Conjugative delivery of CRISPR-Cas9 for the selective depletion of antibiotic-resistant enterococci

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Abstract

The innovation of new therapies to combat multidrug-resistant (MDR) bacteria is being outpaced by the continued rise of MDR bacterial infections. Of particular concern are hospital-acquired infections (HAIs) recalcitrant to antibiotic therapies. The Gram-positive intestinal pathobiont *Enterococcus faecalis* contributes to a high incidence of HAIs with limited antibiotic treatment options. Therefore, novel therapies for the mitigation of *E. faecalis* HAIs is critically needed. We previously characterized an *E. faecalis* Type II CRISPR-Cas system and demonstrated its utility in the sequence-specific removal of antibiotic resistance determinants. Here we present work describing the adaption of this CRISPR-Cas system into a constitutively expressed module encoded on a pheromone-responsive conjugative plasmid that efficiently transfers to *E. faecalis* for the selective removal of antibiotic resistance genes. Using *in vitro* competition assays, we show that these CRISPR-Cas-encoding delivery plasmids, or CRISPR-Cas antimicrobials, can reduce the occurrence of antibiotic resistance in enterococcal populations in a sequence-specific manner. Furthermore, we demonstrate that deployment of CRISPR-Cas antimicrobials in the murine intestine reduces the occurrence of antibiotic-resistant *E. faecalis* by several orders of magnitude. Finally, we show that *E. faecalis* donor strains harboring CRISPR-Cas antimicrobials are immune to uptake of antibiotic resistance determinants *in vivo*. Our results demonstrate that conjugative delivery of CRISPR-Cas antimicrobials may be adaptable for future deployment from probiotic bacteria for exact targeting of defined MDR bacteria or for precision engineering of polymicrobial communities in the mammalian intestine.

53 **Introduction**

54 Disruption of the intestinal microbiota can predispose individuals to infection by opportunistic
55 pathogens [1, 2]. Antibiotics facilitate such disturbances leading to the development of hospital-
56 acquired infections (HAIs) [3, 4]. *Enterococcus faecalis*, a normal constituent of the healthy human
57 intestinal microbiota and historically used in probiotics and during food fermentation, is now a
58 leading cause of HAIs [5-7]. *E. faecalis* is an adept opportunist that can proliferate in the dysbiotic
59 intestine following antibiotic perturbation [8]. Antibiotic-resistant clinical isolates of *E. faecalis*
60 typically possess expanded genomes compared to susceptible isolates due to the acquisition of
61 mobile genetic elements [9, 10], and patients colonized with these multidrug-resistant (MDR) *E.*
62 *faecalis* are at increased risk of acquiring bloodstream infections [11]. Thus, there is a need for
63 novel strategies to decolonize high-risk individuals of MDR *E. faecalis* [12].

64

65 CRISPR-Cas systems are protective barriers in prokaryotes that function in the adaptive
66 immunity to mobile genetic elements [13-15]. The well-studied Type II CRISPR-Cas system
67 consists of a DNA endonuclease (Cas9) that uses a programmable RNA guide to cleave a double-
68 stranded DNA target sequence [16]. Double-stranded DNA breaks can be lethal to bacteria [17],
69 therefore deploying Type II CRISPR-Cas as a sequence-specific antimicrobial is an attractive
70 alternative to conventional antibiotic therapy. Previous studies have explored this concept using
71 phages to deliver engineered CRISPR systems targeting specific genes [18-21]. Although
72 effective, this method of delivery is likely to be challenging for many species of bacteria including
73 *E. faecalis* due to phage receptor and other cell wall mutations that arise readily in response to
74 lytic phage infection [22-25].

75

76 We previously demonstrated the utility of CRISPR-Cas9 as a sequence-specific *in vitro*
77 antimicrobial in *E. faecalis* by delivering CRISPR guide sequences on mobilizable cloning vectors
78 into *E. faecalis* cells that chromosomally encode *cas9* [26]. However, since MDR *E. faecalis*

79 strains lack complete CRISPR-Cas systems, and specifically *cas9* [27], efficient delivery of the
80 entire CRISPR-Cas9 machinery to MDR *E. faecalis* would likely be required. An alternative
81 method for the delivery of CRISPR-Cas is the use of pheromone-responsive plasmids (PRPs),
82 which naturally achieve high rates of conjugation, have a narrow host range limited to *E. faecalis*,
83 are capable of comprehensively infiltrating *E. faecalis* populations, and can disseminate within
84 intestinal *E. faecalis* in the absence of antibiotic selection [28-31]. The transmission of PRPs is
85 tightly regulated, so the deployment of a PRP CRISPR-Cas antimicrobial could be conditionally
86 tuned for precision targeting and delivery [32].
87
88 PRPs respond to pheromones (short peptides) that are secreted by plasmid-free *E. faecalis*
89 cells [33]. When donor cells detect excess pheromones, indicating the presence of plasmid-free
90 cells in the vicinity [34], they synthesize aggregation substance which facilitates contact between
91 plasmid-bearing and plasmid-free cells [35]. This enables the rapid and efficient transfer of PRPs
92 from donor to recipient. In addition, many PRPs encode accessory genes such as antibiotic
93 resistance and bacteriocins [36, 37]. Previous work demonstrated that the PRP pPD1 conjugated
94 at high rates in the intestines of mice, and pPD1 transfer to plasmid-free *E. faecalis* was beneficial
95 since recipients that failed to acquire the plasmid were killed by the pPD1 encoded bac-21
96 bacteriocin [29].
97
98 We recently discovered that *E. faecalis* cells possessing self-targeting CRISPR-Cas systems
99 are able to transiently tolerate this self-targeting, albeit at a fitness cost, and that antibiotic
100 selection determines the outcome of this conflict by selecting for CRISPR mutants or loss of the
101 CRISPR-targeted gene [26, 38]. We observed CRISPR-Cas self-targeting lethality when the
102 expression of *cas9* was increased using a constitutive promoter [39]. In this study, we engineered
103 pPD1 with a complete, constitutively expressed CRISPR-Cas9 targeting cassette, specific for the
104 enterococcal antibiotic resistance genes *ermB* (encoding erythromycin resistance) and *tetM*

105 (encoding tetracycline resistance). We chose pPD1 as it lacks natively encoded antibiotic
106 resistance yet it harbors the fitness-enhancing bac-21 bacteriocin [40, 41]. Using this engineered
107 PRP, we successfully depleted tetracycline and erythromycin resistance from *E. faecalis*
108 populations *in vitro*. Crucially, this approach worked in the absence of externally applied selection
109 for pPD1 in donor strains, showing practicality for its usage as a potential antimicrobial. Using an
110 *in vivo* intestinal colonization model, we show that these constructs are conjugated to
111 erythromycin-resistant recipient cells *in vivo*. Donors carrying the engineered PRP targeting *ermB*
112 significantly reduced the prevalence of erythromycin-resistant intestinal *E. faecalis*, supporting the
113 utility of the engineered PRP in mitigating MDR *E. faecalis*. Furthermore, we show that these
114 donors are immune to the uptake of *ermB*-encoding PRPs harbored by recipient *E. faecalis* cells.
115 This work is a first step in utilizing conjugative elements for specific delivery of CRISPR-Cas
116 antimicrobials to precisely target antibiotic-resistant bacteria in the intestinal microbiota.
117

118 **Results**

119 **Delivery of CRISPR-Cas9 effectively removes antibiotic resistance *in vitro* by targeting**
120 **plasmid-borne resistance genes**

121 To assess the ability of CRISPR-Cas9 to target and remove antibiotic resistance in *E. faecalis*,
122 we modified the PRP pPD1 by introducing the *cas9* gene and a CRISPR guide RNA under the
123 control of the constitutive *bacA* promoter (Fig. 1A and S1). To facilitate detection of the plasmid
124 we included a chloramphenicol resistance marker (*cat*) for selection. We generated two pPD1
125 derivatives, referred to as pKH88[sp-*tetM*] and pKH88[sp-*ermB*], which carry guide RNAs that
126 target the enterococcal *tetM* and *ermB* genes conferring tetracycline and erythromycin resistance,
127 respectively. These plasmids retain pheromone response functions and bac-21 bacteriocin
128 production (Fig. 1A).
129

130 PRPs are vehicles for the transmission of antibiotic resistance genes in *E. faecalis* populations
131 [30], thus we first assessed the ability of our engineered pPD1 derivatives to eliminate antibiotic
132 resistance genes present on other PRPs. We reasoned that bacteriocin production by pKH88
133 derivatives would promote maintenance of these plasmids in enterococcal populations. The donor
134 strain used in these experiments was *E. faecalis* CK135 and the recipient was *E. faecalis* OG1SSp
135 possessing either pAM771 which carries the erythromycin resistance gene *ermB* or pCF10 which
136 carries the tetracycline resistance gene *tetM* (Fig. 1B). Both CK135 and OG1SSp are isogenic
137 derivatives of the human oral commensal isolate OG1 [42]. We mixed donors and recipients and
138 plated them on Brain Heart Infusion (BHI) agar. After overnight growth we recovered the biofilm
139 and enumerated colony forming units (CFU) of the different cell types (passage 0 in Fig. 1C and
140 1D). We observed a significant reduction in the number of erythromycin- and tetracycline-resistant
141 transconjugants only when recipient cells received a pKH88 derivative encoding a cognate
142 CRISPR guide (Fig. 1C and 1D). Non-cognate guides were ineffective at reducing antibiotic
143 resistance. This loss of antibiotic resistance was maintained after growth of the community in
144 fresh BHI broth overnight (passage 1 in Fig. 1C and 1D), demonstrating this loss is sustained
145 within the transconjugant population.
146
147 **Conjugation of pKH88[sp-*ermB*] to MDR *E. faecalis* resolves erythromycin resistance.**
148 To determine whether the pKH88 plasmids would be effective against MDR *E. faecalis*, we
149 used pKH88[sp-*ermB*] to target *ermB* in *E. faecalis* V583 (Fig. 2A), a vancomycin-resistant
150 hospital-adapted isolate [43]. V583 *ermB* is encoded on the plasmid pTEF1, and V583 does not
151 encode a *tetM* gene [10, 44]. Initially, we observed poor conjugation frequencies of pKH88
152 derivatives into V583 when using CK135 as the donor (Fig. S2A and S2B). *E. faecalis* V583
153 encodes a Type IV restriction endonuclease that restricts entry of 5-methylcytosine-modified DNA
154 and has been characterized as a barrier to DNA uptake in enterococci [39, 45, 46]. We reasoned
155 that this barrier could account for the reduced dissemination of pKH88 to *E. faecalis* V583.

156 Therefore, we utilized the *E. faecalis* OG1 variant strain OG1RF deleted for a 5-methylcytosine
157 DNA methyltransferase (ΔEfaRFI) as the donor strain. This strain cannot methylate plasmid DNA
158 and the plasmid will not be cleaved by the recipient cell following conjugation [46]. We observed
159 that delivery of pKH88[sp-*ermB*] from *E. faecalis* OG1RF(ΔEfaRFI) to *E. faecalis* V583 decreased
160 total erythromycin resistance in the population (Fig. 2B). In addition, *E. faecalis* V583 did not
161 tolerate CRISPR cleavage of *ermB* on pTEF1, indicated by a reduction in viable *E. faecalis* V583
162 cells (Fig. 2B) and which is consistent with our previous findings [26]. This was specific to *ermB*
163 cleavage, as transconjugants that received the nonspecific targeting plasmid pKH88[sp-*tetM*]
164 retained cell viability and erythromycin resistance (Fig. 2C).
165
166 **CRISPR-Cas9 selectively depletes erythromycin resistance within enterococcal**
167 **populations in the dysbiotic murine intestine**
168 We next evaluated the efficacy of pKH88[sp-*ermB*] for the removal of erythromycin resistance
169 using a mouse model of *E. faecalis* intestinal colonization. Mice were administered an antibiotic
170 cocktail in their drinking water for seven days. Antibiotic water was removed for 24 hours and the
171 mice were gavaged with *E. faecalis* OG1SSp(pAM771) recipients, modeling a bloom of antibiotic-
172 resistant *E. faecalis* in the intestine. After an additional 24 hours, mice were gavaged with donor
173 *E. faecalis* CK135 carrying either pKH88[sp-*ermB*] (targeting) or pKH88[sp-*tetM*] (non-targeting),
174 and fecal samples were collected over the course of 23 days. A schematic of this experiment is
175 depicted in Fig. S3. Whereas *E. faecalis* CK135 donors colonized the intestine to a sustained high
176 density, *E. faecalis* OG1SSp(pAM771) recipient cells gradually declined in abundance over time
177 (Fig. S4A and S4B). We attribute this gradual reduction in the recipient population to competitive
178 killing by bacteriocin-producing donors as previously described for pPD1 carrying *E. faecalis* [29],
179 and/or competition from the reemerging native microbiota that was initially displaced by the
180 antibiotic treatment. Robust numbers of pKH88-containing transconjugants (i.e. chloramphenicol
181 resistant OG1SSp) appeared two days post-colonization of the donor strains for both the

182 pKH88[sp-*ermB*] and pKH88[sp-*tetM*] groups (Fig. S4C). However, the initial frequency of
183 transconjugants per recipient reached only ~10^{-5}, indicating that overall conjugation frequency
184 was relatively low (Fig. 3A). This was followed by a gradual increase in the transconjugant to
185 recipient ratio, approaching 1:1 (Fig. 3A), as the total recipient population decreased over time
186 (Fig. S4A). This suggests that the initial transconjugant population remains fixed due to a pKH88
187 bacteriocin-mediated fitness advantage upon restoration of the indigenous microbiota.

188 A significant reduction in the number of erythromycin-resistant recipients compared to the total
189 recipient population was observed in the pKH88[sp-*ermB*] targeting group and not the pKH88[sp-
190 *tetM*] non-targeting group (Figs. 4A and 4B). This indicates that introduction of the *ermB* targeting
191 spacer in conjunction with *cas9* on a conjugative plasmid is sufficient to reduce erythromycin
192 resistance from *E. faecalis* OG1SSp(pAM771) transconjugants in the intestine. To our surprise,
193 the difference in erythromycin-resistant recipients between the pKH88[sp-*ermB*] and pKH88[sp-
194 *tetM*] groups was significantly less than the difference between the total erythromycin resistant
195 intestinal *E. faecalis,* which includes both donors and recipients. The magnitude of this difference
196 was ~3 log by the end of the experiment (Fig 5A). This indicated that there was likely a substantial
197 amount of conjugation by pAM771 into the donor population. Indeed, in as little as two days,
198 donors acquired erythromycin resistance, but only in the pKH88[sp-*tetM*] non-targeting group (Fig.
199 5B). These data indicate that *E. faecalis* CK135(pKH88[sp-*tetM*]) donors are susceptible to
200 counter-conjugation of pAM771 from recipients but that CK135(pKH88[sp-*ermB*]) donors are
201 immune, presumably due to CRISPR-Cas targeting of *ermB* on pAM771 upon counter-
202 conjugation. We conclude that our CRISPR antimicrobial dramatically reduces the prevalence of
203 a targeted conjugative plasmid within the input *E. faecalis* population, likely through a
204 multifactorial mechanism involving a bacteriocin-dependent competitive advantage, cleavage of
205 the targeted plasmid, and prevention of counter-plasmid acquisition. This in turn substantially
206 impacts the distribution of drug resistance, as mice colonized with pKH88[sp-*ermB*] donors had

207 0.1% of the total levels of erythromycin-resistant *E. faecalis* possessed by mice colonized with
208 pKH88[sp-*tetM*] donors.
209
210 We next assessed whether the microbial complexity of the intestinal environment plays a role
211 in the efficacy of the CRISPR-Cas antimicrobial, since curtailing antibiotic therapy spontaneously
212 restores the microbiota in the antibiotic dysbiosis model [47]. We first colonized germ-free mice
213 with *E. faecalis* OG1SSp(pAM771) recipient cells and 24 hours later introduced donor *E. faecalis*
214 CK135 carrying either pKH88[sp-*ermB*] or pKH88[sp-*tetM*]. Gnotobiotic mice maintained a high
215 intestinal burden of both donors and recipients for the entire 23 days, in contrast to the antibiotic
216 dysbiosis model where the recipient population is reduced over time (Fig. S5A, S5B and S4A).
217 Despite sustained high density colonization of both donors and recipients, the total number of
218 transconjugants was similar to those observed in the antibiotic dysbiosis model (Fig. S5C and
219 S4C) but the transconjugant per recipient ratio remained stably low at 10^{-5} (Fig. 3B). Sustained
220 high recipient density indicated that the apparent competitive advantage possessed by pKH88-
221 containing donors via bac-21 does not manifest in gnotobiotic mice. These data suggest that even
222 with prolonged high densities of both donors and recipients in the absence of microbial
223 complexity, there may be biogeographical barriers that restrict the dissemination of pKH88
224 derivatives. This also implies that the competitive advantage possessed by the donor is most
225 prevalent in the presence of the microbiota, and therefore some degree of microbial complexity
226 is likely necessary for the functionality of our CRISPR-Cas antimicrobial. Interestingly, similar to
227 observations in the antibiotic dysbiosis model, pKH88 donors acquired erythromycin resistance,
228 but only in the pKH88[sp-*tetM*] non-targeting group (Fig. 5C).
229 These results demonstrate that our engineered CRISPR-Cas antimicrobial is a robust barrier
230 to *in vivo* plasmid acquisition and reduces the *in vivo* prevalence of a specifically targeted drug
231 resistance trait. From a technological viewpoint this is the ideal situation of a prospective

232 “probiotic” donor; a strain that can effectively disseminate a CRISPR targeting plasmid while being
233 protected from acquisition of undesirable traits.
234
235 **CRISPR-Cas antimicrobial prevents antibiotic-mediated intestinal expansion of**
236 **transconjugants.**
237 A hallmark of enterococcal intestinal colonization is its ability to expand its population upon
238 antibiotic perturbation of the indigenous microbiota [4, 25]. This antibiotic-mediated expansion is
239 attributed to both intrinsic and acquired antibiotic resistance. To determine the ability of our
240 CRISPR-Cas antimicrobial to minimize the outgrowth of erythromycin resistant *E. faecalis* in the
241 intestine following antibiotic therapy, we utilized both our antibiotic dysbiosis and gnotobiotic
242 mouse models. We treated the mice 27 days post co-colonization with a single 40 µg dose of oral
243 erythromycin and determined the abundance of erythromycin resistant transconjugants and total
244 erythromycin resistant *E. faecalis*. Prior to antibiotic treatment there were no erythromycin
245 resistant transconjugants recovered from mice receiving donors carrying the targeting plasmid
246 pKH88[sp-*ermB*], whereas two of eight mice receiving donors carrying the non-targeting plasmid
247 pKH88[sp-*tetM*] had recoverable erythromycin resistant transconjugants (Fig. 6). Upon oral
248 erythromycin treatment, there was a ~4 log expansion of erythromycin resistant transconjugants
249 in the pKH88[sp-*tetM*] non-targeting group, and no erythromycin resistant transconjugants
250 emerged in the mice colonized with *E. faecalis* CK135 pKH88[sp-*ermB*] donors (Fig. 6).
251 Additionally, the total erythromycin-resistant *E. faecalis* in the pKH88[sp-*tetM*] non-targeting group
252 was significantly higher compared to the pKH88[sp-*ermB*] targeting group (Fig. 6). Together these
253 data indicate that transconjugants receiving pKH88[sp-*ermB*] have been depleted of erythromycin
254 resistance and that pKH88[sp-*ermB*] can significantly reduce the overall total erythromycin
255 resistance within intestinal *E. faecalis*, pre- and post-antibiotic therapy. Interestingly, any
256 remaining viable transconjugants in the pKH88[sp-*ermB*] targeting group were impaired in their
257 ability to increase in cell density compared to mice receiving pKH88[sp-*tetM*] non-targeting donors

258 (Fig. 6), further indicating that targeted *E. faecalis* cells fail to expand their population due to loss
259 of pAM771 encoded erythromycin resistance.

260 In gnotobiotic mice, following oral erythromycin treatment, again we did not recover any
261 erythromycin resistant transconjugants in the pKH88[sp-*ermB*] targeting group (Fig. S6).
262 Interestingly, in gnotobiotic mice, we did not observe outgrowth of erythromycin resistant
263 transconjugants in the pKH88[sp-*tetM*] non-targeting group, even though we did not recover any
264 erythromycin resistant transconjugants in the pKH88[sp-*ermB*] targeting group, similar to the
265 antibiotic dysbiosis model (Fig. S6). We attribute this to the high level of both donors and
266 recipients in the gnotobiotic intestine that were unaffected by oral erythromycin treatment (Fig.
267 S5A and S5B). Intestinal colonization saturation by donors and recipients may be spatially
268 restricting, thus promoting colonization resistance against transconjugant expansion upon
269 erythromycin treatment in the pKH88[sp-*tetM*] non-targeting group.

270

271 **Discussion**

272 This study presents a strategy for the delivery of a CRISPR-based antimicrobial that targets
273 antibiotic-resistant *E. faecalis*. We show that this conjugative antimicrobial significantly reduces
274 antibiotic resistance from *in vitro* and *in vivo* enterococcal communities in the absence of
275 externally applied selection for donor strains. Our approach for the delivery of a CRISPR-Cas
276 antimicrobial differs from studies which employed either transformable plasmids or phages for the
277 recognition and delivery to target cells [18-21]. Although these studies provided the foundation for
278 the use of CRISPR antimicrobial activity against bacteria, limitations were noted. In the case of
279 phage-mediated CRISPR antimicrobial dissemination, this technique suffered from incomplete
280 phage dissemination. This scenario is expected due to selective pressures imposed on bacteria
281 by phages during infection, including receptor mutations [22-25]. Such issues have recently been
282 addressed using multi-phage delivery systems or by co-opting phage particles for the delivery of
283 CRISPR-Cas targeting modules embedded within mobilizable genome islands [48, 49]. Although

284 the use of a phage-based delivery system for *E. faecalis* should be possible, enterococcal phage
285 biology is understudied and limited information exists concerning phage host ranges and genetics.
286 Therefore, we chose to engineer a PRP for the dissemination of an *E. faecalis*-specific CRISPR-
287 Cas antimicrobial because the conditions governing PRP regulation have been extensively
288 studied, PRPs are specific for *E. faecalis,* PRPs achieve high conjugation rates*,* PRPs can be
289 genetically modified to diversify host tropism by altering pheromone responsiveness, and PRPs
290 are stably maintained and disseminate in the absence of selection [50, 51].
291
292 The intestine is a reservoir for MDR *E. faecalis*, and *E. faecalis* infections often follow
293 exposure to broad spectrum antibiotics. We applied our CRISPR-Cas antimicrobial method for
294 the removal of an antibiotic resistance trait from intestinal *E. faecalis*. The mouse models of
295 intestinal colonization used were designed to mimic enterococcal overgrowth in the human
296 intestine following antibiotic therapy [3, 4]. Antibiotics disrupt the intestinal microbiota, allowing
297 antibiotic-resistant enterococci to become the dominant colonizer. In our study, we used *E.*
298 *faecalis* donor strains for the conjugative delivery of CRISPR-Cas to remove erythromycin
299 resistance from an abundant intestinal *E. faecalis* target population. The conjugative transfer of
300 an *ermB*-specific CRISPR-Cas targeting system into recipient *E. faecalis* cells showed a modest
301 reduction in erythromycin resistant intestinal *E. faecalis*. However, within this erythromycin
302 resistant population, all transconjugants that acquired the cognate CRISPR-Cas targeting plasmid
303 were fully depleted of erythromycin resistance. This indicates that, although the frequency of
304 conjugation was low compared to the total available recipient population and not as robust as *in*
305 *vitro* conjugation frequencies, the specificity of our CRIPSR-Cas targeting system in the intestine
306 was absolute. If successful conjugation occurs there is complete loss of erythromycin resistance.
307 In addition, we observed that donors carrying a cognate CRISPR antimicrobial plasmid were
308 protected from acquisition of erythromycin resistance from recipients. This is an important feature

309 of our deliverable system since the transmission of undesirable target traits to donor cells would
310 impede the usefulness of such a delivery strategy within complex microbial environments.
311
312 There are several reasons why intestinal conjugation frequency might be suboptimal in our
313 study. First, our analysis measured transconjugants from fecal samples, not the intestinal
314 mucosa. The spatial distribution of donor and recipient *E. faecalis* cells colonizing the intestinal
315 mucosal layer is unknown and evidence suggests that conjugation frequency could be impacted
316 by the regional location of *E. faecalis* intestinal colonization [28]. Second, pKH88 derivatives
317 require recipient-derived cPD1 pheromone to induce conjugation [52]. We know little about the
318 kinetics of pheromone signaling in the intestine. Low rates of conjugation could reflect low local
319 concentrations of cPD1 in the vicinity of donors resulting in few donors that effectively conjugate,
320 thereby minimizing conjugation frequencies. Alternatively, excess donors can inhibit conjugation
321 to recipient cells [53]. This is due to the production of inhibitory self-signaling peptides produced
322 by the donors that compete with recipient pheromones and reduce conjugation frequencies.
323 Exploring the efficiency of conjugation upon altering donor to recipient ratios could lead to more
324 permissive conjugation in the intestine.
325
326 Moving forward, improvements to our current system are justified if an engineered conjugative
327 CRISPR antimicrobial is to be clinically effective for precision removal of antibiotic resistance
328 genes in *E. faecalis* or other MDR bacteria. Modifications include multiplexing CRISPR guide
329 RNAs to target different resistance genes and/or the same genes at multiple sites, as well as
330 increasing the overall conjugation frequency by altering PRP regulation, dosing of donor strains,
331 or the plasmid replicon itself. Theoretically, resistance to bac-21 could compromise the efficacy
332 of our system, and future iterations should utilize alternative bacteriocins as well as bacteriocin-
333 free constructs. Since our model system has relied on laboratory-domesticated strains of *E.*
334 *faecalis* OG1 [42], it will be imperative to determine the efficacy of this system against more “wild”

335 clinical isolates which are the intended targets of such a therapy. For safe and effective delivery
336 of the CRISPR antimicrobial in the clinic, the use of an attenuated *E. faecalis* strain or a related
337 member of the Lactobacillales approved for probiotic use would be essential. Finally, this system
338 could be adapted for the targeted removal of antibiotic resistance from bacteria other than *E.*
339 *faecalis.* Gram-positive conjugative plasmids like pIP501, which belongs to the rep_1 family, are
340 broadly disseminated conjugative plasmids [50, 51, 54]. pIP501 is transferrable among
341 enterococci, other lactic acid bacteria, staphylococci, and clostridia [54]. The modification of a
342 promiscuous mobilizable plasmid such as pIP501 with CRISPR-Cas antimicrobial function could
343 expand the capabilities of our current system by facilitating broad targeting of multiple Gram-
344 positive pathogens.
345

346 **Materials and Methods**

347 **Bacterial culture methods and molecular biology techniques**

348 *Escherichia coli* cultures were incubated with shaking at 220 RPM at 37°C in Lysogeny Broth
349 (LB). *E. faecalis* cultures were grown in Brain Heart Infusion (BHI) broth at 37°C without shaking.
350 PCR was performed using Taq Polymerase (New England Biolabs), and Q5 DNA Polymerase
351 (New England Biolabs) for molecular cloning. The PureLink PCR Purification kit (Invitrogen) was
352 used to purify DNA fragments and the GeneJet Plasmid Purification kit (Fisher) was used for
353 plasmid purification. Primers were obtained from Sigma Aldrich. Sanger sequencing was
354 performed at the Massachusetts General Hospital DNA Core Facility. *E. coli* EC1000 was used
355 for routine plasmid propagation [55]. *E. faecalis* and *E. coli* competent cells were prepared as
356 described previously [26]. Genomic DNA was extracted using the MO BIO Microbial DNA Isolation
357 Kit (Qiagen). Antibiotics were used at the following concentrations: chloramphenicol, 15 µg/ml;
358 streptomycin, 500 µg/ml; spectinomycin, 500 µg/ml; vancomycin, 10 µg/ml; erythromycin, 50
359 µg/ml; rifampicin, 50 µg/ml; fusidic acid, 25 µg/ml; tetracycline, 10 µg/ml.
360

361 **Strain and plasmid construction**

362 All strains used in this study can be found in Table S1. The donor strain used for competition
363 experiments was a rifampicin and fusidic acid-resistant derivative of CK135 [56]. All plasmids
364 used in this study can be found in Table S2. Fragments obtained from the plasmids pHA101 [57],
365 pPD1 (24), P_{bacA}-pG19, pKH12 [26] and pGR *tetM*/*ermB* [39] were amplified using Q5 DNA
366 polymerase and assembled using NEB HiFi DNA Assembly Master Mix (New England Biolabs).
367 For simplicity, assembly of the CRISPR-targeting construct (P_{bacA}-*cas9*-*cat*-guide RNA) was
368 divided into two steps. First, P_{bacA}*cas9* was introduced into pPD1 by homologous recombination.
369 Then the *cat* gene from pKH12 and the P_{bacA}-driven CRISPR guide RNA was integrated
370 downstream of P_{bacA}-*cas9*. Following the generation of this construct, we discovered that the first
371 integration site obstructed the pheromone response, therefore the entire engineered region (P_{bacA}-
372 *cas9*-*cat*-guide RNA) was amplified as one fragment and integrated into native pPD1 between
373 orfs *ppd4* and *ppd5* using the integration plasmid pCOP88 (Fig. S1). The modified pPD1
374 derivatives are designated as pKH88[sp-*tetM*] and pKH88[sp-*ermB*], which target *tetM* or *ermB*
375 respectively. Primer sequences are listed in Table S3.

376

377 ***In vitro* Competition Assays**

378 Overnight cultures of donors and recipients were pelleted, resuspended in fresh BHI broth,
379 and cultured for 1.5 hours. Donors and recipients were mixed in a volume ratio of 1:9, and the
380 mixtures were spread on BHI agar. Following overnight incubation, lawns were scraped into sterile
381 PBS and serial dilutions (10 µl each) were spotted on BHI plates supplemented with antibiotics to
382 enumerate colony forming units (CFU) of donors, recipients, and transconjugants as well as total
383 CFU of tetracycline- and erythromycin-resistant cells. For individual passages, the recovered
384 conjugation mixture was diluted 1:1000 in fresh BHI broth without selection and incubated
385 overnight, after which the constituents of the mixture were again enumerated.

386

387 **Mouse models of *E. faecalis* colonization**

388 All animal protocols were approved by the Institutional Animal Care and Use Committee of
389 the University of Colorado School of Medicine (protocol number 00253).

390

391 For the antibiotic dysbiosis model, 7 days prior to colonization recipients, 6-8 week old
392 C57BL6/J mice were gavaged with 150 µL of an antibiotic cocktail (streptomycin 1 mg/mL,
393 gentamicin 1 mg/mL, erythromycin 200 µg/mL) and given a water bottle *ad libitum* with the same
394 antibiotic cocktail for 6 days following the initial gavage. 24 hours prior to colonization recipients,
395 antibiotic water was removed and replaced with standard sterile reverse osmosis water. Donor
396 and recipient strains were cultured overnight in BHI and mice were gavaged with ~10^9 CFU in
397 100 µL of sterile PBS. All mice were first gavaged with *E. faecalis* OG1SSp(pAM771). After 24
398 hours, mice (n=13, 7 female (F) and 6 male (M)) were gavaged with CK135(pKH88[sp-*ermB*])
399 while the second group (n=12, 5 F and 7 M) were gavaged with CK135(pKH88[sp-*tetM*]). Fecal
400 samples were collected at the designated time points, resuspended in 1 mL of sterile PBS, and
401 plated on BHI agar supplemented with rifampicin, fusidic acid, erythromycin, chloramphenicol,
402 streptomycin and spectinomycin in combinations that would select for the desired populations and
403 using the concentrations described above. After 27 days, 8 mice (4 F and 4 M) from the pKH88[sp-
404 *ermB*] targeting group and 7 mice (3 F and 4 M) from the pKH88[sp-*tetM*] non-targeting group
405 were gavaged with erythromycin (40 µg). Fecal samples were collected from all mice pre-
406 erythromycin gavage. Post-erythromycin gavage, fecal samples were collected from all 8 mice
407 from the pKH88[sp-*ermB*] targeting group and from 6 of 7 mice from the pKH88[sp-*tetM*] non-
408 targeting group due to the inability of a single mouse from this group to defecate.

409

410 All germ-free mice were gavaged with *E. faecalis* OG1SSp(pAM771). 24 hours later these
411 gnotobiotic mice were gavaged with *E. faecalis* CK135(pKH88[sp-*ermB*]) (n=8, 4 F and 4 M) or
412 *E. faecalis* CK135(pKH88[sp-*tetM*]) (n=8, 4 F and 4 M). Fecal samples were collected at the

413 designated time points and donors, recipients, and transconjugants were enumerated on selective
414 media as described above. After 27 days, all 8 gnotobiotic mice from each group were gavaged
415 with 40 µg of erythromycin.
416
417 **Statistical analysis**
418 GraphPad-Prism version 7.02 was used to determine statistical significance using a two-tailed
419 paired or unpaired Student's t-test with or without a Mann-Whitney correction.
420
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425 for providing *E. faecalis* OG1SSp(pAM771), and Michael Gilmore for providing *E. coli* EC1000
426 and *E. faecalis* V583.
427
428 **Figure Legends**
429 **Figure 1. CRISPR-Cas engineered conjugative pPD1 derivative plasmids target and**
430 **eliminate antibiotic resistance genes in *in vitro* co-culture. (A)** Schematic of the pPD1
431 derivative pKH88. pKH88 encodes *cas9* and a CRISPR guide RNA under the control of the
432 constitutive enterococcal *bacA* promoter. The guide RNA consists of a CRISPR repeat region and
433 a unique spacer sequence with complementarity to the *ermB* gene (pKH88[sp-*ermB*]) of pAM771
434 or the *tetM* gene (pKH88[sp-*tetM*]) of pCF10. pKH88 derivatives encode genes necessary for
435 conjugative transfer, chloramphenicol resistance (*cat*) and biosynthesis of the bacteriocin bac-21.
436 **(B)** Cartoon depicting the delivery of pKH88 derivatives to target recipient *E. faecalis* cells.
437 Abundance of erythromycin **(C)** or tetracycline **(D)** resistant *E. faecalis* OG1SSp recipients
438 following acquisition of pKH88[sp-*ermB*] or pKH88[sp-*tetM*]. Removal of erythromycin and

439 tetracycline resistance from the transconjugant population depends on the introduction of a
440 cognate spacer targeting pKH88 derivative. Markers; R– rifampicin resistance, F – fusidic acid
441 resistance, Cam – chloramphenicol resistance. Abx – antibiotic resistance. *p=0.01, **p=0.009,
442 ***p=0.006 by unpaired Student's t-test.
443
444 **Figure 2. Engineered CRISPR-Cas targeting plasmid specifically reduces drug resistance**
445 **in MDR *E. faecalis*. (A)** Cartoon depicting the transfer, targeting and removal of erythromycin
446 resistance from MDR *E. faecalis* V583. Conjugation of *E. faecalis* V583 with pKH88[sp-*ermB*] **(B)**
447 but not pKH88[sp-*tetM*] **(C)** reduces erythromycin resistant *E. faecalis* V583 over the course of
448 four serial passages in BHI broth. Markers; Erm – erythromycin resistance, Van – vancomycin
449 resistance, R – rifampicin resistance, F – fusidic acid resistance, S – streptomycin resistance, Sp
450 – spectinomycin resistance, Cam – chloramphenicol resistance.
451
452 **Figure 3. Saturation of the *E. faecalis* OG1SSp(pAM771) recipient population by pKH88 is**
453 **time-dependent.** The ratio of transconjugants to recipients was calculated for both the pKH88[sp-
454 *ermB*] targeting group (*ermB*) and the pKH88[sp-*tetM*] non-targeting group (*tetM*) for 23 days of
455 co-colonization in antibiotic-treated **(A)** and gnotobiotic **(B)** mice.
456
457 **Figure 4. pKH88[sp-*ermB*] reduces erythromycin-resistant *E. faecalis* in the murine**
458 **intestine. (A)** Antibiotic treated mice co-colonized with *E. faecalis* OG1SSp(pAM771) and
459 CK135(pKH88[sp-*ermB]*) – targeting group. **(B)** Antibiotic treated mice co-colonized with *E.*
460 *faecalis* OG1SSp(pAM771) and CK135(pKH88[sp-*tetM*]) – non-targeting group. The total
461 OG1SSp(pAM771) recipient population and recipients that are erythromycin resistant were
462 enumerated by selective plating of mouse feces over the course or 23 days of co-colonization.
463 The solid horizontal line indicates the limit of detection. Markers; S – streptomycin, Sp –

464 spectinomycin, Erm – erythromycin resistance. *p=0.02, **p=0.03, ***p=0.009 by paired Student's
465 t-test. NS – not significant.
466
467 **Figure 5. *E. faecalis* CK135 pKH88[sp-*ermB*] donors are immune to acquisition of**
468 **erythromycin resistance from recipients in the murine intestine. (A)** Total intestinal load of
469 erythromycin resistance among input *E. faecalis* donors and recipients from antibiotic-treated
470 mice. Erythromycin resistance is acquired by donors only in the pKH88[sp-*tetM*] non-targeting
471 group in both antibiotic-treated **(A)** and gnotobiotic **(B)** mice. The solid horizontal line indicates
472 the limit of detection. Markers; Erm – erythromycin. *p=0.0003, **p=<0.0001, by unpaired
473 Student's t-test with a Mann Whitney correction.
474
475 **Figure 6. Transconjugants that receive pKH88[sp-*ermB*] do not bloom in the intestines of**
476 **dysbiotic mice following oral erythromycin treatment.** 27 days post co-colonization with *E.*
477 *faecalis* OG1SSp(pAM771) and CK135(pKH88[sp-*ermB*]) (targeting group) or with *E. faecalis*
478 OG1SSp(pAM771) and CK135(pKH88[sp-*tetM*]) (non-targeting group), mice received a single 40
479 µg dose of oral erythromycin. The number of recipients, transconjugants and erythromycin
480 resistant transconjugants were enumerated from fecal pellets before and after oral erythromycin
481 treatment. The solid horizontal line indicates the limit of detection. Markers; Erm – erythromycin,
482 S – streptomycin, Sp – spectinomycin, Cam – chloramphenicol. *p=0.01, **p=0.004, by unpaired
483 Student's t-test with a Mann Whitney correction.
484
485 **Figure S1. The allelic exchange vector pCOP88 integrates the P_{bacA}-*cas9*-*cat*-guide RNA**
486 **module into pPD1.** pCOP88 derivatives contain elements from the allelic exchange vector pLT06
487 [58]. These include a temperature sensitive *repA* (*repA*^{ts}) allele that facilitates forced integration
488 of pCOP88 derivatives into target DNA sequences under non-permissive temperature (42°C) and
489 a P-*pheS* cassette that allows for the cellular utilization of *p*-chloro-phenylalanine as a counter-

490 selectable marker. The P*bacA-cas9-cat*-guide RNA module is flanked by 1-kb pPD1 homology
491 regions used for the homologous recombination of the module into native pPD1.
492
493 **Figure S2. *E. faecalis* V583 restricts conjugation of pKH88 derivatives originating from *E.***
494 ***faecalis* CK135. (A)** The ratio of *E. faecalis* V583 and OG1SSp transconjugants following *in vitro*
495 co-culture with *E. faecalis* CK135(pKH88) derivative donors. **(B)** Comparison of normalized *E.*
496 *faecalis* V583 transconjugant and *E. faecalis* V583 recipient numbers following *in vitro* co-culture
497 with *E. faecalis* CK135(pKH88) derivative donors.
498
499 **Figure S3. Schematic cartoon of the antibiotic dysbiosis mouse model.**
500
501 **Figure S4. Intestinal colonization of *E. faecalis* populations following co-colonization with**
502 **OG1SSp(pAM771) and CK135(pKH88) donors in antibiotic-treated mice. (A)** Recipients. **(B)**
503 Donors. **(C)** Transconjugants. Markers; S – streptomycin, Sp – spectinomycin, R – rifampicin
504 resistance, F – fusidic acid resistance, Cam – chloramphenicol.
505
506 **Figure S5. Intestinal colonization of *E. faecalis* populations following co-colonization with**
507 **OG1SSp(pAM771) and CK135(pKH88) donors in gnotobiotic mice. (A)** Recipients. **(B)**
508 Donors. **(C)** Transconjugants. The solid horizontal line indicates the limit of detection. Markers; S
509 – streptomycin, Sp – spectinomycin, R – rifampicin resistance, F – fusidic acid resistance, Cam –
510 chloramphenicol.
511
512 **Figure S6. Non-targeted *E. faecalis* intestinal transconjugant populations do not bloom**
513 **following oral erythromycin treatment of gnotobiotic mice.** 27 days post co-colonization with
514 *E. faecalis* OG1SSp(pAM771) and CK135(pKH88[sp-*ermB*]) (targeting group) or with *E. faecalis*
515 OG1SSp(pAM771) and CK135(pKH88[sp-*tetM*]) (non-targeting group), mice received a single 40

516 µg dose of oral erythromycin. The number of recipients, transconjugants and erythromycin
517 resistant transconjugants were enumerated from fecal pellets before and after oral erythromycin
518 treatment. The solid horizontal line indicates the limit of detection. Markers; Erm – erythromycin,
519 S – streptomycin, Sp – spectinomycin, Cam – chloramphenicol.
520

521 **Table S1. Strains used in this study**

Organism	Strain Name	Description	Ref
E. coli	EC1000[a]	*E. coli* cloning host, providing *repA in trans.*F- , *araD139 (ara ABC-leu)7679, galU, galK, lacX74, rspL, thi, repA* of pWV01 in *glgB, km*	(51)
E. faecalis	V583[a]	MDR bloodstream isolate, resistant to vancomycin, gentamicin, and erythromycin	(41)
	OG1SSp[b]	Spectinomycin-streptomycin-resistant derivative of OG1	(57)
	CK135[c]	OG1 $rpoB_{H486Y}$ (spontaneous Rif^r derivative)	(52)
	CK135RF	Spontaneous fusidic acid-resistant derivative of CK135	This study
	OG1RF ΔEfaRFI	OG1RF EfaRFI (OG1RF_11622-11621) deletion mutant	(43)

[a] Strain provided by Michael Gilmore, Massachusetts Eye and Ear Infirmary and Harvard Medical School

[b] Strain provided by Gary Dunny, University of Minnesota, Minneapolis

[c] Strain provided by Christopher Kristich, Medical College of Wisconsin

522 **Table S2. Plasmids used in this study**

Plasmid	Description	Ref

pLT06[a]	Encodes temperature-sensitive *repA* and *pheS** counter-selection	(55)
pHA101	pLT06 + *oriT*	(54)
pCOP88[sp-*tetM*]	pLT06 derivative used to knock in CRISPR-targeting construct with a spacer targeting *tetM*	This study
pCOP88[sp-*ermB*]	pLT06 derivative used to knock in CRISPR-targeting construct with a spacer targeting *ermB*	This study
pKH88[sp-*ermB*]	pPD1 derivative with CRISPR-targeting cassette for *ermB*; also encodes *cat*	This study
pKH88[sp-*tetM*]	pPD1 derivative with CRISPR-targeting cassette for *tetM*; also encodes *cat*	This study
pAM771[a]	Non-cytolytic derivative of the PRP pAD1 mutagenized with Tn*917,* encodes erythromycin resistance via *ermB*	[59]
pCF10	PRP; encodes tetracycline resistance via *tetM*	(58)
pPD1[b]	PRP; encodes Bac-21 bacteriocin	(59)

[a] Plasmid provided by Michael Gilmore, Massachusetts Eye and Ear Infirmary and Harvard Medical School

[b] Plasmid provided by Christopher Kristich, Medical College of Wisconsin

523 **Table S3. Primers used in this study**

Primer Name	**Sequence (5'-3')**	**Use**
pCOP88 Ori for	TGCAGCGTTTCTTTGAATAG	Create pCOP88
pCOP88 Ori rev	GCTTTGCAAAGTCTGAAAAC	Create pCOP88

pCOP88 PheS cat for	TGCCACCTTCGTTTTCAGACTTTGCAAAGCCAAGTTAAGGGATGCAGTTTAAAAATG	Create pCOP88
pCOP88 PheS cat rev	GGCATGATGGTTGCCGGTCGATAAACCCAGCGAAC	Create pCOP88
pCOP88 cas9 for	AAACATTACTCTATAGCAAACACAGTTAACCACG	Create pCOP88
pCOP88 cas9-cat-PSRT rev	CAATATCAGAATCAATCCACTCCTGAATCCCATTC	Create pCOP88
pCOP88 Arm 1 for	CTGGGTTTATCGACCGGCAACCATCATGCCTAAATTTTTATC	Create pCOP88
pCOP88 Arm 1 rev	GTTAACTGTGTTTGCTATAGAGTAATGTTTTAATTTTTTCTCTTTTTCAG	Create pCOP88
pCOP88 Arm 2 for	GGATTCAGGAGTGGATTGATTCTGATATTGCCAATC	Create pCOP88
pCOP88 Arm 2 rev	CTAAAACGTCCTATTCAAAGAAACGCTGCAAGTCAACTAGAATCTGCTG	Create pCOP88
cas9 rev	TTTATTAAAGTTCATCTAGTCGACAACTTTACGGCGTGTTTC	Create pCOP88
cat for	AAAGTTGTCGACTAGATGAACTTTAATAAAATTGATTTAGACAAT	Create pCOP88
cat rev	TCAACAAACTGGCCCGTTTGTTGAACTACTTTATAAAAGCCAGTCATTAGGC	Create pCOP88
PSRT for	AGTAGTTCAACAAACGGGCC	Create pCOP88 modification

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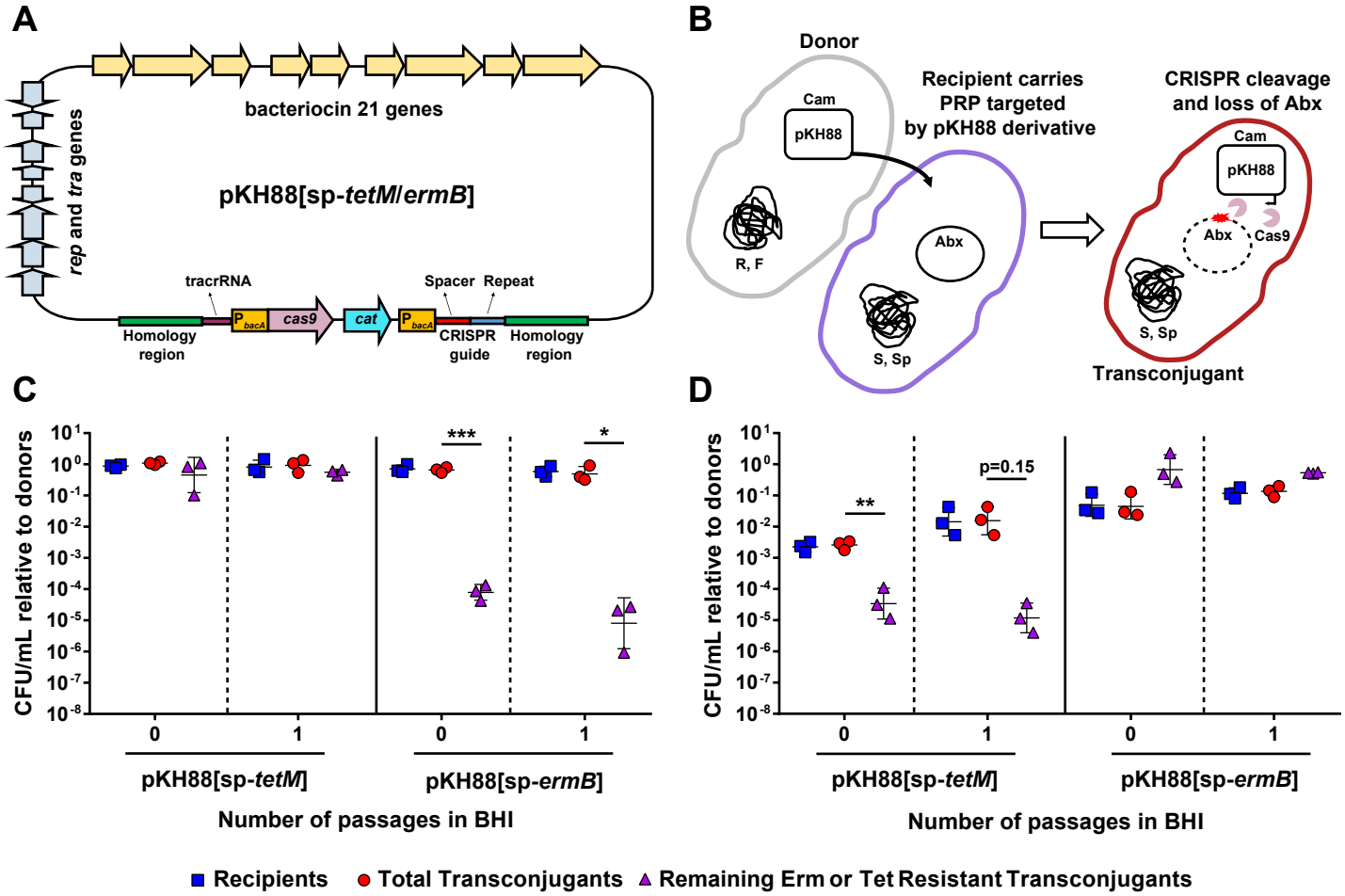
A
bacteriocin 21 genes
rep and tra genes
pKH88[sp-tetM/ermB]
tracrRNA
Spacer
Repeat
Homology region
cas9
cat
CRISPR guide
Homology region
B
Donor
Cam
pKH88
R, F
Recipient carries PRP targeted by pKH88 derivative
Abx
S, Sp
CRISPR cleavage and loss of Abx
Cas9
Transconjugant
C
CFU/mL relative to donors
***
*
pKH88[sp-tetM]
pKH88[sp-ermB]
Number of passages in BHI
D
**
p=0.15
Recipients
Total Transconjugants
Remaining Erm or Tet Resistant Transconjugants

Figure 1

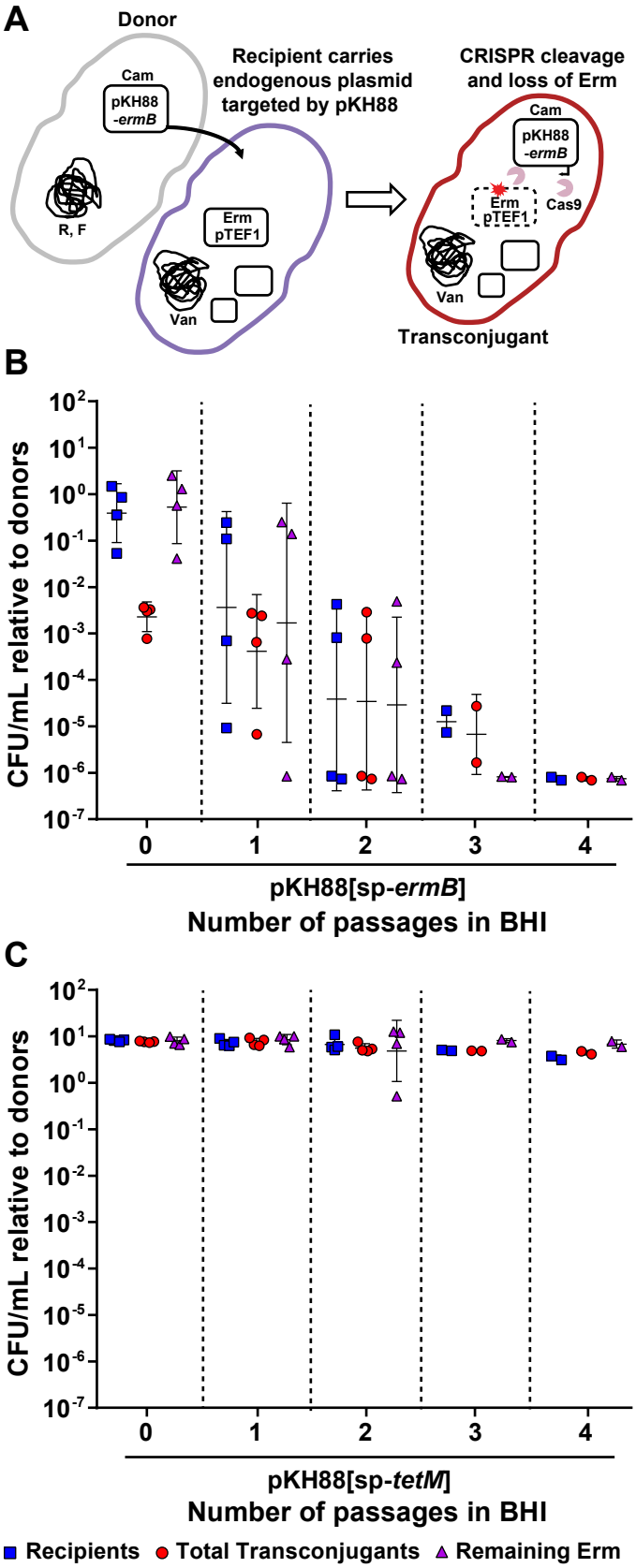
A
Donor
Cam
pKH88
-ermB
R, F
Recipient carries
endogenous plasmid
targeted by pKH88
Erm
pTEF1
Van
CRISPR cleavage
and loss of Erm
Cam
pKH88
-ermB
Erm
pTEF1
Cas9
Van
Transconjugant
B
CFU/mL relative to donors
pKH88[sp-ermB]
Number of passages in BHI
C
CFU/mL relative to donors
pKH88[sp-tetM]
Number of passages in BHI
Recipients
Total Transconjugants
Remaining Erm

Figure 2

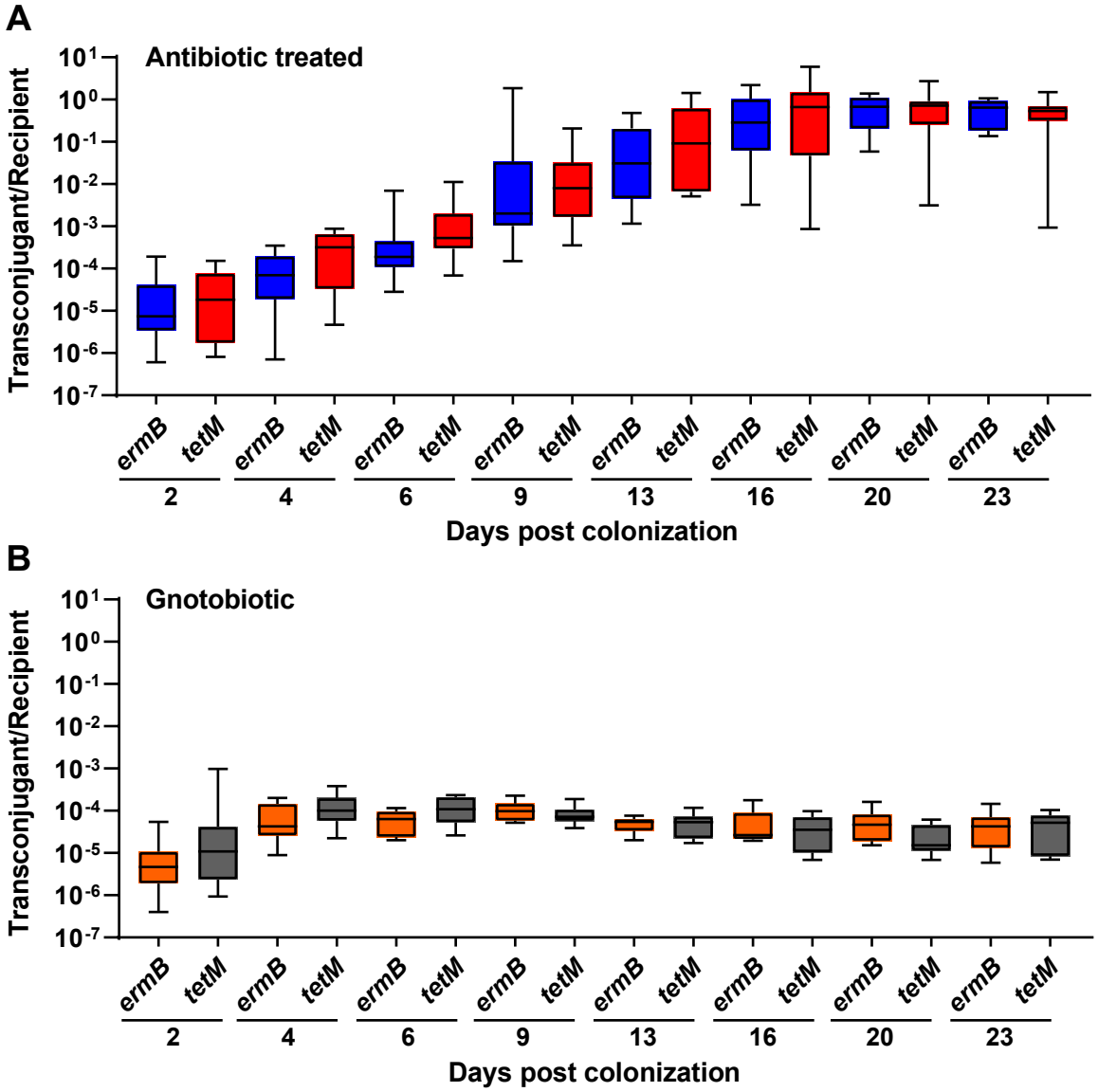
A
Antibiotic treated
Transconjugant/Recipient
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
ermB
tetM
2
4
6
9
13
16
20
23
Days post colonization
B
Gnotobiotic
Transconjugant/Recipient
Days post colonization

Figure 3

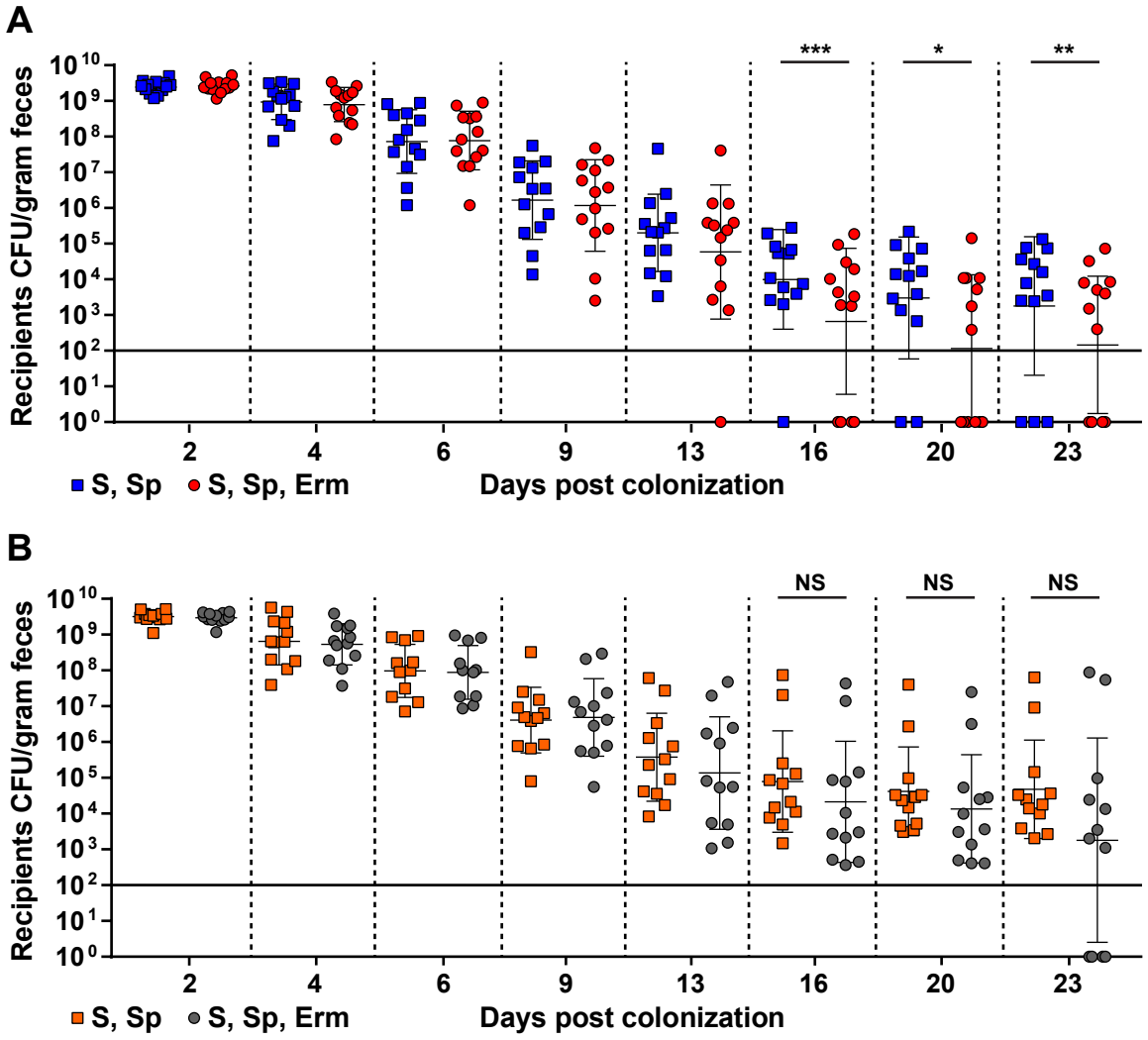
A
Recipients CFU/gram feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
***
*
**
2
4
6
9
13
16
20
23
S, Sp
S, Sp, Erm
Days post colonization
B
Recipients CFU/gram feces
NS
NS
NS
S, Sp
S, Sp, Erm
Days post colonization

Figure 4

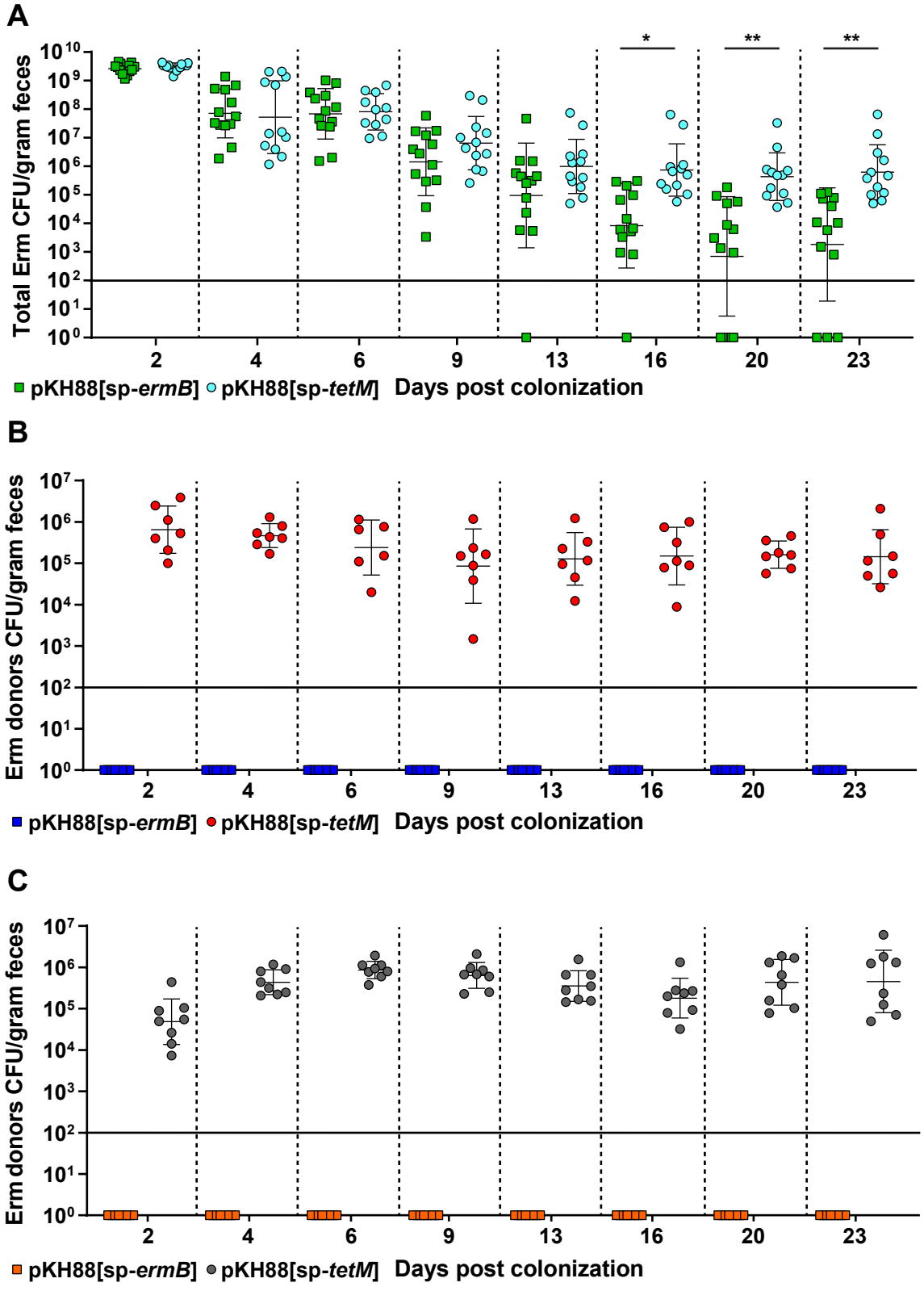
A
Total Erm CFU/gram feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
*
**
**
2
4
6
9
13
16
20
23
pKH88[sp-*ermB*] pKH88[sp-*tetM*] Days post colonization
B
Erm donors CFU/gram feces
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
pKH88[sp-*ermB*] pKH88[sp-*tetM*] Days post colonization
C
Erm donors CFU/gram feces
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
pKH88[sp-*ermB*] pKH88[sp-*tetM*] Days post colonization

Figure 5

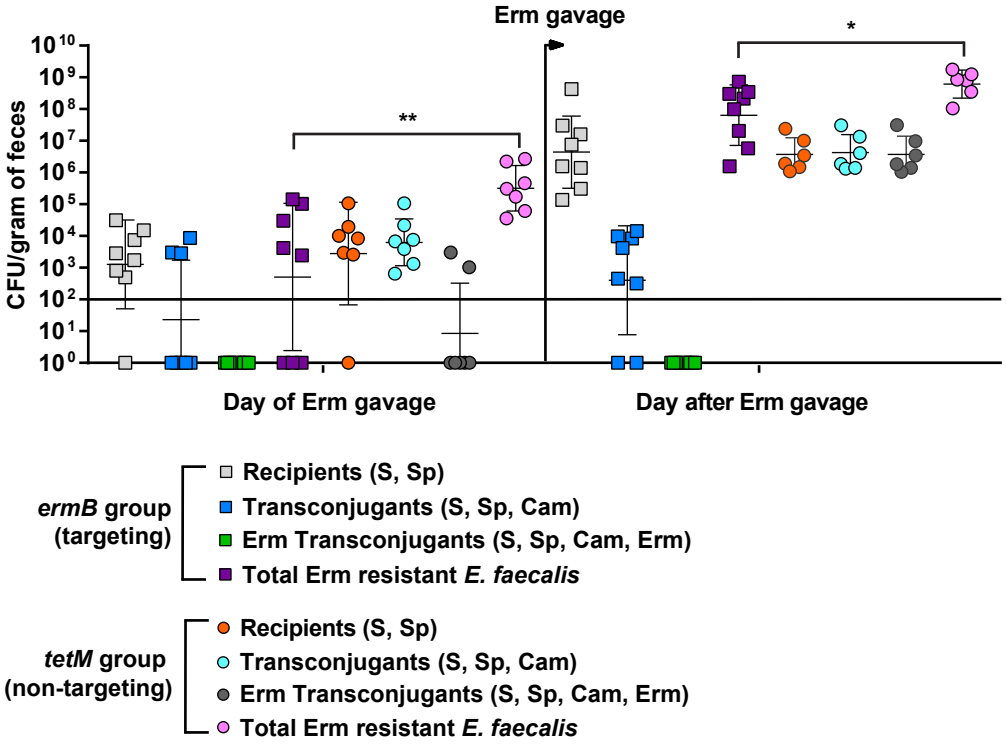
Erm gavage
CFU/gram of feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
**
*
Day of Erm gavage
Day after Erm gavage
ermB group (targeting)
Recipients (S, Sp)
Transconjugants (S, Sp, Cam)
Erm Transconjugants (S, Sp, Cam, Erm)
Total Erm resistant *E. faecalis*
tetM group (non-targeting)
Recipients (S, Sp)
Transconjugants (S, Sp, Cam)
Erm Transconjugants (S, Sp, Cam, Erm)
Total Erm resistant *E. faecalis*

Figure 6

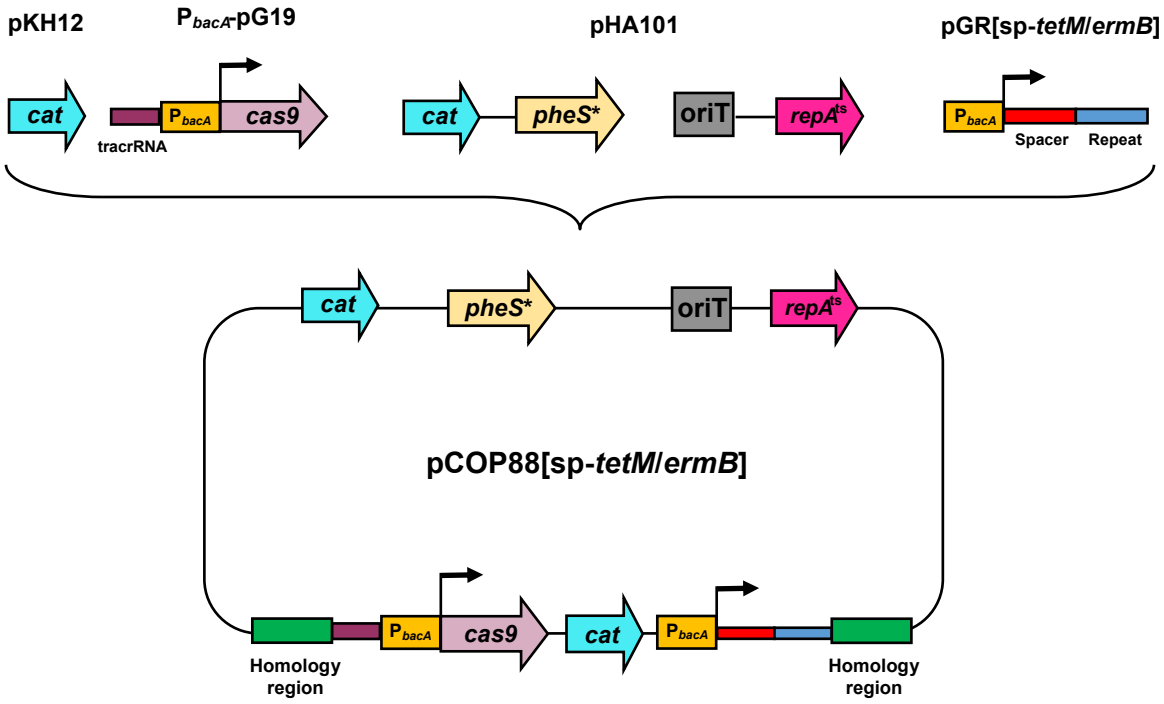
pKH12
PbacA-pG19
pHA101
pGR[sp-tetM/ermB]
cat
tracrRNA
PbacA
cas9
cat
pheS*
oriT
repAts
PbacA
Spacer
Repeat
cat
pheS*
oriT
repAts
pCOP88[sp-tetM/ermB]
Homology region
PbacA
cas9
cat
PbacA
Homology region

Figure S1

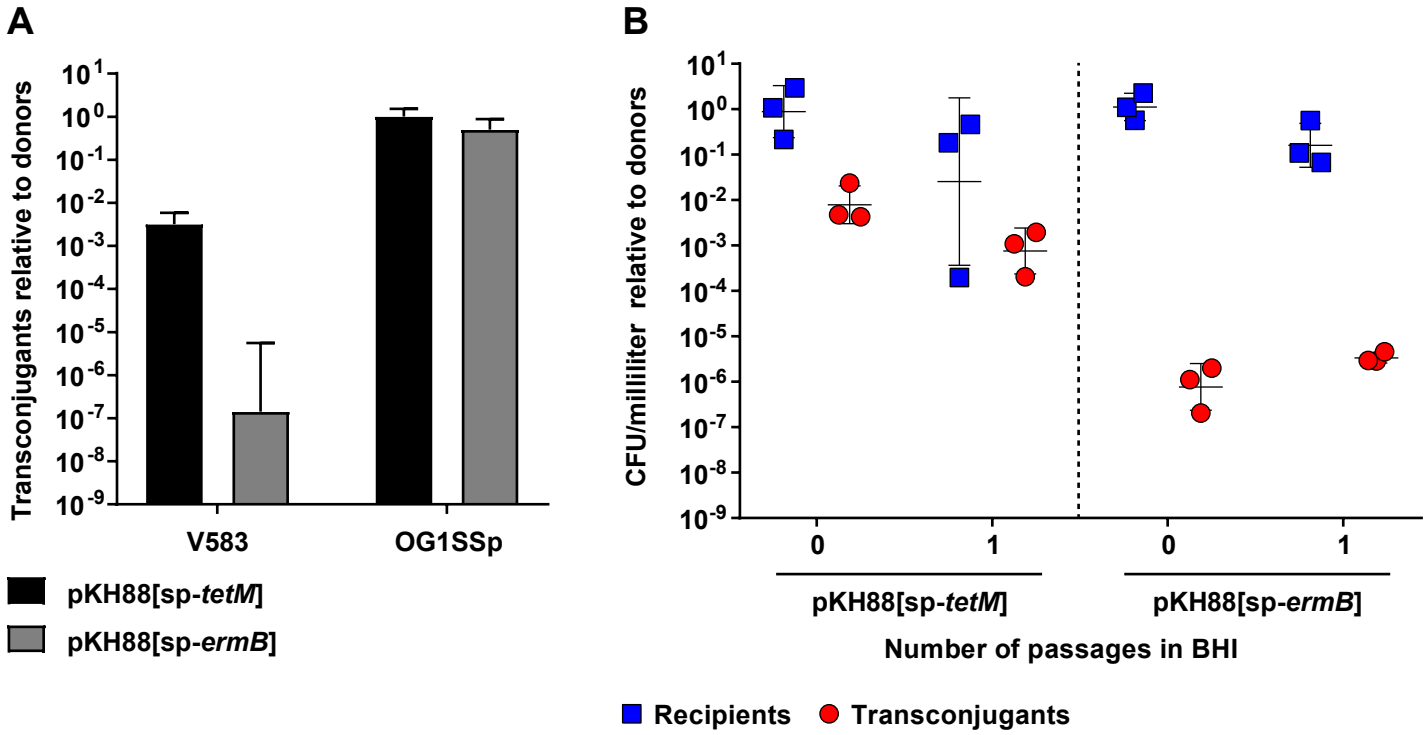
A
Transconjugants relative to donors
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
V583
OG1SSp
pKH88[sp-*tetM*]
pKH88[sp-*ermB*]
B
CFU/milliliter relative to donors
0
1
0
1
pKH88[sp-*tetM*]
pKH88[sp-*ermB*]
Number of passages in BHI
Recipients
Transconjugants

Figure S2

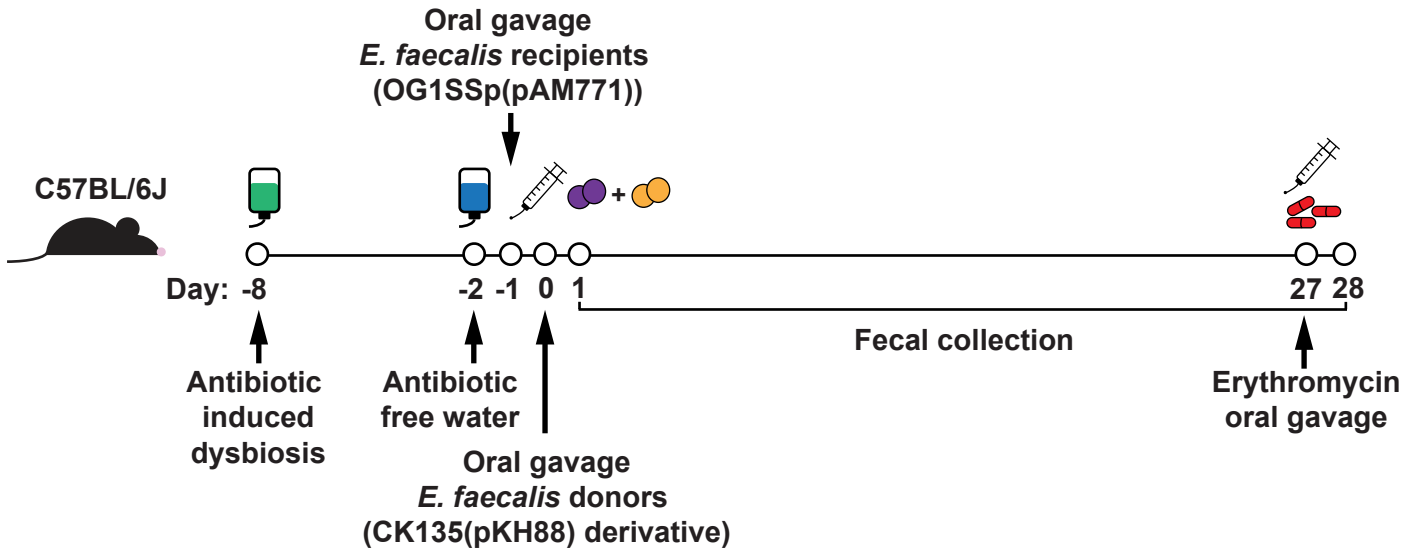
Oral gavage
E. faecalis recipients
(OG1SSp(pAM771))
C57BL/6J
Day: -8
-2 -1 0 1
27 28
Fecal collection
Antibiotic
induced
dysbiosis
Antibiotic
free water
Oral gavage
E. faecalis donors
(CK135(pKH88) derivative)
Erythromycin
oral gavage

Figure S3

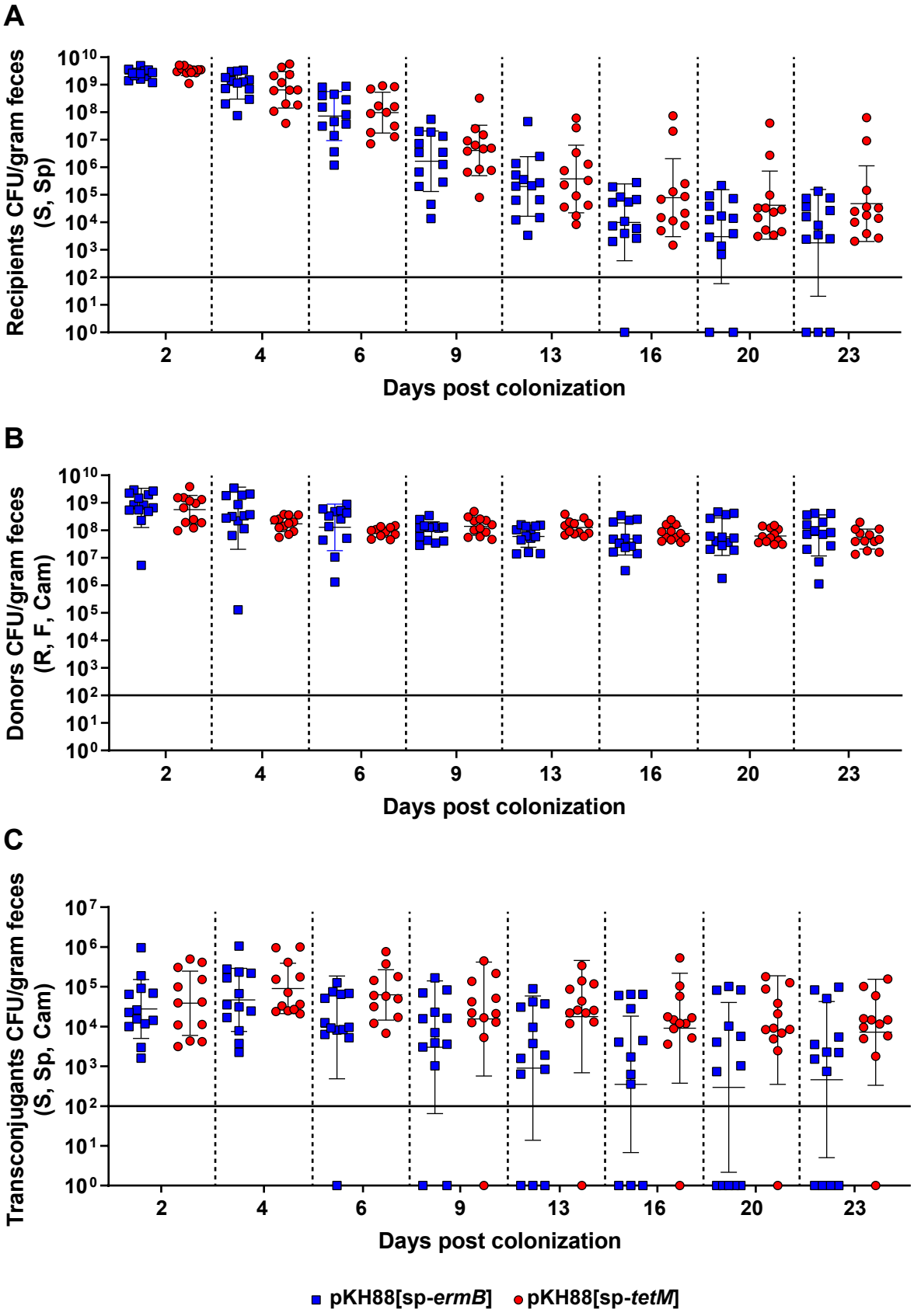
A
Recipients CFU/gram feces (S, Sp)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
B
Donors CFU/gram feces (R, F, Cam)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
C
Transconjugants CFU/gram feces (S, Sp, Cam)
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
pKH88[sp-*ermB*]
pKH88[sp-*tetM*]

Figure S4

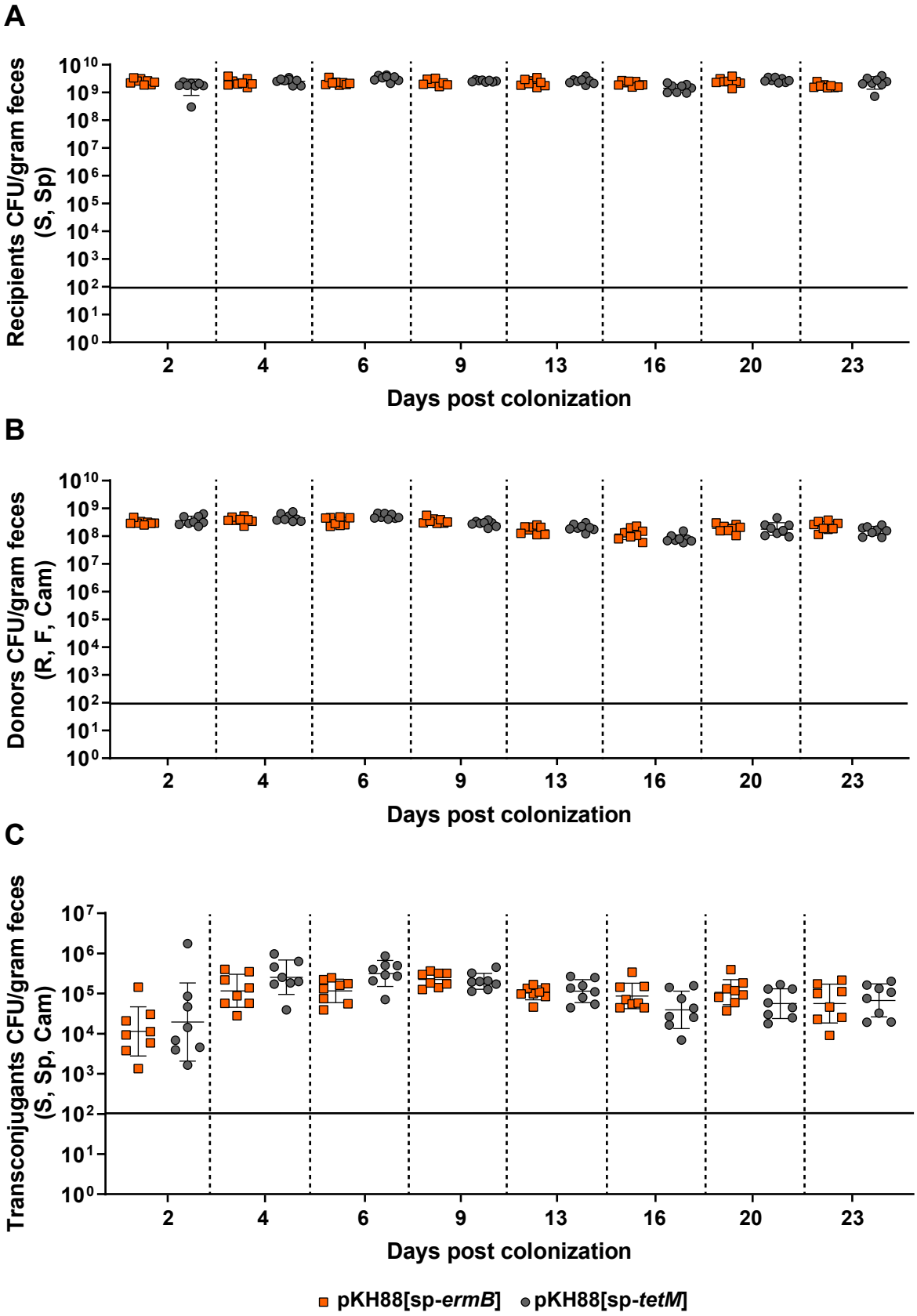
A
Recipients CFU/gram feces (S, Sp)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
B
Donors CFU/gram feces (R, F, Cam)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
C
Transconjugants CFU/gram feces (S, Sp, Cam)
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
pKH88[sp-*ermB*]
pKH88[sp-*tetM*]

Figure S5

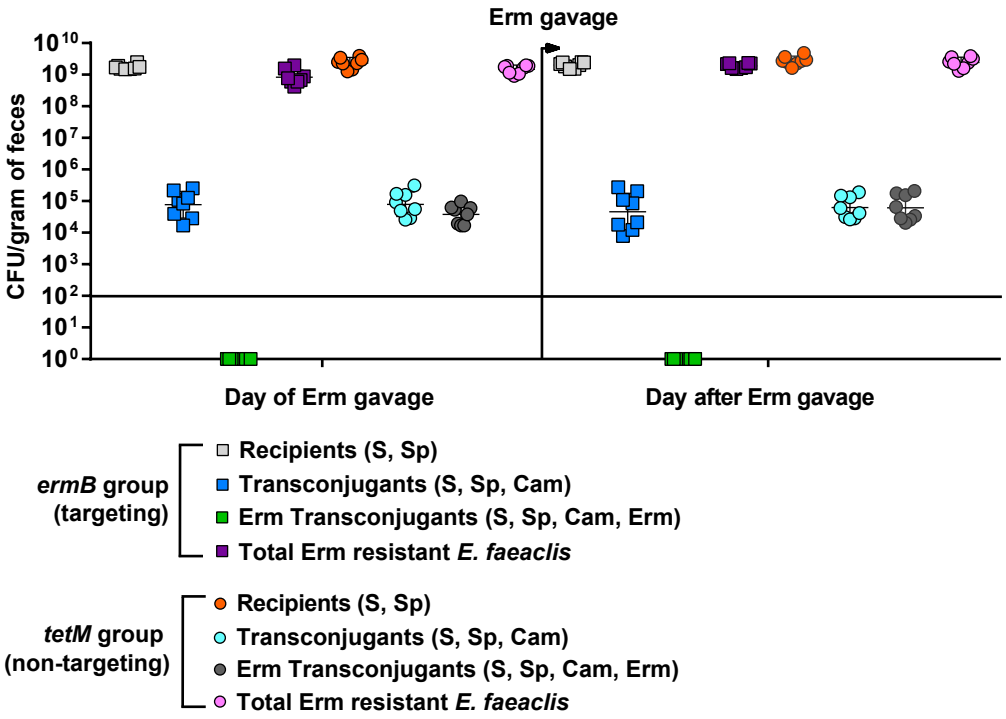
Erm gavage
CFU/gram of feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
Day of Erm gavage
Day after Erm gavage
ermB group (targeting)
Recipients (S, Sp)
Transconjugants (S, Sp, Cam)
Erm Transconjugants (S, Sp, Cam, Erm)
Total Erm resistant *E. faeaclis*
tetM group (non-targeting)
Recipients (S, Sp)
Transconjugants (S, Sp, Cam)
Erm Transconjugants (S, Sp, Cam, Erm)
Total Erm resistant *E. faeaclis*

Figure S6