1 *Enterococcus faecalis* strains with compromised CRISPR-Cas defense emerge under antibiotic
2 selection for a CRISPR-targeted plasmid
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Abstract

Enterococcus faecalis is a Gram-positive bacterium that natively colonizes the human gastrointestinal tract and opportunistically causes life-threatening infections. Multidrug-resistant (MDR) *E. faecalis* strains have emerged that are replete with mobile genetic elements (MGEs). Non-MDR *E. faecalis* strains frequently possess CRISPR-Cas systems, which reduce the frequency of mobile genetic element (MGE) acquisition. We demonstrated in previous studies that *E. faecalis* populations can transiently maintain both a functional CRISPR-Cas system and a CRISPR-Cas target. In this study, we used serial passage and deep sequencing to analyze these populations. In the presence of antibiotic selection for the plasmid, mutants with compromised CRISPR-Cas defense and enhanced ability to acquire a second antibiotic resistance plasmid emerged. Conversely, in the absence of selection, the plasmid was lost from wild-type *E. faecalis* populations, but not *E. faecalis* populations that lacked the *cas9* gene. Our results indicate that *E. faecalis* CRISPR-Cas can become compromised under antibiotic selection, generating populations with enhanced abilities to undergo horizontal gene transfer.

Importance

Enterococcus faecalis is a leading cause of hospital-acquired infections and disseminator of antibiotic resistance plasmids among Gram-positive bacteria. We have previously shown that *E. faecalis* strains with an active CRISPR-Cas system can prevent plasmid acquisition and thus limit the transmission of antibiotic resistance determinants. Yet, CRISPR-Cas was not a perfect barrier. In this study, we observed populations of *E. faecalis* with transient co-existence of CRISPR-Cas and one of its plasmid targets. Our experimental data demonstrate that antibiotic selection results in compromised *E. faecalis* CRISPR-Cas function, thereby facilitating the acquisition of additional resistance plasmids by *E. faecalis*.

35 **Introduction**

36 *Enterococcus faecalis* is a Gram-positive bacterium that natively colonizes the human
37 gastrointestinal tract and opportunistically causes life-threatening infections (1-4). Antibiotic
38 resistance is a major concern for treatment of these infections. *E. faecalis* can acquire antibiotic
39 resistance through horizontal gene transfer (HGT), mediated primarily by plasmids, transposons,
40 and integrative conjugative elements (5-7). One of the most clinically relevant forms of HGT in *E.*
41 *faecalis* is afforded through the pheromone-responsive plasmids. The pheromone-responsive
42 plasmids are narrow host range conjugative plasmids that disseminate antibiotic resistance and
43 virulence genes among *E. faecalis* (5, 8-10) and mobilize resistance genes to other pathogens
44 (2, 11-14).

45

46 Some *E. faecalis* encode CRISPR-Cas systems. We have demonstrated that these systems
47 reduce the spread of antibiotic resistance plasmids among *E. faecalis in vitro,* and *in vivo* in the
48 murine intestine (15-19). CRISPR-Cas systems confer sequence-specific genome defense
49 against mobile genetic elements (MGEs) (20, 21). Type II CRISPR-Cas systems occur in *E.*
50 *faecalis* (22-24). They consist of a CRISPR array and CRISPR-associated (*cas*) genes. In *E.*
51 *faecalis*, the CRISPR is an array of 36 base pair (bp) repeat sequences interspersed by 30 bp
52 sequences referred to as spacers. When cells are challenged with a MGE, some incorporate a
53 short segment (protospacer) of the invading MGE genome into the CRISPR as a novel spacer
54 (20, 25). By this mechanism, the CRISPR serves as a heritable molecular memory of MGE
55 encounters. Short sequence motifs adjacent to protospacers, called protospacer adjacent motifs
56 (PAMs), as well as the Cas9, Cas1, and Cas2 nucleases are important for adaptation (26-31).
57 The CRISPR is transcribed and processed into small RNAs called crRNAs; one crRNA
58 corresponds to one spacer (32). If a MGE possessing the protospacer and PAM enters the cell,
59 the Cas9 nuclease is directed to the MGE genome by the crRNA. Cas9 cleaves the invading
60 MGE, generating a double-stranded DNA break, thereby inhibiting its entry (33-36).

61

62 Several of our studies (17, 18, 37) have focused on the CRISPR3-Cas locus of the *E. faecalis*
63 strain T11RF, which natively lacks horizontally acquired antibiotic resistance genes (38, 39). *E.*
64 *faecalis* T11RF is closely related to the model multidrug-resistant *E. faecalis* strain V583, but lacks
65 the ~620 kb of horizontally acquired genome content that V583 possesses (39). T11RF has a
66 CRISPR-Cas system, while V583 does not (24). In general, multidrug-resistant *E. faecalis* lack
67 CRISPR-Cas. The *E. faecalis* T11RF CRISPR3-Cas possesses 21 spacers. Spacer 6 is identical
68 to the *repB* gene of the model pheromone-responsive plasmid pAD1. pAD1 encodes a haemolytic
69 bacteriocin referred to as cytolysin and the virulence factor aggregation substance (8, 40). We
70 previously demonstrated that T11RF CRISPR3-Cas interferes with acquisition of the pAD1
71 derivative pAM714, which encodes erythromycin resistance via *ermB* on Tn*917* (41, 42). Deletion
72 of *cas9* or spacer 6 in *E. faecalis* T11RF resulted in up to a 150-fold increase in pAM714
73 acquisition in *in vitro* mating with *E. faecalis* OG1SSp(pAM714) donors (17). This increase in
74 conjugation frequency was not observed for the pheromone-responsive plasmid pCF10, which is
75 not natively targeted by the T11RF CRISPR3 spacers (17). These experiments confirmed that
76 CRISPR3-Cas is a sequence-specific anti-plasmid defense system in *E. faecalis* T11RF.

77

78 Despite *E. faecalis* T11RF CRISPR3-Cas acting as a barrier to pAM714, up to 10^5
79 T11RF(pAM714) transconjugants still arose in *in vitro* conjugation reactions (17). In this study, we
80 tested the investigated the fate of these transconjugants in serial passage experiments with and
81 without selection for the plasmid.

82

83 **Results**

84 **Serial passage experiments to study transconjugant evolution over time.** We mated *E.*
85 *faecalis* OG1SSp(pAM714) donors and T11RF or T11RF Δ*cas9* recipients for 18 hours on agar
86 plates without erythromycin selection (17). After 18 hours, mating reactions were recovered and

87 plated on agar selective for transconjugants, donors, and recipients. These data were previously
88 reported and demonstrated that CRISPR-Cas significantly reduces pAM714 acquisition by T11RF
89 (17). We randomly selected 6 T11RF(pAM714) and 6 T11RFΔ*cas9*(pAM714) transconjugants for
90 further analysis in this study. The 6 T11RF(pAM714) transconjugants are referred to hereafter as
91 WT1-WT6, and the 6 T11RFΔ*cas9*(pAM714) transconjugants are referred to as Δ1-Δ6. The
92 transconjugant colonies were each resuspended in brain heart infusion (BHI) broth. Samples were
93 removed from the colony resuspensions for serial dilution and plating to determine the percentage
94 of erythromycin-resistant cells, and for PCR and sequencing to determine the size and sequence
95 of the CRISPR3. These data are referred to as "Day 0." Next, each transconjugant colony
96 resuspension was split equally into BHI broth and BHI broth with erythromycin and passaged daily
97 for 14 days. At every passage, broth samples were removed to determine the percentage of
98 erythromycin-resistant cells and the size (by agarose gel electrophoresis) and sequence (by
99 Illumina sequencing) of the CRISPR3 amplicon. As a control, wild-type T11RF was also passaged
100 for 14 days in BHI broth.
101
102 At Day 0, the WT1-WT6 populations, except for WT4, were a mix of erythromycin-sensitive
103 (primarily) and erythromycin-resistant cells (Figure S1A). The Δ1-Δ6 populations were comprised
104 of erythromycin-resistant cells (Figure S1A). The CRISPR3 arrays of all populations were wild-
105 type size (1.76 kb) based on PCR amplification and agarose gel electrophoresis analysis of
106 products (Figure S1B).
107
108 **Erythromycin resistance is lost from most WT but no Δ*cas9* populations during passage**
109 **in non-selective medium.** The frequency of erythromycin-resistant cells decreased in the WT1,
110 WT2, WT3, WT5, and WT6 populations over the course of serial passage in BHI broth lacking
111 erythromycin (Figure 1A). In contrast, erythromycin resistance was stably maintained at high
112 frequencies in the WT4 population and all of the Δ1-Δ6 populations in the absence of erythromycin

113 selection (Figure 1A). The CRISPR3 arrays of all populations were of wild-type size based on
114 PCR amplification and agarose gel electrophoresis analysis of products (Figure 1B and Figure
115 S2).
116
117 We analyzed the WT4 population further because it was an outlier compared to the other WT
118 populations. We sequenced the *cas9* coding region of the WT4 population from Day 0 and
119 identified a mutation resulting in an Ala749Thr substitution. Ala749 occurs within the RuvC
120 nuclease domain and is conserved in the well-studied Cas9 of *Streptococcus pyogenes* (17). The
121 Ala749Thr substitution may result in loss in Cas9 function, causing WT4 to phenocopy the Δ1-Δ6
122 populations.
123
124 **CRISPR-Cas mutants emerge in WT populations during passage with erythromycin.** We
125 observed stable maintenance of erythromycin resistance in all WT1-WT6 and Δ1-Δ6 populations
126 during passage with erythromycin selection (Figure 1A). By Day 14 of passage with erythromycin,
127 CRISPR3 arrays in all WT populations except WT1 and WT4 were smaller in size than the wild-
128 type T11RF control (Figure 1B). The variation in array size initiated sporadically over the 14 days,
129 and each transconjugant had a unique pattern (Figure S2). Sanger sequencing of the CRISPR3
130 array amplicons from Day 1 and Day 14 erythromycin-passaged populations revealed that the
131 CRISPR3 spacer 6 was either deleted from the array or the spacer 6 region had poor sequence
132 quality (Table 2). We will refer to spacer 6 as S_6 hereafter, and use this subscript nomenclature
133 for the other 20 spacers of the T11RF CRISPR3 array (e.g., S_1, S_2, etc.).
134
135 In contrast to WT populations, CRISPR3 array sizes for the Δ1-Δ6 populations were unchanged
136 after passage in medium with erythromycin (Figure 1B and Table 2). We chose the Δ4 population
137 as a representative of the Δ1-Δ6 populations for future analyses.
138

139 To investigate whether mutations were present outside the CRISPR3 array, we performed whole
140 genome Illumina sequencing on select populations. We observed variation in the *cas9* sequence
141 of the WT1, WT2, and WT3 populations relative to the T11RF wild-type (Table 3). All of the
142 mutations led to nonsynonymous changes (Table 3). Some of the populations possessed
143 variations in one or more additional genes (Table S2). No mutations were identified in the S_6
144 protospacer or the PAM region of the *repB* gene in pAM714. However, we did identify a mutation
145 within *repB*, not associated with the protospacer or PAM, in the WT2 population (Table S2). We
146 observed no evidence of Tn*917* movement from pAM714 into the T11 chromosome as all reads
147 overlapping the ends of Tn*917* also overlapped the pAD1 reference sequence.
148
149 **CRISPR3 alleles with spacer deletions are present in most WT populations after serial**
150 **passage with erythromycin selection.** To attain greater resolution of CRISPR3 alleles, we used
151 Illumina sequencing to analyze CRISPR3 amplicons. We analyzed the CRISPR3 amplicons
152 obtained for the WT1-WT6 and Δ4 populations after Day 1 and Day 14 of serial passage with
153 erythromycin. We also analyzed the wild-type T11RF control passaged in BHI broth. We achieved
154 an average of 16 million reads for our amplicons (Table S3).
155
156 To identify specific CRISPR3 alleles in the amplicon sequencing, we created a pool of references
157 by sliding a contiguous and non-overlapping window of 96 bp in length along CRISPR3 reference,
158 which allows each reference to contain exactly two adjacent spacers connected by one repeat.
159 The sliding window starts at 30 bp upstream of the first repeat and ends at 30 bp downstream of
160 the terminal repeat. In total, 22 references were generated to represent wild type alleles. Then,
161 we manually constructed artificial CRISPR3 reference sequences for every possible spacer
162 deletion and rearrangement event with a length of 96 bp. In total, 462 references were constructed
163 to represent all mutant alleles. We then mapped CRISPR amplicon reads to wild-type and mutant
164 references (see Figure S3). The mapping was performed with stringency to allow unique mapping

165 only. After mapping, the number of mapped reads per 96-bp reference was calculated and
166 normalized by the total number of mapped reads per all references. To further evaluate the
167 normalized reads per reference, we calculated z-score using mean and standard deviation from
168 the T11RF Day 1 and Day 14 mapping result. The higher the z-score, the more abundant reads
169 per reference. The significance of z-score was calculated using t-test with a degree of freedom of
170 483. By applying a p-value cutoff of 0.05, we identified the most abundant CRISPR3 alleles in
171 each population (Figure 2 and Table 2). Of the five T11RF transconjugant populations analyzed,
172 all other than WT1 possessed at least one significantly enriched mutant CRISPR3 allele after 14
173 days of passage with antibiotic selection, and each of those alleles lacked S_6 (Table 2).
174
175 One drawback for our method is that it is difficult to set a p-value cutoff. Here we used canonical
176 definition of 0.05. However, the normalized reads per reference for wild-type alleles is expected
177 to be much higher than that from mutant alleles, and in fact, it is much higher in control samples
178 (T11RF control Day 1 and Day 14). Such variation results in large standard deviation values which
179 skew the calculation of z-scores and hence underestimates the abundance of mutant alleles. We
180 predict that more mutant alleles existed in Day 14 WT transconjugant samples, which is supported
181 by an examination of the normalized reads per reference plot (Figure S4), but we are uncertain
182 about their significance as measured by p-value. Supporting our approach, control samples
183 yielded results largely as expected. In control samples (T11RF and the representative Δ*cas9*
184 transconjugant), only wild type alleles were significantly abundant with p-value < 0.05. However,
185 we also observed uneven distribution among different 96-bp references representing wild-type
186 alleles, which resulted in a “loss of spacer16-17” call for the Day 14 Δ*cas9* transconjugant at the
187 p-value of 0.057. When looking at the normalized reads per reference heatmap (Figure S4), we
188 believe spacer16-17 was still largely intact within the Δ*cas9* transconjugant population, the
189 abundance of which is about 1000 times more than that from a mutant 96-bp reference. In short,
190 our statistical approach has strengths and weaknesses, which have been discussed here.

191

192 **Functional assays confirm that CRISPR-Cas defense was compromised after antibiotic**
193 **selection.** Our sequencing analyses suggested that the WT transconjugants passaged with
194 erythromycin had become deficient for CRISPR-Cas defense either by deletion of spacer 6 or
195 inactivation of Cas9 by mutation. For a functional assessment, we tested whether the
196 transconjugant populations could still defend against sequences targeted by T11RF CRISPR3-
197 Cas. To do this, we utilized the pheromone-responsive plasmid pCF10 (43), which is not natively
198 targeted by the T11RF CRISPR3-Cas system. We previously demonstrated that pCF10 transfer
199 into wild-type and Δ*cas9* T11RF strains is equivalent (17). In this study, we modified pCF10 to be
200 targeted by the T11RF CRISPR3-Cas system. We generated three derivatives of *E. faecalis*
201 OG1SSp(pCF10), each with an insertion of a T11RF CRISPR3 spacer (S_1, S_6, or S_7) and
202 CRISPR3 consensus PAM in the pCF10 *uvrB* gene (Figure 3A). Disruption of *uvrB* does not
203 impact pCF10 conjugation (17). We then compared conjugation of wild-type pCF10 and these
204 derivatives into the control T11RF population that had been passaged in BHI broth for 14 days.
205 As expected, conjugation frequencies were significantly lower for all pCF10 derivatives bearing
206 CRISPR3 targets, as compared to the wild-type pCF10 (Figure 3B).

207

208 We then evaluated pCF10 transfer into the WT5 Day 14 populations. For WT5 passaged without
209 erythromycin, we observed similar results to the T11RF control (Figure 3B), demonstrating that
210 the CRISPR3-Cas system in this population is still functional. For WT5 passaged with
211 erythromycin, conjugation frequency of only the pCF10 derivative bearing S_1 was reduced relative
212 to wild-type pCF10 (Figure 3B). This is consistent with the deletion of S_6 and S_7 in this population
213 (Figure 2), and demonstrates that the sequencing data are accurate and not the result of PCR
214 amplification bias. We note that we observed a ~3 log higher conjugation frequency of pCF10 into
215 this population compared to the WT5 population passaged without erythromycin (Figure 3B). This
216 is due to comparatively low donor numbers (averages of 7.1×10^6 and 5.6×10^9 CFU/mL,

217 respectively; raw transconjugant and donor numbers for Figure 2 are presented in Dataset S1).
218 pAM714 encodes the bacteriocin, cytolysin (8). We previously reported that *E. faecalis* with
219 pAM714 kill *E. faecalis* cells that lack pAM714 (18). The probable explanation is that the WT5
220 population passaged with erythromycin, which has a very high carriage rate of pAM714 (Figure
221 1) killed OG1SSp(pCF10) donors using cytolysin.
222
223 We also evaluated pCF10 transfer into the WT4 Day 14 populations. WT4 was unique among the
224 6 WT transconjugants in that it maintained erythromycin resistance at high frequencies in the
225 absence of erythromycin selection (Figure 1A). We identified a probable loss-of-function mutation
226 in the RuvC domain-encoding region of *cas9*. The WT4 populations did not interfere with any of
227 the three pCF10 derivatives bearing CRISPR3 targets (Figure 3B). This is consistent with a loss
228 of CRISPR-Cas function in the WT4 populations conferred by a loss of function mutation in *cas9*.
229
230 **Analysis of *E. faecalis* genomes identifies a strongly supported instance of *in situ* CRISPR-**
231 **Cas compromise.** Our data demonstrate that loss-of-function mutations in CRISPR-Cas arise
232 that promote plasmid acquisition (for WT4) or plasmid maintenance (for WT2-3 and WT5-6) in *E.*
233 *faecalis*. We used genome data available for *E. faecalis* to identify potential instances where this
234 may have occurred in nature. We focused on published MDR *E. faecalis* strains that possess
235 CRISPR-Cas, reasoning that CRISPR-Cas function may have been compromised, allowing for
236 the accumulation of acquired resistance genes. More specifically, we narrowed our focus to
237 strains that both encode CRISPR-Cas as well as resistance to the last-line antibiotics vancomycin
238 or linezolid, which are often plasmid-borne. Using these strict criteria, we analyzed the literature
239 and identified one strongly supported occurrence of CRISPR-Cas compromise. The MDR *E.*
240 *faecalis* strain KUB3006, for which a completely closed genome is available (44), possesses 4
241 plasmids, including one that encodes linezolid resistance. Yet, it also encodes CRISPR3-Cas
242 (44). The *cas9* gene is frameshifted, and the frameshift occurs within the codon for one of the two

243 Cas9 active sites that we previously experimentally confirmed in *E. faecalis* (17). We conclude
244 that CRISPR3-Cas function is likely to be compromised in this strain, which requires experimental
245 confirmation.
246
247 **Discussion**
248
249 In this study we investigated the fates of *E. faecalis* transconjugants that acquired a CRISPR-
250 targeted plasmid, using serial passage with and without antibiotic selection for the plasmid.
251
252 We observed that 5 of 6 wild-type T11RF(pAM714) transconjugants lost the plasmid over the
253 course of antibiotic-free serial passage, while T11RFΔ*cas9*(pAM714) transconjugants maintained
254 the plasmid. This is indicative of active CRISPR-Cas defense in the T11RF(pAM714)
255 transconjugant populations. An important caveat is that erythromycin is a bacteriostatic antibiotic,
256 and we cannot be certain that plasmid-free T11RF surviving on the transconjugant selective agar
257 were not carried over with the initial transconjugant colonies that were picked. At least two
258 explanations for our observations are possible. The first is that CRISPR-Cas 'scrubs' pAM714
259 from T11RF over the course of antibiotic-free serial passage. The second is that T11RF cells
260 lacking pAM714 (either carried over or sporadically arising) outcompete T11RF(pAM714) cells.
261 This competitive effect is due to the growth defect of cells that simultaneously possess CRISPR-
262 Cas and one of its plasmid targets, as we have previously reported (15, 16).
263
264 On the other hand, when the same T11RF(pAM714) transconjugants were passaged with
265 antibiotic selection, the plasmid was maintained, as expected, but variants emerged that lacked
266 the spacer targeting the plasmid. Variations in *cas9* were also observed, but not experimentally
267 investigated, so their impacts on CRISPR-Cas function remain undefined. Importantly, one of
268 these transconjugant populations was found to have lost previously programmed sequence-

269 specific defense against resistance plasmids. This supports our CRISPR amplicon sequence
270 analysis by demonstrating that spacer losses identified by sequencing corresponded with loss of
271 functional CRISPR-Cas defense. We also identified one very strongly supported example of
272 CRISPR-Cas compromise in an independent *E. faecalis* isolate with a closed genome. We posit
273 that the interplay of CRISPR-Cas, plasmids, and antibiotic selection should be further investigated
274 for a comprehensive understanding of the roles of genome defense systems such as CRISPR-
275 Cas in mitigating the antibiotic resistance crisis.
276
277 **Materials and Methods**
278
279 **Strains, reagents, and routine molecular biology procedures.** Bacterial strains and plasmids
280 used in this study are listed in Table 1. *E. faecalis* strains were grown in Brain Heart Infusion (BHI)
281 broth or on agar plates at 37°C unless otherwise noted. Antibiotics were used for *E. faecalis* at
282 the following concentrations: erythromycin, 50 µg/mL; chloramphenicol, 15 µg/mL; streptomycin,
283 500 µg/mL; spectinomycin, 500 µg/mL; rifampicin, 50 µg/mL; fusidic acid, 25 µg/mL; tetracycline,
284 10 µg/mL. *Escherichia coli* strains used for plasmid propagation and were grown in lysogeny broth
285 (LB) broth or on agar plates at 37°C. Chloramphenicol was used at 15 µg/mL for *E. coli*. PCR was
286 performed using *Taq* (New England Biolabs) or Phusion (Fisher Scientific) polymerases. Primer
287 sequences used are in Table S1. Routine Sanger sequencing was carried out at the
288 Massachusetts General Hospital DNA core facility (Boston, MA). *E. faecalis* electrocompetent
289 cells were made using the lysozyme method as previously described (45).
290
291 **Generation of pCF10 derivatives**. To insert the T11 CRISPR3 spacer 1 (S_1), S_6, and S_7
292 sequences and CRISPR3 PAM (TTGTA) into pCF10, 47 bp and 39 bp single stranded DNA oligos
293 were annealed to each other to generate dsDNA with restriction enzyme overhangs for BamHI
294 and PstI. The annealed oligos were ligated into the pLT06 derivative pWH107 that includes

295 sequence from pCF10 *uvrB*, to insert these sequences into the *uvrB* gene of pCF10 by
296 homologous recombination. A knock-in protocol was performed as previously described (17).
297
298 **Conjugation experiments.** *E. faecalis* donor and recipient strains were grown in BHI overnight
299 to stationary phase. A 1:10 dilution was made for both donor and recipient cultures in fresh BHI
300 broth and incubated for 1.5 hr to reach mid-exponential phase. A mixture of 100 µL donor cells
301 and 900 µL recipient cells was pelleted and plated on non-selective BHI agar to allow conjugation.
302 After 18 h incubation, the conjugation mixture was scraped from the plate using 2 mL 1X PBS
303 supplemented with 2 mM EDTA. Serial dilutions were prepared from the conjugation mixture and
304 plated on BHI agars selective for transconjugants or donors. After 24-48 h incubation, colony
305 forming units per milliliter (CFU/mL) was determined using plates with 30 - 300 colonies. The
306 conjugation frequency was calculated as the CFU/mL of transconjugants divided by the CFU/mL
307 of donors.
308
309 **Serial passage.** Transconjugant colonies were suspended in 50 µL BHI broth. The 50 µL
310 suspension was used as follows: 3 µL was used for PCR to confirm the integrity of the CRISPR
311 array, 10 µL was inoculated into plain BHI broth, another 10 µL was inoculated into selective BHI
312 broth for plasmid selection, and another 10 µL was used for serial dilution and plating on selective
313 medium to enumerate the initial number of plasmid-containing cells in the transconjugant
314 colonies. Broth cultures were incubated for 24 h, followed by 1:1000 dilution into either fresh plain
315 BHI or fresh selective BHI. At each 24 h interval, 3 µL of each culture from the previous incubation
316 was used for PCR to check CRISPR array integrity, and 10 µL was used for serial dilution and
317 plating on agars to determine CFU/mL for total viable cells and plasmid-containing cells. The
318 cultures were passaged in this manner for 14 days; cryopreserved culture stocks were made daily
319 in glycerol. To use the Day 14 transconjugant populations in conjugation reactions, the glycerol
320 stocks were completely thawed on ice, and 20 µL was inoculated into plain BHI broth. The cultures

321 were incubated for 6-8 h to allow them to reach mid-exponential phase (OD_{600nm} ≈ 0.5–0.7), and
322 900 µL was used as recipient in conjugation reactions as described above.
323
324 **Deep sequencing of CRISPR3 amplicons and genomic DNA.** For CRISPR3 amplicon
325 sequencing, 3 µL from a broth culture was used as template in PCR using Phusion Polymerase
326 with CR3_seq_F/R primers (Table S1). The PCR products were purified using the Thermo
327 Scientific PCR purification kit (Thermo Scientific). Genomic DNA was isolated using the phenol-
328 chloroform method (46). The purified PCR amplicons and genomic DNA samples were
329 sequenced using 2 x 150 bp paired end sequencing chemistry by Molecular Research LP (MR
330 DNA; Texas).
331
332 **Whole genome sequencing analysis.** T11 supercontig and pAD1 plasmid contig references
333 were downloaded from NCBI (accession numbers: T11: NZ_GG688637.1-NZ_GG688649; pAD1:
334 AB007844, AF394225, AH011360, L01794, L19532, L37110, M84374, M87836, U00681,
335 X17214, X62657, X62658, M11180 [Tn*917*]). Reads were aligned to these references using
336 default parameters in CLC Genomics Workbench (Qiagen) where ≥50% of each mapped read
337 has ≥80% sequence identity to the reference. Variations occurring with ≥35% frequency at
338 positions with ≥10X coverage between our samples and the reference contigs were detected
339 using the Basic Variant Detector. At the same time, local realignment was performed, followed by
340 Fixed Ploidy variant detection using default parameters and variants probability ≥90% in CLC
341 Genomics Workbench. The basic variants and fixed ploidy variants were combined for each
342 sequencing sample and subjected to manual inspection. The variants that were detected in the
343 T11 genome from all samples were inferred to be variants in our parent T11 stock and were
344 manually removed. The variants that were detected in pAD1 genome from all transconjugant
345 samples were inferred to be variants in our pAM714 stock, hence were also manually removed.
346 Next, variants within the CRISPR3 array were removed as we analyzed CRISPR3 alleles using a

347 different approach (amplicon deep sequencing; see below). All variants detected from all
348 populations were manually checked for coverage depth to eliminate the detection bias. The
349 variants detected in all samples are shown in Table S2. To detect the insertion site of Tn*917*, the
350 mapped reads on reference M11180 were inspected. Reads immediately adjacent to the 5' and
351 3' ends of Tn*917* shared consensus sequences which were later used for blast against T11 and
352 pAD1 (Figure S5). No evidence for Tn*917* insertions other than in the expected position in
353 pAM714 was detected.
354
355 **Analysis of CRISPR3 amplicon sequencing.** To analyze the amplicon sequencing, we first
356 created a pool of references with 96-bp in length to represent both wild type and mutant CRISPR3
357 alleles. In the wild type reference pool, each 96-bp reference contains two adjacent spacers
358 connected by one direct repeat (5'-S_x-R-S_{x+1}-3' where x is from 1 to 20). To include the leader end
359 and terminal end, wild type reference pool also contains 30 bp upstream of the first repeat (S_0)
360 and 30 bp downstream of the terminal repeat (S_T), generating two additional wild type references:
361 5'-S_0-R-S_1-3' and 5'-S_{21}-TR-S_T-3'. In total, 22 references were created to represent wild type
362 CRISPR3 allele.
363
364 To detect CRISPR3 spacer deletions and rearrangements, we manually created an additional
365 462 references with 96-bp in length to represent mutant CRISPR3 alleles. Each mutant reference
366 contains two spacers connected by a direct repeat, where the reference was not already
367 represented in the wild type reference pool. Schematically, the mutant references can be
368 expressed by 5'-spacer[x]-repeat-spacer[y]-3' (5'-S_xRS_y-3') where $y \neq x$. The selection of
369 spacer[x] is from S_0 to S_{21} while the selection of spacer[y] is from S_1 to S_T. Additionally, we assume
370 that if spacer[y] is ST then the repeat would be terminal repeat TR, generating 5'-S_xTRS_T-3' (x is
371 from 0 to 21). In total, 462 references were created with 96-bp in length.
372

373 Sequencing reads were first clipped into 75 bp fragments to enhance mapping efficiency, allowing
374 for retrieval of maximal sequence information. The clipped reads were used to map to the pool of
375 96-bp references using stringent mapping parameters in CLC Genomics Workbench (Qiagen).
376 The stringent mapping parameters require 100% of each mapped read to be ≥95% identical to
377 one unique reference. Thus, the sequencing reads from different CRISPR alleles will be
378 distinguished. The number of mapped reads per each reference was calculated and normalized
379 by the total number of mapped reads to all references, generating normalized reads per reference.
380 The total number of mapped reads to pools of wild type references and mutant references were
381 summarized in Table S3.
382
383 To visualize the normalized reads per reference, a heatmap was generated (Figure S4). On the
384 heatmap, each row name represents 5'-S_x (x is from 0 to 20) and each column name represents
385 R-S_y-3' (y is from 1 to 21) or TR-S_T-3'. The diagonal cells are empty due to the assumption of no
386 repetitive spacers (or 5'-Sx-R-Sx-3'). Intuitively, the cells adjacent to the diagonal cells on the
387 upper side represents wild type pool of references, 5'-Sx-R-Sy-3' where y=x+1 (Figure S3). The
388 cell color represents normalized reads per reference (from white to red to black is lowest to
389 intermediate to the highest abundance).
390
391 To evaluate the statistical significance of normalized mapped reads, we calculated z-score using
392 the mean and standard deviation calculated from T11RF control. Combining the normalized reads
393 per reference from Day 1 and Day 14 T11RF control, we obtained an average normalized reads
394 per reference of 0.002 or 2%) with standard deviation of 0.0096 or 9.6%. These numbers were
395 used to calculated z-score for normalized reads per reference for all samples. To assess the
396 significance, the z-score is further transformed into p-value. A p-value of 0.05 was used as a
397 significant cutoff.
398

399 To visualize the p-value and derive mutant alleles, a heatmap was plotted using the same method
400 as above. The cells with p-value < 0.05 were color coded. To derive the mutant allele, we assume
401 that S_0 and S_T are intact. The sequence of spacers was derived based on the idea of forward
402 algorithm and Viterbi algorithm. Each p-value was considered as a conditional probability: p-value
403 (5'-Sx-R-Sy-3') ~ P(spacer y $|_{\text{spacer x}}$).
404
405 **Accession number.** The sequencing data for amplicon and whole genome sequencing analysis
406 of transconjugant populations has been deposited in the NCBI Sequence Read Archive under
407 PRJNA418345**.**
408
409 **Acknowledgement.** This work was supported by Public Health Service grant R01AI116610 to
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411 data analysis methods.
412
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558

559 **Table 1. Bacterial strains and plasmids used.**
560

Name	Description	Reference
***E. faecalis* strains**		
T11RF	Rifampicin- and fusidic acid-resistant derivative of the human urine isolate T11	(17, 39)
T11RFΔ*cas9*	Derivative of T11RF with *cas9* deleted	(17)
OG1RF	Rifampicin- and fusidic acid-resistant derivative of the human oral isolate OG1	(22, 47)
OG1SSp	Spectinomycin- and streptomycin-resistant derivative of OG1; donor strain for conjugation assays	(41-43)
Plasmids		
pAM714	65 kb PRP encoding erythromycin on Tn*917*, derivative of pAD1	(41)
pCF10	67 kb PRP encoding tetracycline resistance on Tn*925*	(43)
pLZ12	Broad host range shuttle vector encoding chloramphenicol resistance	(48)
pKH12	pLZ12 with *oriT*	(16)
pKHS5	pKH12 with CRISPR2 protospacer S5 and CRISPR1/2 PAM	This study
pKHS96	pKH12 with CRISPR1 protospacer S96 and CRISPR1/2 PAM	(16)
pVP107	pLT06 with T11CR2 protospacer and CRISPR1/2 PAM	(17)
pWH107	pVP107 digested with XbaI/SphI and re-ligated with primers pVP107_ XbaI _For/pVP107_SphI_Rev to remove PstI enzyme site	This study
pWH107.S1	pWH107 with T11CR3 protospacer S1 and CRISPR3 PAM inserted between BamHI/PstI	This study
pWH107.S6	pWH107 with T11CR3 protospacer S6 and CRISPR3 PAM inserted between BamHI/PstI	This study
pWH107.S7	pWH107 with T11CR3 protospacer S10 and CRISPR3 PAM inserted between BamHI/PstI	This study

561

562

563 **Table 2. CRISPR alleles.**
564

Sample name	Day 1 Sanger[c]	Day 14 Sanger[c]	Day 1 Amplicon[d]	Day 14 Amplicon[d]
T11RF control[a]	WT	WT	WT	WT
Δ4[b]	WT	WT	WT	WT
WT1[b]	Poor quality at S_6-S_7	WT	WT	WT
WT2[b]	WT	ΔS_2-S_{11}	WT	ΔS_2-S_{11}
WT3[b]	Poor quality at S_6-S_7	ΔS_5-S_7	WT	ΔS_5-S_7
WT5[b]	Poor quality at S_3-S_8	Poor quality at S_3-S_9	WT	ΔS_5-S_8
WT6[b]	Poor quality at S_1-S_7	Poor quality at S_5-S_6	ΔS_5-S_6	ΔS_6

565 [a]T11RF without pAM714 passaged for 14 days in BHI medium.
566 [b]pAM714 transconjugants passaged for 14 days in BHI medium with erythromycin.
567 [c]CRISPR3 alleles detected by Sanger sequencing.
568 [d]CRISPR3 alleles detected by Illumina amplicon deep sequencing. Abundant alleles with p-value
569 < 0.05 are shown.
570

571

572 **Table 3. Nonsynonymous *cas9* mutations detected by whole genome sequencing.**
573

Position	Ref	Allele	Amino acid change	WT1[a]	WT2[a]	WT3[a]
652983	G	A	Gln506*	31.6% (689x)	ND (590x)	ND (577x)
653184	C	T	Glu439Lys	ND (640x)	ND (536x)	24.2% (594x)
653180	AG	T	Leu440fs	ND (640x)	ND (528x)	23.5% (590x)
654165	G	-	Arg112fs	ND (676x)	48.4% (659x)	ND (772x)

574 [a]Shown are variation frequency and coverage at the indicated nucleotide position on *E. faecalis* T11
575 contig 1.11.
576 ND, not detected.

577

578

579 **Figure Legends**

580

581 **Figure 1. Antibiotic resistance phenotypes and qualitative assessment of CRISPR3**
582 **genotypes in serially passaged transconjugant populations.** a) Erythromycin resistant
583 CFU/mL (expressed as percent of total CFU/mL) remaining in *E. faecalis* populations over the
584 course of passage without (left) and with (right) antibiotic selection. WT transconjugant
585 populations are shown in green or red and Δ*cas9* transconjugant populations are shown in black.
586 b) CRISPR3 amplicon size from early (Day 1) and late (Day 14) passage dates for six WT
587 transconjugant populations and a representative Δ*cas9* transconjugant population (Δ4). The
588 CRISPR3 region was PCR-amplified using aliquots from passaged cultures as template. As a
589 control, T11RF without pAM714 was passaged for 14 days and the CRISPR3 locus amplified
590 (T11RF). P: positive control, T11RF genomic DNA. L: DNA ladder.

591

592 **Figure 2. Detection of mutant CRISPR3 array alleles using amplicon sequencing.** Heat maps
593 show the mutant alleles with significant abundance (p-value <0.05) present at Day 1 and Day 14
594 of T11RF control (a) and antibiotic-passaged transconjugant populations (b-g). Each cell within
595 heatmap represents one of the 484 artificial references where a 5' spacer (rows) is linked with a
596 3' spacer (columns) by a repeat. White cells represent mutant alleles that were not significantly
597 abundant.

598

599 **Figure 3. Experimental confirmation of compromised CRISPR-Cas function in antibiotic-**
600 **passaged WT5 and in WT4.** Day 14 transconjugant populations passaged in BHI and
601 erythromycin (Erm) were used as recipients in conjugation reactions with OG1SSp(pCF10) and
602 derivatives of pCF10. a) Schematic of where the T11 CRSPR3 spacer 1, spacer 6 and spacer 7
603 sequences with corresponding PAM (underlined) were introduced into pCF10. Wild-type pCF10
604 is not natively targeted by T11RF CRISPR3-Cas. b) Conjugation frequency (ratio of

605 transconjugants to donors) from mating reactions. A749T refers to the *cas9* mutation detected in
606 the WT4 population. Statistical significance was determined using a student's t-test; P-values: **
607 ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001.
608
609

610 **Supplemental Material Legends.**

611

612 **Dataset S1. Raw transconjugant and donor CFU/mL data.**

613

614 **Table S1. Primers used in this study.**

615

616 **Table S2. SNP detection in all genomic DNA sequencing samples.**

617

618 **Table S3. Mapping statistics of the amplicon sequencing.**

619

620 **Figure S1. Initial phenotypes of transconjugants chosen for serial passage experiments.**
621 a) Frequency of erythromycin resistance in transconjugant colonies used to initiate serial passage
622 experiments. Erythromycin resistance is used to track pAM714 maintenance in the population. b)
623 CRISPR3 amplicon PCR results for transconjugant colonies used to initiate serial passage
624 experiments. Shown are CRISPR3 amplicon sizes for six T11RF pAM714 transconjugants (WT
625 1-6), a representative T11RF∆*cas9* pAM714 transconjugant (∆4), T11RF genomic DNA as a
626 positive control (P), and a reagent control (N).

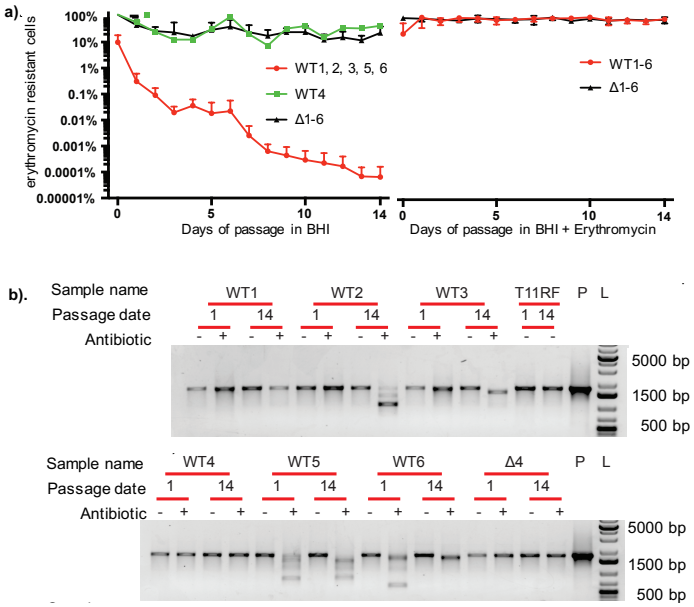
627

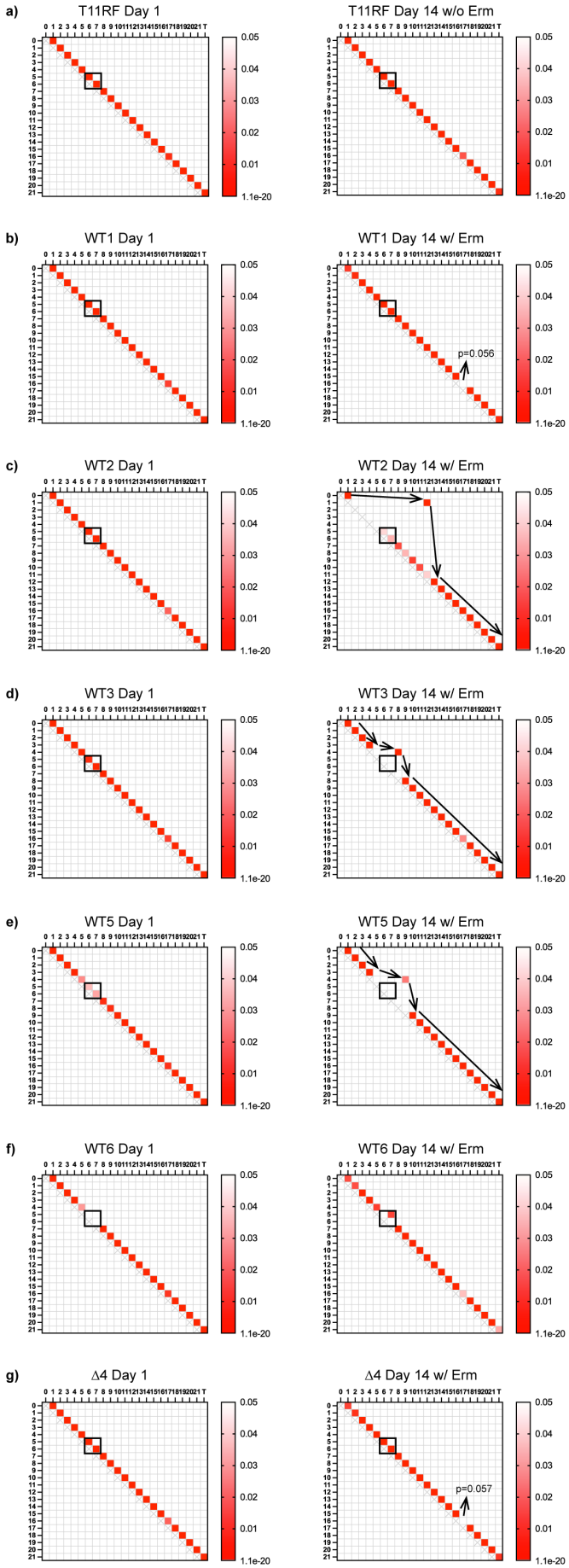
628 **Figure S2. Agarose gel electrophoresis analysis of CRISPR3 amplicons in six**
629 **T11RF(pAM714) transconjugant populations.** Six T11RF(pAM714) transconjugants were
630 serially passaged for 14 days in BHI (left panel) or BHI with erythromycin (right panel). The size
631 of the CRISPR3 array was monitored using PCR and gel electrophoresis on each passage day.

632

633 **Figure S3. Demonstration of mutant alleles and visualization of heatmap matrix.** A cartoon
634 of the T11 CRISPR3 array is shown below the heatmap. The array contains 21 unique spacer

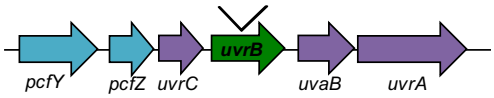
635 sequences, of which spacer 6 targets pAM714. Four scenarios (a-d) corresponding to four cells
636 indicating different wild-type (b) or mutant (a,c,d) allele types in the matrix are shown.
637
638 **Figure S4. Less abundant mutant alleles can be observed in a heatmap of normalized read**
639 **counts.** Heat maps show the abundance of each mutant allele per sample. Day 1 and Day 14
640 populations were analyzed and T11RF was used as a control. Each cell within the heat map
641 represents one of the 484 artificial references. The color scale represents low (white) to
642 intermediate (ref) to high (black) abundance alleles. Black boxes indicate the expected wild type
643 allele at the S5-S6 region. Pink boxes surround rare alleles that did not meet the significance
644 cutoff but can be visible here**.**
645
646 **Figure S5. Mapping of Tn*917* location.** The consensus sequences were extracted from 5' and
647 3' ends of transposon Tn*917* and mapped to pAD1 sequences.
648
649
650


a).
erythromycin resistant cells
100%
10%
1%
0.1%
0.01%
0.001%
0.0001%
0.00001%
WT1, 2, 3, 5, 6
WT4
Δ1-6
WT1-6
Δ1-6
0
5
10
14
Days of passage in BHI
Days of passage in BHI + Erythromycin
b).
Sample name
Passage date
Antibiotic
WT1
WT2
WT3
T11RF
P
L
5000 bp
1500 bp
500 bp
WT4
WT5
WT6
Δ4

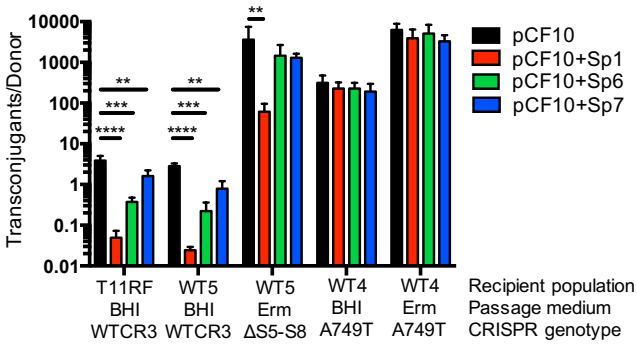

a)
T11RF Day 1
T11RF Day 14 w/o Erm
b)
WT1 Day 1
WT1 Day 14 w/ Erm
p=0.056
c)
WT2 Day 1
WT2 Day 14 w/ Erm
d)
WT3 Day 1
WT3 Day 14 w/ Erm
e)
WT5 Day 1
WT5 Day 14 w/ Erm
f)
WT6 Day 1
WT6 Day 14 w/ Erm
g)
Δ4 Day 1
Δ4 Day 14 w/ Erm
p=0.057
0.05
0.04
0.03
0.02
0.01
1.1e-20

a)

pCF10	WT *uvrB* sequence; not targeted by CRISPR3-*cas*
pCF10+Sp1	AAAAGTTGAATATGTTTCGCTTTGGTGTAATTGTA
pCF10+Sp6	CCGATACAGCTCTTTATTCTTCTAATTACATTGTA
pCF10+Sp7	TGCTTTTGGAGGAATACAAATGAGAAGATTTTGTA



b)



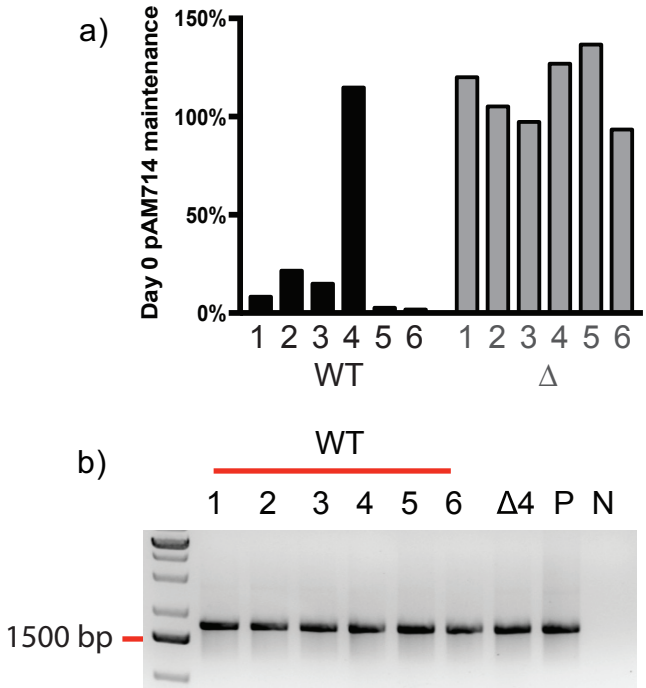


Figure S1. Initial phenotypes of transconjugants chosen for serial passage experiments. a) Frequency of erythromycin resistance in transconjugant colonies used to initiate serial passage experiments. Erythromycin resistance is used to track pAM714 maintenance in the population. b) CRISPR3 amplicon PCR results for transconjugant colonies used to initiate serial passage experiments. Shown are CRISPR3 amplicon sizes for six T11RF pAM714 transconjugants (WT 1-6), a representative T11RFΔ*cas9* pAM714 transconjugant (Δ4), T11RF genomic DNA as a positive control (P), and a reagent control (N).

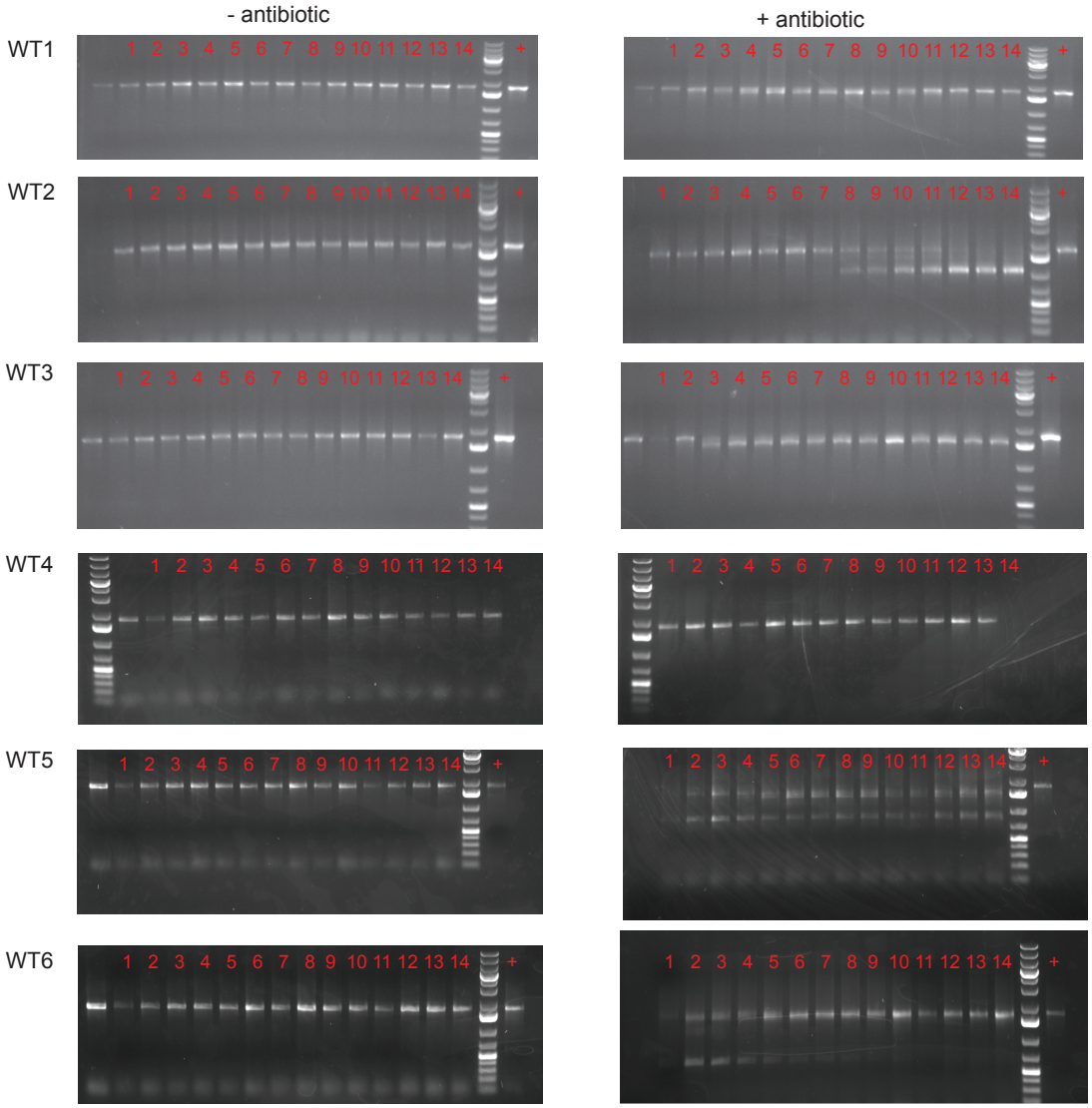


Figure S2. Agarose gel electrophoresis analysis of CRISPR3 amplicons in six T11RF(pAM714) transconjugant populations. Six T11RF(pAM714) transconjugants were serially passaged for 14 days in BHI (left panel) or BHI with erythromycin (right panel). The size of the CRISPR3 array was monitored using PCR and gel electrophoresis on each passage day.

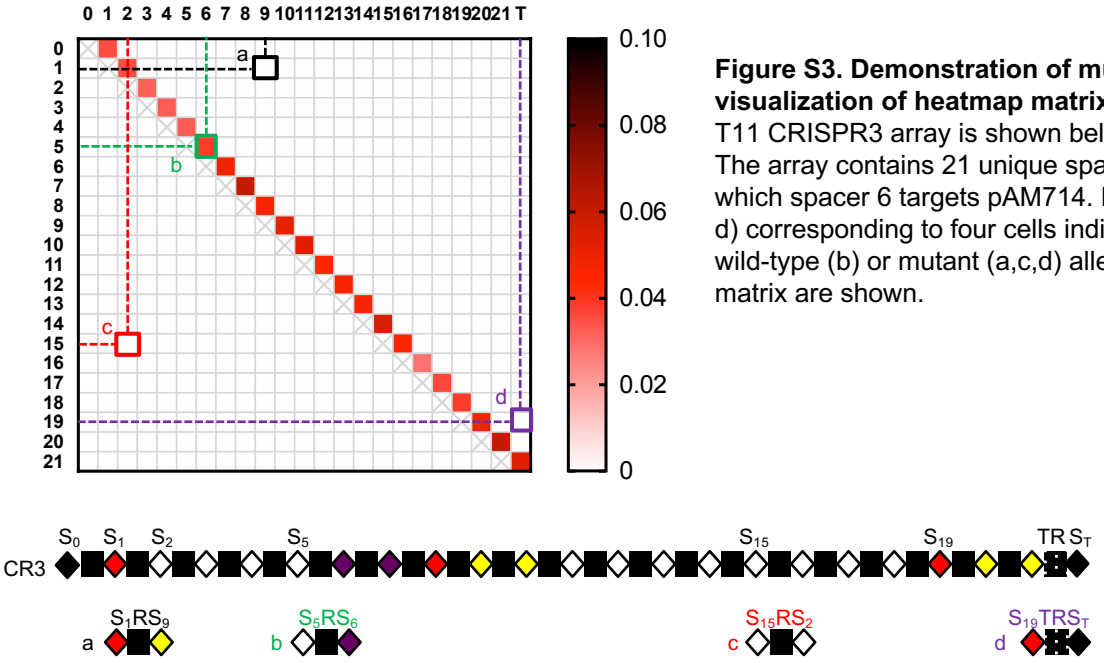


Figure S3. Demonstration of mutant alleles and visualization of heatmap matrix. A cartoon of the T11 CRISPR3 array is shown below the heatmap. The array contains 21 unique spacer sequences, of which spacer 6 targets pAM714. Four scenarios (a-d) corresponding to four cells indicating different wild-type (b) or mutant (a,c,d) allele types in the matrix are shown.

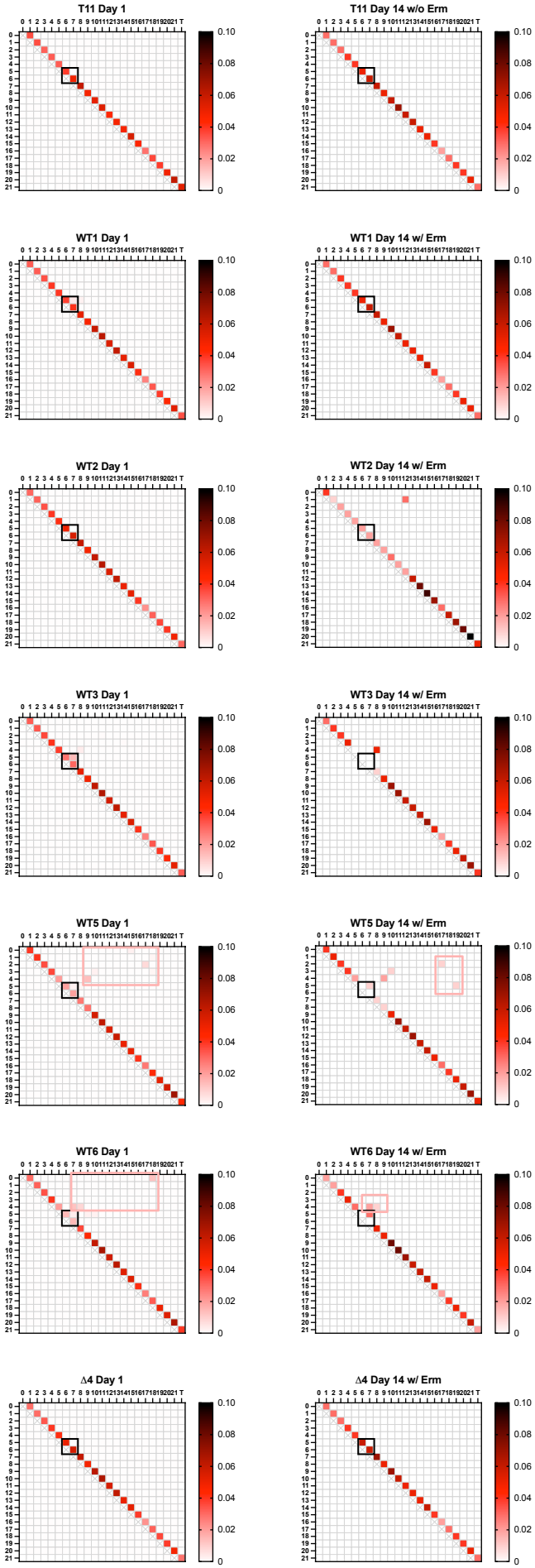


Figure S4. Less abundant mutant alleles can be observed in a heatmap of normalized read counts. Heat maps show the abundance of each mutant allele per sample. Day 1 and Day 14 populations were analyzed and T11RF was used as a control. Each cell within the heat map represents one of the 484 artificial references. The color scale represents low (white) to intermediate (ref) to high (black) abundance alleles. Black boxes indicate the expected wild type allele at the S5-S6 region. Pink boxes surround rare alleles that did not meet the significance cutoff but can be visible here.

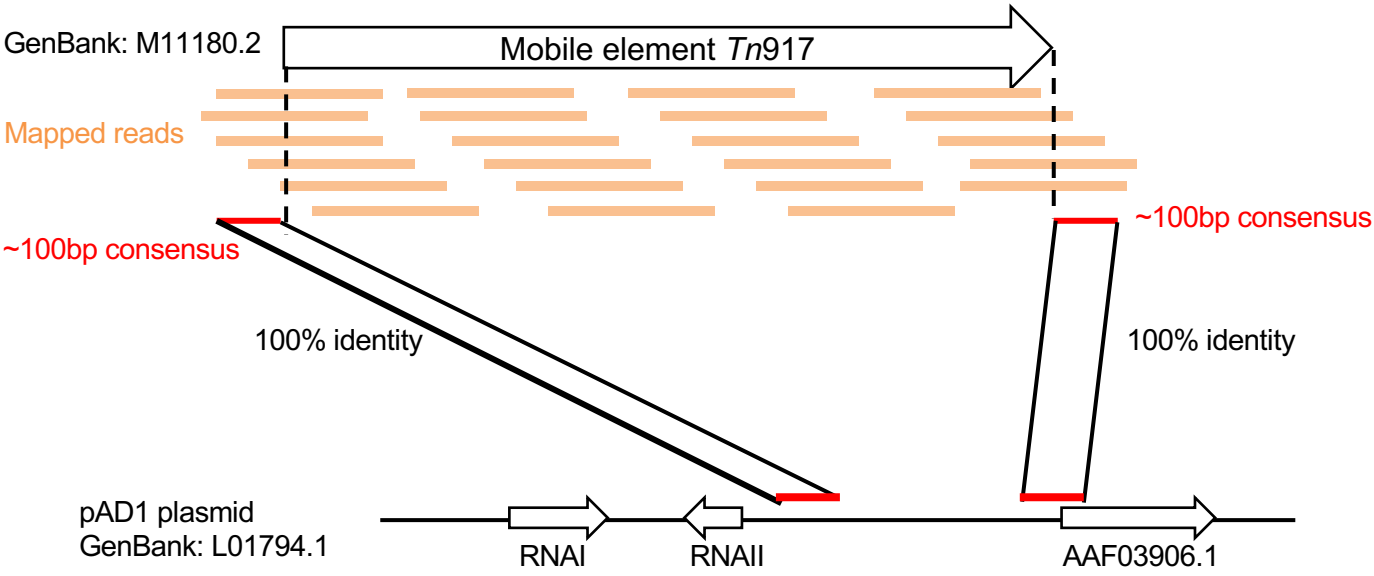


Figure S5. Mapping of Tn*917* location. The consensus sequences were extracted from 5' and 3' ends of transposon Tn*917* and mapped to pAD1 sequences.

Table S1. Primers used in this study.

Name	Primer sequence
CRISPR seq primers	
CRISPR1 seq For	CGTATTTGACAGAGGATGAAG
CRISPR1 seq Rev	CGAATATGCCTGTGGTGAAA
CRISPR2 seq For	TGCTGTTACAGCTACTAAA
CRISPR2 seq Rev	GCCAATGTTACAATATCAAACA
CRISPR3 seq For	GCTCACTGTATTGGAAGAAC
CRISPR3 seq Rev	CATCGATTCATTATTCCTCCAA
pWH107 and derivatives	
pVP107_XbaI_For	CTAGAGATAATATATCTTTTATATAGAAGATGGGTACCATGGCATG
pVP107_SphI_Rev	CCATGGTACCCATCTTCTATATAAAAGATATATTATCT
T11CR3sp6_BamPst_F	GATCCTCCCGATACAGCTCTTTATTCTTCTAATTACATTGTACTGCA
T11CR3sp6_BamPst_R	GTACAATGTAATTAGAAGAATAAAGAGCTGTATCGGGAG
T11CR3sp1_BamPst_F	GATCCTCAAAAGTTGAATATGTTTCGCTTTGGTGTAATTGTACTGCA
T11CR3sp1_BamPst_R	GTACAATTACACCAAAGCGAAACATATTCAACTTTTGAG
T11CR3sp7_BamPst_F	GATCCTCTGCTTTTGGAGGAATACAAATGAGAAGATTTTGTACTGCA
T11CR3sp7_BamPst_R	GTACAAAATCTTCTCATTTGTATTCCTCCAAAAGCAGAG
T11CR2S1 seq arm1 F	CGAAATCAGCACATGGAACA
T11CR2S1 seq arm2 R	CCAGTAACTGTATCAACTAC

Table S2. SNP detection in all genomic DNA sequencing samples.

Contig	Position	Ref	Allele	Annotation	Amino acid change	T11RF[a]	WT3[b]	WT1[c]	WT2[c]	WT3[c]	WT5[c]	WT6[c]	Δ4[c]
AD1REP ABC	1904	C	T	Replication protein	AAB00504.1: p.Pro100Leu				45.49				
1.11	51613	C	T	Collagen adhesin								25.00	
1.11	51674	G	A	Collagen adhesin	WP_002379313.1: p.Gly392Ser						33.90	33.39	
1.11	51745	A	G	Collagen adhesin							33.02		
1.11	337315	G	T	Helicase	WP_002382285.1: p.Glu425*							37.16	
1.11	652983	G	A	Cas9	WP_002379510.1: p.Gln506*			31.64					
1.11	653180	AG	T	Cas9	WP_002379510.1: p.Leu440fs		22.74			23.52			
1.11	653184	C	T	Cas9	WP_002379510.1: p.Glu439Lys		23.21			24.17			
1.11	654165	G	-	Cas9	WP_002379510.1: p.Arg112fs				48.41				
1.11	685523	A	T	Conserved hypothetical protein	WP_002382413.1: p.Glu5Val		23.24						
1.11	685938	A	T	Conserved hypothetical protein	WP_002382413.1: p.Glu143Asp		26.00						
1.11	974866	AT	GC	Cell wall surface anchor family protein	WP_002379620.1: p.Ile972Ala							23.41	
1.11	974991	A	G	Cell wall surface anchor family protein								22.67	
1.2	2903	C	T	Peptidylprolyl isomerase	WP_002355150.1: p.Val167Ile				46.03				

[a]Day 14 BHI-passaged
[b]Day 1 Erythromycin-passaged population WT3 to confirm presence of *cas9* mutations prior to Day 14
[c]Day 14 Erythromycin-passaged

Table S3. Mapping statistics of the amplicon sequencing.

Sample name	# of Total reads	# mapped to WT pool of references	# mapped to mutant pool of references
Day 1			
T11RF	19,082,652	7,273,291	125,204
Δ4	20,808,404	8,995,910	77,551
WT1	16,736,162	6,808,760	168,051
WT2	16,859,152	7,222,339	59,609
WT3	17,357,970	7,136,664	250,783
WT5	17,744,230	6,784,881	430,500
WT6	16,179,400	6,337,706	406,347
Day 14			
T11RF	17,973,946	7,760,594	68,900
Δ4	14,403,868	5,369,063	55,037
WT1	14,158,158	5,219,558	55,730
WT2	14,538,836	4,489,642	254,615
WT3	17,174,946	5,653,212	385,906
WT5	15,200,646	5,384,356	460,061
WT6	12,325,042	4,201,804	303,973