Genome-wide mutagenesis identifies factors involved in *Enterococcus faecalis* vaginal adherence and persistence.

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RUNNING TITLE: ***Enterococcus faecalis* Vaginal Colonization**

21 **ABSTRACT**

22 *Enterococcus faecalis* is a Gram-positive commensal bacterium native to the gastrointestinal tract and an
23 opportunistic pathogen of increasing clinical concern. *E. faecalis* also colonizes the female reproductive
24 tract and reports suggest vaginal colonization increases following antibiotic treatment or in patients with
25 aerobic vaginitis. Currently, little is known about specific factors that promote *E. faecalis* vaginal
26 colonization and subsequent infection. We developed a mouse model of *E. faecalis* vaginal carriage and
27 demonstrate that both vancomycin resistant and sensitive strains colonize the murine vaginal tract.
28 Following vaginal colonization, we observed *E. faecalis* in vaginal, cervical and uterine tissue. A mutant
29 lacking endocarditis- and biofilm-associated pili (Ebp) exhibited a decreased ability to associate with
30 human vaginal and cervical cells *in vitro* as well as ascend to the cervix and uterus *in vivo*. To further
31 identify novel factors that promote vaginal colonization, we screened a low-complexity transposon (Tn)
32 mutant library to identify genes important for *E. faecalis* persistence in the vaginal tract. This screen
33 revealed 383 mutants that were underrepresented during vaginal colonization at all time points compared
34 to growth in culture medium. We confirmed that mutants deficient in ethanolamine catabolism and the
35 type VII secretion system were attenuated in persisting during vaginal colonization. These results reveal
36 the complex nature of vaginal colonization and suggest that multiple factors contribute to *E.*
37 *faecalis* persistence in the reproductive tract.

38

39 **IMPORTANCE**

40 Despite increasing prevalence and association of *E. faecalis* with aerobic vaginitis, essentially nothing is
41 known about the bacterial factors that influence *E. faecalis* vaginal colonization. We have established an
42 animal model of vaginal colonization that supports colonization of multiple *E. faecalis* strains.
43 Additionally, we determined that pili, ethanolamine utilization, and type VII secretion system genes

44 contribute to vaginal persistence and ascension to reproductive tract tissues. Identification of factors
45 important for vaginal colonization and persistence provides potential targets for the development of
46 therapeutics. This study is the first to identify key determinants that promote vaginal colonization by *E.*
47 *faecalis*, which may represent an important reservoir for antibiotic resistant enterococci.
48

49 **INTRODUCTION**

50 *Enterococcus faecalis* is an opportunistic pathogen that resides in the human gastrointestinal and
51 urogenital tracts [1, 2]. While *E. faecalis* colonization is normally asymptomatic, certain populations are
52 at risk for severe disease including urinary tract infections (UTIs), wound infections, pelvic inflammatory
53 disease (PID), infective endocarditis, and adverse birth effects during pregnancy [3, 4]. Enterococcal
54 infections are often associated with the production of biofilms, assemblages of microbes enclosed in an
55 extracellular polymeric matrix that exhibit cell-to-cell interactions. These biofilms have been observed on
56 catheters, diabetic ulcers, and wounds resulting in severe infection. Treatment of enterococcal infections
57 is becoming increasingly problematic due to their augmented resistance to antibiotics, including "last-
58 line-of-defense" antibiotics such as vancomycin [5, 6]. Recently, there has been an increase in the
59 emergence of vancomycin resistant enterococci [7], putting immunocompromised individuals at risk for
60 developing severe chronic enterococcal infections. The emergence of vancomycin resistant enterococci
61 (VRE) and its prevalence in both community and nosocomial settings is concerning and necessitates the
62 development of alternative therapeutics to treat enterococcal infections.
63

64 *E. faecalis* encodes a multitude of virulence factors that allow the bacterium to colonize and persist in
65 different sites of the human body. Surface proteins such as the adhesin to collagen (Ace), enterococcal
66 fibronectin binding protein A (EfbA), aggregation substance (AS), and the endocarditis-and biofilm-
67 associated pilin (Ebp) have been previously shown to play important roles in infective endocarditis and

68 UTIs [8]. Secreted factors such as gelatinase (GelE), autolysin A (AtlA), and serine protease (SprE) are
69 biofilm-associated factors that are involved in the degradation of host substrates, including collagen, fibrin
70 and certain complement components [9]. Many of these virulence factors are regulated via quorum
71 sensing, which may be responsible for the switch from a commensal to pathogenic state [10-12].
72
73 Certain risk factors are associated with the transition of *E. faecalis* from commensalism to pathogenicity
74 such as immune status, prolonged hospital stay, and the use of antibiotics [13]. *E.*
75 *faecalis* colonization and infection is often polymicrobial and these interactions have been observed in the
76 intestine, bloodstream, and wounds [14]. Furthermore, *E. faecalis* is frequently found in the vaginal tract
77 of healthy women [15, 16] and its prevalence is increased in women diagnosed with aerobic vaginitis
78 (AV), an inflammatory response accompanied by depletion of commensal *Lactobacillus*
79 *sp.* and increased presence of opportunistic pathogens such as *E. faecalis*, Group
80 B *Streptococcus* (GBS), *Staphylococcus aureus*, and *Escherichia coli* [17, 18]. Symptoms of AV include
81 malodor and discomfort, but AV can transition to more serious complications such as PID, severe UTIs,
82 and complications during pregnancy. While it is evident that *E. faecalis* colonizes the human vaginal tract,
83 the molecular determinants that allow enterococci to colonize and persist in the vaginal tract remain to be
84 identified.
85
86 In this study, we developed a murine model of *E. faecalis* vaginal colonization and persistence. We
87 determined that *E. faecalis* OG1RF (a rifampicin and fusidic acid derivative of strain OG1) and
88 vancomycin resistant *E. faecalis* V583 can colonize and persist in the vaginal tract of CD1 and C57BL/6
89 mouse strains. We detected fluorescent *E. faecalis* in the vaginal lumen as well as the cervical and uterine
90 tissues of colonized mice. Further, we demonstrate that an *E. faecalis* strain lacking Ebp pili is attenuated
91 in both *in vitro* and *in vivo* models of vaginal colonization. Lastly, we screened an *E. faecalis* OG1RF

92 transposon (Tn) mutant library for mutants that are underrepresented in the vaginal tract compared the
93 culture input, revealing multiple factors for *E. faecalis* persistence within the vagina. These factors include
94 the Ebp pili, ethanolamine utilization genes, and genes involved in type VII secretion system (T7SS)
95 machinery. We confirmed that mutant strains lacking these genes are significantly attenuated in their
96 ability to colonize and ascend in the female reproductive tract. This work is an important first step in
97 identifying factors required for enterococcal vaginal colonization and will provide insight into potential
98 therapeutic targets aimed at mitigating *E. faecalis* vaginal colonization in at-risk individuals.
99

100 **RESULTS**

101 ***E. faecalis* colonization of the female reproductive tract.**

102 To characterize the ability of *E. faecalis* to interact with the epithelial cells of the lower female
103 reproductive tract, we performed *in vitro* quantitative adherence assays using *E. faecalis* strains V583
104 [19] and OG1RF [20]. An inoculum of 10^5 CFU/well (multiplicity of infection [MOI] = 1) was added to
105 a confluent monolayer of immortalized human vaginal and endocervical epithelial cells. Following 30
106 minutes of incubation, the cells were washed to remove non-adherent bacteria, the epithelial cells were
107 detached from the plates, and adherent bacteria were plated on agar. Data are expressed as percent
108 recovered cell-associated *E. faecalis* relative to the initial inoculum. Both strains exhibited substantial
109 adherence to both cell lines (Fig. 1A). Next, we assessed the ability of *E. faecalis* to establish colonization
110 of the murine vaginal tract. C57BL/6 mice were treated with β-estradiol 1 day prior to inoculation with
111 10^7 CFU of *E. faecalis* V583 or OG1RF. After 1 day post-inoculation, the vaginal lumen was swabbed
112 and bacteria were plated to enumerate *E. faecalis* V583 and OG1RF vaginal colonization levels (Fig. 1B).
113 Swabs were plated on selective agar to ensure quantification of only the enterococcal strains of interest,
114 restricting growth of native enterococcus. To determine whether *E. faecalis* ascends into reproductive

115 tissues during colonization, murine vaginal, cervical, and uterine tissues were collected and homogenized
116 to enumerate *E. faecalis* V583 abundance. *E. faecalis* was recovered from all mice 1 day post-inoculation
117 in all tissues tested (Fig. 1C) and the CFU recovered from the vaginal swabs were similar to the total CFU
118 counts from the vaginal tissue homogenates (Fig. 1B, C). This level and range of recovered *E. faecalis*
119 CFU is similar to what we have observed using this mouse model for GBS and *S. aureus* vaginal
120 colonization [21, 22]. To visualize *E. faecalis* within the murine reproductive tract, mice were inoculated
121 with *E. faecalis* V583 expressing green fluorescent protein (GFP) [23]. We harvested the female
122 reproductive tract 1 day post-inoculation, made serial sections of these tissues, and performed fluorescent
123 microscopy to visualize *E. faecalis* (Fig. 1D, E, F). We observed numerous fluorescent bacteria in the
124 vaginal and uterine lumen (Fig. 1D, F) and embedded in the cervical lamina propria (Fig. 1E).
125
126 ***E. faecalis* persists in the vaginal tract.**
127 To assess vaginal persistence, C57BL/6 mice were colonized with *E. faecalis* V583 or OG1RF and
128 swabbed to determine bacterial load over time. We observed that both strains exhibited similar persistence
129 within the mouse vagina (Fig. 2A, B). The mean CFU recovered remained constant for the first week and
130 then declined as mice eventually cleared both strains by 11-13 days. To determine if enterococcal vaginal
131 persistence differs across mouse strains, C57BL/6 and CD1 mice were inoculated with V583 and swabbed
132 over time. Both mouse strains were initially colonized with V583, but bacteria in C57BL/6 mice persisted
133 longer (Fig. 2C, D). By day six only 20% of CD1 mice remained colonized compared to 85% of C57BL/6
134 mice. Differences in vaginal persistence may be due to differences in the native vaginal microbiota or
135 immune status between mouse strains. These results show that mouse strain background influences *E.*
136 *faecalis* vaginal colonization and that C57BL/6 mice are a sufficient model to assess prolonged *E. faecalis*
137 vaginal colonization and persistence.
138

139 **Enterococcal pili contribute to interaction with reproductive tract tissues.**

140 The endocarditis- and biofilm-associated pilin (Ebp) of *E. faecalis* mediates infective endocarditis and
141 UTIs, thus we hypothesized that Ebp may similarly contribute to vaginal colonization. To determine
142 whether Ebp is important for facilitating interaction with the vaginal epithelium, we used a deletion mutant
143 of *E. faecalis* OG1RF lacking all pilin structural components (Δ*ebpABC*) [24]. We observed that the pilus
144 mutant exhibited significantly reduced adherence to human vaginal and endocervical cells *in vitro*
145 (Fig. 3A, B). To determine if Ebp is important for *in vivo* vaginal colonization and persistence, mice were
146 inoculated with either WT OG1SSp (an isogenic derivative of OG1 that is resistant to streptomycin and
147 spectinomycin) or OG1RF Δ*ebpABC* and colonization was quantified over the course of 13 days. We note
148 that both WT strains, OG1RF and OG1SSp, persist in the vaginal lumen and colonize reproductive tract
149 tissues at similar levels (Fig. S1A, B). We observed no differences in the CFU recovered from the vaginal
150 lumen between WT OG1SSp and OG1RF Δ*ebpABC* strains when inoculated together in competition (Fig.
151 3C). However, in a subsequent experiment, we recovered significantly less of the Δ*ebpABC* mutant in
152 vaginal and cervical tissues and slightly less Δ*ebpABC* in uterine tissue compared to the WT strain 4 days
153 post-inoculation (Fig. 3D, E). Taken together these data suggest that Ebp contributes to *E. faecalis*
154 attachment to reproductive tract tissues, but additional factors are likely required for persistence in the
155 vaginal lumen.

156

157 **Identification of additional vaginal colonization factors by transposon mutagenesis analysis.**

158 Although the Ebp pilus mutant showed reduced association with reproductive tract tissues, we observed
159 no significant difference in the ability of this mutant to persist in the mouse vagina (Fig. 3C). Thus, we
160 hypothesized that additional bacterial factors are involved in promoting *E. faecalis* vaginal colonization.
161 To identify genetic determinants that confer vaginal persistence, we used sequence-

162 defined *mariner* technology transposon sequencing (SMarT TnSeq) to screen an *E. faecalis* OG1RF Tn
163 library consisting of 6,829 unique mutants [25]. The library was grown to mid-log phase in triplicate and
164 10^7 CFU of each replicate was vaginally inoculated into a group of 5 C57BL/6 mice (Fig. 4A). Vaginal
165 swabs were plated on selective media daily for 8 days and CFU were quantified to assess colonization of
166 the OG1RF Tn library compared to WT OG1RF (Fig. 4A and B). Genomic DNA was isolated from pooled
167 Tn libraries recovered on days 1, 5, and 8 post-inoculation and Tn insertions in *E. faecalis* genomic DNA
168 were sequenced as described by Dale *et al.* [25]. Sequenced reads were mapped to the *E. faecalis* OG1RF
169 genome to identify genes that are necessary for *E. faecalis* vaginal colonization.
170
171 We observed that the *in vivo* vaginal environment altered the abundance of select mutants from the Tn
172 library pool compared to the original culture input (Fig. 4C, D, E) (Suppl. Tables 1 - 6). At day 1, a total
173 of 667 depleted mutants were identified, along with 544 and 507 at days 5 and 8 respectively; 383 of these
174 mutants were identified at all three time points (Fig. 5A). Classification by clusters of orthologous groups
175 of proteins (COGs) could be identified in 196 mutants from all 3 time points, the majority of which are
176 involved in carbohydrate, amino acid, lipid and nucleotide transport/metabolism, as well as those involved
177 in transcription and defense mechanisms (Fig. 5B). Interestingly the *ebpC* transposon mutant
178 (*OG1RF_10871*, which encodes the shaft component of Ebp) was underrepresented at all time points (Fig.
179 4C, D, E, Suppl Table 1). Additional mutants of interest included those involved in ethanolamine
180 catabolism as well as T7SS components.
181
182 Potential gain-of-function mutations have also been discovered during genome-wide library screens of
183 fitness determinants in other bacteria [26-30]. In addition to mutations that adversely impact vaginal
184 colonization, our data shows that Tn insertions in 11 protein coding genes and 11 intergenic regions

185 potentially enhance bacterial fitness *in vivo*. Nineteen of the 22 enriched mutants were common between
186 days 1 and 5 whereas the other 3 were unique to day 8 (Suppl Table 5 and 6). Since the majority of the
187 mutants with increased fitness encode hypothetical proteins, the relationship between these genes and
188 vaginal colonization is currently unclear and requires further investigation.
189

190 **Ethanolamine utilization and T7SS genes contribute to *E. faecalis* persistence in the reproductive**
191 **tract.**

192 Our TnSeq analysis revealed many potential mutants that exhibited decreased colonization in the murine
193 vaginal tract. We sought to confirm these results by analyzing mutants from systems with multiple
194 affected genes. One significantly affected operon was ethanolamine (EA) utilization (*eut*) which consists
195 of 19 genes in *E. faecalis* [31]. Mutations in 4 *eut* genes were significantly underrepresented *in vivo*
196 compared to the culture input at all time points. These included transposon mutants of the genes encoding
197 both subunits for ethanolamine ammonia lyase, *eutB (OG1RF_11344)* and *eutC (OG1RF_11343)* [32], a
198 carboxysome structural protein, *eutL (OG1RF_11342)*[33], and the response regulator, *eutV*
199 *(OG1RF_11347)*, of the two-component system involved in the regulation of EA utilization [34] (Suppl
200 Table 1). To assess the importance of EA utilization on *E. faecalis* vaginal colonization, we co-colonized
201 mice with *E. fae*calis OG1SSp and an OG1RF Δ*eutBC* mutant [35]. Compared to the WT OG1SSp strain,
202 the Δ*eutBC* mutant was cleared significantly faster from the mouse vagina as seen by the mean CFU
203 recovered and competitive index (CI) over time (Fig. 6A, B, C). These results suggest that the utilization
204 of ethanolamine is important for enterococcal persistence in the vaginal tract.
205

206 We also observed that Tn insertion mutants in the T7SS locus were significantly underrepresented at all
207 time points *in vivo* compared to the culture input. The T7SS has been shown to play an important role in

208 virulence in multiple bacterial species such as *Staphylococcus*, *Listeria* and *Bacillus* [36-38]. In *E.*
209 *faecalis*, genes in the T7SS locus have been shown to be induced during phage infection [39]. We observed
210 that transposon mutants for *esaB* (*OG1RF_11103)*, a putative cytoplasmic accessory protein;
211 *OG1RF_11109* and *OG1RF_11111*, putative toxin effector proteins; *OG1RF_11113*, a putative toxin,
212 *OG1RF_11114,* a transmembrane protein; and *OG1RF_11122,* a potential antitoxin protein were all
213 significantly underrepresented (Suppl Table 1). To confirm the role of the T7SS in vaginal colonization,
214 we utilized an *esaB* (*esaB*-Tn) mutant, in which *esaB* is disrupted by a transposon and thus Tn-mediated
215 polar effects may exist for this strain. Following co-colonization with *E. faecalis* OG1RF and the OG1RF
216 *esaB*-Tn mutant strain, we observed that while there were no differences in initial colonization, fewer
217 *esaB*-Tn mutant bacteria were recovered from the vaginal lumen at later time points (Fig. 6D). We
218 repeated this experiment and harvested tissues at an earlier time point, before there were any colonization
219 differences observed between the two strains (day 11). Here we observed that WT OG1RF outcompeted
220 the *esaB*-Tn mutant strain and was better able to access reproductive tract tissues (Fig. 6E, F), indicating
221 that the T7SS may be involved in vaginal persistence and ascension in the female reproductive tract.
222

223 **DISCUSSION**

224 *E. faecalis* is associated with a wide spectrum of infections, particularly under immunocompromised states
225 and during compositional shifts in the host microbiota [40, 41]. Although an increasing body of evidence
226 links enterococci with bacterial vaginosis (BV) and aerobic vaginitis (AV) [17, 18, 42-44], the molecular
227 determinants that facilitate *E. faecalis* colonization and persistence in the vaginal tract are largely
228 unknown. Here, we established *in vitro* and *in vivo* systems to acquire genome-scale interactions that
229 confer *E. faecalis* fitness within the female reproductive tract. We show that both vancomycin sensitive
230 enterococci (VSE) and VRE adhere to cell types of vaginal and cervical origin, a signature of bacterial

231 colonization that precedes tissue invasion and systemic spread. Further, genetic features involved in
232 biofilm formation, ethanolamine utilization and polymicrobial interactions influence *E. faecalis* vaginal
233 carriage.
234
235 Previous studies have demonstrated the importance of Ebp pili in enterococcal virulence and biofilm
236 formation [14]. We found that deletion of *ebpABC* attenuated binding to human vaginal and endocervical
237 cells but did not influence bacterial burden in the vaginal lumen, similar to the observed function of Ebp
238 pili in the intestine[45]. Enhanced *E. faecalis* adherence in tissue culture compared to *in vivo* colonization
239 may reflect the lack of liquid and mucus flow that bacteria encounter within the vaginal tract, emphasizing
240 the significance of our animal model for to investigating *E. faecalis*-vaginal interactions. Consistent with
241 this observation, an *in vivo* Tn library screen revealed only two underrepresented biofilm-associated
242 mutants, *ebpC-Tn* and *OG1RF_10506-Tn*, encoding a putative polysaccharide deacetylase homolog
243 implicated in low biofilm formation *in vitro* [46, 47]. Together, these results show that individual
244 mutations in *ebp* or other well characterized biofilm genes are not sufficient to impair vaginal niche
245 establishment and/or persistence of enterococci, which likely depends on the concerted effort of multiple
246 factors.
247
248 Transition from nutrient rich laboratory media to the vaginal tract likely imparts dramatic alterations in *E.*
249 *faecalis* metabolism. In support of this hypothesis, our high-throughput Tn mutant screen showed that
250 mutations in carbohydrate, amino acid and nucleotide metabolic pathways were indispensable in the
251 vaginal tract. Specifically, we showed that WT bacteria outcompete a *eut* locus mutant during vaginal
252 colonization. In contrast, Kaval and colleagues demonstrated that mutations in *eut* genes leads to a slight
253 increase in fitness within the intestine [48]. This observation likely reflects the metabolic requirements of

254 different host environments. While a number of reports exist on the contributions of EA catabolism in
255 host-bacteria interactions within the intestine [49], studies are lacking for the relevance of this EA
256 metabolism in other host-associated environments. Our results raise important questions regarding EA
257 utilization in the female reproductive tract. Although commensal microbes and the epithelium are rich
258 sources of EA, the composition and source of EA in the vaginal tract remains unknown. A recent report
259 showed that *E. faecalis* EA utilization attenuates intestinal colitis in IL10 knockout mice in the presence
260 of a defined microbiota [50]. Whether EA utilization promotes virulence or commensalism for enterococci
261 in the context of vaginal tissue remains to be determined. Considering that the by-product of EA
262 metabolism, acetate, is anti-inflammatory and promotes IgA production in the intestine [51, 52], it is
263 intriguing to consider that enterococcal EA catabolism might modulate immune responses within the
264 female reproductive tract.
265
266 T7SSs have been implicated in the maintenance of bacterial membrane integrity, virulence, and
267 interbacterial antagonism [36, 53-59]. In *S. aureus*, T7SS encoded proteins confer protection from
268 membrane damage caused by host fatty acids [58, 59]. Although *E. faecalis* T7SS genes were shown to
269 be induced in response to phage driven membrane damage, direct contributions of these genes in cell
270 envelop barrier function and/or virulence in the context of animal models are poorly defined. Our TnSeq
271 analysis revealed that insertional mutations in six T7SS genes diminished early and late vaginal
272 colonization by *E. faecalis* OG1RF. In vaginal co-colonization competition experiments, an *esaB-Tn* strain
273 reached WT colonization levels early and showed a colonization defect in long term persistence. The
274 incongruence in the colonization kinetics of T7SS mutant strains compared to T7SS-Tn library mutants
275 which were observed early after inoculation, presumably stem from the inherent differences in the vaginal
276 milieu in these two experiments. The TnSeq library employed in this screen is a complex population of

277 approximately 7,000 unique mutants, but it is still very likely that direct or indirect interactions between
278 mutants influences fitness. LXG–domain toxins, which are part of the T7SS, have been shown to
279 antagonize neighboring non-kin bacteria [56]. The fact that two mutants with LXG–domain encoding gene
280 mutations, *OG1RF_11109* and *OG1RF_11111*, were underrepresented across all time points during
281 vaginal colonization suggests that these putative antibacterial proteins may influence enterococcal
282 interactions with the resident microbes of the vaginal tract.
283
284 Here, we report the development of a novel mouse model for investigating host-enterococcal interactions
285 in the vaginal tract. This will be a useful model for analyzing the bacterial and host factors that govern
286 enterococcal vaginal colonization, as well as characterizing the polymicrobial interactions that may
287 contribute to *E. faecalis* niche establishment and persistence. Transposon library screening of *E.*
288 *faecalis* recovered from the mouse vagina has revealed new insights into our understanding of
289 enterococcal vaginal carriage. Our results emphasize the importance of pili, ethanolamine utilization, and
290 T7SS components for successful *E. faecalis* colonization of the female reproductive tract, highlighting the
291 complex nature of this niche. Continued studies will further elucidate the molecular mechanisms of
292 vaginal persistence, and may represent an important reservoir for antibiotic resistant enterococci.
293
294 **METHODS**
295 **Bacterial strains and culture conditions**.
296 A detailed list of bacterial strains can be found in Suppl Table 7. *E. faecalis* strains V583 [60] and OG1-
297 (RF and SSp) [20, 61] were used for these experiments. *E. faecalis* was grown in brain heart infusion (BHI
298 [62]) broth at 37℃ with aeration and growth was monitored by measuring the optical density at 600nm
299 (OD_{600}). For selection of *E. faecalis* V583, BHI agar was supplemented with gentamicin (100 µg/ml). For

300 selection of *E. faecalis* OG1RF, OG1RF Δ*ebpABC* [24] and OG1RF Δ*eutBC* [35], BHI agar was
301 supplemented with rifampicin (50 µg/ml) and fusidic acid (25 µg/ml). For selection of *E. faecalis*
302 OG1SSp, BHI agar was supplemented with streptomycin (150 µg/ml) and spectinomycin (100 µg/ml). *E.*
303 *faecalis* OG1RF *esaB*-Tn [25] was grown on BHI agar supplemented with rifampicin (50 µg/ml), fusidic
304 acid (25 µg/L), and chloramphenicol (15 µg/ml).
305
306 ***In vitro* adherence assays**
307 Immortalized VK2 human vaginal epithelial cells and End1 human endocervical epithelial cells were
308 obtained from the American Type Culture Collection (VK2.E6E7, ATCC CRL-2616 and End1/E6E7,
309 ATCC CRL-2615) and were maintained in keratinocyte serum-free medium (KSFM; Gibco) with 0.1
310 ng/mL human recombinant epidermal growth factor (EGF; Gibco) and 0.05 mg/ml bovine pituitary extract
311 (Gibco) at 37°C with 5% CO2. Assays to determine cell surface-adherent *E. faecalis* were performed as
312 described previously when quantifying GBS adherence [63]. Briefly, bacteria were grown to mid-log
313 phase (OD_{600} = 0.4 - 0.6) and added to cell monolayers (multiplicity of infection [MOI] = 1). After a 30-
314 min incubation, cells were washed with PBS three times following detachment with 0.1 mL of 0.25%
315 trypsin-EDTA solution and lysed with addition of 0.4 mL of 0.025% TritonX-100 in PBS by vigorous
316 pipetting. The lysates were then serially diluted and plated on Todd Hewitt agar (THA) to enumerate
317 the bacterial CFU. Experiments were performed at least three times with each condition in triplicate, and
318 results from a representative experiment are shown.
319
320 **Murine vaginal colonization model**.
321 Animal experiments were approved by the Institutional Animal Care and Use Committee at University of
322 Colorado-Anschutz Medical Campus protocols #00316 and #00253 and performed using accepted
323 veterinary standards. A mouse vaginal colonization model for GBS was adapted for our studies [21]. 8-

324 week old female CD1 (Charles River) or C57BL/6 (Jackson) mice were injected intraperitoneally with 0.5
325 mg 17β-estradiol (Sigma) 1 day prior to colonization with *E. faecalis*. Mice were vaginally inoculated
326 with 10^7 CFU of *E. faecalis* in 10µL PBS and on subsequent days the vaginal lumen was swabbed with a
327 sterile ultrafine swab (Puritan). For co-colonization, mice were inoculated with *E. faecalis* OG1SSp,
328 OG1RF or deletion mutants in the OG1RF background. To assess tissue CFU, mice were euthanized
329 according to approved veterinary protocols and the female reproductive tract tissues were dissected and
330 placed into 500µL PBS and bead beat for 2 min to homogenize the tissues. The resulting homogenate was
331 serially diluted and *E. faecalis* CFU enumerated on BHI agar supplemented with antibiotics to select for
332 the strain of interest.
333
334 **Histology.**
335 Mice were inoculated with *E. faecalis* V583 containing a plasmid that expresses *gfp* (pMV158GFP) and
336 contains resistance to tetracycline (15 µg/mL) [23]. After 1 day post-inoculation, the murine female
337 reproductive tract was harvested, embedded into Optimal Cutting Temperature (OCT) compound
338 (Sakura), and sectioned at 7µm with a CM1950 freezing cryostat (Leica). For fluorescence microscopy,
339 coverslips were mounted with VECTASHIELD mounting medium containing 4',6-diamidino-2-
340 phenylindole (DAPI, Vector Labs). Images were taken with a BZ-X710 microscope (Keyence) [22].
341
342 **Transposon mutant library growth and vaginal colonization.**
343 The *E. faecalis* OG1RF transposon mutant library was generated previously [25]. The E. *faecalis* OG1RF
344 pooled transposon library was inoculated into 5 ml of BHI at a total of 10^8 CFU and grown with aeration
345 to an OD_{600} of 0.5. The library was inoculated into the vaginal tracts of C57BL/6 mice at 10^7 CFU. The
346 library was plated on BHI agar to confirm the inoculum for all groups of mice. Mice were swabbed daily
347 and swabs were plated on BHI supplemented with rifampicin (50 µg/ml), fusidic acid (25 µg/ml) and

348 chloramphenicol (20 μg/ml) to quantify CFU. On days 1, 5, and 8, undiluted swabs were plated on BHI
349 agar with antibiotics and grown to a bacterial lawn. Bacteria were scraped and re-suspended in PBS and
350 pelleted. DNA from days 1, 5, 8, and the input culture was extracted from pellets using a
351 ZymoBIOMICS™ DNA MiniPrep kit (Zymo Research).
352
353 **Transposon library sequencing and data analysis.**
354 Transposon-junction DNA library preparation and Illumina NovaSeq 6000 DNA sequencing (150 base
355 paired-end mode) was performed by the Microarray and Genomics Core at the University of Colorado
356 Anschutz Medical Campus as previously described [39]. For downstream analysis of transposon-
357 junctions, we used only the R1 reads generated by paired end sequencing. Illumina adapter trimmed raw
358 reads were mapped to the *E. faecalis* OG1RF reference sequence (NC_017316.1) and differentially
359 abundant transposon mutants were identified using statistical analysis scripts established by Dale *et*
360 *al.*[25]. The abundance of Tn mutants in culture was compared to input library used for culture inoculation
361 and mutants that are not significantly different ($p > 0.05$) between these two samples were considered for
362 the next steps of the analysis. For comparisons between *in vivo* and *in vitro* samples, mutants were
363 considered significantly different if the adjusted P value was < 0.05 and a $\log_2$ (fold change) > 1. The
364 DNA sequencing reads associated with this study have been deposited at the European Nucleotide Archive
365 under accession number PRJEB37929.
366
367 **Data analysis**.
368 GraphPad Prism version 7.0 was used for statistical analysis and statistical significance was accepted at P
369 values of < 0.05 (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; **** $P < 0.00005$). Specific tests are indicated
370 in figure legends.

371

372 **ACKNOWLEGMENTS**

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379

380 **FIGURE LEGENDS**

381 **Figure 1: *E. faecalis* colonizes the murine female reproductive tract.** (A) Adherence of *E. faecalis*
382 V583 and OG1RF to human vaginal and endocervical cells. Experiments were performed in triplicate and
383 error bars represent standard deviations (SDs); the results of a representative experiment are shown. (B)
384 CFU counts of V583 and OG1RF recovered from vaginal swabs 1 day post-inoculation. (C) CFU counts
385 of V583 from vaginal, cervical and uterine tissue 1 day post-inoculation. (D, E, F) Mice were inoculated
386 with V583 expressing *gfp* and 7μm sections of vaginal (D), cervical (E) and uterine (F) tissue were stained
387 with DAPI for fluorescence microscopy. White arrows indicate green fluorescent bacteria present in tissue
388 sections. Images were all taken at 40x magnification. LP = lamina propria, EP = epithelial layer, Lu =
389 lumen.

390

391 **Figure 2: *E. faecalis* persists in the murine vaginal tract.** (A and B) *E. faecalis* V583 and OG1RF in
392 the murine vaginal tract. C57BL/6 mice were inoculated with 10^7 V583 or OG1RF CFU and the vaginal
393 lumen of each mouse was swabbed daily and swabs were serially diluted and plated on selective media to

394 quantify CFU. Data are presented as recovered CFU per swab (A) and mean recovered CFU (B). (C and
395 D) CD1 (n = 10) and C57BL/6 (n = 7) mice were inoculated with V583 and the vaginal lumen of each
396 mouse was swabbed daily, serially diluted, and plated on selective media to quantify CFU. Data are
397 presented as recovered CFU per swab (C) and mean recovered CFU (D)
398
399 **Figure 3: Enterococcal pili contribute to colonization of the female reproductive tract.** (A and B) *E.*
400 *faecalis* OG1RF WT and OG1RF Δ*ebpABC* adherence to human vaginal (A) and endocervical (B) cells.
401 Experiments were performed with four technical replicates and error bars represent SDs; the results of a
402 representative experiment are shown and analyzed using an unpaired t-test; ***P < 0.0001; ****P <
403 0.00001. (C) C57BL/6 mice were co-inoculated with OG1SSp WT and OG1RF Δ*ebpABC* and the vaginal
404 lumen was swabbed daily. (D and E) C57BL/6 mice were co-inoculated with OG1SSp WT and OG1RF
405 Δ*ebpABC* and reproductive tissue was collected and CFU/gram was quantified. Data are presented as
406 recovered Log_{10}CFU/gram (D) and competitive index (CI) between OG1SSp WT and OG1RF Δ*ebpABC*
407 (E). CI is enumerated by calculating the ratio of WT to mutant *E. faecalis* recovered from the mouse
408 reproductive tract. A CI >1 indicates an advantage to WT *E. faecalis*. Values below the limit of detection
409 were enumerated as one-half the limit of detection. Data were analyzed by a paired t-test; *P < 0.05.
410
411 **Figure 4: Identification of additional factors required for vaginal colonization and persistence by**
412 **transposon mutant library screen.** (A) Schematic representing experimental approach. C57BL/6 mice
413 were treated with 17β-estradiol prior to inoculation with the OG1RF Tn library in triplicate groups or WT
414 OG1RF. The vaginal lumen was swabbed and CFU was enumerated daily. DNA from recovered OG1RF
415 Tn mutants was sequenced on days 1, 5 and 8. (B) CFU recovered from vaginal swabs of triplicate groups

416 of mice colonized with OG1RF Tn mutagenesis library (T1, T2, T3) and OG1RF WT (n=5 mice per
417 group). (C, D and E) Volcano plots depicting underrepresented and overrepresented mutants *in vivo*
418 compared to culture on day 1 (C), day 5 (D) and day 8 (E) post-inoculation. Colored dots represent mutants
419 of interest. Pink = *ebpC* Tn mutant, Orange = *eut* Tn mutant, Green = T7SS gene Tn mutants. (F)
420
421 **Figure 5: Classification of transposon insertion mutants by Cluster of Orthologous Groups (COGs).**
422 (A) Euler plot representing number of underrepresented mutants at all time points. (B) Cluster of
423 Orthologous Groups (COGs) from underrepresented mutants common to all three time points categorized
424 into functional categories. Total = 196 represents all common mutants that were assigned a COG domain.
425
426 **Figure 6: Ethanolamine utilization and type VII secretion system genes contribute to enterococcal**
427 **persistence in the vaginal tract.** (A, B and C) C57BL/6 mice were co-inoculated with OG1SSp WT and
428 OG1RF Δ*eutBC* and vaginal lumen was swabbed to quantify CFU. Data are presented as recovered CFU
429 per swab (A), mean recovered CFU (B) and CI between WT and mutant strains (C). Data was analyzed
430 used a Two-way ANOVA with multiple comparisons; $*P < 0.05$, $**P < 0.005$, $***P < 0.00005$. (D)
431 C57BL/6 mice were co-inoculated with OG1RF WT and OG1RF *esaB*-Tn and vaginal lumen was
432 swabbed to quantify CFU. Data was analyzed using a Two-way ANOVA with multiple comparisons; $**P$
433 < 0.005, $***P < 0.0005$ (E and F) C57BL/6 mice were co-inoculated with OG1RF WT and OG1RF *esaB*-
434 Tn and reproductive tissue was collected at 11 days post-inoculation. Data are presented as recovered
435 Log_{10} CFU/gram (E) and CI between WT and Tn-mutant strain (F). CI is enumerated as described in
436 Figure 4. Data was analyzed using a paired t-test; $*P < 0.05$, $**P < 0.005$.

437

438 **Supplemental Figure 1: Colonization and persistence of *E. faecalis* OG1- (RF and SSp).** (A and B)
439 C57BL/6 mice were co-inoculated with OG1RF WT and OG1SSp WT and the vaginal lumen was
440 swabbed to quantify CFU. Reproductive tissue was collected at 8 days post-inoculation. Data are
441 presented as recovered CFU per swab (A) and Log_{10} CFU/gram (B). Data was analyzed using a Two-way
442 ANOVA with multiple comparisons; $P > 0.05$ (A) and a paired t-test; $P > 0.05$ (B).

443

444 **Supplemental Table 1: Common underrepresented *E. faecalis* OG1RF transposon mutants during**
445 **vaginal colonization day 1, 5 and 8 post-inoculation.** All significantly underrepresented Tn mutants
446 common to all time points are listed along with their functional categorization from COGs and log_2
447 adjusted P values comparing *in vitro* to *in vivo* samples.

448

449 **Supplemental Table 2: Underrepresented *E. faecalis* OG1RF transposon mutants during vaginal**
450 **colonization day 1 post-inoculation.** All significantly underrepresented Tn mutants from *in vivo* samples
451 1 day post-inoculation are listed along with their functional categorization from COGs and log_2 adjusted
452 P values comparing *in vitro* to *in vivo* samples.

453

454 **Supplemental Table 3: Underrepresented *E. faecalis* OG1RF transposon mutants during vaginal**
455 **colonization day 5 post-inoculation.** All significantly underrepresented Tn mutants from *in vivo* samples
456 5 days post-inoculation are listed along with their functional categorization from COGs and log_2 adjusted
457 P values comparing *in vitro* to *in vivo* samples.

458

459 **Supplemental Table 4: Underrepresented *E. faecalis* OG1RF transposon mutants during vaginal**
460 **colonization day 8 post-inoculation.** All significantly underrepresented Tn mutants from *in vivo* samples
461 8 days post-inoculation are listed along with their functional categorization from COGs and $\log_2$ adjusted
462 P values comparing *in vitro* to *in vivo* samples.
463
464 **Supplemental Table 5: Overrepresented *E. faecalis* OG1RF transposon mutants during vaginal**
465 **colonization day 1 and 5 post-inoculation.** All significantly overrepresented Tn mutants from *in vivo*
466 samples 1 and 5 days post-inoculation are listed along with their functional categorization from COGs and
467 $\log_2$ adjusted P values comparing *in vitro* to *in vivo* samples.
468
469 **Supplemental Table 6: Overrepresented *E. faecalis* OG1RF transposon mutants during vaginal**
470 **colonization day 8 post-inoculation.** All significantly overrepresented Tn mutants from *in vivo* samples
471 8 days post-inoculation are listed along with their functional categorization from COGs and $\log_2$ adjusted
472 P values comparing *in vitro* to *in vivo* samples.
473
474 **Supplemental Table 7:** List of bacterial strains and method of selection used in this study.
475
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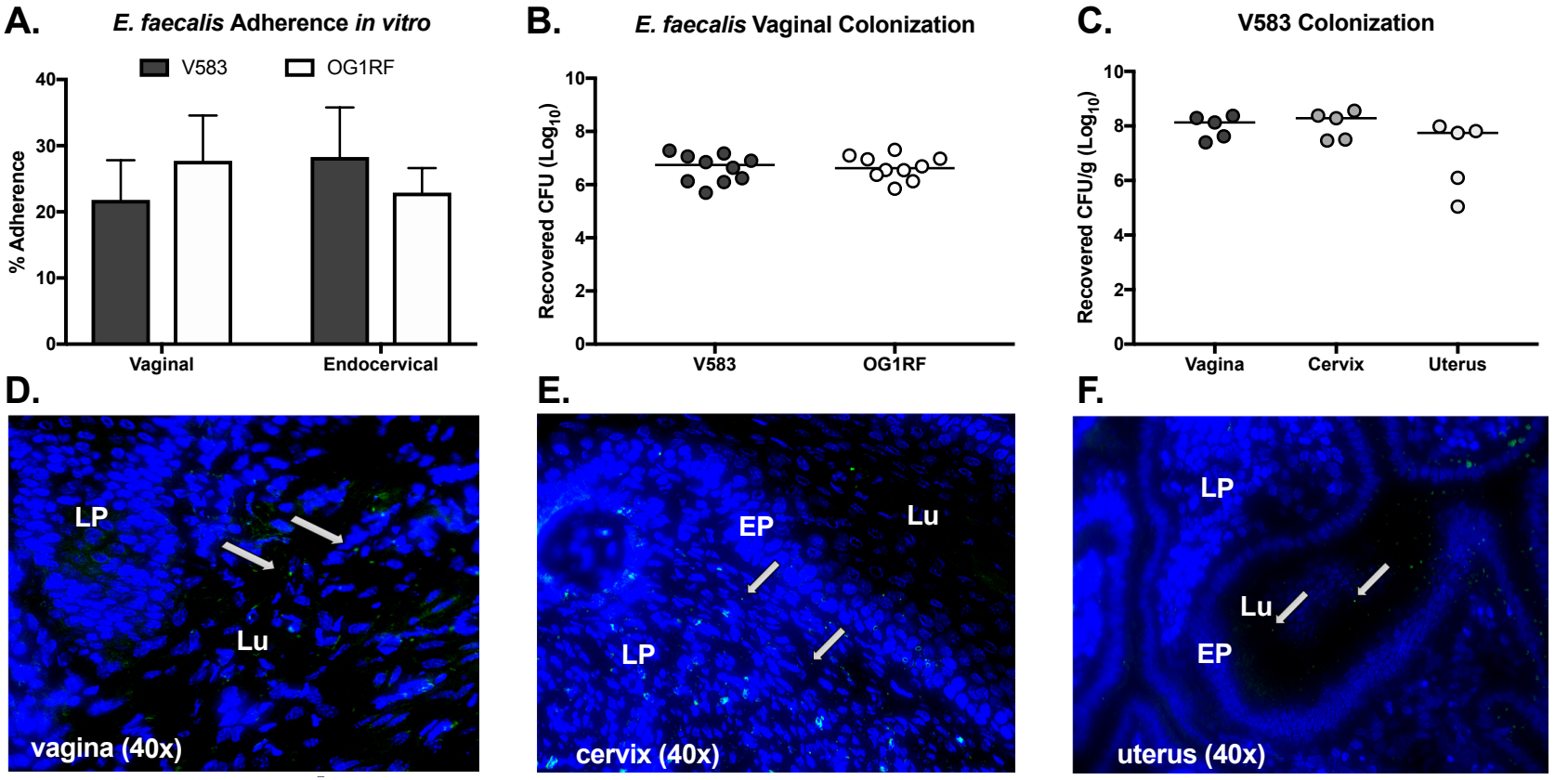
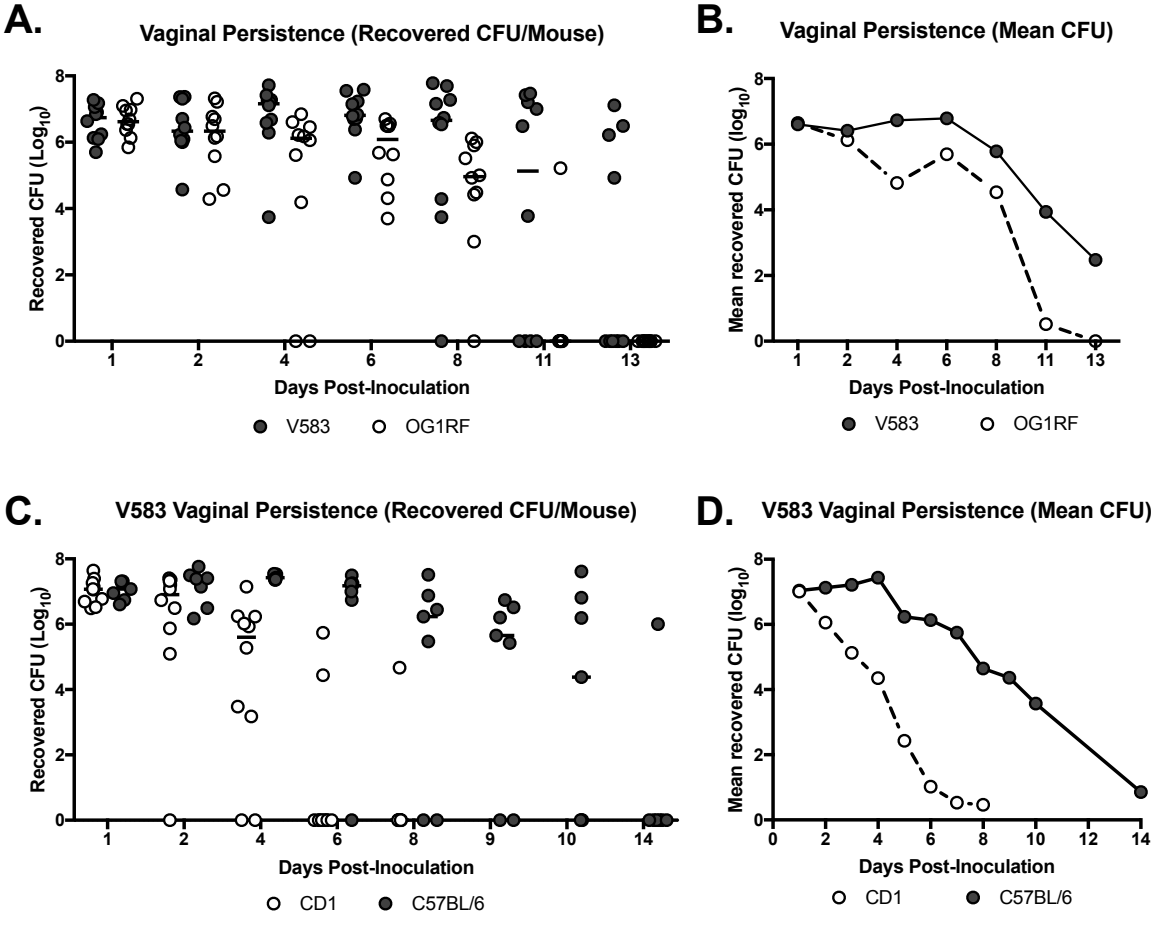
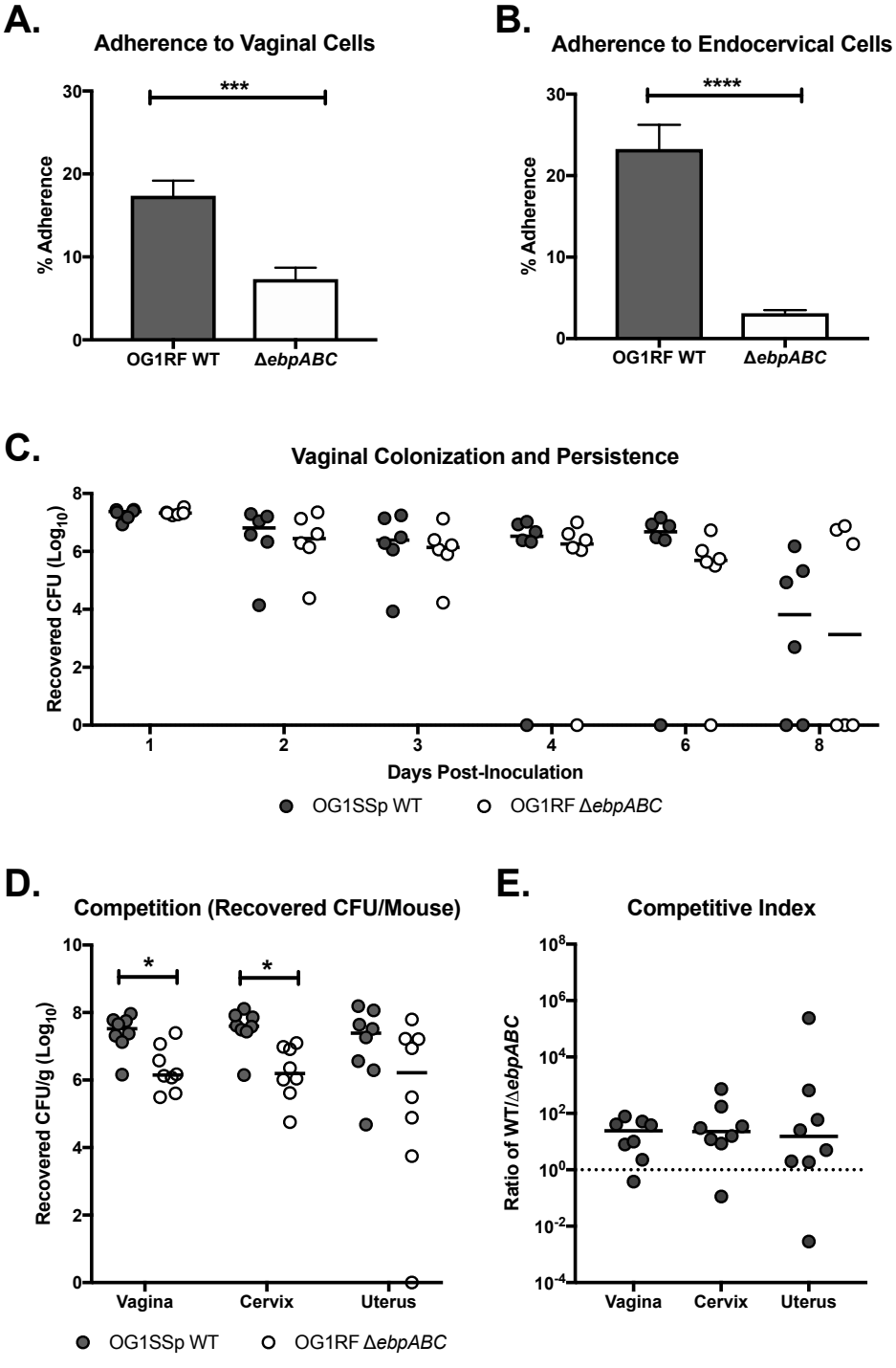
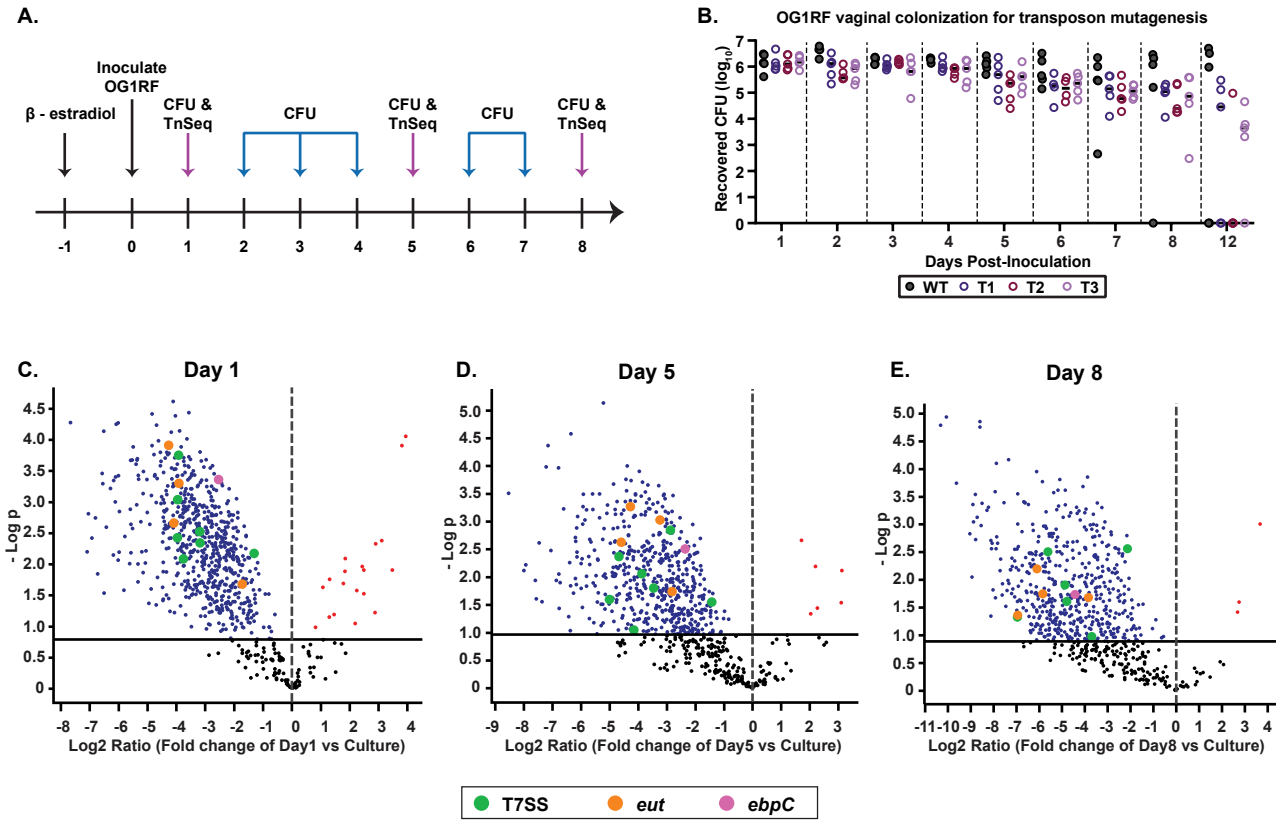
621

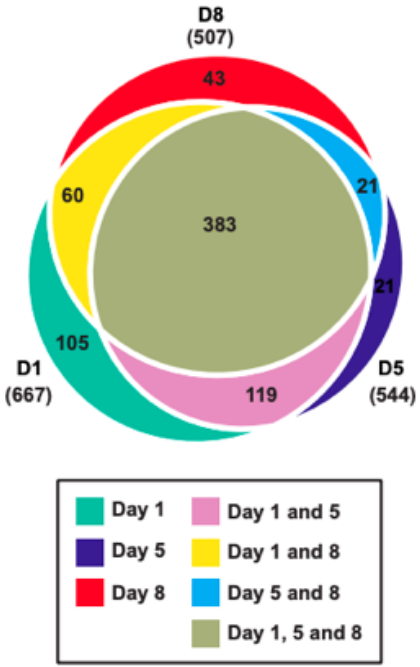

A.
E. faecalis Adherence in vitro
V583
OG1RF
% Adherence
40
30
20
10
0
Vaginal
Endocervical
B.
E. faecalis Vaginal Colonization
Recovered CFU (Log10)
10
8
6
4
2
0
V583
OG1RF
C.
V583 Colonization
Recovered CFU/g (Log10)
10
8
6
4
2
0
Vagina
Cervix
Uterus
D.
LP
Lu
vagina (40x)
E.
EP
Lu
LP
cervix (40x)
F.
LP
Lu
EP
uterus (40x)


A.
Vaginal Persistence (Recovered CFU/Mouse)
Recovered CFU (Log10)
Days Post-Inoculation
V583
OG1RF
B.
Vaginal Persistence (Mean CFU)
Mean recovered CFU (log10)
Days Post-Inoculation
V583
OG1RF
C.
V583 Vaginal Persistence (Recovered CFU/Mouse)
Recovered CFU (Log10)
Days Post-Inoculation
CD1
C57BL/6
D.
V583 Vaginal Persistence (Mean CFU)
Mean recovered CFU (log10)
Days Post-Inoculation
CD1
C57BL/6

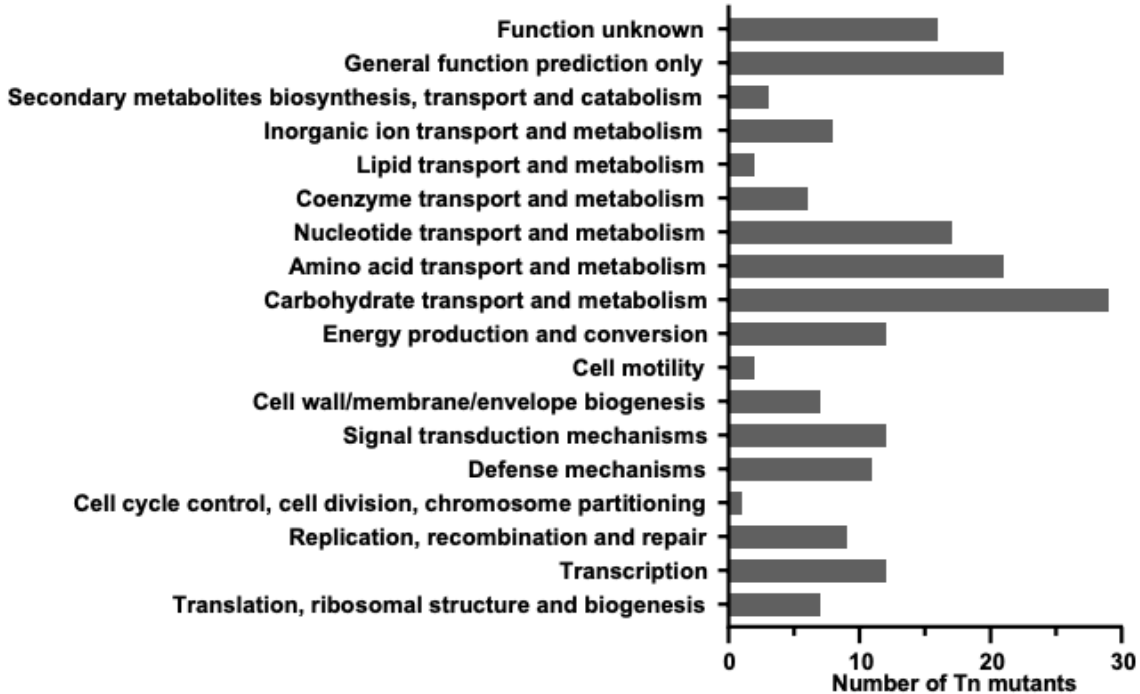

A.
Adherence to Vaginal Cells
***
% Adherence
30
20
10
0
OG1RF WT
ΔebpABC
B.
Adherence to Endocervical Cells
****
% Adherence
30
20
10
0
OG1RF WT
ΔebpABC
C.
Vaginal Colonization and Persistence
Recovered CFU (Log10)
8
6
4
2
0
1
2
3
4
6
8
Days Post-Inoculation
OG1SSp WT
OG1RF ΔebpABC
D.
Competition (Recovered CFU/Mouse)
*
*
Recovered CFU/g (Log10)
10
8
6
4
2
0
Vagina
Cervix
Uterus
OG1SSp WT
OG1RF ΔebpABC
E.
Competitive Index
Ratio of WT/ΔebpABC
10^8
10^6
10^4
10^2
10^0
10^-2
10^-4
Vagina
Cervix
Uterus

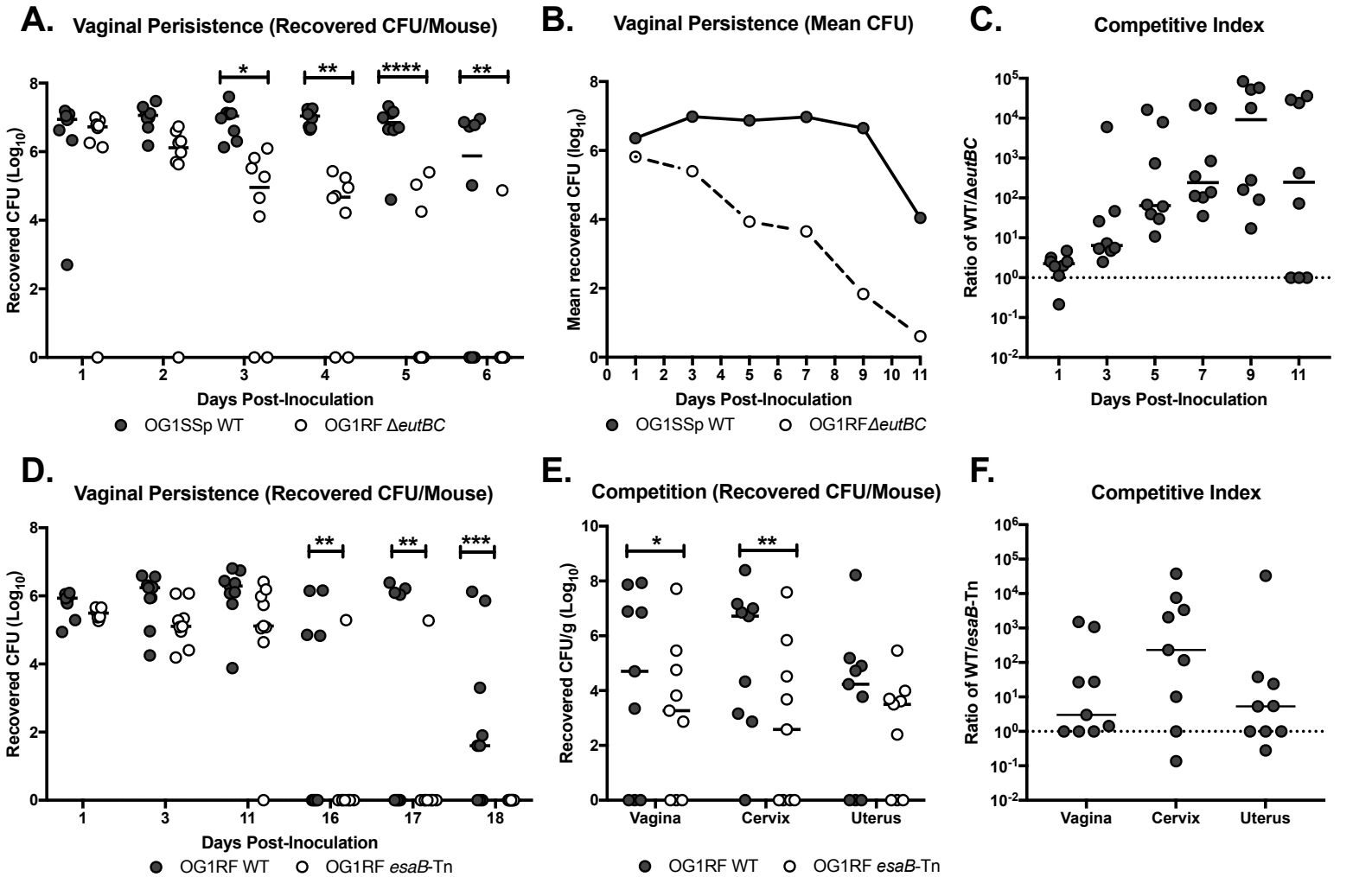

A.
β - estradiol
Inoculate OG1RF
CFU & TnSeq
CFU
CFU & TnSeq
CFU
CFU & TnSeq
-1 0 1 2 3 4 5 6 7 8
B.
OG1RF vaginal colonization for transposon mutagenesis
Recovered CFU (log10)
Days Post-Inoculation
WT T1 T2 T3
C.
Day 1
- Log p
Log2 Ratio (Fold change of Day1 vs Culture)
D.
Day 5
- Log p
Log2 Ratio (Fold change of Day5 vs Culture)
E.
Day 8
- Log p
Log2 Ratio (Fold change of Day8 vs Culture)
T7SS
eut
ebpC

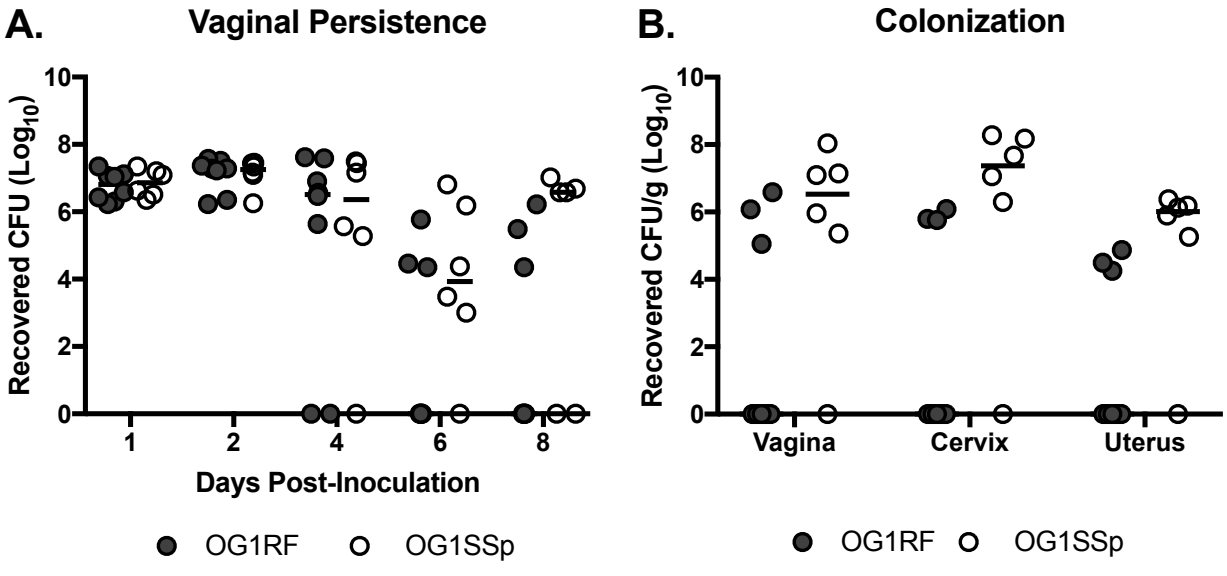
A.


D8
(507)
43
60
21
383
21
105
D1
(667)
119
D5
(544)
Day 1
Day 1 and 5
Day 5
Day 1 and 8
Day 8
Day 5 and 8
Day 1, 5 and 8

B.


Function unknown
General function prediction only
Secondary metabolites biosynthesis, transport and catabolism
Inorganic ion transport and metabolism
Lipid transport and metabolism
Coenzyme transport and metabolism
Nucleotide transport and metabolism
Amino acid transport and metabolism
Carbohydrate transport and metabolism
Energy production and conversion
Cell motility
Cell wall/membrane/envelope biogenesis
Signal transduction mechanisms
Defense mechanisms
Cell cycle control, cell division, chromosome partitioning
Replication, recombination and repair
Transcription
Translation, ribosomal structure and biogenesis
0
10
20
30
Number of Tn mutants


A.
Vaginal Perisistence (Recovered CFU/Mouse)
Recovered CFU (Log10)
Days Post-Inoculation
OG1SSp WT
OG1RF ΔeutBC
B.
Vaginal Persistence (Mean CFU)
Mean recovered CFU (log10)
Days Post-Inoculation
OG1SSp WT
OG1RFΔeutBC
C.
Competitive Index
Ratio of WT/ΔeutBC
Days Post-Inoculation
D.
Vaginal Persistence (Recovered CFU/Mouse)
Recovered CFU (Log10)
Days Post-Inoculation
OG1RF WT
OG1RF esaB-Tn
E.
Competition (Recovered CFU/Mouse)
Recovered CFU/g (Log10)
Vagina
Cervix
Uterus
OG1RF WT
OG1RF esaB-Tn
F.
Competitive Index
Ratio of WT/esaB-Tn
Vagina
Cervix
Uterus


A.
Vaginal Persistence
Recovered CFU (Log10)
10
8
6
4
2
0
1
2
4
6
8
Days Post-Inoculation
OG1RF
OG1SSp
B.
Colonization
Recovered CFU/g (Log10)
10
8
6
4
2
0
Vagina
Cervix
Uterus
OG1RF
OG1SSp