TITLE

Reactive Oxygen Detoxification Contributes to *Mycobacterium abscessus* Antibiotic Survival

AUTHORS

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ABSTRACT

When a population of bacteria encounter a bactericidal antibiotic most cells die rapidly. However, a sub-population, known as “persister cells”, can survive for prolonged periods in a non-growing, but viable, state. Persister cell frequency is dramatically increased by stresses such as nutrient deprivation, but it is unclear what pathways are required to maintain viability, and how this process is regulated. To identify the genetic determinants of antibiotic persistence in mycobacteria, we

carried out transposon mutagenesis high-throughput sequencing (Tn-Seq) screens in *Mycobacterium abscessus* (*Mabs*). This analysis identified genes essential in both spontaneous and stress-induced persister cells, allowing the first genetic comparison of these states in mycobacteria, and unexpectedly identified multiple genes involved in the detoxification of reactive oxygen species (ROS). We found that endogenous ROS were generated following antibiotic exposure, and that the KatG catalase-peroxidase contributed to survival in both spontaneous and starvation-induced persisters. We also found that that hypoxia significantly impaired bacterial killing, and notably, in the absence of oxygen, KatG became dispensable. Thus, the lethality of some antibiotics is amplified by toxic ROS accumulation, and persister cells depend on detoxification systems to remain viable.

INTRODUCTION

A key goal of antibiotic therapy is the complete eradication of the pathogen. While many common infections respond rapidly to antibiotics, and high cure rates are achieved with 1-2 weeks of therapy[1,2], there are also infections where bacterial clearance is either very slow, or frequently incomplete. This challenge is exemplified by mycobacterial infections, where treatment courses extend from months to years to prevent relapse.

While the ability of mycobacteria to escape antibiotic-mediated killing is multifactorial, the phenomenon of antibiotic “persistence” is likely an important contributor[3-6]. Studies on penicillin dating from the 1940s noted that when a population of susceptible bacteria were exposed to a bactericidal antibiotic, the majority of the population died within a few hours, but that a small sub-population of ‘persisters’ remained viable for over a week[7]. Importantly, these persister cells had not acquired a mutation conferring heritable antibiotic resistance, and do not grow in the presence of the antibiotic. Rather, they have entered into a readily-reversable epigenetic state[8-10] where, despite antibiotic-mediated inhibition of critical processes, they are able to survive. Virtually all

53 bacterial species display the ability to form persister cells, and their development is strongly
54 induced in response to stresses such as nutrient deprivation or acidic pH[6-7,11-12]. Notably, these
55 same stresses are encountered in the lysosome of an activated immune cell[13], and studies of
56 pathogens isolated from activated macrophages indeed show a strong immune-mediated
57 increase in persister cells[5,14]. Thus, paradoxically, the immune system may actually impede
58 bacterial eradication by antibiotics.
59
60 Persister formation has been studied extensively in model systems such as *Escherichia coli*,
61 which has provided important insights, but also highlighted uncertainties of current models.
62 Several different pathways have been implicated in *E. coli* persister formation, including the HipBA
63 toxin-antitoxin system[15], guanosine pentaphosphate ((p)ppGpp) synthesis by RelA/SpoT
64 enzymes[16,17], and Lon protease[16]. In each of these models, the postulated mechanism is to halt
65 cell division and render the process targeted by antibiotics non-essential. However, important
66 questions remain unanswered. It is unclear how persister cells remain viable when critical
67 processes such as transcription or translation are arrested by antibiotics, as well as how the
68 process is regulated and induced by stress.
69
70 Even the mechanism of cell death following antibiotic exposure itself remains uncertain, and
71 somewhat controversial. Historically, antibiotics have been presumed to kill bacteria as a direct
72 result of inhibition of their target molecule, such as β-lactam antibiotics disrupting cell wall integrity,
73 directly leading to mechanical cell lysis[18]. However, a number of studies, largely from *E. coli*, have
74 suggested that reactive oxygen species (ROS) accumulation triggered by antibiotic stress might
75 also contribute to cell death[10,19-20]. Conversely, other studies have found no such association[21,22],
76 leaving the role of ROS in antibiotic-mediated cell death unresolved.
77

78 Studying persister cell physiology in mycobacterial pathogens offers several advantages.
79 Mycobacterial persister cells are particularly resilient, as *Mycobacterium smegmatis* (*Msmeg*) and
80 *Mycobacterium tuberculosis (Mtb)* persisters can endure many weeks of antibiotic exposure[10,23-
81 24]. Clinically, mycobacterial infections are difficult to eradicate. Fully-susceptible *Mtb* requires
82 multiple antibiotics for four months or longer[25-26], whereas non-tuberculous mycobacteria typically
83 require treatment for 12-18 months and have a relapse rates of roughly 50%[27]. *Mycobacterium*
84 *abscessus* (*Mabs*) is among the most difficult of all bacterial pathogens to treat, because in
85 addition to the possibility of forming persister cells, it is intrinsically resistant to many classes of
86 antibiotics, leaving few treatment options[28]. This leads to the use of antibiotics with greater toxicity
87 to patients, and a need to use these agents for prolonged periods to prevent relapse. Thus,
88 identifying the genes that *Mabs* persister cells rely on for survival could be beneficial by
89 highlighting pathways that might be targeted therapeutically to eliminate persister cells.
90
91 Previous genetic screens have studied antibiotic responses in mycobacteria, with some
92 evaluating heritable resistance, and others investigating persister cell formation. Several studies
93 of genetic resistance have successfully used either transposon mutagenesis with high-throughput
94 sequencing of insertion sites (Tn-Seq) or CRISPR-based transcriptional repression with high-
95 throughput sequencing of guide RNAs (CRISPRi) to identify genes promoting growth in sub-
96 inhibitory concentrations of antibiotic. These studies have provided insights such as the
97 importance of cell membrane permeability controlling antibiotic penetration into the cytoplasm[29-
98 31]. Persister cell formation has proven challenging to study, likely because their low frequency leads
99 to population bottlenecks that confound genetic analysis. Although screens in *Mtb* have been
100 conducted in macrophages and mice, and genes such as *glpK* and *cinA* identified, overall the
101 number of mutants isolated in these screens has been low[32-34]. There has been one effective in
102 vitro Tn-Seq study of rifampin survival in *Mtb* that isolated over 100 mutants[19], demonstrating the

103 feasibility of genetic screening in this context. However, whether these phenotypes seen with
104 rifampin in *Mtb* extend to other organisms and other antibiotics remains to be determined.
105
106 Here, we study persister cell formation in *Mabs*, and describe the results of genome-wide Tn-seq
107 screens seeking to identify the genes required for both spontaneous and starvation-induced
108 antibiotic persistence. We identified diverse pathways contributing to persister cell survival, and
109 unexpectedly, observed a prominent role for ROS detoxifying factors such as the catalase-
110 peroxidase enzyme KatG, which contributed to both spontaneous and starvation-induced
111 persistence. We found that endogenous ROS were generated following antibiotic exposure, and
112 that hypoxia significantly impaired bacterial killing. Thus, the lethality of some antibiotics is
113 amplified by toxic ROS accumulation, and persister cells require detoxification systems.
114
115 **RESULTS**
116 **Starvation-induced persister cell formation in mycobacteria**
117 We first sought to develop conditions suitable for genetic analysis of antibiotic persistence in
118 mycobacteria. Genetic screens examining persister cell physiology faces two inherent obstacles.
119 First, persister cells are rare in unstressed bacterial populations, and antibiotic-mediated cell
120 death creates population bottlenecks that obscure mutant phenotypes. Second, most
121 mycobacterial populations contain spontaneous drug-resistant mutants that can expand if the
122 population is exposed to a single antibiotic. To overcome these obstacles, we sought to establish
123 large-scale, high-density culture conditions to prevent genetic bottlenecks, and used multiple
124 antibiotics to suppress expansion of spontaneous drug-resistant mutants. We began by assessing
125 the feasibility of this approach using wild-type *Msmeg.* We exposed the cells to either the
126 combination of rifampin, isoniazid, and ethambutol (RIF/INH/EMB) used to treat *Mtb*, or to the
127 combination of tigecycline and linezolid (TIG/LZD), two translation-inhibiting antibiotics frequently
128 used to treat *Mabs*[27,35], and empirically determined the minimum-inhibitory concentrations (MICs)

129 each antibiotic under the high-density culture conditions that would be needed for genetic analysis
130 of antibiotic persister cells. Both antibiotic combinations reduced the bacterial population >1000-
131 fold within 72h (Figure 1A). We then evaluated both spontaneous and stress-induced persister
132 cell formation under these conditions in *Msmeg*. We compared logarithmically growing (mid-log)
133 cultures in 7H9 rich media to cultures starved for 2 days in PBS prior to addition of antibiotics.
134 Consistent with expectations, we found a marked increase in the frequency of persister cells in
135 starved cultures, with a 100-fold increase in survival following TIG/LZD exposure and a 10,000-
136 fold increase following RIF/INH/EMB exposure (Figure 1A).
137
138 We next examined two species of pathogenic mycobacteria to similarly assess stress-induced
139 persister formation under these conditions, as has been reported previously[36-39]. We again
140 compared cells starved in PBS to mid-log cells growing 7H9, and found that cultures of wild-type
141 *Mabs* (ATCC 19977 strain) and *Mtb* (Erdman) also displayed dramatic increases in the frequency
142 of persister cells in nutrient-deprived cultures (Figure 1B,C). Notably, for *Mabs* and *Msmeg,* the
143 development of these persister cells required an adaptation period of several days under
144 starvation conditions, as formation of persister cells was dramatically impaired if cells were shifted
145 immediately into nutrient-deficient conditions with antibiotics, suggesting that a regulated process
146 needed to be completed (Figure 1D-F).
147
148 **Identification of pathways needed for persister formation in *Mabs***
149 We used these conditions to carry out Tn-Seq screens in *Mabs* to identify genes necessary for
150 forming both spontaneous and starvation-induced persister cells. We conducted the screen using
151 a *Mabs Himar1* Tn library comprised of ~55,000 mutations across all 4,992 non-essential *Mabs*
152 genes in strain ATCC 19977[29]. We maintained cells in log-phase growth in 7H9 rich media, or
153 starved cells in PBS as described above, and then exposed them to TIG/LZD for 6 days (Figure
154 2A), a point at which spontaneous persister cells comprise the majority of the population (Figure

155 1C). Following antibiotic exposure cells were then washed and placed in antibiotic-free liquid
156 media to recover, genomic DNA was isolated, and Tn insertion sites sequenced. We then used
157 TRANSIT software[40] to quantify the abundance of each Tn mutant across different conditions and
158 identify mutants with statistically-significant differences in distribution, in order to identify essential
159 genes in both spontaneous and stress-induced antibiotic persister cells. We identified 277 *Mabs*
160 genes required for surviving TIG/LZD exposure in rich media, 271 genes required for survival
161 during starvation and 362 genes required to survive the combined exposure to antibiotics and
162 starvation using criteria for significance of Log_2 fold-change > 0.5 and Benjamini–Hochberg
163 adjusted p-value (p-adj.) ≤ 0.05 (Figure 2B-E). Of the genes required for antibiotic persistence,
164 ~60% were required in both nutrient-replete and starvation states, although condition-specific
165 determinants were also seen (Figure 2F). As expected, we identified genes with already-
166 established functions in antibiotic responses, including MAB_2752 and MAB_2753 which are both
167 homologs of known antibiotic transporters in *Mtb,* and tetracycline-responsive transcription factors
168 such as MAB_4687 and MAB_0314c (Table S1), indicating an ability of these Tn-Seq conditions
169 to identify physiologically relevant antibiotic-response genes.
170
171 To identify other cellular processes necessary for persister survival we performed pathway
172 enrichment analysis on the set of genes identified by Tn-Seq. We used the DAVID[41] analysis tool
173 to perform systematic queries of the KEGG, GO, and Uniprot databases and identify over-
174 represented processes and pathways. Interestingly, although cells were exposed to translation-
175 inhibiting antibiotics, and no exogenous oxidative or nitrosative stress was applied to the cells, we
176 identified a number of factors needed to combat these stresses. This includes *bfrB* (bactoferritin),
177 *ahpe* (peroxiredoxin) and *katG* (catalase/peroxidase) as well as 5 components of the bacterial
178 proteasome pathway, known to mediate resistance to nitrosative stress in *Mtb*[42]. We also
179 identified multiple members of DNA-damage response pathways including *recF*, *recG*, *uvrA*, *uvrB*
180 and *uvrC* (Figure 2G, Table S2). Examining starvation-induced persisters, a number of the same

181 pathways were again seen, and the mutant with the greatest persister defect in this context was
182 *mntH*, a redox-regulated Mn/Zn transporter implicated in peroxide resistance in other organisms[43-
183 44]. Taken together, these findings suggest an unexpected scenario whereby translation inhibition
184 triggers accumulation of reactive oxygen or nitrogen species, with damage to macromolecules
185 such as DNA occurring.
186
187 We next sought to independently confirm a role in persister survival for genes identified by Tn-
188 Seq. We selected a set of genes with strong defects in persister formation, representing several
189 of the functional pathways identified, and used oligonucleotide-mediated recombineering
190 (ORBIT)[45] to disrupt their open reading frames. The initial genes selected were *pafA* (proteasome
191 pathway), *katG* (catalase-peroxidase), *recR* (DNA repair), *blaR* (β-lactam sensing), and
192 *MAB_1456c* (cobalamin synthesis). To control for non-specific effects of antibiotic selection during
193 the recombineering process, we created a control strain using ORBIT to target a non-coding
194 intergenic region downstream of a redundant tRNA gene (*MAB_t5030c*). We then individually
195 screened each of these mutants to determine if they displayed deficits in persister cell formation
196 by exposing cells to TIG/LZD, either in rich 7H9 media or under starvation conditions, as had been
197 done in the pooled Tn-Seq screen. For Δ*katG* we detected clear defects in persister cell survival
198 following 6 days of antibiotic exposure in either rich media or under starvation-induced conditions,
199 corroborating the results of our Tn-Seq analysis (Figure 3A). We observed smaller defects in the
200 Δ*pafA* and Δ*MAB_1456c* mutants (Figure 3B,C), and in *blaR* found no loss of overall viability, but
201 instead observed a delayed resumption of growth after removal of antibiotics (Figure 3D, data not
202 shown).
203 To further confirm the role of *katG* and *pafA,* and exclude off-target effects of recombineering, we
204 performed genetic complementation analysis by restoring a wild-type copy of each gene into the
205 respective Δ*pafA* and Δ*katG* mutants. In each case, we integrated a single copy of the wild-type

206 gene, under the control of its endogenous promoter, into the genome at the L5 *attB* site (hereafter
207 *pafA+, katG+* strains), and constructed isogenic control strains with empty vector integrated at
208 the same site (hereafter *pafA-, katG-* strains). We confirmed expression of the re-introduced copy
209 of each gene by RT-qPCR in the *pafA+*, and *katG+* strains, and found expression within roughly
210 2-fold of endogenous wild-type levels (Figure 4A,D). We then challenged these strains with
211 TIG/LZD as before. In rich media, where the Δ*katG* mutants have a moderate persistence defect,
212 the *katG+* strain had roughly a 50-fold increase in viable cells relative to the *katG-* strain. We then
213 exposed cells to antibiotics under starvation conditions, where the Δ*katG* mutant phenotype is
214 more severe. Under these conditions the *katG-* cells succumbed rapidly between 3d and 10d after
215 antibiotic exposure, with a 1,000-fold decrease in viable cells relative to control cells, whereas the
216 *katG+* strain showed a near-complete restoration of persistence (Figure 4B). We analogously
217 examined complementation of Δ*pafA* mutants, and although the phenotype of the Δ*pafA* mutant
218 is less severe overall than a Δ*katG* mutant, we saw a similar restoration of survival in *pafA+* cells
219 relative to *pafA-* cells (Figure 4E). We next evaluated whether the *pafA-*, and *katG-* strains were
220 overall more sensitive to the growth inhibitory effects of TIG/LZD, or whether they had specific
221 defects in survival above the mean bactericidal concentration. We performed MIC determination
222 for TIG and LZD individually for each strain, comparing the *katG+*/*katG-* and *pafA+/pafA-* strains.
223 We found that that the MICs for each of these strains were unchanged, demonstrating that these
224 mutants were not more readily inhibited by these antibiotics. Instead, they have more rapid
225 kinetics of cell death at bactericidal concentrations, consistent with a specific defect in antibiotic
226 persistence (Figure 4C,F).
227

228 **Reactive oxygen contributes to antibiotic lethality in *Mabs***

229 We next investigated the role of KatG in persister cell cells more broadly. We began by assessing
230 whether *katG* conferred protection from other antibiotics with diverse mechanisms of action,

231 selecting antibiotics that are used clinically for mycobacterial infections. Because *katG*- mutants
232 showed the greatest defects in starvation-induced persistence, we analyzed survival of *katG*+ and
233 *katG*- strains in starvation-adapted cultures exposed to a panel of different antibiotics. Because
234 both TIG and LZD both act by inhibiting translation, we began by exposing cells to either TIG or
235 LZD alone. As expected, the degree of bacterial killing was significantly less with either agent
236 alone than when they are added in combination. Upon exposure to either of these antibiotics the
237 *katG*- cells died more rapidly than *katG*+ cells, though we note that the final proportion of
238 persisters in the population was unchanged in *katG*- cells (Figure 5A). When we exposed cells to
239 rifabutin (RFB), an RNA polymerase inhibitor, we saw a similar effect, with a 100-fold loss of
240 viability in *katG*- cells relative to the *katG*+ cells (Figure 5B). In contrast, when we exposed
241 cultures to either levofloxacin (gyrase inhibitor) or cefoxitin (β-lactam inhibitor of peptidoglycan
242 cross-linking), *katG* had little to no effect on cell viability (Figure 5C-D). Thus, the role of KatG is
243 context-dependent, suggesting that some antibiotics generate oxidative stress that can be
244 ameliorated by KatG catalase/peroxidase activity while others do not.
245
246 The identification of *katG* as essential for persister cells to survive exposure to TIG/LZD suggests
247 that ROS are present and causing damage. Although TIG/LIN are translation inhibitors that do not
248 directly generate ROS we evaluated whether they might nonetheless be triggering ROS
249 accumulation as a secondary effect. We examined ROS levels in *katG*+ *Mabs* using the ROS
250 indicator dye cellROX, that is retained in cells when it becomes oxidized[46]. At baseline, during
251 log-phase growth in rich media < 3% of cells had ROS accumulation (Figure 5E). We saw a
252 moderate increase in ROS accumulation in starved cultures, with roughly 10% of the population
253 cellROX+. However, when starved cultures were exposed to the TIG/LZD we saw a dramatic
254 increase in ROS accumulation with over 30% cellROX+ cells. Taken together, these data indicate
255 that translation inhibition does indeed have unanticipated downstream effects on cellular redox
256 balance, with ROS accumulation that could be contributing to cell death.

257

258 We next tested whether ROS were contributing to cell death by reducing ROS production and
259 assessing the impact on cell viability. A well-established system for studying hypoxia in
260 mycobacteria is the Wayne Model of gradual-onset hypoxia, whereby low-density cultures are
261 inoculated in sealed vessels with minimal headspace. As the culture slowly grows, the soluble O_2
262 is consumed, resulting in the onset of hypoxia over several days, a process that can be monitored
263 by the decolorization of Methylene Blue dye in the media[47]. Under aerobic conditions in rich
264 media, we observed the expected rapid killing of *Mabs* over the first 5 days with the combination
265 of TIG/LZD, with more rapid loss of viability in KatG- cells. However, under hypoxic conditions,
266 where ROS production is suppressed, we saw much slower bacterial killing. Importantly, under
267 hypoxic conditions *katG*- cells no longer had a survival defect relative to *katG*+ cells, supporting
268 the hypothesis that translation-inhibiting antibiotics also cause secondary accumulation of lethal
269 ROS in antibiotic-treated cells that need to be detoxified by KatG (Figure 5F).

270

271 **Incomplete penetrance of *katG* phenotype among *Mabs* strains**

272 Finally, we tested the role of KatG in *Mabs* clinical strains to determine how widely the role of *katG*
273 is conserved among different *Mabs* strains. We obtained 2 clinical isolates of *Mabs*, used ORBIT
274 to disrupt the *katG* locus, and evaluated the ability of these strains to form both spontaneous and
275 starvation-induced persister cells upon exposure to TIG/LZD. For clinical strain-1, the Δ*katG*
276 mutant showed no defects in either condition (Figure 6B). In contrast, for clinical strain-2, *katG*
277 contributed to starvation-induced persistence, as the Δ*katG* mutant rapidly lost viability in a
278 manner similar to the ATCC 19977 reference strain. However, unlike ATCC 19977, this phenotype
279 only manifested in starvation-induced persister cells, and was not seen in the absence of stress
280 (Figure 6C). Thus, the *katG* phenotype displays incomplete penetrance, and the degree of
281 protection it confers falls along a continuum among *Mabs* strains.

282 In summary, the results of these studies point to an important effect of ROS in amplifying the
283 lethality of some antibiotics in *Mabs*. Through genetic analysis we identified a number of ROS
284 detoxification factors, including KatG, as necessary for persisters to remain viable. This suggested
285 that antibiotics might induce an oxidative state in cells, and direct measurement of ROS following
286 antibiotic exposure indicated that this was indeed the case. Further supporting the deleterious
287 effects of ROS in this context, we found that removal of oxygen both slowed bacterial killing and
288 rendered KatG dispensable. Taken together these results suggest that antibiotic lethality is
289 accelerated by toxic ROS accumulation, and persister survival requires active detoxification
290 systems.
291
292 **DISCUSSION:**
293 **Pathways necessary for persister formation in *Mabs.***
294 The phenomenon of bacterial persistence has been recognized for decades, and has been
295 observed in a broad array of bacterial species. Despite this, a unifying mechanism of persister
296 cell formation has not emerged, suggesting that different pathways may play a role in different
297 contexts. A large body of work comes from *E. coli* where toxin-antitoxin systems such as HipBA[15],
298 Lon protease[16,48] and the (p)ppGpp synthase RelA all contribute to the formation of persister
299 cells[49-52]. However, even within this species other mechanisms seem to function, as RelA
300 contributes to persister cell formation following exposure to β-lactams, but not quinolones[53]. In
301 addition, how exactly these pathways maintain cell viability remains unclear. For example, while
302 (p)ppGpp produced by RelA seems to act through Lon protease[16], the critical substrates in this
303 process are note unknown. It is also unclear how mechanisms identified in one bacterial species
304 may relate to the mechanisms in another. *RelA* plays a role in multiple species of bacteria,
305 including *Pseudomonas aeruginosa*[54], *Staphylococcus aureus*[55], and *Mtb*[56]. However, the role of
306 this pathway does not seem to be universal, as deletion of neither *relA* nor *lon* had an effect on
307 persister formation in *Msmeg*[57]. Interestingly, in our Tn-Seq analysis, we did not identify *relA*.

308 However, a prior study of the *Mabs relA* mutant demonstrates that this strain still synthesizes
309 (p)ppGpp[58], suggesting genetic redundancy and a need to disrupt additional genes in a *relA*
310 mutant in order to study the role of (p)ppGpp in *Mabs*.
311
312 **Mechanisms of antibiotic lethality**
313 The mechanisms of bacterial cell death following antibiotic exposure remains somewhat
314 controversial. The most straightforward explanation is that antibiotics kill by directly disrupting the
315 function of their targets. However, more recently it was suggested that antibiotics kill through a
316 final common pathway of lethal ROS generation, leading to oxidative damage of DNA and other
317 macromolecules[59]. Since that time, there have been studies, both supporting[10,19-20] and refuting
318 the role of ROS[21-22] in antibiotic-mediated cell death.
319
320 Our analysis of *Mabs* persister cells provides new insights. The identification of multiple genes
321 involved in ROS detoxification through an unbiased genome-wide screen suggests that ROS also
322 promotes antibiotic lethality in *Mabs*. This idea is further supported by the detection of elevated levels
323 of ROS after exposure to translation inhibitors, and that removal of oxygen slowed antibiotic killing.
324 Taken together, these data strongly support the idea that ROS are a significant contributor to
325 antibiotic lethality in some contexts. These findings are supported by other published studies in
326 mycobacteria. In *Mabs* and *Msmeg,* other groups have also observed reduced antibiotic-mediated
327 killing in hypoxic conditions[10,36]. In *Mtb*, exposure to rifampin also generates ROS[19,60], and *katG*
328 contributes to survival in rifampin-treated cells[19].
329
330 The source of ROS under these conditions is uncertain. In principle, any of several derangements
331 could lead to ROS accumulation. One of the major sources of cellular ROS is oxidative
332 phosphorylation, as hydrogen peroxide, superoxide, and hydroxyl radicals are natural byproducts.
333 Thus, increased ROS generation by oxidative phosphorylation is an attractive hypothesis.

334 Alternatively, particularly under starvation conditions, it is possible that antioxidants and ROS
335 scavengers may become depleted, creating a more oxidizing environment. Our Tn-Seq analysis
336 provides additional insight on this. We noted a small class of Tn mutants that were paradoxically
337 protected from antibiotic lethality (Figure 2B). Prominent among this class of mutants were several
338 independent components of the NADH dehydrogenase complex (Table S1). Also known as
339 Complex I of the electron transport chain, it is one of the key entry points for electrons into the
340 oxidative phosphorylation pathway. The observation that mutants in this complex are protected
341 suggests that decreasing flux through oxidative phosphorylation, with a concomitant decrease in
342 ROS generation, may enhance survival during antibiotic exposure.
343
344 However, antibiotic-induced ROS accumulation is not a universal phenomenon. With some
345 antibiotics we examined in *Mabs,* loss of *katG* had no effect. In addition, ROS detoxification does
346 not always play a protective role, as loss of catalase and peroxidase activities in *E. coli* also had
347 no effect on persister survival[22]. Taken together, this suggests a model in which antibiotics cause
348 direct toxicity by acting on their target, but in addition, some antibiotics, notably transcription
349 inhibitors and translation inhibitors, also appear to have a secondary toxic effect of causing ROS
350 accumulation. How exactly transcription or translation blockade leads to increased ROS levels is
351 currently unclear and will require further investigation, although our data suggest that the electron
352 transport chain might play an important role.
353
354 **Therapeutic implications**
355 *Mabs* infections are particularly challenging to eradicate, with relapse rates over 50%[28]. Our
356 results highlight several bacterial processes such as the bacterial proteasome or ROS
357 detoxification that might be targeted therapeutically to reduce the development or survival of
358 persister cells. Agents targeting these process might not have any intrinsic antimicrobial activity
359 alone, but might act to target the unique physiology of persister cells that develop upon antibiotic

360 exposure. This would represent a new therapeutic class of “persistence inhibitors” that might act
361 synergistically with traditional antibiotics to eliminate the subpopulation of persister cells that
362 would otherwise remain viable despite prolonged antibiotic exposure in *Mabs* and other chronic
363 infections.
364
365 **Limitations**
366 Tn-Seq has inherent drawbacks, including an inability to identify mutants in essential genes, or in
367 cases of genetic redundancy. Thus, there are likely genes needed for antibiotic persistence in
368 *Mabs* that were not identified in this study. In addition, we studied the response to a single class
369 of antibiotic, focusing on the translation inhibitors often used to treat *Mabs* infections, and we
370 studied only spontaneous and starvation-induced persister cells. It is likely that studying other
371 antibiotics, with different mechanisms of action, or different stresses that induce persister cells,
372 would identify additional genes contributing to persister formation and would allow better
373 identification of core survival pathways that might be shared among different forms of stress.
374
375 **MATERIALS AND METHODS**
376 **Key Reagent Table**

Reagent	**Source/Reference**	**Identifier**
***Mabs* ATCC 19977**	ATCC	19977
***Msmeg* MC2 155**	ATCC	700084
***Mtb* Erdman**	ATCC	35801
***Mabs* clinical strains**	Sacramento Department of Public Health	NA
pkm444	Addgene	108319
pkm496	Addgene	109301

pmv306	Ref. 61	
Middlebrook 7H9	BD	271310
Middlebrook 7H10	BD	262710
Glycerol	Invitrogen	15514011
Tween-80	Fisher	BP338-500
Middlebrook OADC	BD	212351
DPBS (-Ca/Mg)	Gibco	14190144
Tyloxapol	Sigma Aldrich	T8761
Methylene blue	Sigma Aldrich	28514
Tigecycline	Chem Impex	29737
Linezolid	Chem Impex	29723
Levofloxacin	Sigma Aldrich	28266
Cefoxitin	Chem Impex	1490
Rifabutin	Cayman Chemical	16468
Rifampin	Sigma Aldrich	R7382
Isoniazid	Supelco	I3377
Ethambutol	Thermo Scientific	J6069506
DMSO	Sigma Aldrich	D2650
Trizol	Invitrogen	15596018
0.1mm zirconia beads	Biospec	11079101z
PureLink RNA Mini Kit	Invitrogen	12183025
Kanamycin	VWR	75856-686
Zeocin	Invivogen	ANTZN1P
Glycine	Fisher Bioreagents	BP381
Sucrose	Sigma Aldrich	S1888

Anhydrotetracycline	Cayman Chemical	10009542
cellROX green	Invitrogen	C10444
Paraformaldehyde	Sigma Aldrich	P6148
DAPI	Sigma Aldrich	D9542
DNAseI	New England Biolabs	M0303
Maxima H minus reverse transcriptase	Thermo Scientific	EP0752
Taq Polymerase	New England Biolabs	M0273
EvaGreen	Biotium	31019

377

378 Bacterial strains and culture conditions:

379 *Mabs* ATCC 19977, clinical *Mabs* strains and *Msmeg* (MC2 155) were grown in BD Middlebrook
380 7H9 media (liquid) or 7H10 media (solid) supplemented with 0.5% glycerol (Sigma) and 0.2%
381 Tween-80 (Fisher) but without any OADC supplementation except for transformations.
382 Sacramento clinical isolates were obtained from the Sacramento County Department of Public
383 Health Mycobacteriology Laboratory. Confirmation of clinical isolates as *Mabs* was performed by
384 amplifying the 16s rRNA locus and Sanger sequencing. *Mtb* (Erdman) was grown in 7H9 (liquid)
385 or 7H10 (solid) supplemented with 0.5% glycerol, 0.1% Tween-80, and 10% OADC (BD). All
386 cultures were grown at 37°C with gentle shaking. Except for specific hypoxia conditions, all liquid
387 cultures were grown with 90% container headspace or using a gas permeable cap to ensure
388 culture oxygenation. PBS starvation was achieved by washing OD0.5-1.0 *Mabs* 1X in DPBS (-
389 Ca/Mg, Gibco) and resuspending in DPBS at OD=1, supplemented with 0.1% tyloxapol (Sigma).

390

391 *Mabs* antibiotic experiments:

392 For PBS starvation experiments, stocks of *Mabs* were grown for 48 hours in 7H9 passaging
393 continuously in log phase, then PBS starved or passaged in log phase for an additional 48h. Log
394 phase or PBS starved *Mabs* were then resuspended in antibiotic containing media at OD1.0. For
395 experiments with hypoxia, *Mabs* in mid-log aerobic growth was adjusted to OD0.001 in media
396 with 1.5ug/ml methylene blue and added to a rubber septum sealed glass vial with 50%
397 headspace. Methylene blue discoloration was observed at 3d and antibiotics were added at 5d.
398 We empirically determined the half-life of each antibiotic in 7H9 media at 37-deg and for those
399 with half-lives shorter than the experiment, supplemented cultures to with additional antibiotic to
400 maintain the concentration of active antibiotic. Antibiotics were used at the following
401 concentrations: tigecycline (Chem-Impex) at 10ug/ml (8 fold above MIC, re-administered every 3
402 days), linezolid (Chem-Impex) at 100ug/ml (20 fold above MIC), levofloxacin (Sigma) at 40ug/ml
403 (8 fold above MIC), cefoxitin (Chem Impex) at 80ug/ml (8 fold above MIC, re-administered every
404 3 days), and rifabutin (Cayman) at 40ug/ml (4 fold above MIC). After antibiotic administration,
405 colony forming units over time were measured.
406
407 *Msmeg* antibiotic experiments:
408 Individual colonies were picked and grown for 48 hours in log phase before being PBS starved or
409 passaged in log phase for 48h. Log phase or PBS starved *Msmeg* were then resuspended in
410 antibiotic containing media at OD1.0. Antibiotics were used at the following concentrations:
411 tigecycline (Chem-Impex) at 1.25ug/ml (8 fold above MIC, re-administered every 3 days), linezolid
412 (Chem-Impex) at 2.5ug/ml (8 fold above MIC), rifampin (Sigma) at 32ug/ml (8 fold above MIC, re-
413 administered every 6 days), isoniazid (Sigma) at 32ug/ml (8 fold above MIC, re-administered
414 every 6 days), and ethambutol (Thermo) at 4ug/ml (8 fold above MIC, re-administered every 3
415 days). After antibiotic administration, colony forming units over time were measured.
416
417 *Mtb* antibiotic experiments:

418 Freezer stocks of *Mtb* were thawed and grown for 5-7 days in log phase before being starved for
419 14d or longer. Non-starved control *Mtb* were thawed such that they were also grown for 5-7 days
420 in log phase before experimental use. Log phase or PBS starved *Mtb* was then resuspended in
421 antibiotic containing media and adjusted to OD1.0. Antibiotics were used at the following
422 concentrations: rifampin (Sigma) at 0.1ug/ml (4 fold above MIC, re-administered every 6 days),
423 isoniazid (Sigma) at 0.1ug/ml (4 fold above MIC, re-administered every 6 days), and ethambutol
424 (Thermo) at 8ug/ml (4 fold above MIC, re-administered every 6 days). After antibiotic
425 administration, colony forming units over time were measured.
426
427 Transposon insertion sequencing:
428 The construction of this *Himar1* transposon Tn library has been described previously [29]. Screening
429 was performed by growing a freezer stock of the library for 2.5 days in log phase before 48-hour
430 PBS starvation or further continuous log-phase growth. Samples were then resuspended in media
431 containing either tigecycline/linezolid or an equal volume of DMSO solvent and incubated for 6
432 days, with a re-administration of tigecycline or matching DMSO on day 3. The samples were then
433 washed 2X in antibiotic free liquid media, resuspended in antibiotic free liquid media (10X the
434 original culture volume), and grown until OD0.5-1.0. Subsequently, the samples underwent three
435 more rounds of 100-fold passaging in liquid media to amplify surviving bacteria before the
436 samples were collected in Trizol (Invitrogen). A sample taken at the time of the commencement
437 of PBS starvation was collected in Trizol and used as the input control. Three independent trials
438 of this experiment were submitted to the UC Davis DNA Technologies Core, where Tn insertion
439 site flanking sequences were amplified as described previously [29] and sequenced on sequenced
440 on an Illumina AVITI. Sequence reads were mapped to the ATCC 19977 genome and analyzed
441 using TRANSIT software with the following parameters: 0% of N/C termini ignored, 10,000
442 samples, TTR normalization, LOESS correction, include sites with all zeros, site restricted

443 resampling. Genes with significant changes were defined as those with adjusted p-value (p-adj.)
444 <0.05 and $\log_2$ fold change >0.5. P-adj. was calculated using the Benjamini-Hochberg correction.
445
446 Pathway enrichment analysis
447 To improve gene annotation, *Mabs* orthologs to *Mtb* genes were identified. *Mabs* genes were first
448 converted into protein sequences using Mycobrowser, and protein sequences were then used to
449 perform reciprocal BLASTp searches. *Mabs* genes and *Mtb* genes that mapped to each other
450 using independent one-way BLASTp searches with a maximum e-value cutoff of 0.1 were
451 considered orthologs. For pathway analysis, gene lists (*Mtb* orthologs) were then imported into
452 the DAVID knowledgebase[41] and pathway enrichment analysis performed for Gene Ontology
453 biological process, Uniprot keyword and KEGG databases with statistical analysis Fisher's exact
454 test and nominal p-value reported.
455
456 Gene deletion and complementation:
457 Knockout strains were generated using ORBIT[45]. Briefly, *Mabs* was transformed with the
458 kanamycin-resistant ORBIT recombineering plasmid pkm444. 20ml *Mabs* at OD0.5-1.0 was
459 washed 2X in 10% glycerol and resuspended in 200ul 10% glycerol. 500ng plasmid was added
460 and electroporated at 2.5kV in 0.2cm cuvettes. The bacteria were allowed to recover overnight
461 before plating on 150ug/ml kanamycin plates. Clones were selected and regrown in liquid media
462 supplemented with 150ug/ml kanamycin and 10% OADC (BD) to OD0.5-1.0. For recombineering,
463 the pkm444-*Mabs* was grown to mid-log, then diluted to OD0.1 and 200mM glycine (Fisher) was
464 added to the media. 16 hours later, 500mM sucrose (Sigma) and 500ng/ml anhydrotetracycline
465 (Cayman) were added and incubated for an additional 4 hours. Subsequently, the *Mabs* was
466 washed 2X in ice cold 10% glycerol + 500mM sucrose. 200ul of 10X concentrated *Mabs* was then
467 electroporated with 600ng of the zeocin-R ORBIT payload pkm496 plasmid and 2ug of targeting
468 oligonucleotide (Table S3) at 2.5kV in 0.2cm cuvettes. The *Mabs* was then allowed to recover

469 overnight in liquid media with 10% OADC and 500ng/ml anhydrotetracycline before being plated
470 on 150ug/ml zeocin plates. Mutants were then selected and screened for gene deletion by PCR
471 amplification and Sanger sequencing. For genetic complementation, the endogenous loci
472 including promoter and terminator sequences were amplified by PCR and cloned into the EcoRV
473 site of pmv306 with kanamycin resistance[61]. In the case of *katG,* the upstream gene *furA* was
474 also included in the complementation construct to achieve optimal *katG* expression.
475
476 MIC analysis
477 Two-fold serial dilutions of antibiotics were prepared in a 96 well plate in 100ul volume. 100ul of
478 2X bacteria were added (for *Mabs*: used a final OD of 0.001, *Msmeg*: OD0.001, *Mtb*: OD0.01),
479 making a final volume of 200ul. The plates were incubated until there was visible growth in the no
480 antibiotic control well. At this time, the bacteria were transferred to a new plate with 20ul of 40%
481 paraformaldehyde and OD620 measurements were taken with a FilterMax F3 plate reader
482 (Molecular Devices).
483
484 Flow cytometry
485 OD1 Mab was stained with cellROX green (Invitrogen) at a final concentration of 5uM for 1hr at
486 37C. The cells were then washed in PBS and resuspended in PBS with 4% paraformaldehyde
487 and 5ug/ml DAPI (Sigma). The samples were run on a LSRII flow cytometer (BD). Fluorophores
488 were excited with the 405nm (DAPI) and 488nm (cellROX) lasers. Detection was performed using
489 the 450/50 (505LP) filter for DAPI and a 525/50 (555LP) filter for cellROX. Data were analyzed
490 with FlowJo software (BD).
491
492
493 DNA/RNA Purification:

494 Samples were resuspended in 5 volumes of Trizol and were bead beat with 0.1mm zirconia beads
495 (Biospec) 6x2min at 4°C in a Mini-Beadbeater-16 (Biospec). Chloroform was added and RNA in
496 the aqueous phase removed. For DNA isolation, a second RNA extraction was performed with
497 0.8M guanidine thiocyanate and 0.5M guanidine hydrochloride, 60mM Acetate pH 5.2, 1mM
498 EDTA. DNA was then isolated with back-extraction buffer (4M Guanidine Thiocyanate, 50mM
499 Sodium Citrate, 1M Tris base (without pH adjustment ~pH 11) and DNA purified using a PureLink
500 RNA Mini Kit (Invitrogen).
501
502 RT-qPCR
503 RNA was purified using PureLink RNA Mini Kit per manufacturer's instructions. The samples were
504 DNAseI (NEB) treated for 15min/37°C before stopping the reaction by adding 3.5mM EDTA and
505 heating for 10min/75°C. cDNA was synthesized from 500ng total RNA using random hexamers
506 and Maxima H minus reverse transcriptase (Thermo). No reverse-transcription controls were also
507 included and used to confirm the lack of genomic DNA-driven amplification. qPCR reactions used
508 Taq polymerase (NEB) and EvaGreen (Biotium) and were run on Biorad CFX Opus 96 Real-Time
509 PCR System. Melt curves were included for each sample to confirm uniform amplicon identity
510 between samples. Gene-specific amplification was quantified by comparison to a standard curve
511 generated from 3-fold serial dilutions of a control sample, then normalized to 16S rRNA within
512 each sample.
513
514 **FIGURE LEGENDS**
515 **Figure 1. Starvation induces antibiotic persister cells across diverse mycobacteria.** (A)
516 *Msmeg,* (B) *Mtb* or (C) *Mabs* were grown in 7H9 rich media or starved in PBS prior to the addition
517 of the indicated antibiotics. Cells were allowed to adapt to starvation for a period of 48h for *Msmeg*
518 and *Mabs* and 14-21d for *Mtb* prior to the addition of antibiotics in PBS where indicated. (D-F) As
519 above, but with/without a period of adaptation in PBS prior to antibiotics as indicated. Antibiotic

520 concentrations were: *Msmeg* - Isoniazid (INH) 32ug/ml (8 x MIC), rifampin (RIF) 32ug/ml (8 x
521 MIC), ethambutol (EMB) 4ug/ml (8 x MIC), tigecycline (TIG) 1.25ug/ml (8 x MIC), linezolid (LZD)
522 2.5ug/ml (8 x MIC). *Mtb* – RIF 0.1ug/ml (4 x MIC), INH at 0.1ug/ml (4 x MIC), EMB at 8ug/ml (4 x
523 MIC). *Mabs* – TIG (8 x MIC), LZD 100ug/ml (20 x MIC). Antibiotics with half-lives shorter than the
524 duration of experiment were re-added at the following intervals: TIG, EMB every 3d; RIF, INH
525 every 6d. Error bars represent SEM, statistical significance is calculated at each time point using
526 student's t test. ****: $p<0.0001$, ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, ns: $p>0.05$. Data are combined
527 from 3 independent experiments.
528
529 **Figure 2. Tn-Seq identifies genes required for antibiotic persistence in *Mabs*.** (A)
530 Experimental design. (B-E) Tn-Seq analysis showing relative abundance of individual genes
531 under the indicted conditions. All cultures were fully aerated throughout the experiment and
532 cultures without antibiotics received and equal volume of DMSO. For (B-D) gene abundance in
533 the indicated condition is measured relative the input log-phase population. In (E) an additional
534 comparison is made relative to the PBS condition. Genes with significant decreases in abundance
535 are shown in color (p-adj. < 0.05 and $\log_2$ fold-change > 0.5) using the Benjamini–Hochberg
536 adjustment for multiple hypothesis testing. (F) Number of genes essential in each condition
537 relative to the input population. (G) Pathway enrichment analysis of the essential genes in each
538 condition using the DAVID knowledgebase ($p <0.05$). Screens were run as 3 independent
539 experiments and the combined results analyzed. Antibiotic conditions were used as described in
540 Figure 1.
541
542 **Figure 3. Independent deletions of *katG* and *pafA* confirm Tn-Seq results.** (A-E) ORBIT
543 recombineering was used to disrupt the indicated genes, or to generate a control strain with an
544 intergenic region targeted distal to tRNA gene *MAB_t5030c*. Each mutant was either grown in
545 7H9 rich media or starved in PBS for 48 prior to the addition of antibiotics. Error bars represent

546 SEM, statistical significance is calculated at each time point using student's t test. ****: p<0.0001,
547 ***: p<0.001, **: p<0.01, *: p<0.05, ns: p>0.05. Antibiotics were added as described above. Data
548 are combined from 4 independent experiments.
549
550 **Figure 4. Complementation analysis *katG* and *pafA* mutants confirms their role in persister**
551 **survival.** (A) RT-qPCR analysis of *katG* expression in *katG- (Δ katG::pmv306), katG+*
552 *(Δ katG::pmv306 katG),* and control strain (ORBIT intergenic*::pmv306).* (B) CFU over time for
553 *katG+/katG-* strains. (C) MICs for *katG+/katG-* strains. (D) Expression of *pafA in pafA-*
554 *(Δ pafA::pmv306), pafA+ (pafA::pmv306 pafA)* and control strains. (E) CFU over time of
555 *pafA+/pafA-* strains. (F) MICs for *pafA+/pafA-* strains. Antibiotic concentrations in (A-B, D-E) are
556 as described above. Error bars represent SEM, statistical significance is calculated at each time
557 point using student's t test between *katG+/katG-* strains in (B) and between *pafA+/pafA-* strains
558 in (E). ****: p<0.0001, ***: p<0.001, **: p<0.01, *: p<0.05, ns: p>0.05. Antibiotics were added as
559 described above.
560
561 **Figure 5. ROS-mediated toxicity following antibiotic exposure.** (A-D) Analysis of *katG+/katG-*
562 cells challenged with different antibiotics. Cells were starved in PBS for 48h and then exposed to
563 the indicated antibiotic. (E) Flow cytometry of cells *katG*$^{+}$ cells stained with DAPI and the ROS-
564 sensitive dye cellROX after 72h in the indicated conditions. Percentage cellROX-positive cells are
565 shown. (F) Persister survival over time for aerated and hypoxic and cultures of *Mabs* after
566 exposure to TIG/LIN. Error bars represent SEM, statistical significance is calculated at each time
567 point using student's t test. ****: p<0.0001, ***: p<0.001, **: p<0.01, *: p<0.05, ns: p>0.05. (A-D)
568 are combined data from 3 independent experiments. (E) are representative (median) data from 3
569 independent experiments. (F) are combined data from 4 independent experiments. Antibiotics
570 were added as described above.

571

572 **Figure 6. Incomplete penetrance of *katG* phenotype among *Mabs* strains.** Δ*katG* strains and
573 control strains targeting an intergenic region were constructed on the indicated *Mabs*
574 backgrounds using ORBIT recombineering: (A) ATCC 19977, (B-C) clinical strains. Bacteria were
575 cultured in 7H9 or starved for 48h in PBS and then treated with antibiotics where indicated.
576 Cultures without antibiotics received an equal volume of DMSO as a control. Survival over time
577 is shown. Error bars represent SEM, statistical significance is calculated at each time point using
578 student's t test. ****: $p<0.0001$, ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, ns: $p>0.05$.Combined data
579 from 4 independent experiments are shown. Antibiotics were added as described above.

580

581 **Table S1. Tn-Seq results**

582 **Table S2. DAVID functional pathway analysis**

583 **Table S3. Oligonucleotide sequences**

584

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601 **CONFLICTS OF INTEREST**

602 BHP and SAS serve on the scientific advisory board of X-Biotics Therapeutics.

603

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804

805

FIGURE 1

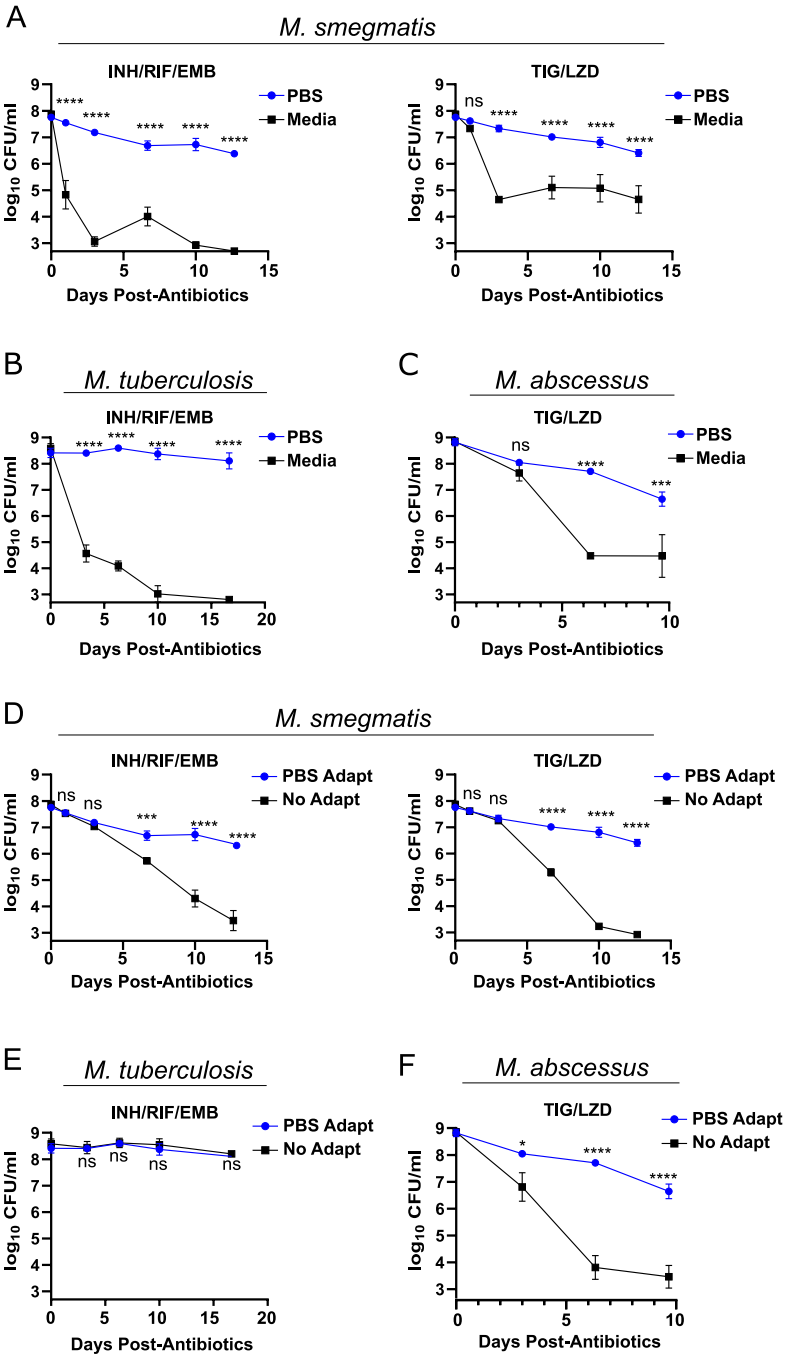

A
M. smegmatis
INH/RIF/EMB
TIG/LZD
PBS
Media
log10 CFU/ml
Days Post-Antibiotics
B
M. tuberculosis
C
M. abscessus
D
PBS Adapt
No Adapt
E
F

FIGURE 2

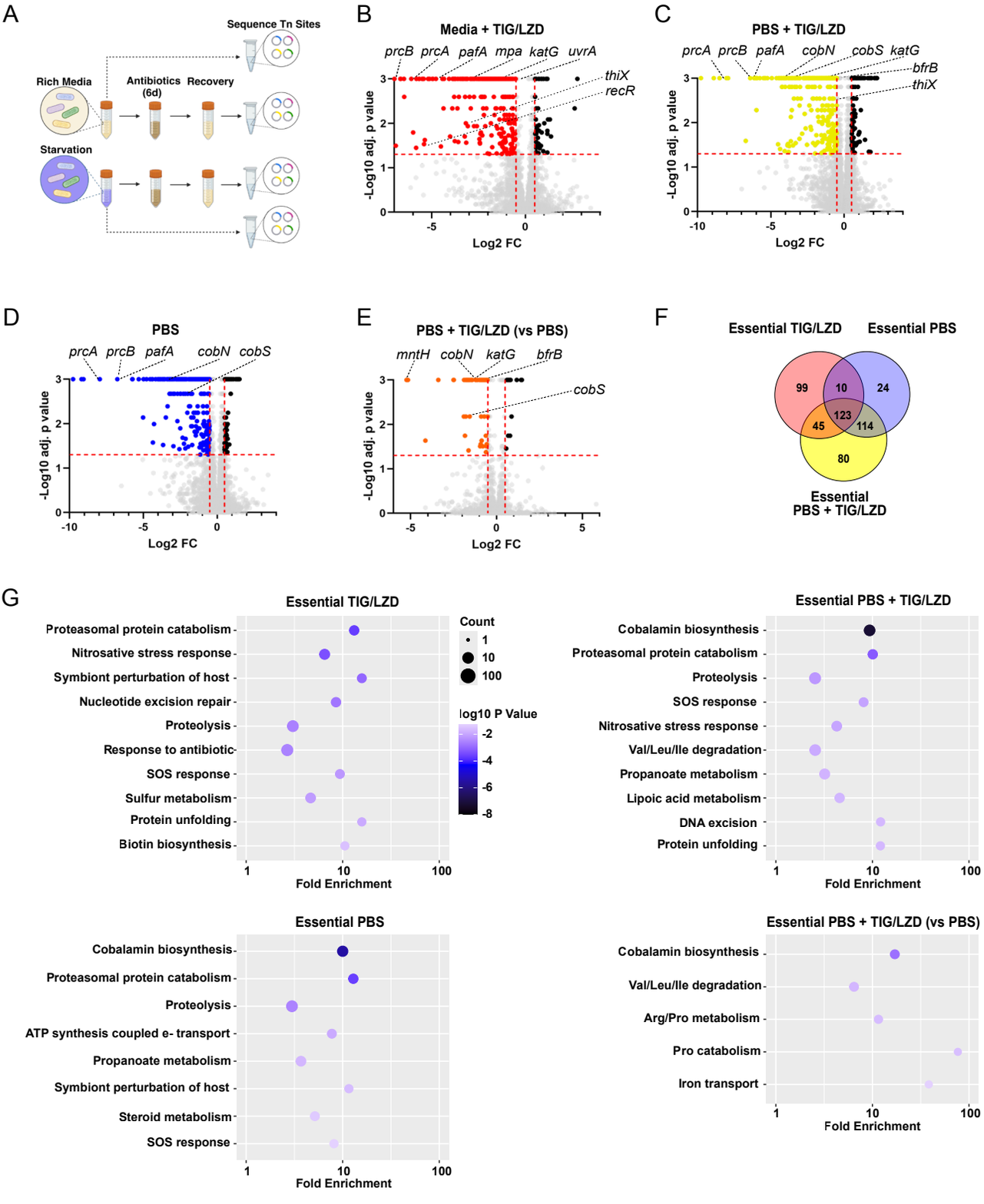

A
Sequence Tn Sites
Rich Media
Antibiotics (6d)
Recovery
Starvation
B
Media + TIG/LZD
prcB prcA pafA mpa katG uvrA
thiX
recR
-Log10 adj. p value
Log2 FC
C
PBS + TIG/LZD
prcA prcB pafA cobN cobS katG
bfrB
thiX
-Log10 adj. p value
Log2 FC
D
PBS
prcA prcB pafA cobN cobS
-Log10 adj. p value
Log2 FC
E
PBS + TIG/LZD (vs PBS)
mntH cobN katG bfrB
cobS
-Log10 adj. p value
Log2 FC
F
Essential TIG/LZD
Essential PBS
99
10
24
123
45
114
80
Essential PBS + TIG/LZD
G
Essential TIG/LZD
Proteasomal protein catabolism
Nitrosative stress response
Symbiont perturbation of host
Nucleotide excision repair
Proteolysis
Response to antibiotic
SOS response
Sulfur metabolism
Protein unfolding
Biotin biosynthesis
Fold Enrichment
Count
1
10
100
log10 P Value
-2
-4
-6
-8
Essential PBS + TIG/LZD
Cobalamin biosynthesis
Proteasomal protein catabolism
Proteolysis
SOS response
Nitrosative stress response
Val/Leu/Ile degradation
Propanoate metabolism
Lipoic acid metabolism
DNA excision
Protein unfolding
Fold Enrichment
Essential PBS
Cobalamin biosynthesis
Proteasomal protein catabolism
Proteolysis
ATP synthesis coupled e- transport
Propanoate metabolism
Symbiont perturbation of host
Steroid metabolism
SOS response
Fold Enrichment
Essential PBS + TIG/LZD (vs PBS)
Cobalamin biosynthesis
Val/Leu/Ile degradation
Arg/Pro metabolism
Pro catabolism
Iron transport
Fold Enrichment

FIGURE 3

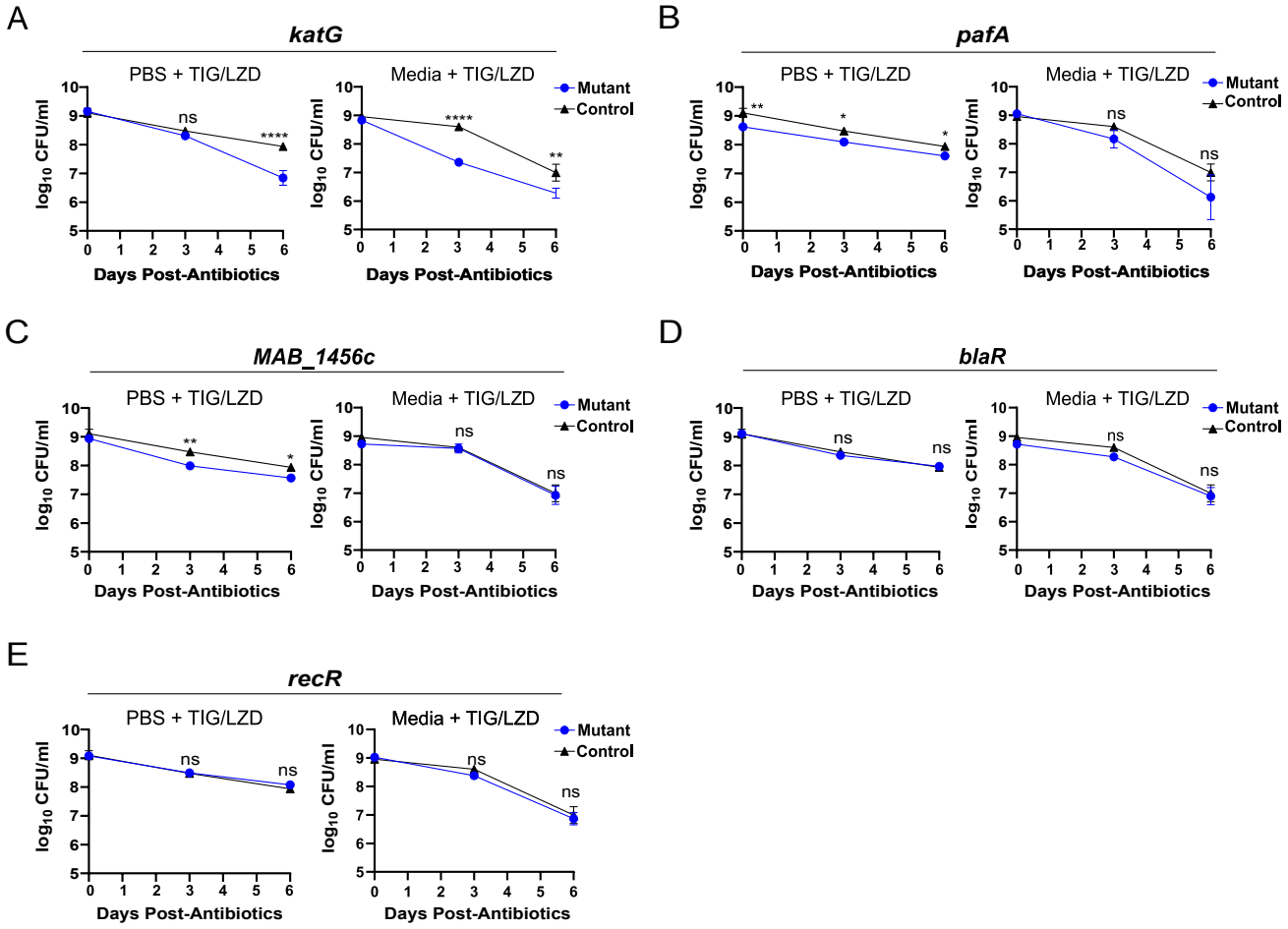

A
katG
PBS + TIG/LZD
Media + TIG/LZD
Mutant
Control
log10 CFU/ml
Days Post-Antibiotics
ns
****
**
B
pafA
PBS + TIG/LZD
Media + TIG/LZD
Mutant
Control
log10 CFU/ml
Days Post-Antibiotics
**
*
ns
C
MAB_1456c
PBS + TIG/LZD
Media + TIG/LZD
Mutant
Control
log10 CFU/ml
Days Post-Antibiotics
**
*
ns
D
blaR
PBS + TIG/LZD
Media + TIG/LZD
Mutant
Control
log10 CFU/ml
Days Post-Antibiotics
ns
E
recR
PBS + TIG/LZD
Media + TIG/LZD
Mutant
Control
log10 CFU/ml
Days Post-Antibiotics
ns

FIGURE 4

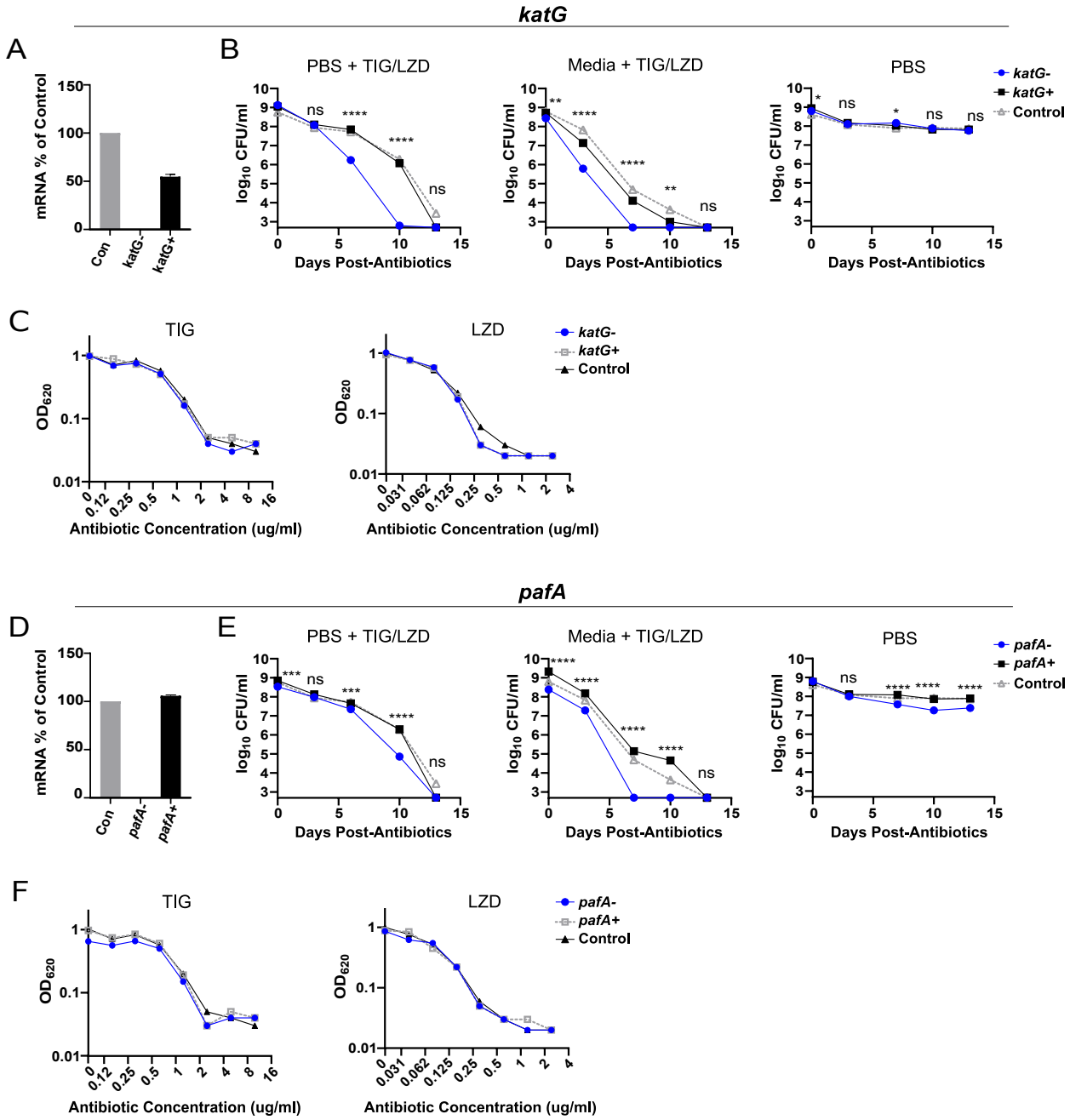

katG
A
mRNA % of Control
Con
katG-
katG+
B
PBS + TIG/LZD
Media + TIG/LZD
PBS
log10 CFU/ml
Days Post-Antibiotics
katG-
katG+
Control
C
TIG
LZD
OD620
Antibiotic Concentration (ug/ml)
pafA
D
E
pafA-
pafA+
F

FIGURE 5

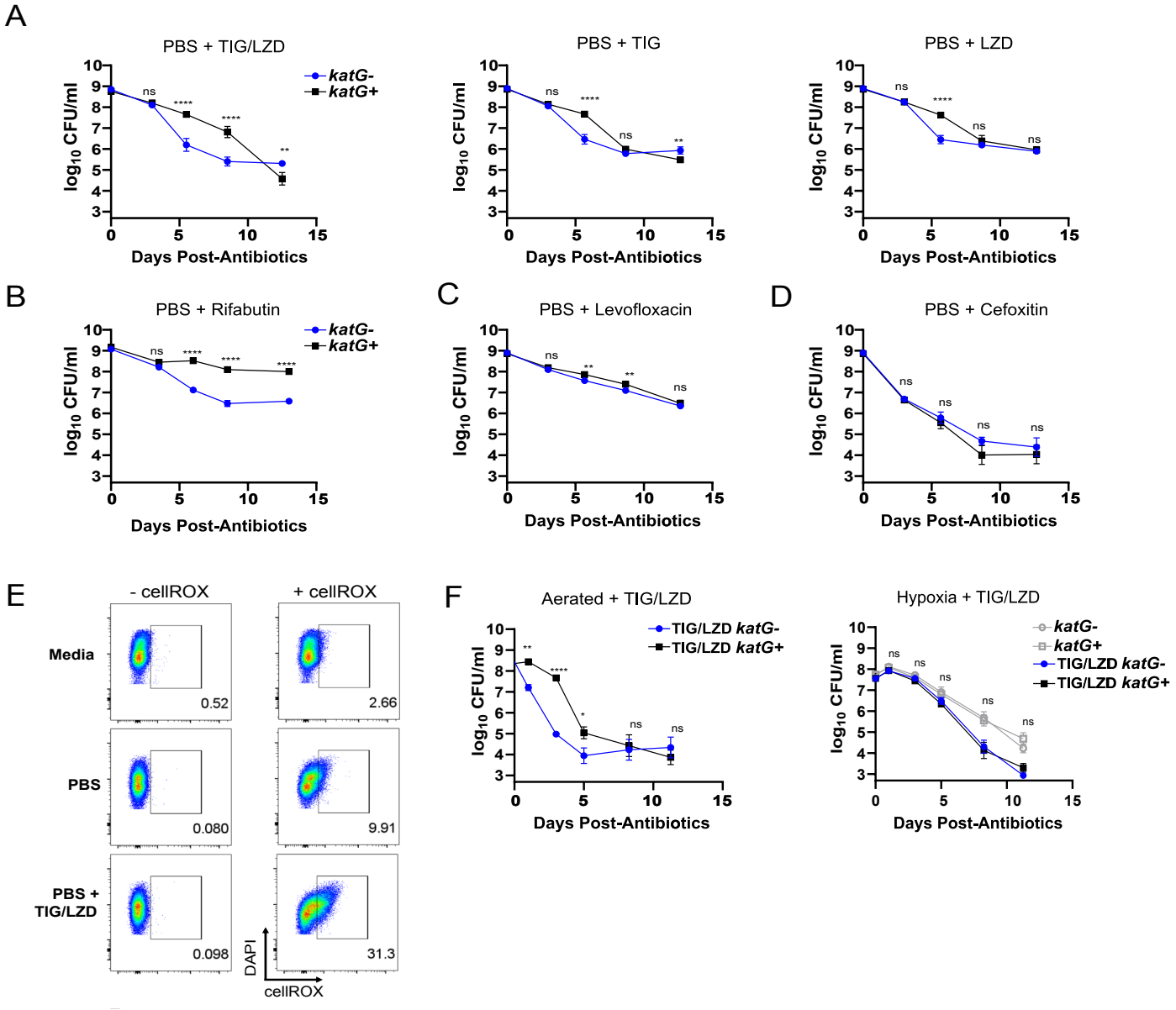
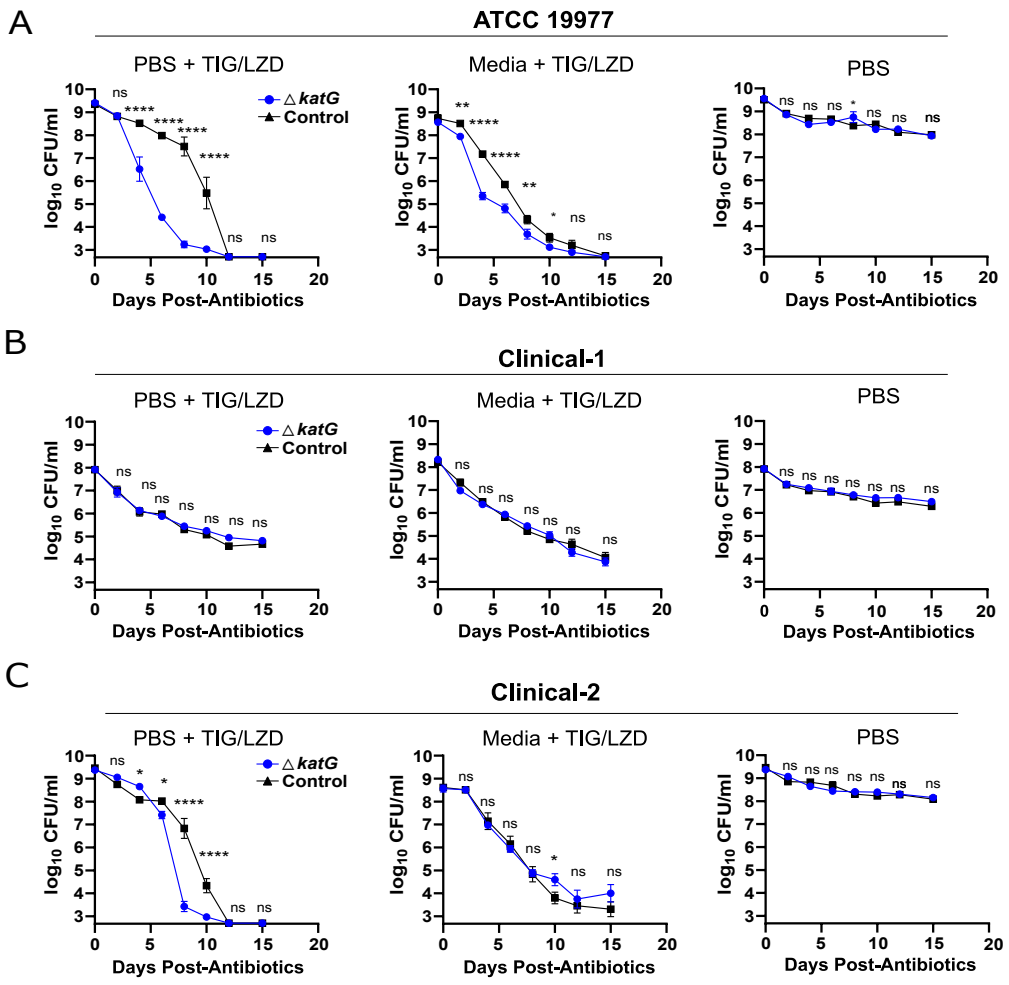

A
PBS + TIG/LZD
PBS + TIG
PBS + LZD
katG-
katG+
log10 CFU/ml
Days Post-Antibiotics
ns
****
**
B
PBS + Rifabutin
C
PBS + Levofloxacin
D
PBS + Cefoxitin
E
- cellROX
+ cellROX
Media
PBS
PBS + TIG/LZD
0.52
2.66
0.080
9.91
0.098
31.3
DAPI
cellROX
F
Aerated + TIG/LZD
Hypoxia + TIG/LZD
TIG/LZD katG-
TIG/LZD katG+
*

FIGURE 6


A
ATCC 19977
PBS + TIG/LZD
Media + TIG/LZD
PBS
△ katG
Control
log10 CFU/ml
Days Post-Antibiotics
B
Clinical-1
C
Clinical-2