CRISPR-Cas blocks antibiotic resistance plasmid transfer between *Enterococcus faecalis* strains in the gastrointestinal tract

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Running head: Impact of CRISPR-Cas on plasmid transfer \textit{in vivo}

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Abstract

The emergence of antibiotic resistant bacteria is a major public health issue. Antibiotic-resistant bacteria can emerge via the horizontal acquisition of antibiotic resistance genes. This process is especially relevant in the emergence of antibiotic-resistant enterococci, which are among the leading causes of nosocomial infections in the United States. *Enterococcus faecalis* is host to a class of plasmids, the pheromone-responsive plasmids (PRPs), that mediate inter- and intraspecies transfer of antibiotic resistance genes and other virulence traits. Interestingly, hospital-associated *E. faecalis* generally lack complete CRISPR-Cas genome defense systems, which reduce the acquisition frequency of PRPs in commensal isolates. The absence of CRISPR-Cas likely contributes to the emergence of multidrug-resistant *E. faecalis*. Here, we assessed the impacts of *in vitro* growth conditions (planktonic versus biofilm), production of a PRP-encoded bacteriocin, and *in vivo* mouse gastrointestinal colonization on CRISPR-Cas efficacy. We found that bacteriocin production significantly impacts PRP transfer efficiency and CRISPR-Cas efficacy under *in vitro* but not *in vivo* conditions. Strikingly, we observed that CRISPR-Cas completely prevented PRP acquisition in 85% of mice, with or without a PRP-encoded bacteriocin, a much more pronounced effect than that observed *in vitro*. Our data suggest that the *in vitro* and *in vivo* activities of CRISPR-Cas in *E. faecalis* are differentially regulated. These results demonstrate that native CRISPR-Cas in *E. faecalis* confers robust *in vivo* defense against antibiotic resistance plasmids. Our results are significant because they demonstrate that CRISPR-Cas has a profound effect on the dissemination of antibiotic resistance genes in the gastrointestinal tract.
Importance

The emergence of antibiotic resistant microorganisms is a threat to public health. Horizontal transfer of antibiotic resistance genes contributes to the emergence of multidrug resistant bacteria capable of causing untreatable infections. Some bacteria encode a barrier to horizontal gene transfer, referred to as CRISPR-Cas. We demonstrate that the CRISPR-Cas genome defense system of Enterococcus faecalis blocks the acquisition of a horizontally transferred antibiotic resistance determinant in the murine gastrointestinal tract. This indicates that barriers to horizontal gene transfer encoded by the normal human microbiota have significant impacts on the dissemination of antibiotic resistance. Moreover, our data suggest that, during antibiotic therapy, E. faecalis strains possessing CRISPR-Cas are penalized and depleted from the GI microbiota, resulting in outgrowth of populations lacking genome defense.
Introduction

Enterococcus faecalis is a gram-positive bacterium and native inhabitant of the gastrointestinal tracts (GI) of humans and other animals (1). E. faecalis is also an opportunistic pathogen that is among the leading causes of hospital-acquired infections in the United States (2-4). Hospital-associated E. faecalis strains are often multidrug-resistant (MDR) and can encode resistance to vancomycin, leaving few treatment options (3, 5).

E. faecalis strains acquire novel genetic traits, including antibiotic resistance, via horizontal gene transfer (HGT) (6-8). A common method of HGT in E. faecalis is mediated by the narrow host range pheromone-responsive plasmids (PRPs). The PRPs are a unique class of plasmids that are highly co-evolved with E. faecalis (9, 10). PRPs are large (~60 kb) and conjugate efficiently, resulting in transfer frequencies reaching 1 transconjugant for every 10-100 donor cells (11). PRPs are often identified in the genomes of clinical isolates of E. faecalis where they provide accessory antibiotic resistance genes (8, 12-16). In addition to antibiotic resistance, some PRPs encode bacteriocins, which are a class of antimicrobials produced by E. faecalis that can confer a competitive advantage in polymicrobial environments (17). The model PRP, pAD1, encodes a bacteriocin called cytolysin (18). Cytolysin is a lantibiotic-like antimicrobial peptide with activity against a number of gram-positive bacteria and mammalian cells (19, 20). The bactericidal and hemolytic properties of cytolysin enhance the virulence of E. faecalis in some infections (21-24).
Bacteria can employ genome defense strategies to prevent the acquisition of plasmids (25). Adaptive immunity conferred by Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR associated proteins (CRISPR-Cas) is one strategy by which bacteria protect themselves from mobile genetic elements (MGEs). CRISPR-Cas systems contain short segments of spacer DNA sequences that serve as a molecular memory of past exposure to MGEs (26, 27). Spacers are transcribed into RNA molecules that, when bound to an effector nuclease, identify complementary foreign DNA and target it for cleavage (28-31). Interestingly, there is a strong correlation between the absence of CRISPR-Cas systems and the presence of multidrug resistance traits in hospital-adapted *E. faecalis* (32). This, in association with the MGE-rich nature of multidrug-resistant strains, led to the hypothesis that MDR *E. faecalis* emerge due to the absence of barriers to HGT (32).

*E. faecalis* possesses type II CRISPR-Cas systems (CRISPR1-Cas and CRISPR3-Cas) (6, 32), which are defined by the presence of the type-specific gene, *cas9* (26). *E. faecalis* also has an orphan locus, CRISPR2, that is not associated with *cas* genes. CRISPR2 has been identified in all *E. faecalis* isolates, whereas CRISPR1-Cas and CRISPR3-Cas have variable distribution among the species (33). Interestingly, CRISPR2 is usually the only CRISPR locus present in hospital-adapted strains of *E. faecalis* (32, 33). We recently demonstrated that both CRISPR1-Cas and CRISPR3-Cas are active for genome defense *in vitro*, and that the orphan CRISPR2 locus confers genome defense only in the presence of CRISPR1 Cas9 (34, 35). In one of these studies, CRISPR3-Cas was shown to interfere with the acquisition of pAM714, a
derivative of pAD1 that confers erythromycin resistance (36, 37). Specifically, in *E. faecalis* T11RF, a prototypical non-MDR commensal *E. faecalis* strain which possesses a CRISPR3 spacer with perfect complementarity to pAM714, pAM714 conjugation frequency in biofilms was significantly reduced, compared to a *cas9* deletion mutant (35).

Little is known about how different environmental conditions affect CRISPR-Cas activity. To further investigate the effects of CRISPR-Cas defense on PRP transfer, we monitored the transfer kinetics of bacteriocin (cytolysin)-positive and -negative derivatives of pAD1 over time in both planktonic and biofilm matings. We determined that these factors significantly affect the overall efficacy of CRISPR-Cas defense. Because *E. faecalis* are native gastrointestinal colonizers and reservoirs for antibiotic resistance in the human microbiota, we also assessed CRISPR-Cas anti-plasmid activity in a mouse model of gastrointestinal colonization. We discovered that CRISPR-Cas is a strikingly robust barrier to plasmid acquisition *in vivo*, with a magnitude of effect much more pronounced than that observed for any *in vitro* condition tested. These results illustrate the role of the native microbiota (in this case, commensal, non-MDR *E. faecalis* natively possessing CRISPR-Cas) in preventing the horizontal transmission of antibiotic resistance determinants.

**Results**

**Conjugative transfer of pAM714 and impact of CRISPR-Cas in planktonic and biofilm matings.** Spacer 6 of the CRISPR3-Cas locus of *E. faecalis* T11RF has 100%
sequence identity with the repB gene of pAM714, and acquisition of pAM714 is increased by ~80-fold in E. faecalis T11RFΔcas9 after 18 hours of biofilm mating on an agar surface (35). To gain insight into the kinetics of plasmid transfer, and whether or not culture condition impacts CRISPR-Cas defense, we sampled planktonic and biofilm (agar spread plate) mating reactions over an 18-hour period (Figure 1). All mating reactions were inoculated at a donor to recipient ratio of 1:9. The donor strain used in these experiments was E. faecalis OG1SSp(pAM714). The recipient strains were T11RF and a T11RF derivative with an in-frame deletion of cas9, T11RFΔcas9 (see Table 1 for a list of strains).

The number of pAM714 transconjugants (i.e., the number of T11RF or T11RFΔcas9 cells that acquired pAM714) was used to compare the kinetics of conjugative plasmid transfer in the presence and absence of CRISPR-Cas defense. After only 30 minutes of mating, we observed ~$10^3$-$10^4$ transconjugants for both recipient strains (T11RF and Δcas9) under both planktonic and biofilm conditions (Figure 1A and 1B). Under planktonic mating conditions, the number of T11Δcas9(pAM714) transconjugants remained stable over all time points while the number of T11RF(pAM714) transconjugants decreased (Figure 1A). This suggests that CRISPR-Cas in T11RF is actively targeting pAM714 in planktonic conjugation by preventing plasmid acquisition and/or by curing T11RF transconjugants of pAM714 (38). The number of transconjugants produced in biofilm matings were comparatively higher (Figure 1B). Both recipient strains achieved transconjugant yields similar to those previously reported for an 18-hour conjugation reaction in a biofilm (35). CRISPR-Cas had a
significant impact on transconjugant yield in only a subset of time points, with the most pronounced effect observed at 18 hours (Figure 1B).

**Impact of cytolysin on CRISPR-Cas efficacy.** PRPs can encode bacteriocins that promote their maintenance in polymicrobial communities (17, 39). pAM714 encodes a bacteriocin, cytolysin, that is bactericidal against gram-positive bacteria including *E. faecalis* (20). The operon encoding cytolysin also encodes a membrane-associated immunity factor that protects the cytolysin-producing cell from the activity of cytolysin (40). Therefore, pAM714-containing cells (and by extension, newly generated transconjugants) are immune to the bacteriocin. To determine if cytolysin production impacts CRISPR-Cas anti-plasmid defense, we utilized another derivative of pAD1, pAM771, which is cytolysin-negative (41, 42). Under planktonic conditions using OG1SSp(pAM771) as a plasmid donor, we obtained a similar number of T11RF(pAM771) and T11RFΔcas9(pAM771) transconjugants across all time points (Figure 1C). Moreover, we observed a statistically significant impact of CRISPR-Cas on pAM771 acquisition at only one time point, and the magnitude of the effect was small. These results are in contrast to those observed for pAM714 planktonic matings (Figure 1A). These results suggest that cytolysin production influences CRISPR-Cas efficacy at a population level under planktonic mating conditions. In pAM771 biofilm matings, we observed an overall increase in transconjugant numbers by at least one log for all time points and for both wild-type and Δcas9 recipient strains (Figure 1D), as compared to pAM714 (Figure 1B). However, the impact of CRISPR-Cas was similar and did not appear to be influenced by cytolysin production.
Recipient strain densities are substantially impacted by cytolysin in planktonic mating reactions. Under planktonic mating conditions, we observed a decrease in pAM714 transconjugants over time (Figure 1A) and a difference in the overall number of T11RF(pAM714) transconjugants compared to T11RF(pAM771) transconjugants after 5 and 18 hours of conjugation (Figure 1A and 1C). We hypothesized that pAM714-encoded cytolysin impacts plasmid transfer kinetics under planktonic mating conditions.

Cells possessing pAM714 (i.e., donor strains) are protected from the action of cytolysin through production of an immunity factor (40). T11RF and T11RFΔcas9 recipients do not encode the immunity factor and are vulnerable to the activity of cytolysin. We analyzed total T11RF or T11RFΔcas9 densities in our conjugation reactions (the same reactions as shown in Figure 1). In planktonic mating reactions, T11RF density remained high (~10⁸ CFU/mL) and stable when the donor strain harbored pAM771 (Figure 2A). However, in the presence of pAM714 donors, there was a linear decrease in T11RF density that initiated after only 30 minutes of mating (Figure 2A). A similar trend was also observed for the T11RFΔcas9 recipient strain (Figure 2B). We conclude that under planktonic mating conditions, cytolysin production from pAM714 continuously depletes the recipient cell population, and this is independent of CRISPR-Cas.

We performed the same analysis for biofilm matings and found that the degree of cell death attributed to cytolysin production from pAM714 was less robust (Figure 2C and D). Significant changes in T11RF and T11RFΔcas9 did not occur until the later time...
points of conjugation. From this, we conclude that cytolysin activity has a greater impact on the population dynamics of conjugation reactions under planktonic mating conditions as compared to biofilm mating conditions.

To obtain better resolution of the recipient cell sensitivity to cytolysin activity, we utilized a cytolysin immunity assay (40). We sought to determine the time point in our pAM714 matings at which the recipient populations became immune to cytolysin activity. To isolate only recipient cells from mating reactions, we sampled from our conjugation reactions at set time points, and then performed an outgrowth for 18 hours in antibiotic medium that would eliminate OG1SSp(pAM714) donors (see Materials and Methods). These cultures were then assessed for cytolysin susceptibility in the cytolysin immunity assay. We note that, during this outgrowth, it is possible that additional pAM714 transfer may occur between recipient and transconjugant cells present in these populations.

Under planktonic mating conditions (Figure 3, top panel), the T11RF population was susceptible to cytolysin throughout the mating reaction, consistent with CRISPR-Cas impeding acquisition of pAM714 in this population. Conversely, T11RFΔcas9 populations became immune to cytolysin activity. These results suggest that in the absence of active CRISPR-Cas, pAM714 is transferred into the recipient population at a level sufficient to render the population immune to the activity of cytolysin. In biofilm conjugation reactions, the T11RF population becomes immune to cytolysin after 5 h of mating and the Δcas9 strain is at least partially immune after 1 h of mating on a solid surface (Figure 3, bottom panel). These data likely reflect the overall enhanced
conjugation rate and higher number of transconjugants in a biofilm as compared to planktonic matings. Overall these data indicate that the absence of CRISPR-Cas leads to more rapid acquisition of cytolysin immunity at a population level, presumably through unrestricted plasmid acquisition. Further, the presence of active CRISPR-Cas defense against pAM714 in the recipient population results in prolonged susceptibility to the activity of cytolysin in this population.

**CRISPR-Cas has robust activity against PRPs in vivo.** There is little understanding of the impact of CRISPR-Cas systems on the dissemination of antibiotic resistance plasmids in vivo. Here, we assessed *E. faecalis* CRISPR3-Cas activity against antibiotic resistance plasmids in a mouse model of *E. faecalis* intestinal dysbiosis. To establish antibiotic-induced dysbiosis, mice were administered a cocktail of antibiotics in their drinking water for seven days, followed by their placement on regular water for 24 hours. The mice were colonized sequentially with recipient and donor *E. faecalis* strains. Fecal pellets were collected at 24, 48, and 96 hours post co-colonization, homogenized, and the number of transconjugants obtained at each time was compared across groups. Three experimental groups consisting of different combinations of donor and recipient strains were used: OG1SSp with T11RF as a plasmid-free control, OG1SSp(pAM714) donors with T11RF recipients, and OG1SSp(pAM714) donors with T11RFΔcas9 recipients. In separate experiments of similar design, OG1SSp(pAM771) donors were used.
At 24 hours post co-colonization, we observed pAM714 transfer in only one of ten mice when T11RF was the recipient (Figure 4A). Transconjugants were not recovered in this mouse at subsequent time points. Conversely, pAM714 transconjugants at densities up to $10^6$ CFU/g of feces were observed for eight of ten mice colonized with T11RFΔcas9 recipients over the course of the experiment (Figure 4A).

To assess the potential for cytolysin to influence plasmid transfer in the intestine, we performed experiments with OG1SSp(pAM771). At 24 and 48-hours post co-colonization, only one out of ten mice with T11RF as a recipient experienced pAM771 plasmid transfer (Figure 4B) whereas T11RFΔcas9 recipients in all mice tested acquired pAM771 and retained the plasmid over 96 hours (Figure 4B). These data show that there is a significant impact of CRISPR-Cas on plasmid transfer between E. faecalis strains in the mouse intestine. Moreover, cytolysin production does not appear to impact the efficacy of CRISPR-Cas defense in vivo.

In a few instances we observed that the control (no plasmid) mice had colony growth on media with selection for transconjugants (i.e. media supplemented with rifampicin, fusidic acid, and erythromycin). This growth did not occur until 48 or 96 hours post-co-colonization (Figure 4). Sporadic native microbiota in the mouse intestine may have possessed resistance to these antibiotics. Alternatively, T11RF recipients could have acquired erythromycin resistance from native microbiota present in a subset of mice.
Cytolysin-independent *in vivo* colonization benefit to strains possessing a PRP.

We next explored the ability of cytolysin to impact the colonization of *E. faecalis* in the mouse intestine. To assess PRP recipient cell density in mice, fecal samples were plated on media to select for recipient populations (i.e., media supplemented with rifampicin and fusidic acid). If cytolysin production impacted the viability of recipient cell populations in the mouse intestine, we expected to see a decline in T11RF and T11RFΔcas9 densities in mice when OG1SSp(pAM714) was the donor and stable colonization when cytolysin-deficient OG1SSp(pAM771) was the donor. This is not what was observed. At 24 hours post-co-colonization of donors and recipients, the densities of recipient strains from plasmid-free control, pAM714, and pAM771 test groups were similar (Figure 5A and 5B). At both 48 and 96 hours post co-colonization, the density of T11RF recipients from the plasmid-free control group was significantly higher than the T11RF and T11RFΔcas9 densities in both the pAM714 and pAM771 test groups. Moreover, the densities of T11RF and T11RFΔcas9, when co-colonized with either OG1SSp(pAM714) or OG1SSp(pAM771), decreased by > 1 log between the 24 and 96-hour time points. Finally, there was no difference in T11RF and T11RFΔcas9 colonization, showing that the presence or absence of cas9 does not impact colonization (Figure 5A and 5B). From these data we conclude that the cytolysin-independent reduction in recipient cell viability observed during *in vitro* PRP transfer (Figure 2) does not occur in the mouse intestinal environment tested here. However, our data suggest that there is a cytolysin-independent negative effect of pAM714/pAM771 on recipient cell density *in vivo.*
We next explored the impact of cytolysin production on donor strain densities by plating feces on media selective for donors (i.e., spectinomycin and streptomycin). The density of OG1SSp with and without plasmid at 24 hours post co-colonization differed by ~2 log, regardless of the plasmid (Figure 5C and D). Additionally, the control group with no plasmid had a significantly reduced donor density compared to plasmid-bearing donors at all time points. We do not attribute this observation to residual erythromycin present after microbiota depletion (thereby inhibiting OG1SSp in the control group but not the plasmid-containing donors), because the erythromycin-sensitive recipient populations would also have been affected. On the other hand, the densities of donor strains harboring either plasmid remained stable throughout the experiment and were consistent across all mice in each group. These data suggest that there was a cytolysin-independent colonization benefit for OG1SSp strains harboring pAM714 or pAM771.

Discussion

To our knowledge, our study is the first to directly assess the impact of CRISPR-Cas genome defense on antibiotic resistance dissemination in the gastrointestinal tract. We observed a profound effect of CRISPR-Cas defense on in vivo transfer of a pheromone-responsive plasmid conferring antibiotic resistance in E. faecalis. Native CRISPR-Cas defense in E. faecalis T11RF was a near-complete barrier to the in vivo acquisition of an antibiotic resistance plasmid. These results are significant because they suggest that CRISPR-Cas systems can have profound effects, more broadly, on horizontal gene transfer in the gastrointestinal microbiota.
We hypothesized that the absence of CRISPR-Cas in the progenitors of hospital-adapted *E. faecalis* allowed for their rapid evolution of multidrug resistance via horizontal gene transfer (32). The results of our current study demonstrate that CRISPR-Cas is, in fact, a robust barrier to plasmid acquisition *in vivo*. In this study, we did not provide erythromycin during the *in vivo* mating period, therefore we did not determine the impact of antibiotic selection for the plasmid on experimental outcomes. We predict that there would be very strong selection for CRISPR-Cas mutants or fully CRISPR-Cas-deficient strains that lack this barrier to plasmid acquisition. Further, the impact of CRISPR-Cas should be assessed in multiple *in vivo* models. Here, we induced gastrointestinal dysbiosis with antibiotics, allowed the mice to recover for one day, and then colonized them with *E. faecalis*. This models what can occur in patients after receiving antibiotic therapy. Another model, recently developed, establishes long-term colonization of *E. faecalis* without major disruption of normal gastrointestinal microbiota (17). In this model system, production of the Bac-21 bacteriocin from the PRP pPD1 significantly enhanced *E. faecalis* colonization of the gastrointestinal tract (17). In addition, another recent study utilized a germ-free mouse model to examine plasmid transfer between *E. faecalis* strains throughout the mouse intestinal tract (43). The impact of CRISPR-Cas and cytolysin production on *in vivo* PRP transfer should be assessed in these models, as well.

Our study identified key differences concerning CRISPR-Cas defense efficacy, depending on the experimental conditions used and the accessory factors (in this case, bacteriocin production) encoded by the plasmid studied. From the five conditions tested
in our study, we observed that CRISPR-Cas activity can range from having no impact on plasmid acquisition to completely blocking plasmid acquisition between the same donor and recipient pair of *E. faecalis* strains (see Fig S1 for a comparison of conjugation frequencies between conditions). We confirmed our previously published result that CRISPR-Cas defense in T11RF has a significant impact on pAM714 acquisition after 18 hours of biofilm mating. However, CRISPR-Cas did not have a significant impact on plasmid transfer at most earlier time points sampled. CRISPR-Cas also had a significant impact on plasmid acquisition in planktonic matings, but only if the plasmid encoded a bacteriocin. The bacteriocin had profound effects on recipient cell density in *in vitro* planktonic matings, and to a lesser extent in biofilm matings. The bacteriocin did not impact the *in vivo* efficacy of CRISPR-Cas. This is consistent with a previous study that analyzed *in vivo* transfer of pAM714 and pAM771 among *E. faecalis* in the hamster gastrointestinal tract where bacteriocin production also did not impact *in vivo* plasmid transfer (44). That study also determined that the Tn917 insertions in pAM714 and pAM771 were stable after *in vivo* passage (44).

There is a clear difference in the *in vitro* and *in vivo* efficacies of CRISPR-Cas defense in *E. faecalis*. We recently reported that, under *in vitro* conditions, *E. faecalis* CRISPR-Cas is attenuated (45). Specifically, *E. faecalis* can transiently tolerate both an active CRISPR-Cas system and one of its targets, albeit at a fitness cost (34, 38, 45). Overexpression of *cas9* overcomes this phenotype, greatly increasing the efficacy of defense and preventing CRISPR/target co-maintenance (45). Little is known about the regulation of CRISPR-Cas systems in *E. faecalis*. Our results suggest that they are
differentially regulated in vitro and in vivo. There are several possible mechanisms for our observations, including differential transcriptional regulation of cas genes in vitro and in vivo, or differential expression or activity of anti-CRISPR regulatory factors (46) in E. faecalis, although to our knowledge none have yet been identified in this species. Future studies will seek to elucidate the mechanisms underlying the robust in vivo anti-plasmid activity of E. faecalis CRISPR-Cas.

Materials and Methods

Bacteria and reagents used. Strains used in this study are shown in Table 1. E. faecalis strains were cultured in brain heart infusion (BHI) broth or on BHI agar at 37°C. Antibiotic concentrations used were as follows: rifampicin, 50 µg/mL; fusidic acid, 25 µg/mL; spectinomycin, 500 µg/mL; streptomycin, 500 µg/mL; erythromycin, 50 µg/mL. Antibiotics were purchased from Sigma-Aldrich or Research Products International (RPI).

Conjugation experiments. Donor and recipient strains were cultured overnight in BHI broth in the absence of antibiotic selection. The following day, cultures were diluted 1:10 into fresh BHI and incubated at 37°C for 1.5 hours. For planktonic conjugations, 2 mL of donor and 18 mL of recipient were mixed in a flask and incubated without agitation at 37°C for 30 min to 18 h. At each time point, 1 mL of the mating reaction was removed and used for serial dilutions and plating on selective media. For biofilm mating reactions, 100 µL of donor was mixed with 900 µL of recipient. The mixture was centrifuged for 1 min at 16,000 x g. After centrifugation, 100 µL supernatant was used to
resuspend the pellet, which was then spread-plated on non-selective BHI agar. To allow for sampling of multiple time points of biofilms, multiple identical conjugation reactions were generated using the same donor and recipient inocula. The conjugation reactions were incubated at 37°C for 30 min to 18 h. At each time point, cells were collected by washing and scraping an agar plate using 2 mL 1X phosphate buffered saline (PBS) supplemented with 2 mM EDTA, and serial dilutions were plated on selective media. For all matings, BHI agar supplemented with antibiotics was used to quantify the donor (spectinomycin, streptomycin, and erythromycin), recipient (rifampicin and fusidic acid), and transconjugant (rifampicin, fusidic acid, and erythromycin) populations. Plates were incubated for 36-48 h at 37°C. Plates with 30 to 300 colonies were used to calculate CFU/mL. The limit of detection for transconjugants was $10^1$ CFU.

**Cytolysin immunity assay.** Recipient strain susceptibility to cytolysin was assessed by a previously described method (40), with slight modifications. To eliminate residual cytolysin activity due to the presence of the donor strain, 50 µL of mating reactions from each time point during conjugation were incubated overnight in BHI broth supplemented with rifampicin and fusidic acid to select for recipient and transconjugant strains. The following day, these donor-deficient cultures were used in a soft agar overlay on normal BHI agar. After allowing the overlay to dry, 5 µL of OG1SSp(pAM714) was spotted on the overlay as an indicator for cytolysin susceptibility. Plates were incubated overnight at 37°C and were inspected for zones of inhibition the following day.
Mouse model of *E. faecalis* colonization. Seven days prior to bacterial colonization, 6-8 week old C57BL/6 mice (Jackson laboratories) were gavaged with 100 µL of an antibiotic cocktail (streptomycin 1 mg/mL, gentamicin 1 mg/mL, erythromycin 200 µg/mL), and given a water bottle *ad libitum* with the same antibiotic cocktail for 6 days following gavage. 24 h prior to bacterial inoculation, antibiotic water was removed and replaced with standard sterile antibiotic-free water. Bacteria were grown overnight in BHI, and mice were gavaged with 1e⁹ CFU/mL in PBS of each bacterial strain as experimental groups indicated. Samples used for gavage were plated on BHI to confirm that inocula were equal across strains. Fecal samples from mice were collected at 0 h, 24 h, 48 h and 96 h. Fecal samples were resuspended in 1 mL of sterile PBS and dilutions were plated on BHI agar supplemented with antibiotics to quantify the donor (spectinomycin, streptomycin, and erythromycin), recipient (rifampicin and fusidic acid), and transconjugant (rifampicin, fusidic acid, and erythromycin) populations. Plates were incubated for 36-48 h at 37°C. Plates with 30 to 300 colonies were used to calculate CFU/gram of feces. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus (protocol number B-113916(09)1E).

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Table 1. *E. faecalis* strains used in this study.

| Strain Name            | Description                                                                 | Reference |
|------------------------|-----------------------------------------------------------------------------|-----------|
| T11RF                  | Rifampicin-fusidic acid resistant derivative of strain T11                  | (35, 47)  |
| T11RFΔcas9             | T11RF with an in-frame deletion of cas9                                     | (35)      |
| OG1SSp(pAM714)         | Spectinomycin-streptomycin resistant derivative of strain OG1 harboring pAM714, conferring erythromycin resistance via Tn917 insertion upstream of the par locus; cyl⁺ | (36, 48)  |
| OG1SSp(pAM771)         | Spectinomycin-streptomycin resistant derivative of strain OG1 harboring pAM771, conferring erythromycin resistance via Tn917 insertion disrupting cylL of the cytolysin operon; cyl⁻ | (22, 41, 42) |
Figure 1. Mating condition impacts effectiveness of CRISPR-Cas on plasmid transfer in vitro. The CFU/mL of transconjugants obtained in mating reactions sampled over an 18-hour period is shown for T11RF (squares) and T11RFΔcas9 (triangles) recipient strains. Conjugation was performed under planktonic conditions in broth (A and C) and biofilm conditions on an agar plate (B and D) utilizing OG1SSp as a donor strain for plasmids pAM714 (cyl+; open, red symbols) and pAM771 (cyl-; closed, green symbols). These plasmids are isogenic except for the location of Tn917 insertion; in pAM771, bacteriocin production is disrupted by Tn917 insertion into cylL, whereas in pAM714 the Tn917 occurs in an intergenic region upstream of the par locus. Data
shown are the average and standard deviation from a minimum of three independent
trials for each time point for both mating conditions. Statistical significance was
assessed using a two-tailed Student’s t-Test; \( P \)-values, \(<0.05\), \(<0.01\) and \(<0.001\).
cyl = cytolysin.
Figure 2. Production of cytolysin from pAM714 alters recipient cell density in vitro. T11RF (squares) and T11RFΔcas9 (triangles) recipient cell densities in mating reactions were determined by plating reactions on selective media for the recipients. The recipient CFU/mL was determined for both planktonic (A and B) and biofilm (C and D) mating reactions with pAM714 (cyl+; open, red symbols) and pAM771 (cyl-; closed green symbols) donors. Data shown are the average and standard deviation from a minimum of three independent trials for each time point for both mating conditions. Statistical significance was assessed using a two-tailed Student’s t-Test; P-values, <0.05, <0.01 and <0.001. cyl = cytolysin.
Figure 3. Cytolysin immunity assays. A cytolysin immunity assay was performed as previously described on mating reactions where OG1SSp(pAM714) was used as a donor (40). Samples of planktonic and biofilm mating reactions were collected at each time point and inoculated into broth that would select for recipient cells only for an outgrowth period of 18 hrs. 5 µL of an overnight culture of strain OG1SSp(pAM714) was spotted onto an overlay of recipient strains to assay for cytolysin susceptibility. A zone of inhibition indicates susceptibility to cytolysin; S, susceptible and I, immune. Pictures presented are representative of results obtained from at least two independent trials of the assay.
Figure 4. CRISPR-Cas has a strong impact on plasmid transfer in the mouse gut.

The number of transconjugant CFU/g of feces for each individual mouse was determined by plating feces on rifampicin-fusidic acid-erythromycin agar; each symbol represents one mouse. At least ten mice from two independent experiments were used for each experimental group. Black horizontal bars represent the geometric mean of data in each group. Panel A shows data for OG1SSp(pAM714) donors with T11RF (open squares) or T11RFΔcas9 (open triangles) recipients, and a no-plasmid control (OG1SSp with T11RF; open circles). Panel B shows data for OG1SSp(pAM771) donors with T11RF (closed squares) or T11RFΔcas9 (closed triangles) recipients, and a no-
plasmid control (OG1SSp with T11RF; closed circles), Statistical significance was assessed using a two-tailed Student’s t-Test; $P$-value, **$<0.01$ and ***$<0.001$. 
Figure 5. Cytolysin-independent colonization phenotypes in the mouse gut. The densities of recipient (A and B) and donor (C and D) strains are shown as the CFU/g of feces on the appropriate antibiotic selection. These experiments were performed on the same test groups described in Figure 4, where least ten mice from two independent experiments were used for each experimental group. Groups with OG1SSp(pAM714) donors (A and C) are represented with open, red symbols and experimental groups with OG1SSp(pAM771) donors (B and D) are denoted by closed, green symbols. Statistical significance was assessed using a two-tailed Student’s t-Test; P-values, **<0.01 and ***<0.001.
Supporting Information

**Figure S1. Frequency of conjugation in vivo and in vitro.** Conjugation frequencies for plasmid transfer *in vivo* (A and B) and *in vitro* (C-F) were calculated as the number of transconjugants per donor. A-B) The conjugation frequency of pAM714 (A) and pAM771 (B) transfer in the mouse gut is shown above; each symbol represents one mouse from two independent experiments where at least ten mice were used for each experimental group. No symbol means that a frequency could not be calculated because one or both of the values (donor CFU/g or transconjugant CFU/g) were zero. Black horizontal bars
represent the geometric mean of data in each group. A-F) Conjugation frequencies of pAM714 and pAM771 transfer in vitro under planktonic (C and D) and biofilm (E and F) mating conditions. Data shown are the average and standard deviation from a minimum of three independent trials for each time point for both mating conditions. Statistical significance was assessed using a two-tailed Student’s t-Test; P-values, *<0.05, **<0.01 and ***<0.001.