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; laboratory model strains as donor versus recent human isolates as donor; and the biofilm substrate utilized 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). We also identified that *E. faecalis* Δ*cas9* has altered biofilm structure and thickness relative to the wild-type strain when cultured on a plastic substrate, but equivalent growth in the agar plate biofilms widely used for conjugation studies. 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.

IMPORTANCE

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

INTRODUCTION

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

Horizontal gene transfer (HGT) disseminates antibiotic resistance genes among bacterial pathogens, including *E. faecalis* (9). Many studies have identified mobile genetic elements (MGEs) such as pheromone-responsive plasmids (PRPs), mobilizable plasmids, and transposons as means of HGT in *E. faecalis* (9–11). Conjugation is the most studied form of HGT in *E. faecalis* (12–15). PRPs are conjugative plasmids that can achieve very high conjugation frequencies due to their transfer mechanism which capitalizes on sex pheromone production by recipient cells to facilitate cell contact with donors (16–18). Thus, *E. faecalis* donor strains harboring a PRP can transfer the plasmid to a recipient cell via conjugation and produce a transconjugant (16, 19). PRPs were first identified in *E. faecalis* and appear to exclusively replicate within the enterococci (20, 21). Examples of well-studied, model PRPs are pAD1 and pCF10 (20, 22, 23).

Clustered regularly interspaced short palindromic repeat and CRISPR associated proteins (CRISPR-Cas) systems, can provide sequence-specific genome defense against HGT (24–26). This system preserves a genetic memory of past encounters with MGEs via short sequences called spacers. Upon spacer-dependent recognition of a targeted MGE, Cas-encoded nucleases can cleave and deactivate it (26–28). In *E. faecalis*, predominantly type IIA CRISPR-Cas systems are found and characterized by the presence of type II specific nuclease Cas9 (formerly known as Csn1) (29–31). Genome analysis has also identified presence of type IIC CRISPR-Cas system in *E. faecalis* recently (32). Thus far, four different CRISPR-Cas loci namely CRISPR1-Cas, CRISPR2, CRISPR3-Cas and CRISPR4 have been identified in *E. faecalis* (29, 30, 33). The

plasmid recipient strain used in this study, *E. faecalis* urinary tract isolate T11RF (34, 35) possesses a native CRISPR3-Cas system (30). The T11RF CRISPR3-Cas spacer 6 has sequence identity to the model PRP pAD1 and reduces acquisition frequency of this plasmid in T11RF (30, 34).

From bioinformatic studies, we know that many MDR *E. faecalis* are "immunocompromised" and lack functional CRISPR-Cas systems, which likely allows for accumulation of antibiotic resistance-encoding MGEs in these strains (30, 36, 37). Using the non-MDR *E. faecalis* T11RF as a model recipient, previous works demonstrated that *E. faecalis* CRISPR-Cas confers defense against pAD1 derivatives both *in vitro* and *in vivo* (in the murine intestine) (34, 38). These experimental studies established conclusively that CRISPR-Cas can serve as an anti-plasmid defense system in *E. faecalis*. Yet, a limitation of these studies is that they assessed CRISPR-Cas efficacy against model antibiotic resistance plasmids and used laboratory model donor strains derived from the natively plasmid-free 1975 oral isolate OG1 (39). *E. faecalis* MDR clinical isolates can possess up to six plasmids (2, 40, 41), most of which have not been investigated beyond sequencing. These *E. faecalis* clinical isolates are plasmid reservoirs and donors from which antibiotic resistance spreads. Theoretically, plasmids in these donor cells may collaborate to enhance their transfer rates and/or overcome anti-plasmid defense in the recipient cell. Indeed, it has been reported that anti-CRISPR phages can cooperate to defeat CRISPR-Cas defense in host bacteria (42). Hence, the number and genotype of plasmids harbored by an *E. faecalis* donor are factors that need to be explored to determine their impact on CRISPR-Cas anti-plasmid defense. Other recent research has also demonstrated that the effectiveness of a CRISPR-based antimicrobial against *E. faecalis* fecal surveillance isolates can be affected by competitive factors

(such as bacteriocins) produced by the isolates (43). This emphasizes the need to incorporate clinical isolates in research to understand CRISPR-Cas efficacy against them.

Biofilm formation is a critical contributor to *E. faecalis* virulence (44, 45). *In vivo*, *E. faecalis* forms biofilms in the gastrointestinal tract (46), on heart valves during endocarditis (47), and on in-dwelling urinary and bloodstream catheters (48–50). In addition, PRPs promote *E. faecalis* cellular aggregation and biofilm formation, facilitating their transfer by conjugation (17, 51). In laboratory settings, there are various ways to culture and investigate *E. faecalis* biofilms, the simplest of which is an agar plate biofilm. For these, *E. faecalis* strains are spread-plated on an agar plate, incubated, and then collected for quantification of colony-forming units or other analyses (34). More complex models include flow-cell biofilms coupled with confocal imaging (52) and *in vitro* synthetic endocarditis models (53). To our knowledge, the only biofilm model used to study *E. faecalis* CRISPR-Cas anti-plasmid defense is the agar plate biofilm method. In this study, we lay the groundwork for using confocal imaging to study CRISPR-Cas in *E. faecalis*, making the unexpected discovery that *E. faecalis* T11RFΔ*cas9* has a biofilm formation defect on a plastic substrate.

Overall, in this study we incorporated both laboratory model strains and clinical isolates to examine the correlation between the number of plasmids present in a donor cell and the ability of CRISPR-Cas to enact its defense mechanism in the recipient. We found that presence of multiple plasmids in a donor strain and the lack of active CRISPR-Cas system in a recipient strain can additively confer strikingly high conjugative transfer frequencies of antibiotic resistance

plasmids. Our results can be leveraged to develop enhanced CRISPR-based antimicrobials. Our research also underscores the importance of investigating additional potential factors that may influence the effectiveness of CRISPR-Cas anti-plasmid defense, including biofilm growth substrates.

RESULTS

CRISPR-Cas defense in the recipient is less effective when pTEF1 and pTEF2 are present in the donor strain, compared to pTEF1 alone. We first examined the correlation between the number of plasmids present in the donor cell and the ability of CRISPR-Cas in the recipient cell to enact its defense mechanism. We hypothesized that the presence of multiple plasmids in an *E. faecalis* donor would increase the conjugative transfer of antibiotic resistance and negatively impact CRISPR-Cas genome defense in recipients. To generate isogenic plasmid donors with varying number of plasmids, we used *E. faecalis* clinical isolate V583 as the plasmid donor and *E. faecalis* laboratory model strain OG1SSp (16, 54) as the recipient strain in a conjugation assay. We chose V583 because it was among the first vancomycin-resistant clinical isolates reported in the United States (55) and contains 3 plasmids, pTEF1, pTEF2 and pTEF3 (2). pTEF1 is a PRP encoding erythromycin resistance (2). pTEF2 is also a PRP but does not encode antibiotic resistance genes (2). pTEF1 and pTEF2 share sequence features with the model PRPs pAD1 (56) and pCF10 (18), respectively, which respond to different peptide pheromones (2). pTEF3 is a nonconjugative broad host range plasmid (2). Conjugation assays were conducted as described previously (34) (Figure 1). Antibiotic-containing agar plates were used to select for OG1SSp(pTEF1) transconjugants. Eight transconjugants were selected for PCR to confirm their plasmid content. Two of these transconjugants had one plasmid - OG1SSp(pTEF1) - and 6

transconjugants had two plasmids - OG1SSp(pTEF1, pTEF2). Transfer of pTEF3 was not observed (Figure S1). Thus, we generated our first controlled set of donors with varying number of plasmids.

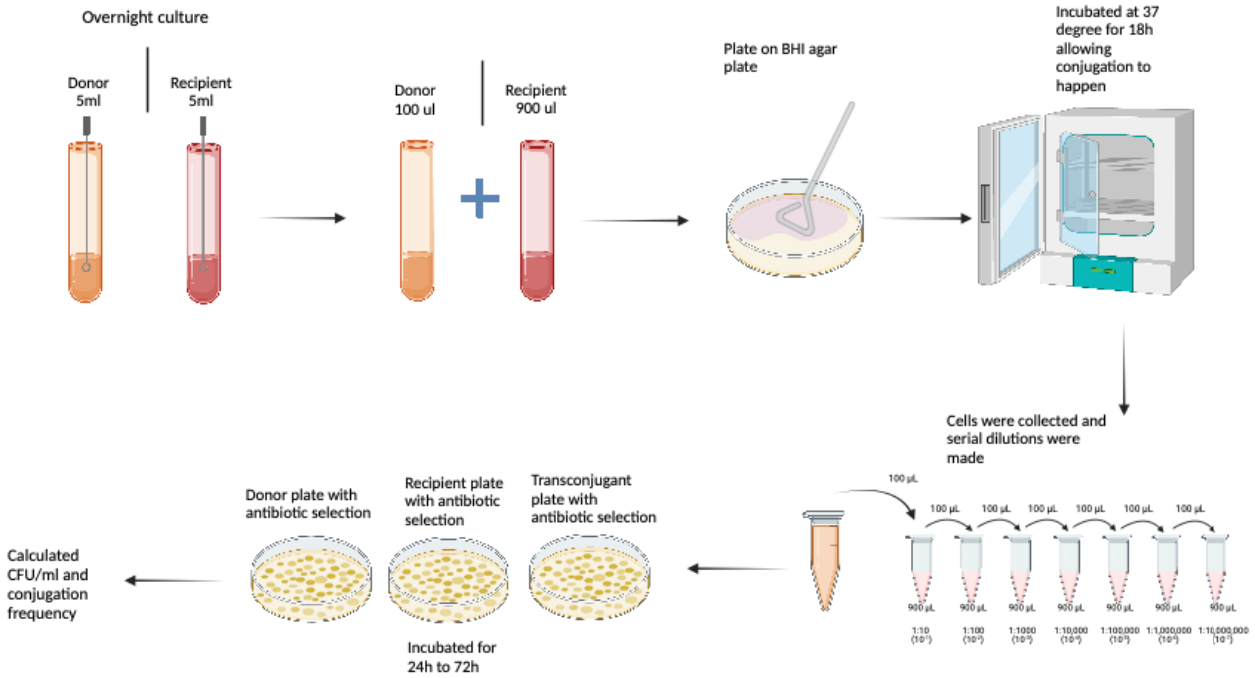

Figure 1: Overview of conjugation assays. In day 1, donor and recipients were inoculated in 5 mL BHI without antibiotic selection and incubated overnight at 37°C. The next day, the cultures were diluted 1:10 into fresh BHI and incubated for 1.5 h at 37°C or until log phase reached. A 100 µL volume of donor culture was mixed with 900 µL volume of recipient culture, and the mixture was pelleted at 13,000 rpm for 1 min. 900 µL supernatant was discarded, and the remaining 100 µL of supernatant was used to resuspend the pellet. This was then plated on a BHI agar plate and incubated at 37°C for 18 h. Cells were collected from the plate with 2 mL 1X PBS supplemented with 2 mM EDTA. This was serially diluted (10^{-1} to 10^{-8}). Dilutions were plated

on BHI agar plates supplemented with appropriate antibiotics to quantify donors, recipients and transconjugants. CFU/mL was calculated after 48 h to 72 h incubation at 37°C and used for calculating conjugation frequency (34).

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

We then compared the pTEF1 conjugation frequency for the two donors. We expected that, if CRISPR-Cas was equally effective against pTEF1 irrespective of pTEF2 presence, the pTEF1 conjugation frequency would be unchanged for wild-type T11RF recipients. However, we

observed a ~2 log fold increase in pTEF1 conjugation frequency for wild-type T11RF when the donor strain had both pTEF1 and pTEF2 present, compared with only pTEF1 (Figure 2B-c). Moreover, in the case of T11RF∆*cas9* as the recipient strain, a striking ~4 log fold increase in pTEF1 conjugation frequency was observed when the donor strain had both pTEF1 and pTEF2 present, compared with only pTEF1 (Figure 2B-d). We analyzed the plasmid content of 6 T11RF and 6 T11RF∆*cas9* transconjugants using PCR, finding that both pTEF1 and pTEF2 transferred to all 6 T11RF transconjugants and to 5 of 6 T11RF∆*cas9* transconjugants (Figure S2).

Our results support our hypothesis and demonstrate that CRISPR-Cas efficacy against pTEF1 is diminished when another plasmid, pTEF2, is present in the donor cell and co-transfers with pTEF1. Moreover, pTEF1 transfer frequency is extraordinarily high (~6-log increase) when donors possess both pTEF1 and pTEF2, and recipients lack functional CRISPR-Cas defense. This plasmid cooperation phenomenon may result from pTEF2 enhancing the transfer frequency of pTEF1, pTEF2 inhibiting CRISPR-Cas or other genome defense against pTEF1 in recipients, or both.

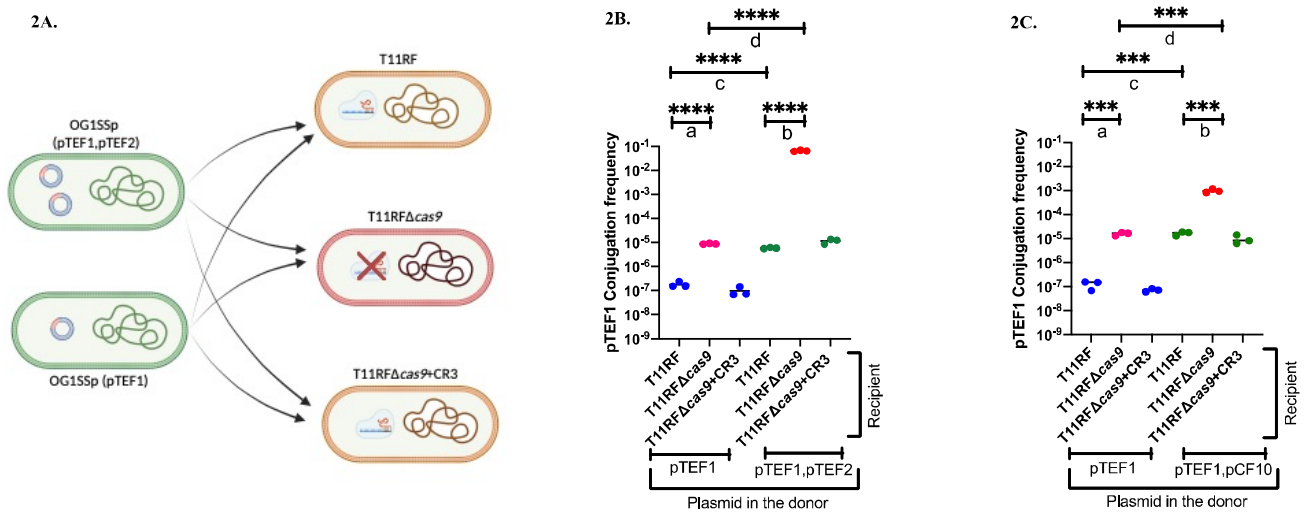

Figure 2: T11RF CRISPR3-Cas efficacy against pTEF1 for OG1SSp(pTEF1) and OG1SSp(pTEF1,pTEF2) or OG1SSp(pTEF1,pCF10) donor strains. Figure 2A shows the donors OG1SSp(pTEF1, pTEF2) or OG1SSp(pTEF1) and the recipients T11RF, T11RFΔ*cas9*, and T11RFΔ*cas9*+CR3 that are used in the conjugation assays. In 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). 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) (Unpaired student t test, ****P-value = .0001). T11RF CRISPR3-Cas can also provide sequence specific genome defense against OG1SSp (pTEF1) and OG1SSp (pTEF1,pCF10) (2C-a, 2C-b) (Unpaired student t test, ***P-value = 0.0002). 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) (Unpaired student t test, ***P-value = .0003, ***P-value = 0.0002) . When pTEF2 is present

instead of pCF10 in donor, ~1.5 log increase of pTEF1 transfer was observed in the absence of *cas9* in recipient.

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

As stated above, we expected that if CRISPR-Cas was equally effective against pTEF1 irrespective of pCF10 presence, the pTEF1 conjugation frequency would be unchanged for wild-type T11RF recipients. However, an ~2 log fold increase in pTEF1 conjugation frequency was observed when the donor strain had both pTEF1 and pCF10 present, compared with only pTEF1 (Figure 2C-c). During the conjugation assay, we only tracked pTEF1 using erythromycin-containing agar. Hence, we screened transconjugants for the presence of both pTEF1 and pCF10 plasmids using erythromycin- and tetracycline-containing agars, respectively. For all transconjugants screened, pTEF1 and pCF10 plasmids were transferred together (Figure S3). Overall, these observations for pCF10 are consistent with those for pTEF2; both plasmids assisted pTEF1 in evading CRISPR-Cas defense.

We noted that Figure 2B (for effect of pTEF2 in donors) and 2C (for effect of pCF10 in donors) were nearly superimposable, with the exception of the results for T11RFΔ*cas9* recipients. The presence of pTEF2 in donors conferred an additional ~2-log increase in pTEF1 conjugation frequency into T11RFΔ*cas9* recipients, compared to the presence of pCF10 in donors (Figure 2B-2C). One possible explanation is that pTEF2 suppresses an additional genome defense system that becomes activated in T11RFΔ*cas9*. We previously reported that a restriction-modification system and CRISPR3-Cas act additively in T11RF to reduce plasmid entry (34), providing support for multiple, co-occurring defense systems in plasmid recipient cells. pTEF2 possesses 21 genes (in two clusters) that pCF10 lacks (Supplemental Dataset S1). Conserved domain analysis identified putative functions including plasmid partitioning, pheromone response, and modification-dependent DNA nicking for 15 of the genes; no conserved domains were identified for 6 of the genes. Further experimentation will be required to establish the basis for the extraordinarily high pTEF2-assisted pTEF1 transfer rate for T11RFΔ*cas9* recipients.

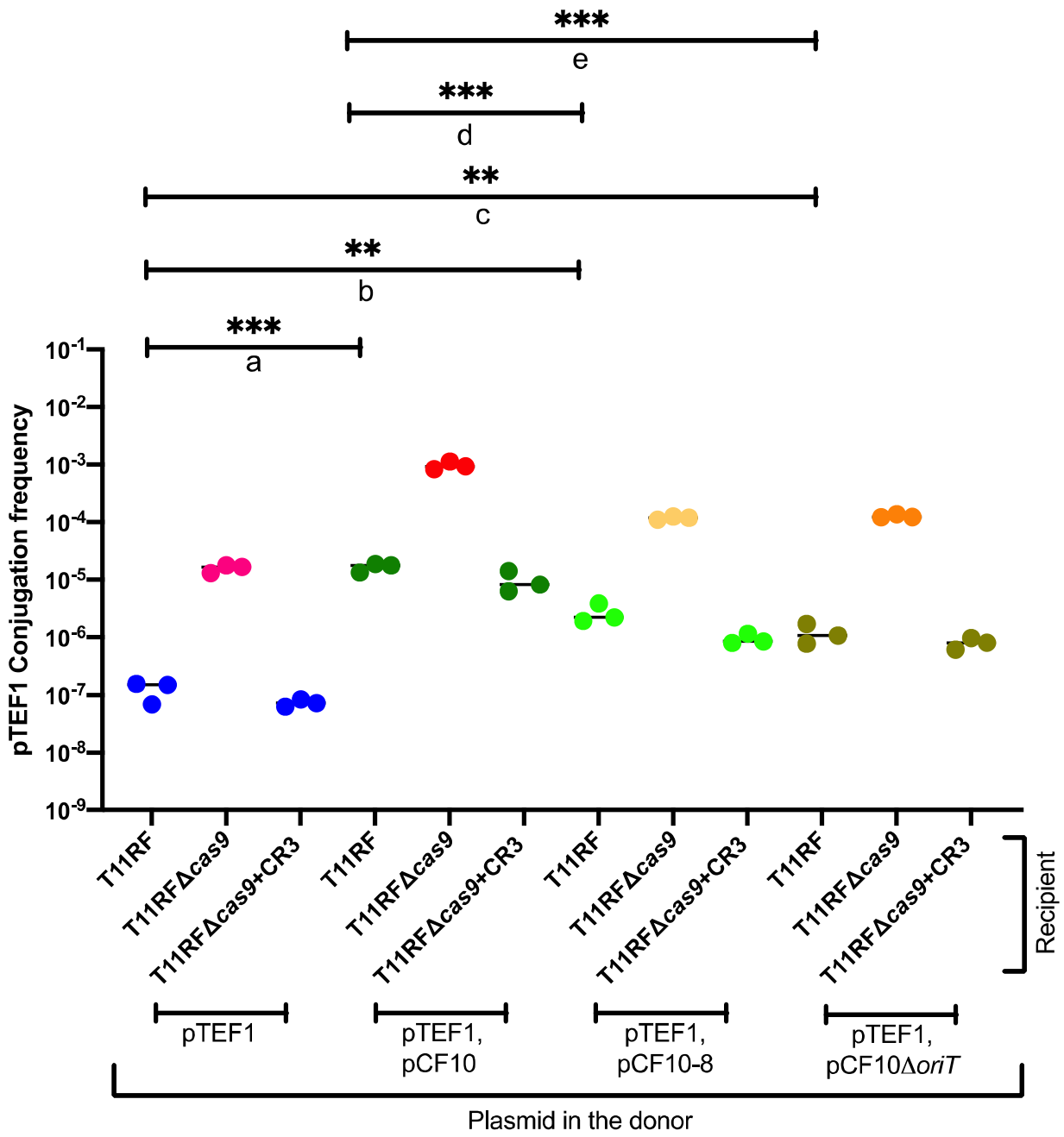

Figure 3: T11RF CRISPR3-Cas efficacy against pTEF1 plasmid in OG1SSp(pTEF1), OG1SSp(pTEF1,pCF10), OG1SSp(pTEF1,pCF10-8) and OG1SSp(pTEF1,pCF10Δ*oriT*) donor strains. Higher conjugation frequency was observed when the donor strain had both pTEF1 and pCF10 present, compared with only pTEF1 in T11RF recipient. T11RF CRISPR3-Cas can provide more sequence specific genome defense to same plasmid donor with one

targeted plasmid compared with two plasmids. pCF10 plasmid was able to increase pTEF1 conjugation frequency and help escaping T11RF CRISPR3-Cas defense like pTEF2 plasmid (a) (Unpaired student t test, ****P*-value = .0003). Higher conjugation frequency was observed when the donor strain had both pTEF1 and pCF10-8 or pTEF1 and pCF10Δ*oriT* present, compared with only pTEF1 in T11RF recipient. Even with the deletion of aggregation substance gene *prgB* or *oriT*, pCF10 plasmid can still help pTEF1 plasmid escaping T11RF CRISPR3-Cas defense (b,c) (Unpaired student t test, ***P*-value = 0.0066, ***P*-value = 0.0093). Conjugation frequency of pTEF1 was compared in the presence of pCF10 or pCF10-8 and pCF10 or pCF10Δ*oriT* plasmids. When the recipient is T11RF, a log fold decrease in conjugation frequency of pTEF1 was observed when in the donor pCF10-8 or pCF10Δ*oriT* present instead of pCF10. Aggregation substance gene *prgB* and *oriT* of pCF10 is important for increased conjugation frequency of pTEF1 (d,e) (Unpaired student t test, ****P*-value = 0.0007, ****P*-value = 0.0004).

Deletion of aggregation substance gene *prgB* from pCF10 decreases pTEF1 conjugation frequency from a multi-plasmid donor. When we observed that pCF10 was able to increase pTEF1 conjugation frequency and help escaping T11RF CRISPR3-Cas defense, we wanted to explore which region of pCF10 might be important for this plasmid cooperativity. The pCF10 gene *prgB* encodes the aggregation substance protein Asc10 and plays an important role during conjugative transfer of pCF10 via intracellular aggregation or clumping (16, 57, 58). From previous studies, we know that deletion of *prgB* contributes to decreased virulence and also decreased conjugation frequency of pCF10 in *E. faecalis* (57, 59, 60). Hence, we obtained a previously reported variant of pCF10 with the aggregation substance gene *prgB* deleted, referred to as pCF10-8 (57). We generated the donor strain OG1SSp(pTEF1, pCF10-8), and then used

OG1SSp(pTEF1) and OG1SSp(pTEF1, pCF10-8) donor strains in conjugation assays. CRISPR-Cas provided genome defense against pTEF1 from both donors, as higher pTEF1 conjugation frequency was observed in the absence of *cas9* in recipient (Figure S4-a, S4-c). When we compared the conjugation frequency of pTEF1 from the two donors to recipient T11RF (Figure 3-b) or T11RFΔ*cas9* (Figure S4-f), we still observed a log fold increase in conjugation frequency of pTEF1, when the donor strain had both pTEF1 and pCF10-8 present, compared with only pTEF1. This means that even with the deletion of aggregation substance gene *prgB*, pCF10-8 can still increase pTEF1 conjugation frequency and help pTEF1 escape T11RF CRISPR3-Cas defense. However, a log fold decrease in pTEF1 conjugation frequency was observed when pCF10-8 was present in the donor instead of pCF10 for T11RF (Figure 3-d) or T11RFΔ*cas9* (Figure S4-h) recipients. Thus, we concluded that the pCF10 aggregation substance gene *prgB* is important for the increased conjugation frequency of pTEF1 from a multi-plasmid donor, but it is not the only important region under the conditions tested.

Deletion of *oriT* from pCF10 decreases pTEF1 conjugation frequency from a multi-plasmid donor. The origin of transfer region, or *oriT*, is the region on plasmid DNA where conjugation initiates, hence playing an important role during conjugative transfer of plasmids (61). A previous study demonstrated that deletion of *oriT* from pCF10 significantly decreases its conjugation frequency (62). Therefore, we obtained an *oriT*-deleted mutant of pCF10, pCF10Δ*oriT* (62), and generated the donor strain OG1SSp (pTEF1, pCF10Δ*oriT*). We used OG1SSp (pTEF1) and OG1SSp (pTEF1, pCF10Δ*oriT*) donor strains in conjugation assays with the same recipients as before. CRISPR-Cas provided genome defense against pTEF1 from both donors, as higher pTEF1 conjugation frequency was observed in the absence of *cas9* in

recipients (Figure S4-a, S4-d). When we compared the conjugation frequency of pTEF1 in two donors to recipient T11RF (Figure 3-c) or T11RFΔ*cas9* (Figure S4-g), we still observed a log fold increase in conjugation frequency of pTEF1, when the donor strain had both pTEF1 and pCF10Δ*oriT* present, compared with only pTEF1. These results were similar to what we observed with pCF10-8 and indicate that even with the deletion *oriT*, pCF10Δ*oriT* can still increase pTEF1 conjugation frequency. However, similar to pCF10-8 results, a log fold decrease in pTEF1 conjugation frequency was observed when pCF10Δ*oriT* was present in the donor instead of pCF10 for T11RF (Figure 3-e) or T11RFΔ*cas9* (Figure S4-i) recipients. Thus, we concluded that, the *oriT* of pCF10 is also important for increased conjugation frequency of pTEF1 from a multi-plasmid donor. The potential additive effect of *prgB/oriT* double deletion was not investigated in our study but would be informative.

T11RF CRISPR3-Cas can target clinical isolates and provide defense against resistance plasmids harbored by those clinical isolates. We included 10 previously reported hospital fecal surveillance isolates (43) and 25 previously reported urine isolates (41) in this study (Supplemental Dataset S2). At first, we checked if spacers in the T11RF CRISPR3-Cas system could target the chromosomes or plasmids of these clinical isolates. We found that 6 of 10 fecal surveillance isolates (Table 1) and 17 of 25 urine isolates (Table 2) were targeted by the T11RF CRISPR3-Cas system as evidenced by 100% nucleotide sequence identity between spacer and target, and the presence of a suitable protospacer adjacent motif (previously defined in reference 34). The targeted plasmids and antibiotic resistance genes encoded by those plasmids are shown in Table 1 and Table 2. Moving forward, we will denote plasmids targeted by the T11RF CRISPR3-Cas system in bold text.

Fecal surveillance 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**
142-1 (**Chr**) (77kb, 59kb)	Spacer 14	***tetM***, *ermB*	rep9b, rep9c

Table 1: Selection of fecal surveillance isolates for conjugation assays. 6 of 10 clinical fecal surveillance isolates possessed plasmids or chromosomal sequence targeted by the T11RF CRISPR3-Cas system. Targeted plasmids or chromosome are shown in bold in column 1, T11RF CRISPR3-array spacer that target the plasmid or the chromosome are shown in column 2 and resistance gene encoded in the targeted plasmids or chromosome are shown in bold in column 3 (43). Rep family of the plasmids are shown in column 4 and the targeted plasmid rep family is indicated in bold. Strains used for conjugation experiments are highlighted in grey.

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

EfsC8 (**52kb**)	spacer 7	***ermB***	**rep9c**
EfsC9 (**94kb,** 44kb)	spacer 6,7		**rep9a,** rep9b
EfsC12 (**65kb**)	spacer 6		**rep9a**
EfsC13 (**80kb,** 70kb)	spacer 7		**rep9c,** rep9b
EfsC17 (**61kb**)	spacer 7		**rep9b**
EfsC23 (**53kb**)	spacer 6,7		**rep9a**
EfsC33 (**104kb,** 72kb)	spacer 6	***ermB***	**rep9a,** rep9b
EfsC49 (**Chr**) (79kb)	spacer 9	***tetM***	repA_N
EfsC61 (**57kb**)	spacer 7	***ant(6)-la***	**rep2**
EfsC94 (**Chr**) (78kb, 13kb, 9kb)	spacer 19	***lsa(A)***	repUS11, repUS41, rep11b
EfsC116 (72kb, **42kb,** 5kb)	spacer 6		rep9b, **rep9a**, rep6
EfsC130 (**86kb**)	spacer 6,7		**rep9a**
EfsPF15 (**65kb**)	spacer 6		**rep9a**
EfsPF20 (**53kb**)	spacer 6,7		**rep9a**
EfsPF23 (68kb, **52kb**)	spacer 6,7		rep9b, **rep9a**
EfsPF24-2 (74kb, **53kb**)	spacer 6,7		repUS11, **rep9a**,
EfsPF27 (**53kb**)	spacer 6,7		**rep9a**,

Table 2: Selection of urine isolates for conjugation experiments. 17 of 25 urine isolates possessed plasmids or chromosome targeted by the T11RF CRISPR3-Cas system. Targeted plasmids or chromosome are shown in bold in column 1, T11RF CRISPR3-array spacers that target the plasmid or the chromosome are shown in column 2 and resistance gene encoded in the targeted plasmids or chromosome are shown in bold in column 3. Rep family of the plasmids are

shown in column 4 and the targeted plasmid rep family is indicated in bold (41). Strains used for conjugation experiments are highlighted in grey.

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

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

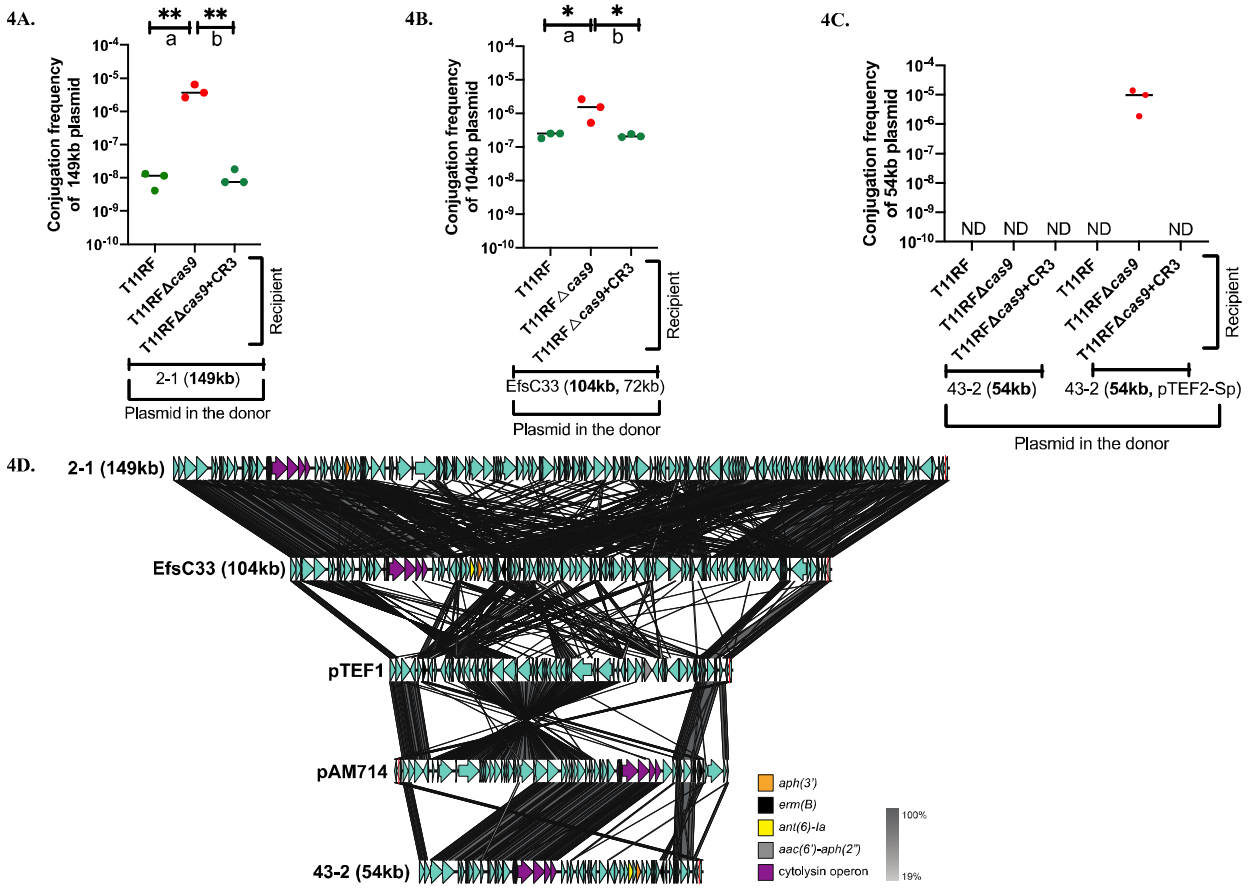

Figure 4: Clinical isolate plasmid donors targeted by T11RF CRISPR3-Cas spacer 6. T11RF CRISPR3-Cas decreased the rate of transfer of the **149 kb** resistance plasmid from fecal surveillance isolate 2-1 (4A) (unpaired student t test; ***P*-value=0.0099) and the **104 kb** plasmid from urine isolate EfsC33 (4B) (unpaired student t test; **P*-value=0.0458, **P*-value=0.0449). 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).

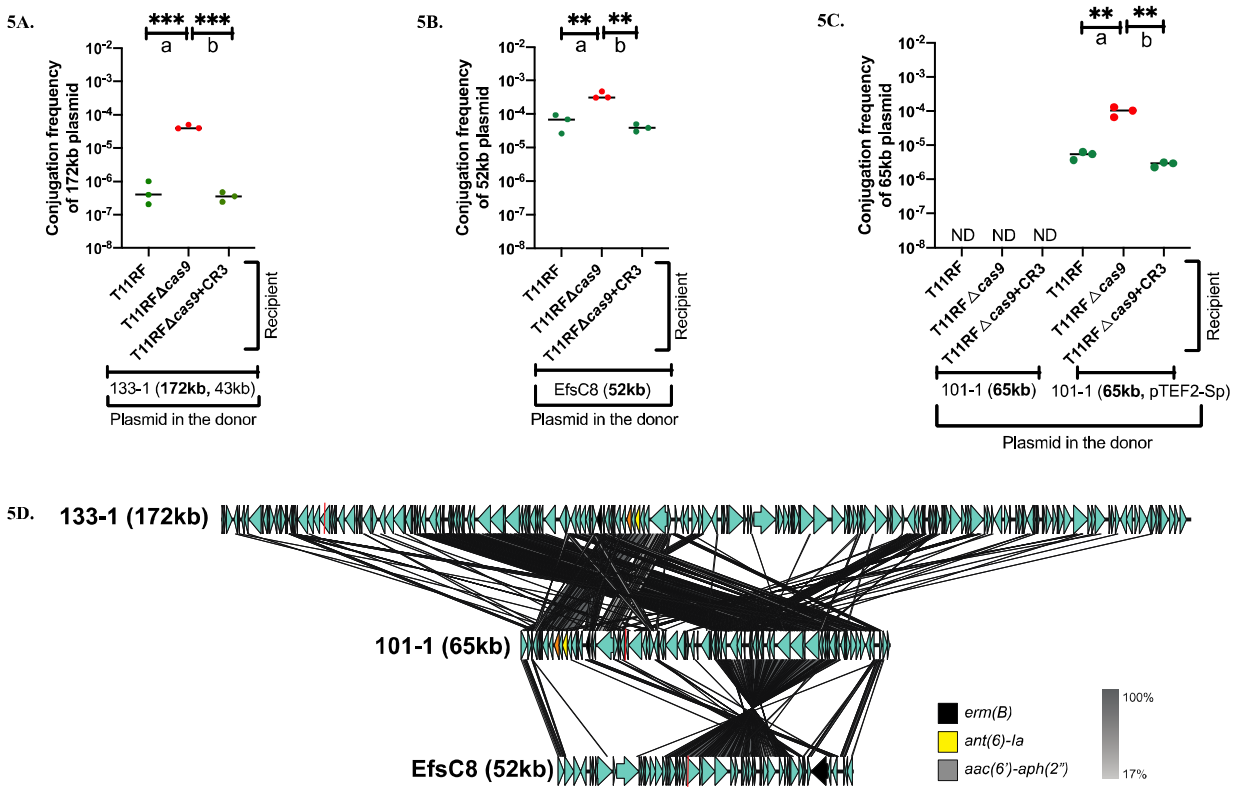

Figure 5: Clinical isolate plasmid donors targeted by T11RF CRISPR3-Cas spacer 7. T11RF CRISPR3-Cas was able to decrease the rate of transfer of the resistance plasmid **172kb** from clinical fecal surveillance isolate 133-1 (5A) (unpaired student t test; ****P*-value=0.0002) and **52kb** from clinical urine isolate EfsC8 (5B) (unpaired student t test; ***P*-value=0.0030, ***P*-value=0.0019). Conjugation was not detected for fecal isolate 101-1 with the recipients when present alone in the donor. pTEF2-Sp plasmid made the clinical resistant plasmid **65kb** from fecal isolate 101-1 mobilizable. However, T11RF CRISPR3-Cas was still able to decrease the rate of transfer of the resistance plasmid **65kb** as higher conjugation frequency was observed in the absence of *cas9* gene (5C) (unpaired student t test; ***P*-value=0.0035, ***P*-value=0.0032). tblastx alignment of plasmids targeted by T11RF CRISPR3 spacer 7 sequence is presented in

5D. Location of spacer 7 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 (%).

pTEF2 increases conjugation frequency of resistance plasmids from two fecal isolates and helps them escape T11RF CRISPR3-Cas defense. Conjugation was not detected for the fecal isolates 43-2 (**54kb**), and 101-1 (**65kb**) with T11RF (Figure 4C, Figure 5C). We investigated what effect pTEF2 would have on the transfer of these plasmids, given our results demonstrating that pTEF2 could assist pTEF1 with transfer and apparent evasion of CRISPR-Cas defense (Figure 2B). We made new donor strains with pTEF2-Sp (pTEF2 modified to encode spectinomycin resistance (63)). We used these new donor strains, 43-2 (**54kb**, pTEF2-Sp), and 101-1 (**65kb**, pTEF2-Sp) in conjugation assays with our previous recipients. We observed that pTEF2-Sp can make the clinical resistant plasmids mobilizable, as they conjugated to the recipients. However, T11RF CRISPR3-Cas still provided defense against the **54 kb** plasmid of the 43-2 strain (Figure 4C) and the **65 kb** plasmid of the 101-1 strain (Figure 5C), as in the absence of T11RF *cas9*, higher conjugation frequencies were observed. For the 43-2 donor strain, conjugation of the **54 kb** plasmid was detected only when *cas9* was deleted from the recipient strain. We did not perform the same experiment with pCF10, as pCF10 encodes tetracycline resistance, and 43-2 and 101-1 also encode tetracycline resistance in their chromosome. Therefore, we concluded that pTEF2 can increase conjugation frequency of other resistance plasmids and help them in escaping T11RF CRISPR3-Cas defense. Our findings underscore the importance of considering plasmid cooperativity when refining CRISPR-based

antimicrobial designs. Our work suggests that targeting pTEF2 may specifically be useful for mitigating antibiotic resistance plasmid spread.

***cas9* deletion alters *E. faecalis* biofilm structure on a plastic substrate.** Biofilms are important sites for HGT and are major contributors to *E. faecalis* virulence (44, 45). We sought to expand our studies of CRISPR-Cas defense by utilizing a different biofilm model to study plasmid transfer. Here, we cultured biofilms on plastic coverslips for 72 hours and observed the biofilm structure using confocal microscopy. For these experiments, we cultured only the recipient strains in monocultures, to determine whether deletion of *cas9* had an effect on biofilm formation. This was important to confirm, as deletion of *cas9* could have phenotypic effects beyond genome defense; for example, *cas9* contributes significantly to tissue invasion and meningitis virulence in Group B *Streptococcus* (64). Confocal microscopic analysis of the biofilms revealed that T11RF and T11RFΔ*cas9*+CR3 (Figure 6A, 6C) can form significantly thicker biofilms compared with T11RFΔ*cas9* (Figure 6B). However, there is also a significant difference between the wild type and complement strains, meaning that the biofilm formation defect from *cas9* deletion was only partially complemented (Figure 6D-c). Hence, we concluded that T11RF, T11RFΔ*cas9* and T11RFΔ*cas9*+CR3 have different biofilm structures when grown on plastic.

Regarding our agar plate biofilm experiments, we analyzed the recipient CFU/mL from the experiments with OG1-derived donors (Supplemental Dataset S3) to test whether total recipient yield was affected by *cas9* deletion. We observed no significant difference among the recipients

number for the experiments (Figure 6E). Note, we excluded all clinical isolate donors from this analysis because some encode bacteriocins that killed recipients (Supplemental Dataset S3, see isolate 2-1 as an example), as we have previously noted (38, 43). We conclude that total recipient number in agar plate biofilms is not affected by *cas9*; however, we cannot exclude the possibility that the agar plate biofilms have different overall structure when *cas9* is deleted. This difference in biofilm structure may have an effect on CRISPR-Cas efficacy, but this would need further investigation.

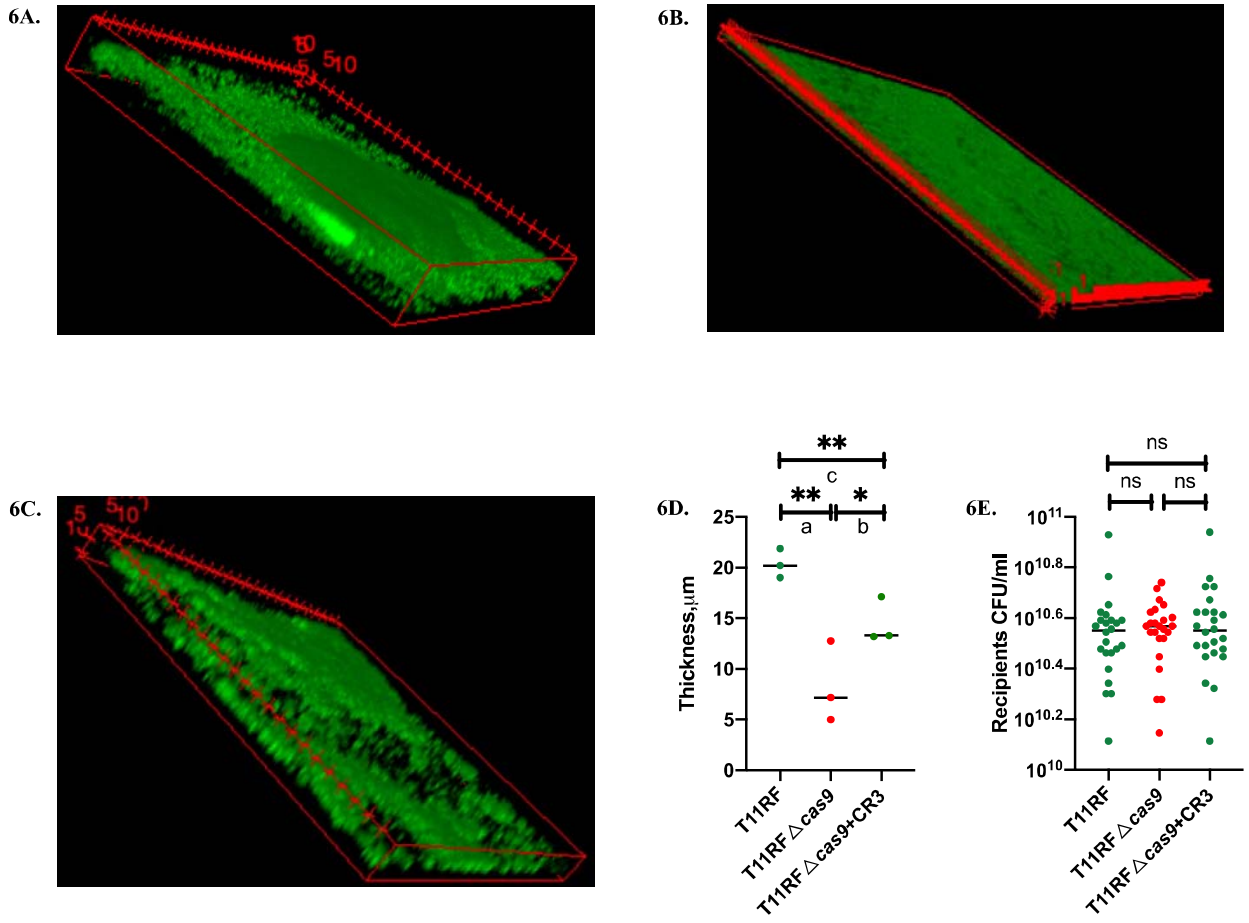

Figure 6: T11RF, T11RFΔ*cas9* and T11RFΔ*cas9*+CR3 have different biofilm structure. 72h old biofilm of T11RF, T11RFΔ*cas9* and T11RFΔ*cas9*+CR3 strains were stained and analyzed via confocal microscopy. (A) Representative biofilm image of T11RF is showing and the thickness is 20.19 μm. (B) Representative biofilm image of T11RFΔ*cas9* is showing and the thickness is 5 μm. (C) Representative biofilm image of T11RFΔ*cas9*+CR3 is showing, and the thickness is 13.19 μm. (D) Statistical analysis showing T11RF and T11RFΔ*cas9*+CR3 can form thicker biofilm compared with T11RFΔ*cas9* (a,b). T11RFΔ*cas9*+CR3 can partially complement the phenotype (c) (unpaired student t test; ***P*-value=0.0040, **P*-value=0.0390, ** *P*-value=0.0097).

(E) CFU/mL of the recipients from agar plate biofilm assay are shown. There is no significant difference among the recipient numbers (unpaired student t test; ns *P*-value=0.4578, ns *P*-value=0.2957, ns *P*-value=0.2899).

DISCUSSION

In this era of antibiotic resistance, *E. faecalis* is considered a serious threat as it can acquire a large number of resistance genes via HGT (2, 9). Bacterial CRISPR-Cas systems may be leveraged as an alternative treatment approach, providing sequence-specific cleavage of antibiotic resistance plasmids in *E. faecalis* (34, 38). Most available studies of CRISPR-Cas efficacy have used laboratory model strains and laboratory model plasmids. However, clinical isolates can behave differently than the laboratory model strains, and CRISPR-Cas efficacy can be affected by many factors (43). Therefore, for this study we included both laboratory model strains and clinical isolates to study previously unexplored factors that might affect CRISPR-Cas

efficacy. Our hypothesis was that the number of plasmids present in a donor strain is an important factor that might affect CRISPR-Cas efficacy. Through our experiments we showed our hypothesis is supported and found two plasmid pairs (i)pTEF1,pTEF2 and (ii) pTEF1, pCF10 that showed plasmid cooperativity. These plasmid pairs worked together to escape T11RF CRISPR3-Cas defense. From our experiments we saw pTEF2 or pCF10 were able to increase pTEF1 conjugation frequency and together they were able to evade CRISPR3-Cas defense. We identified two genes (i) aggregation substance gene *prgB* and (ii) *oriT* of pCF10 plasmid potentially important for the plasmid cooperativity as deletion of these genes resulted in decreased conjugation frequency of pTEF1.

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

cooperativity results indicate that the first conjugative resistance plasmid lacks something essential for robust transfer, to which they are getting "help" from the second plasmid, and together, they escape T11RF CRISPR3-Cas defense by dint of higher conjugation frequency. A limitation of our experimental design is, we did not pinpoint the exact mechanism of this plasmid cooperativity. It could be the helper plasmid enhancing the transfer frequency of the first plasmid, the helper plasmid inhibiting the CRISPR-Cas defense against the first plasmid, or both.

Overall, we conclude that the presence of multiple interacting plasmids in donor strains and the lack of an active CRISPR-Cas system in a recipient strain act additively to confer extremely high plasmid transfer frequencies in *E. faecalis*. To date, a few multi-plasmid studies have shown the effect of an ecological setting on the maintenance of individual plasmids in a multi-plasmid community or how a conjugative helper plasmid aids a nonconjugative virulence plasmid to become mobilized (65, 66). Hence, our study opens a new direction of plasmid cooperativity and contributes towards designing novel CRISPR antimicrobials.

Apart from the plasmid number present in donor strains, there may be other factors affecting CRISPR-Cas efficacy, such as biofilm formation capacity or internal CRISPR-Cas regulation in the recipient strains. For example, transcriptional regulation of *cas9* was reported as a major factor which can influence CRISPR-Cas genome defense in *S. pyogenes* (67). In this study, we established a protocol to visualize bacterial biofilm structure using confocal microscope and studied the biofilm structure of our recipients. We found that T11RF and T11RFΔ*cas9*+CR3 can form thicker biofilm compared with T11RFΔ*cas9*. However, whether this difference in biofilm

structure also occurs in agar plate assays where the donors and recipients are mixed prior to plating, and/or whether this difference in biofilm structure has any effect on CRISPR-Cas efficacy, need further exploration.

Overall, our research is significant in its application towards designing improved CRISPR-based antimicrobials as an alternative approach to combat HGT and antibiotic resistance. Our research also emphasizes to study factors affecting CRISPR-Cas efficacy and conducting research using clinical isolates. The more information we gather on the efficacy of CRISPR-Cas, the more effectively we can harness it as an alternative treatment approach.

MATERIALS AND METHODS

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

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

Selection of fecal surveillance isolates and urine isolates. 10 fecal surveillance isolates (43), and 25 urine isolates (41) were analyzed for this study (Supplemental Dataset S2). First, a database was created in Geneious with the previously reported genome sequences of the 10 fecal surveillance isolates or 25 urine isolates. All CRISPR3 spacer sequences from T11RF were queried against the database using BLASTn. 6 of 10 fecal surveillance isolates (Table 1) and 17 of 25 urine isolates (Table 2) were identified with T11RF CRISPR3-Cas targets in either plasmids or in the chromosome with 100% nucleotide sequence identity. The presence of the expected protospacer adjacent motif sequence (34) adjacent to the target sequence was also confirmed.

Generation of 43-2 (54kb, pTEF2-Sp) and 101-1 (65kb, pTEF2-Sp) donor strains and conjugation assays. *E. faecalis* V19 (pTEF2-Sp) (63) was used as donor and fecal isolates 43-2 (**54kb**), 101-1 (**65kb**) were used as recipients in conjugation assays to generate 43-2 (**54kb**, pTEF2-Sp) and 101-1 (**65kb**, pTEF2-Sp) donor strains. PCR was performed to confirm the plasmids present in the transconjugant. Once confirmed, 43-2 (**54kb**, pTEF2-Sp) and 101-1

(**65kb**, pTEF2-Sp)**,** were used as donors in conjugation assays using T11RF, T11RFΔ*cas9* and T11RFΔ*cas9*+CR3 as recipients. Conjugation assays were done in biological triplicate using the previously described method (34). Antibiotic selection plates were prepared according to the resistance gene present in the donor and recipients (Table S1). All the CFU/mL calculation and data analysis were done as described above.

Plasmid alignment and analysis. Plasmid tblastx alignments were performed using EasyFig 3.0.0 at default parameters. Antimicrobial resistance genes were identified using ABRicate v1.0.1 querying the ResFinder database at default parameters. pTEF2-specific genes (in comparison to pCF10) were analyzed using NCBI Conserved Domains (68), PSORTb 3.0.3 (69), and InterPro (70). Plasmid replicon types were predicted using PlasmidFinder v2.1 at default parameters. Where plasmid replicon type was not identified using PlasmidFinder, plasmids were analyzed using NCBI Conserved Domains to identify the *rep* gene. Further typing was completed using blastn and PlasmidFinder queries at 70% identity threshold (41).

Biofilm assay. *E. faecalis* was struck on BHI agar and incubated at 37^0 C. Next day, a single colony was picked and resuspended in 1 mL BHI broth. A plastic cover slip (Fisher Scientific) was sterilized with 100% ethanol in a biosafety cabinet. Sterilized cover slips were placed in each well of a six well culture plate (sterilized, nontreated) (VWR). 4 mL BHI was then added to each well and 1 mL prepared culture was mixed to each. The final volume in each well was 5 mL. The six well plate was covered and incubated statically for 72 h at 37^0 C. At 24 h and 48 h timepoints, the medium was removed very carefully by pipetting without disturbing the biofilm

growing at the bottom of the well, and 5 mL fresh BHI was added. After 72 h, the medium was removed from each well, and the biofilm was washed twice with 1X PBS (Thermo Scientific). 1 mL 5% paraformaldehyde (PFA; diluted from 16% PFA, Fisher Scientific) was added to each well to cover the coverslip and incubated 1 hr at room temperature. Then, the PFA was removed, and each well was washed with 1X PBS twice. 1 mL prepared SYTO9 (Thermo Fisher Scientific) was added to each well. To prepare the SYTO9 for staining, 1 µl of 5 mM SYTO9 was mixed in 1 mL 1X PBS. After adding the SYTO9, the plate was incubated for 30 min at room temperature. Then the stain was removed from each well and washed with 1X PBS twice. All the washing was done very carefully without disturbing the biofilm. A slide was taken and a drop of antifade solution (Invitrogen) was added. Using a tweezer, the coverslip was removed from the well and placed upside down on the antifade solution. Any air bubble was removed by pressing with tweezer. The slide was air dried and imaged using confocal microscope (Zeiss LSM 880). If the imaging was done later, the prepared slides were stored at 4^0 C. Z-stack was captured from 4 different spots for each sample, and the thickness of the biofilm was considered as the average of range from z-stack. The images were analyzed using Fiji software (version 2.9.0/1.54f). Biofilm formation assays were performed in biological triplicate.

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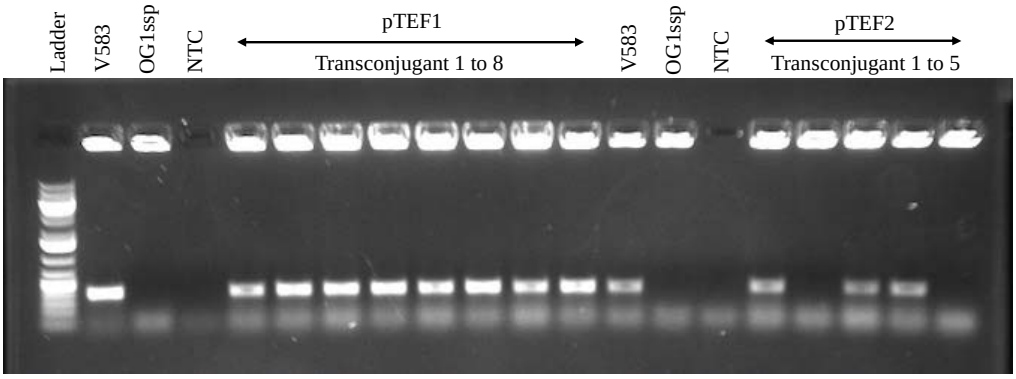
SUPPLEMENTAL MATERIALS

A.



B.

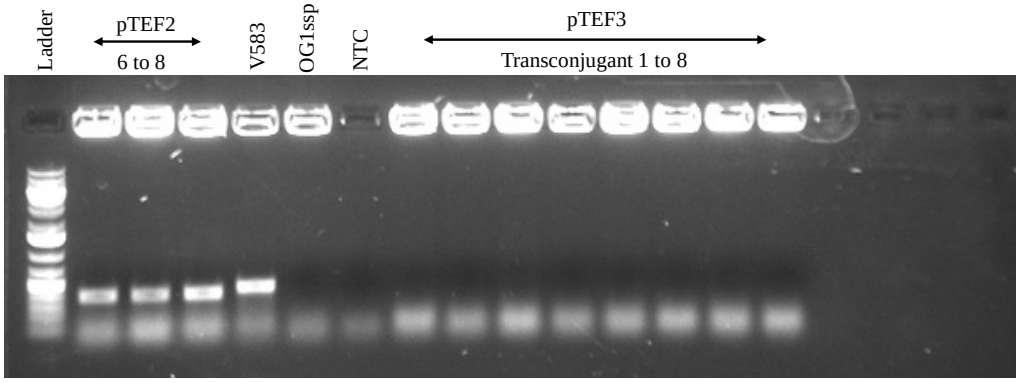
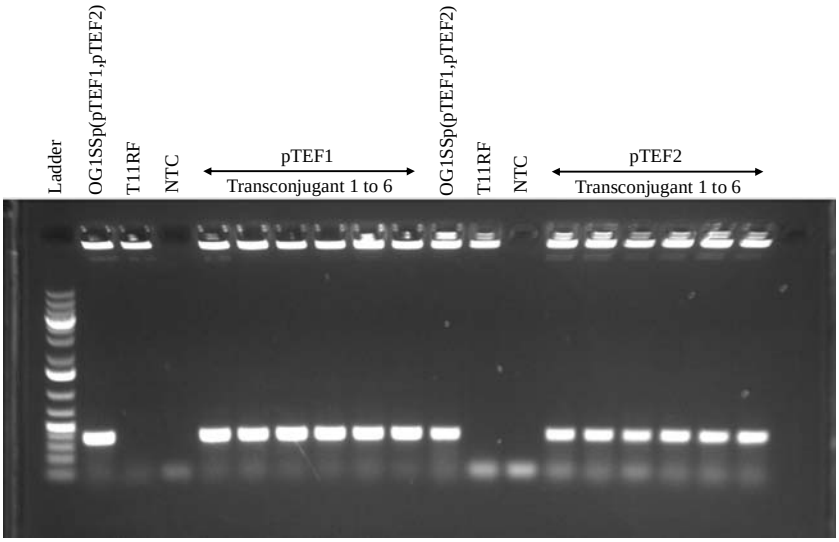


Figure S1: Identification of plasmid pTEF1, pTEF2 and pTEF3 by PCR. V583 was used as positive control and gel image showing it contains all the three plasmids. OG1SSp used as negative control and gel image showing it contains none of the plasmid. No template control (NTC) was also added. Designed primers for pTEF1, pTEF2 and pTEF3 were used to screen the transconjugants. Gel image showing all the 8 selected transconjugants contain pTEF1 plasmid (A); 6 transconjugants contain pTEF2 plasmid (A,B) and none of the transconjugants contain pTEF3 plasmid (B).

A.



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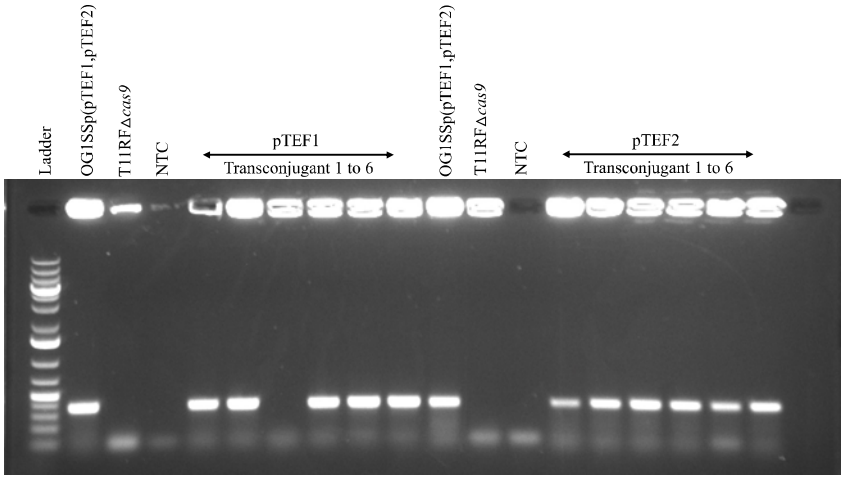

Figure S2: Identification of plasmid pTEF1 and pTEF2 in transconjugants by PCR. OG1SSp was used as positive control and gel image showing it contains both pTEF1 and pTEF2 plasmids in all cases. T11RF and T11RFΔ*cas9* were used as negative control and gel image showing they contain none of the plasmids. No template control (NTC) was also added.

Designed primers for pTEF1 and pTEF2 were used to screen the transconjugants. Gel image showing all the 6 selected transconjugants contain pTEF1 and pTEF2 plasmids when T11RF was recipient (A). 5 transconjugants contain pTEF1 plasmid and all 6 transconjugants contain pTEF2 plasmid when T11RFΔ*cas9* was recipient (B).

A. **B.**

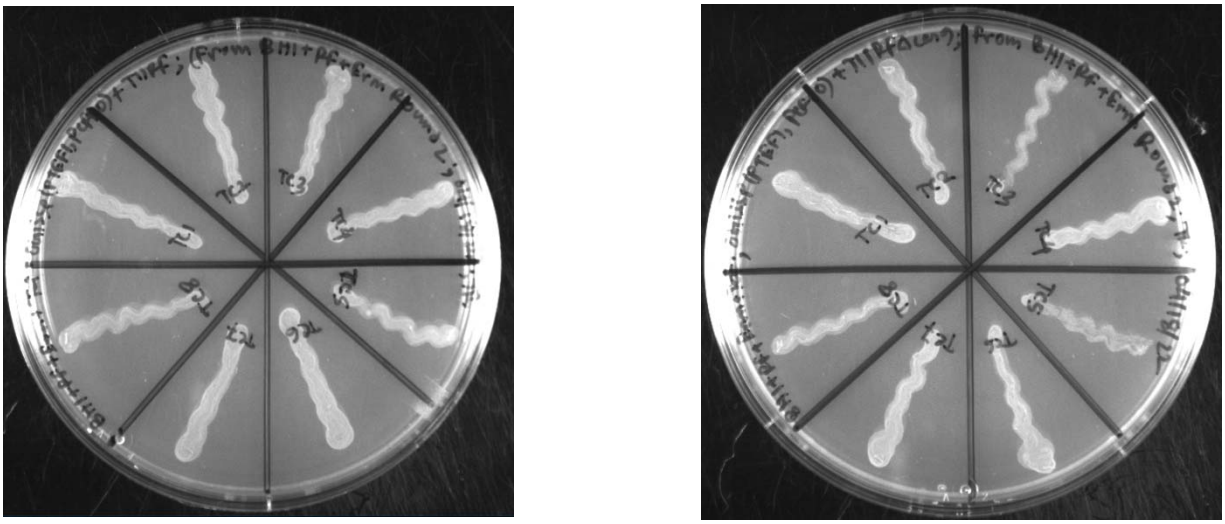

Figure S3: Identification of plasmid pTEF1 and pCF10 in transconjugants. 8 transconjugants were picked from transconjugant plates where OG1SSp (pTEF1,pCF10) was donor and either T11RF (A) or T11RFΔ*cas9* (B) were recipients. In the first transconjugant plate only pTEF1 was selected by erythromycin antibiotic.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

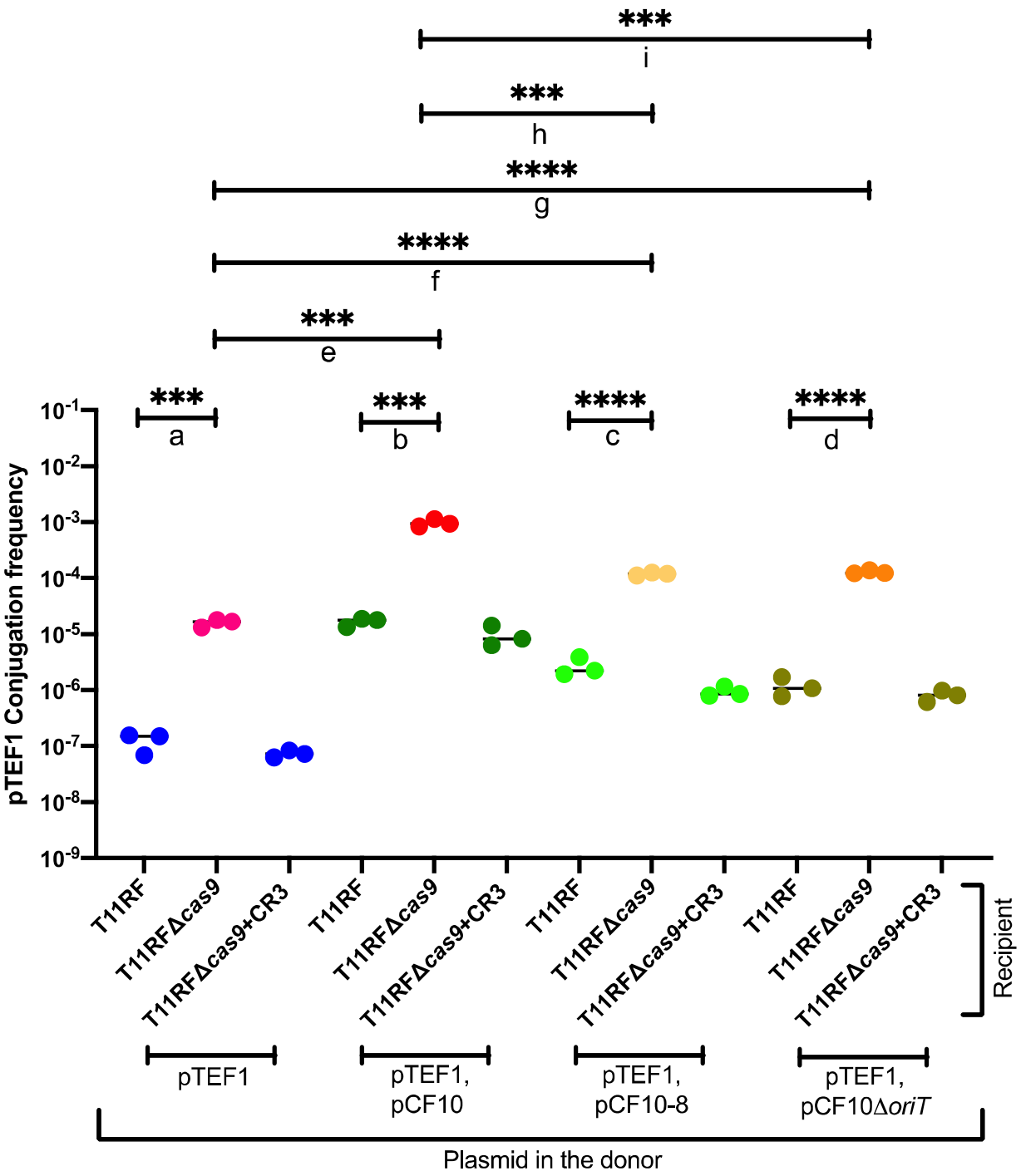

Figure S4: T11RF CRISPR3-Cas efficacy against pTEF1 plasmid in OG1SSp (pTEF1), OG1SSp (pTEF1,pCF10), OG1SSp (pTEF1,pCF10-8) and OG1SSp (pTEF1,pCF10Δ*oriT*) donor strains. T11RF CRISPR3-Cas was able to provide sequence specific genome defense

against pTEF1 in all donors as higher conjugation frequency was observed in the absence of *cas9* in recipients (a,b,c,d) (Unpaired student t test, ***P-value = 0.0002, ****P-value <0.0001). Higher conjugation frequency was observed when the donor strain had two plasmids pTEF1 and pCF10 (e) or pTEF1 and pCF10-8 (f) or pTEF1 and pCF10Δ*oriT* (g) present, compared with only pTEF1 in T11RFΔ*cas9* recipient (Unpaired student t test, ***P-value = .0002, ****P-value <0.0001). Conjugation frequency of pTEF1 was compared in the presence of pCF10 or pCF10-8 and pCF10 or pCF10Δ*oriT* plasmids. When the recipient is T11RFΔ*cas9*, a log fold decrease in conjugation frequency of pTEF1 was observed when in the donor pCF10-8 (h) or pCF10Δ*oriT* (i) present instead of pCF10. Aggregation substance gene *prgB* and *oriT* of pCF10 is important for increased conjugation frequency of pTEF1 (Unpaired student t test, ***P-value = 0.0003).

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Strain	Description	Reference(s)
V583	Vancomycin-resistant *E. faecalis* clinical isolate harboring pTEF1, pTEF2 and pTEF3 plasmids	(2, 55)
OG1SSp	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring no plasmids	(16, 54)
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)
T11RF∆*cas9*	T11RF CRISPR3-*cas9* deletion mutant	(34)
T11RF∆*cas9*+CR3	T11RF∆*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, 71, 72)
OG1SSp (pAM771)	Spectinomycin- and streptomycin-resistant derivative of strain OG1 harboring pAM771 plasmid, conferring erythromycin resistance via Tn*917* insertion disrupting *cylL* of the cytolysin operon	(38, 73–75)
OG1SSp (pAM771,pCF10)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pAM771 and pCF10	This study
OG1SSp (pTEF1,pCF10)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pTEF1 and pCF10	This study
CK104 (pCF10-8)	OG1RF∆*upp* strain harboring pCF10-8 plasmid (*prgB* deletion mutant retaining first three and last three codons, Asc10-)	(57, 76)
OG1SSp (pTEF1,pCF10-8)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pTEF1 and pCF10-8 plasmids	This study
CK104 (pCF10∆*oriT*)	OG1RF∆*upp* strain harboring pCF10∆*oriT* plasmid.	(62)
OG1SSp (pTEF1, pCF10∆*oriT*)	Spectinomycin- and streptomycin-resistant derivative of OG1 *E. faecalis* strain harboring pTEF1 and pCF10∆*oriT*	This study
59 (43kb, 88kb)	Fecal surveillance *E. faecalis* isolate 59 harboring vancomycin resistant 43 kb plasmid and erythromycin	(43)

	resistant 83 kb plasmid	
43-2 (54kb)	Fecal surveillance *E. faecalis* isolate 43_2 harboring erythromycin resistant 54kb plasmid	(43)
2-1 (149kb)	Fecal surveillance *E. faecalis* isolate 2_1 harboring erythromycin resistant 149kb plasmid	(43)
133-1 (172kb)	Fecal surveillance *E. faecalis* isolate 133_1 harboring erythromycin resistant 172kb plasmid	(43)
101-1 (65kb)	Fecal surveillance *E. faecalis* isolate 101_1 harboring erythromycin resistant 65kb plasmid	(43)
142-1 (59kb,77kb)	Fecal surveillance *E. faecalis* isolate 142_1 harboring erythromycin resistant 59kb plasmid and 77kb plasmid	(43)
V19 (pTEF2-Sp)	Plasmid-free derivative of V583 Em^S, Gm^S harboring Spectinomycin resistant pTEF2 plasmid	(63)
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 C8UK3 harboring erythromycin resistant 52kb plasmid	(41)
EfsC33 (72kb, 104kb)	*E. faecalis* urine isolate C33 harboring erythromycin resistant 104kb plasmid and 72kb plasmid	(41)
EfsC61 (57kb)	*E. faecalis* urine isolate C61 harboring streptomycin resistant 57kb plasmid	(41)

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

Primer name	Description	Sequence	Reference
FpTEF1	Forward primer for pTEF1 and pAM771	ACAAACGCTGAATCCCTTGG	This study
RpTEF1	Reverse primer for pTEF1 and pAM771	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

Table S2: Primers used in this study.