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* is associated with HAIs and some strains are MDR. Therefore, novel strategies to control *E. faecalis* populations are 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 **Importance**

54 CRISPR-Cas nucleic acid targeting systems hold promise for the amelioration of multidrug-
55 resistant enterococci, yet the utility of such tools in the context of the intestinal environment where
56 enterococci reside is understudied. We describe the development of a CRISPR-Cas antimicrobial,
57 deployed on a conjugative plasmid, for the targeted removal of antibiotic resistance genes from
58 intestinal *Enterococcus faecalis*. We demonstrate that CRISPR-Cas targeting reduces antibiotic
59 resistance of *E. faecalis* by several orders of magnitude in the intestine. Although barriers exist
60 that influence the penetrance of the conjugative CRISPR-Cas antimicrobial among target recipient
61 *E. faecalis* cells, the removal of antibiotic resistance genes in *E. faecalis* upon uptake of the
62 CRISPR-Cas antimicrobial is absolute. In addition, cells that obtain the CRISPR-Cas antimicrobial
63 are immunized against the acquisition of new antibiotic resistance genes. This study suggests a
64 potential path toward plasmid based CRISPR-Cas therapies in the intestine.
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79 **Introduction**

80 Disruption of the intestinal microbiota can predispose individuals to infection by opportunistic
81 pathogens (1, 2). Antibiotics facilitate such disturbances leading to the development of hospital-
82 acquired infections (HAIs) (3, 4). *Enterococcus faecalis*, a normal constituent of the healthy
83 human intestinal microbiota and historically used in probiotics and during food fermentation, is
84 now a leading cause of HAIs (5-7). *E. faecalis* is an adept opportunist that can proliferate in the
85 dysbiotic intestine following antibiotic perturbation (8). Antibiotic-resistant clinical isolates of *E.*
86 *faecalis* typically possess expanded genomes compared to susceptible isolates due to the
87 acquisition of mobile genetic elements (9, 10), and patients colonized with these multidrug-
88 resistant (MDR) *E. faecalis* are at increased risk of acquiring bloodstream infections (11). Thus,
89 there is a need for novel strategies to decolonize high-risk individuals of MDR *E. faecalis* (12).
90

91 CRISPR-Cas systems are protective barriers in prokaryotes that function in the adaptive
92 immunity to mobile genetic elements (13-15). The well-studied Type II CRISPR-Cas system
93 consists of a DNA endonuclease (Cas9) that uses a programmable RNA guide to cleave a double-
94 stranded DNA target sequence (16). Double-stranded DNA breaks can be lethal to bacteria (17),
95 therefore deploying Type II CRISPR-Cas as a sequence-specific antimicrobial is an attractive
96 alternative to conventional antibiotic therapy. Previous studies have explored this concept using
97 phages to deliver engineered CRISPR systems targeting specific genes (18-21). Although
98 effective, this method of delivery is likely to be challenging for many species of bacteria including
99 *E. faecalis* due to phage receptor and other cell wall mutations that arise readily in response to
100 lytic phage infection (22-25).
101

102 We previously demonstrated the utility of CRISPR-Cas9 as a sequence-specific *in vitro*
103 antimicrobial in *E. faecalis* by delivering CRISPR guide sequences on mobilizable cloning vectors
104 into *E. faecalis* cells that chromosomally encode *cas9* (26). However, since MDR *E. faecalis*

105 strains lack complete CRISPR-Cas systems, and specifically *cas9* (27), efficient delivery of the
106 entire CRISPR-Cas9 machinery to MDR *E. faecalis* would likely be required. An alternative
107 method for the delivery of CRISPR-Cas is the use of pheromone-responsive plasmids (PRPs),
108 which naturally achieve high rates of conjugation, have a narrow host range limited to *E. faecalis*,
109 are capable of comprehensively infiltrating *E. faecalis* populations, and can disseminate within
110 intestinal *E. faecalis* in the absence of antibiotic selection (28-31). The transmission of PRPs is
111 tightly regulated, so the deployment of a PRP CRISPR-Cas antimicrobial could be conditionally
112 tuned for precision targeting and delivery (32).
113
114 PRPs respond to pheromones (short peptides) that are secreted by plasmid-free *E. faecalis*
115 cells (33). When donor cells detect excess pheromones, indicating the presence of plasmid-free
116 cells in the vicinity (34), they synthesize aggregation substance which facilitates contact between
117 plasmid-bearing and plasmid-free cells (35). This enables the rapid and efficient transfer of PRPs
118 from donor to recipient. In addition, many PRPs encode accessory genes such as antibiotic
119 resistance and bacteriocins, small ribosomally synthesized antimicrobial proteins that target
120 diverse bacteria and for which producing strains must encode immunity genes (36-38). Previous
121 work demonstrated that the PRP pPD1 conjugated at high rates in the intestines of mice, and
122 pPD1 transfer to plasmid-free *E. faecalis* was beneficial since recipients that failed to acquire the
123 plasmid were killed by the pPD1 encoded bac-21 bacteriocin (29).
124
125 We recently discovered that *E. faecalis* cells possessing self-targeting CRISPR-Cas systems
126 are able to transiently tolerate this self-targeting, albeit at a fitness cost, and that antibiotic
127 selection determines the outcome of this conflict by selecting for CRISPR mutants or loss of the
128 CRISPR-targeted gene (26, 39). We observed CRISPR-Cas self-targeting lethality when the
129 expression of *cas9* was increased using a constitutive promoter (40). In this study, we engineered
130 pPD1 with a complete, constitutively expressed CRISPR-Cas9 targeting cassette, specific for the

131 enterococcal antibiotic resistance genes *ermB* (encoding erythromycin resistance) and *tetM*
132 (encoding tetracycline resistance). We chose pPD1 as it lacks natively encoded antibiotic
133 resistance yet it harbors the fitness-enhancing bac-21 bacteriocin (41, 42). Using this engineered
134 PRP, we successfully depleted tetracycline and erythromycin resistance from *E. faecalis*
135 populations *in vitro*. Crucially, this approach worked in the absence of externally applied selection
136 for pPD1 in donor strains, showing practicality for its usage as a potential antimicrobial. Using an
137 *in vivo* intestinal colonization model, we show that these constructs are conjugated to
138 erythromycin-resistant recipient cells *in vivo*. Donors carrying the engineered PRP targeting *ermB*
139 significantly reduced the prevalence of erythromycin-resistant intestinal *E. faecalis*, supporting the
140 utility of the engineered PRP in mitigating MDR *E. faecalis*. Furthermore, we show that these
141 donors are immune to the uptake of *ermB*-encoding PRPs harbored by recipient *E. faecalis* cells.
142 This work is a first step in utilizing conjugative elements for specific delivery of CRISPR-Cas
143 antimicrobials to precisely target antibiotic-resistant bacteria in the intestinal microbiota.
144

145 **Results**

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

148 To assess the ability of CRISPR-Cas9 to target and remove antibiotic resistance in *E. faecalis*,
149 we modified the PRP pPD1 by introducing the *cas9* gene and a CRISPR guide RNA under the
150 control of the constitutive *bacA* promoter (Fig. 1A and S1). To facilitate detection of the plasmid
151 we included a chloramphenicol resistance marker (*cat*) for selection. We generated two pPD1
152 derivatives, referred to as pKH88[sp-*tetM*] and pKH88[sp-*ermB*], which carry guide RNAs that
153 target the enterococcal *tetM* and *ermB* genes conferring tetracycline and erythromycin resistance,
154 respectively. These plasmids retain pheromone response functions and bac-21 bacteriocin
155 production (Fig. 1A).
156

157 PRPs are vehicles for the transmission of antibiotic resistance genes in *E. faecalis* populations
158 (30), thus we first assessed the ability of our engineered pPD1 derivatives to eliminate antibiotic
159 resistance genes present on other PRPs. We reasoned that bacteriocin production by pKH88
160 derivatives would promote maintenance of these plasmids in enterococcal populations. The donor
161 strain used in these experiments was *E. faecalis* CK135 and the recipient was *E. faecalis* OG1SSp
162 possessing either pAM771 which carries the erythromycin resistance gene *ermB* or pCF10 which
163 carries the tetracycline resistance gene *tetM* (Fig. 1B). Both CK135 and OG1SSp are isogenic
164 derivatives of the human oral commensal isolate OG1 (43). We mixed donors and recipients and
165 plated them on Brain Heart Infusion (BHI) agar. After overnight growth we recovered the biofilm
166 and enumerated colony forming units (CFU) of the different cell types (passage 0 in Fig. 1C and
167 1D). We observed a significant reduction in the number of erythromycin- and tetracycline-resistant
168 transconjugants only when recipient cells received a pKH88 derivative encoding a cognate
169 CRISPR guide (Fig. 1C and 1D). Non-cognate guides were ineffective at reducing antibiotic
170 resistance. This loss of antibiotic resistance was maintained after growth of the community in
171 fresh BHI broth overnight for the pKH88[sp-*ermB*] targeting group (passage 1 in Fig. 1C), and
172 trended toward maintenance of antibiotic resistance loss for the pKH88[sp-*tetM*] targeting group
173 (passage 1 in Fig. 1D).
174
175 **Conjugation of pKH88[sp-*ermB*] to MDR *E. faecalis* resolves erythromycin resistance.**
176 To determine whether the pKH88 plasmids would be effective against MDR *E. faecalis*, we
177 used pKH88[sp-*ermB*] to target *ermB* in *E. faecalis* V583 (Fig. 2A), a vancomycin-resistant
178 hospital-adapted isolate (44). V583 *ermB* is encoded on the plasmid pTEF1, and V583 does not
179 encode a *tetM* gene (10, 45). Initially, we observed poor conjugation frequencies of pKH88
180 derivatives into V583 when using CK135 as the donor (Fig. S2A and S2B). *E. faecalis* V583
181 encodes a Type IV restriction endonuclease that restricts entry of 5-methylcytosine-modified DNA
182 and has been characterized as a barrier to DNA uptake in enterococci (40, 46, 47). We reasoned

183 that this barrier could account for the reduced dissemination of pKH88 to *E. faecalis* V583.
184 Therefore, we utilized the *E. faecalis* OG1 variant strain OG1RF deleted for a 5-methylcytosine
185 DNA methyltransferase (ΔEfaRFI) as the donor strain. This strain cannot methylate plasmid DNA
186 and the plasmid will not be cleaved by the recipient cell following conjugation (47). We observed
187 that delivery of pKH88[sp-*ermB*] from *E. faecalis* OG1RF(ΔEfaRFI) to *E. faecalis* V583 decreased
188 total erythromycin resistance in the population (Fig. 2B). In addition, *E. faecalis* V583 did not
189 tolerate CRISPR cleavage of *ermB* on pTEF1, indicated by a reduction in viable *E. faecalis* V583
190 cells (Fig. 2B) and which is consistent with our previous findings (26). This was specific to *ermB*
191 cleavage, as transconjugants that received the nonspecific targeting plasmid pKH88[sp-*tetM*]
192 retained cell viability and erythromycin resistance (Fig. 2C).
193
194 **CRISPR-Cas9 selectively depletes erythromycin resistance within enterococcal**
195 **populations in the dysbiotic murine intestine**
196 We next evaluated the efficacy of pKH88[sp-*ermB*] for the removal of erythromycin resistance
197 using a mouse model of *E. faecalis* intestinal colonization. Mice were administered an antibiotic
198 cocktail in their drinking water for seven days. Antibiotic water was removed for 24 hours and the
199 mice were gavaged with *E. faecalis* OG1SSp(pAM771) recipients, modeling a bloom of antibiotic-
200 resistant *E. faecalis* in the intestine. After an additional 24 hours, mice were gavaged with donor
201 *E. faecalis* CK135 carrying either pKH88[sp-*ermB*] (targeting) or pKH88[sp-*tetM*] (non-targeting),
202 and fecal samples were collected over the course of 23 days. A schematic of this experiment is
203 depicted in Fig. S3. Whereas *E. faecalis* CK135 donors colonized the intestine to a sustained high
204 density, *E. faecalis* OG1SSp(pAM771) recipient cells gradually declined in abundance over time
205 (Fig. S4A and S4B). We attribute this gradual reduction in the recipient population to competitive
206 killing by bacteriocin-producing donors as previously described for pPD1 carrying *E. faecalis* (29),
207 and/or competition from the reemerging native microbiota that was initially displaced by the
208 antibiotic treatment. Robust numbers of pKH88-containing transconjugants (i.e. chloramphenicol

209 resistant OG1SSp) appeared two days post-colonization of the donor strains for both the
210 pKH88[sp-*ermB*] and pKH88[sp-*tetM*] groups (Fig. S4C). However, the initial frequency of
211 transconjugants per recipient reached only ~10^{-5}, indicating that overall conjugation frequency
212 was relatively low (Fig. 3A). This was followed by a gradual increase in the transconjugant to
213 recipient ratio, approaching 1:1 (Fig. 3A), as the total recipient population decreased over time
214 (Fig. S4A). This suggests that the initial transconjugant population remains fixed due to a pKH88
215 bacteriocin-mediated fitness advantage upon restoration of the indigenous microbiota.
216
217 Although there appeared to be a trend of reduced erythromycin resistance within the recipient
218 population for the pKH88[sp-*ermB*] group, no statistically significant reduction in the number of
219 erythromycin-resistant recipients compared to the total recipient population was observed in the
220 pKH88[sp-*ermB*] targeting group (Fig. 4A). Similarly no significant difference in the erythromycin
221 resistant recipients compared to total recipients was observed for the pKH88[sp-*tetM*] non-
222 targeting group (Fig. 4B). However, to our surprise, the difference in erythromycin-resistant
223 recipients between the pKH88[sp-*ermB*] and pKH88[sp-*tetM*] groups was considerably less than
224 the difference between the total erythromycin resistant intestinal *E. faecalis,* which includes both
225 donors and recipients. The magnitude of this difference was ~3 log by the end of the experiment
226 (Fig 5A). This indicated that there was likely a substantial amount of conjugation by pAM771 into
227 the donor population. Indeed, in as little as two days, donors acquired erythromycin resistance,
228 but only in the pKH88[sp-*tetM*] non-targeting group (Fig. 5B). These data indicate that *E. faecalis*
229 CK135(pKH88[sp-*tetM*]) donors are susceptible to counter-conjugation of pAM771 from recipients
230 but that CK135(pKH88[sp-*ermB*]) donors are immune, presumably due to CRISPR-Cas targeting
231 of *ermB* on pAM771 upon counter-conjugation. We conclude that our CRISPR antimicrobial
232 dramatically reduces the prevalence of a targeted conjugative plasmid within the input *E. faecalis*
233 population, likely through a multifactorial mechanism involving a bacteriocin-dependent
234 competitive advantage, cleavage of the targeted plasmid, and prevention of counter-plasmid

235 acquisition. This in turn substantially impacts the distribution of drug resistance, as mice colonized
236 with pKH88[sp-*ermB*] donors had 0.1% of the total levels of erythromycin-resistant *E. faecalis*
237 possessed by mice colonized with pKH88[sp-*tetM*] donors.
238
239 We next assessed whether the microbial complexity of the intestinal environment plays a role
240 in the efficacy of the CRISPR-Cas antimicrobial, since curtailing antibiotic therapy spontaneously
241 restores the microbiota in the antibiotic dysbiosis model (48). We first colonized germ-free mice
242 with *E. faecalis* OG1SSp(pAM771) recipient cells and 24 hours later introduced donor *E. faecalis*
243 CK135 carrying either pKH88[sp-*ermB*] or pKH88[sp-*tetM*]. Gnotobiotic mice maintained a high
244 intestinal burden of both donors and recipients for the entire 23 days, in contrast to the antibiotic
245 dysbiosis model where the recipient population is reduced over time (Fig. S5A, S5B and S4A).
246 Despite sustained high density colonization of both donors and recipients, the total number of
247 transconjugants was similar to those observed in the antibiotic dysbiosis model (Fig. S5C and
248 S4C) but the transconjugant per recipient ratio remained stably low at 10^{-5} (Fig. 3B). Sustained
249 high recipient density indicated that the apparent competitive advantage possessed by pKH88-
250 containing donors via bac-21 does not manifest in gnotobiotic mice. These data suggest that even
251 with prolonged high densities of both donors and recipients in the absence of microbial
252 complexity, there may be biogeographical barriers that restrict the dissemination of pKH88
253 derivatives. This also implies that the competitive advantage possessed by the donor is most
254 prevalent in the presence of the microbiota, and therefore some degree of microbial complexity
255 is likely necessary for the functionality of our CRISPR-Cas antimicrobial. Interestingly, similar to
256 observations in the antibiotic dysbiosis model, pKH88 donors acquired erythromycin resistance,
257 but only in the pKH88[sp-*tetM*] non-targeting group (Fig. 5C).
258
259 These results demonstrate that our engineered CRISPR-Cas antimicrobial is a robust barrier
260 to *in vivo* plasmid acquisition and reduces the *in vivo* prevalence of a specifically targeted drug

261 resistance trait. From a technological viewpoint this is the ideal situation of a prospective
262 "probiotic" donor; a strain that can effectively disseminate a CRISPR targeting plasmid while being
263 protected from acquisition of undesirable traits.
264
265 **CRISPR-Cas antimicrobial prevents antibiotic-mediated intestinal expansion of**
266 **transconjugants.**
267 A hallmark of enterococcal intestinal colonization is its ability to expand its population upon
268 antibiotic perturbation of the indigenous microbiota (4, 25). This antibiotic-mediated expansion is
269 attributed to both intrinsic and acquired antibiotic resistance. To determine the ability of our
270 CRISPR-Cas antimicrobial to minimize the outgrowth of erythromycin resistant *E. faecalis* in the
271 intestine following antibiotic therapy, we utilized both our antibiotic dysbiosis and gnotobiotic
272 mouse models. We treated the mice 27 days post co-colonization with a single 40 µg dose of oral
273 erythromycin and determined the abundance of erythromycin resistant transconjugants and total
274 erythromycin resistant *E. faecalis*. Prior to antibiotic treatment there were no erythromycin
275 resistant transconjugants recovered from mice receiving donors carrying the targeting plasmid
276 pKH88[sp-*ermB*], whereas two of eight mice receiving donors carrying the non-targeting plasmid
277 pKH88[sp-*tetM*] had recoverable erythromycin resistant transconjugants (Fig. 6). Upon oral
278 erythromycin treatment, there was a ~4 log expansion of erythromycin resistant transconjugants
279 in the pKH88[sp-*tetM*] non-targeting group, and no erythromycin resistant transconjugants
280 emerged in the mice colonized with *E. faecalis* CK135 pKH88[sp-*ermB*] donors (Fig. 6). This
281 suggests that the perceived absence of erythromycin resistant transconjugants in six of the eight
282 mice in the pKH88[sp-*tetM*] non-targeting group was due to their abundance being below the limit
283 of detection prior to antibiotic treatment. Additionally, the total number of erythromycin-resistant
284 *E. faecalis* in the pKH88[sp-*tetM*] non-targeting group was significantly higher compared to the
285 pKH88[sp-*ermB*] targeting group (Fig. 6). Together these data indicate that transconjugants
286 receiving pKH88[sp-*ermB*] have been depleted of erythromycin resistance and that pKH88[sp-

287 *ermB*] can significantly reduce the overall total erythromycin resistance within intestinal *E.*
288 *faecalis*, pre- and post-antibiotic therapy. Interestingly, any remaining viable transconjugants in
289 the pKH88[sp-*ermB*] targeting group were impaired in their ability to increase in cell density
290 compared to mice receiving pKH88[sp-*tetM*] non-targeting donors (Fig. 6), further indicating that
291 targeted *E. faecalis* cells fail to expand their population due to loss of pAM771 encoded
292 erythromycin resistance.
293
294 In gnotobiotic mice, following oral erythromycin treatment, again we did not recover any
295 erythromycin resistant transconjugants in the pKH88[sp-*ermB*] targeting group (Fig. S6).
296 Interestingly, in gnotobiotic mice, we did not observe outgrowth of erythromycin resistant
297 transconjugants in the pKH88[sp-*tetM*] non-targeting group, even though we did not recover any
298 erythromycin resistant transconjugants in the pKH88[sp-*ermB*] targeting group, similar to the
299 antibiotic dysbiosis model (Fig. S6). We attribute this to the high level of both donors and
300 recipients in the gnotobiotic intestine that were unaffected by oral erythromycin treatment (Fig.
301 S5A and S5B). Intestinal colonization saturation by donors and recipients may be spatially
302 restricting, thus promoting colonization resistance against transconjugant expansion upon
303 erythromycin treatment in the pKH88[sp-*tetM*] non-targeting group.
304
305 **Discussion**
306 This study presents a strategy for the delivery of a CRISPR-based antimicrobial that targets
307 antibiotic-resistant *E. faecalis*. We show that this conjugative antimicrobial significantly reduces
308 antibiotic resistance from *in vitro* and *in vivo* enterococcal communities in the absence of
309 externally applied selection for donor strains. Our approach for the delivery of a CRISPR-Cas
310 antimicrobial differs from studies which employed either transformable plasmids or phages for the
311 recognition and delivery to target cells (18-21). Although these studies provided the foundation
312 for the use of CRISPR antimicrobial activity against bacteria, limitations were noted. In the case

313 of phage-mediated CRISPR antimicrobial dissemination, this technique suffered from incomplete
314 phage dissemination. This scenario is expected due to selective pressures imposed on bacteria
315 by phages during infection, including receptor mutations (22-25). Such issues have recently been
316 addressed using multi-phage delivery systems or by co-opting phage particles for the delivery of
317 CRISPR-Cas targeting modules embedded within mobilizable genome islands (49, 50). Although
318 the use of a phage-based delivery system for *E. faecalis* should be possible, enterococcal phage
319 biology is understudied and limited information exists concerning phage host ranges and genetics.
320 Therefore, we chose to engineer a PRP for the dissemination of an *E. faecalis*-specific CRISPR-
321 Cas antimicrobial for several reasons. First, the conditions governing PRP regulation have been
322 extensively studied. Second, PRPs are specific for *E. faecalis*. Third, PRPs achieve high
323 conjugation rates and can be genetically modified to diversify host tropism by altering pheromone
324 responsiveness. Fourth, PRPs are stably maintained and disseminate in the absence of selection
325 (51, 52).
326
327 The intestine is a reservoir for MDR *E. faecalis*, and *E. faecalis* infections often follow
328 exposure to broad spectrum antibiotics. We applied our CRISPR-Cas antimicrobial method for
329 the removal of an antibiotic resistance trait from intestinal *E. faecalis*. The mouse models of
330 intestinal colonization used were designed to mimic enterococcal overgrowth in the human
331 intestine following antibiotic therapy (3, 4). Antibiotics disrupt the intestinal microbiota, allowing
332 antibiotic-resistant enterococci to become the dominant colonizer. In our study, we used *E.*
333 *faecalis* donor strains for the conjugative delivery of CRISPR-Cas to remove erythromycin
334 resistance from an abundant intestinal *E. faecalis* target population. The conjugative transfer of
335 an *ermB*-specific CRISPR-Cas targeting system into recipient *E. faecalis* cells showed little to no
336 reduction in erythromycin resistance from the total intestinal *E. faecalis* recipient population.
337 However, within the recipient population that did receive the CRISPR targeting plasmid carrying
338 a cognate spacer, all transconjugants were fully depleted of erythromycin resistance. This

339 indicates that, although the frequency of conjugation was low compared to the total available
340 recipient population and not as robust as *in vitro* conjugation frequencies, the specificity of our
341 CRIPSR-Cas targeting system in the intestine was absolute. If successful conjugation occurs,
342 there is complete loss of erythromycin resistance. In addition, we observed that donors carrying
343 a cognate CRISPR antimicrobial plasmid were protected from acquisition of erythromycin
344 resistance from recipients. This is an important feature of our deliverable system since the
345 transmission of undesirable target traits to donor cells would impede the usefulness of such a
346 delivery strategy within complex microbial environments. The discovery that *E. faecalis* donor
347 cells carrying the CRISPR-Cas antimicrobial are immunized against the acquisition of antibiotic
348 resistance suggests that donors could be used as a probiotic. These probiotic *E. faecalis* cells
349 would be protected from the acquisition of antibiotic resistance traits and could pre-emptively
350 occupy the intestine to outcompete or inhibit invading multidrug resistant strains.
351
352 There are several reasons why intestinal conjugation frequency might be suboptimal in our
353 study. First, our analysis measured transconjugants from fecal samples, not the intestinal
354 mucosa. The spatial distribution of donor and recipient *E. faecalis* cells colonizing the intestinal
355 mucosal layer is unknown and evidence suggests that conjugation frequency could be impacted
356 by the regional location of *E. faecalis* intestinal colonization (28). Second, pKH88 derivatives
357 require recipient-derived cPD1 pheromone to induce conjugation (53). We know little about the
358 kinetics of pheromone signaling in the intestine. Low rates of conjugation could reflect low local
359 concentrations of cPD1 in the vicinity of donors resulting in few donors that effectively conjugate,
360 thereby minimizing conjugation frequencies. Alternatively, excess donors can inhibit conjugation
361 to recipient cells (54). This is due to the production of inhibitory self-signaling peptides produced
362 by the donors that compete with recipient pheromones and reduce conjugation frequencies.
363 Exploring the efficiency of conjugation upon altering donor to recipient ratios could lead to more
364 permissive conjugation in the intestine. Another possibility is that conjugation in the intestine may

365 be more efficient at a precise density of donors, recipients, or both, which may require future
366 optimization for effective plasmid delivery.
367
368 Moving forward, improvements to our current system are justified if an engineered conjugative
369 CRISPR antimicrobial is to be clinically effective for precision removal of antibiotic resistance
370 genes in *E. faecalis* or other MDR bacteria. Modifications include multiplexing CRISPR guide
371 RNAs to target different resistance genes and/or the same genes at multiple sites, as well as
372 increasing the overall conjugation frequency by altering PRP regulation, dosing of donor strains,
373 or the plasmid replicon itself. Theoretically, resistance to bac-21 could compromise the efficacy
374 of our system, and future iterations should utilize alternative bacteriocins as well as bacteriocin-
375 free constructs. In this study we focused on targeting antibiotic resistance that resides on
376 plasmids. However, many *E. faecalis* antibiotic resistance genes are chromosomally encoded.
377 Future iterations of this system should also be engineered for the specific targeting of
378 chromosomal antibiotic resistance genes. Recently we showed that CRISPR-Cas targeting of the
379 *vanB* locus on the *E. faecalis* V583 chromosome is lethal (40). Targeting chromosomal antibiotic
380 resistance genes could have the added feature of bacterial lethality. Such a strategy may be
381 preferable depending on whether the intended use of the CRISPR-Cas antimicrobial is for
382 reshaping the microbiota or for targeting drug resistant strains during infection.
383
384 Our model system has relied on laboratory-domesticated strains of *E. faecalis* OG1 (43), it
385 will be imperative to determine the efficacy of this system against more “wild” clinical isolates
386 which are the intended targets of such a therapy. For safe and effective delivery of the CRISPR
387 antimicrobial in the clinic, the use of an attenuated *E. faecalis* strain or a related member of the
388 Lactobacillales approved for probiotic use would be essential. Finally, this system could be
389 adapted for the targeted removal of antibiotic resistance from bacteria other than *E. faecalis.*
390 Gram-positive conjugative plasmids like pIP501, which belongs to the rep_1 family, are broadly

391 disseminated conjugative plasmids (51, 52, 55). pIP501 is transferrable among enterococci, other
392 lactic acid bacteria, staphylococci, and clostridia (55). The modification of a promiscuous
393 mobilizable plasmid such as pIP501 with CRISPR-Cas antimicrobial function could expand the
394 capabilities of our current system by facilitating broad targeting of multiple Gram-positive
395 pathogens.
396

397 **Materials and Methods**

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

399 *Escherichia coli* cultures were incubated with shaking at 220 RPM at 37°C in Lysogeny Broth
400 (LB). *E. faecalis* cultures were grown in Brain Heart Infusion (BHI) broth at 37°C without shaking.
401 PCR was performed using Taq Polymerase (New England Biolabs), and Q5 DNA Polymerase
402 (New England Biolabs) for molecular cloning. The PureLink PCR Purification kit (Invitrogen) was
403 used to purify DNA fragments and the GeneJet Plasmid Purification kit (Fisher) was used for
404 plasmid purification. Primers were obtained from Sigma Aldrich. Sanger sequencing was
405 performed at the Massachusetts General Hospital DNA Core Facility. *E. coli* EC1000 was used
406 for routine plasmid propagation (56). *E. faecalis* and *E. coli* competent cells were prepared as
407 described previously (26). Genomic DNA was extracted using the MO BIO Microbial DNA
408 Isolation Kit (Qiagen). Antibiotics were used at the following concentrations: chloramphenicol, 15
409 µg/ml; streptomycin, 500 µg/ml; spectinomycin, 500 µg/ml; vancomycin, 10 µg/ml; erythromycin,
410 50 µg/ml; rifampicin, 50 µg/ml; fusidic acid, 25 µg/ml; tetracycline, 10 µg/ml.
411

412 **Strain and plasmid construction**

413 All strains used in this study can be found in Table S1. The donor strain used for competition
414 experiments was a rifampicin and fusidic acid-resistant derivative of CK135 (57). All plasmids
415 used in this study can be found in Table S2. Fragments obtained from the plasmids pHA101 (58),
416 pPD1 (24), P_{bacA}-pG19, pKH12 (26) and pGR *tetM*/*ermB* (40) were amplified using Q5 DNA

417 polymerase and assembled using NEB HiFi DNA Assembly Master Mix (New England Biolabs).
418 For simplicity, assembly of the CRISPR-targeting construct (P_{bacA}-*cas9*-*cat*-guide RNA) was
419 divided into two steps. First, P_{bacA}*cas9* was introduced into pPD1 by homologous recombination.
420 Then the *cat* gene from pKH12 and the P_{bacA}-driven CRISPR guide RNA was integrated
421 downstream of P_{bacA}-*cas9*. Following the generation of this construct, we discovered that the first
422 integration site obstructed the pheromone response, therefore the entire engineered region (P_{bacA}-
423 *cas9*-*cat*-guide RNA) was amplified as one fragment and integrated into native pPD1 between
424 orfs *ppd4* and *ppd5* using the integration plasmid pCOP88 (Fig. S1). The modified pPD1
425 derivatives are designated as pKH88[sp-*tetM*] and pKH88[sp-*ermB*], which target *tetM* or *ermB*
426 respectively. Primer sequences are listed in Table S3.
427
428 **_In vitro_ competition assays**
429 Overnight cultures of donors and recipients were pelleted, resuspended in fresh BHI broth,
430 and cultured for 1.5 hours. Donors and recipients were mixed in a volume ratio of 1:9, and the
431 mixtures were spread on BHI agar. Following overnight incubation, lawns were scraped into sterile
432 PBS and serial dilutions (10 µl each) were spotted on BHI plates supplemented with antibiotics to
433 enumerate colony forming units (CFU) of donors, recipients, and transconjugants as well as total
434 CFU of tetracycline- and erythromycin-resistant cells. For individual passages, the recovered
435 conjugation mixture was diluted 1:1000 in fresh BHI broth without selection and incubated
436 overnight, after which the constituents of the mixture were again enumerated.
437
438 **Mouse models of _E. faecalis_ colonization**
439 All animal protocols were approved by the Institutional Animal Care and Use Committee of
440 the University of Colorado School of Medicine (protocol number 00253).
441

442 For the antibiotic dysbiosis model, 7 days prior to colonization, 6-8 week old C57BL6/J mice
443 were gavaged with 150 µL of an antibiotic cocktail (streptomycin 1 mg/mL, gentamicin 1 mg/mL,
444 erythromycin 200 µg/mL) and given a water bottle *ad libitum* with the same antibiotic cocktail for
445 6 days following the initial gavage. 24 hours prior to colonization with recipient *E. faecalis*,
446 antibiotic water was removed and replaced with standard sterile reverse osmosis water. Donor
447 and recipient strains were cultured overnight in BHI and mice were gavaged with ~10^9 CFU in
448 100 µL of sterile PBS. All mice were first gavaged with *E. faecalis* OG1SSp(pAM771). After 24
449 hours, mice (n=13, 7 female (F) and 6 male (M)) were gavaged with CK135(pKH88[sp-*ermB*])
450 while the second group (n=12, 5 F and 7 M) were gavaged with CK135(pKH88[sp-*tetM*]). Fecal
451 samples were collected at the designated time points, resuspended in 1 mL of sterile PBS, and
452 plated on BHI agar supplemented with rifampicin, fusidic acid, erythromycin, chloramphenicol,
453 streptomycin and spectinomycin in combinations that would select for the desired populations and
454 using the concentrations described above. After 27 days, 8 mice (4 F and 4 M) from the pKH88[sp-
455 *ermB*] targeting group and 7 mice (3 F and 4 M) from the pKH88[sp-*tetM*] non-targeting group
456 were gavaged with erythromycin (40 µg). Fecal samples were collected from all mice pre-
457 erythromycin gavage. Post-erythromycin gavage, fecal samples were collected from all 8 mice
458 from the pKH88[sp-*ermB*] targeting group and from 6 of 7 mice from the pKH88[sp-*tetM*] non-
459 targeting group due to the inability of a single mouse from this group to defecate.
460
461 All germ-free mice were gavaged with *E. faecalis* OG1SSp(pAM771. 24 hours later these
462 gnotobiotic mice were gavaged with *E. faecalis* CK135(pKH88[sp-*ermB*]) (n=8, 4 F and 4 M) or
463 *E. faecalis* CK135(pKH88[sp-*tetM*]) (n=8, 4 F and 4 M). Fecal samples were collected at the
464 designated time points and donors, recipients, and transconjugants were enumerated on selective
465 media as described above. After 27 days, all 8 gnotobiotic mice from each group were gavaged
466 with 40 µg of erythromycin.
467

468 **Statistical analysis**

469 GraphPad-Prism version 8.2.0 was used to determine statistical significance and the following
470 tests were employed based on the experimental set up. For *in vitro* competition assays a log
471 normal distribution was assumed and a Student's t-test was performed. For intestinal colonization
472 studies, due to a higher degree of variation in the colonization kinetics between individual mice,
473 a normal distribution was not assumed and thus a nonparametric Mann-Whitney test was
474 performed. Values below the limit of detection for in vivo animal experiments were represented
475 as constant (one half of the value of the limit of detection). The limit of detection for all animal
476 experiments was 100 colony forming units per gram of feces. Error bars represent the geometric
477 mean +/- the geometric standard deviation. p-values indicate the following: * 0.01 – 0.05, ** 0.001
478 - <0.01, *** 0.0001 - <0.001, **** <0.0001.

479

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483 manuscript. We thank Christopher Kristich for providing *E. faecalis* CK135(pPD1).

484

485 **Figure Legends**

486 **Figure 1. CRISPR-Cas engineered conjugative pPD1 derivative plasmids target and**
487 **eliminate antibiotic resistance genes in *in vitro* co-culture. (A)** Schematic of the pPD1
488 derivative pKH88. pKH88 encodes *cas9* and a CRISPR guide RNA under the control of the
489 constitutive enterococcal *bacA* promoter. The guide RNA consists of a CRISPR repeat region and
490 a unique spacer sequence with complementarity to the *ermB* gene (pKH88[sp-*ermB*]) of pAM771
491 or the *tetM* gene (pKH88[sp-*tetM*]) of pCF10. pKH88 derivatives encode genes necessary for
492 conjugative transfer, chloramphenicol resistance (*cat*) and biosynthesis of the bacteriocin bac-21.
493 **(B)** Cartoon depicting the delivery of pKH88 derivatives to target recipient *E. faecalis* cells.

494 Abundance of erythromycin **(C)** or tetracycline **(D)** resistant *E. faecalis* OG1SSp recipients
495 following acquisition of pKH88[sp-*ermB*] or pKH88[sp-*tetM*]. Removal of erythromycin and
496 tetracycline resistance from the transconjugant population depends on the introduction of a
497 cognate spacer targeting pKH88 derivative. Markers; R – rifampicin, F – fusidic acid, Cam –
498 chloramphenicol. Abx – antibiotic resistance. **p=0.006 (for passage 0 in C) **p=0.003 (for
499 passage 1 in C), **p=0.009 (for passage 0 in D).
500
501 **Figure 2. Engineered CRISPR-Cas targeting plasmid specifically reduces drug resistance**
502 **in MDR *E. faecalis*. (A)** Cartoon depicting the transfer, targeting and removal of erythromycin
503 resistance from MDR *E. faecalis* V583. Conjugation of *E. faecalis* V583 with pKH88[sp-*ermB*] **(B)**
504 but not pKH88[sp-*tetM*] **(C)** reduces erythromycin resistant *E. faecalis* V583 over the course of
505 four serial passages in BHI broth. Markers; Erm – erythromycin, Van – vancomycin, R – rifampicin,
506 F – fusidic acid, S – streptomycin, Sp – spectinomycin, Cam – chloramphenicol.
507
508 **Figure 3. Saturation of the *E. faecalis* OG1SSp(pAM771) recipient population by pKH88 is**
509 **time-dependent.** The ratio of transconjugants to recipients was calculated for both the pKH88[sp-
510 *ermB*] targeting group (*ermB*) and the pKH88[sp-*tetM*] non-targeting group (*tetM*) for 23 days of
511 co-colonization in antibiotic-treated **(A)** and gnotobiotic **(B)** mice.
512
513 **Figure 4. Assessment of erythromycin-resistant transconjugants from pKH88[sp-*ermB*]**
514 **targeting and pKH88[sp-*tetM*] non-targeting populations in the murine intestine. (A)**
515 Antibiotic treated mice co-colonized with *E. faecalis* OG1SSp(pAM771) and CK135(pKH88[sp-
516 *ermB]*) – targeting group. **(B)** Antibiotic treated mice co-colonized with *E. faecalis*
517 OG1SSp(pAM771) and CK135(pKH88[sp-*tetM*]) – non-targeting group. The total
518 OG1SSp(pAM771) recipient population and recipients that are erythromycin resistant were
519 enumerated by selective plating of mouse feces over the course or 23 days of co-colonization.

520 The solid horizontal line indicates the limit of detection. Markers; S – streptomycin, Sp –
521 spectinomycin, Erm – erythromycin resistance.
522
523 **Figure 5. *E. faecalis* CK135 pKH88[sp-*ermB*] donors are immune to acquisition of**
524 **erythromycin resistance from recipients in the murine intestine. (A)** Total intestinal load of
525 erythromycin resistance among input *E. faecalis* donors and recipients from antibiotic-treated
526 mice. Erythromycin resistance is acquired by donors only in the pKH88[sp-*tetM*] non-targeting
527 group in both antibiotic-treated **(B)** and gnotobiotic **(C)** mice. The solid horizontal line indicates
528 the limit of detection. Markers; Erm – erythromycin. ***p=0.0003, ****p=<0.0001.
529
530 **Figure 6. Transconjugants that receive pKH88[sp-*ermB*] do not bloom in the intestines of**
531 **dysbiotic mice following oral erythromycin treatment.** 27 days post co-colonization with *E.*
532 *faecalis* OG1SSp(pAM771) and CK135(pKH88[sp-*ermB*]) (targeting group) or with *E. faecalis*
533 OG1SSp(pAM771) and CK135(pKH88[sp-*tetM*]) (non-targeting group), mice received a single 40
534 µg dose of oral erythromycin. The number of recipients, transconjugants and erythromycin
535 resistant transconjugants were enumerated from fecal pellets before and after oral erythromycin
536 treatment. The solid horizontal line indicates the limit of detection. Markers; Erm – erythromycin,
537 S – streptomycin, Sp – spectinomycin, Cam – chloramphenicol. *p=0.01, **p=0.004.
538
539 **Figure S1. The allelic exchange vector pCOP88 integrates the P_{bacA}-*cas9*-*cat*-guide RNA**
540 **module into pPD1.** pCOP88 derivatives contain elements from the allelic exchange vector pLT06
541 (59). These include a temperature sensitive *repA* (*repA*^{ts}) allele that facilitates forced integration
542 of pCOP88 derivatives into target DNA sequences under non-permissive temperature (42°C) and
543 a P-*pheS* cassette that allows for the cellular utilization of *p*-chloro-phenylalanine as a counter-
544 selectable marker. The P*bacA*-*cas9*-*cat*-guide RNA module is flanked by 1-kb pPD1 homology
545 regions used for the homologous recombination of the module into native pPD1.

546

547 **Figure S2. *E. faecalis* V583 restricts conjugation of pKH88 derivatives originating from *E.***
548 ***faecalis* CK135. (A)** The ratio of *E. faecalis* V583 and OG1SSp transconjugants following *in vitro*
549 co-culture with *E. faecalis* CK135(pKH88) derivative donors. **(B)** Comparison of normalized *E.*
550 *faecalis* V583 transconjugant and *E. faecalis* V583 recipient numbers following *in vitro* co-culture
551 with *E. faecalis* CK135(pKH88) derivative donors.

552

553 **Figure S3. Schematic cartoon of the antibiotic dysbiosis mouse model.**

554

555 **Figure S4. Intestinal colonization of *E. faecalis* populations following co-colonization with**
556 **OG1SSp(pAM771) and CK135(pKH88) donors in antibiotic-treated mice. (A)** Recipients. **(B)**
557 Donors. **(C)** Transconjugants. Markers; S – streptomycin, Sp – spectinomycin, R – rifampicin, F
558 – fusidic acid, Cam – chloramphenicol.

559

560 **Figure S5. Intestinal colonization of *E. faecalis* populations following co-colonization with**
561 **OG1SSp(pAM771) and CK135(pKH88) donors in gnotobiotic mice. (A)** Recipients. **(B)**
562 Donors. **(C)** Transconjugants. The solid horizontal line indicates the limit of detection. Markers; S
563 – streptomycin, Sp – spectinomycin, R – rifampicin, F – fusidic acid, Cam – chloramphenicol.

564

565 **Figure S6. Non-targeted *E. faecalis* intestinal transconjugant populations do not bloom**
566 **following oral erythromycin treatment of gnotobiotic mice.** 27 days post co-colonization with
567 *E. faecalis* OG1SSp(pAM771) and CK135(pKH88[sp-*ermB*]) (targeting group) or with *E. faecalis*
568 OG1SSp(pAM771) and CK135(pKH88[sp-*tetM*]) (non-targeting group), mice received a single 40
569 µg dose of oral erythromycin. The number of recipients, transconjugants and erythromycin
570 resistant transconjugants were enumerated from fecal pellets before and after oral erythromycin

571 treatment. The solid horizontal line indicates the limit of detection. Markers; Erm – erythromycin,
572 S – streptomycin, Sp – spectinomycin, Cam – chloramphenicol.
573

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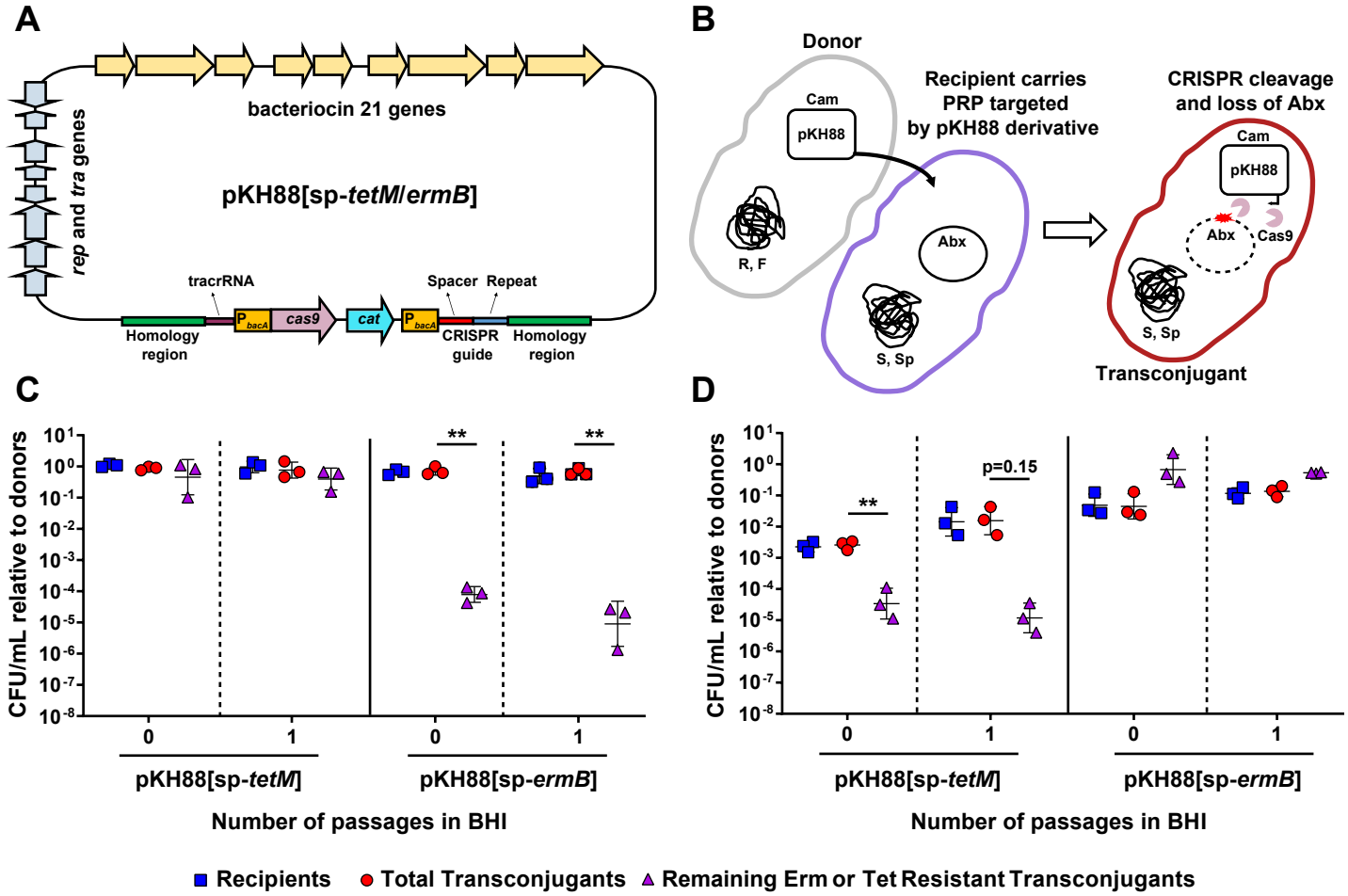
A
bacteriocin 21 genes
rep and tra genes
pKH88[sp-tetM/ermB]
tracrRNA
Spacer
Repeat
Homology region
cas9
cat
CRISPR guide
Homology region
B
Donor
Cam
pKH88
R, F
Recipient carries PRP targeted by pKH88 derivative
Abx
S, Sp
CRISPR cleavage and loss of Abx
Cas9
Transconjugant
C
CFU/mL relative to donors
**
**
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

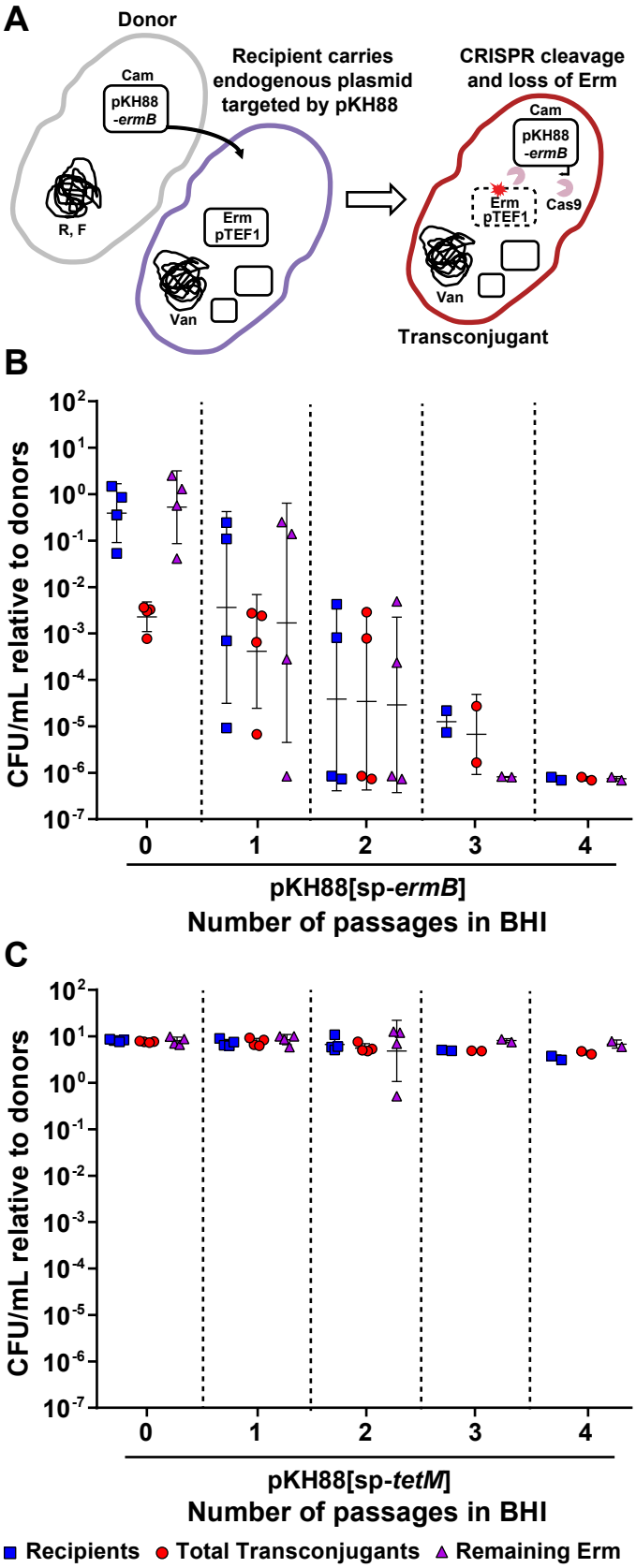
A
Donor
Cam
pKH88
-ermB
R, F
Recipient carries
endogenous plasmid
targeted by pKH88
Erm
pTEF1
Van
CRISPR cleavage
and loss of Erm
Cam
pKH88
-ermB
Erm
pTEF1
Cas9
Van
Transconjugant
B
CFU/mL relative to donors
pKH88[sp-ermB]
Number of passages in BHI
C
CFU/mL relative to donors
pKH88[sp-tetM]
Number of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

Figure 2

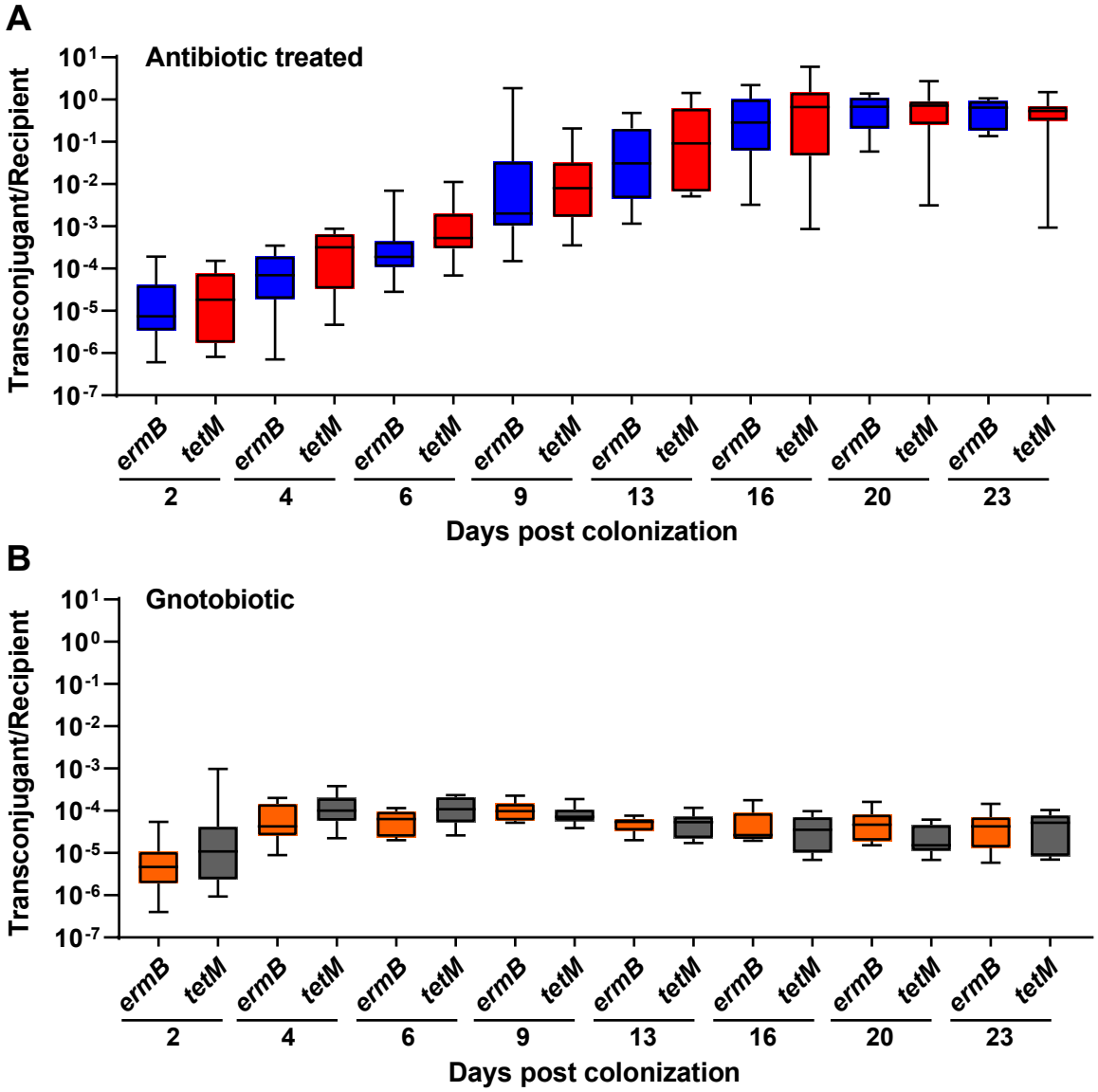
A
Antibiotic treated
Transconjugant/Recipient
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
ermB
tetM
2
4
6
9
13
16
20
23
Days post colonization
B
Gnotobiotic
Transconjugant/Recipient
Days post colonization

Figure 3

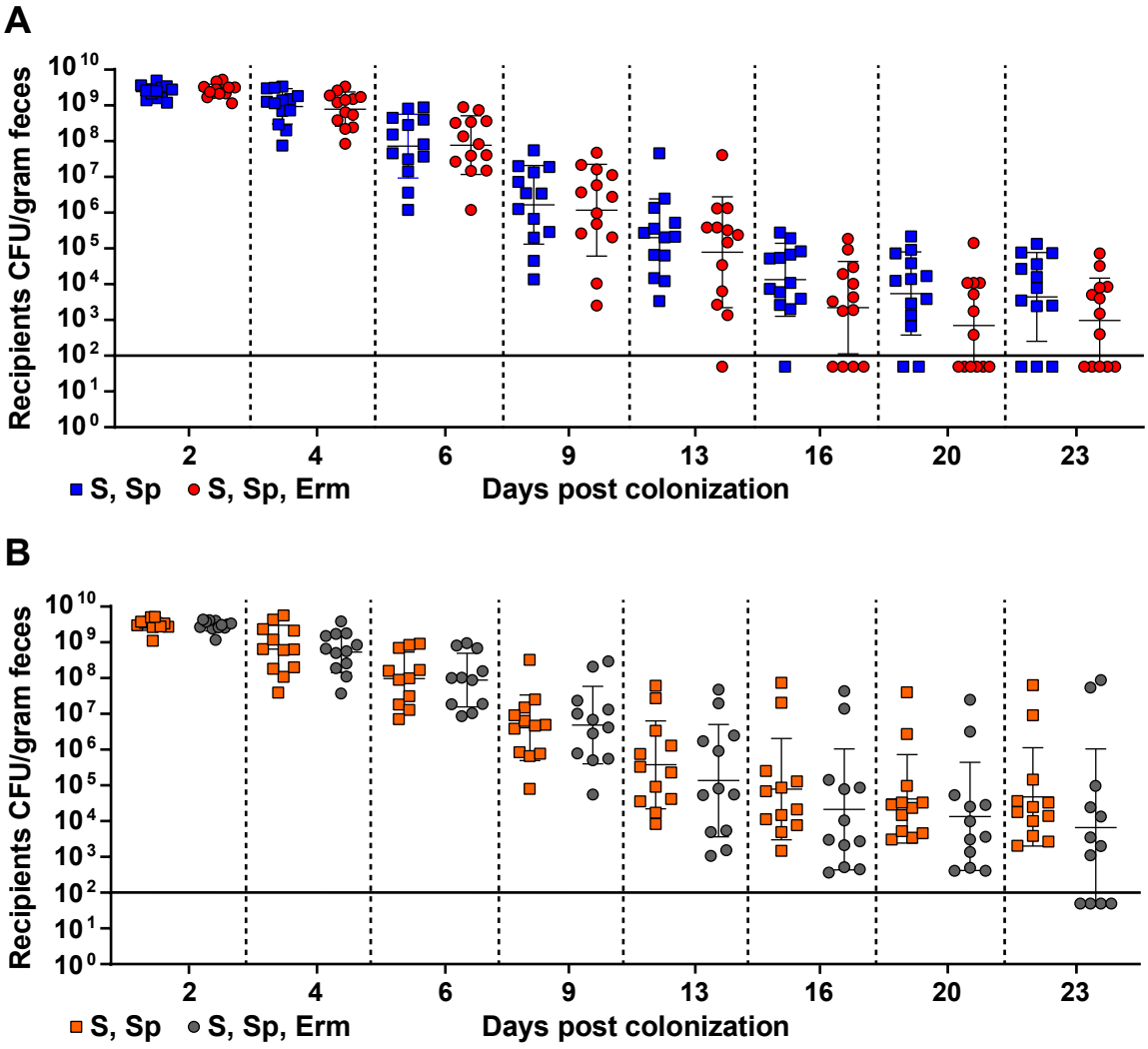
A
Recipients CFU/gram feces
10 10
10 9
10 8
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
2
4
6
9
13
16
20
23
S, Sp
S, Sp, Erm
Days post colonization
B
Recipients CFU/gram feces
10 10
10 9
10 8
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
2
4
6
9
13
16
20
23
S, Sp
S, Sp, Erm
Days post colonization

Figure 4

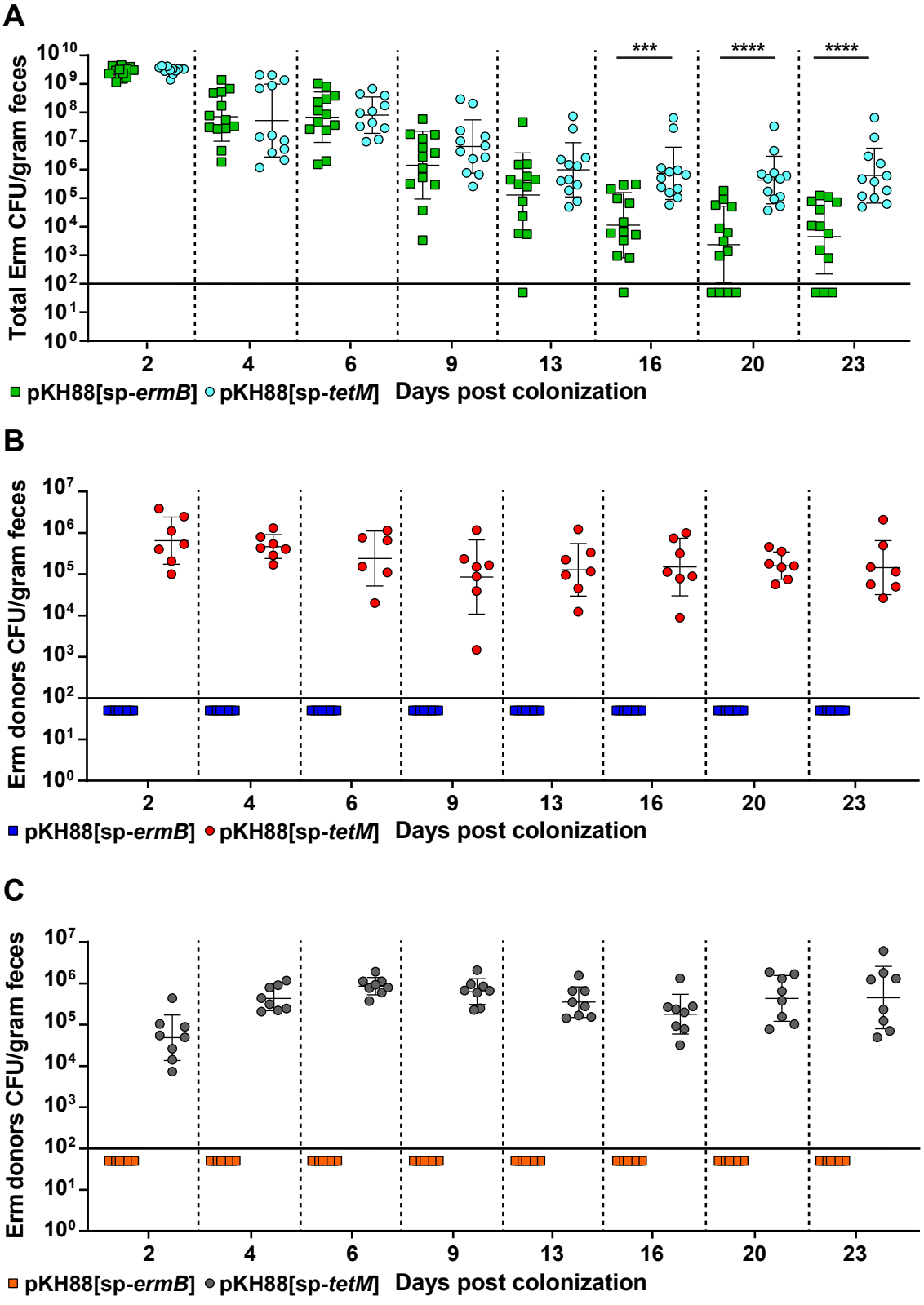
A
Total Erm CFU/gram feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
***
****
****
2
4
6
9
13
16
20
23
pKH88[sp-*ermB*] pKH88[sp-*tetM*] Days post colonization
B
Erm donors CFU/gram feces
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
pKH88[sp-*ermB*]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

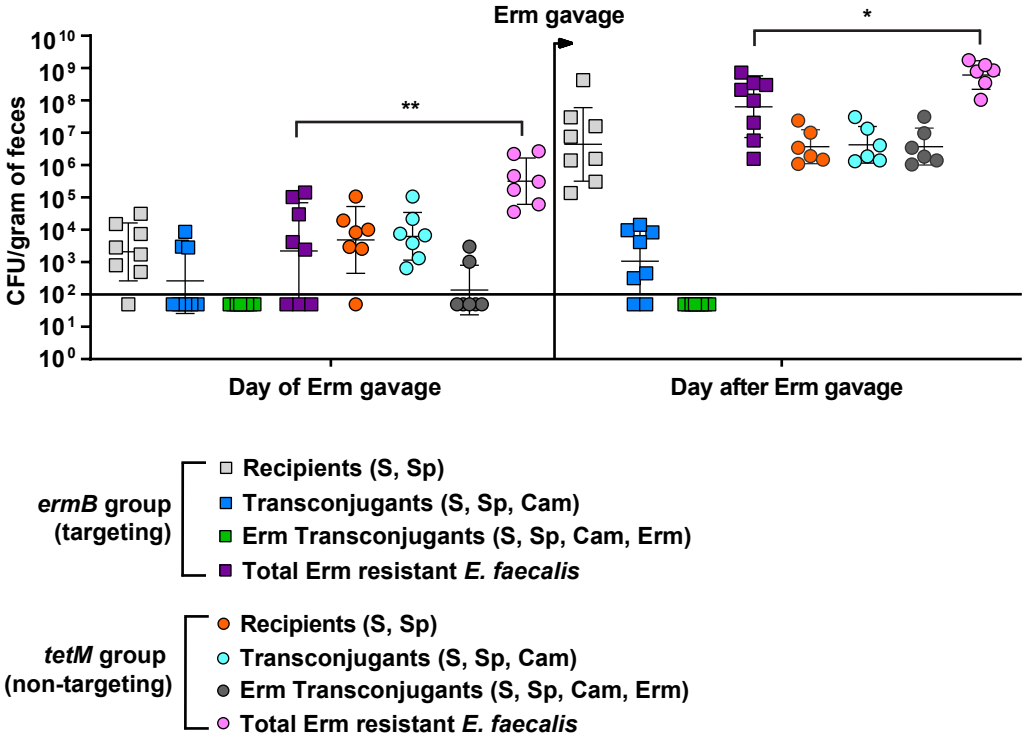
Erm gavage
*
**
CFU/gram of feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
Day of Erm gavage
Day after Erm gavage
ermB group (targeting)
Recipients (S, Sp)
Transconjugants (S, Sp, Cam)
Erm Transconjugants (S, Sp, Cam, Erm)
Total Erm resistant E. faecalis
tetM group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

Figure 6

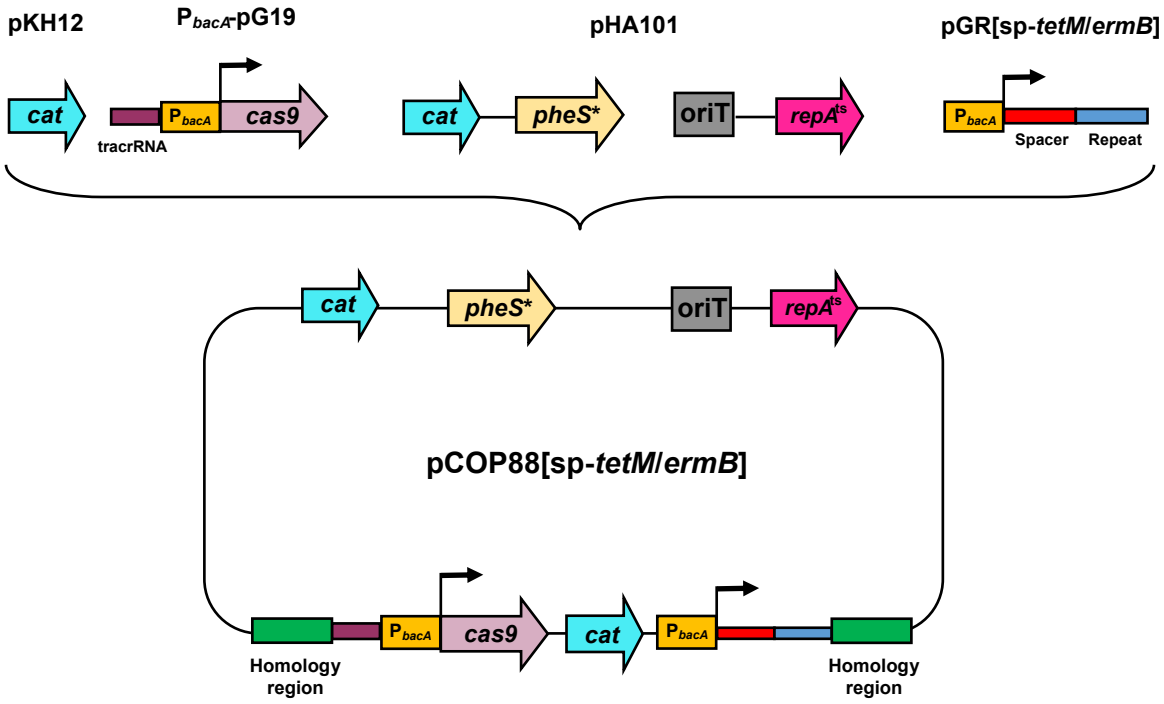
pKH12
PbacA-pG19
pHA101
pGR[sp-tetM/ermB]
cat
tracrRNA
PbacA
cas9
cat
pheS*
oriT
repAts
PbacA
Spacer
Repeat
cat
pheS*
oriT
repAts
pCOP88[sp-tetM/ermB]
Homology region
PbacA
cas9
cat
PbacA
Homology region

Figure S1

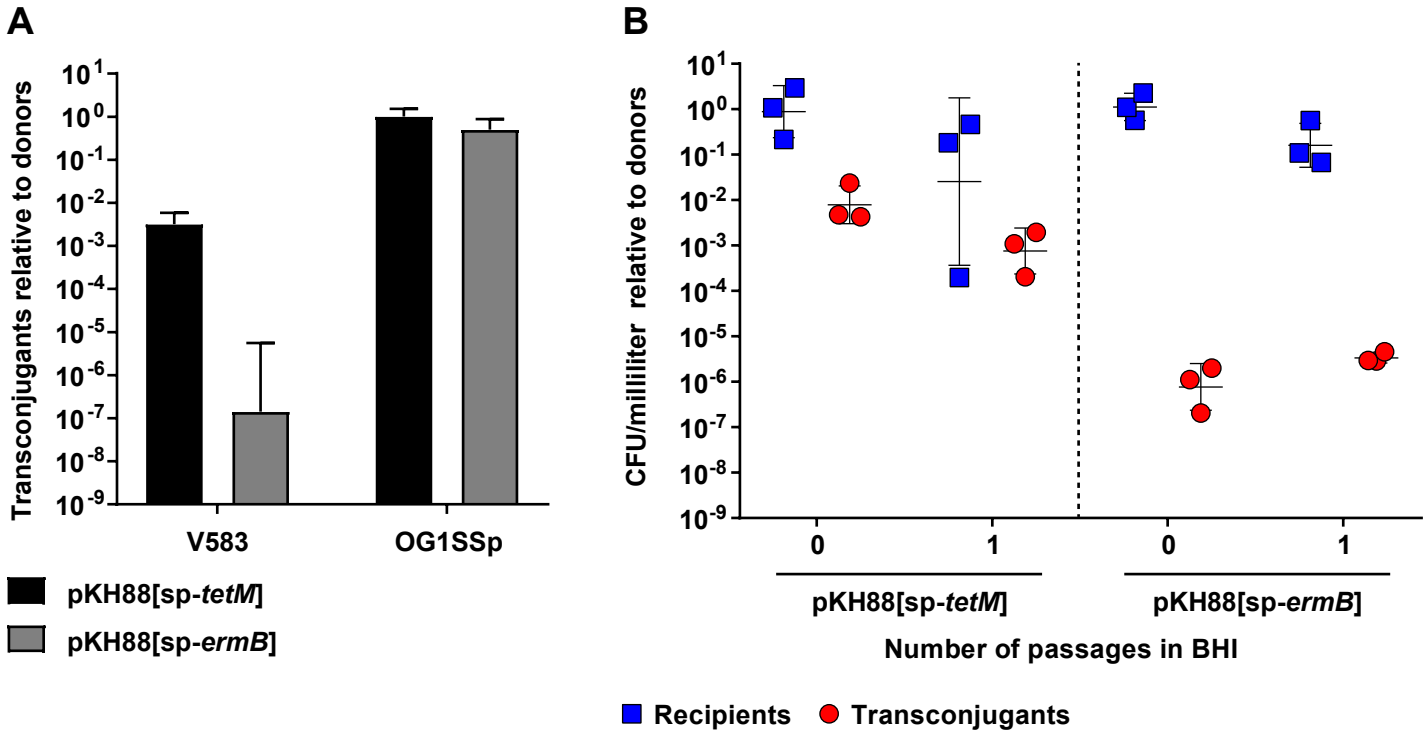
A
Transconjugants relative to donors
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
V583
OG1SSp
pKH88[sp-*tetM*]
pKH88[sp-*ermB*]
B
CFU/milliliter relative to donors
0
1
0
1
pKH88[sp-*tetM*]
pKH88[sp-*ermB*]
Number of passages in BHI
Recipients
Transconjugants

Figure S2

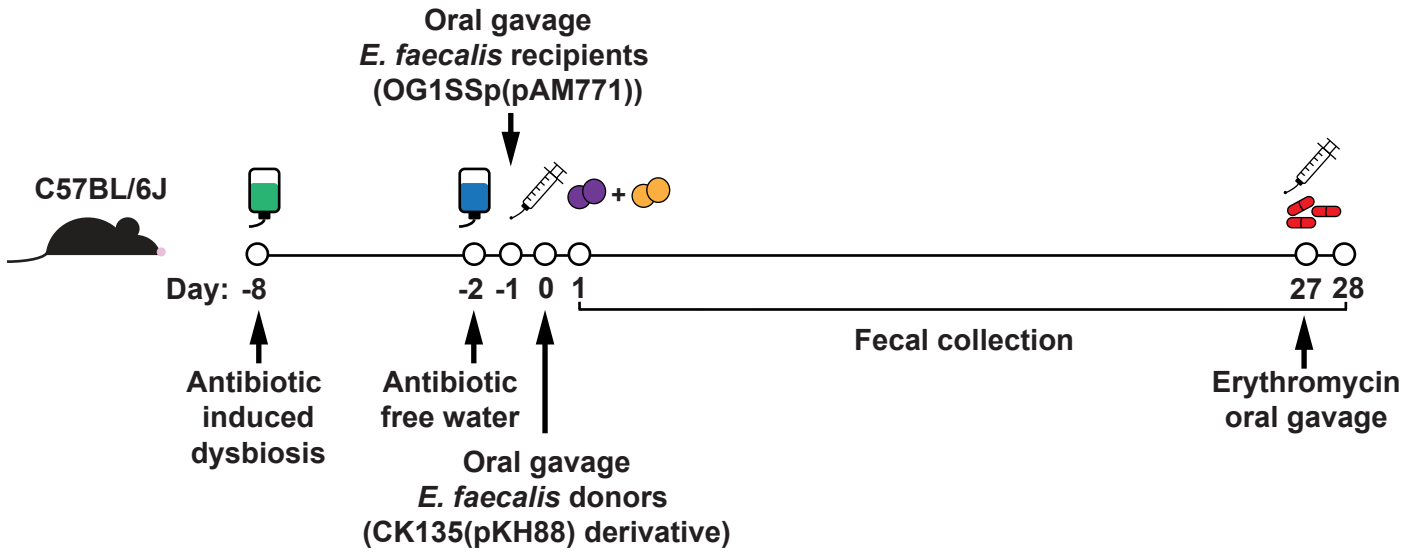
Oral gavage
E. faecalis recipients
(OG1SSp(pAM771))
C57BL/6J
Day: -8
-2 -1 0 1
27 28
Fecal collection
Antibiotic
induced
dysbiosis
Antibiotic
free water
Oral gavage
E. faecalis donors
(CK135(pKH88) derivative)
Erythromycin
oral gavage

Figure S3

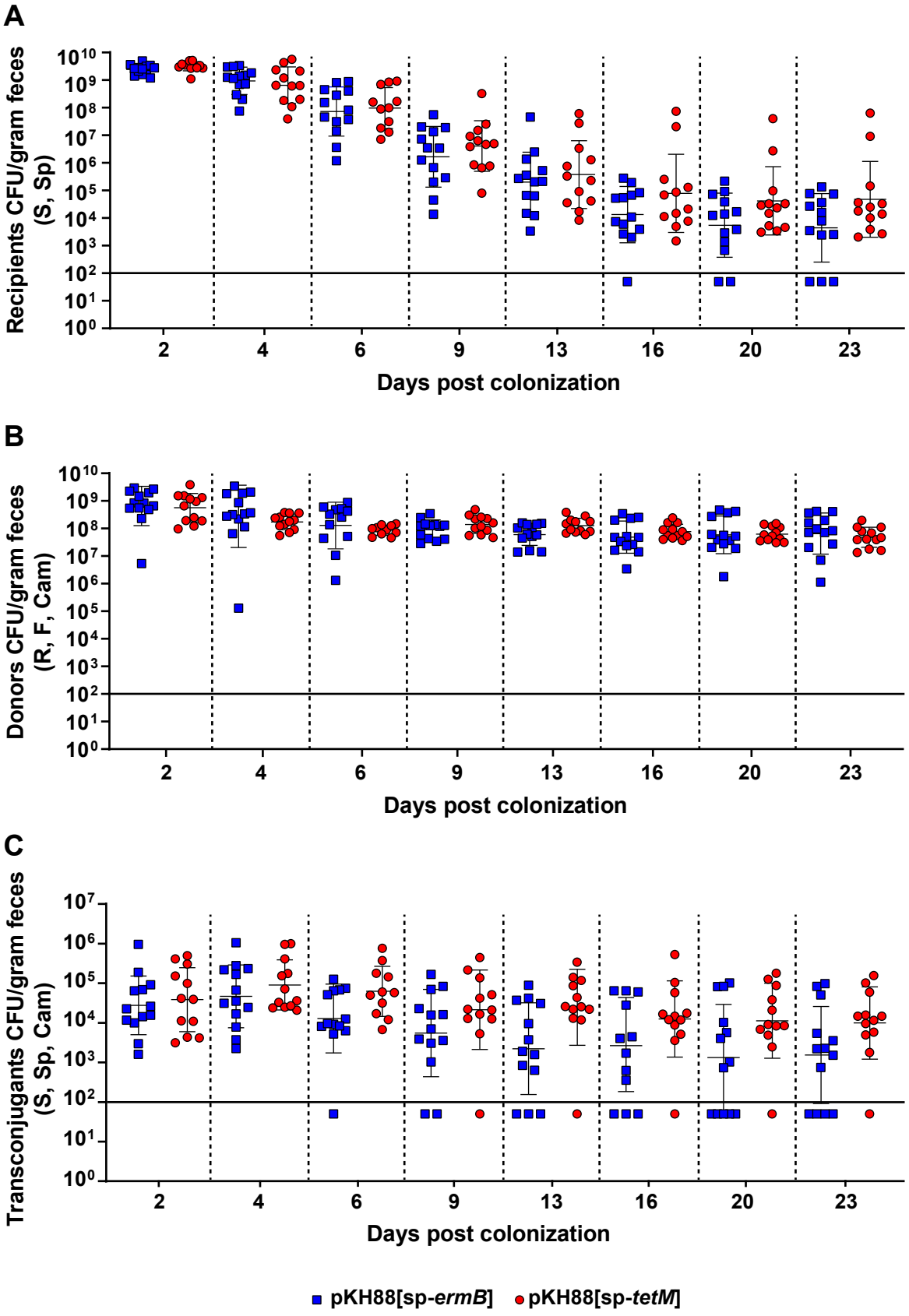
A
Recipients CFU/gram feces (S, Sp)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
B
Donors CFU/gram feces (R, F, Cam)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post 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

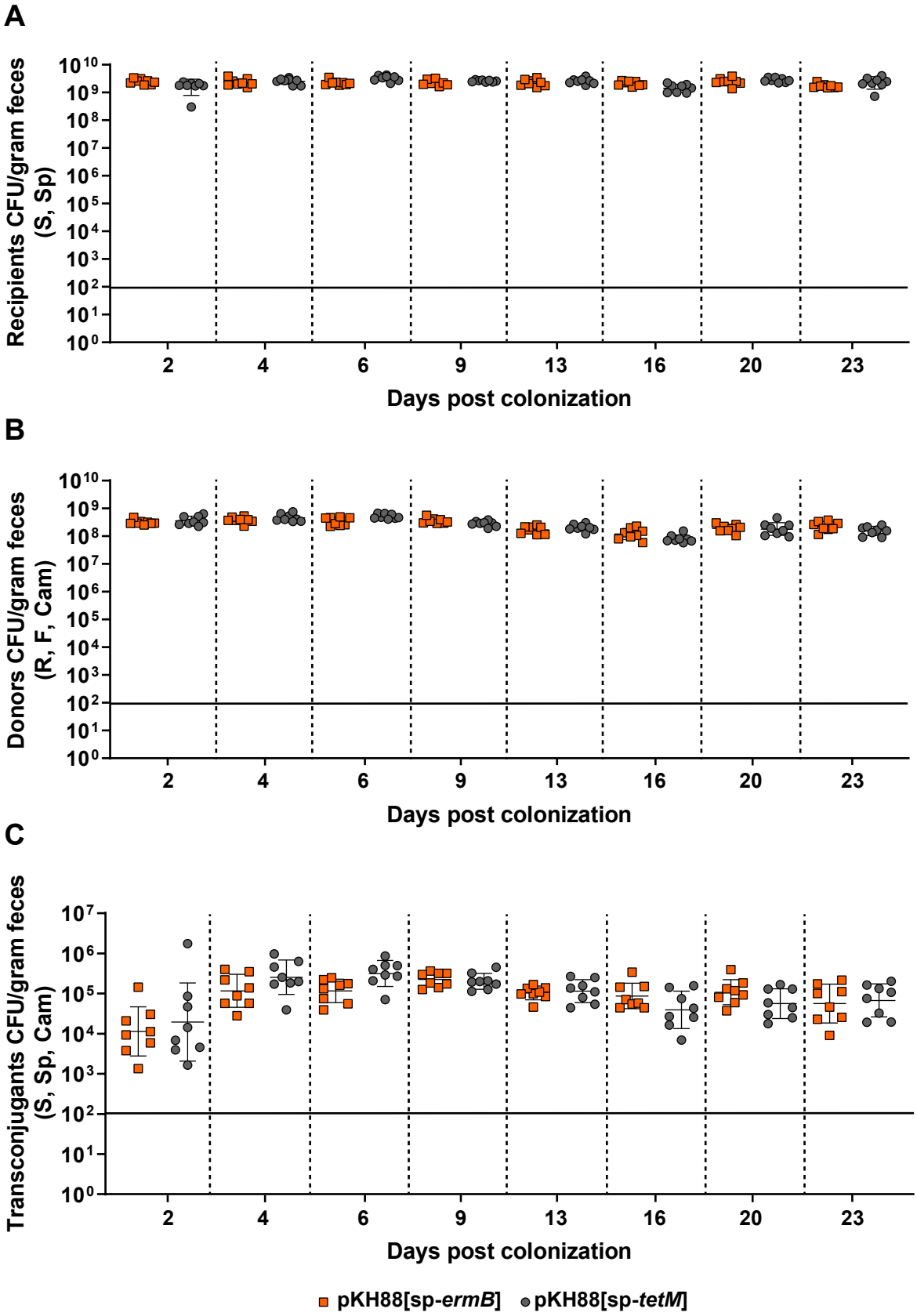
A
Recipients CFU/gram feces (S, Sp)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post colonization
B
Donors CFU/gram feces (R, F, Cam)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
2
4
6
9
13
16
20
23
Days post 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

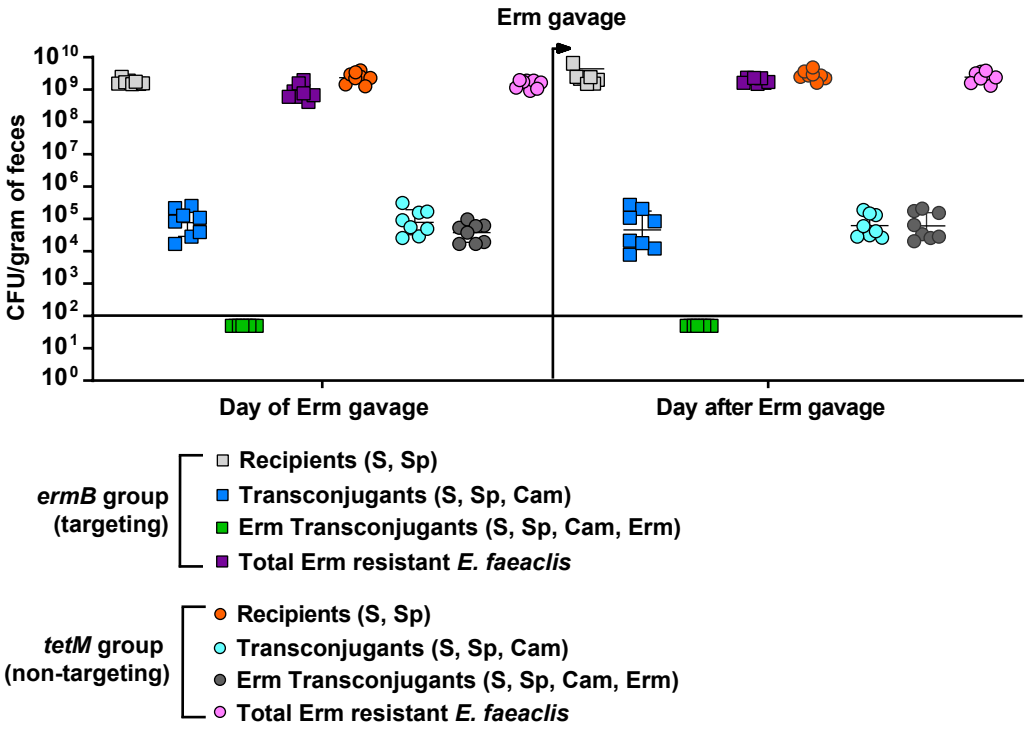
Erm gavage
CFU/gram of feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
Day of Erm gavage
Day after Erm gavage
ermB group (targeting)
Recipients (S, Sp)
Transconjugants (S, Sp, Cam)
Erm Transconjugants (S, Sp, Cam, Erm)
Total Erm resistant *E. faeaclis*
tetM group (non-targeting)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

Figure S6

Table S1. Strains used in this study

Organism	Strain Name	Description	Ref
E. coli	EC1000	*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*	(1)
E. faecalis	V583	MDR bloodstream isolate, resistant to vancomycin, gentamicin, and erythromycin	(2)
	OG1SSp	Spectinomycin-streptomycin-resistant derivative of OG1	(3)
	CK135	OG1 $rpoB_{H486Y}$ (spontaneous Rif^r derivative)	(4)
	CK135RF	Spontaneous fusidic acid-resistant derivative of CK135	This study
	OG1RF ΔEfaRFI	OG1RF EfaRFI (OG1RF_11622-11621) deletion mutant	(5)

Table S2. Plasmids used in this study

Plasmid	Description	Ref
pLT06	Encodes temperature-sensitive *repA* and *pheS** counter-selection	(6)
pHA101	pLT06 + *oriT*	(7)
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	Non-cytolytic derivative of the PRP pAD1 mutagenized with Tn*917,* encodes erythromycin resistance via *ermB*	(8)
pCF10	PRP; encodes tetracycline resistance via *tetM*	(9)
pPD1	PRP; encodes Bac-21 bacteriocin	(10)

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	GTTAACTGTGTTTGCTATAGAGTAATGTTTTAATTTTTTTCTCTTTTTCAG	Create pCOP88
pCOP88 Arm 2 for	GGATTCAGGAGTGGATTGATTCTGATATTGCCAATC	Create pCOP88
pCOP88 Arm 2 rev	CTAAAACGTCCTATTCAAAGAAACGCTGCAAGTCAACTAGAATCTGCTG	Create pCOP88
cas9 rev	TTTATTAAAGTTCATCTAGTCGACAACTTTACGGCGTGTTTC	Create pCOP88

cat for	AAAGTTGTCGACTAGATGAACTTTAATAAAATTG ATTTAGACAAT	Create pCOP88
cat rev	TCAACAAACTGGCCCGTTTGTTGAACTACTTTAT AAAAGCCAGTCATTAGGC	Create pCOP88
PSRT for	AGTAGTTCAACAAACGGGCC	Create pCOP88

References

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2. Sahm DF, Kissinger J, Gilmore MS, Murray PR, Mulder R, Solliday J, Clarke B. 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob Agents Chemother 33:1588-91.

3. Dunny GM, Brown BL, Clewell DB. 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc Natl Acad Sci USA 75:3479-3483.

4. Kristich CJ, Little JL. 2012. Mutations in the beta subunit of RNA polymerase alter intrinsic cephalosporin resistance in enterococci. Antimicrob Agents Chemother 56:2022-7.

5. Huo W, Adams HM, Trejo C, Badia R, Palmer KL. 2019. A type I restriction-modification system associated with *Enterococcus faecium* subspecies separation. Appl Environ Microbiol 85.

6. Thurlow LR, Thomas VC, Hancock LE. 2009. Capsular polysaccharide production in *Enterococcus faecalis* and contribution of CpsF to capsule serospecificity. J Bacteriol 191:6203-10.

7. Bhardwaj P, Ziegler E, Palmer KL. 2016. Chlorhexidine induces VanA-type vancomycin resistance genes in enterococci. Antimicrob Agents Chemother 60:2209-21.

8. Ike Y, Clewell DB. 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn917 as an insertional mutagen. J Bacteriol 158:777-783.

9. Dunny G, Funk C, Adsit J. Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis*. Plasmid 6:270-278.

10. Fujimoto S, Tomita H, Wakamatsu E, Tanimoto K, Ike Y. 1995. Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. J Bacteriol 177:5574-5581.