory concentration (MIC) of tobramycin
769 was determined for each strain carrying intact *lasR* ($LasR^+$, black bars) or Δ*lasR* ($LasR^-$, white
770 bars). Results are the average of three independent experiments and the vertical bars show standard
771 deviation. In pair-wise comparisons with the $LasR^{+/-}$ PA14 strains there was a significant
772 interaction between the effects of strain and *lasR* allele for T1 ($p<0.01$ and $F_{(1,8)} = 29.01$ for panel
773 B and $p<0.005$ and $F(1,8) = 17.99$ for panel C) but not for the other strains. In all three panels,
774 pairwise comparisons of $LasR^+$ and $LasR^-$ of each strain was determined using Sidak's post-hoc
775 test with p-values adjusted for multiple comparisons. *, $p<0.05$; **, $p<0.01$, ***, $p < 0.001$****,
776 $p < 0.0001$, ns = not significant.

777