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 *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.

38 **Importance**

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

50 **Introduction**

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

57

58 *E. faecalis* strains acquire novel genetic traits, including antibiotic resistance, via
59 horizontal gene transfer (HGT) (6-8). A common method of HGT in *E. faecalis* is
60 mediated by the narrow host range pheromone-responsive plasmids (PRPs). The PRPs
61 are a unique class of plasmids that are highly co-evolved with *E. faecalis* (9, 10). PRPs
62 are large (~60 kb) and conjugate efficiently, resulting in transfer frequencies reaching 1
63 transconjugant for every 10-100 donor cells (11). PRPs are often identified in the
64 genomes of clinical isolates of *E. faecalis* where they provide accessory antibiotic
65 resistance genes (8, 12-16). In addition to antibiotic resistance, some PRPs encode
66 bacteriocins, which are a class of antimicrobials produced by *E. faecalis* that can confer
67 a competitive advantage in polymicrobial environments (17). The model PRP, pAD1,
68 encodes a bacteriocin called cytolysin (18). Cytolysin is a lantibiotic-like antimicrobial
69 peptide with activity against a number of gram-positive bacteria and mammalian cells
70 (19, 20). The bactericidal and hemolytic properties of cytolysin enhance the virulence of
71 *E. faecalis* in some infections (21-24).

72

73 Bacteria can employ genome defense strategies to prevent the acquisition of plasmids
74 (25). Adaptive immunity conferred by Clustered Regularly Interspaced Short
75 Palindromic Repeats and CRISPR associated proteins (CRISPR-Cas) is one strategy
76 by which bacteria protect themselves from mobile genetic elements (MGEs). CRISPR-
77 Cas systems contain short segments of spacer DNA sequences that serve as a
78 molecular memory of past exposure to MGEs (26, 27). Spacers are transcribed into
79 RNA molecules that, when bound to an effector nuclease, identify complementary
80 foreign DNA and target it for cleavage (28-31). Interestingly, there is a strong correlation
81 between the absence of CRISPR-Cas systems and the presence of multidrug
82 resistance traits in hospital-adapted *E. faecalis* (32). This, in association with the MGE-
83 rich nature of multidrug-resistant strains, led to the hypothesis that MDR *E. faecalis*
84 emerge due to the absence of barriers to HGT (32).
85
86 *E. faecalis* possesses type II CRISPR-Cas systems (CRISPR1-Cas and CRISPR3-Cas)
87 (6, 32), which are defined by the presence of the type-specific gene, *cas9* (26). *E.*
88 *faecalis* also has an orphan locus, CRISPR2, that is not associated with *cas* genes.
89 CRISPR2 has been identified in all *E. faecalis* isolates, whereas CRISPR1-Cas and
90 CRISPR3-Cas have variable distribution among the species (33). Interestingly,
91 CRISPR2 is usually the only CRISPR locus present in hospital-adapted strains of *E.*
92 *faecalis* (32, 33). We recently demonstrated that both CRISPR1-Cas and CRISPR3-Cas
93 are active for genome defense *in vitro,* and that the orphan CRISPR2 locus confers
94 genome defense only in the presence of CRISPR1 Cas9 (34, 35). In one of these
95 studies, CRISPR3-Cas was shown to interfere with the acquisition of pAM714, a

96 derivative of pAD1 that confers erythromycin resistance (36, 37). Specifically, in *E.*
97 *faecalis* T11RF, a prototypical non-MDR commensal *E. faecalis* strain which possesses
98 a CRISPR3 spacer with perfect complementarity to pAM714, pAM714 conjugation
99 frequency in biofilms was significantly reduced, compared to a *cas9* deletion mutant
100 (35).
101
102 Little is known about how different environmental conditions affect CRISPR-Cas activity.
103 To further investigate the effects of CRISPR-Cas defense on PRP transfer, we
104 monitored the transfer kinetics of bacteriocin (cytolysin)-positive and -negative
105 derivatives of pAD1 over time in both planktonic and biofilm matings. We determined
106 that these factors significantly affect the overall efficacy of CRISPR-Cas defense.
107 Because *E. faecalis* are native gastrointestinal colonizers and reservoirs for antibiotic
108 resistance in the human microbiota, we also assessed CRISPR-Cas anti-plasmid
109 activity in a mouse model of gastrointestinal colonization. We discovered that CRISPR-
110 Cas is a strikingly robust barrier to plasmid acquisition *in vivo*, with a magnitude of effect
111 much more pronounced than that observed for any *in vitro* condition tested. These
112 results illustrate the role of the native microbiota (in this case, commensal, non-MDR *E.*
113 *faecalis* natively possessing CRISPR-Cas) in preventing the horizontal transmission of
114 antibiotic resistance determinants.
115
116 **Results**
117 **Conjugative transfer of pAM714 and impact of CRISPR-Cas in planktonic and**
118 **biofilm matings.** Spacer 6 of the CRISPR3-Cas locus of *E. faecalis* T11RF has 100%

119 sequence identity with the *repB* gene of pAM714, and acquisition of pAM714 is
120 increased by ~80-fold in *E. faecalis* T11RF∆*cas9* after 18 hours of biofilm mating on an
121 agar surface (35). To gain insight into the kinetics of plasmid transfer, and whether or
122 not culture condition impacts CRISPR-Cas defense, we sampled planktonic and biofilm
123 (agar spread plate) mating reactions over an 18-hour period (Figure 1). All mating
124 reactions were inoculated at a donor to recipient ratio of 1:9. The donor strain used in
125 these experiments was *E. faecalis* OG1SSp(pAM714). The recipient strains were
126 T11RF and a T11RF derivative with an in-frame deletion of *cas9*, T11RF∆*cas9* (see
127 Table 1 for a list of strains).
128
129 The number of pAM714 transconjugants (i.e., the number of T11RF or T11RF∆*cas9*
130 cells that acquired pAM714) was used to compare the kinetics of conjugative plasmid
131 transfer in the presence and absence of CRISPR-Cas defense. After only 30 minutes of
132 mating, we observed ~10^3-10^4 transconjugants for both recipient strains (T11RF and
133 ∆*cas9*) under both planktonic and biofilm conditions (Figure 1A and 1B). Under
134 planktonic mating conditions, the number of T11∆*cas9*(pAM714) transconjugants
135 remained stable over all time points while the number of T11RF(pAM714)
136 transconjugants decreased (Figure 1A). This suggests that CRISPR-Cas in T11RF is
137 actively targeting pAM714 in planktonic conjugation by preventing plasmid acquisition
138 and/or by curing T11RF transconjugants of pAM714 (38). The number of
139 transconjugants produced in biofilm matings were comparatively higher (Figure 1B).
140 Both recipient strains achieved transconjugant yields similar to those previously
141 reported for an 18-hour conjugation reaction in a biofilm (35). CRISPR-Cas had a

142 significant impact on transconjugant yield in only a subset of time points, with the most
143 pronounced effect observed at 18 hours (Figure 1B).
144
145 **Impact of cytolysin on CRISPR-Cas efficacy.** PRPs can encode bacteriocins that
146 promote their maintenance in polymicrobial communities (17, 39). pAM714 encodes a
147 bacteriocin, cytolysin, that is bactericidal against gram-positive bacteria including *E.*
148 *faecalis* (20). The operon encoding cytolysin also encodes a membrane-associated
149 immunity factor that protects the cytolysin-producing cell from the activity of cytolysin
150 (40). Therefore, pAM714-containing cells (and by extension, newly generated
151 transconjugants) are immune to the bacteriocin. To determine if cytolysin production
152 impacts CRISPR-Cas anti-plasmid defense, we utilized another derivative of pAD1,
153 pAM771, which is cytolysin-negative (41, 42). Under planktonic conditions using
154 OG1SSp(pAM771) as a plasmid donor, we obtained a similar number of
155 T11RF(pAM771) and T11RF∆*cas9*(pAM771) transconjugants across all time points
156 (Figure 1C). Moreover, we observed a statistically significant impact of CRISPR-Cas on
157 pAM771 acquisition at only one time point, and the magnitude of the effect was small.
158 These results are in contrast to those observed for pAM714 planktonic matings (Figure
159 1A). These results suggest that cytolysin production influences CRISPR-Cas efficacy at
160 a population level under planktonic mating conditions. In pAM771 biofilm matings, we
161 observed an overall increase in transconjugant numbers by at least one log for all time
162 points and for both wild-type and ∆*cas9* recipient strains (Figure 1D), as compared to
163 pAM714 (Figure 1B). However, the impact of CRISPR-Cas was similar and did not
164 appear to be influenced by cytolysin production.

165

166 **Recipient strain densities are substantially impacted by cytolysin in planktonic**
167 **mating reactions.** Under planktonic mating conditions, we observed a decrease in
168 pAM714 transconjugants over time (Figure 1A) and a difference in the overall number of
169 T11RF(pAM714) transconjugants compared to T11RF(pAM771) transconjugants after 5
170 and 18 hours of conjugation (Figure 1A and 1C). We hypothesized that pAM714-
171 encoded cytolysin impacts plasmid transfer kinetics under planktonic mating conditions.

172

173 Cells possessing pAM714 (i.e., donor strains) are protected from the action of cytolysin
174 through production of an immunity factor (40). T11RF and T11RF∆*cas9* recipients do
175 not encode the immunity factor and are vulnerable to the activity of cytolysin. We
176 analyzed total T11RF or T11RF∆*cas9* densities in our conjugation reactions (the same
177 reactions as shown in Figure 1). In planktonic mating reactions, T11RF density
178 remained high (~10^8 CFU/mL) and stable when the donor strain harbored pAM771
179 (Figure 2A). However, in the presence of pAM714 donors, there was a linear decrease
180 in T11RF density that initiated after only 30 minutes of mating (Figure 2A). A similar
181 trend was also observed for the T11RF∆*cas9* recipient strain (Figure 2B). We conclude
182 that under planktonic mating conditions, cytolysin production from pAM714 continuously
183 depletes the recipient cell population, and this is independent of CRISPR-Cas.

184

185 We performed the same analysis for biofilm matings and found that the degree of cell
186 death attributed to cytolysin production from pAM714 was less robust (Figure 2C and
187 D). Significant changes in T11RF and T11RF∆*cas9* did not occur until the later time

188 points of conjugation. From this, we conclude that cytolysin activity has a greater impact
189 on the population dynamics of conjugation reactions under planktonic mating conditions
190 as compared to biofilm mating conditions.
191
192 To obtain better resolution of the recipient cell sensitivity to cytolysin activity, we utilized
193 a cytolysin immunity assay (40). We sought to determine the time point in our pAM714
194 matings at which the recipient populations became immune to cytolysin activity. To
195 isolate only recipient cells from mating reactions, we sampled from our conjugation
196 reactions at set time points, and then performed an outgrowth for 18 hours in antibiotic
197 medium that would eliminate OG1SSp(pAM714) donors (see Materials and Methods).
198 These cultures were then assessed for cytolysin susceptibility in the cytolysin immunity
199 assay. We note that, during this outgrowth, it is possible that additional pAM714 transfer
200 may occur between recipient and transconjugant cells present in these populations.
201
202 Under planktonic mating conditions (Figure 3, top panel), the T11RF population was
203 susceptible to cytolysin throughout the mating reaction, consistent with CRISPR-Cas
204 impeding acquisition of pAM714 in this population. Conversely, T11RF∆*cas9*
205 populations became immune to cytolysin activity. These results suggest that in the
206 absence of active CRISPR-Cas, pAM714 is transferred into the recipient population at a
207 level sufficient to render the population immune to the activity of cytolysin. In biofilm
208 conjugation reactions, the T11RF population becomes immune to cytolysin after 5 h of
209 mating and the ∆*cas9* strain is at least partially immune after 1 h of mating on a solid
210 surface (Figure 3, bottom panel). These data likely reflect the overall enhanced

211 conjugation rate and higher number of transconjugants in a biofilm as compared to
212 planktonic matings. Overall these data indicate that the absence of CRISPR-Cas leads
213 to more rapid acquisition of cytolysin immunity at a population level, presumably through
214 unrestricted plasmid acquisition. Further, the presence of active CRISPR-Cas defense
215 against pAM714 in the recipient population results in prolonged susceptibility to the
216 activity of cytolysin in this population.
217
218 **CRISPR-Cas has robust activity against PRPs *in vivo*.** There is little understanding
219 of the impact of CRISPR-Cas systems on the dissemination of antibiotic resistance
220 plasmids *in vivo*. Here, we assessed *E. faecalis* CRISPR3-Cas activity against antibiotic
221 resistance plasmids in a mouse model of *E. faecalis* intestinal dysbiosis. To establish
222 antibiotic-induced dysbiosis, mice were administered a cocktail of antibiotics in their
223 drinking water for seven days, followed by their placement on regular water for 24
224 hours. The mice were colonized sequentially with recipient and donor *E. faecalis* strains.
225 Fecal pellets were collected at 24, 48, and 96 hours post co-colonization, homogenized,
226 and the number of transconjugants obtained at each time was compared across groups.
227 Three experimental groups consisting of different combinations of donor and recipient
228 strains were used: OG1SSp with T11RF as a plasmid-free control, OG1SSp(pAM714)
229 donors with T11RF recipients, and OG1SSp(pAM714) donors with T11RF∆*cas9*
230 recipients. In separate experiments of similar design, OG1SSp(pAM771) donors were
231 used.
232

233 At 24 hours post co-colonization, we observed pAM714 transfer in only one of ten mice
234 when T11RF was the recipient (Figure 4A). Transconjugants were not recovered in this
235 mouse at subsequent time points. Conversely, pAM714 transconjugants at densities up
236 to 10^6 CFU/g of feces were observed for eight of ten mice colonized with T11RF∆*cas9*
237 recipients over the course of the experiment (Figure 4A).
238
239 To assess the potential for cytolysin to influence plasmid transfer in the intestine, we
240 performed experiments with OG1SSp(pAM771). At 24 and 48-hours post co-
241 colonization, only one out of ten mice with T11RF as a recipient experienced pAM771
242 plasmid transfer (Figure 4B) whereas T11RF∆*cas9* recipients in all mice tested acquired
243 pAM771 and retained the plasmid over 96 hours (Figure 4B). These data show that
244 there is a significant impact of CRISPR-Cas on plasmid transfer between *E. faecalis*
245 strains in the mouse intestine. Moreover, cytolysin production does not appear to impact
246 the efficacy of CRISPR-Cas defense *in vivo*.
247
248 In a few instances we observed that the control (no plasmid) mice had colony growth on
249 media with selection for transconjugants (i.e. media supplemented with rifampicin,
250 fusidic acid, and erythromycin). This growth did not occur until 48 or 96 hours post-co-
251 colonization (Figure 4). Sporadic native microbiota in the mouse intestine may have
252 possessed resistance to these antibiotics. Alternatively, T11RF recipients could have
253 acquired erythromycin resistance from native microbiota present in a subset of mice.
254

255 **Cytolysin-independent *in vivo* colonization benefit to strains possessing a PRP.**
256 We next explored the ability of cytolysin to impact the colonization of *E. faecalis* in the
257 mouse intestine. To assess PRP recipient cell density in mice, fecal samples were
258 plated on media to select for recipient populations (i.e., media supplemented with
259 rifampicin and fusidic acid). If cytolysin production impacted the viability of recipient cell
260 populations in the mouse intestine, we expected to see a decline in T11RF and
261 T11RF∆*cas9* densities in mice when OG1SSp(pAM714) was the donor and stable
262 colonization when cytolysin-deficient OG1SSp(pAM771) was the donor. This is not what
263 was observed. At 24 hours post-co-colonization of donors and recipients, the densities
264 of recipient strains from plasmid-free control, pAM714, and pAM771 test groups were
265 similar (Figure 5A and 5B). At both 48 and 96 hours post co-colonization, the density of
266 T11RF recipients from the plasmid-free control group was significantly higher than the
267 T11RF and T11RF∆*cas9* densities in both the pAM714 and pAM771 test groups.
268 Moreover, the densities of T11RF and T11RF∆*cas9*, when co-colonized with either
269 OG1SSp(pAM714) or OG1SSp(pAM771), decreased by > 1 log between the 24 and 96-
270 hour time points. Finally, there was no difference in T11RF and T11RF∆*cas9*
271 colonization, showing that the presence or absence of *cas9* does not impact
272 colonization (Figure 5A and 5B). From these data we conclude that the cytolysin-
273 dependent reduction in recipient cell viability observed during *in vitro* PRP transfer
274 (Figure 2) does not occur in the mouse intestinal environment tested here. However, our
275 data suggest that there is a cytolysin-independent negative effect of pAM714/pAM771
276 on recipient cell density *in vivo*.
277

278 We next explored the impact of cytolysin production on donor strain densities by plating
279 feces on media selective for donors (i.e., spectinomycin and streptomycin). The density
280 of OG1SSp with and without plasmid at 24 hours post co-colonization differed by ~2 log,
281 regardless of the plasmid (Figure 5C and D). Additionally, the control group with no
282 plasmid had a significantly reduced donor density compared to plasmid-bearing donors
283 at all time points. We do not attribute this observation to residual erythromycin present
284 after microbiota depletion (thereby inhibiting OG1SSp in the control group but not the
285 plasmid-containing donors), because the erythromycin-sensitive recipient populations
286 would also have been affected. On the other hand, the densities of donor strains
287 harboring either plasmid remained stable throughout the experiment and were
288 consistent across all mice in each group. These data suggest that there was a cytolysin-
289 independent colonization benefit for OG1SSp strains harboring pAM714 or pAM771.
290

291 **Discussion**

292 To our knowledge, our study is the first to directly assess the impact of CRISPR-Cas
293 genome defense on antibiotic resistance dissemination in the gastrointestinal tract. We
294 observed a profound effect of CRISPR-Cas defense on *in vivo* transfer of a pheromone-
295 responsive plasmid conferring antibiotic resistance in *E. faecalis*. Native CRISPR-Cas
296 defense in *E. faecalis* T11RF was a near-complete barrier to the *in vivo* acquisition of an
297 antibiotic resistance plasmid. These results are significant because they suggest that
298 CRISPR-Cas systems can have profound effects, more broadly, on horizontal gene
299 transfer in the gastrointestinal microbiota.
300

301 We hypothesized that the absence of CRISPR-Cas in the progenitors of hospital-
302 adapted *E. faecalis* allowed for their rapid evolution of multidrug resistance via
303 horizontal gene transfer (32). The results of our current study demonstrate that
304 CRISPR-Cas is, in fact, a robust barrier to plasmid acquisition *in vivo*. In this study, we
305 did not provide erythromycin during the *in vivo* mating period, therefore we did not
306 determine the impact of antibiotic selection for the plasmid on experimental outcomes.
307 We predict that there would be very strong selection for CRISPR-Cas mutants or fully
308 CRISPR-Cas-deficient strains that lack this barrier to plasmid acquisition. Further, the
309 impact of CRISPR-Cas should be assessed in multiple *in vivo* models. Here, we
310 induced gastrointestinal dysbiosis with antibiotics, allowed the mice to recover for one
311 day, and then colonized them with *E. faecalis*. This models what can occur in patients
312 after receiving antibiotic therapy. Another model, recently developed, establishes long-
313 term colonization of *E. faecalis* without major disruption of normal gastrointestinal
314 microbiota (17). In this model system, production of the Bac-21 bacteriocin from the
315 PRP pPD1 significantly enhanced *E. faecalis* colonization of the gastrointestinal tract
316 (17). In addition, another recent study utilized a germ-free mouse model to examine
317 plasmid transfer between *E. faecalis* strains throughout the mouse intestinal tract (43).
318 The impact of CRISPR-Cas and cytolysin production on *in vivo* PRP transfer should be
319 assessed in these models, as well.
320
321 Our study identified key differences concerning CRISPR-Cas defense efficacy,
322 depending on the experimental conditions used and the accessory factors (in this case,
323 bacteriocin production) encoded by the plasmid studied. From the five conditions tested

324 in our study, we observed that CRISPR-Cas activity can range from having no impact
325 on plasmid acquisition to completely blocking plasmid acquisition between the same
326 donor and recipient pair of *E. faecalis* strains (see Fig S1 for a comparison of
327 conjugation frequencies between conditions). We confirmed our previously published
328 result that CRISPR-Cas defense in T11RF has a significant impact on pAM714
329 acquisition after 18 hours of biofilm mating. However, CRISPR-Cas did not have a
330 significant impact on plasmid transfer at most earlier time points sampled. CRISPR-Cas
331 also had a significant impact on plasmid acquisition in planktonic matings, but only if the
332 plasmid encoded a bacteriocin. The bacteriocin had profound effects on recipient cell
333 density in *in vitro* planktonic matings, and to a lesser extent in biofilm matings. The
334 bacteriocin did not impact the *in vivo* efficacy of CRISPR-Cas. This is consistent with a
335 previous study that analyzed *in vivo* transfer of pAM714 and pAM771 among *E. faecalis*
336 in the hamster gastrointestinal tract where bacteriocin production also did not impact *in*
337 *vivo* plasmid transfer (44). That study also determined that the Tn*917* insertions in
338 pAM714 and pAM771 were stable after *in vivo* passage (44).
339
340 There is a clear difference in the *in vitro* and *in vivo* efficacies of CRISPR-Cas defense
341 in *E. faecalis*. We recently reported that, under *in vitro* conditions, *E. faecalis* CRISPR-
342 Cas is attenuated (45). Specifically, *E. faecalis* can transiently tolerate both an active
343 CRISPR-Cas system and one of its targets, albeit at a fitness cost (34, 38, 45).
344 Overexpression of *cas9* overcomes this phenotype, greatly increasing the efficacy of
345 defense and preventing CRISPR/target co-maintenance (45). Little is known about the
346 regulation of CRISPR-Cas systems in *E. faecalis*. Our results suggest that they are

347 differentially regulated *in vitro* and *in vivo*. There are several possible mechanisms for
348 our observations, including differential transcriptional regulation of *cas* genes *in vitro*
349 and *in vivo*, or differential expression or activity of anti-CRISPR regulatory factors (46) in
350 *E. faecalis*, although to our knowledge none have yet been identified in this species.
351 Future studies will seek to elucidate the mechanisms underlying the robust *in vivo* anti-
352 plasmid activity of *E. faecalis* CRISPR-Cas.
353

354 **Materials and Methods**

355 **Bacteria and reagents used.** Strains used in this study are shown in Table 1. *E.*
356 *faecalis* strains were cultured in brain heart infusion (BHI) broth or on BHI agar at 37°C.
357 Antibiotic concentrations used were as follows: rifampicin, 50 µg/mL; fusidic acid, 25
358 µg/mL; spectinomycin, 500 µg/mL; streptomycin, 500 µg/mL; erythromycin, 50 µg/mL.
359 Antibiotics were purchased from Sigma-Aldrich or Research Products International
360 (RPI).
361

362 **Conjugation experiments.** Donor and recipient strains were cultured overnight in BHI
363 broth in the absence of antibiotic selection. The following day, cultures were diluted 1:10
364 into fresh BHI and incubated at 37°C for 1.5 hours. For planktonic conjugations, 2 mL of
365 donor and 18 mL of recipient were mixed in a flask and incubated without agitation at
366 37°C for 30 min to 18 h. At each time point, 1 mL of the mating reaction was removed
367 and used for serial dilutions and plating on selective media. For biofilm mating
368 reactions, 100 µL of donor was mixed with 900 µL of recipient. The mixture was
369 centrifuged for 1 min at 16,000 x g. After centrifugation, 100 µL supernatant was used to

370 resuspend the pellet, which was then spread-plated on non-selective BHI agar. To allow
371 for sampling of multiple time points of biofilms, multiple identical conjugation reactions
372 were generated using the same donor and recipient inocula. The conjugation reactions
373 were incubated at 37°C for 30 min to 18 h. At each time point, cells were collected by
374 washing and scraping an agar plate using 2 mL 1X phosphate buffered saline (PBS)
375 supplemented with 2 mM EDTA, and serial dilutions were plated on selective media. For
376 all matings, BHI agar supplemented with antibiotics was used to quantify the donor
377 (spectinomycin, streptomycin, and erythromycin), recipient (rifampicin and fusidic acid),
378 and transconjugant (rifampicin, fusidic acid, and erythromycin) populations. Plates were
379 incubated for 36-48 h at 37°C. Plates with 30 to 300 colonies were used to calculate
380 CFU/mL. The limit of detection for transconjugants was 10^1 CFU.
381
382 **Cytolysin immunity assay.** Recipient strain susceptibility to cytolysin was assessed by
383 a previously described method (40), with slight modifications. To eliminate residual
384 cytolysin activity due to the presence of the donor strain, 50 μL of mating reactions from
385 each time point during conjugation were incubated overnight in BHI broth supplemented
386 with rifampicin and fusidic acid to select for recipient and transconjugant strains. The
387 following day, these donor-deficient cultures were used in a soft agar overlay on normal
388 BHI agar. After allowing the overlay to dry, 5 μL of OG1SSp(pAM714) was spotted on
389 the overlay as an indicator for cytolysin susceptibility. Plates were incubated overnight
390 at 37°C and were inspected for zones of inhibition the following day.
391

392 **Mouse model of *E. faecalis* colonization.** Seven days prior to bacterial colonization,
393 6-8 week old C57BL/6 mice (Jackson laboratories) were gavaged with 100 μL of an
394 antibiotic cocktail (streptomycin 1 mg/mL, gentamicin 1 mg/mL, erythromycin 200
395 μg/mL), and given a water bottle *ad libitum* with the same antibiotic cocktail for 6 days
396 following gavage. 24 h prior to bacterial inoculation, antibiotic water was removed and
397 replaced with standard sterile antibiotic-free water. Bacteria were grown overnight in
398 BHI, and mice were gavaged with $1e^9$ CFU/mL in PBS of each bacterial strain as
399 experimental groups indicated. Samples used for gavage were plated on BHI to confirm
400 that inocula were equal across strains. Fecal samples from mice were collected at 0 h,
401 24 h, 48 h and 96 h. Fecal samples were resuspended in 1 mL of sterile PBS and
402 dilutions were plated on BHI agar supplemented with antibiotics to quantify the donor
403 (spectinomycin, streptomycin, and erythromycin), recipient (rifampicin and fusidic acid),
404 and transconjugant (rifampicin, fusidic acid, and erythromycin) populations. Plates were
405 incubated for 36-48 h at 37°C. Plates with 30 to 300 colonies were used to calculate
406 CFU/gram of feces. All animal protocols were approved by the Institutional Animal Care
407 and Use Committee of the University of Colorado Anschutz Medical Campus (protocol
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409

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416

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564
565

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

Strain Name	Description	Reference
T11RF	Rifampicin-fusidic acid resistant derivative of strain T11	(35, 47)
T11RF∆*cas9*	T11RF with an in-frame deletion of *cas9*	(35)
OG1SSp(pAM714)	Spectinomycin-streptomycin resistant derivative of strain OG1 harboring pAM714, conferring erythromycin resistance via Tn*917* insertion upstream of the *par* locus; *cyl*$^{+}$	(36, 48)
OG1SSp(pAM771)	Spectinomycin-streptomycin resistant derivative of strain OG1 harboring pAM771, conferring erythromycin resistance via Tn*917* insertion disrupting *cylL* of the cytolysin operon; *cyl*$^{-}$	(22, 41, 42)

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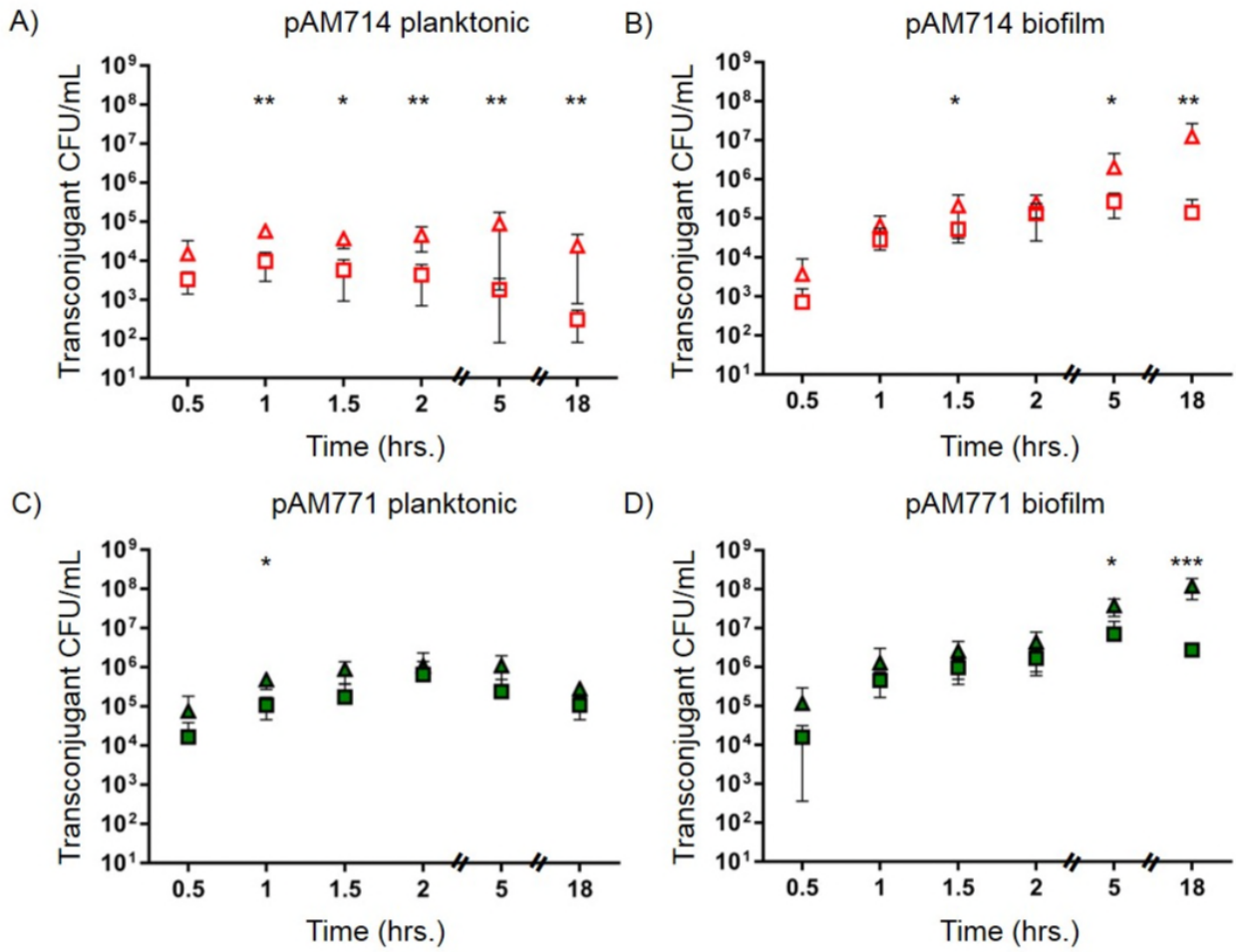
568

569 **Figures**

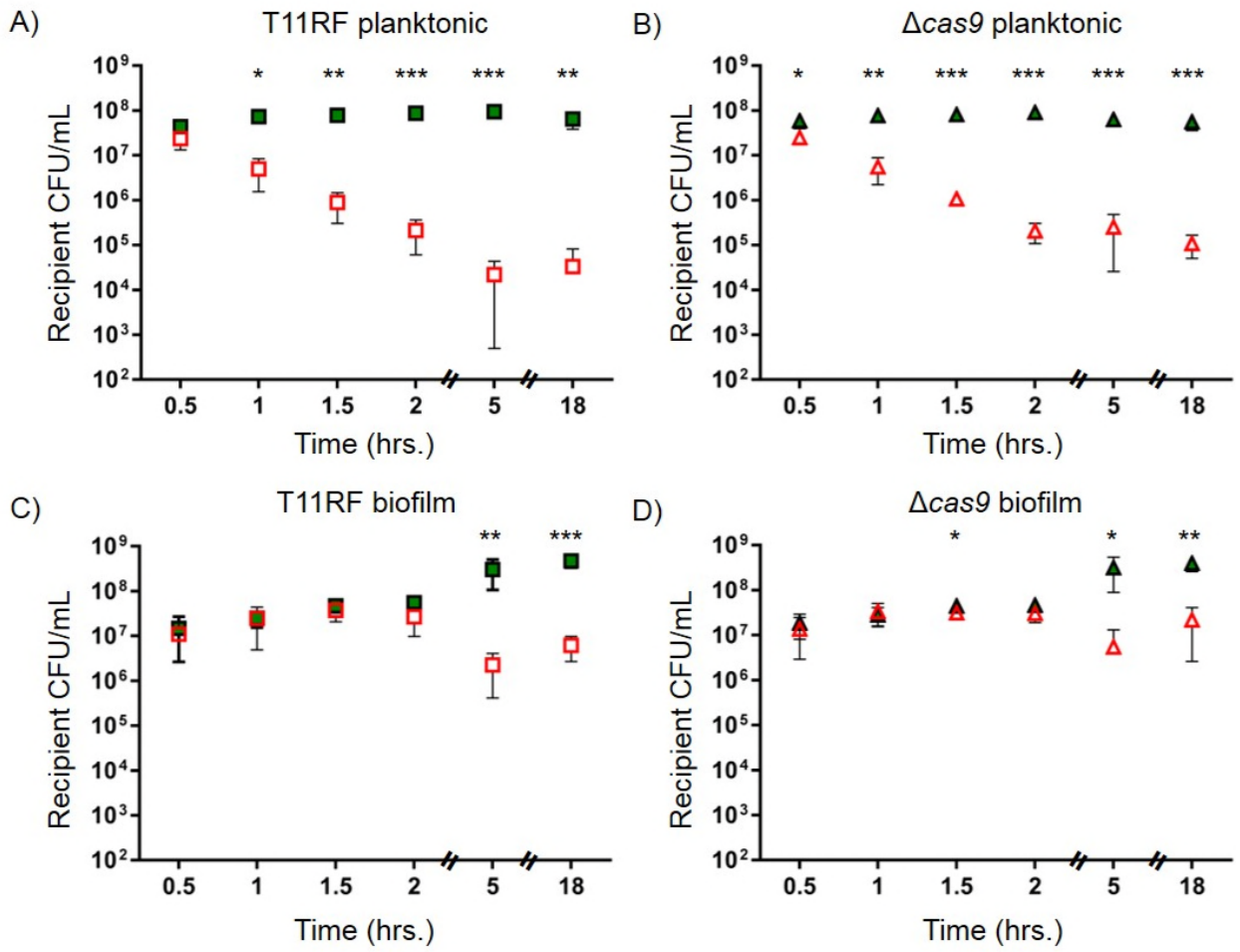


570

571 **Figure 1. Mating condition impacts effectiveness of CRISPR-Cas on plasmid**
572 **transfer *in vitro*.** The CFU/mL of transconjugants obtained in mating reactions sampled
573 over an 18-hour period is shown for T11RF (squares) and T11RFΔ*cas9* (triangles)
574 recipient strains. Conjugation was performed under planktonic conditions in broth (A
575 and C) and biofilm conditions on an agar plate (B and D) utilizing OG1SSp as a donor
576 strain for plasmids pAM714 (cyl+; open, red symbols) and pAM771 (cyl-; closed, green
577 symbols). These plasmids are isogenic except for the location of Tn*917* insertion; in
578 pAM771, bacteriocin production is disrupted by Tn*917* insertion into *cylL*, whereas in
579 pAM714 the Tn*917* occurs in an intergenic region upstream of the *par* locus. Data

580 shown are the average and standard deviation from a minimum of three independent
581 trials for each time point for both mating conditions. Statistical significance was
582 assessed using a two-tailed Student's t-Test; *P*-values, *<0.05, **<0.01 and ***<0.001.
583 cyl = cytolysin.
584

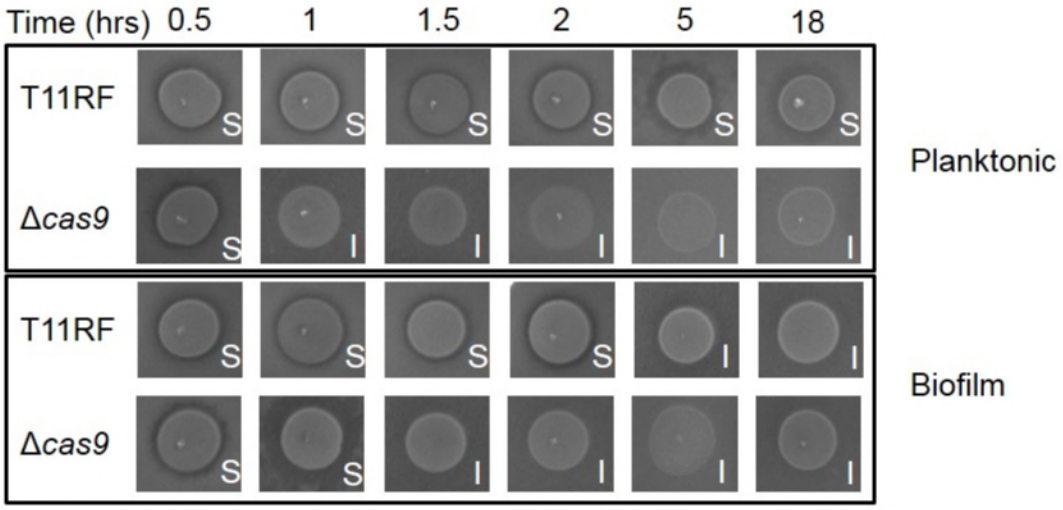
585



586

587 **Figure 2. Production of cytolysin from pAM714 alters recipient cell density *in*
588 *vitro*.** T11RF (squares) and T11RFΔ*cas9* (triangles) recipient cell densities in mating
589 reactions were determined by plating reactions on selective media for the recipients.
590 The recipient CFU/mL was determined for both planktonic (A and B) and biofilm (C and
591 D) mating reactions with pAM714 (cyl+; open, red symbols) and pAM771 (cyl-; closed
592 green symbols) donors. Data shown are the average and standard deviation from a
593 minimum of three independent trials for each time point for both mating conditions.
594 Statistical significance was assessed using a two-tailed Student's t-Test; *P*-values,
595 *<0.05, **<0.01 and ***<0.001. cyl = cytolysin.

596



597

598 **Figure 3. Cytolysin immunity assays.** A cytolysin immunity assay was performed as
599 previously described on mating reactions where OG1SSp(pAM714) was used as a
600 donor (40). Samples of planktonic and biofilm mating reactions were collected at each
601 time point and inoculated into broth that would select for recipient cells only for an
602 outgrowth period of 18 hrs. 5 μL of an overnight culture of strain OG1SSp(pAM714) was
603 spotted onto an overlay of recipient strains to assay for cytolysin susceptibility. A zone
604 of inhibition indicates susceptibility to cytolysin; S, susceptible and I, immune. Pictures
605 presented are representative of results obtained from at least two independent trials of
606 the assay.

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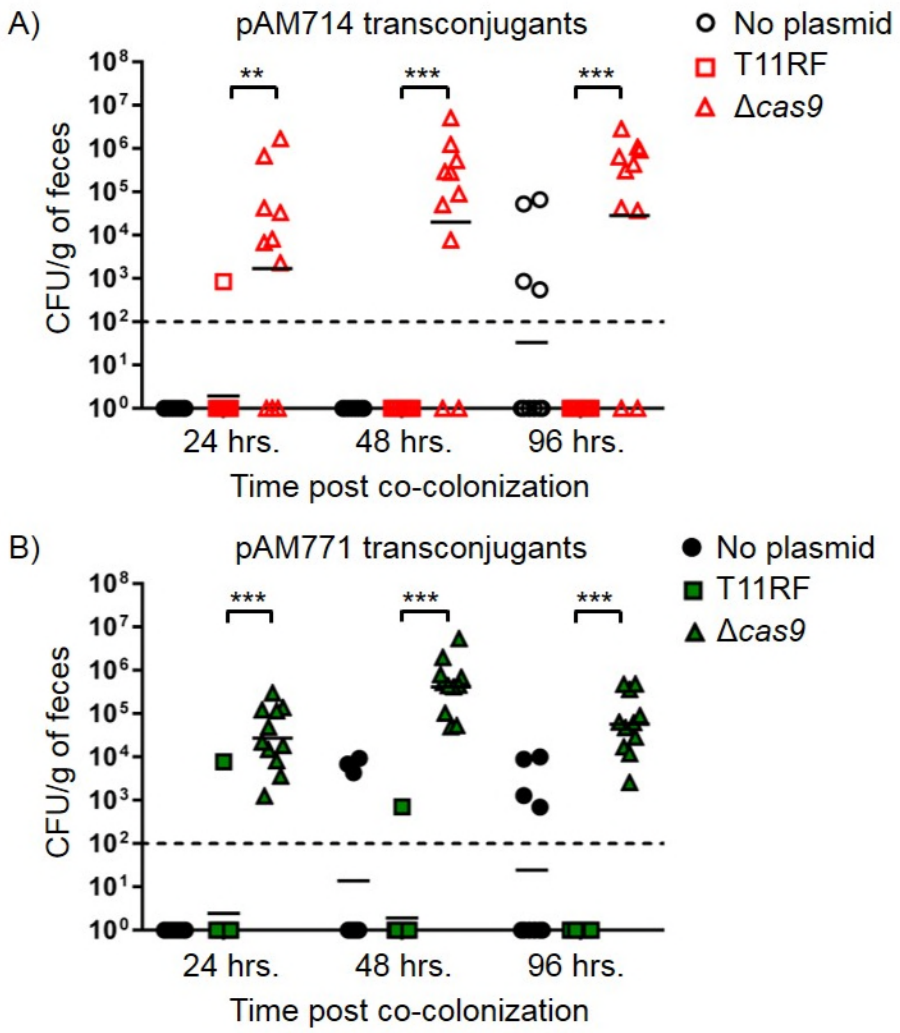

609

610 **Figure 4. CRISPR-Cas has a strong impact on plasmid transfer in the mouse gut.**
611 The number of transconjugant CFU/g of feces for each individual mouse was
612 determined by plating feces on rifampicin-fusidic acid-erythromycin agar; each symbol
613 represents one mouse. At least ten mice from two independent experiments were used
614 for each experimental group. Black horizontal bars represent the geometric mean of
615 data in each group. Panel A shows data for OG1SSp(pAM714) donors with T11RF
616 (open squares) or T11RFΔ*cas9* (open triangles) recipients, and a no-plasmid control
617 (OG1SSp with T11RF; open circles). Panel B shows data for OG1SSp(pAM771) donors
618 with T11RF (closed squares) or T11RFΔ*cas9* (closed triangles) recipients, and a no-

619 plasmid control (OG1SSp with T11RF; closed circles), Statistical significance was
620 assessed using a two-tailed Student's t-Test; *P*-value, **<0.01 and ***<0.001.
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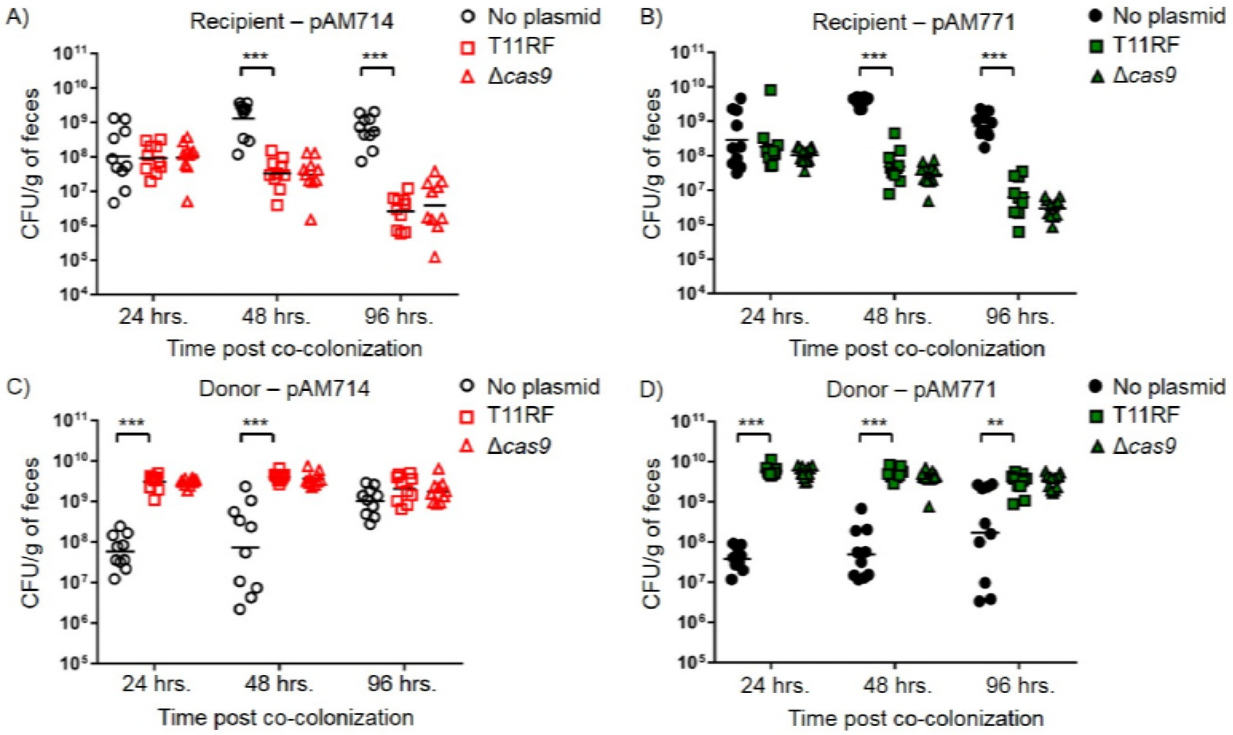


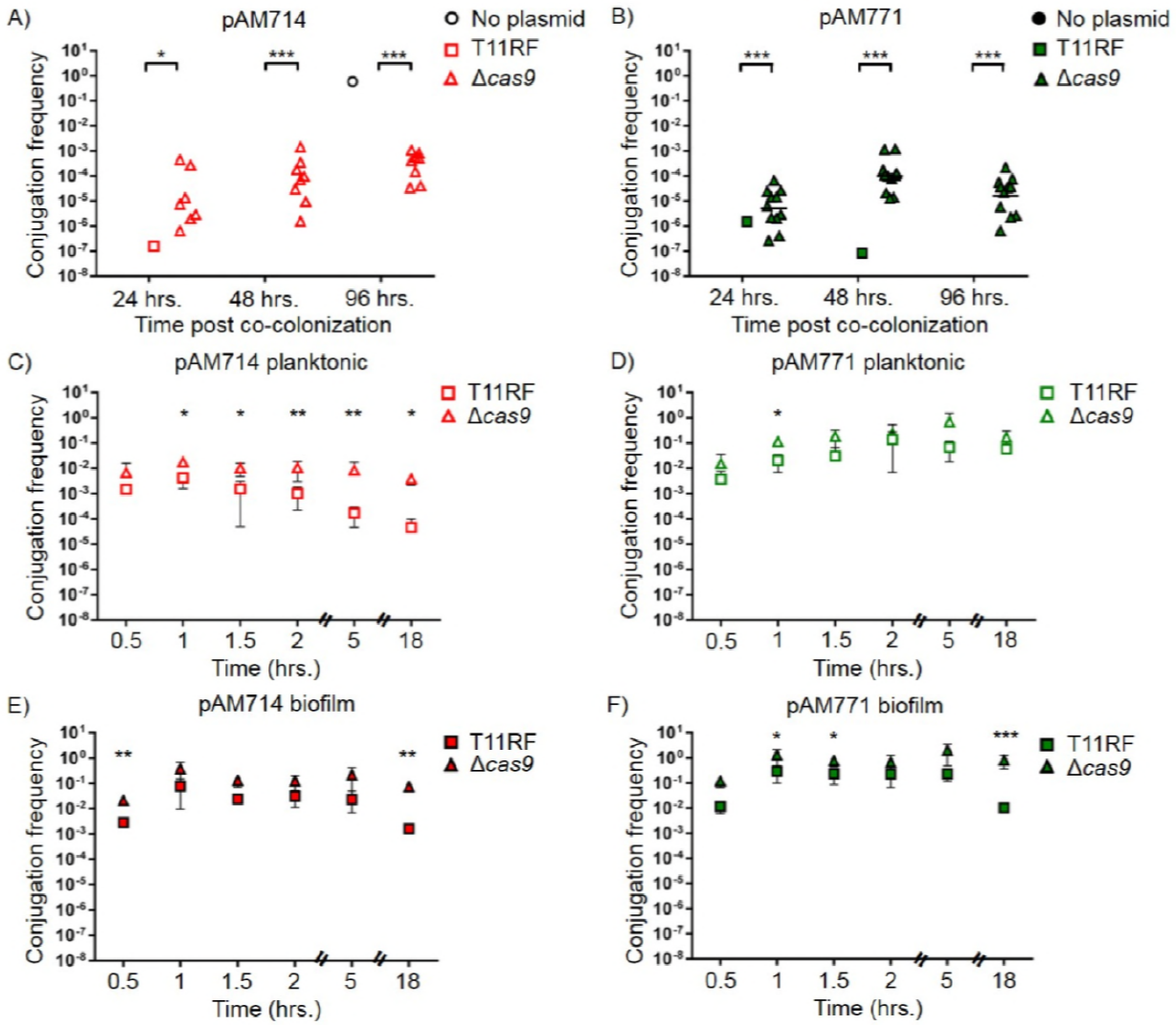
623

624 **Figure 5. Cytolysin-independent colonization phenotypes in the mouse gut.** The
625 densities of recipient (A and B) and donor (C and D) strains are shown as the CFU/g of
626 feces on the appropriate antibiotic selection. These experiments were performed on the
627 same test groups described in Figure 4, where least ten mice from two independent
628 experiments were used for each experimental group. Groups with OG1SSp(pAM714)
629 donors (A and C) are represented with open, red symbols and experimental groups with
630 OG1SSp(pAM771) donors (B and D) are denoted by closed, green symbols. Statistical
631 significance was assessed using a two-tailed Student's t-Test; *P*-values, **<0.01 and
632 ***<0.001.

633

634

635 **Supporting Information**



636

637 **Figure S1. Frequency of conjugation *in vivo* and *in vitro*.** Conjugation frequencies
638 for plasmid transfer *in vivo* (A and B) and *in vitro* (C-F) were calculated as the number of
639 transconjugants per donor. A-B) The conjugation frequency of pAM714 (A) and pAM771
640 (B) transfer in the mouse gut is shown above; each symbol represents one mouse from
641 two independent experiments where at least ten mice were used for each experimental
642 group. No symbol means that a frequency could not be calculated because one or both
643 of the values (donor CFU/g or transconjugant CFU/g) were zero. Black horizontal bars

644 represent the geometric mean of data in each group. A-F) Conjugation frequencies of
645 pAM714 and pAM771 transfer *in vitro* under planktonic (C and D) and biofilm (E and F)
646 mating conditions. Data shown are the average and standard deviation from a minimum
647 of three independent trials for each time point for both mating conditions. Statistical
648 significance was assessed using a two-tailed Student's t-Test; *P*-values, *<0.05, **<0.01
649 and ***<0.001.