1 **Differential ampicillin/ceftriaxone susceptibility among diverse *Enterococcus***

2 ***faecalis* from infective endocarditis**

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8 Running Head: *Enterococcus faecalis* from infective endocarditis

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10 **Abstract**

11 *Enterococcus faecalis* is a leading cause of infective endocarditis (IE), especially

12 among older patients with comorbidities. Here we investigated the genomic diversity

13 and antimicrobial susceptibility of 33 contemporary *E. faecalis* isolates from definite or

14 probable IE cases at the University of Pittsburgh Medical Center (UPMC) between 2018

15 and 2020. Isolates belonging to two multi-locus sequence types (STs), ST6 and ST179,

16 were isolated from nearly 40% of IE patients. Both of these dominant STs carried known

17 beta-lactam resistance-associated mutations affecting the low-affinity penicillin-binding

18 protein 4 (PBP4). We assessed the ability of ampicillin and ceftriaxone (AC) both alone

19 and in combination to inhibit genetically diverse *E. faecalis* IE isolates in checkerboard

20 synergy assays and an *in vitro* one-compartment pharmacokinetic-pharmacodynamic

21 (PK-PD) model of AC treatment. ST6 isolates as well as an isolate with a mutation in

22 the PP2C-type protein phosphatase IreP had higher ceftriaxone MICs compared to

23 other isolates, and showed diminished *in vitro* synergy of AC. Additionally, both ST6 and

24 ST179 isolates exhibited regrowth after 48 hours of humanized exposures to AC.

25 Overall, we found evidence for diminished *in vitro* AC activity among *E. faecalis* IE

26 isolates with PBP4 and IreP mutations. This study highlights the need to evaluate

27 alternate antibiotic combinations in clinical practice against diverse contemporary *E.*

28 *faecalis* IE isolates.

Introduction

29

30   Enterococci are commensal inhabitants of the human gastrointestinal tract (1). In

31 the antibiotic era, *Enterococcus faecalis* has emerged as a leading cause of infection,

32 especially in immunocompromised patients (2). The ability of *E. faecalis* to cause

33 infection is due in part to the bacteria's ability to tolerate a variety of stresses, including

34 desiccation, starvation, and disinfectants (3). *E. faecalis* also possesses intrinsic

35 resistance to a variety of antibiotics, including cephalosporins (4). While the degree of

36 cephalosporin resistance varies between *E. faecalis* isolates, some factors that

37 contribute to this variability include penicillin-binding proteins, two-component systems,

38 and the RNA polymerase beta-subunit (5–8).

39   *E. faecalis* is a common cause of infective endocarditis (IE) (9), and the

40 prevalence of *E. faecalis* IE is steadily increasing (10). Despite the use of *in vitro* active

41 antibiotic combination therapy for at least six weeks, mortality from *E. faecalis* IE

42 remains as high as 30% (11, 12). Treatment guidelines for *E. faecalis* IE include using

43 the combination of ampicillin and gentamicin (AG) or ampicillin and ceftriaxone (AC)

44 (13, 14). AC is a promising alternative to AG because it avoids the nephrotoxicity and

45 the need for therapeutic drug monitoring associated with gentamicin use (15).

46 Observational clinical studies in Europe have suggested that treatment of *E. faecalis* IE

47 with AC is as efficacious as treatment with AG, and results in lower rates of renal

48 toxicity (16, 17). Based upon these findings, AC is now widely used to treat *E. faecalis*

49 IE (18). Nevertheless, *E. faecalis* IE mortality rates have not improved in decades (10).

50 We propose that the optimal treatment approach for this disease has not yet been

51 implemented in clinical practice.

52   Treatment practice for *E. faecalis* IE has largely shifted from AG to AC without

53   investigating how genetically diverse *E. faecalis* isolates respond to AC. Prior studies

54   documenting AC synergy have utilized a limited number of laboratory strains of *E.*

55   *faecalis*, which are unlikely to represent the large genetic diversity of this species (15,

56   19, 20). We recently reviewed 190 *E. faecalis* IE cases from 2010-2017 at the University

57   of Pittsburgh Medical Center (UPMC), and found higher overall 90-day mortality rates

58   among patients treated with AC vs. AG (21% vs. 8%, *P*=0.02) (21). While baseline

59   differences between patients influenced these findings, nonetheless it seems that for

60   some patients, the use of AC is not optimal. In this study, we investigated the genetic

61   and phenotypic diversity of 33 contemporary *E. faecalis* isolates collected from patients

62   with IE. We focused on characterizing the genomic diversity of *E. faecalis* sampled from

63   IE, identifying differences in *in vitro* AC susceptibilities, and correlating these

64   susceptibility differences with mutations in known beta-lactam resistance-associated

65   genes.

Results

Clinical presentation, diagnosis, and treatment of patients with E. *faecalis* IE

68   A total of 33 isolates were collected from patients diagnosed with either probable

69   (42.4%) or definite (57.6%) IE based on modified Duke's criteria (22, 23). The median

70   age of the patients was 63 years (range, 24-91 years) and 79% were male (Table 1).

71   AC combination therapy was the first-line treatment for 26 patients (78.8%). The

72   remaining 7 patients received various combinations of cefepime, ceftaroline,

73   daptomycin, and vancomycin. Three patients (9.1%) required alterations in their

74   antibiotic treatment courses, and two patients (6.1%) had positive blood cultures for *E.*

75   *faecalis* within three months of the initiation of antibiotic treatment. The 30-day mortality

76   rate among patients was 27.3%, and the 90-day mortality rate was 33.3% (Table 1).

77   **Genomic diversity and biofilm formation among *E. faecalis* IE isolates**

78   The genomes of all 33 *E. faecalis* IE isolates were sequenced and a core

79   genome phylogeny was constructed (Table S1, Fig. 1). Isolates were found to belong to

80   16 distinct multi-locus sequence types (STs), with an additional three isolates belonging

81   to STs that have not yet been defined. ST6 (n=7) and ST179 (n=6) were the most

82   prevalent STs observed, accounting for 36% of collected isolates. Isolates belonging to

83   ST16, ST40, and ST59 were cultured from two patients each. *E. faecalis* from all other

84   patients belonged to unique STs.

85   Mutations in the low-affinity penicillin-binding protein 4 (PBP4) have been

86   previously described in *E. faecalis*, and have been shown to affect the activity of beta-

87   lactam agents, including cephalosporins and carbapenems (24–26). We observed

88   PBP4 mutations in all isolates belonging to ST6 and ST179 (Fig. 1). All seven ST6

89   isolates carried a single base deletion 82 nucleotides upstream of *pbp4* (-82delT) while

90   all ST179 isolates as well as two additional isolates (isolates 683 and 808) harbored a

91   non-synonymous P520S mutation in PBP4. In addition, we identified one isolate with a

92   9-bp deletion in the PP2C-type protein phosphatase gene *ireP*. Mutations in *ireP* have

93   been previously found in *E. faecalis* exhibiting high-level resistance to cephalosporins

94   (27).

95   We screened all 33 *E. faecalis* genomes for acquired antimicrobial resistance

96   genes using the ResFinder tool available through the Center for Genomic Epidemiology

97   (CGE) (28) (Fig. 1). Aminoglycoside resistance genes were found almost exclusively in

98   ST6 isolates (100%) and were otherwise rare in other STs (7%). All isolates belonging

99   to ST6 and ST179 carried the erythromycin resistance gene *erm*(B) and the tetracycline

100   resistance gene *tet*(M). The two ST16 isolates carried the antifolate resistance gene

101   *dfrG.* All genomes were also screened for plasmid *rep* genes using PlasmidFinder (29),

102   which identified five different *rep* genes that together were detected in 24 (70.6%)

103   isolate genomes (Fig. 1). Among these, five genomes had only one *rep* gene identified,

104   while the remaining 19 genomes each had 2-4 *rep* genes. ST6 and ST179 isolates

105   carried significantly more *rep* genes than isolates belonging to non-ST6 and non-ST179

106   STs (ST6, mean 2.71 vs. 0.75 *rep* genes, p<0.0001; ST179, mean 2.17 vs. 0.75 *rep*

107   genes, p=0.0007). Finally, we examined all isolates for the presence of the *E. faecalis*

108   cytolysin, a pore-forming exotoxin that lyses bacterial and eukaryotic cells (30). An

109   intact cytolysin operon was found in two ST6 isolates and four ST179 isolates (Fig. 1).

110   One additional ST6 isolate had a disrupted operon. Cytolysin operons were not found in

111   isolates belonging to any other STs.

112   *E. faecalis* IE is often associated with biofilm formation (31), and it is well known

113   that biofilms protect bacteria from antibiotic-mediated killing (32). To assess differences

114   in biofilm formation among the different *E. faecalis* IE isolates collected, we quantified

115   the ability of each isolate to adhere to polystyrene plates using a standard *in vitro* crystal

116   violet-based biofilm assay (33) (Fig. S1). Biofilm formation was found to be highly

117   variable, even among isolates belonging to the same ST (Fig. S1A). Additionally, no

118   significant differences in biofilm formation were found between isolates belonging to

119   different STs (Fig. S1B).

Susceptibility to ampicillin and ceftriaxone, alone and in combination, is variable

among *E. faecalis* from IE

We investigated the susceptibilities of the 33 *E. faecalis* IE isolates to ampicillin and ceftriaxone by first measuring the minimum inhibitory concentration (MIC) of each isolate against each antibiotic (Fig. 2). To understand how different resistance-associated mutations in PBP4 affect antibiotic susceptibility, we separated isolates into groups based on their PBP4 genotypes. Ampicillin MICs were ≤ 4 μg/mL for all isolates, and did not vary significantly between PBP4 genotypes (Fig. 2A). ST6 isolates, all of which carried the PBP4 -82delT mutation, were more susceptible to ampicillin compared to other isolates, but the difference was not statistically significant (Fig. 2A). ST6 isolates were more resistant to ceftriaxone compared to both PBP4 wild type isolates ($p<0.0001$) as well as to isolates carrying the PBP4 P520S mutation ($p=0.0006$) (Fig. 2B). We also examined whether there was a correlation between ampicillin and ceftriaxone MICs by plotting them together on the same plot (Fig. 2C). We found no significant correlation between ampicillin and ceftriaxone MICs. However, the isolate with a 9-bp deletion in *ireP* had the highest ampicillin and ceftriaxone MICs among isolates lacking PBP4 mutations (Fig. 2C).

To investigate how effectively AC could synergize to inhibit the *in vitro* growth of genetically diverse *E. faecalis* from IE, we performed standard two-antibiotic checkerboard assays on the 33 *E. faecalis* IE isolates collected (34). We found that AC demonstrated synergy against nearly all isolates, with average FIC values below 0.5 for all but three isolates (Fig. 3A). Nonetheless, FIC values among the ST6 isolates carrying the PBP4 -82delT mutation were significantly higher than both PBP4 wild type

143   (p=0.001) and PBP4 P520S (p=0.0005) isolates. We also examined average bacterial

144   growth across the checkerboard assay plates among isolates encoding different

145   resistance-associated mutations (Fig. 3B). Bacterial growth was similar between PBP4

146   wild type and P520S isolates, while isolates encoding the -82delT mutation and the 9bp

147   deletion in *ireP* (IrePdel) showed more growth at higher concentrations of both

148   antibiotics. These data suggest that AC may be less active against isolates encoding

149   *pbp4* and *ireP* mutations.

150   ***In vitro* pharmacokinetic/pharmacodynamic responses to AC among *E. faecalis***

151   **from IE**

152   Next, we tested whether diminished *in vitro* synergy observed among isolates

153   carrying PBP4 mutations would be recapitulated in the presence of dynamic drug

154   exposures that mimic those achieved in patients. To do so, we employed a one-

155   compartment pharmacokinetic/pharmacodynamic (PK/PD) model to measure the killing

156   activity and suppression of resistance following exposures to ampicillin and ceftriaxone

157   either alone or in combination (Fig. 4). Three representative *E. faecalis* IE isolates

158   encoding wild type PBP4 (isolate 685, ST869), PBP4 P520S (isolate 684, ST179) or

159   PBP4 -82delT (isolate 871, ST6) were selected. Simulated antibiotic exposures were

160   selected based on those recommended by consensus guidelines (Ampicillin 2g q 4h,

161   Ceftriaxone 2g q 12h) (13) (Fig. 4A). Following exposure to ceftriaxone alone, isolates

162   with PBP4 wild type or the P520S mutation showed initial killing within two hours,

163   followed by rapid regrowth to the same levels of drug-free control arms (Fig. 4B). The

164   isolate with the PBP4 -82delT mutation was not inhibited by ceftriaxone at any point

165   during the experiment. By comparison, ampicillin alone resulted in rapid killing of all

166   three isolates, with log-kills ranging from 2.48 to 4.76 $\log_{10}$ CFU/mL at 12 hours;

167   however, each of the three isolates demonstrated regrowth after 12 hours. When

168   administered in combination, AC was rapidly bactericidal and suppressed bacterial

169   populations to 10^1-10^2 CFU/mL at 24 hours. After 24 hours, three distinct patterns were

170   identified. Against the isolate with wild type PBP4, viable bacteria were not observed

171   after 24 hours and bactericidal activity was maintained until the end of the experiment at

172   72 hours. Against the ST179 isolate encoding the PBP4 P520S mutation, late regrowth

173   occurred, which was observed only at the final 72-hour experimental time point. Finally,

174   against the ST6 isolate encoding the PBP4 -82delT mutation, early regrowth occurred

175   after 24 hours (Fig. 4B). These data indicate that mutations in PBP4 decrease bacterial

176   susceptibility to AC synergy under assay conditions and at drug concentrations that

177   more closely reflect what the bacteria are likely to experience *in vivo* during infection.

178   **Discussion**

179   In this study, we characterized the genomic diversity and antibiotic susceptibility

180   of 33 *E. faecalis* isolates from patients with probable or definite IE treated at a single

181   medical center between 2018 and 2020. Because AC is the current front-line therapy

182   regimen for *E. faecalis* IE, we focused on the ability of ampicillin and ceftriaxone to

183   inhibit *E. faecalis* growth *in vitro*, using MIC and checkerboard assays as well as a one-

184   compartment PK/PD model of bacterial killing and suppression of regrowth. Our results

185   suggest that while AC is synergistic *in vitro* against the vast majority of *E. faecalis* IE

186   isolates, antibiotic susceptibility levels vary widely among isolates. Notably, we

187   observed diminished AC synergy among isolates carrying a mutation upstream of the

188   low-affinity penicillin binding protein gene *pbp4*, as well as against an isolate with a

189   deletion in the PP2C-type protein phosphatase gene *ireP.* We also detected bacterial

190   regrowth within 72 hours following humanized antibiotic exposures to AC for isolates

191   with, but not without, PBP4 mutations. These findings have potential implications for the

192   clinical management of *E. faecalis* IE.

193   Among *E. faecalis* isolates collected from patients with IE, we observed abundant

194   genetic diversity, as well as enrichment of a small number of specific genetic lineages.

195   ST6 and ST179 were the most frequently observed lineages at our center, accounting

196   for nearly 40% of all *E. faecalis* IE cases. This is notable because all ST6 and ST179

197   isolates were found to carry mutations affecting the low-affinity penicillin-binding protein

198   gene *pbp4*, which are predicted to impact beta-lactam susceptibility (25, 26). All ST6

199   isolates carried a mutation 82 nucleotides upstream of *pbp4* (-82delT) that was

200   previously shown to cause overexpression of PBP4 and increased beta-lactam

201   resistance (25, 26). This correlated with our observation that ST6 isolates were less

202   susceptible to ceftriaxone compared to isolates with wild type *pbp4* sequences. We also

203   observed a non-synonymous, P520S-encoding mutation in *pbp4* among eight different

204   isolates. In *E. faecalis*, PBP4 is a 680-amino acid membrane protein with three

205   penicillin-binding motifs (STFK, SDN and KTG), which are located in a penicillin-binding

206   module between amino acids 350-680 (35). The P520S substitution falls within the

207   region between the active site-defining SDN and KTG motifs, and this mutation has

208   been previously found to cause an increase in beta-lactam resistance (24, 25, 36). All

209   ST179 isolates as well as two additional isolates (isolates 683 and 808) in our dataset

210   harbored this point mutation. While we did not find significant differences in ampicillin or

211   ceftriaxone MIC or FIC values between wild type isolates versus those with the PBP4

212 P520S mutation, we did observe late regrowth of the ST179 isolate that was tested in

213 the one-compartment PK/PD experiment. This suggests that the PBP4 P520S mutation

214 may provide increased tolerance of AC (as measured in a bacterial killing assay)

215 without affecting overt resistance (as measured with MIC and checkerboard assays).

216 Most available data on AC synergy against enterococci are from *in vitro* testing

217 (37, 38), a rabbit model of IE (15, 39), and prior clinical studies (40–43). In addition to *in*

218 *vitro* susceptibility testing, we also used a one-compartment PK/PD model to monitor

219 the efficacy of AC exposure on *E. faecalis* bacterial viability over time. This experiment

220 is likely to more accurately reflect the *in vivo* scenario during AC treatment of patients

221 with IE, because antibiotic exposures are modeled to simulate human dosing regimens

222 and serum-free drug concentrations. The results of the PK/PD experiments showed that

223 AC was rapidly bactericidal, and decreased the number of viable bacteria in all assays

224 from 10^6 CFU/mL to 10^2 CFU/mL or less after 24 hours. In the case of the wild type

225 isolate that was tested, the culture had no detectable viable bacteria after 27 hours, and

226 viable bacteria remained below the limit of detection for the remainder of the assay. This

227 was in contrast to the ST179 and ST6 isolates that were tested, which both showed

228 bacterial regrowth after initial clearance of the culture. In the case of the ST179 isolate,

229 viable bacteria were not detected after 48 hours, but they were detected again at the

230 final, 72-hour assay time point. Regrowth of the ST6 isolate was first detected at 27

231 hours, and 10^2-10^3 CFU/mL were observed at all remaining assay time points. These

232 data suggest that patients infected with ST6 and ST179 *E. faecalis* may experience

233 delayed clearance of bacteremia, or recurrence of infection, compared to patients

234 infected by *E. faecalis* with wild type PBP4 genotypes.

235   Prior clinical studies have shown that combination therapy with AC is non-inferior

236   to AG therapy (16, 17). Because the use of ceftriaxone avoids the nephrotoxicity and

237   therapeutic drug monitoring associated with gentamicin use (15), AC is now used

238   almost universally to treat *E. faecalis* IE (18). Nearly 80% of the patients in this study

239   were treated with AC, and no patients in this study received AG. Our findings of variable

240   susceptibility to ceftriaxone and diminished AC synergy among a subset of isolates

241   suggest that AC may not be the optimal treatment for all patients with *E. faecalis* IE. AG

242   is not an ideal alternative, given similar clinical outcomes with additional toxicity.

243   Furthermore, all ST6 isolates that we collected displayed high-level aminoglycoside

244   resistance, meaning that gentamicin treatment would be ineffective for the same

245   patients who may be at higher risk for delayed clearance of bacteremia or recurrent

246   infection due to decreased AC susceptibility. Therapeutic alternatives to AC that were

247   given to patients in this study included ceftaroline, daptomycin, and vancomycin,

248   however these agents (and combinations of them) have not been rigorously assessed

249   for the treatment of *E. faecalis* IE. Nonetheless, our findings suggest that alternative

250   therapies to AC, particularly those that are effective against AC non-susceptible and

251   AC-tolerant isolates, are needed.

252   This study had several limitations. Despite nearly two years of isolate collection

253   from a tertiary medical center, we collected a relatively small number of *E. faecalis*

254   isolates. While we hypothesize that patients with IE due to *E. faecalis* ST6 and ST179

255   may experience worse outcomes due to diminished AC synergy mediated by mutations

256   in *pbp4*, our sample size is currently too small to test this hypothesis. We plan to

257   continue to collect *E. faecalis* IE isolates from our center and other locations, and to

258   ultimately build a cohort that is large enough to identify associations between bacterial

259   genotypes, *in vitro* antibiotic susceptibilities, and clinical outcomes. Another potential

260   limitation is that the checkerboard and one-compartment PK/PD assays we conducted

261   may not accurately mirror bacterial responses to these antimicrobial combinations *in*

262   *vivo*. For example, biofilm formation is likely to play a role in modulating antibiotic

263   susceptibility during endocarditis, however in this study we did not measure how

264   antibiotic susceptibility and biofilm formation might be linked to one another. This will be

265   a focus of our future work, in which we also plan to employ relevant animal models of

266   infection to assess the effectiveness of AC (as well as other alternative combinations)

267   against genetically diverse *E. faecalis* isolates from patients with IE.

268   Overall, this study provides an initial description of the genomic diversity and

269   variability in *in vitro* AC susceptibility among contemporary *E. faecalis* causing IE at a

270   single medical center. Our analysis shows that bacteria belonging to two different STs

271   dominate the *E. faecalis* IE population at our center. Both of these STs carry mutations

272   that alter the *E. faecalis* PBP4, and our *in vitro* data suggest that ST6 isolates in

273   particular may be difficult to effectively treat with AC. Further studies are needed to

274   understand whether and how these findings impact clinical outcomes in patients with *E.*

275   *faecalis* IE. Such studies could lead to improved treatment strategies for *E. faecalis* IE,

276   which would ideally help address the high morbidity and mortality associated with these

277   infections.

278

279   **Materials and Methods**

Collection of isolates

E. faecalis isolates were collected from patients with definite or probable IE at the University of Pittsburgh Medical Center between 2018 and 2020. Clinical demographics and outcomes were collected through a retrospective search of patients' electronic medical records (EMR). Blood cultures and bacterial isolates were collected as part of routine clinical care. This study was approved by the Institutional Review Board at the University of Pittsburgh under protocol #20020039.

Whole genome sequencing and analysis

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) from 1-mL bacterial cultures grown overnight in Brain Heart Infusion (BHI) media. Next-generation sequencing libraries were prepared with a Nextera XT kit (Illumina, San Diego, CA), and libraries were sequenced on an Illumina MiSeq or NextSeq using 150-bp (NextSeq) or 300-bp (MiSeq) paired-end reads. Genomes were assembled with SPAdes v3.13.0 (44), annotated with prokka (45), and were compared to one another with Roary (46). A core genome phylogenetic tree was generated using RAxML with the GTRCAT substitution model and 100 iterations (47). Sequence types, antimicrobial resistance genes, virulence factors, and plasmid replicons were identified using online tools provided by the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk). Illumina read data for isolates sequenced in this study have been submitted to NCBI under BioProject PRJNA729754, with accession numbers listed in Table S1.

Biofilm assay

302   Microtiter plate-based biofilm assays were performed as previously described (33).

303   Briefly, an overnight culture of each *E. faecalis* IE isolate was diluted 100-fold into BHI

304   broth supplemented with 0.25% glucose. 200 µL of this dilution was plated into eight

305   replicate wells of a 96-well untreated polystyrene microtiter plate, and plates were

306   incubated for 24 hours at 37°C under static conditions. Planktonic cells were discarded

307   and plates were washed three times with 250 µL 1xPBS, after which adherent bacteria

308   in each well were stained with 200 µL 0.1% crystal violet (CV) prepared in water. After

309   incubation for 30 minutes at 4°C, wells were washed twice with 250 µL 1xPBS to

310   remove excess stain. Plates were dried and then 250 µL of 4:1 ethanol:acetone was

311   added to each well to solubilize the CV-stained biofilms. After incubation for 45 minutes

312   at room temperature, the absorbance in each well was measured at 550 nm using a

313   Synergy H1 microplate reader (Biotek, Winooski, VT). Negative control wells contained

314   200 µL of BHI broth supplemented with 0.25% glucose and no bacteria.

315   **Antimicrobial susceptibility and synergy testing**

316   Ampicillin and ceftriaxone susceptibilities and synergy were determined by 96-well plate

317   checkerboard assay (34). Briefly, 96-well plates were prepared containing two-fold

318   serial dilutions of each antibiotic, either alone or in combination, in 100 µL of Mueller

319   Hinton Broth (MHB). Overnight cultures of each *E. faecalis* isolate grown in MHB were

320   diluted to an OD_{600} of 0.1, were then diluted 1:1000 into fresh MHB, and 100 µL of this

321   dilution was transferred into each well of the 96-well plate, yielding approximately 10^5

322   CFU per well. Plates were incubated for 24 hours at 37°C under static conditions, and

323   growth in each well was analyzed by both visual inspection and by OD_{600} measurement

324   using a Synergy H1 microplate reader (Biotek, Winooski, VT). Assays were conducted

325   in biological replicates. Minimum inhibitory concentrations (MICs) were determined

326   according to methods established by the Clinical and Laboratory Standards Institute

327   (48). Fractional inhibitory concentrations (FICs) were calculated with the following

328   equation: FIC = (C_A/MIC_A) + (C_C/MIC_C), where MIC_A and MIC_C are the MICs of

329   ampicillin and ceftriaxone alone, and C_A and C_C are the concentrations of each drug in

330   the well with the strongest inhibition. Synergy was defined as FIC<0.5. The same

331   concentration ranges of each antibiotic were tested against each isolate. Two isolates

332   did not yield FIC values because they did not grow in any assay wells containing both

333   antibiotics.

334   **In vitro one-compartment pharmacokinetic-pharmacodynamic (PK-PD) model**

335   A well-established one-compartment PK-PD model was employed to simulate free

336   serum concentrations of ampicillin 2 grams (g) every 4 hours (*f*Cmax: 120 µg/mL, half-

337   life: 1h, protein binding: 20%) and ceftriaxone 2 g every 12 hours (*f*Cmax: 25.7 µg/mL,

338   half-life: 6h, protein binding: 90%) (13). A working volume of 250 mL of cation-adjusted

339   Mueller-Hinton (CAMH) broth in a glass model with 4 ports (inflow, outflow, sampling,

340   and ventilation) was used and maintained at 37°C. Peristaltic pumps controlled the flow

341   of media to achieve desired antibiotic concentrations. In combination studies, flow rates

342   were set to achieve ampicillin exposures given a shorter half-life and supplemental

343   ceftriaxone was added using methods described by Blaser et al. (49). All drugs were

344   infused over 30 minutes by programmable syringe pumps. Samples were removed at 0,

345   2, 6, 10, 24, 27, 30, 48, 51, 54, and 72 hours, centrifuged at 5,000 rpm for 5 minutes,

346   washed in cold saline, and resuspended to the original volume to prevent antibiotic

347   carryover. Each sample was then serially 10-fold diluted and plated on CAMH agar to

348   enumerate surviving bacteria. A starting inoculum of 1×10^6 CFU/mL was used;

349   bactericidal activity was defined as a ≥ 3-$\log_{10}$ CFU/mL decrease.

Statistics

351   Differences in *rep* gene abundance, antibiotic MICs, and FICs were assessed with

352   unpaired two-tailed t-tests.

Acknowledgements

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536

537 **Table 1. Demographic and clinical features of *E. faecalis* IE patients.**

Variable	N=33
Age at admission[1], years	63 (43, 72)
Male sex	26 (78.8)
White race	24 (72.7)
Definite IE[2]	19 (57.6)
Length of Bacteremia[1], hours	66.3 (42.0, 105.5)
Native valve IE	17 (51.5)
Prosthetic valve IE	3 (9.1)
Received AC, once cultures showed *E. faecalis* bacteremia	26 (78.8)
Length of hospitalization[1], days	12 (9, 31)
Treatment failure requiring antibiotic change	3 (9.1)
Relapse, positive *E. faecalis* bacteremia within 3 months of treatment	2 (6.1)
Death during hospital admission	8 (24.2)
30-day mortality	9 (27.3)
90-day mortality	11 (33.3)
Readmission within 3 months, any cause[3]	17 (68)

538 [1]Median value (interquartile range) is reported. For all other variables number of

539 patients (% of patients) is reported. AC = Ampicillin Ceftriaxone.

540 [2]Definite IE based on the modified Duke Criteria for endocarditis.

541 [3]Percent readmission was calculated for patients that survived initial hospitalization.

542   **FIGURE LEGENDS**

543   **Figure 1. Core genome phylogeny of *E. faecalis* IE isolates.** A single-copy core

544   genome phylogeny was constructed for 33 *E. faecalis* isolates from patients with

545   probable or definite IE. The midpoint-rooted RAxML tree was built from single

546   nucleotide polymorphisms (SNPs) in 1980 single copy core genes identified with Roary.

547   Tips are annotated with isolate name, multi-locus sequence type (ST), *pbp4* and *ireP*

548   mutations, acquired drug resistance-associated genes, plasmid *rep* genes, and

549   cytolysin operon presence (red = intact operon, pink = disrupted operon). NF = ST not

550   found in database; *aac(6')-aph(2")*, *aph(2')-Ic, aph(3')-III*, and *ant(6)-Ia* = aminoglycoside

551   resistance (colored purple); *erm*(B) = erythromycin resistance (colored pink); *tet*(M) and

552   *tet*(L) = tetracycline resistance (colored blue); *dfrG* = antifolate resistance (colored

553   green).

554   **Figure 2. Variability in ampicillin and ceftriaxone susceptibility among *E. faecalis***

555   **from IE.** Minimum inhibitory concentrations (MICs) of (A) ampicillin and (B) ceftriaxone

556   among 33 *E. faecalis* IE isolates. MIC values were averaged between biological

557   replicate experiments, and isolates are grouped based on their PBP4 genotype. Bars

558   show mean MIC values and error bars show standard error of the mean. P-values are

559   from two-tailed t-tests. ns = not significant. (C) Ceftriaxone MIC versus ampicillin MIC

560   for each tested isolate. Isolates are colored based on their PBP4 genotypes as in (A)

561   and (B). IrePdel refers to isolate 957, which possesses a 9-bp deletion in the PP2C-type

562   protein phosphatase gene *ireP*.

563 **Figure 3. Variability in AC synergy between *E. faecalis* IE isolates.** (A) FIC values

564 calculated from AC checkerboard assays. FIC values were averaged between biological

565 replicate experiments, and isolates are grouped based on their PBP4 genotype. Bars

566 show mean FIC values, and error bars denote standard error of the mean. IrePdel

567 refers to isolate 957, which possesses a 9bp deletion in *ireP*. P-values are from two-

568 tailed t-tests. (B) Average bacterial growth in checkerboard assays. Results are

569 separated by *pbp4* and *ireP* genotype, and growth was averaged among all assays

570 conducted for isolates possessing each genotype. WT = wild type. Colored triangles

571 show decreasing ampicillin (yellow) and ceftriaxone (blue) concentrations across and

572 down each plate, respectively. The same antibiotic concentrations were tested against

573 all isolates. Green shading indicates bacterial growth after 24 hours in each assay well,

574 with darker shading indicating more growth.

575 **Figure 4. One-compartment pharmacodynamic modeling of ampicillin,**

576 **ceftriaxone, and their combination against three different *E. faecalis* IE isolates**

577 **belonging to different STs.** (A) Simulated free serum exposures of ampicillin and

578 ceftriaxone to achieve peak concentrations of 120 and 25.7 μg/mL, respectively. (B)

579 Growth and killing of three different *E. faecalis* IE isolates in the presence of no drug

580 (Control, black curves), ampicillin (AMP, yellow curves), ceftriaxone (CRO, blue curves),

581 or ampicillin and ceftriaxone (green curves). Log_{10} CFU/mL is plotted over time for each

582 condition. Assay limit of detection was 10 CFU/mL.

583 **Figure S1. Variable biofilm production among *E. faecalis* IE isolates.** (A) *In vitro*

584 biofilm production of 33 *E. faecalis* IE isolates, organized by sequence type (ST). Biofilm

585   formation was measured as the OD at 550nm using a standard crystal violet-based

586   assay. Bars show mean crystal violet absorbance, and error bars show standard

587   deviation among three biological replicate assays, each with eight technical replicates.

588   STs with more than one isolate are colored; singleton STs are white. Neg. = negative

589   control. (B) Biofilm formation among isolates grouped by their PBP4 genotypes. Bars

590   show mean crystal violet absorbance values and error bars denote standard error of the

591   mean. ns = not significant.
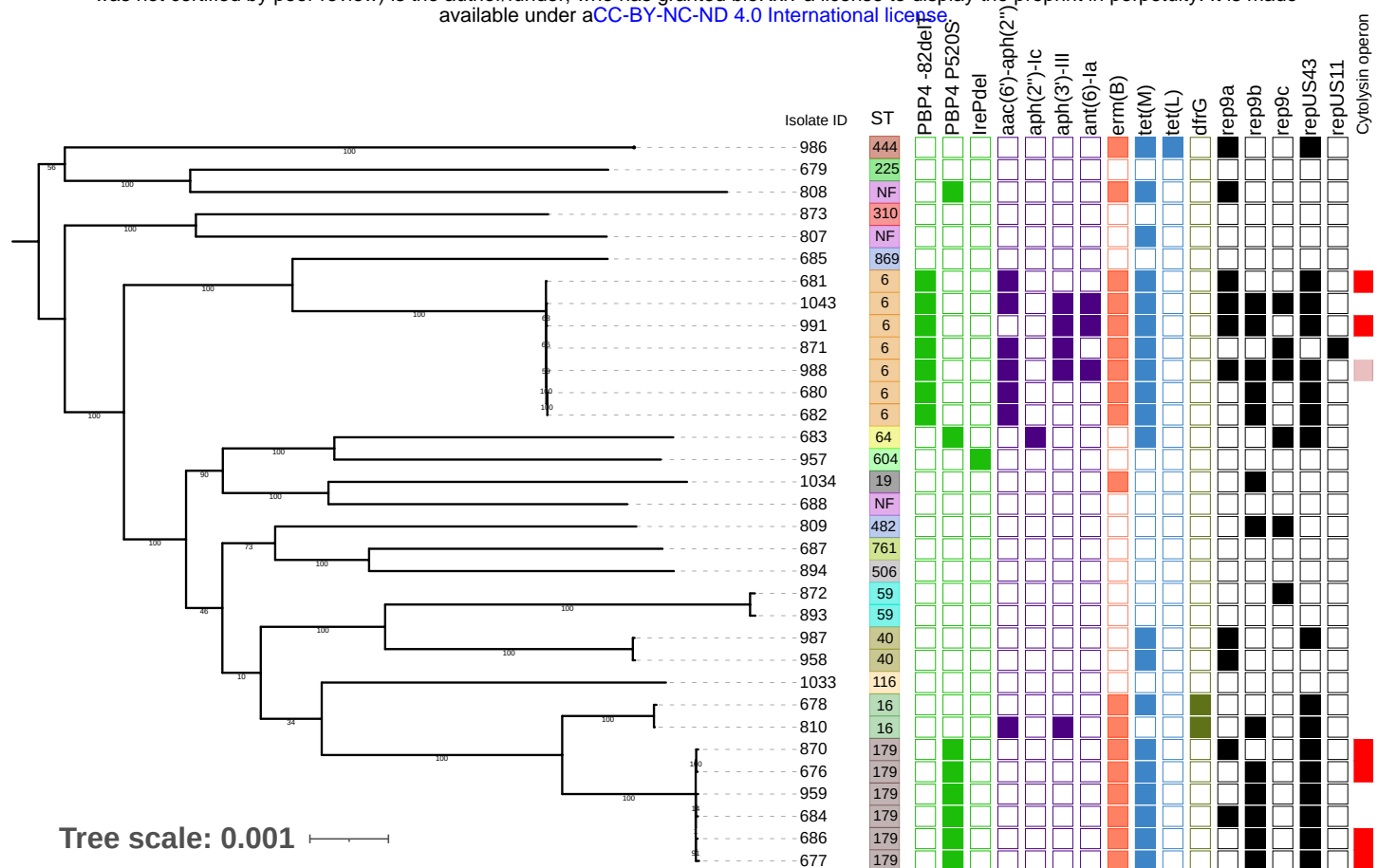
Figure 1. Core genome phylogeny of *E. faecalis* IE isolates. A single-copy core genome phylogeny was constructed for 33 *E. faecalis* isolates from patients with probable or definite IE. The midpoint-rooted RAxML tree was built from single nucleotide polymorphisms (SNPs) in 1980 single copy core genes identified with Roary. Tips are annotated with isolate name, multi-locus sequence type (ST), *pbp4* and *ireP* mutations, acquired drug resistance-associated genes, plasmid *rep* genes, and cytolysin operon presence (red = intact operon, pink = disrupted operon). NF = ST not found in database; *aac(6')-aph(2")*, *aph(2')-Ic, aph(3')-III*, and *ant(6)-Ia* = aminoglycoside resistance (colored purple); *erm*(B) = erythromycin resistance (colored pink); *tet*(M) and *tet*(L) = tetracycline resistance (colored blue); *dfrG* = antifolate resistance (colored green).
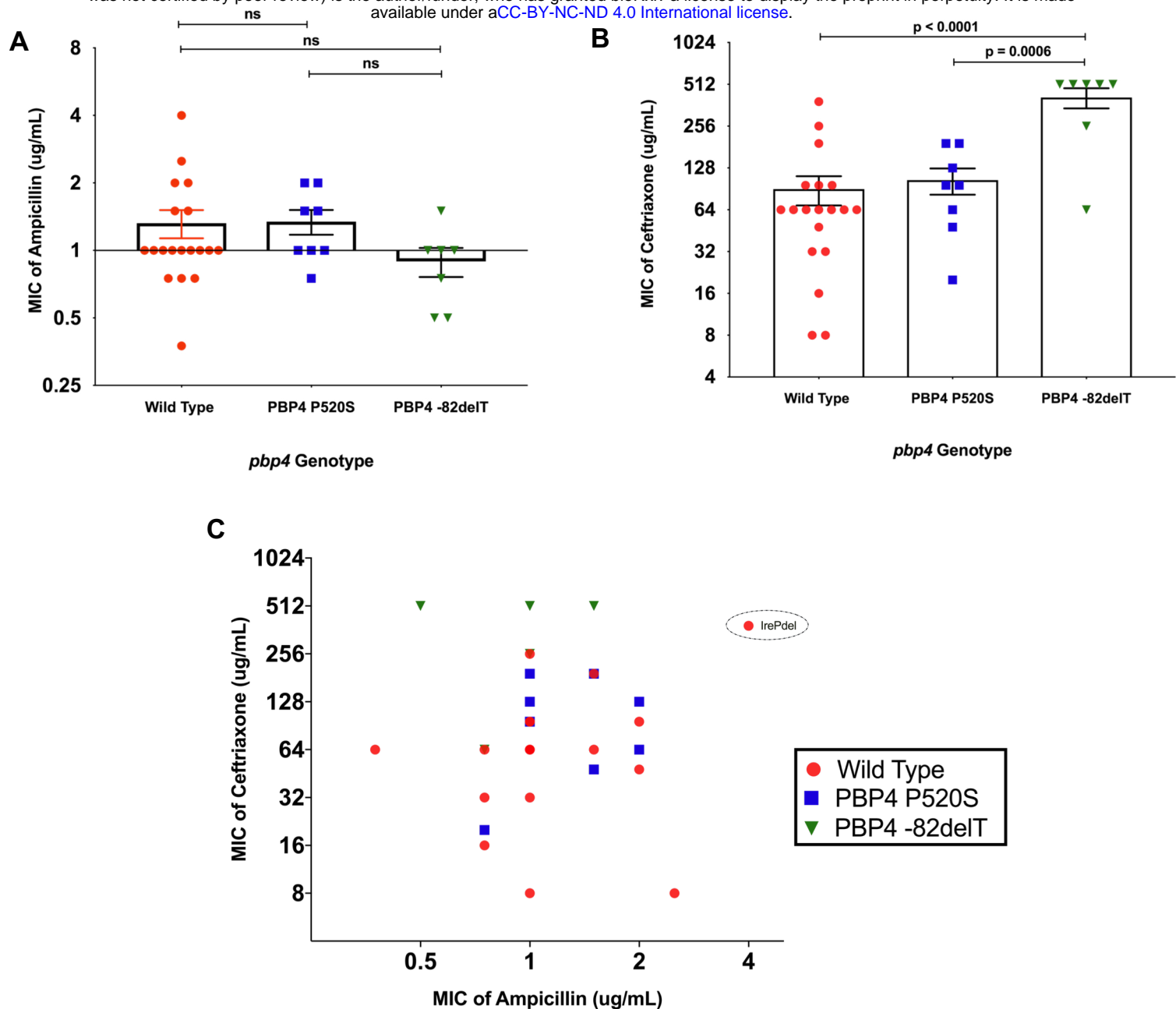
Figure 2. Variability in ampicillin and ceftriaxone susceptibility among *E. faecalis* from IE. Minimum inhibitory concentrations (MICs) of (A) ampicillin and (B) ceftriaxone among 33 *E. faecalis* IE isolates. MIC values were averaged between biological replicate experiments, and isolates are grouped based on their PBP4 genotype. Bars show mean MIC values and error bars show standard error of the mean. P-values are from two-tailed t-tests. ns = not significant. (C) Ceftriaxone MIC versus ampicillin MIC for each tested isolate. Isolates are colored based on their PBP4 genotypes as in (A) and (B). IrePdel refers to isolate 957, which possesses a 9-bp deletion in the PP2C-type protein phosphatase gene *ireP*.
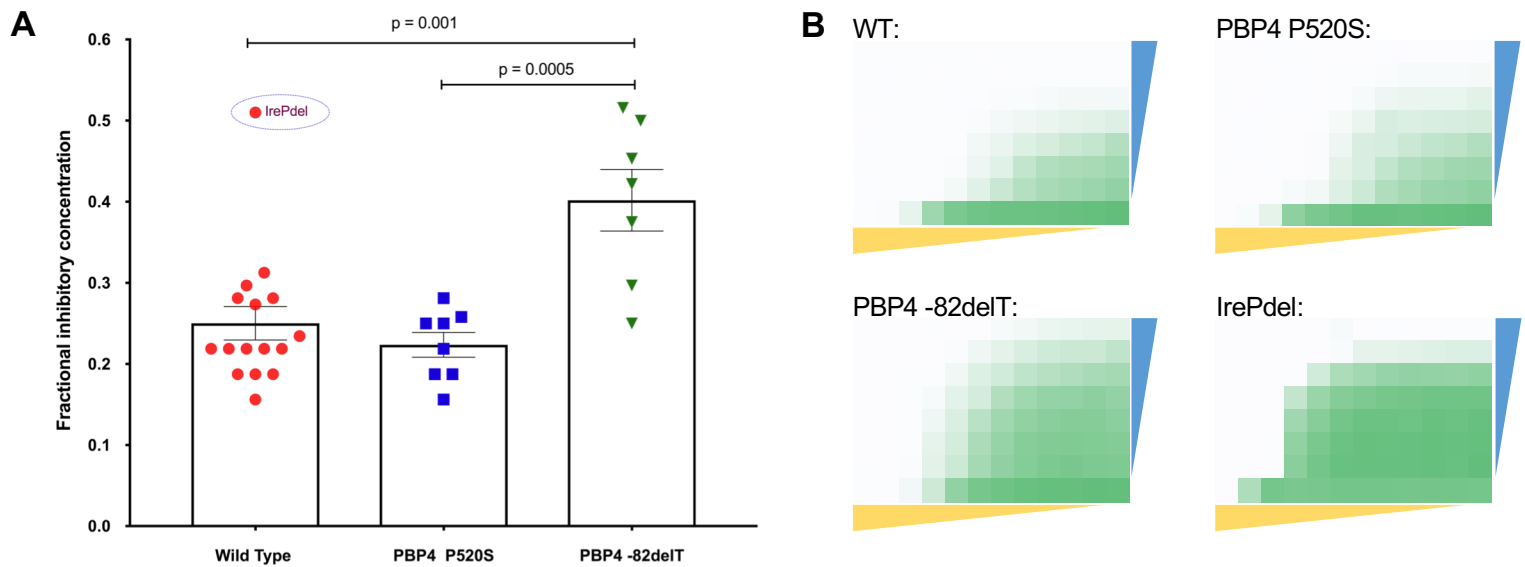
Figure 3. Variability in AC synergy between *E. faecalis* IE isolates. (A) FIC values calculated from AC checkerboard assays. FIC values were averaged between biological replicate experiments, and isolates are grouped based on their PBP4 genotype. Bars show mean FIC values, and error bars denote standard error of the mean. IrePdel refers to isolate 957, which possesses a 9bp deletion in *ireP*. P-values are from two-tailed t-tests. (B) Average bacterial growth in checkerboard assays. Results are separated by *pbp4* and *ireP* genotype, and growth was averaged among all assays conducted for isolates possessing each genotype. WT = wild type. Colored triangles show decreasing ampicillin (yellow) and ceftriaxone (blue) concentrations across and down each plate, respectively. The same antibiotic concentrations were tested against all isolates. Green shading indicates bacterial growth after 24 hours in each assay well, with darker shading indicating more growth.
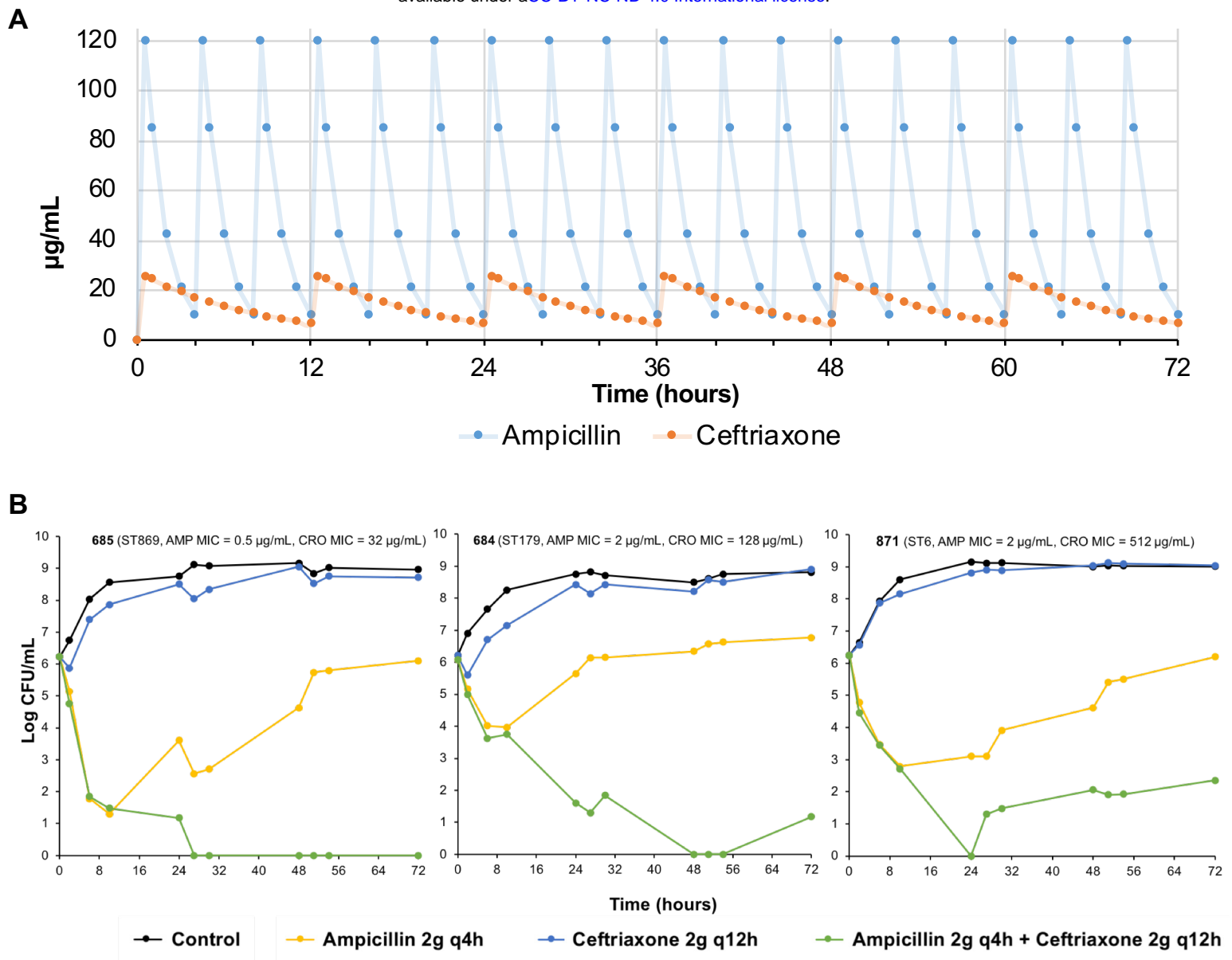
Figure 4. One-compartment pharmacodynamic modeling of ampicillin, ceftriaxone, and their combination against three different _E. faecalis_ IE isolates belonging to different STs. (A) Simulated free serum exposures of ampicillin and ceftriaxone to achieve peak concentrations of 120 and 25.7 µg/mL, respectively. (B) Growth and killing of three different _E. faecalis_ IE isolates in the presence of no drug (Control, black curves), ampicillin (AMP, yellow curves), ceftriaxone (CRO, blue curves), or ampicillin and ceftriaxone (green curves). Log$_{10}$ CFU/mL is plotted over time for each condition. Assay limit of detection was 10 CFU/mL.
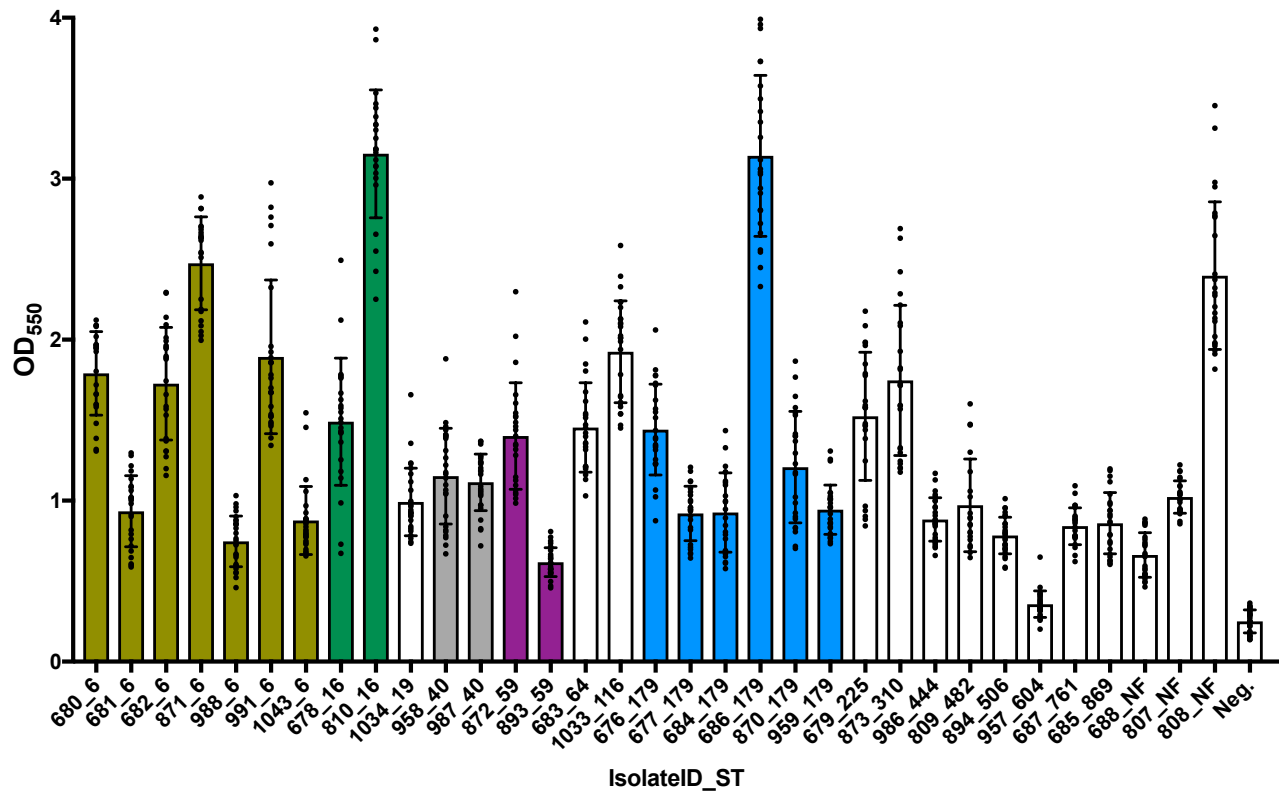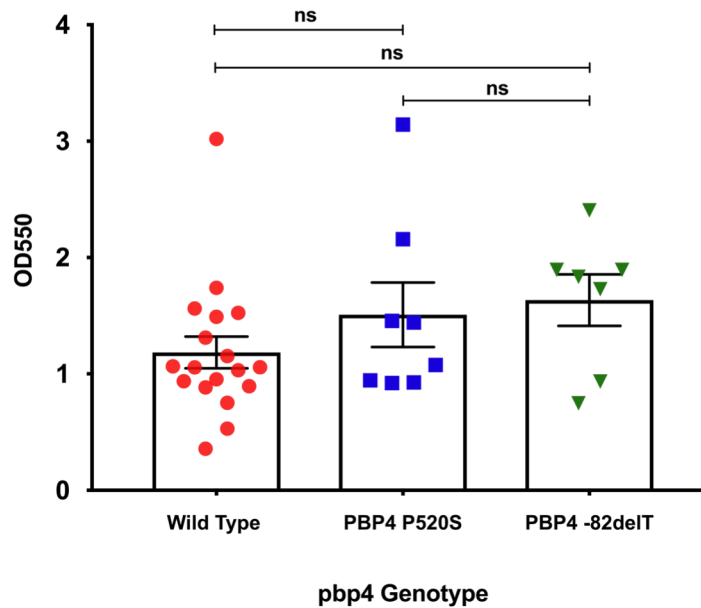
Figure S1. Variable biofilm production among *E. faecalis* IE isolates. (A) *In vitro* biofilm production of 33 *E. faecalis* IE isolates, organized by sequence type (ST). Biofilm formation was measured as the OD at 550nm using a standard crystal violet-based assay. Bars show mean crystal violet absorbance, and error bars show standard deviation among three biological replicate assays, each with eight technical replicates. STs with more than one isolate are colored; singleton STs are white. Neg. = negative control. (B) Biofilm formation among isolates grouped by their PBP4 genotypes. Bars show mean crystal violet absorbance values and error bars denote standard error of the mean. ns = not significant.