Factors affecting CRISPR-Cas defense against antibiotic resistance plasmids harbored by *Enterococcus faecalis* laboratory model strains and clinical isolates

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ABSTRACT

Enterococcus faecalis is a Gram-positive bacterium and opportunistic pathogen that acquires resistance to a wide range of antibiotics by horizontal gene transfer (HGT). The rapid increase of multidrug-resistant (MDR) bacteria including MDR *E. faecalis* necessitates the development of alternative therapies and a deeper understanding of the factors that impact HGT. CRISPR-Cas systems provide sequence-specific defense against HGT. From previous studies, we know that *E. faecalis* CRISPR-Cas provides sequence-specific anti-plasmid defense during agar plate biofilm mating and in the murine intestine. Those studies were mainly conducted using laboratory model strains with a single, CRISPR-targeted plasmid in the donor. MDR *E. faecalis* typically possess multiple plasmids that are diverse in sequence and may interact with each other to impact plasmid transfer and CRISPR-Cas efficacy. Here, we altered multiple parameters of our standard *in vitro* conjugation assays to assess CRISPR-Cas efficacy, including the number and genotype of plasmids in the donor; and laboratory model strains as donor versus recent human isolates as donor during conjugation. We found that the plasmids pTEF2 and pCF10, which are not targeted by CRISPR-Cas in our recipient, enhance the conjugative transfer of the CRISPR-targeted plasmid pTEF1 into both wild-type and CRISPR-Cas-deficient (via deletion of *cas9*) recipient cells. However, the effect of pTEF2 on pTEF1 transfer is much more pronounced, with a striking 6-log increase in pTEF1 conjugation frequency when pTEF2 is also present in the donor and recipients are deficient for CRISPR-Cas (compared to 4-log for pCF10). Overall, this study provides insight about the interplay between plasmids and CRISPR-Cas defense, opening avenues for developing novel therapeutic strategies to curb HGT among bacterial pathogens, and highlighting pTEF2 as a plasmid for additional mechanistic study.

35 **IMPORTANCE**

36 The emergence of MDR bacteria, including MDR *E. faecalis,* limits treatment options and
37 necessitates development of alternative therapeutics. In these circumstances, bacterial CRISPR-
38 Cas systems are being explored by the field to develop CRISPR-based antimicrobials. However,
39 in many cases CRISPR-Cas efficacy has only been assessed using laboratory model strains. More
40 studies are required that investigate clinical isolates, as those are the intended targets for CRISPR
41 antimicrobials. Here, we demonstrated how the number of plasmids harbored by an *E. faecalis*
42 donor strain can affect the apparent efficacy of CRISPR-Cas anti-plasmid defense in a recipient
43 strain. Overall, our research is important to develop improved CRISPR-based antimicrobials to
44 combat the spread and accumulation of antibiotic resistance determinants.

45

46 **INTRODUCTION**

47 *E. faecalis* is a Gram-positive opportunistic pathogen (1, 2). Despite being a natural inhabitant of
48 the mammalian gastrointestinal tract, due to fecal contamination, this pathogen is frequently found
49 in soil, sewage, water, and food (3, 4). *E. faecalis* is also a leading cause of hospital-acquired
50 infections in the United States, especially in immunocompromised patients (5). *E. faecalis* is
51 considered a serious threat by the United States Centers for Disease Control and Prevention due to
52 high occurrence of resistance to a variety of antibiotics including vancomycin, a last resort
53 antibiotic, leaving few treatment options (6–8).

54

55 Horizontal gene transfer (HGT) disseminates antibiotic resistance genes among bacterial
56 pathogens, including *E. faecalis* (9). Many studies have identified mobile genetic elements

57 (MGEs) such as pheromone-responsive plasmids (PRPs), mobilizable plasmids, and transposons
58 as means of HGT in *E. faecalis* (9–11). Conjugation is the most studied form of HGT in *E. faecalis*
59 (12–15). PRPs are conjugative plasmids that can achieve very high conjugation frequencies due to
60 their transfer mechanism which capitalizes on sex pheromone production by recipient cells to
61 facilitate cell contact with donors (16–18). Thus, *E. faecalis* donor strains harboring a PRP can
62 transfer the plasmid to a recipient cell via conjugation and produce a transconjugant (16, 19). PRPs
63 were first identified in *E. faecalis* and appear to exclusively replicate within the enterococci (20,
64 21). Examples of well-studied, model PRPs are pAD1 and pCF10 (20, 22, 23).

65

66 Clustered regularly interspaced short palindromic repeat and CRISPR associated proteins
67 (CRISPR-Cas) systems, can provide sequence-specific genome defense against HGT (24–26).
68 This system preserves a genetic memory of past encounters with MGEs via short sequences called
69 spacers. Upon spacer-dependent recognition of a targeted MGE, Cas-encoded nucleases can cleave
70 and deactivate it (26–28). In *E. faecalis*, predominantly type IIA CRISPR-Cas systems are found
71 and characterized by the presence of type II specific nuclease Cas9 (formerly known as Csn1) (29–
72 31). Genome analysis has also identified presence of type IIC CRISPR-Cas system in *E. faecalis*
73 recently (32). Thus far, four different CRISPR-Cas loci namely CRISPR1-Cas, CRISPR2,
74 CRISPR3-Cas and CRISPR4 have been identified in *E. faecalis* (29, 30, 33). The plasmid recipient
75 strain used in this study, *E. faecalis* urinary tract isolate T11RF (34, 35) possesses a native
76 CRISPR3-Cas system (30). The T11RF CRISPR3-Cas spacer 6 has sequence identity to the model
77 PRP pAD1 and reduces acquisition frequency of this plasmid in T11RF (30, 34).

78

79 From bioinformatic studies, we know that many MDR *E. faecalis* are "immunocompromised" and
80 lack functional CRISPR-Cas systems, which likely allows for accumulation of antibiotic
81 resistance-encoding MGEs in these strains (30, 36, 37). Using the non-MDR *E. faecalis* T11RF as
82 a model recipient, previous works demonstrated that *E. faecalis* CRISPR-Cas confers defense
83 against pAD1 derivatives both *in vitro* and *in vivo* (in the murine intestine) (34, 38). These
84 experimental studies established conclusively that CRISPR-Cas can serve as an anti-plasmid
85 defense system in *E. faecalis*. Yet, a limitation of these studies is that they assessed CRISPR-Cas
86 efficacy against model antibiotic resistance plasmids and used laboratory model donor strains
87 derived from the natively plasmid-free 1975 oral isolate OG1 (39). *E. faecalis* MDR clinical
88 isolates can possess up to six plasmids (2, 40, 41), most of which have not been investigated
89 beyond sequencing. These *E. faecalis* clinical isolates are plasmid reservoirs and donors from
90 which antibiotic resistance spreads. Theoretically, plasmids in these donor cells may collaborate
91 to enhance their transfer rates and/or overcome anti-plasmid defense in the recipient cell.
92 Conjugation efficiency is influenced by a complex interplay of intracellular and intercellular
93 interactions between distinct plasmids (42). Indeed, it has also been reported that anti-CRISPR
94 phages can cooperate to defeat CRISPR-Cas defense in host bacteria (43). Hence, the number and
95 genotype of plasmids harbored by an *E. faecalis* donor are factors that need to be explored to
96 determine their impact on CRISPR-Cas anti-plasmid defense. Other recent research has also
97 demonstrated that the effectiveness of a CRISPR-based antimicrobial against *E. faecalis* fecal
98 surveillance isolates can be affected by competitive factors (such as bacteriocins) produced by the
99 isolates (44). This emphasizes the need to incorporate clinical isolates in research to understand
100 CRISPR-Cas efficacy against them.

101

102 Overall, in this study we incorporated both laboratory model strains and clinical isolates to
103 examine the correlation between the number of plasmids present in a donor cell and the ability of
104 CRISPR-Cas to enact its defense mechanism in the recipient. We found that presence of multiple
105 plasmids in a donor strain and the lack of active CRISPR-Cas system in a recipient strain can
106 additively confer strikingly high conjugative transfer frequencies of antibiotic resistance plasmids.
107 Our results can be leveraged to develop enhanced CRISPR-based antimicrobials. Our research also
108 underscores the importance of investigating additional potential factors that may influence the
109 effectiveness of CRISPR-Cas anti-plasmid defense.

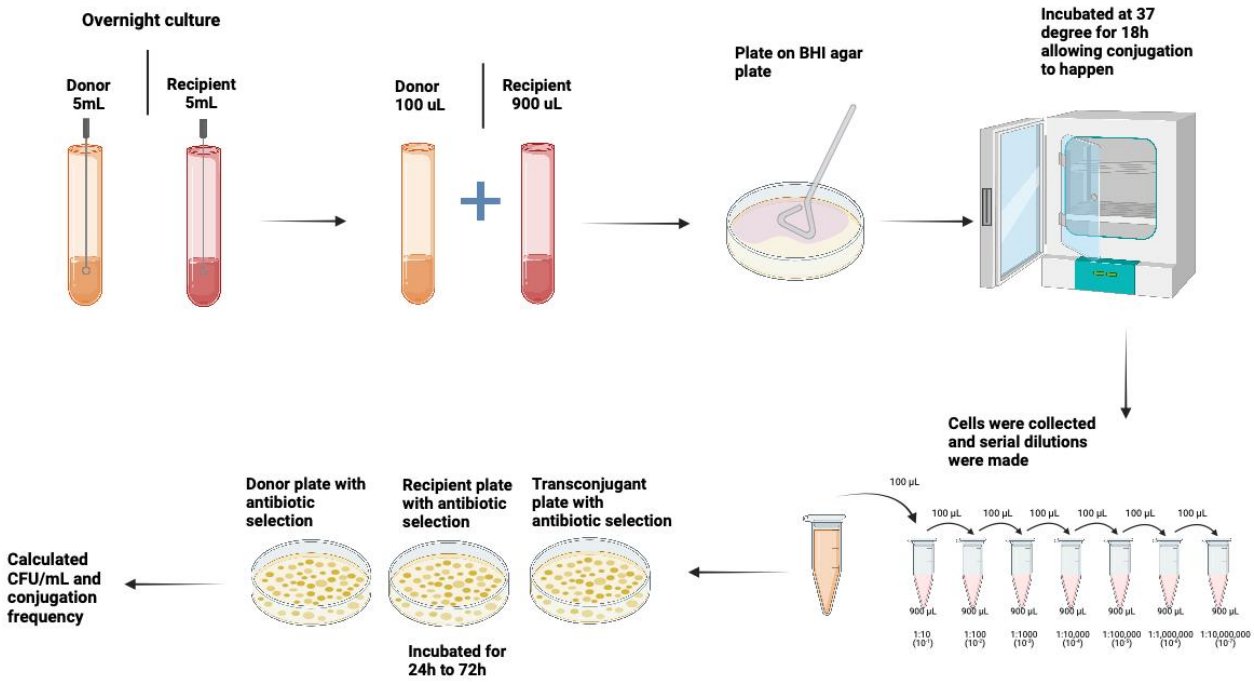
110

111 **RESULTS**

112 **CRISPR-Cas defense in the recipient is less effective when pTEF1 and pTEF2 are present in**
113 **the donor strain, compared to pTEF1 alone.** We first examined the correlation between the
114 number of plasmids present in the donor cell and the ability of CRISPR-Cas in the recipient cell
115 to enact its defense mechanism. We hypothesized that the presence of multiple plasmids in an *E.*
116 *faecalis* donor would increase the conjugative transfer of antibiotic resistance and negatively
117 impact CRISPR-Cas genome defense in recipients. To generate isogenic plasmid donors with
118 varying number of plasmids, we used *E. faecalis* clinical isolate V583 as the plasmid donor and *E.*
119 *faecalis* laboratory model strain OG1SSp (16, 45) as the recipient strain in a conjugation assay.
120 We chose V583 because it was among the first vancomycin-resistant clinical isolates reported in
121 the United States (46) and contains 3 plasmids, pTEF1, pTEF2 and pTEF3 (2). pTEF1 is a PRP
122 encoding erythromycin resistance (2). pTEF2 is also a PRP but does not encode antibiotic
123 resistance genes (2). pTEF1 and pTEF2 share sequence features with the model PRPs pAD1 (47)
124 and pCF10 (18), respectively, which respond to different peptide pheromones (2). pTEF3 is a

125 nonconjugative broad host range plasmid (2). Conjugation assays were conducted as described
126 previously (34) (Figure 1). Antibiotic-containing agar plates were used to select for
127 OG1SSp(pTEF1) transconjugants. For all the conjugation assays, conjugation frequency was
128 calculated by dividing the CFU/mL of the transconjugants by the CFU/mL of the donors. Eight
129 transconjugants were selected for PCR to confirm their plasmid content. Two of these
130 transconjugants had one plasmid - OG1SSp(pTEF1) - and 6 transconjugants had two plasmids -
131 OG1SSp(pTEF1, pTEF2). Transfer of pTEF3 was not observed (Figure S1). Thus, we generated
132 our first controlled set of donors with varying number of plasmids.

133



134

135 **Figure 1: Overview of conjugation assays.** In day 1, donor and recipients were inoculated in 5
136 mL BHI without antibiotic selection and incubated overnight at 37°C. The next day, the cultures
137 were diluted 1:10 into fresh BHI and incubated for 1.5 h at 37°C or until log phase reached. A 100

138 μL volume of donor culture was mixed with 900 μL volume of recipient culture, and the mixture
139 was pelleted at 13,000 rpm for 1 min. 900 μL supernatant was discarded, and the remaining 100
140 μL of supernatant was used to resuspend the pellet. This was then plated on a BHI agar plate and
141 incubated at 37°C for 18 h. Cells were collected from the plate with 2 mL 1X PBS supplemented
142 with 2 mM EDTA. This was serially diluted (10^{-1} to 10^{-8}). Dilutions were plated on BHI agar plates
143 supplemented with appropriate antibiotics to quantify donors, recipients and transconjugants.
144 CFU/mL was calculated after 48 h to 72 h incubation at 37°C and used for calculating conjugation
145 frequency (34) (Created in https://BioRender.com).

146

147 We used the newly established OG1SSp(pTEF1) and OG1SSp(pTEF1, pTEF2) donors in further
148 conjugation assays (Figure 2A). *E. faecalis* urinary tract isolate T11RF (34, 35) was used as the
149 recipient strain for these experiments (Figure 2A). T11RF is vancomycin-susceptible and often
150 used in comparative analyses with V583 (11, 30, 34). Though T11RF and V583 share 99.5%
151 nucleotide identity in their core genome, T11RF lacks ~620 kb of horizontally acquired genome
152 content present in V583 (11, 30). Spacer 6 of T11RF CRISPR3-Cas system has sequence identity
153 with a region in pTEF1; none of the spacers have sequence identity with pTEF2, indicating that
154 this plasmid is not targeted by the T11RF CRISPR3-Cas system. The other two recipients for this
155 study were T11RFΔ*cas9,* where the *cas9* gene was deleted to deactivate the CRISPR-Cas system,
156 and T11RFΔ*cas9*+CR3, where the *cas9* gene was complemented back to the Δ*cas9* mutant into a
157 neutral site on the chromosome (34) (Figure 2A). Conjugation assays were conducted as described
158 previously (34) (Figure 1). As expected, T11RF CRISPR3-Cas provided genome defense against
159 pTEF1 for both OG1SSp(pTEF1) and OG1SSp(pTEF1,pTEF2) donors, as significantly higher
160 pTEF1 conjugation frequency was observed in the absence of *cas9* for both (Figure 2B-a,2B-b).

161

162 We then compared the pTEF1 conjugation frequency for the two donors. We expected that, if
163 CRISPR-Cas was equally effective against pTEF1 irrespective of pTEF2 presence, the pTEF1
164 conjugation frequency would be unchanged for wild-type T11RF recipients. However, we
165 observed a 32-fold increase in pTEF1 conjugation frequency for wild-type T11RF when the donor
166 strain had both pTEF1 and pTEF2 present, compared with only pTEF1 (Figure 2B-c)**.** Moreover,
167 in the case of T11RFΔ*cas9* as the recipient strain, a striking 7278-fold increase in pTEF1
168 conjugation frequency was observed when the donor strain had both pTEF1 and pTEF2 present,
169 compared with only pTEF1 (Figure 2B-d). We analyzed the plasmid content of 6 T11RF and 6
170 T11RFΔ*cas9* transconjugants using PCR, finding that both pTEF1 and pTEF2 transferred to all 6
171 T11RF transconjugants and to 5 of 6 T11RFΔ*cas9* transconjugants (Figure S2).

172

173 Our results support our hypothesis and demonstrate that CRISPR-Cas efficacy against pTEF1 is
174 diminished when another plasmid, pTEF2, is present in the donor cell and co-transfers with pTEF1.
175 Moreover, pTEF1 transfer frequency is extraordinarily high (a 6-log increase) when donors
176 possess both pTEF1 and pTEF2, and recipients lack functional CRISPR-Cas defense compared to
177 donor where only pTEF1 is present and the recipient possesses active CRISPR-Cas.

178

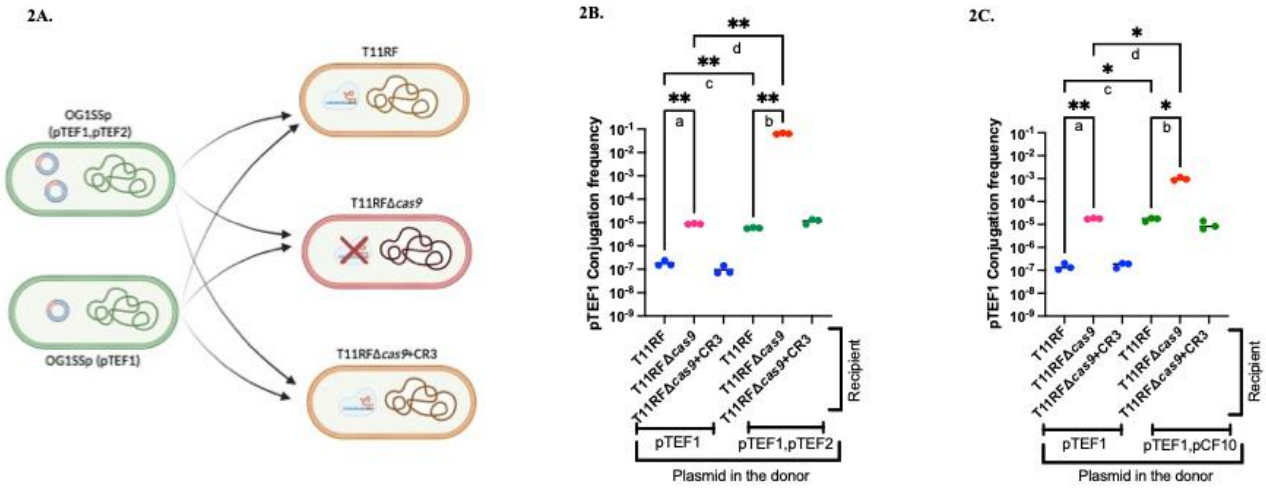


Figure 2: T11RF CRISPR3-Cas efficacy against pTEF1 for OG1SSp(pTEF1) and OG1SSp(pTEF1,pTEF2) or OG1SSp(pTEF1,pCF10) donor strains. Figure 2A shows the donors OG1SSp(pTEF1, pTEF2) or OG1SSp(pTEF1) and the recipients T11RF, T11RFΔ*cas9*, and T11RFΔ*cas9*+CR3 that are used in the conjugation assays for figure 2B (Created in https://BioRender.com). In Figure 2B and 2C, conjugation frequency of pTEF1 and statistical analysis are showing. T11RF CRISPR3-Cas can provide sequence specific genome defense against OG1SSp (pTEF1) and OG1SSp (pTEF1,pTEF2) (2B-a, 2B-b) (Dunnett's T3 multiple comparisons test, ***P*-value = 0.0029 (2B-a), ***P*-value = 0.0067 (2B-b)). However, higher conjugation frequency of pTEF1 was observed when the donor strain had both pTEF1 and pTEF2 present, compared with only pTEF1 (2B-c, 2B-d) (Dunnett's T3 multiple comparisons test, ***P*-value = 0.0049 (2B-c), ***P*-value = 0.0067 (2B-d)) . T11RF CRISPR3-Cas can also provide sequence specific genome defense against OG1SSp (pTEF1) and OG1SSp (pTEF1,pCF10) (2C-a, 2C-b) (Dunnett's T3 multiple comparisons test, ***P*-value = 0.0053 (2C-a), **P*-value = 0.0360 (2C-b)) . Higher conjugation frequency of pTEF1 was observed when the donor strain had both pTEF1 and pCF10 present, compared with only pTEF1 (2C-c, 2C-d) (Dunnett's T3 multiple comparisons test, **P*-value = 0.0438 (2C-c), **P*-value = 0.0361 (2C-d)).

196

197 **pCF10 increased pTEF1 conjugation frequency when both plasmids were present together**
198 **in a donor.** pCF10 is a well-studied model PRP that shares identical conjugation genes with
199 pTEF2 (2, 18). pCF10 encodes tetracycline resistance and is not targeted by T11RF CRISPR3-Cas
200 (34). Therefore, we explored whether pCF10 can "help" pTEF1 in a manner similar to that
201 observed for pTEF2. We generated another donor strain, OG1SSp(pTEF1, pCF10), and then used
202 OG1SSp(pTEF1) and OG1SSp(pTEF1, pCF10) donor strains in conjugation assays. As expected,
203 T11RF CRISPR3-Cas provided genome defense against pTEF1 from both donors, as higher
204 pTEF1 conjugation frequencies were observed in the absence of *cas9* (Figure 2C-a, 2C-b).

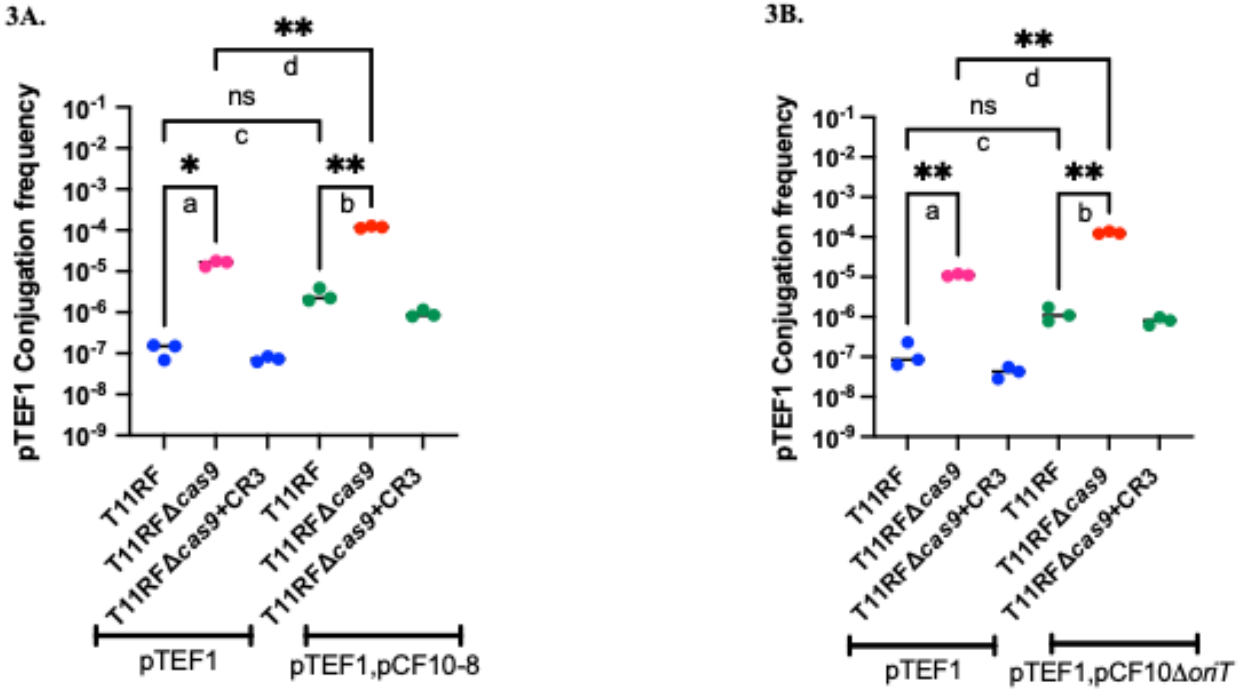
205

206 We then compared the pTEF1 conjugation frequency for the two donors. A 114-fold increase in
207 pTEF1 conjugation frequency was observed when the donor strain had both pTEF1 and pCF10
208 present, compared with only pTEF1 (Figure 2C-c). In the case of T11RFΔ*cas9* as the recipient
209 strain, a 54-fold increase in pTEF1 conjugation frequency was observed when the donor strain had
210 both pTEF1 and pCF10 present, compared with only pTEF1 (Figure 2C-d). During the
211 conjugation assay, we only tracked pTEF1 using erythromycin-containing agar. Hence, we
212 screened transconjugants for the presence of both pTEF1 and pCF10 plasmids using erythromycin-
213 and tetracycline-containing agars, respectively. For all transconjugants screened, pTEF1 and
214 pCF10 plasmids were transferred together (Figure S3). Overall, from these observations we can
215 say that pCF10 also increased pTEF1 conjugation frequency when present together in a donor.

216

217 We noted that Figure 2B (for effect of pTEF2 in donors) and 2C (for effect of pCF10 in donors)
218 were nearly superimposable, with the exception of the results for T11RFΔ*cas9* recipients. The
219 presence of pTEF2 in donors conferred an additional 2-log increase in pTEF1 conjugation
220 frequency into T11RFΔ*cas9* recipients, compared to the presence of pCF10 in donors (Figure 2B-
221 2C). pTEF2 possesses 21 genes (in two clusters) that pCF10 lacks (Supplemental Dataset S1).
222 Conserved domain analysis identified putative functions including plasmid partitioning,
223 pheromone response, and modification-dependent DNA nicking for 15 of the genes; no conserved
224 domains were identified for 6 of the genes. Further experimentation will be required to establish
225 the basis for the extraordinarily high pTEF2-assisted pTEF1 transfer rate for T11RFΔ*cas9*
226 recipients.

227



228

229 **Figure 3: T11RF CRISPR3-Cas efficacy against pTEF1 plasmid in OG1SSp(pTEF1) and**
230 **OG1SSp(pTEF1,pCF10-8) or OG1SSp(pTEF1,pCF10Δ*oriT*) donor strains.** Figure 3A and 3B
231 show the conjugation frequency of pTEF1 and statistical analysis. CRISPR-Cas was able to
232 provide sequence specific genome defense against pTEF1 in both donors as higher conjugation
233 frequency was observed in the absence of *cas9* in recipients (3A-a, 3A-b) (Dunnett's T3 multiple
234 comparisons test, **P*-value = 0.0358 (3A-a), ***P*-value = 0.0064 (3A-b)). Higher conjugation
235 frequency was observed when the donor strain had both pTEF1 and pCF10-8 present, compared
236 with only pTEF1 to T11RF (3A-c) or T11RFΔ*cas9* (3A-d) (Dunnett's T3 multiple comparisons
237 test, ns *P*-value = 0.2139 (3A-c), ***P*-value = 0.0088 (3A-d)). Even with the deletion of
238 aggregation substance gene *prgB*, pCF10 plasmid can still increase the conjugation frequency of
239 pTEF1. CRISPR-Cas was able to provide sequence specific genome defense against pTEF1 in
240 both donors as higher conjugation frequency was observed in the absence of *cas9* in recipients
241 (3B-a, 3B-b) (Dunnett's T3 multiple comparisons test, ***P*-value = 0.0063 (3B-a), ***P*-value =
242 0.0074 (3B-b). Higher conjugation frequency was observed when the donor strain had both pTEF1
243 and pCF10Δ*oriT* present, compared with only pTEF1 to T11RF (3B-c) or T11RFΔ*cas9* (3B-d)
244 (Dunnett's T3 multiple comparisons test, ns *P*-value = 0.2598 (3B-c), ***P*-value = 0.0087 (3B-
245 d)). Even with the deletion *oriT*, pCF10 plasmid can still increase pTEF1 conjugation frequency.

246

247 **Deletion of aggregation substance gene *prgB* from pCF10 decreases pTEF1 conjugation**
248 **frequency from a multi-plasmid donor.** When we observed that pCF10 was able to increase
249 pTEF1 conjugation frequency when present together in a donor, we wanted to explore which
250 region of pCF10 might be important for this plasmid cooperativity. The pCF10 gene *prgB* encodes
251 the aggregation substance protein Asc10 and plays an important role during conjugative transfer

252 of pCF10 via intracellular aggregation or clumping (16, 48, 49). From previous studies, we know
253 that deletion of *prgB* contributes to decreased virulence and also decreased conjugation frequency
254 of pCF10 in *E. faecalis* (48, 50, 51). Hence, we obtained a previously reported variant of pCF10
255 with the aggregation substance gene *prgB* deleted, referred to as pCF10-8 (48). We generated the
256 donor strain OG1SSp(pTEF1, pCF10-8) and then used OG1SSp(pTEF1) and OG1SSp(pTEF1,
257 pCF10-8) donor strains in conjugation assays. CRISPR-Cas provided genome defense against
258 pTEF1 from both donors, as higher pTEF1 conjugation frequency was observed in the absence of
259 *cas9* in recipient (Figure 3A-a, 3A-b). When we compared the conjugation frequency of pTEF1
260 from the two donors, we still observed a 21-fold increase in conjugation frequency of pTEF1 to
261 recipient T11RF (Figure 3A-c) or a 7-fold increase to T11RFΔ*cas9* (Figure 3A-d), when the donor
262 strain had both pTEF1 and pCF10-8 present, compared with only pTEF1. This means that even
263 with the deletion of aggregation substance gene *prgB*, pCF10-8 can still increase pTEF1
264 conjugation frequency. However, for wild type pCF10 we observed higher fold increase in
265 conjugation frequency of pTEF1 (Figure 2C-c, 2C-d) than pCF10-8. Thus, we concluded that the
266 pCF10 aggregation substance gene *prgB* is important for the increased conjugation frequency of
267 pTEF1 from a multi-plasmid donor, but it is not the only important region under the conditions
268 tested.

269

270 **Deletion of *oriT* from pCF10 decreases pTEF1 conjugation frequency from a multi-plasmid**
271 **donor.** The origin of transfer region, or *oriT,* is the region on plasmid DNA where conjugation
272 initiates, hence playing an important role during conjugative transfer of plasmids (52). A previous
273 study demonstrated that deletion of *oriT* from pCF10 significantly decreases its conjugation
274 frequency (53). Therefore, we obtained an *oriT*-deleted mutant of pCF10, pCF10Δ*oriT* (53), and

275 generated the donor strain OG1SSp (pTEF1, pCF10Δ*oriT*). We used OG1SSp (pTEF1) and
276 OG1SSp (pTEF1, pCF10Δ*oriT*) donor strains in conjugation assays with the same recipients as
277 before. CRISPR-Cas provided genome defense against pTEF1 from both donors, as higher pTEF1
278 conjugation frequency was observed in the absence of *cas9* in recipients (Figure 3B-a, 3B-b).
279 When we compared the conjugation frequency of pTEF1 in two donors we still observed a 9-fold
280 increase in conjugation frequency of pTEF1 to recipient T11RF (Figure 3B-c) or a 11-fold increase
281 to T11RFΔ*cas9* (Figure 3B-d), when the donor strain had both pTEF1 and pCF10Δ*oriT* present,
282 compared with only pTEF1. These results were similar to what we observed with pCF10-8 and
283 indicate that even with the deletion *oriT*, pCF10Δ*oriT* can still increase pTEF1 conjugation
284 frequency. However, for wild type pCF10 we observed higher fold increase in conjugation
285 frequency of pTEF1 (Figure 2C-c, 2C-d) than pCF10Δ*oriT*. Thus, we concluded that, the *oriT* of
286 pCF10 is also important for increased conjugation frequency of pTEF1 from a multi-plasmid
287 donor. The potential additive effect of *prgB/oriT* double deletion was not investigated in our study
288 but would be informative.

289

290 **T11RF CRISPR3-Cas can target clinical isolates and provide defense against resistance**
291 **plasmids harbored by those clinical isolates.** We included 10 previously reported hospital fecal
292 surveillance isolates (44) and 25 previously reported urine isolates (41) in this study. At first, we
293 checked if spacers in the T11RF CRISPR3-Cas system could target the chromosomes or plasmids
294 of these clinical isolates. We found that 6 of 10 fecal surveillance isolates (Supplemental Dataset
295 S2) and 17 of 25 urine isolates (Supplemental Dataset S2) were targeted by the T11RF CRISPR3-
296 Cas system as evidenced by 100% nucleotide sequence identity between spacer and target, and the
297 presence of a suitable protospacer adjacent motif (previously defined in reference 34). The targeted

298 plasmids and antibiotic resistance genes encoded by those plasmids are shown in Table 1 and
299 Supplemental Dataset S2. Moving forward, we will denote plasmids targeted by the T11RF
300 CRISPR3-Cas system in bold text (for each isolate, we denote their plasmid content by their sizes
301 in kilobases [kb] after the isolate name).

302

Fecal surveillance isolates and urine isolates	**T11RF CRISPR3-array spacer that targets the plasmid or chromosome of the isolates**	**Resistance gene encoded in the targeted plasmids or chromosome**	**Rep family of the plasmids**
59 (88kb, **43kb**)	Spacer 6	***vanHAX***, *ermB*	rep9c, **rep9a**
43-2 (**54kb**)	Spacer 6	***ermB***	**rep9a**, rep2
2-1 (**149kb**)	Spacer 6	***ermB***	**rep9a**
133-1 (**172kb,** 43kb)	Spacer 7	***ermB***	**Unknown**, Unknown
101-1 (**65kb**)	Spacer 7	***ermB***	**rep9b**
EfsC8 (**52kb**)	spacer 7	***ermB***	**rep9c**
EfsC33 (**104kb,** 72kb)	spacer 6	***ermB***	**rep9a,** rep9b
EfsC61 (**57kb**)	spacer 7	***ant(6)-la***	**rep2**

303

304

305 **Table 1: Fecal surveillance isolates and urine isolates for conjugation assays.** Five fecal
306 surveillance isolates (the first five listed) and three urine isolates (the last three) used for
307 conjugation assays in this study are presented in Table 1. Targeted plasmids or chromosome are
308 shown in bold in column 1, T11RF CRISPR3-array spacer that target the plasmid, or the
309 chromosome are shown in column 2 and resistance gene encoded in the targeted plasmids or
310 chromosome are shown in bold in column 3. The rep family of the plasmids are shown in column

311 4 and the CRISPR-targeted plasmid rep family is indicated in bold (41, 44). Isolates for which
312 conjugation was not detected are highlighted in grey.

313

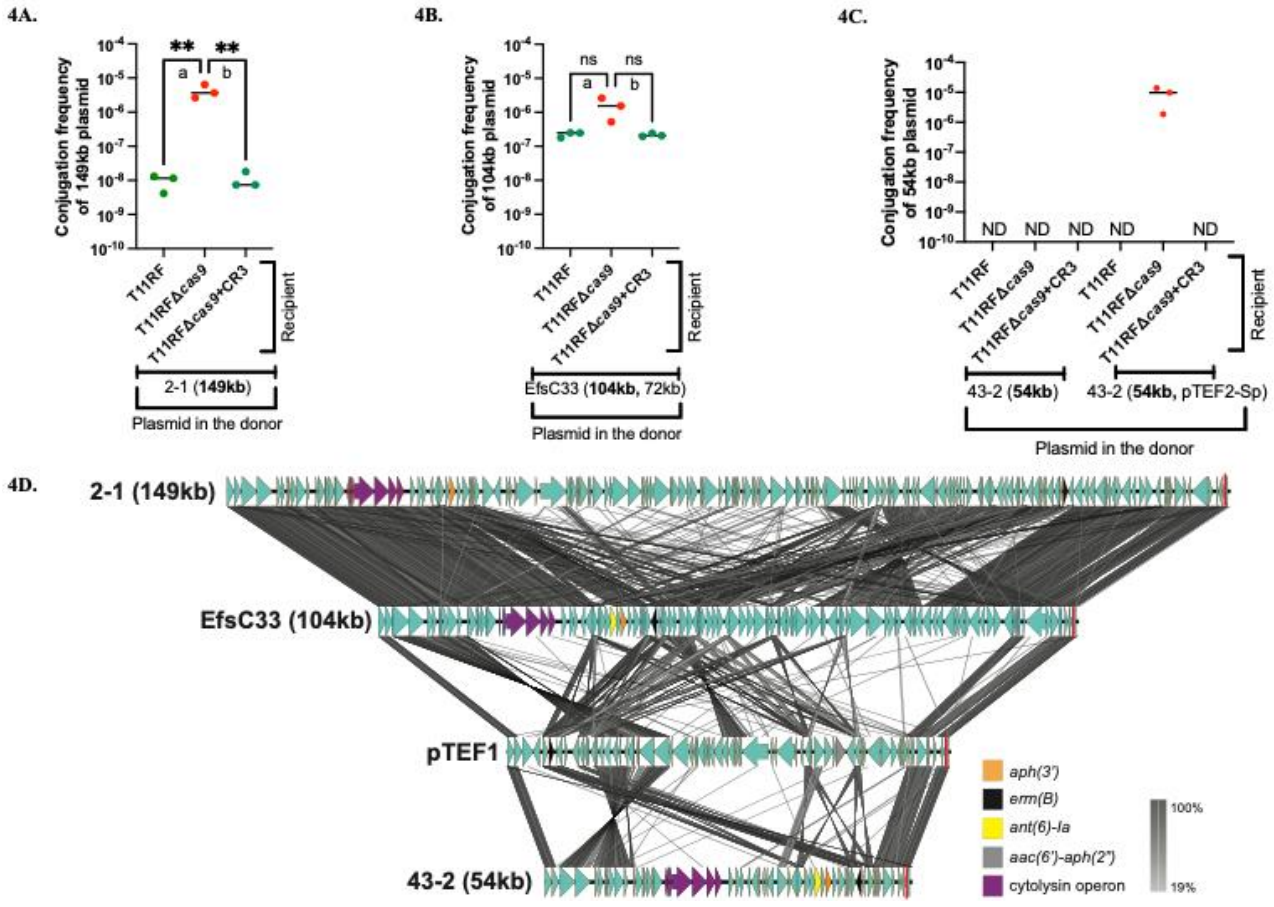
314 The fecal isolates 59 (88 kb**, 43 kb**), 43-2 (**54 kb**), 2-1 (**149 kb**), 133-1 (**172 kb**, 43 kb), 101-1 (**65**
315 **kb**) and urine isolates EfsC8 (**52 kb**), EfsC33 (**104 kb**, 72 kb), EfsC61 (**57 kb**) were used as
316 plasmid donors in conjugation assays with T11RF recipients (Table 1). We chose these clinical
317 isolates as plasmid donors because they harbor CRISPR3-targeted resistance plasmids, and thus
318 we were able to track those plasmids during conjugation assays. Plasmids with sequences targeted
319 by spacer 6 and spacer 7 are shown in Figure 4 and Figure 5, respectively. For fecal isolates 59
320 (88kb, **43kb**), 43-2 (**54kb**), 101-1 (**65Kb**) and urine isolate EfsC61 (**57kb**), conjugation was not
321 detected. Fecal isolates 2-1 (**149 kb**), 133-1 (**172kb**, 43kb) and urine isolates EfsC8 (**52kb**),
322 EfsC33 (**104 kb,** 73kb) conjugated with our recipients at detectable levels. The T11RF CRISPR3-
323 Cas system provided defense against the **149 kb** plasmid from the fecal isolate 2-1 (Figure 4A)
324 with a more modest effectiveness against the **104kb** plasmid from the urine isolate EfsC33 (Figure
325 4B). Interestingly, this was very comparable to T11RF CRISPR3-Cas spacer 7 targeted plasmids
326 (Figure 5). The T11RF CRISPR3-Cas system provided defense against the **172 kb** plasmid in fecal
327 isolate 133-1(Figure 5A) and a weaker protective effect against the **52 kb** plasmid in urine isolate
328 EfsC8 (Figure 5B) as higher conjugation frequencies for these plasmids were observed in the
329 absence of *cas9* in recipients.

330

331 We noted highly variable transfer rates of the CRISPR3-targeted plasmids and the magnitude of
332 CRISPR3-Cas efficacy against them, despite the plasmids being PRPs from similar plasmid rep

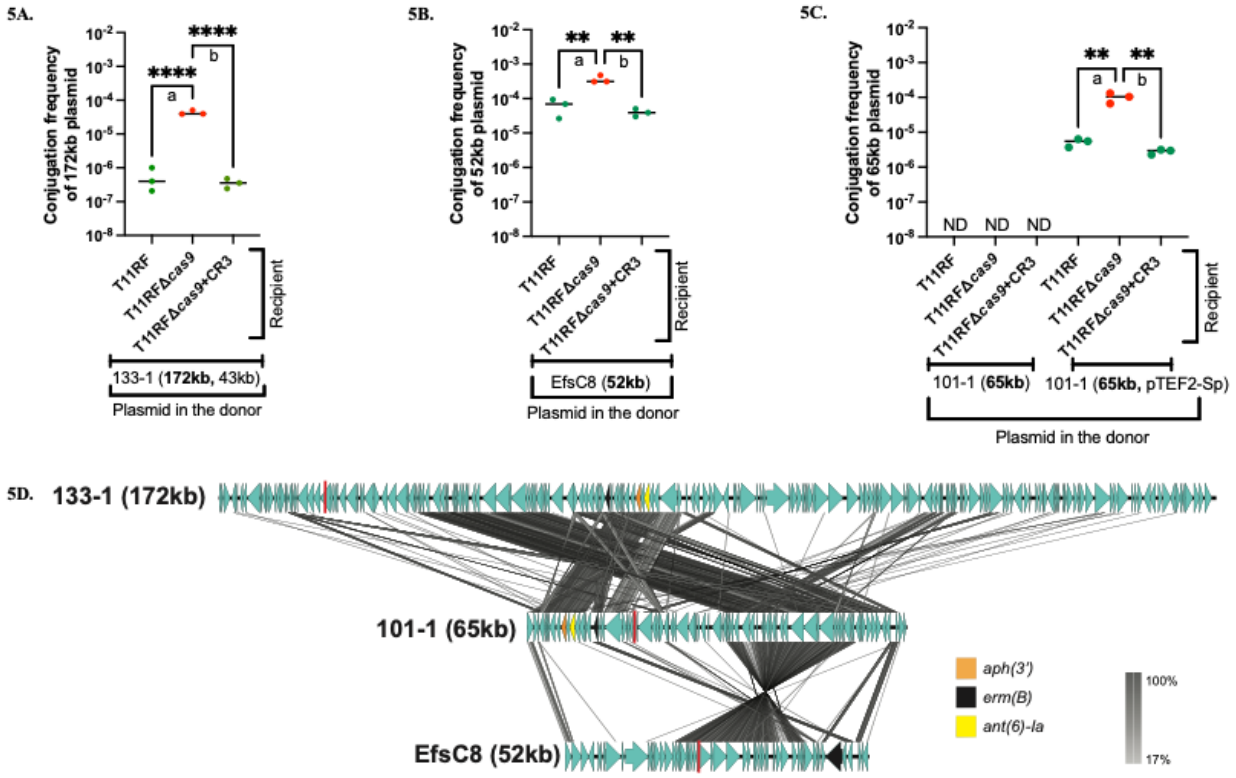
333 families. We performed plasmid alignment of the spacer 6- and spacer 7-targeted plasmids
334 (Figures 4D and 5D, respectively). Our alignments corroborate conclusions reached by prior
335 investigation of the *E. faecalis* urine isolate collection, namely, that plasmid rep typing fails to
336 capture the genetic diversity of PRPs (41). The variability of CRISPR3-Cas defense against these
337 "wild" plasmids is likely due to their genetic diversity and interactions with other MGEs in the
338 donor, which together influence plasmid transfer frequency and interaction with recipient defense
339 systems. This study marks a significant milestone as the first to demonstrate the efficacy of the
340 T11RF CRISPR3-Cas system in targeting plasmids from recent clinical isolates.

341



342

Figure 4: Clinical isolate plasmid donors targeted by T11RF CRISPR3-Cas spacer 6. T11RF CRISPR3-Cas decreased the rate of transfer of the **149 kb** resistance plasmid from fecal surveillance isolate 2-1 (4A) (Tukey's multiple comparisons test, ****P*-value = 0.0088 (4A-a,4A-b)) and the **104 kb** plasmid from urine isolate EfsC33 (4B) (Tukey's multiple comparisons test, ns *P*-value = 0.0786 (4B-a), ns *P*-value = 0.0765 (4B-b)) . Conjugation was not detected for **54 kb** plasmid from fecal isolate 43-2 with the recipients when present alone in the donor. pTEF2-Sp mobilized the **54kb** plasmid from fecal isolate 43-2 in the absence of *cas9* in the recipient (4C). tblastx alignment of plasmids targeted by T11RF CRISPR3 spacer 6 sequence is presented in 4D. Location of spacer 6 sequence is indicated with a red line. Coding sequences are denoted by arrows with antibiotic resistance genes and cytolysin operon genes indicated in unique colors. Lines drawn between plasmid sequences show sequence identity (%) (4D).



356 **Figure 5: Clinical isolate plasmid donors targeted by T11RF CRISPR3-Cas spacer 7.** T11RF
357 CRISPR3-Cas was able to decrease the rate of transfer of the resistance plasmid **172kb** from
358 clinical fecal surveillance isolate 133-1 (5A) (Tukey's multiple comparisons test, ****P-value <
359 0.0001 (5A-a,5A-b)) and **52kb** from clinical urine isolate EfsC8 (5B) (Tukey's multiple
360 comparisons test, **P-value = 0.0016 (5B-a), **P-value = 0.0011 (5B-b)) . Conjugation was not
361 detected for fecal isolate 101-1 with the recipients when present alone in the donor. pTEF2-Sp
362 plasmid mobilized the clinical resistant plasmid **65kb** from the fecal isolate 101-1. However,
363 T11RF CRISPR3-Cas was still able to decrease the rate of transfer of the resistance plasmid **65kb**
364 as higher conjugation frequency was observed in the absence of *cas9* gene (5C) (Tukey's multiple
365 comparisons test, **P-value = 0.0019 (5C-a), **P-value = 0.0017 (5C-b)) . tblastx alignment of
366 plasmids targeted by T11RF CRISPR3 spacer 7 sequence is presented in 5D. Location of spacer 7
367 sequence is indicated with a red line. Coding sequences are denoted by arrows with antibiotic
368 resistance genes indicated in unique colors. Lines drawn between plasmid sequences show
369 sequence identity (%).

370

371 **pTEF2 increases conjugation frequency of resistance plasmids from two fecal isolates and**
372 **helps them escape T11RF CRISPR3-Cas defense.** Conjugation was not detected for the fecal
373 isolates 43-2 (**54kb**), and 101-1 (**65kb**) with T11RF (Figure 4C, Figure 5C). We investigated what
374 effect pTEF2 would have on the transfer of these plasmids, given our results demonstrating that
375 pTEF2 could assist pTEF1 with transfer and apparent evasion of CRISPR-Cas defense (Figure
376 2B). We made new donor strains with pTEF2-Sp (pTEF2 modified to encode spectinomycin
377 resistance (54)). We used these new donor strains, 43-2 (**54kb**, pTEF2-Sp), and 101-1 (**65kb**,
378 pTEF2-Sp) in conjugation assays with our previous recipients. We observed that pTEF2-Sp can

379 mobilize the clinical resistant plasmids, as they conjugated to the recipients. However, T11RF
380 CRISPR3-Cas still provided defense against the **54 kb** plasmid of the 43-2 strain (Figure 4C) and
381 the **65 kb** plasmid of the 101-1 strain (Figure 5C), as in the absence of T11RF *cas9,* higher
382 conjugation frequencies were observed. For the 43-2 donor strain, conjugation of the **54 kb**
383 plasmid was detected only when *cas9* was deleted from the recipient strain. We did not perform
384 the same experiment with pCF10, as pCF10 encodes tetracycline resistance, and 43-2 and 101-1
385 also encode tetracycline resistance in their chromosome. Therefore, we concluded that pTEF2 can
386 increase conjugation frequency of other resistance plasmids and help them in escaping T11RF
387 CRISPR3-Cas defense. Our findings underscore the importance of considering plasmid
388 cooperativity when refining CRISPR-based antimicrobial designs. Our work suggests that targeted
389 depletion of pTEF2 from *E. faecalis* populations may specifically be useful for mitigating
390 antibiotic resistance plasmid spread.

391

392

393 **DISCUSSION**

394 In this era of antibiotic resistance, *E. faecalis* is considered a serious threat as it can acquire a large
395 number of resistance genes via HGT (2, 9). Bacterial CRISPR-Cas systems may be leveraged as
396 an alternative treatment approach, providing sequence-specific cleavage of antibiotic resistance
397 plasmids in *E. faecalis* (34, 38). Most available studies of CRISPR-Cas efficacy have used
398 laboratory model strains and laboratory model plasmids. However, clinical isolates can behave
399 differently than the laboratory model strains, and CRISPR-Cas efficacy can be affected by many
400 factors (44). Therefore, for this study we included both laboratory model strains and clinical

401 isolates to study previously unexplored factors that might affect CRISPR-Cas efficacy. Our
402 hypothesis was that the number of plasmids present in a donor strain is an important factor that
403 might affect CRISPR-Cas efficacy. Through our experiments we showed our hypothesis is
404 supported and found two plasmid pairs (i) pTEF1,pTEF2 and (ii) pTEF1, pCF10 that showed
405 plasmid cooperativity. From our experiments we saw pTEF2 or pCF10 were able to increase
406 pTEF1 conjugation frequency. We identified two regions, (i) aggregation substance gene *prgB* and
407 (ii) *oriT* of pCF10, potentially important for the plasmid cooperativity as deletion of these regions
408 resulted in decreased conjugation frequency of pTEF1.

409

410 We included 10 clinical fecal surveillance isolates (44) and 25 urine isolates (41) in this study. We
411 found that 6 fecal surveillance isolates, and 17 urine isolates can be targeted by the T11RF
412 CRISPR3-Cas system. When we used these clinical isolates as plasmid donors, we found that
413 T11RF CRISPR3-Cas was able to provide defense against the resistance plasmids harbored by
414 those isolates. To the best of our knowledge, this is the first research studying T11RF CRISPR3-
415 Cas efficacy against a collection of clinical isolates. Interestingly, there is a significant impact of
416 CRISPR-Cas on targeted plasmids, but the magnitude of the impact differed for different clinical
417 isolates. There were a few clinical isolates that did not conjugate with our recipient at detectable
418 levels. However, when we conjugated pTEF2-Sp plasmid in those clinical isolates, pTEF2-Sp was
419 able to mobilize two clinical resistant plasmids (i) 54kb, pTEF2-Sp from 43-2 and (ii) 65kb,
420 pTEF2-Sp from 101-1. However, T11RF CRISPR3-Cas system was still able to provide defense
421 against those plasmids once they were mobilized. This finding is similar to our plasmid
422 cooperativity results of pTEF1, pTEF2 and pTEF1, pCF10. Together these plasmid cooperativity
423 results indicate that the first conjugative resistance plasmid lacks something essential for robust

424 transfer, to which they are getting "help" from the second plasmid, and together, they escape
425 T11RF CRISPR3-Cas defense by dint of higher conjugation frequency. A limitation of our
426 experimental design is, we did not pinpoint the exact mechanism of this plasmid cooperativity. It
427 could be the helper plasmid enhancing the transfer frequency of the first plasmid, the helper
428 plasmid inhibiting the CRISPR-Cas defense against the first plasmid, or both.

429

430 Overall, we conclude that the presence of multiple interacting plasmids in donor strains and the
431 lack of an active CRISPR-Cas system in a recipient strain act additively to confer extremely high
432 plasmid transfer frequencies in *E. faecalis*. To date, a few multi-plasmid studies have demonstrated
433 how ecological settings influence the maintenance of individual plasmids within a multi-plasmid
434 community, how a conjugative helper plasmid can mobilize a non-conjugative virulence plasmid,
435 and how interactions between plasmids can affect conjugation efficiency (42, 55, 56). Hence, our
436 study opens a new direction of plasmid cooperativity and contributes towards designing novel
437 CRISPR antimicrobials.

438

439 Apart from the plasmid number present in donor strains, there may be other factors affecting
440 CRISPR-Cas efficacy, such as biofilm formation capacity or internal CRISPR-Cas regulation in
441 the recipient strains. For example, transcriptional regulation of *cas9* was reported as a major factor
442 which can influence CRISPR-Cas genome defense in *S. pyogenes* (57). Hence, it is necessary to
443 continue to study potential factors that might affect CRISPR-Cas efficacy, in future studies.
444 Overall, our research is significant in its application towards designing improved CRISPR-based
445 antimicrobials as an alternative approach to combat HGT and antibiotic resistance. Our research

446 also emphasizes to study factors affecting CRISPR-Cas efficacy and conducting research using
447 clinical isolates. The more information we gather on the efficacy of CRISPR-Cas, the more
448 effectively we can harness it as an alternative treatment approach.

449

450 **MATERIALS AND METHODS**

451 **Bacterial strains and reagents used.** Strains and plasmids used in this study are shown in Table
452 S1. *E. faecalis* strains were cultured in brain heart infusion (BHI) broth or agar at 37°C without
453 shaking, unless otherwise stated. Antibiotics were added at the following concentrations: rifampin
454 (R), 50 μg/mL; fusidic acid (F), 25 μg/mL; streptomycin (S), 500 μg/mL; tetracycline (Tet), 10
455 μg/mL; spectinomycin (Sp), 500 μg/mL; erythromycin (Erm), 50 μg/mL; vancomycin (Van), 10
456 μg/mL; gentamicin (Gent), 10 μg/mL. Routine PCR analysis was performed using *Taq* polymerase
457 (New England Biolabs). Primers (Sigma-Aldrich) used in this study are shown in Table S2.

458

459 **Conjugation assays.** Conjugation assays were conducted as previously described (34). CFU/mL
460 was determined using the following formula: CFU/mL = number of colony/(amount plated (mL)
461 x dilution factor). Conjugation frequency was calculated by dividing the CFU/mL of the
462 transconjugants by the CFU/mL of the donors. Raw CFU/mL data for conjugation experiments are
463 shown in Supplemental Dataset S3. Graphs were prepared and statistical analysis performed using
464 GraphPad Prism (Version 9.0.0).

465

466 **Selection of fecal surveillance isolates and urine isolates.** 10 fecal surveillance isolates (44), and
467 25 urine isolates (41) were analyzed for this study. First, a database was created in Geneious with
468 the previously reported genome sequences of the 10 fecal surveillance isolates or 25 urine isolates.
469 All CRISPR3 spacer sequences from T11RF were queried against the database using BLASTn. 6
470 of 10 fecal surveillance isolates and 17 of 25 urine isolates were identified with T11RF CRISPR3-
471 Cas targets in either plasmids or in the chromosome with 100% nucleotide sequence identity
472 (Supplemental Dataset S2). The presence of the expected protospacer adjacent motif sequence (34)
473 adjacent to the target sequence was also confirmed.

474

475 **Generation of 43-2 (54kb, pTEF2-Sp) and 101-1 (65kb, pTEF2-Sp) donor strains and**
476 **conjugation assays.** *E. faecalis* V19 (pTEF2-Sp) (54) was used as donor and fecal isolates 43-2
477 (**54kb**), 101-1 (**65kb**) were used as recipients in conjugation assays to generate 43-2 (**54kb**,
478 pTEF2-Sp) and 101-1 (**65kb**, pTEF2-Sp) donor strains. PCR was performed to confirm the
479 plasmids present in the transconjugant. Once confirmed, 43-2 (**54kb**, pTEF2-Sp) and 101-1 (**65kb**,
480 pTEF2-Sp)**,** were used as donors in conjugation assays using T11RF, T11RFΔ*cas9* and
481 T11RFΔ*cas9*+CR3 as recipients. Conjugation assays were done in biological triplicate using the
482 previously described method (34). Antibiotic selection plates were prepared according to the
483 resistance gene present in the donor and recipients (Table S1). All the CFU/mL calculation and
484 data analysis were done as described above.

485

486 **Plasmid alignment and analysis**. Plasmid tblastx alignments were performed using EasyFig 3.0.0
487 at default parameters. Antimicrobial resistance genes were identified using ABRicate v1.0.1

488 querying the ResFinder database at default parameters. pTEF2-specific genes (in comparison to
489 pCF10) were analyzed using NCBI Conserved Domains (58), PSORTb 3.0.3 (59), and InterPro
490 (60). Plasmid replicon types were predicted using PlasmidFinder v2.1 at default parameters. Where
491 plasmid replicon type was not identified using PlasmidFinder, plasmids were analyzed using NCBI
492 Conserved Domains to identify the *rep* gene. Further typing was completed using blastn and
493 PlasmidFinder queries at 70% identity threshold (41).

494

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499

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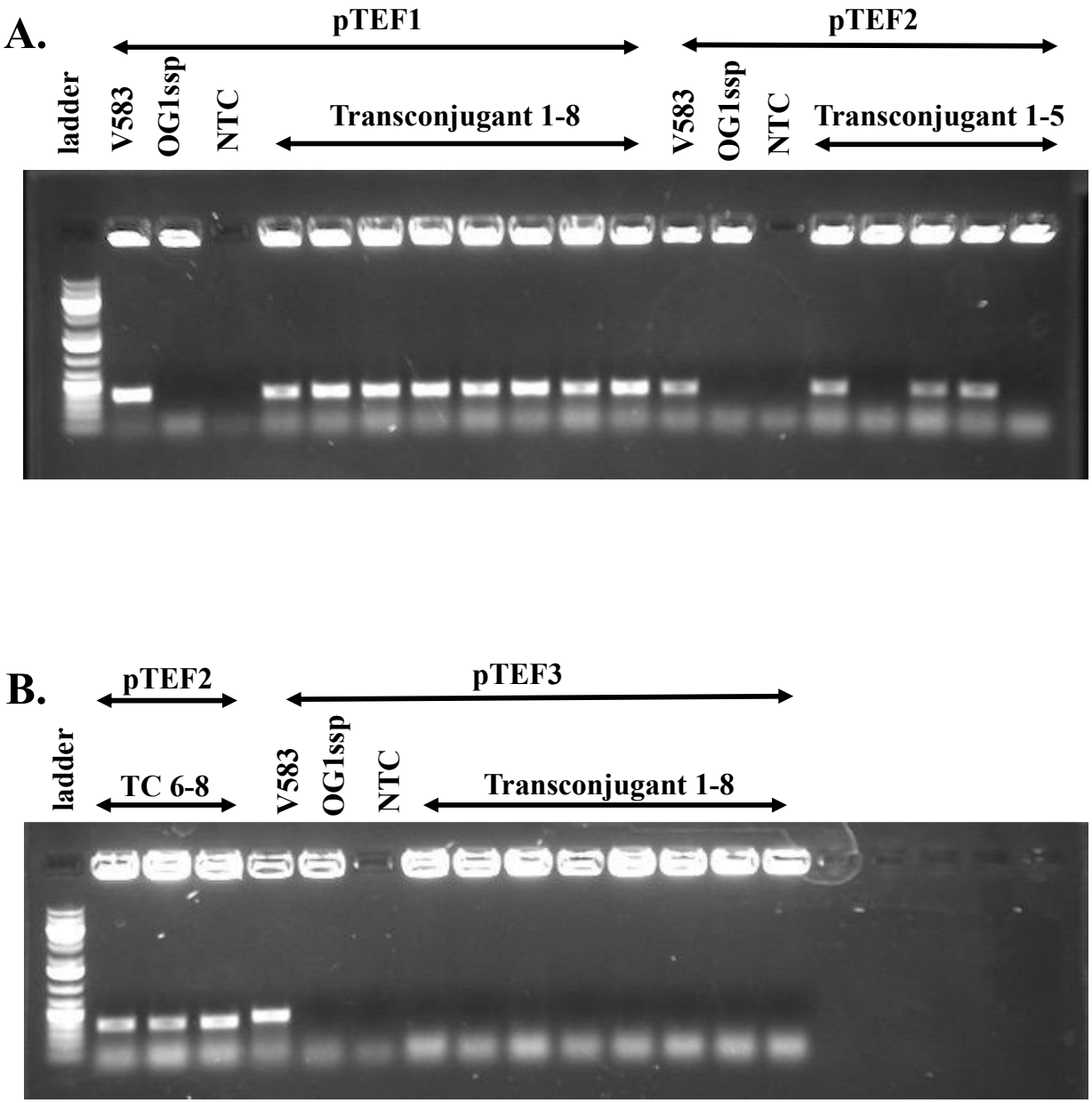
708 **SUPPLEMENTAL MATERIALS**



709

710 **Figure S1: Identification of plasmid pTEF1, pTEF2 and pTEF3 by PCR**. Primers designed
711 for pTEF1, pTEF2 and pTEF3 were used to screen the transconjugants. V583 was used as the
712 positive control, and the amplified plasmid is indicated in each case. The gel image shows that it
713 contains all three plasmids. OG1SSp was used as the negative control and the amplified plasmid

714 is indicated in each case. The gel image shows that it contains none of the plasmid. A no template
715 control (NTC) was also added in each case. The gel image shows that all eight selected
716 transconjugants contain the pTEF1 plasmid (A); six transconjugants contain the pTEF2 plasmid
717 (A,B) and none of the transconjugants contain pTEF3 plasmid (B).

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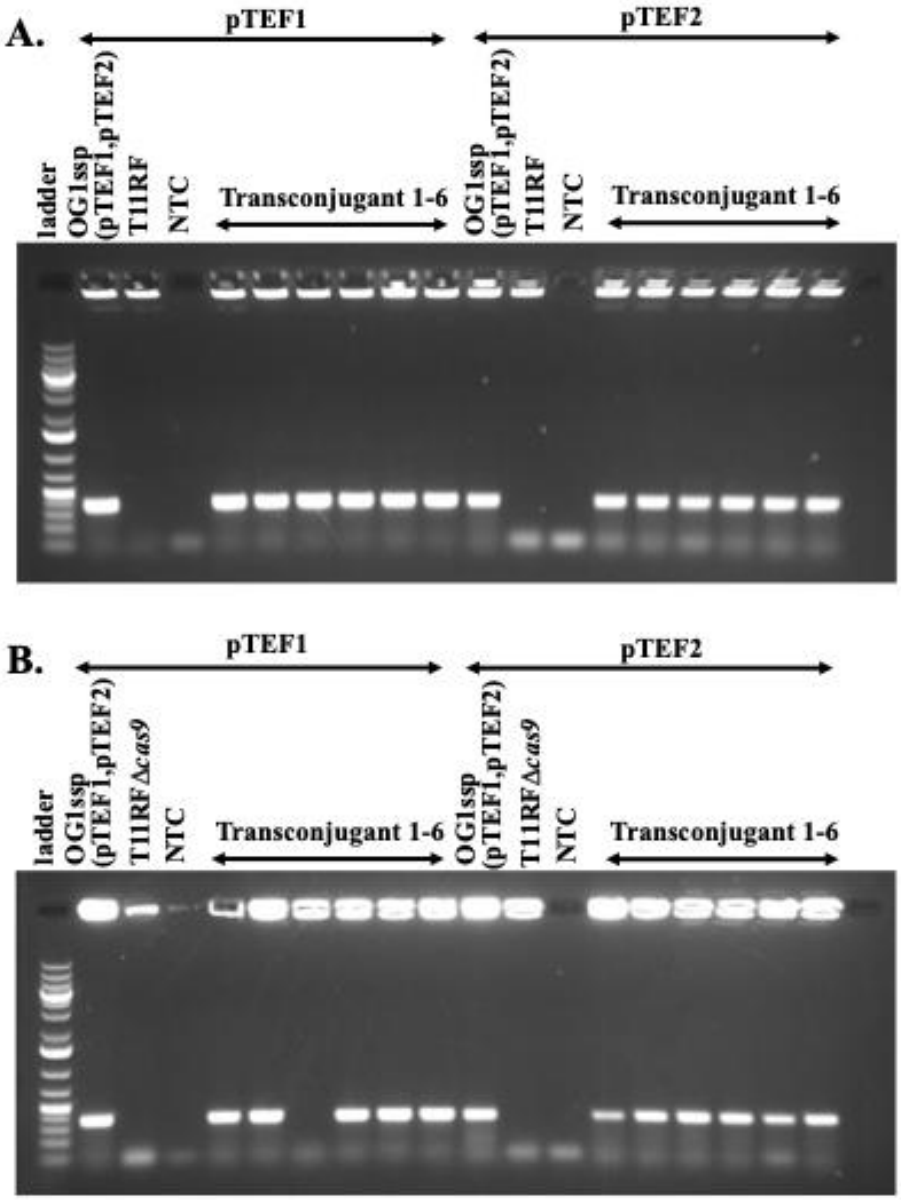

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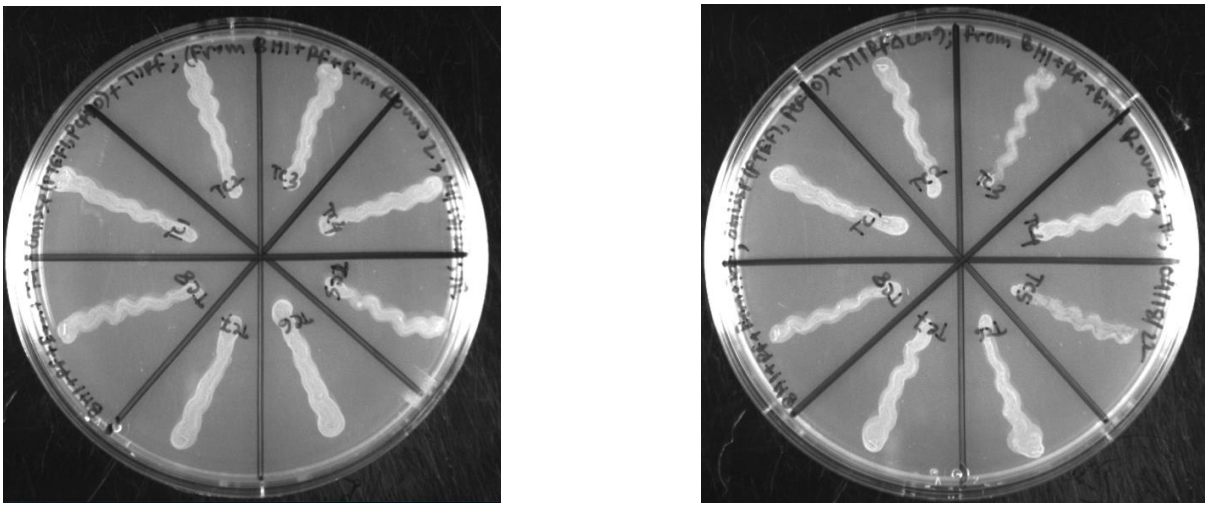
721 **Figure S2: Identification of plasmid pTEF1 and pTEF2 in transconjugants by PCR**. Primers
722 designed for pTEF1 and pTEF2 were used to screen the transconjugants. OG1SSp (pTEF1,
723 pTEF2) was used as the positive control, and the amplified plasmid is indicated in each case. The
724 gel image shows that it contains both pTEF1 and pTEF2 plasmids (A, B). T11RF (A) and
725 T11RFΔ*cas9* (B) were used as the negative control and gel image shows that they contain none of
726 the plasmids. A no template control (NTC) was also added in each case. The gel image shows that
727 all the six selected transconjugants contain pTEF1 and pTEF2 plasmids when T11RF was the
728 recipient (A). Five transconjugants contain pTEF1 plasmid and all six transconjugants contain
729 pTEF2 plasmid when T11RFΔ*cas9* was the recipient (B).

730

731 **A.** **B.**



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734 **Figure S3: Identification of plasmid pTEF1 and pCF10 in transconjugants**. 8 transconjugants
735 were picked from the original transconjugant plates and restreaked where OG1SSp
736 (pTEF1,pCF10) was donor and either T11RF (A) or T11RFΔ*cas9* (B) were recipients. In the
737 original transconjugant plate only pTEF1 was selected by erythromycin antibiotic. In the
738 restreaked plate both pTEF1 and pCF10 were selected by erythromycin and tetracycline
739 respectively. We found that in all cases pTEF1 and pCF10 were transferred together.

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Strain	**Description**	**Reference(s)**
V583	Vancomycin-resistant *E. faecalis* clinical isolate harboring pTEF1, pTEF2 and pTEF3 plasmids	(2, 46)
OG1SSp	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring no plasmids	(16, 45)
OG1SSp (pTEF1)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring erythromycin resistant pTEF1 plasmid	This study
OG1SSp (pTEF1,pTEF2)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pTEF1 and pTEF2 plasmids	This study
T11RF	Rifampin- and fusidic acid-resistant derivative of *E. faecalis* urine isolate T11	(34, 35)
T11RFΔ*cas9*	T11RF CRISPR3-*cas9* deletion mutant	(34)
T11RFΔ*cas9*+CR3	T11RFΔ*cas9* mutant with chromosomal integration of CRISPR3 *cas9* between EFMG_00904 and EFMG_00905	(34)
OG1RF (pCF10)	Rifampin- and fusidic acid-resistant derivative of OG1 *E. faecalis* strain harboring pCF10 plasmid encoding tetracycline resistance on Tn*925*	(16, 61, 62)
OG1SSp (pTEF1,pCF10)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pTEF1 and pCF10	This study
CK104 (pCF10-8)	OG1RFΔ*upp* strain harboring pCF10-8 plasmid (*prgB* deletion mutant retaining first three and last three codons, Asc10-)	(48, 63)

OG1SSp (pTEF1,pCF10-8)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pTEF1 and pCF10-8 plasmids	This study
CK104 (pCF10Δ*oriT*)	OG1RFΔ*upp* strain harboring pCF10Δ*oriT* plasmid.	(53)
OG1SSp (pTEF1, pCF10Δ*oriT*)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pTEF1 and pCF10Δ*oriT*	This study
59 (88kb,43kb)	Fecal surveillance *E. faecalis* isolate 59 harboring vancomycin resistant 88 kb plasmid and erythromycin resistant 43 kb plasmid	(44)
43-2 (54kb)	Fecal surveillance *E. faecalis* isolate 43-2 harboring erythromycin resistant 54kb plasmid	(44)
2-1 (149kb)	Fecal surveillance *E. faecalis* isolate 2-1 harboring erythromycin resistant 149kb plasmid	(44)
133-1 (172kb)	Fecal surveillance *E. faecalis* isolate 133-1 harboring erythromycin resistant 172kb plasmid	(44)
101-1 (65kb)	Fecal surveillance *E. faecalis* isolate 101-1 harboring erythromycin resistant 65kb plasmid	(44)
142-1 (77kb, 59kb)	Fecal surveillance *E. faecalis* isolate 142-1 harboring erythromycin resistant 77kb plasmid and 59kb plasmid	(44)
V19 (pTEF2-Sp)	Plasmid-free derivative of V583 Em^S, Gm^S harboring Spectinomycin resistant pTEF2 plasmid	(54)
43-2 (54kb, pTEF2-Sp)	Fecal surveillance *E. faecalis* isolate 43-2 harboring erythromycin resistant 54kb plasmid and Spectinomycin resistant pTEF2 plasmid	This study
101-1 (65kb, pTEF2-Sp)	Fecal surveillance *E. faecalis* isolate 101-1 harboring erythromycin resistant 65kb plasmid Spectinomycin resistant pTEF2 plasmid	This study
EfsC8 (52kb)	*E. faecalis* urine isolate EfsC8 harboring erythromycin resistant 52kb plasmid	(41)
EfsC33 (104kb, 72kb)	*E. faecalis* urine isolate EfsC33 harboring erythromycin resistant 104kb plasmid and 72kb plasmid	(41)
EfsC61 (57kb)	*E. faecalis* urine isolate EfsC61 harboring streptomycin resistant 57kb plasmid	(41)

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743 **Table S1: *E. faecalis* strains and plasmids used in this study.**

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745

Primer name	Description	Sequence	Reference
FpTEF1	Forward primer for pTEF1	ACAAACGCTGAATCCCTTGG	This study
RpTEF1	Reverse primer for pTEF1	TGATTTTATGCATCACTCGTCGC	This study
FpTEF2	Forward primer for pTEF2	TCGGCGTTTCTTTCGAAAGTGGG	This study
RpTEF2	Reverse primer for pTEF2	TGATTTTATGCATCACACGACGC	This study
FpTEF3	Forward primer for pTEF3	CTACCCTACCCCCAGGACGC	This study
RpTEF3	Reverse primer for pTEF3	AGCTAAGACGGGAGATTTAGCCG	This study
prgW_F	Forward primer for pCF10	ACACCTAACCTGCTATATCTCGG	This study
prgW_R	Reverse primer for pCF10	AGTATCGGAAAAGGCAGCAA	This study
149kb_F	Forward primer for 149kb plasmid of 2-1	AGGTGGTGTTGGAAAGTCCA	This study
149kb_R	Reverse primer for 149kb plasmid of 2-1	AACGTAATCACTCGCCACGA	This study

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747 **Table S2: Primers used in this study.**

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