1   # DNA repair is essential for *Vibrio cholerae* growth on

2   # Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Medium

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25 **Abstract**

26 Thiosulfate-citrate-bile salts-sucrose (TCBS) agar is a selective and differential media

27 for the enrichment of pathogenic *Vibrios*. We observed that an exonuclease VII (*exoVII*)

28 mutant of *Vibrio cholerae* failed to grow on TCBS agar, suggesting that DNA repair

29 mutant strains may be hampered for growth in this selective media. Examination of the

30 selective components of TCBS revealed that bile acids were primarily responsible for

31 toxicity of the *exoVII* mutant. Suppressor mutations in DNA gyrase restored growth of

32 the *exoVII* mutants on TCBS, suggesting that TCBS inhibits DNA gyrase similar to the

33 antibiotic ciprofloxacin. To better understand what factors are important for *V. cholerae*

34 to grow on TCBS, we generated a randomly-barcoded TnSeq (RB-TnSeq) library in *V.*

35 *cholerae* and have used it to uncover a range of DNA repair mutants that also fail to

36 grow on TCBS agar. The results of this study suggest that TCBS agar causes DNA

37 damage to *V. cholerae* similarly to the mechanism of action of fluoroquinolones, and

38 overcoming this DNA damage is critical for *Vibrio* growth on this selective medium.

39

40 **Abstract Importance**

41 TCBS is often used to diagnose cholera infection. We found that many mutant *V.*

42 *cholerae* strains are attenuated for growth on TCBS agar, meaning they could remain

43 undetected using this culture-dependent method. Hypermutator strains with defects in

44 DNA repair pathways might be especially inhibited by TCBS. In addition, *V. cholerae*

45 grown successively on TCBS agar develops resistance to ciprofloxacin.

46 **Introduction**

47   *Vibrio cholerae* is the causative agent of cholera, a gastrointestinal infection

48   characterized by profuse watery diarrhea that, when left untreated, causes rapid

49   dehydration and death. This gram-negative bacterium lives a dual lifestyle, alternating

50   between its aquatic environmental niche and human host environment where it is

51   acquired through ingestion of contaminated food or water. Cholera remains endemic in

52   much of the developing world, with sporadic outbreaks occurring in nations with poor

53   sanitation practices and limited access to clean drinking water (1, 2).

54

55   Culture confirmation from stool samples remains the gold standard for the diagnosis of

56   *V. cholerae* infections (3). This is typically achieved using Thiosulfate-Citrate-Bile Salts-

57   Sucrose (TCBS) agar, a highly selective medium for *Vibrios*. Following growth, the

58   identity of a *Vibrio* grown on TCBS agar can be further determined based on its capacity

59   to ferment sucrose— while *V. cholerae* can ferment sucrose, most other medically

60   relevant *Vibrios* cannot (4).

61

62   Aside from sucrose, the major selective factors in TCBS are the medium's alkaline pH

63   and bile salts, which inhibit the growth of non-*Vibrio* enterics. Given that *V. cholerae*

64   encounters bile routinely throughout its pathogenic lifecycle, it has evolved a variety of

65   mechanisms to sense, respond to, and limit the antimicrobial effects of bile. To this end,

66   bile represents a significant environmental signal in the pathogenesis of *V. cholerae. V.*

67   *cholerae* senses bile through an interaction between the inner membrane sensory and

68   regulatory proteins ToxR and ToxS (5, 6). For example, in the human intestinal tract, bile

69   activation of ToxRS adapts *V. cholerae* for bile tolerance by inducing and inhibiting the

70   expression of outer membrane proteins OmpU and OmpT, respectively (7–9). Because

71   bile can enter the cell through OmpT but not OmpU (10, 11), a shift in porin expression

72   protects *V. cholerae* from its cytotoxic effects.

73

74   The bacterial stress response to bile is mostly characterized by DNA damage leading to

75   SOS induction as well as the remodeling of the cell membrane (12–17). Early works in

76   *E. coli* demonstrate that cell death and SOS induction in response to bile mimic that of

77   cells treated with the DNA crosslinking agent mitomycin C, suggesting that bile may

78   cause direct DNA damage in bacteria (18). Another study by Prieto and colleagues

79   indicate that treatment of *Salmonella enterica* with bile causes oxidative DNA damage

80   and increases the frequency of GC → AT transitions. This led the authors to suggest

81   that base excision repair and recombinational repair, but not nucleotide excision repair,

82   are necessary for *S. enterica* tolerance to bile (13).

83

84   In this work we demonstrate that exonuclease VII (*exoVII*) mutant *V. cholerae* fails to

85   grow on TCBS agar, and that the main component in TCBS agar that is responsible for

86   this inhibition is ox bile. We found that mutations in DNA gyrase (encoded by genes

87   *gyrA* and *gyrB*) suppress the toxicity of TCBS in the absence of ExoVII and that some of

88   these mutations confer resistance to the fluoroquinolone ciprofloxacin. Finally, we

89   performed randomly barcoded transposon insertion site sequencing (RB-TnSeq) to

90   identify other *V. cholerae* mutants with growth defects on TCBS agar, and in doing so

91   uncovered mutants with component-dependent growth defects. The results presented

92   here (19) provide an explanation for the inhibition of *exoVII* mutant *V. cholerae* and

93   suggest that bile salts/acids may induce quinolone-like DNA damage in bacteria.

94   Moreover, we show that DNA repair is essential for *V. cholerae* to robustly grow on

95   TCBS which has significant implications on whether TCBS can accurately sample the

96   diversity of *V. cholerae* in clinical isolates.

97

98   **Results**

99   ***xseA* mutant *V. cholerae* cannot grow on TCBS agar.**

100   While conducting previously-described experiments to enhance the efficiency of

101   Multiplex Genome Editing by Natural co-Transformation (MuGENT)(28), we

102   serendipitously discovered that a *V. cholerae* mutant strain derived from the El Tor

103   Biotype E7946 (strain TND0252: Ptac-*tfoX,* Δr*ecJ*501bp, Δ*exoVII*501bp, Δ*lacZ*::*lacIq*,

104   Δ*vc1807*::SpecR), which had been developed to allow MuGENT with minimal homology

105   regions, failed to grow on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar, a

106   selective and differential medium for *Vibrios*. However, the parental E7946 strain and

107   another El Tor isolate that is widely used, C6706, grew well (Fig 1A). TND0252 has

108   three notable changes including Isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducible

109   *tfoX*, which triggers natural competence, and null mutations in the two nucleases *recJ*

110   and *exoVII. vc1807* is a frame-shifted transposase that appears to be neutral in all

111   conditions and is used in the MuGENT protocol and thus likely was not responsible for

112   the lack of growth on TCBS (28). We therefore hypothesized mutation of *tfoX, recJ,* or

113   *exoVII* was responsible for TCBS toxicity. We found that repairing the *tfoX* (strain

114   CW2171, "+P*tfox*") or *recJ* gene (strain CW2173, "+*recJ*") with WT sequence did not

115   restore WT-like growth on TCBS agar. However, restoration of the *xseA* null mutation to

116   wild type sequence (strain CW2172, "+*exoVII*") restored TCBS growth (Fig. 1A). *xseA,*

117   along with *xseB,* form the ExoVII nuclease that has been implicated in DNA repair. We

118   thus conclude that *V. cholerae* mutants lacking functional ExoVII are highly attenuated

119   for growth on TCBS agar.

120

121   We next questioned what component of TCBS agar was responsible for inhibiting the

122   *exoVII* mutant. TCBS agar contains several selective components that inhibit the growth

123   of non-*Vibrio* enteric bacteria (Table 1). We retrieved and cultured a mariner transposon

124   *xseA* mutant (hereafter *xseA::*Tn) from a regenerated ordered *V. cholerae* strain C6706

125   mutant library (21) and spot plated serial dilutions of this mutant and the WT strain over

126   different compositions of TCBS agar. As seen with strain E7946, the *xseA* null mutant

127   of C6706 failed to grow on TCBS (Fig. 1B). We reasoned the non-selective components

128   of TCBS include sucrose, peptone, yeast extract, and sodium chloride, and media

129   containing only these four ingredients was termed "TCBS base" media. Alternatively, the

130   other components of TCBS including sodium citrate, ox bile, sodium cholate, ferric

131   citrate, and thiosulfate likely provide selection for *Vibrios.* To identify which selective

132   component of TCBS inhibited growth of the *xseA*::Tn mutant, we spot plated this mutant

133   and WT on base TCBS containing only one of the selective components. The *xseA::*Tn

134   mutant grew equivalent to WT in TCBS base agar supplemented with sodium cholate,

135   sodium citrate, sodium thiosulfate, or ferric citrate added at the wt/vol listed in Table 1,

136   but was strongly attenuated relative to WT on TCBS base agar supplemented with ox

137   bile (Fig. 1B). Of note, *xseA::*Tn did exhibit limited growth on ox bile. Thus, we

138   concluded the major selective component responsible for inhibiting the *exoVII* mutant *V.*

139   *cholerae* strain is ox bile although growth on complete TCBS was the most attenuated.

140

141   ***exoVII* growth inhibition on TCBS can be suppressed by mutations in DNA**

142   **gyrase.** To better understand the requirement for *exoVII* growth on TBCS, we plated the

143   *xseA*::Tn strain on TCBS agar to select for suppressor mutations that restore growth.

144   We isolated and sequenced the genomes of four suppressor mutants. Three of the four

145   mutants had unique mutations in either of the two subunits of DNA gyrase- *gyrA* and

146   *gyrB* (Fig. 2A, Table S1). Of note, one of the four mutants had mutations in both *gyrA*

147   and gyrB (Fig. 2A, Table S1). As DNA gyrase is essential for growth (29–31), we deduce

148   that these mutations did not produce a null phenotype but rather altered the properties

149   of DNA gyrase restoring TCBS growth. The suppressor that did not encode a mutation

150   in *gyrA* or *gyrB* mapped to the ATP-dependent DNA helicase *dinG* (GPY04_RS07870)

151   (Table S1), but for this study we will focus on the more common DNA gyrase mutations.

152   We used MuGENT to regenerate *the gyrA/gyrB* suppressor mutations in the parent

153   *xseA*::Tn strain and confirmed that promote growth on TCBS agar despite the lack of

154   functional ExoVII (Fig. 2B).

155

156   ***exoVII* and *gyrAB* alter *V. cholerae* ciprofloxacin resistance.** DNA gyrase, also

157   known as bacterial type II topoisomerase, relieves topological strain on unwound DNA

158   by performing negative supercoiling, and is the target of quinolone antibiotics.

159   Quinolone drugs like ciprofloxacin work as topoisomerase poisons by trapping DNA

160   gyrase in a covalently attached DNA complex after it makes double stranded breaks

161 (DSBs), causing cell death by inhibiting DNA replication and transcription while leading

162 to the accumulation of DSBs (32). In *E. coli,* ExoVII mutants are hypersensitive to

163 ciprofloxacin, and thus ExoVII was hypothesized to relieve covalent complexes of DNA

164 gyrase bound to DNA (19). To test whether ExoVII has a similar function in *V. cholerae*,

165 we measured the sensitivity of ExoVII null mutants to ciprofloxacin using a disk diffusion

166 assay. We found that, indeed, mutant *V. cholerae* lacking either subunit of *exoVII*

167 (*xseA*::Tn or *xseB*::Tn) are indeed significantly more sensitive to inhibition by

168 ciprofloxacin than WT exhibiting a larger zone of inhibition (ZOI) (Fig. 3A). We next

169 hypothesized that suppressor mutations in *gyrA* and/or *gyrB* would reduce ciprofloxacin

170 sensitivity in the *xseA*:Tn mutant. However, contrary to our prediction only one of the

171 three suppressor mutant strains (*xseA*::Tn *gyrB*$_{\Delta 1018-1026}$) significantly restored WT

172 ciprofloxacin resistance whereas the other two maintained ciprofloxacin sensitivity

173 similar to *xseA:*Tn (Fig. 3A). Therefore, even though all three DNA gyrase suppressor

174 mutants were able to overcome *xseA*:Tn growth inhibition on TCBS, only one leads to

175 enhanced ciprofloxacin resistance, suggesting that the disruption of DNA gyrase by

176 TCBS is mechanistically distinct from ciprofloxacin.

177

178 Given that all three sets of DNA gyrase mutations suppress *xseA*::Tn TCBS toxicity but

179 only one of the three confers ciprofloxacin resistance, we determined if these

180 suppressor mutations restored growth on bile by plating serial dilutions of the

181 suppressor mutants on TCBS base agar supplemented with only 0.5% (w/v) ox bile (Fig.

182 3B). While the *xseA::*Tn and *xseB::*Tn mutants demonstrate strong attenuation when

183 plated on ox bile, all three suppressor mutants display significantly restored growth

184   although not to the levels observed for WT *V. cholerae*. From these results we conclude

185   that ox bile induces DNA gyrase-mediated DNA damage, similar to but distinct from

186   ciprofloxacin, and the ExoVII null mutants fail to grow on TCBS because they are

187   unable to repair this damage.

188

189   **Passaging *V. cholerae* on TCBS agar selects for ciprofloxacin resistance.** We next

190   hypothesized that if TCBS and ciprofloxacin inhibit the growth of the *exoVII* mutant via

191   DNA gyrase toxicity, cells grown on TCBS should be more susceptible to inhibition by

192   ciprofloxacin than cells grown on LB. We tested this using disc diffusion assays with the

193   WT strain on LB agar and TCBS agar, and as predicted the zones of inhibition by

194   ciprofloxacin of cells plated on TCBS agar was significantly greater than that of cells

195   grown on LB agar (Fig 4A). This suggests that the additional damage caused by TCBS

196   predisposes *V. cholerae* to ciprofloxacin toxicity. Next, because we found that the

197      $gyrB_{\Delta1018-1026}$ suppressor mutation increased ciprofloxacin resistance of the *xseA*::Tn

198   mutant, we tested if this *gyrB* mutations would confer resistance to ciprofloxacin in the

199   WT strain. Our results demonstrated that the $gyrB_{\Delta1018-1026}$ strain produced a

200   substantially smaller zone of inhibition than WT, suggesting that this mutant is resistant

201   to ciprofloxacin even if *xseA* is functional (Fig. 4B).

202

203   Our results thus far suggest that *V. cholerae* growing on TCBS experiences DNA gyrase

204   mediated DNA damage similar to ciprofloxacin treatment. We therefore tested whether

205   continuous growth of the WT strain on TCBS agar would impact ciprofloxacin resistance

206   by serially passaging on either TCBS or LB agar daily for 15 days. Every 3 days we

207   isolated six random clones per lineage to assess their sensitivity to ciprofloxacin by disc

208   diffusion. On days 12 and 15 we observed an increasingly significant difference in the

209   distributions of ZOIs between the TCBS and LB lineages (Fig. 4C). Notably, while the

210   TCBS lineage clones varied widely in their sensitivity to ciprofloxacin at every passaging

211   interval, the LB lineage clones were relatively consistent with the unpassaged Δ*lacZ*

212   parent strain. This result therefore suggests that passaging on TCBS agar potentiates

213   the evolution of ciprofloxacin resistant *V. cholerae*.

214

215   **Deployment of a randomly-barcoded TnSeq (RB-TnSeq) library identifies DNA**

216   **repair mutants attenuated for growth on TCBS agar**. Given that our results thus far

217   suggest TCBS agar causes DNA damage and is selective for *V. cholerae* growth, we

218   hypothesized that other DNA repair pathways would be required for growth on TCBS

219   agar. To explore this hypothesis and more generally assess genes necessary for growth

220   of *V. cholerae* on TCBS, we constructed a randomly barcoded transposon insertion site

221   sequencing (RB-TnSeq) (23) mutant library in *V. cholerae*. Our library contains over

222   36,000 barcoded transposon insertion sites mapped to the *V. cholerae* C6706 genome,

223   with at least one barcoded transposon mapped to 2833/3571 protein-coding genes

224   (79.33%) (Table 2) (33).

225

226   We first used our RB-TnSeq library in experiments to screen for mutants that failed to

227   grow. These experiments were done in triplicate in TCBS broth cultures that contained

228   all components of TCBS (labeled "TCBS complete") to maintain library diversity. By

229   sequencing and counting the barcodes present in the selected mutant pool and

230   comparing those counts to those obtained from multiple uncultured library aliquots (see

231   methods), we calculated scores for 2649 *V. cholerae* genes.

232

233   Our screening approach was validated as we measured highly negative mutant

234   phenotypes for a multitude of genes that are known to be necessary for survival in the

235   presence of bile (Fig 5A). For example, the outer membrane porins OmpU and OmpT,

236   which are known to interact differently with bile, provide a useful test case for the screen

237   (34). OmpT transport is blocked by deoxycholic acid, and as such does not contribute to

238   bile resistance (11). The OmpU porin, on the other hand, is transcriptionally upregulated

239   in response to bile by ToxR and has been long implicated in *V. cholerae* bile resistance

240   (7, 9). Concordantly, while *ompT* had a nearly neutral score (-0.022) for growth in TCBS

241   in our screens, *ompU* had a highly negative score (-6.546) (Fig. 5A). We also measured

242   deleterious phenotypes for mutants in *tolC* (-7.417), *vexAB* (-2.314 and -2.276) and

243   *vexCD* (-1.98 and -1.914) (Fig. 5A). VexAB and VexCD are Resistance Nodulation

244   Division (RND) family efflux pumps that function in conjunction with the outer membrane

245   pore TolC to expel bile acids from the cell and thus, are known to be involved in *V.*

246   *cholerae* resistance to bile (35–38).

247

248   Given the success of our screen in identifying genes that could be predicted to be

249   required for growth in TCBS, we next examined the rest of the genome. To identify

250   genes that are specifically required for TCBS and do not exert general toxicity, we

251   compared the datasets generated from growth in TCBS broth to a similar analysis of the

252   library grown in LB using the same conditions. Genes were filtered for specific defects

253   as described in the materials and methods. Filtering the data using these metrics

254   yielded 111 genes that, when interrupted by Tn insertion, cause strong defects to *V.*

255   *cholerae* grown in TCBS broth relative to LB (Fig. S1A). Gene ontology term enrichment

256   analysis using ShinyGO (25) on these 111 genes revealed enrichment for genes

257   involved in 4 major cellular processes: 1.) phospholipid transport, membrane

258   organization and lipopolysaccharide (LPS) biosynthesis, 2.) phosphate ion transport, 3.)

259   DNA replication and repair, and 4.) carbohydrate import and metabolism (Fig. S1B). Of

260   note, we identified negative scores for many genes implicated in DNA repair (Fig. 5B).

261   We retrieved several mutants in DNA repair genes from the ordered mutant library and

262   demonstrated they had decreased growth on TCBS, but not LB, relative to the WT strain

263   (Fig. 5C).

264

265   **RB-TnSeq reveals both component-dependent and independent growth defects**

266   **for *V. cholerae* mutants in TCBS.** Given that TCBS is a complex media with many

267   components (Table 1), we wondered how each selective ingredient impacted the gene

268   fitness scores observed upon selection with complete TCBS. We once again leveraged

269   the high-throughput qualities of RB-TnSeq by repeating the TCBS ("complete" medium)

270   screens in TCBS broth base ("base" medium) and TCBS broth base ("base+1") media

271   supplemented with either sodium citrate, ox bile, sodium cholate, ferric citrate, or

272   sodium thiosulfate (Table 1).

273

274   Principal component analysis of each replicate dataset from both the TCBS complete,

275   base, and base+1 experiments show strong clustering of replicate experiments (n=3).

276   Interestingly, all the base+1 media datasets cluster together with the exception of base

277   +ox bile (Fig 6A), demonstrating that the addition of ox bile applies a unique, strong

278   selective pressure to the pool of mutants relative to the other selective components.

279   Interestingly, TCBS complete media is distinct from every other dataset, implying that

280   the multiple inhibitory components in TCBS complete apply a combinatorial selective

281   pressure on *V. cholerae* growth that cannot be attributed to individual components

282   themselves.

283

284   Given the observed clustering of the TCBS base +ox bile datasets relative to the rest of

285   the base+1 experiments, we first examined the results of the TCBS base +ox bile

286   dataset using the filtering metrics previously described. Filtering genes and scores by

287   these metrics yielded a list of 60 genes that exhibited specific growth defects in TCBS

288   base + ox bile (Fig. 6B). As expected, we had strong gene fitness scores for *xseA* and

289   *xseB* in TCBS complete and TCBS base +ox bile, confirming our previous results (Fig.

290   6C). Notably, no other strongly negative scores were observed for *xseA* and *xseB* in any

291   other TCBS base+1 condition, consistent with our results from Fig. 1B. This result

292   provides confidence that we have captured genuine component-dependent fitness

293   defects in our barcoded mutant *V. cholerae* strains.

294

295   We next searched for component specific defects by compiling all gene scores across

296   the TCBS base+1 screens and sorted them by their standard deviation across

297   conditions, revealing an assortment of genes with highly negative scores in only one or

298   a few conditions tested (Fig. 6C). *tolC* mutants were highly attenuated in TCBS base

299   supplemented with either ox bile or sodium cholate, but they did not have a fitness

300   defect in sodium citrate, iron citrate, or sodium thiosulfate. Other genes were specifically

301   important for growth in sodium cholate (*vexC* and *vexC*) or ox bile (*xseA*, *xseB*, *vexA*,

302   and *vexB*).

303

304   Additional component-dependent fitness defects were observed for other genes. For

305   example, transposon mutants in *vc2270* (riboflavin synthase alpha subunit, *ribC*) and

306   *vc1655* (magnesium transporter, *mgtE*) did not impact growth in bile acids but showed

307   reduced fitness the base +sodium citrate condition (Fig. 6C). It has been shown

308   previously that *V. cholerae* lacking the peptidoglycan binding protein *csiV* is

309   hypersensitive to the bile acid deoxycholate (39). Thus, we were not surprised to find

310   *csiV* mutants, though selected against in all conditions tested, were most strongly

311   inhibited by sodium cholate and ox bile. These results demonstrate that each selective

312   component of TCBS requires a distinct set of genes to allow growth, supporting that

313   TCBS complete media is the most selective (Fig. 1B) and distinct by PCA analysis (Fig.

314   6A).

315

316   **Discussion**

317   Although TCBS has been used to isolate *V. cholerae* for decades, there is limited

318   understanding of how this media is selective for *Vibrios* and the traits of *V. cholerae* that

319   are critical for growth. Here, using a combination of forward and reverse genetics, we

320   establish that *V. cholerae* experiences significant DNA damage when growing on TCBS

321   and thus DNA repair is a key phenotype that promotes growth.

322

323   We serendipitously found that ExoVII is necessary for *V. cholerae* growth on TCBS

324   agar, and that ox bile is largely responsible for the attenuation of *exoVII* mutants.

325   Additionally, we found that suppression of the *exoVII* TCBS growth defect is through

326   DNA gyrase mutations. Interestingly, some, but not all, of these DNA gyrase mutations

327   also confer ciprofloxacin resistance. Previously, ExoVII had no well-described role

328   outside of functional redundancy with ExoI and SbcCD exonucleases in recombinational

329   repair (40) and ExoI and RecJ in methyl-directed mismatch repair (MMR) (41). Recently,

330   ExoVII of *E. coli* was shown to be the exonuclease that is capable of excising

331   quinolone-induced trapped DNA gyrase cleavage complexes (19). Together, this

332   evidence suggests that the components of TCBS agar (most likely ox bile) cause

333   ciprofloxacin like DNA damage to *V. cholerae*, explaining why *exoVII* mutant *V. cholerae*

334   are inhibited by TCBS.

335

336   We can only speculate how the *gyrA* and *gyrB* mutations suppress the *exoVII* mutant's

337   sensitivity to ox bile. Even more interesting is the nature of the $gyrB_{\Delta1018-1026}$ mutation

338   that confers suppression to *exoVII* mutants on TCBS and ciprofloxacin resistance,

339   considering the most prevalent mechanism for evolving quinolone resistance in bacteria

340   involves missense mutations in the quinolone resistance determining region (QRDR) of

341   *gyrA* (amino acids 67-106 according to *E. coli* KL16 numbering) (42, 43). Because these

342   residues allow targeting of quinolones to DNA gyrase/topoisomerase IV through a

343   conserved water-metal ion bridge (44, 45), it is unlikely that any of the *gyrA* or *gyrB*

344   mutations presented here alter the affinity of ciprofloxacin for DNA gyrase. This could

345   indicate that the inhibition of *exoVII* mutant *V. cholerae* on TCBS occurs in a mechanism

346   distinct from quinolones, though both insults result in lethality due to accumulation of

347   trapped DNA gyrase cleavage complexes. Further mechanistic studies are required to

348   disentangle the similarities and differences of how ox bile and ciprofloxacin cause DNA

349   damage.

350

351   Besides the DNA gyrase mutations, one of the four suppressor mutants was found to

352   have a frameshift mutation in *dinG* that likely results in a null phenotype. DinG is an

353   ATP-dependent, structure specific helicase with 5' → 3' directionality (46). *In vitro*

354   studies using a variety of substrates indicate that DinG is active on structures that mimic

355   replication and homologous recombination intermediates (47). It has been suggested

356   that DinG works semi-redundantly alongside helicases Rep and UvrD to permit DNA

357   replication fork progression through transcribed regions by either displacing R-loops or

358   dislodging RNA polymerase (48). It is currently unclear why a null mutation in *dinG*

359   would suppress the toxicity of TCBS to the *exoVII* mutant. However, *dinG* was found to

360   be upregulated in *E. coli* following treatment with the quinolone nalidixic acid (49).

361   Additionally, *N. meningitidis dinG* mutants were found to be more sensitive to DSBs

362   caused by mitomycin C than WT (50). We speculate that, in the absence of capable

363   ExoVII, DinG may be overactive and thus is deleterious for *V. cholerae* cells grown on

364   TCBS.

365

366   In this work we also confirm that, like in *E. coli* and *S. agalactiae*, *V. cholerae exoVII*

367   mutants are hypersensitive to ciprofloxacin (19, 51), highlighting a common role for

368   ExoVII in excising quinolone-induced trapped DNA gyrase cleavage complexes among

369   these bacteria. In doing so we also report a role for ExoVII in surviving bile-mediated

370   DNA damage. It is of particular interest how ox bile may mimic the DNA-damage caused

371   by quinolones. An additional complication arises considering that ox bile as a poorly

372   defined mixture of 9 individual bile acids, with different suppliers having varying

373   abundances of each bile acid (52).

374

375   It is well understood that bile possesses antimicrobial properties, though our

376   understanding of the exact mechanism(s) through which killing occurs is incomplete. A

377   previous study found that the bile acids chenodeoxycholate and deoxycholate activate

378   the SOS response in *E. coli*. Additionally, the study demonstrates that *E. coli* cells

379   treated with these bile acids induce the SOS response and are killed similar to

380   mitomycin C treatment (18). Other studies have suggested roles for base excision

381   repair, SOS-induced DNA repair, and recombinational repair mediated by RecBCD in *S.*

382   *enterica* for tolerating bile-induced DNA damage (13, 14). Moreover, evidence from both

383   gram positive and negative bacteria suggests that resistance to bile is multifaceted.

384   Several enteric bacteria express efflux pumps capable of expelling bile salts from the

385   cell, thereby preventing extensive membrane damage caused by the detergent-like

386   properties of bile. However, when membranes are disrupted and the cell's permeability

387   barrier is disturbed bile salts may enter cells and cause DNA damage, halting replication

388   and leading to cell death in the absence of appropriate DNA repair mechanisms (12).

389

390   TCBS is routinely used in laboratories to confirm the identity of *V. cholerae* strains. We

391   demonstrate here that extended passage of *V. cholerae* on TCBS agar selects for

392   ciprofloxacin resistant isolates and potentially against mutants that have defects in DNA

393   repair machinery. Therefore, serial passaging on TCBS might be mutagenic and thus

394   should be performed cautiously. We suggest against the practice of screening DNA

395   repair mutant strains over TCBS for confirmation of *Vibrios* since some DNA repair

396   mutants fail to grow completely and may accumulate further mutations when cultured

397   over TCBS.

398

399   More importantly, bacterial pathogens can often evolve to have a mutator phenotype

400   during infection. This is typically a result of deficiencies in MMR (53), and can allow

401   bacteria to gain genetic diversity to allow rapid evolution to new environments.

402   Hypermutators have been observed in enteric pathogens like *E. coli* and *S. enteritidis*

403   (54). Moreover, increased propensity for mutation is likely to play a role in the long-term

404   colonization of cystic fibrosis (CF) patients, from which hypermutable *Burkholderia*

405   *pseudomallei* (55), *Haemophilus influenzae* (56) and *Pseudomonas aeruginosa*(57)

406   have been isolated. For *V. cholerae,* such mutators are not historically thought to evolve

407   during infection. However, a recent metagenomic study of cholera patients and their

408   household contacts indicated the presence of hypermutable *V. cholerae* in clinical

409   isolates from both symptomatic and asymptomatic individuals based on sequence

410   analysis (58). Our results suggest an intriguing possibility that TCBS itself may be

411   selecting against mutator strains that evolve *in vivo* leading their underrepresentation in

412   clinical *V. cholerae* isolates.

413

Materials and Methods

Growth of *V. cholerae* strains.

Unless otherwise noted, wild type (WT) and mutant *V. cholerae* strains were cultivated

from frozen glycerol stocks on shaking incubators set to 37°C with shaking at 210rpm.

Strains to be plated in any derivation of TCBS agar were grown in TCBS base broth

(homebrew) using the wt/vol amounts listed in Table 1, while strains plated on LB agar

were grown in LB broth. All strains are described in Table S2 while all primers are

described in Table S3. When necessary, growth media was supplemented with

kanamycin (100 μg/mL), chloramphenicol (10 μg/mL), and/or trimethoprim (10 μg/mL).

423

Generation or retrieval of mutant *V. cholerae* strains.

Strain TND0252, TND0252, SAD238 and SAD530 were generously gifted to us by

Ankur Dalia, Indiana University. Strains CW2171, CW2172 and CW2173 were

generated by using natural transformation (MuGENT) as previously described (20).

Briefly, *tfox*, *recJ* and *xseA* genes were amplified from WT C6706 gDNA and then used

to repair these three mutations individually in the TND0252 parent background by co-

transformation with a Δ*vc1807::trimR* fragment. The Δ*vc1807::trimR* fragment was

generated by using primers ABD346 and ABD347 to amplify it from strain SAD530.

Strain JBG013 was made using primers JBG019 and CW2709 to amplify the mutant

Δ*lacZ* allele from strain SAD238 (20). We then used MuGENT to transform WT *V.*

cholerae with the Δ*lacZ* allele and Δ*vc1807::trimR*, yielding strain JBG013.

435

436   Unless otherwise noted, transposon mutant strains were retrieved from an ordered

437   mutant library (28). *xseB*::Tn was made by amplifying the region surrounding the *xseB*

438   locus with primers xseB_2.5kb_F and xseB_2.5kb_R (Table S3), and then using an *in*

439   *vitro* Tn5-Kan^R transposon insertion kit (Biosearch Technologies Inc, TNP92110).

440   Competent *V. cholerae* was prepared by growing strain NG001(harboring plasmid

441   pMMB-*tfox-qstR*) in LB broth with chloramphenicol and 100 µg/ml isopropyl β-D-1-

442   thiogalactopyranoside (IPTG) to maintain the plasmid and induce natural competence,

443   respectively. Competent NG001 was diluted 1:4 in 0.5x instant ocean before tDNA was

444   added. We then transformed WT C6706 with the *xseB*::Tn fragment as previously

445   described (21) and confirmed mutation by sanger sequencing before curing the mutant

446   of the competence-inducing plasmid.

447

448   **Curing strains of pMMB-*tfoX-qstR*.**

449   Sequence confirmed mutants were isolated and cultured in LB with the appropriate

450   antibiotic overnight. The following day, the culture was struck over an LB plate to obtain

451   isolated colonies. After incubation overnight, isolated colonies were selected and patch

452   plated over LB + kanamycin agar and LB + chloramphenicol agar. Cured clones were

453   identified as those that grow on LB + kanamycin agar but do not grow on LB +

454   chloramphenicol agar.

455

456   **Spot plating of bacterial strains.**

457   Strains were grown as previously described overnight before being subcultured 1:100 in

458   the same medium. Once reaching exponential growth strains were diluted in PBS to an

459   OD$_{600}$ of 0.5. Serial 10-fold dilutions were made and 2 μL were plated in spots and

460   allowed to dry. Plates were then incubated at 37°C overnight.

461

462   **Generation of *xseA*::Tn suppressor mutants.**

463   *xseA*::Tn *V. cholerae* C6706 was retrieved from an ordered mutant library and cured of

464   the competence-inducing plasmid (28). *xseA*::Tn was then cultured in LB overnight and

465   100 μL of the saturated culture was plated on TCBS agar. The plate was incubated at

466   37°C for 48 hours, after which isolated colonies were selected for follow up

467   experiments. We extracted genomic DNA from the mutants using the Promega Wizard

468   Genomic DNA Purification Kit (A1120). Genome sequencing of mutants was performed

469   by SeqCoast Genomics in 150bp paired-end reads on an Illumina NextSeq 2000 and

470   mutations were predicted using BreSeq (22).

471

472   Suppressor mutations were determined by comparing the BreSeq outputs for our

473   evolved suppressor mutants against the BreSeq outputs generated by sequencing an

474   assortment of repaired ordered library strains. We then amplified the *gyrA* (using

475   primers gyrA_F and gyrA_R, Table S3) and/or *gyrB* (using primers gyrB_F and gyrB_R,

476   Table S3) alleles from these mutants and transformed them into either *xseA*::Tn or WT

477   parent strains using pMMB-*tfox-qstR* as described above. Mutations were confirmed by

478   whole genome sequencing before strains were cured of the competence-inducing

479   plasmid.

480

481   **Disc diffusion assays.**

482 Whatman filter paper was cut into discs using a hole puncher and sterilized by

483 autoclaving. Strains were grown overnight and then back diluted 1:100 before being

484 allowed to grow to exponential phase. Cells were then diluted to OD_{600}=0.1 in PBS and

485 100 μL was plated on LB agar or TCBS agar. Discs were then placed with sterile

486 forceps onto inoculated plates before being impregnated with different dosages of

487 ciprofloxacin or a 0.1N HCl vehicle control. Plates were incubated at 37°C overnight and

488 zones of inhibition were measured the following day.

489

490 **Serial passaging over TCBS agar.**

491 A single culture of $\Delta lacZ$ *V. cholerae* was grown overnight in LB+ trimethoprim. The

492 following morning the culture was back diluted 1:100 before being allowed to grow to

493 exponential phase. Cells were then diluted to OD_{600} to 0.5, and 100 μL of diluted culture

494 was plated on either LB or TCBS agar. Plates were incubated at 37°C overnight. The

495 following day, a sterile 10 μL loop was used to collect samples from bacterial lawns,

496 which were then resuspended in 10 mL PBS. Samples were then diluted further in PBS

497 to OD_{600} of 0.5 and 100 μL of the dilutions were replated on either LB or TCBS agar.

498 Plates were again incubated at 37°C overnight. This process was repeated for 15 days,

499 with passaging happening once per day per lineage (LB lineage and TCBS lineage).

500 Every day 1 mL freezer stocks were made from the PBS resuspensions by mixing 750

501 μL with 250 μL 80% glycerol and storing at -80°C.

502

503 Every three days, the resuspensions were struck on LB + trimethoprim plates that were

504 then incubated at 37°C overnight. The following morning, 6 isolated colonies per lineage

505   were picked and cultured in 1mL LB broth at 37°C. Cultures were allowed to grow to

506   exponential phase, back diluted to OD_{600} of 0.1, and 100 μL of each was plated over LB

507   agar. We then assayed ciprofloxacin sensitivity using the disc diffusion assays

508   described above.

509

510   **Construction and mapping of the RB-TnSeq mutant *V. cholerae* library.**

511   The *E. coli* APA752 donor strain harboring the pKMW3 transposon vector (kindly gifted

512   to us by Aretha Fiebig, Michigan State University) was grown overnight in LB broth with

513   0.3 mM diaminopimelic acid (DAP) and kanamycin. We introduced pKMW3 into WT *V.*

514   *cholerae* through conjugation. The APA752 culture and a WT *V. cholerae* culture were

515   combined in a 9:1 ratio and before 100 μL was plated over LB with 0.3 mM DAP and

516   incubated at 37°C overnight. The following day, bacteria were collected with sterile

517   loops, resuspended in 50 mL LB, and pelleted by centrifugation. The supernatant was

518   removed, and cells were washed in 15 mL LB to remove residual DAP. 5 mL of 80%

519   glycerol was added, and the entire 20 mL volume was split up between 10x2 mL frozen

520   stocks. Transposon mutant titer was measured by plating transconjugants over LB +

521   kanamycin agar. Afterwards, 200 μL of thawed transconjugant stocks were plated over

522   twenty-five 150x15mm petri dishes containing LB agar with kanamycin. Plates were

523   incubated at 37°C for 48 hours.

524

525   Following incubation, three representative plates were selected, and colonies were

526   counted to estimate the total mutant colonies collected between all 25 plates. For each

527   plate, 3 mL of LB was added, and colonies were resuspended using sterile L-shaped

528   spreaders. We collected as much of the 3 mL volume as possible from each plate and

529   pooled it all in a single 50 mL conical. Afterwards, 30 mL of the collected cells were

530   added to a flask containing 270 mL LB with kanamycin, and the mutants were incubated

531   at 37°C with 210 rpm shaking for 1 hour. Glycerol was then added to a 20% final

532   concentration and hundreds of 100 μL library aliquots were frozen for single use, as well

533   as a few dozen 1mL aliquots to facilitate efficient genomic DNA extraction. We extracted

534   genomic DNA from the mutants using the Promega Wizard Genomic DNA Purification

535   Kit.

536

537   For library mapping, we followed the strategy of Wetmore and colleagues (23) with

538   minor modifications to the PCR enrichment of barcode-containing insertion junctions.

539   The modifications are described in detail elsewhere (24). Briefly, 5 μg of library genomic

540   DNA was sheared with a Covaris M220 ultrasonicator to produce ~300 bp fragments.

541   Genomic DNA was then electrophoresed on a 1% TBE gel, stained with SYBR Gold

542   (Invitrogen, S11494), and gel extracted using the Zymoclean Gel DNA Recovery Kit

543   (Zymo Research, D4001) to select for fragments between 150-500 bp. We then

544   performed end repair, A-tailing and adaptor ligation with the NEBNext Ultra II DNA

545   Library Prep Kit for Illumina (E7645S) following the manufacturer's recommended

546   protocol using custom adaptors made by annealing oligos Mod2_TruSeq and

547   Mod2_TS_Univ (Table S3). Adaptor-ligated DNA was cleaned without size selection

548   using NEBNext Sample Purification Beads (0.9x) and eluted in 0.1x TE buffer following

549   the manufacturer's instructions.

550

551 DNA fragments containing the transposons were enriched in a 2-step nested PCR

552 strategy using modified primer sequences based on the original primer sequences used

553 by Wetmore et al (23). This modified enrichment strategy is described by Fiebig and

554 colleagues (24). In the first PCR we used the forward primer TS_pHimar+4_F and the

555 reverse primer TS_R (Table S3). Cycling parameters were 98°C for 2 min, 10x (98°C,

556 30sec; 70°C, 20sec; 72°C, 20sec), 72°C for 5min, and a 4°C hold. After the first PCR,

557 products were cleaned again using NEBNext Sample Purification beads (0.9x) and

558 eluted in 0.1x TE buffer. 15 μL of the cleaned PCR product was used as template in the

559 second transposon enriching PCR. In the second PCR we used the primers

560 P7_MOD_TS_index3 and P5_TS_F (Table S3) to add Illumina P5 and P7 sequences

561 and a 6-bp i7 index. Cycling parameters were 98°C for 3 min, 10x (98°C, 20sec; 70°C,

562 10sec; 72°C, 20sec), 72°C for 5min, and a 4°C hold. Following the second PCR,

563 products were again cleaned using NEBNext Sample Purification beads (0.9x) and

564 eluted in 0.1x TE buffer. Reagents for both PCR reactions were supplied in the

565 NEBNext Ultra II DNA Library Prep Kit for Illumina. For both reactions we used the

566 volumes of reagents as outlined by the kit manual. Prepared reads were submitted to

567 SeqCoast Genomics (Portsmouth, NH) for sequencing on an Illumina NextSeq using a

568 300-cycle NextSeq P1 reagent kit (Illumina 20050264). The sequencing run was

569 supplemented with 25% phiX DNA to aid in clustering. Locations of reliable transposon

570 insertions were aligned and mapped to the *V. cholerae* C6706 genome (GenBank

571 accession numbers CP064350 and CP064351) using custom Perl scripts written and

572 described by Wetmore et al (23) (available at

573   https://bitbucket.org/berkeleylab/feba/src/master/). Mapping statistics are provided in

574   Table 2.

575

576   **Competition of RB-TnSeq *V. cholerae* mutants in liquid media.**

577   For each replicate RB-TnSeq experiment in TCBS broth (including TCBS with or without

578   a selective component), we thawed a single 100 μL aliquot of the RB-TnSeq library on

579   ice before being added to 100 mL of experimental liquid medium in 250 mL unbaffled

580   Erlenmeyer flasks. Initial OD_{600} values were obtained before cultures were incubated for

581   5 hours at 37°C with shaking. OD_{600} values were taken hourly. After 5 hours, cultures

582   were collected in 50 mL conical tubes and pelleted. Supernatants were removed and

583   genomic DNA was extracted from pellets as described above. The comparison

584   experiment performed in LB broth was done almost exactly as described above, except

585   that the cultures were incubated until they had reached OD_{600} of ~1.0 (4 hours and 10

586   minutes)

587

588   The amplification and sequencing of barcodes was performed following the approach

589   described by Wetmore et al (23). 100-200 ng of genomic DNA from each experiment

590   sample was used as template to amplify mutant barcodes by PCR. Barcodes were

591   amplified in 50 μL reaction volumes using Q5 DNA polymerase (New England Biolabs)

592   using 1x Q5 reaction buffer, 1x High GC enhancer, 1.0 units Q5 polymerase, 0.2 mM

593   dNTP and 0.4 μM of each primer. The forward primer was BarSeq_P1, while each

594   reaction used a uniquely indexed reverse primer BarSeq_P2_ITXXX, where "XXX"

595   corresponds to the index number as used by Wetmore and colleagues (Table S3).

596   Reaction conditions were as follows: 98°C for 4 min, 25x (98°C, 30sec; 55°C, 30sec;

597   72°C, 30sec), 72°C for 5min, and a 4°C hold. Following, samples were run on a 2% TAE

598   agarose gel to confirm the presence of PCR products. 5 μL of each PCR was then

599   pooled, and the pool of PCR products was cleaned using a DNA Clean & Concentrator

600   kit (Zymo Research, D4003).

601

602   Barcodes were then sequenced by SeqCenter (Pittsburgh, PA) on a single lane of a

603   NovaSeq X Plus flowcell in 2x150-cycle configuration. Barcodes were supplemented

604   with 50% phiX DNA to aid in clustering and loaded at 110 pM. Reads were trimmed to

605   75 bp before analysis to eliminate unnecessary Illumina adaptors, leaving the barcode

606   sequence. Barcode sequences in each sample were counted and the fitness of each

607   mutant strain was calculated as the normalized log2 difference in barcode counts in the

608   treated sample versus replicate reference samples using the custom scripts developed

609   by Wetmore et al. For our reference samples, we extracted genomic DNA from four

610   individual uncultured 100 μL library aliquots, amplified the barcodes and sequenced

611   them as described above. Gene scores were calculated based on the weighted average

612   of strain scores for mutants with insertions in the central 80% coding region of the gene,

613   again using the scripts developed by Wetmore et al. (available at

614   https://bitbucket.org/berkeleylab/feba/src/master/).

615

616   **Gene Ontology enrichment using ShinyGO.**

617   We wanted to identify genes that were specifically important for TCBS growth and not

618   standard rich media. To identify these genes, we made the following comparison of

619 fitness scores in TCBS to those in LB. Having calculated gene scores based on

620 differences in barcode abundance relative to an uncultured library sample, we then

621 filtered our analysis to exclude genes that have a score in TCBS or TCBS base +ox bile

622 $\leq$-1 (mutant must be attenuated for growth in the experimental condition), $\geq$-2 in LB (to

623 exclude those mutants with general fitness defects in rich media), and a difference in

624 scores between the two conditions (experimental condition score – LB score) $\leq$-1. Using

625 this thresholding strategy, we identified 111 genes necessary for growth in TCBS broth

626 and 60 genes necessary for growth in TCBS base + ox bile broth, relative to their

627 necessity for growth in LB.

628

629 We then performed gene ontology enrichment by comparing the gene IDs against

630 annotations present in the *V. cholerae* N16961 genome (taxonomy ID 243277) using

631 ShinyGO 0.80 (25). To eliminate error in the false discovery rate produced by ShinyGO,

632 we submitted a background list of genes that included the gene IDs for all 2649 scored

633 across the RB-TnSeq experiments. This way, enrichment of genes meeting our filtering

634 criteria isn't biased by the absence of genes we were unable to assign scores to in our

635 RB-TnSeq experiments. ShinyGO options were as follows: FDR cutoff = 0.05, #

636 pathways to show = 10, pathway size minimum = 10. For clarity, we then performed

637 some manual binning of pathways based on redundancy between pathway names and

638 the genes belonging to them (for example, pathways "DNA replication, and DNA repair",

639 "DNA metabolic process, and chromosome segregation" and "DNA repair, and DNA-

640 dependent ATPase activity" contain many of the same genes, so we group them

641 together more broadly under "DNA repair").

642

Statistical analysis and data visualization.

644 All statistical analyses were performed in GraphPad Prism version 10.1.1 Principal

645 component analysis plot was made in R version 4.3.2 (26) using the vegan package

646 (27) version 2.6-6.1.

647

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912

913   **FIGURE LEGENDS**

914   **Figure 1: *exoVII* mutant *V. cholerae* cannot grow on TCBS agar.** (A) Parent strains

915   (left plate) and repaired strains (right plate) were struck over TCBS agar and assayed

916   for growth after 24 hours incubation. Strain E7946 is the parent to TND0252. TND0252

917   is the parent strain to CW2171, CW2172 and CW2173. (B) Serial dilutions of WT and

918   *xseA*::Tn *V. cholerae* spotted over TCBS agar, or TCBS base agar supplemented with a

919   single selective component as indicated using the wt/vol listed in Table 1. For both

920   figures A and B, plates shown are representative of 3 biological replicate experiments.

921

922   **Figure 2: Inhibition of *exoVII* mutants on TCBS agar can be suppressed by**

923   **mutations in DNA gyrase.** (A) Together, *gyrA* and *gyrB* encode DNA gyrase. Color-

924   coded bands within the *gyrA* and *gyrB* reading frames correspond to mutations present

925   among three suppressor mutants generated by plating *xseA*::Tn over TCBS agar and

926   selecting isolated colonies once growth was observed. (B) Serial dilution and spot

927   plating of WT, *xseA*::Tn and *exoVII* suppressor mutants over TCBS agar and LB agar.

928   Plates shown are representative of 3 biological replicate experiments.

929

930   **Figure 3: *exoVII* and *gyrAB* alter *V. cholerae* ciprofloxacin resistance.** (A) WT,

931   *exoVII* and *exoVII* suppressor mutants were assayed for ciprofloxacin sensitivity by disc

932   diffusion with 5 ng of ciprofloxacin on LB agar. Results shown are from 6 biological

933   replicate experiments. The dotted line at 6mm is labeled "LOD" for limit of detection as

934   discs were 6 mm in diameter. Results were analyzed by one-way repeated measures

935   ANOVA with Bonferroni's multiple comparisons test. ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq$

936   0.01; ***, $P \leq 0.001$. (B) Serial dilution and spot plating of WT, *exoVII* and *exoVII*

937   suppressor mutants over TCBS base + ox bile agar. Plates shown are representative of

938   3 biological replicate experiments.

939

940   **Figure 4: Passaging *V. cholerae* on TCBS agar selects for ciprofloxacin**

941   **resistance.** (A) WT *V. cholerae* was assayed for ciprofloxacin sensitivity by disc

942   diffusion with 5ng of ciprofloxacin on LB or TCBS agar. Results shown are from 6

943   biological replicate experiments. Results were analyzed by unpaired T test using the

944   Holm-Sidak method. $P < 0.0001$. (B) WT *V. cholerae* and a strain carrying the $gyrB_{\Delta1018\text{-}}$

945    $_{1026}$ mutation were assayed for ciprofloxacin sensitivity by disc diffusion with 5ng of

946   ciprofloxacin on LB agar. Results shown are from 6 biological replicate experiments.

947   Results were analyzed by unpaired T test with Welch correction using the Holm-Sidak

948   method. $P < 0.0001$. (C) Comparison of ciprofloxacin sensitivity of 6 randomly-selected

949   isolated *V. cholerae* (*lacZ* mutant) colonies following consecutive daily passages on

950   either TCBS or LB agar (see methods). Isolates were assayed for ciprofloxacin

951   sensitivity by disc diffusion with 5ng of ciprofloxacin on LB agar. The red lines

952   correspond to the median ZOI among the 6 isolates. The dashed lines labeled "*lacZ*"

953   and "WT" represent the average (n = 3) ZOIs for the unpassaged *lacZ* parent strain and

954   unpassaged WT *V. cholerae*, respectively. Each data point represents the ZOI of a

955   distinct isolate. Results were analyzed by Mann-Whitney two-tailed T test between the

956   two lineages at each passage shown. Day 12, P = 0.026; Day 15, P = 0.0087. In A, B

957   and C, the dotted line at 6mm is labeled "LOD" for limit of detection as discs were 6mm

958   in diameter.

959

960   **Figure 5: Deployment of an RB-TnSeq library identifies DNA repair mutants**

961   **attenuated for growth on TCBS agar**. (A) Mutant scores for genes known to be

962   required for *V. cholerae* bile tolerance are shown. In each bar, each datapoint

963   represents the gene score measured in 1 of 3 biological replicate experiments. A

964   negative gene score indicates interruption of that gene by transposon insertion had a

965   deleterious effect, while a positive score indicates a growth advantage. (B) DNA repair

966   genes meeting quality thresholds (see methods) identified by gene ontology enrichment

967   using ShinyGO 0.80 are shown. Gene scores are the average of 3 biological replicate

968   experiments. (C) Serial dilution and spot plating of WT and DNA repair mutant strains

969   over TCBS agar and LB agar. Plates shown are representative of 3 biological replicates

970   per strain.

971

972   **Figure 6: RB-TnSeq reveals both component-dependent and independent growth**

973   **defects for *V. cholerae* mutants in TCBS.** (A) Principal component analysis of TCBS

974   complete and TCBS base (with and without individual selective components, Table 1) by

975   gene scores. For each condition, individual experimental replicates are plotted in the

976   same color and connected by lines. Plot was generated in R version 4.3.2 using the

977   package vegan. (B) A wide array of mutants are attenuated for growth in TCBS base

978   supplemented with ox bile. Genes shown in heatmap meet quality thresholds (see

979   methods). Gene scores are the average of 3 biological replicate experiments. (C) Gene

980   scores across media demonstrate component-dependent defects for *V. cholerae*

981   mutants. Each dot in each bar represents the gene's score in one of three biological

982   replicate experiments. Dashed vertical lines were added to separate genes.

983

984   **Figure S1: *V. cholerae* mutants attenuated for growth in TCBS broth**. (A) All genes

985   shown meet score thresholds as described in Methods. Gene scores shown are the

986   average of 3 biological replicate experiments. (B) Genes from S1 that fit into a common

987   cellular process are grouped by black labels. Gene scores shown are the average of 3

988   biological replicate experiments.

989

990

Table 1.) Components of Thiosulfate-Citrate-Bile Salts-Sucrose medium. Individual components masses per 1L of TCBS broth. Components thought to be selective are written in bold. pH ~8.6 at 25°C.	
Sucrose	20g
Peptone	10g
Yeast extract	5g

Sodium chloride	10g
Sodium citrate	10g
Ox bile	5g
Sodium cholate	3g
Ferric citrate	1g
Sodium thiosulfate	10g

991

Table 2.) Barcoded mutant *V. cholerae* library summary.	
Mutants collected	77,678
Mapped barcodes	43,751
Off-by-one barcodes masked	7,361 (16.82%)
Usable, mapped barcodes	36,691 (83.86%)
Unique insertion sites	29,988
Central 80% CDS insertions	23,252
Unique central insertion sites	19,108 (82.18%)
Protein-coding genes in *V. cholerae*	3,571
With central insertions	2,833 (79.33%)

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Figure 1.) *xseA* mutant *V. cholerae* cannot grow on TCBS agar

Figure 2.) inhibition of *exoVII* mutants on TCBS can be suppressed by mutations in DNA gyrase
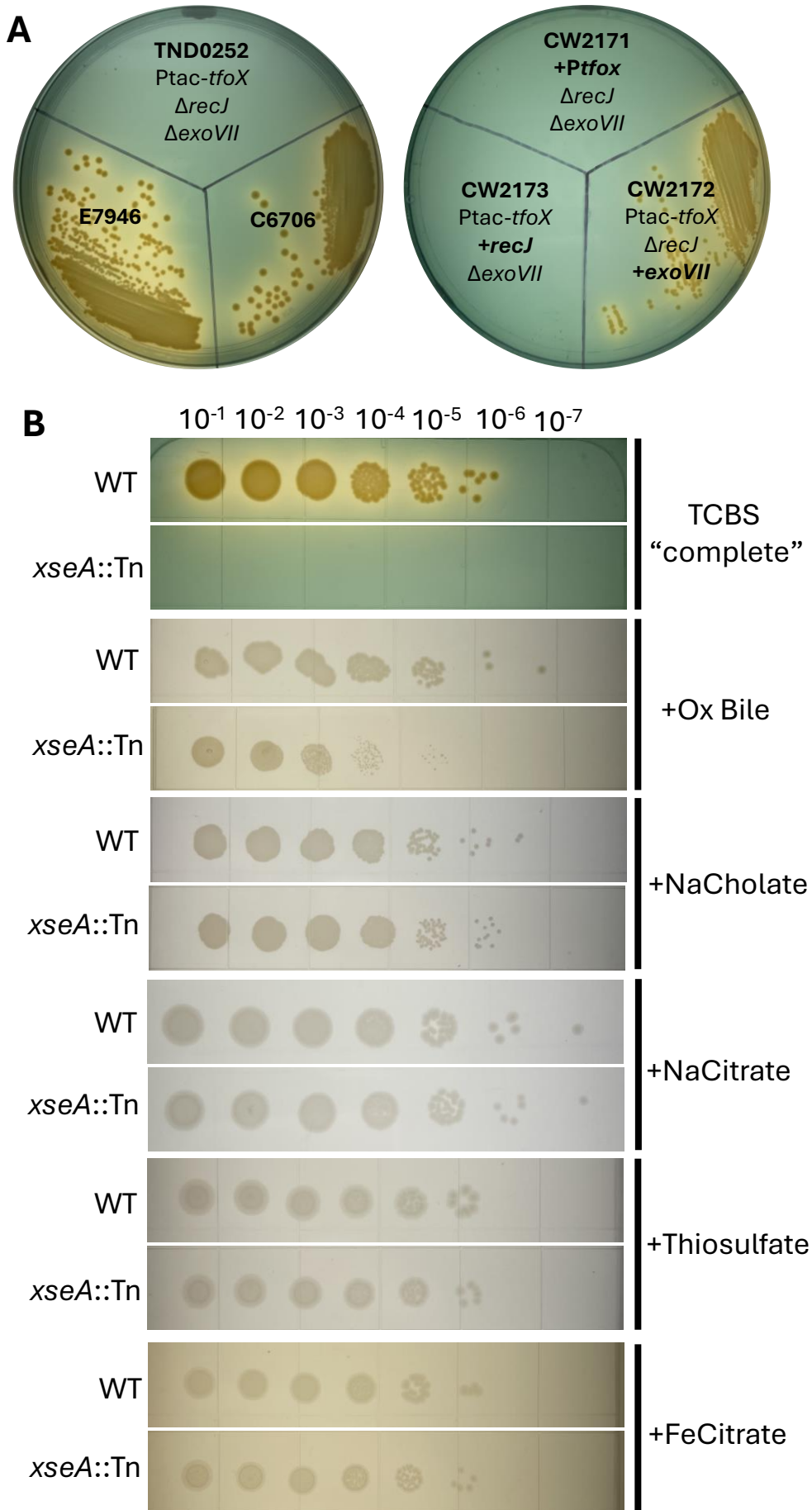
Figure 3.) *exoVII* deficient *V. cholerae* is hypersensitive to ox bile and ciprofloxacin, which can both be suppressed by DNA gyrase mutations.
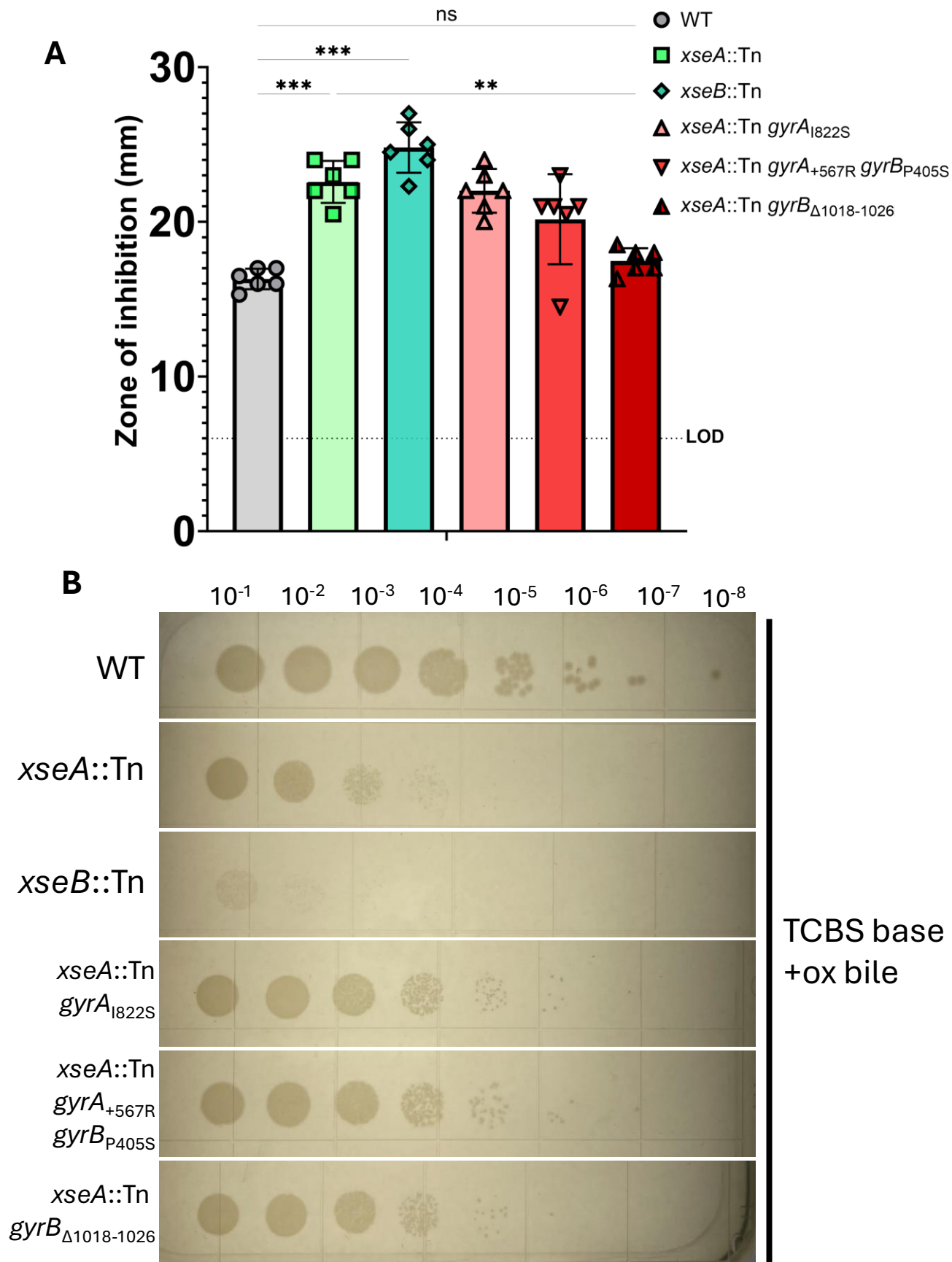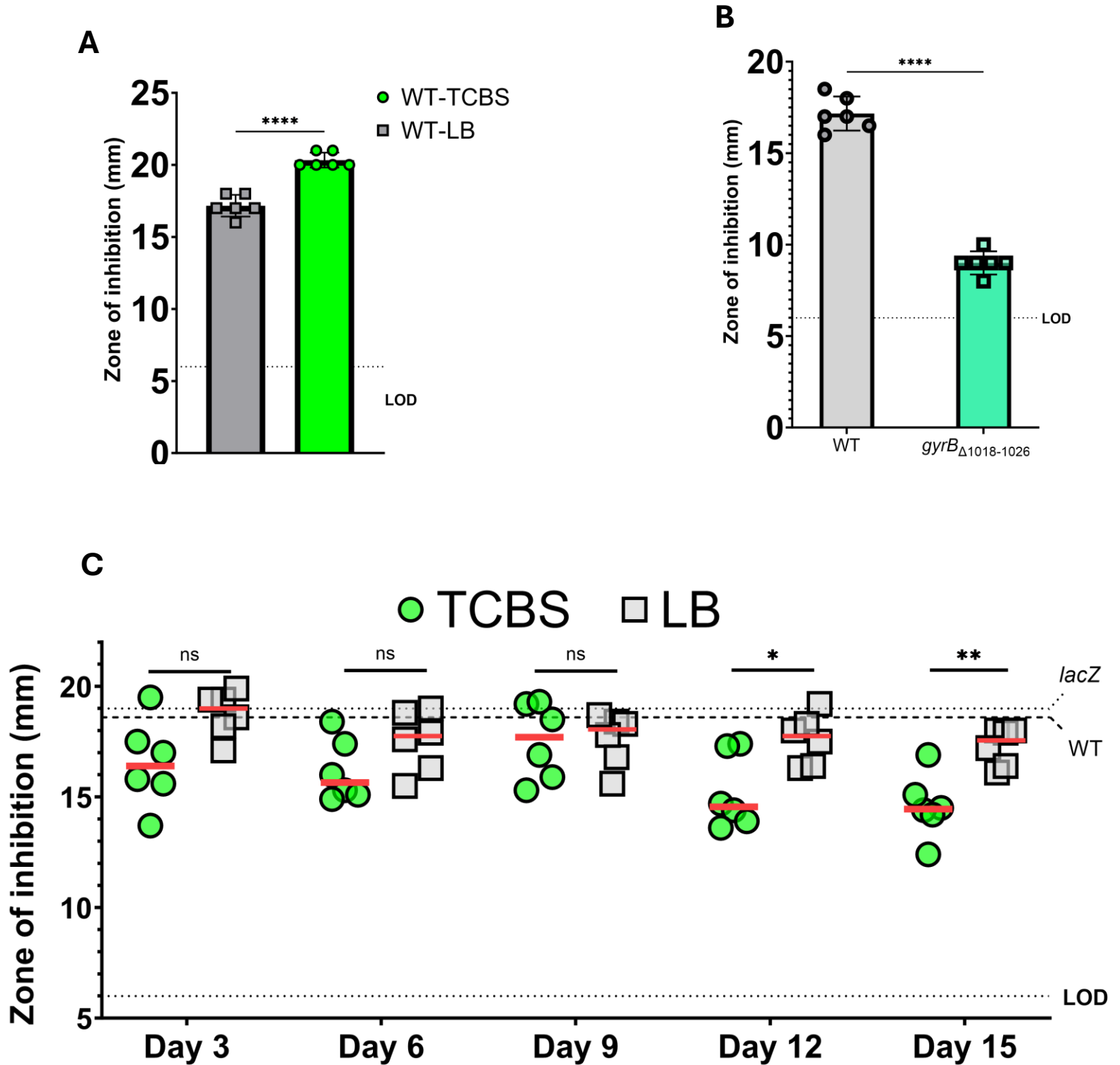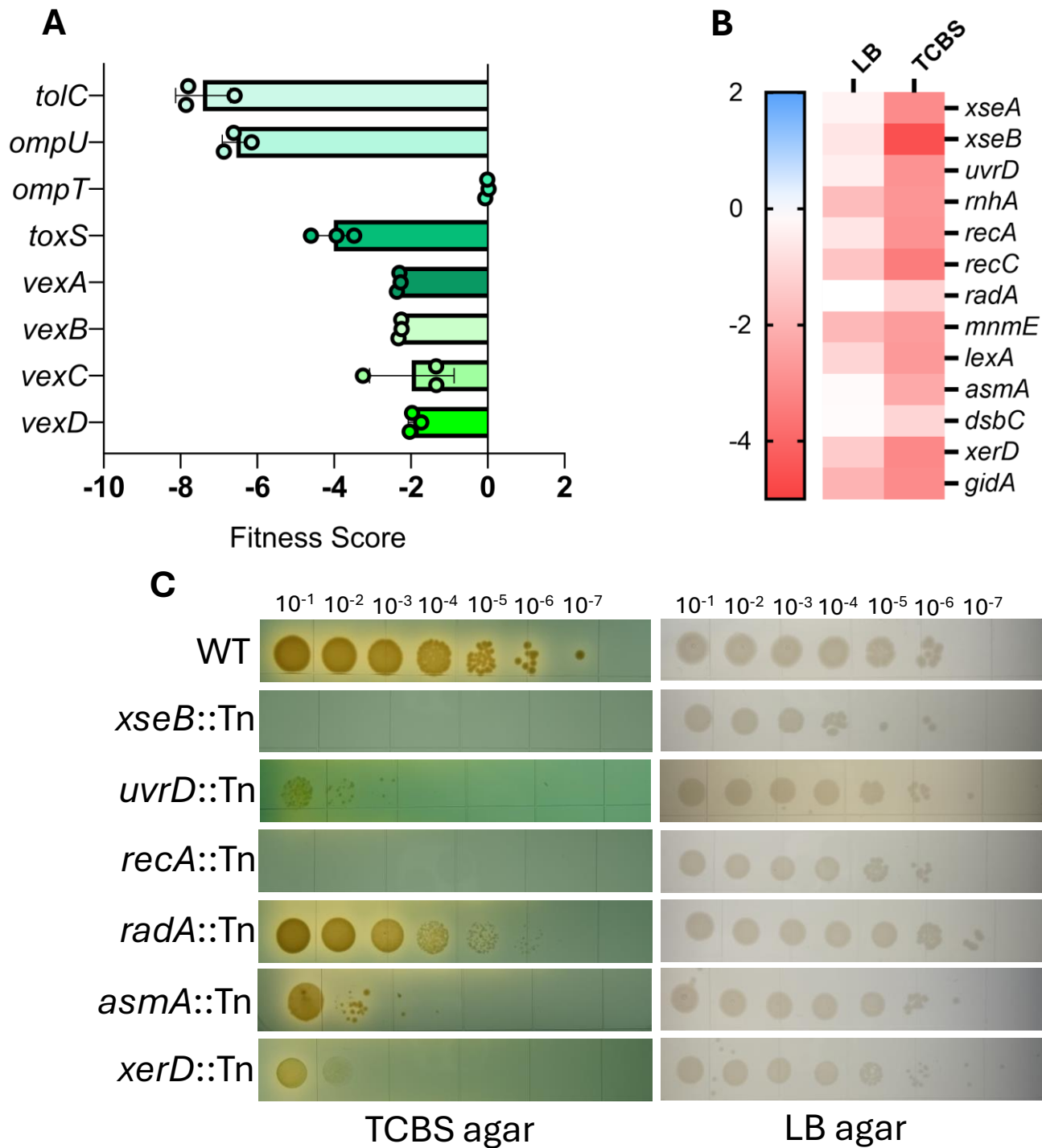
Fig. 4.) Passaging V. cholerae on TCBS agar selects for ciprofloxacin resistance

Figure 5.) Deployment of a randomly-barcoded TnSeq (RB-TnSeq) library identifies DNA repair mutants attenuated for growth in TCBS

Figure 6.) RB-TnSeq reveals component-wise growth defects for *V. cholerae* mutants in TCBS.