Phage infection mediates inhibition of bystander bacteria

Anushila Chatterjee[a]*, Julia L. E. Willett[b]*, Gary M. Dunny[b], Breck A. Duerkop[a,#]

[a]Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, CO, USA, 80045. [b]Department of Microbiology and Immunology, University of Minnesota Medical School, Minneapolis, MN, USA, 55455.

[#]Correspondence: Breck A. Duerkop breck.duerkop@cuanschutz.edu

*A.C. and J.L.E.W. contributed equally to this work.

Running title: Phage induced T7SS promotes antibacterial antagonism

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Abstract

Bacteriophages (phages) are being considered as alternative therapeutics for the treatment of multidrug resistant bacterial infections. Considering phages have narrow host-ranges, it is generally accepted that therapeutic phages will have a marginal impact on non-target bacteria. We have discovered that lytic phage infection induces transcription of type VIIb secretion system (T7SS) genes in the pathobiont *Enterococcus faecalis*. Membrane damage during phage infection induces T7SS gene expression resulting in cell contact dependent antagonism of diverse Gram positive bystander bacteria. Deletion of *essB*, a T7SS structural component abrogates phage-mediated killing of bystanders. A predicted immunity gene confers protection against T7SS mediated inhibition and disruption of an upstream LXG toxin gene rescues growth of *E. faecalis* and *Staphylococcus aureus* bystanders. Phage induction of T7SS gene expression and bystander inhibition requires IreK, a serine/threonine kinase, and OG1RF_11099, a predicted transcriptional activator. Our findings highlight how phage infection of a target bacterium can affect non-target bystander bacteria and implies phage therapy could impose collateral damage to polymicrobial communities.

53 **Introduction**

54 Enterococci constitute a minor component of the healthy human microbiota (1). Enterococci,
55 including *Enterococcus faecalis*, are also nosocomial pathogens that cause a variety of diseases,
56 including sepsis, endocarditis, surgical-site, urinary tract and mixed bacterial infections (2, 3).
57 Over recent decades, enterococci have acquired extensive antibiotic resistance traits, including
58 resistance to "last-resort" antibiotics such as vancomycin, daptomycin, and linezolid (4-8).
59 Following antibiotic therapy, multi-drug resistant (MDR) enterococci can outgrow to become a
60 dominant member of the intestinal microbiota, resulting in intestinal barrier invasion and blood
61 stream infection (7, 9). The ongoing evolution of MDR enterococci in healthcare settings (4-6, 10,
62 11) and their ability to transmit antibiotic resistance among diverse bacteria (9, 12-15), emphasize
63 the immediate need for novel therapeutic approaches to control enterococcal infections.

64 Viruses that infect and kill bacteria (bacteriophages or phages) are receiving attention for their
65 use as antibacterial agents (16). Recent studies have demonstrated the efficacy of anti-
66 enterococcal phages in murine models of bacteremia (17-19) and the administration of phages to
67 reduce *E. faecalis* burden in the intestine gives rise to phage resistant isolates that are
68 resensitized to antibiotics (20). Considering phages are highly specific for their target bacterium,
69 coupled with the self-limiting nature of their host-dependent replication, suggests that unlike
70 antibiotics which have broad off-target antimicrobial activity, phages are likely to have nominal
71 impact on bacteria outside of their intended target strain (21-23). However, our understanding of
72 how phages interact with bacteria and the bacterial response to phage infection is limited.

73 While studying the transcriptional response of phage infected *E. faecalis* cells, we discovered
74 that phage infection induced the expression of genes involved in the biosynthesis of a type VIIb
75 secretion system (T7SS) (24). Firmicutes, including the enterococci, harbor diverse T7SS genes
76 encoding transmembrane and cytoplasmic proteins involved in the secretion of protein substrates
77 (25), and T7SSs promote antagonism of non-kin bacterial cells through contact-dependent
78 secretion of antibacterial effectors and/or toxins (26, 27). The antibacterial activity of T7SSs from

79 *Staphylococcus* and *Streptococcus* are well characterized (25) but T7SS-mediated antibacterial
80 antagonism has not been described in *Enterococcus*. The environmental cues and regulatory
81 pathways that govern T7SS expression and activity are poorly understood, although recent
82 studies indicate that exposure to serum and membrane stresses triggered by pulmonary
83 surfactants, fatty acids and phage infection stimulate T7SS gene expression (24, 28-31). This
84 motivated us to determine if phage induced T7SS gene expression in *E. faecalis* results in the
85 inhibition of non-kin bacterial cells that are not phage targets (bystanders). We discovered that
86 phage infected *E. faecalis* produces potent T7SS antibacterial activity against bystander bacteria.
87 Expression of a T7SS antitoxin (immunity factor) gene in bystander cells and mutation of the LXG
88 domain containing gene located immediately upstream of the immunity factor confer protection
89 against phage mediated T7SS inhibition. Additionally, we discovered that membrane stress during
90 phage infection induces transcription of T7SS genes via a non-canonical IreK signaling pathway.
91 To our knowledge, the enterococcal T7SS is the only example of secretion system induction
92 during phage infection. These data shed light on how phage infection of a cognate bacterial host
93 can influence polymicrobial interactions and raises the possibility that phages may impose
94 unintended compositional shifts among bystander bacteria in the microbiota during phage
95 therapy.
96
97 **Results**
98 **Phage mediated induction of *E. faecalis* T7SS leads to interspecies antagonism.**
99 A hallmark feature of phage therapy is that because phages often have a narrow host range,
100 they will not influence the growth of non-susceptible bacteria occupying the same niche (22). We
101 discovered that infection of *E. faecalis* OG1RF by phage VPE25, induces the expression of T7SS
102 genes (24). The *E. faecalis* OG1RF T7SS locus is absent in the commonly studied vancomycin-
103 resistant strain V583, despite conservation of flanking genes (Fig. 1A) (32, 33). Homologs of the
104 *E. faecalis* T7SS gene *exsA* are found throughout three of the four *Enterococcus* species groups

105 (34), including *Enterococcus faecium*, suggesting a wide distribution of T7SS loci in enterococci
106 (Fig. S1). *In silico* analyses predict that the *E. faecalis* T7SS locus encodes multiple WXG100
107 family effectors and LXG family polymorphic toxins (27, 35). Hence, we hypothesized that
108 induction of T7SS genes during phage infection and consequently the heightened production of
109 T7SS substrates would indirectly influence the growth of non-kin phage-resistant bacterial cells.

110 To investigate if T7SS factors produced during phage infection of *E. faecalis* OG1RF interferes
111 with the growth of phage-resistant bystander bacteria, we generated a strain with an in-frame
112 deletion in the T7SS gene *essB*, encoding a transmembrane protein involved in the transport of
113 T7SS substrates (36). The *essB* mutant is equally susceptible to phage VPE25 infection
114 compared to wild type *E. faecalis* OG1RF (Fig. S2). We performed co-culture experiments where
115 phage susceptible wild type *E. faecalis* OG1RF or Δ*essB* were mixed with a phage resistant
116 bystander, a strain of *E. faecalis* V583 deficient in the production the VPE25 receptor (Δpip_{V583})
117 (37), at a ratio of 1:1 in the absence and presence of phage VPE25 (multiplicity of infection [MOI]
118 = 0.01) (Fig. 1B). Since the *E. faecalis* V583 genome does not contain T7SS genes, this strain
119 should lack immunity to T7SS antagonism (33). The viability of *E. faecalis* Δpip_{V583}, was reduced
120 nearly 100-fold when co-cultured with *E. faecalis* OG1RF in the presence of phage VPE25 (Fig.
121 1C). However, growth inhibition of *E. faecalis* Δpip_{V583} was abrogated during co-culture with phage
122 infected *E. faecalis* Δ*essB*. Phage induced T7SS antagonism could be restored by
123 complementation (Fig. 1C), indicating that inhibition of phage resistant *E. faecalis* Δpip_{V583} is T7SS
124 dependent.

125 T7SS encoded antibacterial toxins secreted by Gram positive bacteria influence intra- and
126 interspecies antagonism (26, 27). While a nuclease toxin produced by *Staphylococcus aureus*
127 targets a closely related *S. aureus* strain (26), *Streptococcus intermedius* exhibits T7SS
128 dependent antagonism against a wide-array of Gram positive bacteria (27). To determine the
129 target range of *E. faecalis* OG1RF T7SS antibacterial activity, we measured the viability of a panel
130 of VPE25 resistant Gram positive and Gram negative bacteria in our co-culture assay (Fig. 1B).

131 Growth inhibition of the distantly related bacterial species *E. faecium* and Gram positive bacteria
132 of diverse genera, including *S. aureus* and *Listeria monocytogenes*, occurred following co-culture
133 with phage infected wild type *E. faecalis* OG1RF but not the Δ*essB* mutant (Fig. 1D). In contrast,
134 Gram positive streptococci were unaffected (Fig. 1D). Similarly, phage induced T7SS activity did
135 not inhibit any Gram negative bacteria tested (Fig. 1D). Collectively, these results show that phage
136 predation of *E. faecalis* promotes T7SS inhibition of diverse bystander bacteria.
137

138 **Molecular basis of *E. faecalis* phage–triggered T7SS antagonism**

139 Our data demonstrate that induction of *E. faecalis* OG1RF T7SS genes during phage infection
140 hinder the growth of select non-kin bacterial species. Antibacterial toxins deployed by Gram
141 negative bacteria via type VI secretion and Gram positive T7SS require physical contact between
142 cells to achieve antagonism (26, 27, 38, 39). Therefore, we investigated if growth inhibition of
143 bystander bacteria is contingent upon direct interaction with phage infected *E. faecalis* using a
144 trans-well assay (27). We added unfiltered supernatants from wild type *E. faecalis* OG1RF and
145 Δ*essB* mutant cultures grown for 24 hrs in the presence and absence of phage VPE25 (MOI =
146 0.01) to the top of a trans well and deposited phage resistant *E. faecalis* Δpip_{V583} in the bottom of
147 the trans well. The 0.4 µm membrane filter that separates the two wells is permeable to proteins
148 and solutes but prevents bacterial translocation. Supernatant from phage infected wild type *E.*
149 *faecalis* OG1RF did not inhibit *E. faecalis* Δpip_{V583} (Fig. 2A) indicating that T7SS mediated growth
150 interference relies on cell to cell contact. To exclude the possibility that T7SS substrates might
151 adhere to the 0.4 µm membrane filter in the trans-well assay, we administered unfiltered culture
152 supernatants directly to *E. faecalis* Δpip_{V583} cells ($5x10^5$ CFU/well) at a ratio of 1:10 and monitored
153 growth over a period of 10 hours. Growth kinetics of *E. faecalis* Δpip_{V583} remained similar
154 irrespective of the presence or absence of conditioned supernatant from wild type *E. faecalis*
155 OG1RF or Δ*essB* mutant cultures (Fig. 2B), further supporting the requirement of contact-
156 dependent engagement of phage mediated T7SS inhibition.

157 We discovered that *E. faecalis* OG1RF inhibits proliferation of non-kin bacterial cells through
158 increased expression of T7SS genes in response to phage infection, but the toxic effectors are
159 unknown. LXG domain containing toxins are widespread in bacteria with a diverse range of
160 predicted antibacterial activities (40, 41). The OG1RF T7SS locus encodes two LXG-domain
161 proteins, expressed from OG1RF_11109 and OG1RF_11121 (Fig. 3A). Polymorphic toxin
162 systems in Gram negative and Gram positive bacteria can encode "orphan" toxins in addition to
163 the primary secreted effectors (42). To identify putative orphan effectors, we aligned
164 OG1RF_11109 and OG1RF_11121 with downstream genes in the T7SS locus. OG1RF_11109
165 shared conserved sequence with OG1RF_11111 and OG1RF_11113, and OG1RF_11121 had
166 homology to OG1RF_11123 (Fig 3A). Homologs to these putative orphan toxins are found as full-
167 length LXG proteins in *E. faecalis* and *Listeria sp*. (Fig. S3), suggesting they are bonafide T7SS
168 effectors. Interestingly, we identified an additional LXG gene product, OG1RF_12414, in a distal
169 locus that is again notably absent from V583 (Fig. S4A and S4B). Although none of the five
170 putative toxins in the T7SS locus have recognizable C-terminal domains, OG1RF_12414 has
171 predicted structural homology to Tne2, a T6SS effector with NADase activity from *Pseudomonas*
172 *protegens* (Fig. S4C) (43). Additionally, we identified numerous C-terminal domains in LXG
173 proteins distributed throughout *Enterococcus* (Fig. S5). These include EndoU and Ntox44
174 nuclease domains (40, 44, 45), which have been characterized in effectors produced by other
175 polymorphic toxin systems.

176 Polymorphic toxins are genetically linked to cognate immunity proteins that neutralize
177 antagonistic activity and prevent self-intoxication (40, 45, 46). Each of the five putative toxins in
178 the OG1RF T7SS locus is encoded directly upstream of a small protein that could function in
179 immunity. Whitney *et al*. demonstrated that the cytoplasmic antagonistic activity of *S. intermedius*
180 LXG toxins TelA and TelB in *E. coli* can be rescued by co-expression of the corresponding
181 immunity factors (27). Therefore, we examined if OG1RF_11110, 11112, or 11122, confer
182 immunity to *E. faecalis* Δpip_{V583} during phage infection of *E. faecalis* OG1RF. Constitutive

183 expression of OG1RF_11122, and not OG1RF_11110 or 11112, partially neutralized phage
184 induced T7SS antagonism (Fig. 3B), confirming an essential role for the OG1RF_11122 gene
185 product in immunity, and suggesting that OG1RF_11121 is at least partly responsible for T7SS
186 mediated intra-species antagonism.

187 To determine the contribution of OG1RF_11121 on intra- and interbacterial antagonism during
188 phage infection, we measured the viability of phage resistant *E. faecalis* Δpip_{V583} and *S. aureus*
189 in co-culture with an *E. faecalis* OG1RF variant carrying a transposon insertion in OG1RF_11121
190 (OG1RF_11121-Tn). OG1RF_11121-Tn is equally susceptible to phage VPE25 infection
191 compared to wild type *E. faecalis* OG1RF (Fig. S2). Similar to the Δ*essB* mutant, phage infected
192 OG1RF_11121-Tn did not inhibit the growth of the bystander bacteria (Fig. 3C – D), implicating
193 that the OG1RF_11121 encoded LXG toxin drives *E. faecalis* T7SS mediated antagonism of
194 bystanders following phage infection.

195

196 **Sub-lethal antibiotic stress promotes T7SS dependent antagonism**

197 Considering two genetically distinct phages trigger the induction of T7SS genes in *E. faecalis*
198 (24), we reasoned that T7SS induction could be a result of phage mediated cellular damage and
199 not specifically directed by a phage encoded protein. Antibiotics elicit a range of damage induced
200 stress responses in bacteria (47-49), therefore, we investigated the effects of subinhibitory
201 concentrations of antibiotics on T7SS expression in *E. faecalis*.

202 To investigate the influence of sublethal antibiotic concentrations on *E. faecalis* OG1RF T7SS
203 transcription, we determined the minimum inhibitory concentrations (MIC) of ampicillin,
204 vancomycin, and daptomycin (Fig.S6A-S6C) and monitored T7SS gene expression in *E. faecalis*
205 OG1RF cells treated with a sub-lethal dose of antibiotic (50% of the MIC). We found that bacterial
206 T7SS genes were significantly upregulated in the presence of the cell membrane targeting
207 antibiotic, daptomycin, relative to the untreated control (Fig. 4A). In contrast, the cell wall
208 biosynthesis inhibitors ampicillin and vancomycin either did not induce or had a minor impact on

209 T7SS mRNA levels, respectively (Fig. 4A). Additionally, induction of T7SS transcription occurred
210 when bacteria are challenged with a sub-inhibitory concentration of the DNA targeting antibiotics
211 ciprofloxacin and mitomycin C (Fig. 4B, Fig. S6D–S6E). Collectively, these data show that T7SS
212 induction in *E. faecalis* occurs in response to cell envelope and DNA stress.

213 We next assessed the influence of daptomycin driven T7SS induction on inter-enterococal
214 antagonism. Since T7SS expression is less robust in the presence of daptomycin compared to
215 phage infection (24), a 10:1 ratio of daptomycin treated *E. faecalis* OG1RF was required for
216 growth inhibition of *E. faecalis* Δpip_{V583} during co-culture (Fig. 4C). Consistent with our previous
217 results, daptomycin induced T7SS inhibition of *E. faecalis* Δpip_{V583} was contact dependent (Fig.
218 4D). To facilitate T7SS mediated contact-dependent killing of the target strain during daptomycin
219 exposure, we performed the inhibition assay on nutrient agar plates. The sub-inhibitory
220 concentration of daptomycin (2.5 µg/ml) used in liquid culture was toxic to the cells on agar plates,
221 so we lowered the daptomycin concentration to 0.5 µg/ml to prevent drug toxicity in the agar-
222 based antagonism assay. Plating T7SS producing *E. faecalis* OG1RF cells and *E. faecalis*
223 Δpip_{V583} bystander cells at a ratio of 10:1 resulted in ~10–fold inhibition of bystander growth (Fig.
224 4E). These data show that in addition to phages, antibiotics can be sensed by *E. faecalis* thereby
225 inducing T7SS antagonism of non-kin bacterial cells. These data also suggest that the magnitude
226 of T7SS gene expression is directly related to the potency of T7SS inhibition.

227

228 **IreK and OG1RF_11099 facilitate T7SS expression in phage infected *E. faecalis* OG1RF via**
229 **a non-canonical signaling pathway**

230 Having established that phage and daptomycin mediated membrane damage stimulates
231 heightened *E. faecalis* OG1RF T7SS gene expression and antagonistic activity, we next sought
232 to identify the genetic determinants that sense this damage and promote T7SS transcription. Two-
233 component systems, LiaR/S and CroS/R, and the PASTA kinase family protein IreK are well-
234 characterized modulators of enterococcal cell envelope homeostasis and antimicrobial tolerance

235 (50-52). Aberrant cardiolipin microdomain remodeling in the bacterial cell membrane in the
236 absence of the LiaR response regulator results in daptomycin hypersensitivity and virulence
237 attenuation (53). CroS/R signaling and subsequent modulation of gene expression govern cell
238 wall integrity and promote resistance to cephalosporins, glycopeptides and beta—lactam
239 antibiotics (54-56). The *ireK* encoded transmembrane Ser/Thr kinase regulates cell wall
240 homeostasis, antimicrobial resistance, and contributes to bacterial fitness during long-term
241 colonization of the intestinal tract (51, 57, 58). Recently it has been shown that direct cross-talk
242 between IreK and the CroS/R system positively impacts enterococcal cephalosporin resistance
243 (59).

244 Wild type *E. faecalis* OG1RF, an *ireK* in-frame deletion mutant (51) and transposon (Tn)
245 insertion mutants of *liaR*, *liaS*, *croR*, and *croS* (60) all display similar growth kinetics in the
246 absence of phage VPE25 infection (Fig. S7A). Although *croR*-Tn and *croS*-Tn exhibit reductions
247 in the plaquing efficiency of VPE25 particles, none of these genetic elements of enterococcal cell
248 wall homeostasis and antibiotic resistance were required for VPE25 infection (Fig. S7B). We
249 queried the expression levels of T7SS genes in these isogenic mutants during phage VPE25
250 infection (MOI = 1). T7SS gene expression was not enhanced in the Δ*ireK* mutant during phage
251 infection (Fig. 5A), whereas *liaR*-Tn, *liaS*-Tn, *croR*-Tn, and *croS*-Tn produced heightened levels
252 of T7SS transcripts similar to the wild type *E. faecalis* OG1RF compared to the uninfected controls
253 (Fig. S8A-S8F). A sub-lethal concentration of the cephalosporin ceftriaxone did not induce T7SS
254 gene expression (Fig. S9), indicating that expression of T7SS genes following phage mediated
255 membrane damage signals through a pathway that is distinct from the IreK response to
256 cephalosporin stress. Additionally, the Δ*ireK* mutant phenocopies the Δ*essB* mutant strain in the
257 interbacterial antagonism co-culture assay, wherein the Δ*ireK* mutant is unable to mediate phage
258 induced T7SS dependent killing of the phage resistant *E. faecalis* Δpip_{V583} non-kin cells (Fig. 5B).
259 Collectively, these results indicate that IreK senses phage mediated membrane damage
260 promoting T7SS transcription independent of the CroS/R pathway.

261 OG1RF_11099, located immediately upstream of the T7SS cluster is predicted to encode a
262 GntR family transcriptional regulator, thus we sought to assess the contribution of *OG1RF_11099*
263 on T7SS transcription and functionality. OG1RF_11099-Tn is equally susceptible to phage VPE25
264 infection compared to wild type *E. faecalis* OG1RF (Fig. S2) In contrast to wild type *E. faecalis*
265 OG1RF, T7SS genes were not induced during phage predation in OG1RF_11099-Tn which
266 harbors a transposon insertion in this potential transcriptional regulatory gene (Fig. 5C).
267 Subsequently, we evaluated the influence of OG1RF_11099-dependent regulation on the activity
268 of enterococcal T7SS in intraspecies antagonism using our co-culture assays. Phage infected
269 OG1RF_11099-Tn behaved similar to the Δ*essB* mutant and was unable to inhibit the *E. faecalis*
270 Δpip_{V583} competitor strain during co-culture (Fig. 5D). Collectively, these results indicate that
271 *OG1RF_11099* encodes a positive regulator of *E. faecalis* T7SS.
272

273 **Discussion**

274 Despite the fact that bacteria exist in complex microbial communities and engage in social
275 interactions (61, 62), phage predation studies have primarily been performed in monoculture (24,
276 63-65). Studies report phage-mediated effects on non-target bacteria linked to interbacterial
277 interactions and evolved phage tropism for non-cognate bacteria (66-68), whereas other studies
278 have identified minimal changes in microbiota diversity during phage therapy (66, 69).

279 Our results extend previous work that observed the induction of *E. faecalis* OG1RF T7SS
280 gene expression in response to phage infection (24). By using an *in vitro* antibacterial antagonism
281 assay, we discovered that phage predation of *E. faecalis* OG1RF has an inhibitory effect on non-
282 phage targeted bacterial species during co-culture. Our work shows that phage mediated
283 inhibition of Gram positive bystander bacteria relies on the expression and activity of T7SS genes.
284 This work emphasizes how phage infection of target bacteria can extend beyond intended phage
285 targets and inhibit other members of a microbial community. This discovery could have profound
286 ramifications on how microbial communities like the microbiota respond to phage therapy.

287 Our data suggest that membrane stress associated with phage infection or sub-lethal
288 daptomycin treatment stimulates T7SS mediated antibacterial antagonism of *E. faecalis* OG1RF.
289 Given that daptomycin is used to target vancomycin-resistant enterococcal infections, this finding
290 provides a model by which antibiotic-resistant enterococci may overgrow and dominate the
291 microbiota after antibiotic treatment. Although further investigation is required to understand how
292 T7SS induction might contribute to enterococcal fitness in polymicrobial environments, disruption
293 of T7SS loci in other bacteria compromises bacterial membrane integrity and attenuates virulence
294 (31, 70). It is possible that environmental conditions encountered in the intestinal tract, including
295 bile salts, antimicrobial proteins, and competition for nutrient resources could influence T7SS
296 activity in *E. faecalis* to facilitate niche establishment and/or persistence within a complex
297 microbial community. Indeed, *E. faecalis* T7SS mutants have a defect in their ability to colonize
298 the murine reproductive tract (manuscript submitted). Further, we discovered that transcriptional
299 activation of the T7SS during phage infection relies on IreK. Previously characterized IreK–
300 mediated stress response pathways, including cephalosporin stress or the CroS/R signaling, did
301 not contribute to T7SS expression. We hypothesize that IreK senses diverse environmental
302 stressors and coordinates distinct outputs in response to specific stimuli. Considering that IreK
303 signaling is important for *E. faecalis* intestinal colonization (58), it is possible that IreK–dependent
304 T7SS expression in response to intestinal cues modulate interbacterial interactions and
305 enterococcal persistence in the intestine. However, the molecular mechanism by which IreK
306 facilitates T7SS transcription remains unanswered.

307 Additionally, we discovered that *E. faecalis* OG1RF T7SS transcription is regulated by the
308 GntR domain containing transcriptional regulator encoded by *OG1RF_11099*, a gene found
309 immediately upstream of the T7SS cluster. Interestingly, *OG1RF_11099* is highly conserved
310 across enterococci, including *E. faecalis* V583 and other strains that lack T7SS. The presence of
311 a conserved transcriptional regulator in the absence of its target genetic region supports the idea
312 that certain strains of enterococci have undergone genome reduction as an evolutionary strategy

313 to adapt to unique host and non-host environments. It is possible that in *E. faecalis* V583, the
314 OG1RF_11099 homolog (EF1328) has been retained to regulate other genes within the regulon
315 that are less dispensable than T7SS.

316 Antibacterial properties of T7SS substrates have been demonstrated (26, 27). Here we
317 provide evidence that the expression of an immunity gene *OG1RF_11122* in T7SS targeted *E.*
318 *faecalis* cells confers protection from inhibition by the upstream LXG toxin encoded by
319 *OG1RF_11121*. Aside from its LXG domain, OG1RF_11121 does not harbor any other
320 recognizable protein domains, hence the mechanism underlying its toxicity is unclear. Whitney *et*
321 *al.* demonstrated that LXG toxin antagonism is contact–dependent, having minimal to no impact
322 on target cells in liquid media (27). Although we found that physical engagement is crucial for *E.*
323 *faecalis* T7SS mediated antagonism, we observed a significant reduction in target cell growth in
324 liquid media both during phage and daptomycin treatment of T7SS proficient *E. faecalis*.
325 Together, these data suggest that heightened T7SS transcription upon phage exposure
326 compared to daptomycin exposure may account for robust *E. faecalis* OG1RF inhibition of other
327 bacteria in liquid culture.

328 Enterococci occupy polymicrobial infections often interacting with other bacteria (71-74).
329 Although commensal *E. faecalis* antagonize virulent *S. aureus* through the production of
330 superoxide (75), the two species also exhibit growth synergy via exchange of critical nutrients
331 (76). Here, we show that phage treatment of *E. faecalis* OG1RF can indirectly impact the growth
332 of neighboring phage-resistant bacteria, including *S. aureus*, in a T7SS–dependent manner,
333 suggesting that phage therapy directed against enterococci driving T7SS activity could be useful
334 for the treatment of polymicrobial infections. However, the counter argument is that phage therapy
335 directed against enterococci could push a bacterial community toward dysbiosis, as phage
336 induced T7SS activity could directly inhibit beneficial bystander bacteria. This raises questions
337 about the consequences of phage mediated off-target effects on bacteria. Could phage induced
338 T7SS activity be used to reduce phage expansion into other closely related strains as a means to

339 dilute phages out of a population, or is it simply that phage induction of the T7SS serves as a
340 mechanism that benefits a select few within a population to aid in their reoccupation of a niche
341 upon overcoming phage infection? Future studies aimed at exploring enterococcal T7SS
342 antagonism in polymicrobial communities should help elucidate the impact of phages on microbial
343 community composition.
344
345 **Materials and Methods**
346 **Bacteria and bacteriophage.** Bacteria and phages used in this study are listed in Table S1.
347 Bacteria were grown with aeration in Todd-Hewitt broth (THB) or on THB agar supplemented with
348 10mM $MgSO_4$ at 37°C. The following antibiotic concentrations were added to media for the
349 selection of specific bacterial strains or species: *E. faecalis* OG1RF (25 µg/ml fusidic acid, 50
350 µg/ml rifampin), *E. faecalis* V583 Δpip_{V583} (100 µg/ml gentamycin), *S. aureus* AH2146 LAC
351 Φ11:LL29 (1 µg/ml tetracyclin), *L. monocytogenes* 10403S (100 µg/ml streptomycin), *V. cholerae*
352 C6706 int I4::TnFL63 and *S. enterica* serovar Typhimurium 140285 put::Kan (50 µg/ml
353 kanamycin). *S. agalactiae* COH1 was distinguished from *E. faecalis* on Chrome indicator Agar
354 (CHROMagar StrepB SB282). We were unable to differentially select *E. coli, S. pyogenes* and *S.*
355 *mitis* from *E. faecalis* based on antibiotic sensitivity. Therefore, colony counts of these bacteria in
356 co-culture experiments were acquired by subtracting the *E. faecalis* colony numbers on selective
357 media from total number of colonies on non-selective media. Strains harboring pLZ12A and its
358 derivatives were grown in the presence of 20 µg/ml chloramphenicol.
359
360 **Bioinformatic analyses.** Genome sequences of *E. faecalis* V583 (NC_004668.1) and OG1RF
361 (NC_017316.1) were obtained from NCBI. Alignments were generated and visualized using
362 EasyFig (77). OG1RF protein domains were identified using KEGG (78) and ExPASy PROSITE
363 (79). Structure modeling of OG1RF_12414 was done with Phyre2 (80). The EsxA phylogenetic
364 tree was constructed in MEGA version X (81) using non-redundant protein sequences obtained

365 from NCBI BLAST (82) with OG1RF_11100 as input and was edited using the Interactive Tree Of
366 Life browser (83). OG1RF_11109 was used as an input for the NCBI Conserved Domain
367 Architecture Retrieval Tool (84) to identify protein domains that co-occur with LXG domains in
368 *Enterococcus*.
369
370 **Antibiotic sensitivity profiles.** Antibiotic susceptibility profiles for ampicillin, vancomycin, and
371 daptomycin were determined using a broth microdilution assay. Overnight (O/N) *E. faecalis*
372 OG1RF cultures were diluted to 5×10^6 CFU/ml and 100 µl was added to each well of a 96-well
373 plate to give a final cell density of 5×10^5 CFU/ml. Antibiotic stocks were added to the first column
374 of each row, mixed thoroughly, and were serially diluted 2-fold across the rows. The last column
375 was used as a no drug control. Cultures containing daptomycin were supplemented with 50 µg/ml
376 $CaCl_2$. Bacterial growth was monitored by measuring absorbance (OD_{600}) using a Synergy H1
377 microplate reader set to 37°C with continuous shaking O/N. Growth curves are presented as the
378 average of three biological replicates. A concentration of antibiotic just below the drug amount
379 that inhibits bacterial growth was deemed sub-lethal and used to examine T7SS genes
380 expression.
381
382 **Co-culture bacterial antagonism assays.** For inter- and intraspecies antagonism assays in
383 liquid media, O/N cultures of different bacteria were diluted in THB containing 10mM $MgSO_4$ to
384 an OD_{600} of 0.2 and mixed together in a 1:1 or 10:1 ratio. The mixed cell suspensions were either
385 left untreated or treated with phage VPE25 (MOI 0.01) / daptomycin (2.5 µg/ml) and grown at
386 37°C with aeration. For antagonism experiments on agar plates, O/N cultures of different strains
387 were diluted to an OD_{600} of 0.2 and mixed together in a 1:1 or 10:1 ratio. A total of 10^7 cells from
388 mixed culture suspension was added to 5 ml THB + 0.35% agar at 55°C and were poured over
389 the surface of a THB agar plate in the absence or presence of daptomycin (0.5 µg/ml). The plates
390 were incubated at 37°C under static conditions for 24 hours. Cells were harvested by scraping off

391 the top agar, resuspending in 5 ml of PBS, and the cfus were obtained by plating serially diluted
392 cell suspension on appropriate selective or differentiating agar plates. Relative viability was
393 calculated from the ratio of target strain cfu in the treated versus the untreated co-culture. The
394 assays were performed in biological triplicates.
395
396 **RNA extraction and quantitative PCR.** RNA was extracted from phage treated or untreated *E.*
397 *faecalis* OG1RF cells by using an RNeasy Mini Kit (Qiagen) with the following published
398 modifications (24). cDNA was generated from 1 µg of RNA using qScript cDNA SuperMix
399 (QuantaBio) and transcript levels were analyzed by qPCR using PowerUp™ SYBR Green Master
400 Mix (Applied Biosystems). Transcript abundances were normalized to the 16S rRNA gene
401 transcripts and fold–change was calculated by comparing to untreated controls. All data are
402 represented as the average of three biological replicates.
403
404 **Bacterial growth curves.** 25 ml of 10mM $MgSO_4$ supplemented THB was inoculated with O/N
405 cultures of *E. faecalis* diluted to an OD_{600} of 0.025 and distributed to a 96-well plate in 0.1 ml
406 volumes. Cultures were incubated at 37° C with aeration. OD_{600} was measured periodically for 18
407 hours in a Synergy H1 microplate reader.
408
409 **Efficiency of plating (EOP) assays.** To investigate if phage VPE25 can infect and lyse *E.*
410 *faecalis* mutants and various other bacterial species, 10^7 PFU/ml of phage was serially diluted
411 and the phage was titered on each strain using a THB agar overlay plaque assay. EOP is
412 expressed as the percentage of phage titer from each strain relative to the wild type *E. faecalis*
413 OG1RF control. Data are presented as the average of three biological replicates.
414
415 **Construction of *E. faecalis* mutants and complementation.** Isolation of *E. faecalis* genomic
416 DNA was performed using a ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research). All PCR

417 reactions used for cloning were performed with high fidelity KOD Hot Start DNA Polymerase (EMD
418 Millipore). *E. faecalis* Δ*essB* was generated by allelic replacement by cloning an in frame *essB*
419 deletion product into pLT06 using Gibson Assembly® Master Mix (New England Biolabs),
420 integrating this construct into the chromosome, and resolving the deletion mutant by homologous
421 recombination (85-87). For ectopic expression of putative immunity proteins, coding regions of
422 OG1RF_11110, OG1RF_11112, and OG1RF_11122 were cloned downstream of the *bacA*
423 promoter (P_{bacA}) by restriction digestion and ligation into the shuttle vector pLZ12A (20). Primer
424 sequences and restriction enzymes used for cloning are listed in Table S1. Plasmids were
425 introduced into electrocompetent *E. faecalis* cells as previously described (20).
426
427 **Statistical analysis.** Statistical tests were performed using GraphPad – Prism version 8.2.1. For
428 qPCR and bacterial competition assays, unpaired Student's t-tests were used. *P* values are
429 indicated in the figure legends.
430
431 **References**

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635

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642

643 **Author Contributions**

644 A.C., J.L.E.W., G.M.D. and B.A.D. designed the study. A.C. and J.L.E.W. perfromed experiemtns
645 and bioinformatic analyses. A.C., J.L.E.W., G.M.D. and B.A.D. analyzed data. A.C., J.L.E.W. and
646 B.A.D. wrote the paper with input from G.M.D.

647

648 **Corresponding author**

649 Address coorespondence to Breck A. Duerkop breck.duerkop@cuanschutz.edu

650

651 **Competing Interests**

652 There are no competing interests to report for this work.

653

654

655 **Figure Legends**

656

657 **Figure 1. Phage mediated inhibition of bystander bacteria is dependent on enterococcal**
658 **T7SS. (A)** Diagram showing the location of T7SS genes in *E. faecalis* OG1RF (NC_017316.1)
659 compared to *E. faecalis* V583 (NC_004668.1). Sequences were obtained from NCBI, and
660 homology comparisons were rendered in EasyFig. Nucleotide alignments generated by Clustal
661 Omega are enlarged for clarity (dashed lines). Stop codons of genes EF1328/OG1RF_11099 and
662 EF1337/OG1RF_11127 are boxed. **(B)** Schematic representation of the co-culture assay used to
663 assess the viability of bystander bacteria during phage induced T7SS activity of wild type *E.*
664 *faecalis* OG1RF and Δ*essB*. Relative viability of bystander strains is calculated by measuring the
665 ratio of bystander cfus in the phage infected culture compared to the bystander cfus from an
666 uninfected control culture. **(C)** The relative abundance of viable bystander bacterium *E. faecalis*
667 Δpip_{V583}. Complementation of the *E. faecalis* Δ*essB* mutant restores T7SS dependent bystander
668 inhibition**. (D)** T7SS inhibition of other bacterial species in the presence and absence of phage
669 infected *E. faecalis* OG1RF or Δ*essB*. Data represent three biological replicates. $^*P < 0.00001$ by
670 unpaired Student's t-test.

671

672 **Figure 2. Phage induced T7SS inhibitory activity of *E. faecalis* is contact dependent.** Intra-
673 species competition experiment performed in the presence of unfiltered supernatant from phage
674 treated and untreated *E. faecalis* wild type OG1RF or Δ*essB* added **(A)** to the top of a well
675 separated by a 0.4 µm membrane from the bottom well containing *E. faecalis* Δpip_{V583} culture or
676 **(B)** directly into *E. faecalis* Δpip_{V583} culture in microtiter plate wells.

677

678 **Figure 3. Identification of *E. faecalis* T7SS toxin and immunity proteins that dictate**
679 **bystander growth inhibition bacteria. (A)** LXG domains in OG1RF_11109 and OG1RF_11121
680 were identified using KEGG and ExPASy PROSITE. Other putative toxic effectors in OG1RF

681 were identified by homology to OG1RF_11109 or OG1RF_11121. Homology diagrams were
682 rendered in EasyFig. **(B)** *E. faecalis* OG1RF T7SS mediated growth inhibition of phage resistant
683 *E. faecalis* Δ*pip*$_{V583}$ during infection is alleviated by expressing OG1RF_11122 in *E. faecalis*
684 Δ*pip*$_{V583}$ but not in the presence of empty vector, or expressing OG1RF_11110, or OG1RF_11112.
685 E, represents empty vector. C, is expression vector. Disruption of OG1RF_11121 by a transposon
686 insertion rescues growth of phage resistant **(C)** *E. faecalis* Δ*pip*$_{V583}$ and **(D)** *S. aureus* strains
687 during co-culture. Data represent three biological replicates. *$P < 0.0001$ by unpaired Student's t-
688 test.
689
690 **Figure 4. Sub-lethal antibiotic treatment enhances T7SS gene expression leading to**
691 **inhibition of bystander bacteria.** Altered expression of T7SS genes upon exposure to sub-
692 inhibitory concentrations of **(A)** ampicillin (0.19 µg/ml), vancomycin (0.78 µg/ml) or daptomycin
693 (6.25 µg/ml) and **(B)** ciprofloxacin (2 µg/ml) or mitomycin C (4 µg/ml) for 40 minutes relative to
694 the untreated control. **(C-E)** Contact–dependent T7SS mediated inhibition of bystander bacteria
695 in the presence of daptomycin. Relative viability of *E. faecalis* Δ*pip*$_{V583}$ was measured during co-
696 culture with *E. faecalis* OG1RF or Δ*essB* antagonists in the presence and absence of daptomycin
697 treatment in **(C)** liquid culture (2.5 µg/ml), **(D)** trans-well plates to prevent physical engagement
698 between cells (2.5 µg/ml) and **(E)** on agar media (0.5 µg/ml). E – empty vector and C –
699 complementation vector. Data represent three biological replicates. *$P < 0.01$, **$P < 0.001$ to
700 0.0001 by unpaired Student's t-test.
701
702 **Figure 5. IreK and OG1RF_11099 control transcription of enterococcal T7SS genes and**
703 **subsequent inhibition of bystander bacteria during phage infection. (A)** Phage infection
704 leads to enhanced expression of T7SS genes in the wild type *E. faecalis* OG1RF but not in an
705 Δ*ireK* mutant strain. **(B)** Growth inhibition of *E. faecalis* Δ*pip*$_{V583}$ during phage infection of *E.*
706 *faecalis* OG1RF is abrogated in an Δ*ireK* mutant of OG1RF. **(C)** Disruption of OG1RF_11099

707 leads to reduced expression of T7SS genes during phage infection. The data are represented as
708 the fold change of normalized mRNA relative to uninfected samples at the same time points. **(D)**
709 T7SS dependent intraspecies antagonism during phage infection is alleviated in the presence of
710 OG1RF_11099-Tn mutant. Data represent three replicates *$P < 0.00001$ by unpaired Student's t-
711 test.
712
713 **Figure S1. Phylogenetic tree of EsxA sequences in *Enterococcus*.** Non-redundant
714 sequences (n=96) were identified using NCBI BLAST with OG1RF EsxA (OG1RF_11100) as the
715 input. The tree was constructed in MEGAX using the Maximum Likelihood method and JTT
716 matrix-based model and is drawn to scale, with branch lengths measured in the number of
717 substitutions per site. The tree with the highest log likelihood (-3544.39) is shown. *E. faecalis*
718 sequences are highlighted in purple, and the GenBank identifier for EsxA from OG1RF
719 (AEA93787.1) is shown in bold red font.
720
721 **Figure S2. Phage VPE25 infects wild type *E. faecalis* OG1RF and Δ*essB* with similar**
722 **efficacy.** The measurement of phage particles released from wild type *E. faeaclis* OG1RF, Δ*essB*,
723 OG1RF_11121-Tn, and OG1RF_11099-Tn mutant strains following phage VPE25 infection. Data
724 represent three biological replicates.
725
726 **Figure S3. Putative orphan toxins in OG1RF are found as full-length LXG-domain proteins**
727 **in other bacteria.** OG1RF_11111, OG1RF_11113, and OG1RF_11123 sequences were used
728 as input for NCBI BLAST. Alignments and homology were rendered in EasyFig.
729
730 **Figure S4. A distal OG1RF locus encodes an additional LXG-domain protein. (A)** Schematic
731 showing homology between V583 (NC_004668.1, top) and OG1RF (NC_017316.1, bottom).
732 Sequences were obtained from NCBI, and homology comparisons were rendered in EasyFig. **(B)**

733 Cartoon depicting the LXG domain of OG1RF_12414 (identified using KEGG and ExPASy
734 PROSITE). **(C)** Predicted structural homology between OG1RF_12414 (lilac) and the
735 *Pseudomonas protogens* Pf-5 Tne2/Tni2 complex (PDB 6B12). Tne2 is shown in green, and Tni2
736 is shown in gray. Structural modeling was done using PHYRE2, and images were rendered in
737 Pymol.
738
739 **Figure S5. Domain architecture of *Enterococcus* LXG proteins.** Domain architectures were
740 identified using the NCBI Conserved Domain Architectural Retrieval Tool (DART) with
741 OG1RF_11109 as an input. Diagrams are drawn to scale.
742
743 **Figure S6. Antibiotic susceptibility of *E. faecalis* OG1RF.** Growth of wild type *E. faecalis*
744 OG1RF was monitored over 18 hours in the presence or absence of **(A)** ampicillin, **(B)**
745 vancomycin and **(C)** daptomycin in microtiter plates. The antibiotic concentrations highlighted with
746 a blue box were deemed sub-inhibitory and used to investigate T7SS expression levels. Early
747 log-phase cultures of *E. faecalis* OG1RF were grown in the presence or absence of **(D)** mitomycin
748 C (4 µg/ml) or **(E)** ciprofloxacin (2 µg/ml) to show that even though these routine concentrations
749 used to promote a stress response in other *E. faecalis*, they do not prevent bacterial growth.
750
751 **Figure S7. *E. faecalis* mutants of cell wall homeostasis show no growth defects and**
752 **respond to phage VPE25 infection. (A)** Optical density of wild type E. *faecalis* and isogenic
753 mutants were monitored for 18 hours. **(B)** While all the strains were susceptible to phage VPE25
754 infection, the proportion of released phage particles was diminished in the *croR* and *croS*
755 transposon mutant background. Data represent three biological replicates. *$P < 0.001$ by unpaired
756 Student's t-test.
757

758 **Figure S8. Quantitative PCR demonstrates that LiaR/S and CroS/R two-component**
759 **systems do not influence T7SS gene expression during phage infection. (A- F)** mRNA
760 transcript levels of T7SS genes are enhanced in the transposon mutants of *liaR*, *liaS*, *croR* and
761 *croS* strains similar to wild type *E. faecalis* OG1RF during phage infection (MOI = 1) compared to
762 untreated controls. Data represent three biological replicates. *$P < 0.01$, **$P < 0.0001$ by unpaired
763 Student's t-test.
764
765 **Figure S9. Sub-lethal ceftriaxone exposure does not influence T7SS gene expression in *E.***
766 ***faecalis* OG1RF.** Transcription of T7SS genes are not elevated 20 minutes post ceftriaxone
767 (128µg/ml) administration relative to the untreated controls.
768
769 **Table S1. List of bacterial strains, phages, plasmids and primers used in this study.**
770

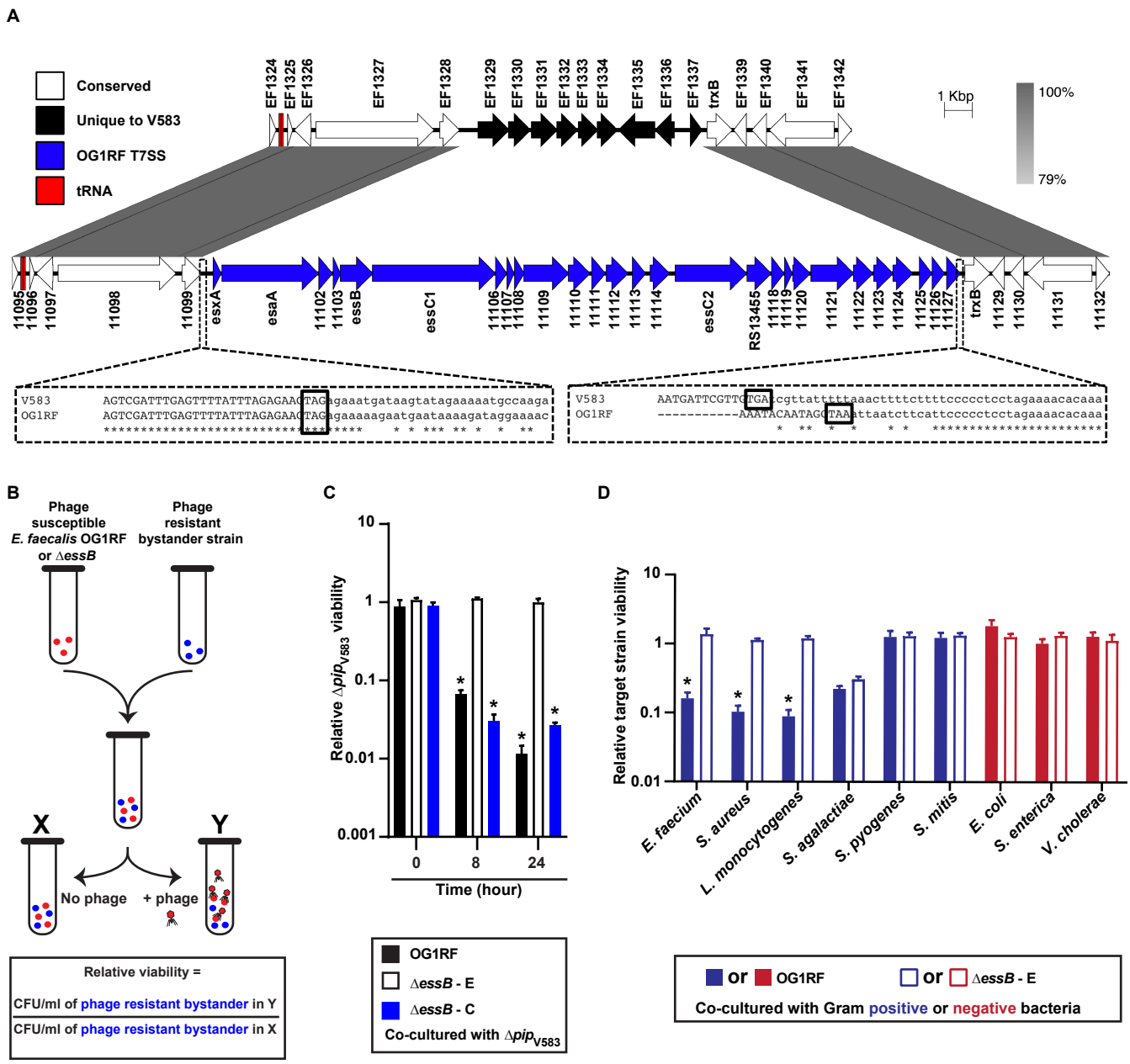

A
Conserved
Unique to V583
OG1RF T7SS
tRNA
EF1324
EF1325
EF1326
EF1327
EF1328
EF1329
EF1330
EF1331
EF1332
EF1333
EF1334
EF1335
EF1336
EF1337
trxB
EF1339
EF1340
EF1341
EF1342
1 Kbp
100%
79%
11095
11096
11097
11098
11099
esxA
esaA
11102
11103
essB
essC1
11106
11107
11108
11109
11110
11111
11112
11113
11114
essC2
RS13455
11118
11119
11120
11121
11122
11123
11124
11125
11126
11127
trxB
11129
11130
11131
11132
V583 AGTCGATTTGAGTTTTATTTAGAGAAGTAGagaaatgataagtatagaaaaatgccaaga
OG1RF AGTCGATTTGAGTTTTATTTAGAGAAGTAGagaaaaagaatgaataaaagataggaaaac
V583 AATGATTCGTTGTGAcgttattttaaacttttcttttcccccctcctagaaaacacaaa
OG1RF ----------AAATACAATAGGTAAttaatcttcattcccccctcctagaaaacacaaa
B
Phage susceptible E. faecalis OG1RF or ΔessB
Phage resistant bystander strain
X
Y
No phage
+ phage
Relative viability =
CFU/ml of phage resistant bystander in Y
CFU/ml of phage resistant bystander in X
C
Relative Δpip V583 viability
10
1
0.1
0.01
0.001
0
8
24
Time (hour)
OG1RF
ΔessB - E
ΔessB - C
Co-cultured with ΔpipV583
D
Relative target strain viability
10
1
0.1
0.01
E. faecium
S. aureus
L. monocytogenes
S. agalactiae
S. pyogenes
S. mitis
E. coli
S. enterica
V. cholerae
or OG1RF
or ΔessB - E
Co-cultured with Gram positive or negative bacteria

Fig. 1

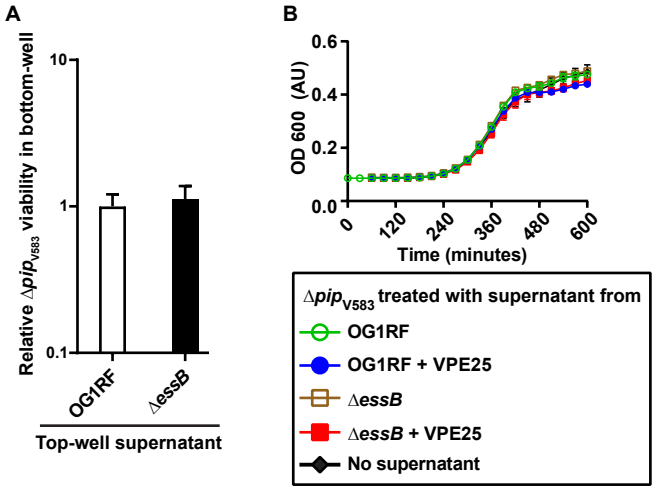

A
Relative Δpip$_{V583}$ viability in bottom-well
10
1
0.1
OG1RF
ΔessB
Top-well supernatant
B
OD 600 (AU)
0.6
0.4
0.2
0.0
0
120
240
360
480
600
Time (minutes)
Δpip$_{V583}$ treated with supernatant from
OG1RF
OG1RF + VPE25
ΔessB
ΔessB + VPE25
No supernatant

Fig. 2

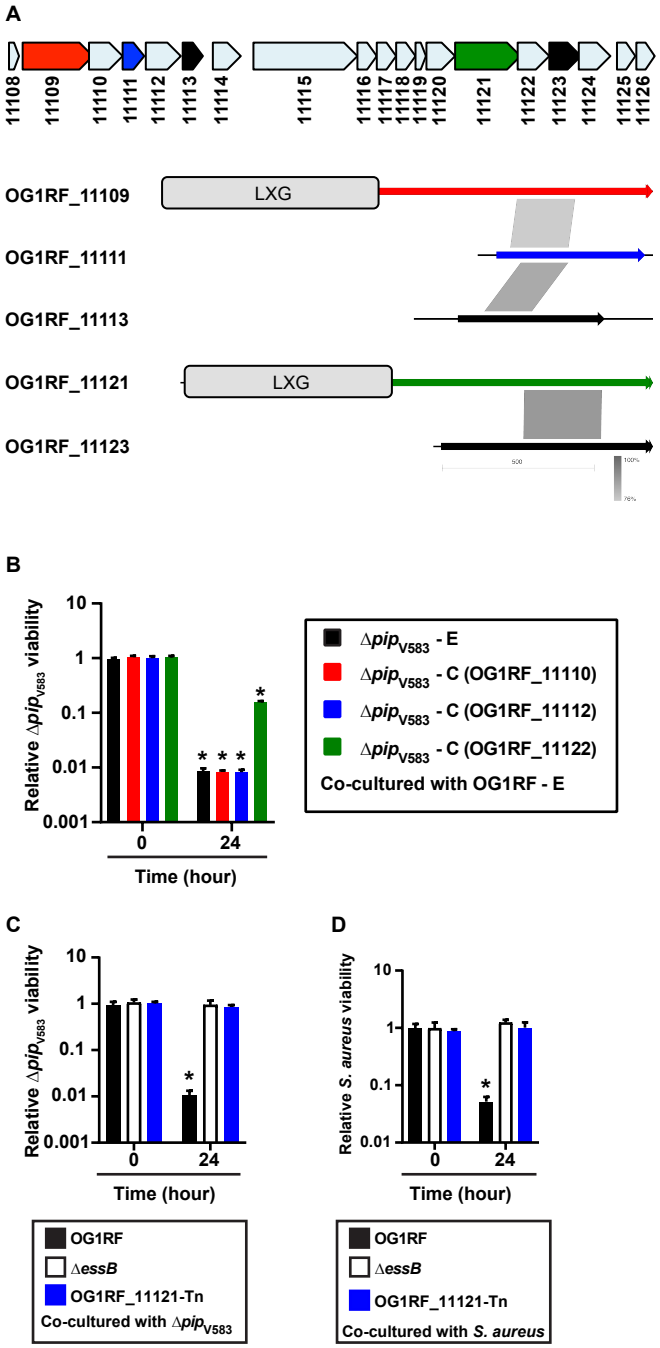

A
11108
11109
11110
11111
11112
11113
11114
11115
11116
11117
11118
11119
11120
11121
11122
11123
11124
11125
11126
OG1RF_11109
LXG
OG1RF_11111
OG1RF_11113
OG1RF_11121
LXG
OG1RF_11123
B
Relative Δpip V583 viability
10
1
0.1
0.01
0.001
0
24
Time (hour)
Δpip V583 - E
Δpip V583 - C (OG1RF_11110)
Δpip V583 - C (OG1RF_11112)
Δpip V583 - C (OG1RF_11122)
Co-cultured with OG1RF - E
C
Relative Δpip V583 viability
10
1
0.1
0.01
0.001
0
24
Time (hour)
OG1RF
ΔessB
OG1RF_11121-Tn
Co-cultured with Δpip V583
D
Relative S. aureus viability
10
1
0.1
0.01
0
24
Time (hour)
OG1RF
ΔessB
OG1RF_11121-Tn
Co-cultured with S. aureus

Fig. 3

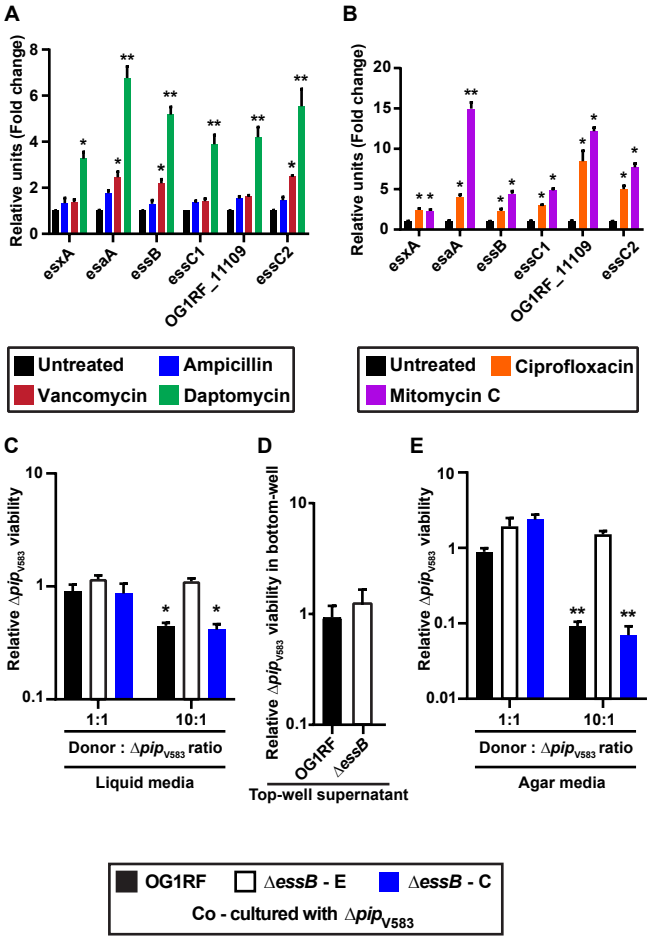

A
Relative units (Fold change)
esxA
esaA
essB
essC1
OG1RF_11109
essC2
Untreated
Ampicillin
Vancomycin
Daptomycin
B
Relative units (Fold change)
Untreated
Ciprofloxacin
Mitomycin C
C
Relative ΔpipV583 viability
1:1
10:1
Donor : ΔpipV583 ratio
Liquid media
D
Relative ΔpipV583 viability in bottom-well
OG1RF
ΔessB
Top-well supernatant
E
Relative ΔpipV583 viability
1:1
10:1
Donor : ΔpipV583 ratio
Agar media
OG1RF
ΔessB - E
ΔessB - C
Co - cultured with ΔpipV583

Fig. 4

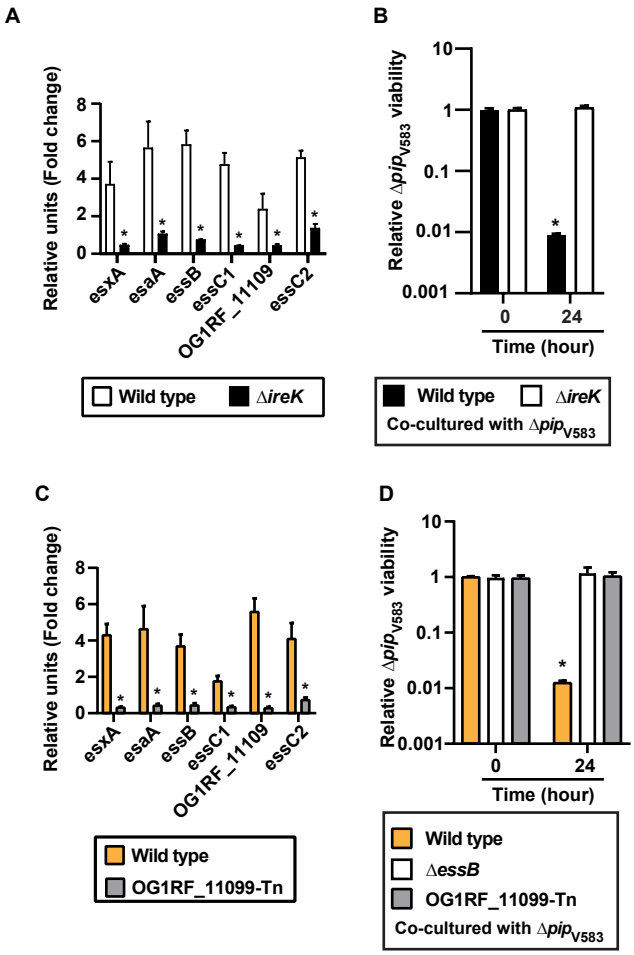
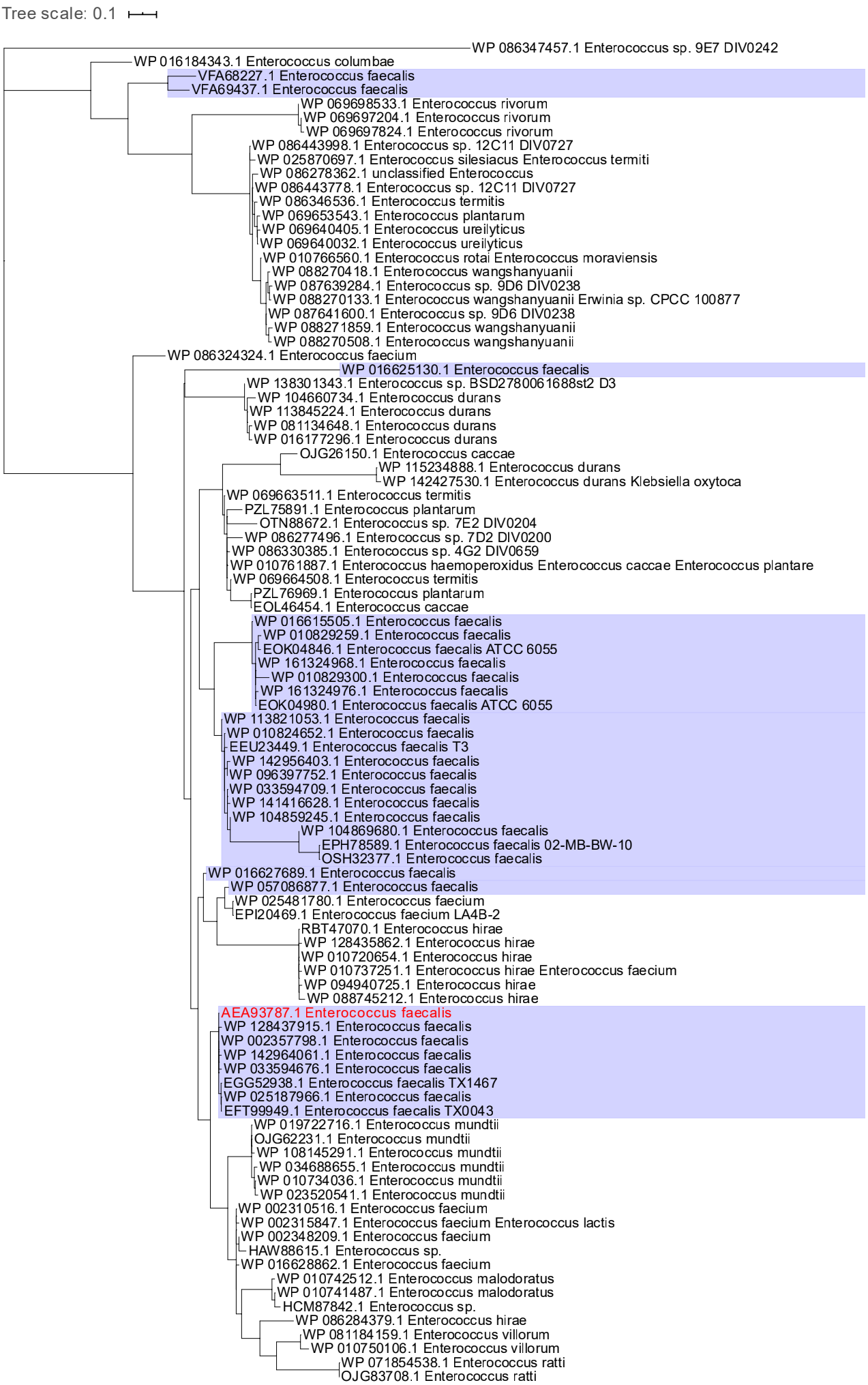

A
Relative units (Fold change)
8
6
4
2
0
esxA
esaA
essB
essC1
OG1RF_11109
essC2
Wild type
ΔireK
B
Relative Δpip V583 viability
10
1
0.1
0.01
0.001
0
24
Time (hour)
Wild type
ΔireK
Co-cultured with Δpip V583
C
Relative units (Fold change)
8
6
4
2
0
esxA
esaA
essB
essC1
OG1RF_11109
essC2
Wild type
OG1RF_11099-Tn
D
Relative Δpip V583 viability
10
1
0.1
0.01
0.001
0
24
Time (hour)
Wild type
ΔessB
OG1RF_11099-Tn
Co-cultured with Δpip V583

Fig. 5


Tree scale: 0.1
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WP 016184343.1 Enterococcus columbae
VFA68227.1 Enterococcus faecalis
VFA69437.1 Enterococcus faecalis
WP 069698533.1 Enterococcus rivorum
WP 069697204.1 Enterococcus rivorum
WP 069697824.1 Enterococcus rivorum
WP 086443998.1 Enterococcus sp. 12C11 DIV0727
WP 025870697.1 Enterococcus silesiacus Enterococcus termiti
WP 086278362.1 unclassified Enterococcus
WP 086443778.1 Enterococcus sp. 12C11 DIV0727
WP 086346536.1 Enterococcus termitis
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WP 069640405.1 Enterococcus ureilyticus
WP 069640032.1 Enterococcus ureilyticus
WP 010766560.1 Enterococcus rotai Enterococcus moraviensis
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WP 088270508.1 Enterococcus wangshanyuanii
WP 086324324.1 Enterococcus faecium
WP 016625130.1 Enterococcus faecalis
WP 138301343.1 Enterococcus sp. BSD2780061688st2 D3
WP 104660734.1 Enterococcus durans
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WP 081134648.1 Enterococcus durans
WP 016177296.1 Enterococcus durans
OJG26150.1 Enterococcus caccae
WP 115234888.1 Enterococcus durans
WP 142427530.1 Enterococcus durans Klebsiella oxytoca
WP 069663511.1 Enterococcus termitis
PZL75891.1 Enterococcus plantarum
OTN88672.1 Enterococcus sp. 7E2 DIV0204
WP 086277496.1 Enterococcus sp. 7D2 DIV0200
WP 086330385.1 Enterococcus sp. 4G2 DIV0659
WP 010761887.1 Enterococcus haemoperoxidus Enterococcus caccae Enterococcus plantare
WP 069664508.1 Enterococcus termitis
PZL76969.1 Enterococcus plantarum
EOL46454.1 Enterococcus caccae
WP 016615505.1 Enterococcus faecalis
WP 010829259.1 Enterococcus faecalis
EOK04846.1 Enterococcus faecalis ATCC 6055
WP 161324968.1 Enterococcus faecalis
WP 010829300.1 Enterococcus faecalis
WP 161324976.1 Enterococcus faecalis
EOK04980.1 Enterococcus faecalis ATCC 6055
WP 113821053.1 Enterococcus faecalis
WP 010824652.1 Enterococcus faecalis
EEU23449.1 Enterococcus faecalis T3
WP 142956403.1 Enterococcus faecalis
WP 096397752.1 Enterococcus faecalis
WP 033594709.1 Enterococcus faecalis
WP 141416628.1 Enterococcus faecalis
WP 104859245.1 Enterococcus faecalis
WP 104869680.1 Enterococcus faecalis
EPH78589.1 Enterococcus faecalis 02-MB-BW-10
OSH32377.1 Enterococcus faecalis
WP 016627689.1 Enterococcus faecalis
WP 057086877.1 Enterococcus faecalis
WP 025481780.1 Enterococcus faecium
EPI20469.1 Enterococcus faecium LA4B-2
RBT47070.1 Enterococcus hirae
WP 128435862.1 Enterococcus hirae
WP 010720654.1 Enterococcus hirae
WP 010737251.1 Enterococcus hirae Enterococcus faecium
WP 094940725.1 Enterococcus hirae
WP 088745212.1 Enterococcus hirae
AEA93787.1 Enterococcus faecalis
WP 128437915.1 Enterococcus faecalis
WP 002357798.1 Enterococcus faecalis
WP 142964061.1 Enterococcus faecalis
WP 033594676.1 Enterococcus faecalis
EGG52938.1 Enterococcus faecalis TX1467
WP 025187966.1 Enterococcus faecalis
EFT99949.1 Enterococcus faecalis TX0043
WP 019722716.1 Enterococcus mundtii
OJG62231.1 Enterococcus mundtii
WP 108145291.1 Enterococcus mundtii
WP 034688655.1 Enterococcus mundtii
WP 010734036.1 Enterococcus mundtii
WP 023520541.1 Enterococcus mundtii
WP 002310516.1 Enterococcus faecium
WP 002315847.1 Enterococcus faecium Enterococcus lactis
WP 002348209.1 Enterococcus faecium
HAW88615.1 Enterococcus sp.
WP 016628862.1 Enterococcus faecium
WP 010742512.1 Enterococcus malodoratus
WP 010741487.1 Enterococcus malodoratus
HCM87842.1 Enterococcus sp.
WP 086284379.1 Enterococcus hirae
WP 081184159.1 Enterococcus villorum
WP 010750106.1 Enterococcus villorum
WP 071854538.1 Enterococcus ratti
OJG83708.1 Enterococcus ratti

Fig. S1

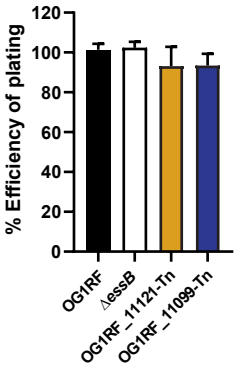
% Efficiency of plating
120
100
80
60
40
20
0
OG1RF
ΔessB
OG1RF_11121-Tn
OG1RF_11099-Tn

Fig. S2

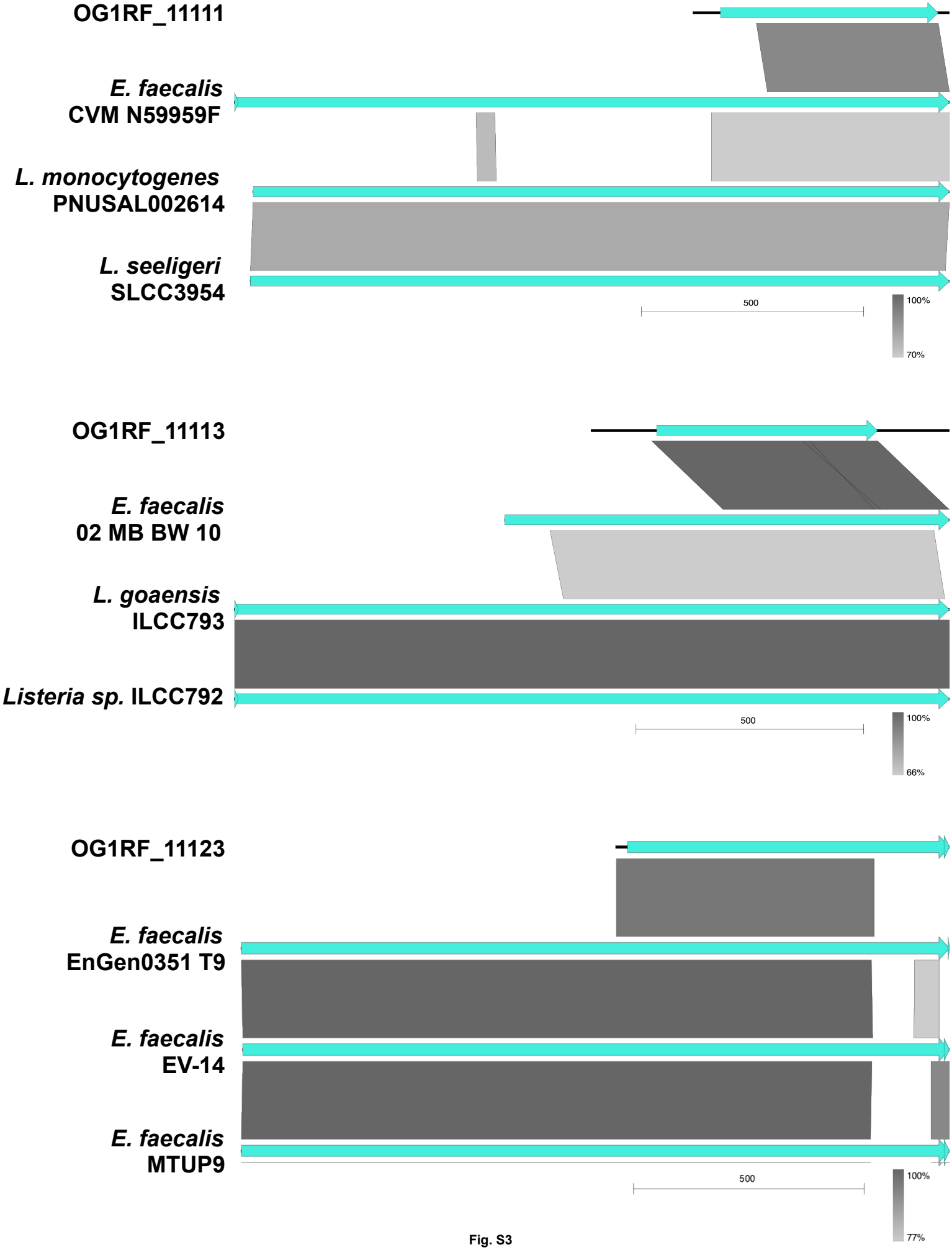
OG1RF_11111
E. faecalis
CVM N59959F
L. monocytogenes
PNUSAL002614
L. seeligeri
SLCC3954
500
100%
70%
OG1RF_11113
E. faecalis
02 MB BW 10
L. goaensis
ILCC793
Listeria sp. ILCC792
500
100%
66%
OG1RF_11123
E. faecalis
EnGen0351 T9
E. faecalis
EV-14
E. faecalis
MTUP9
500
100%
77%

Fig. S3

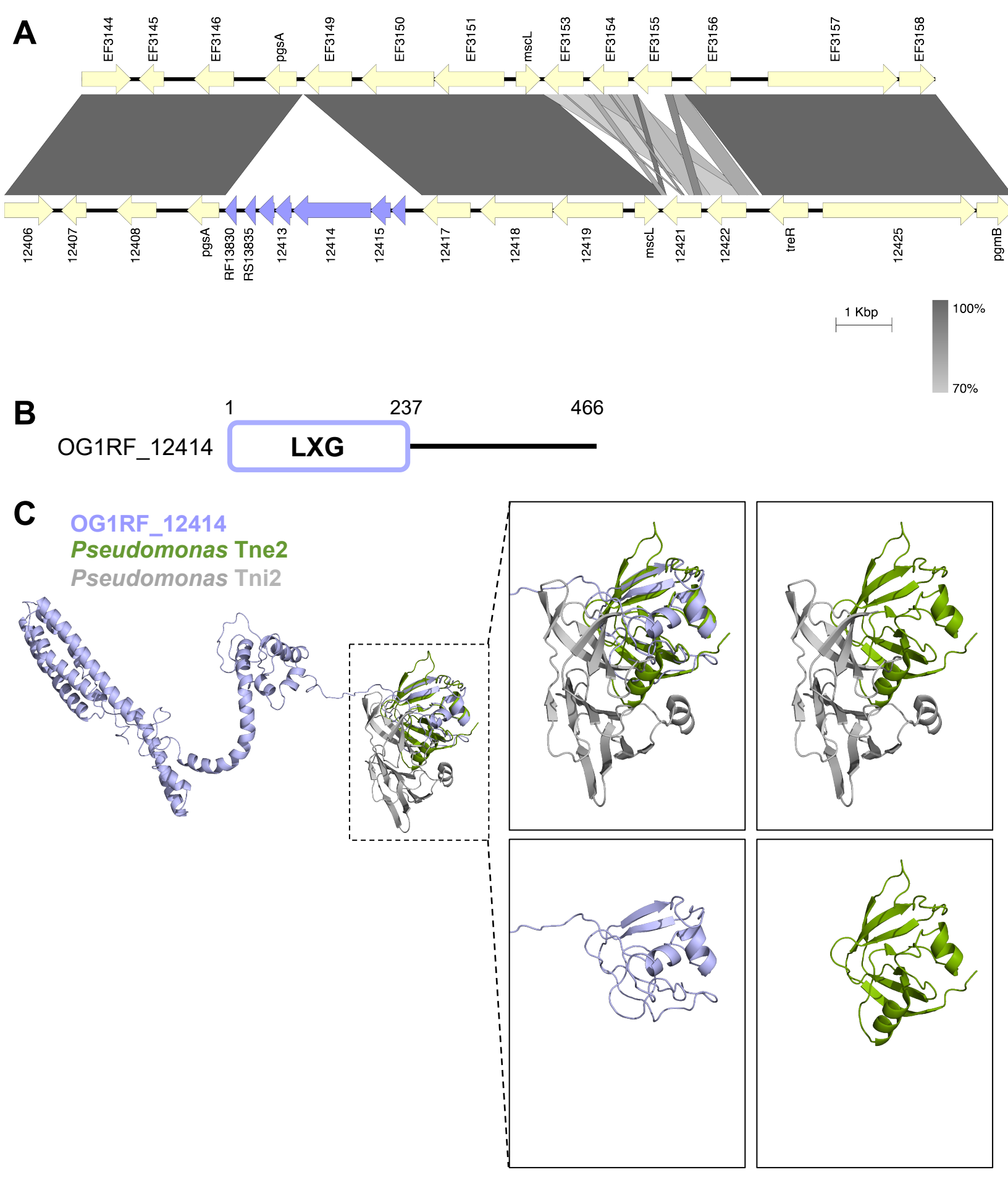
A
EF3144
EF3145
EF3146
pgsA
EF3149
EF3150
EF3151
mscL
EF3153
EF3154
EF3155
EF3156
EF3157
EF3158
12406
12407
12408
pgsA
RF13830
RS13835
12413
12414
12415
12417
12418
12419
mscL
12421
12422
treR
12425
pgmB
1 Kbp
100%
70%
B
1
237
466
OG1RF_12414
LXG
C
OG1RF_12414
Pseudomonas Tne2
Pseudomonas Tni2

Fig. S4

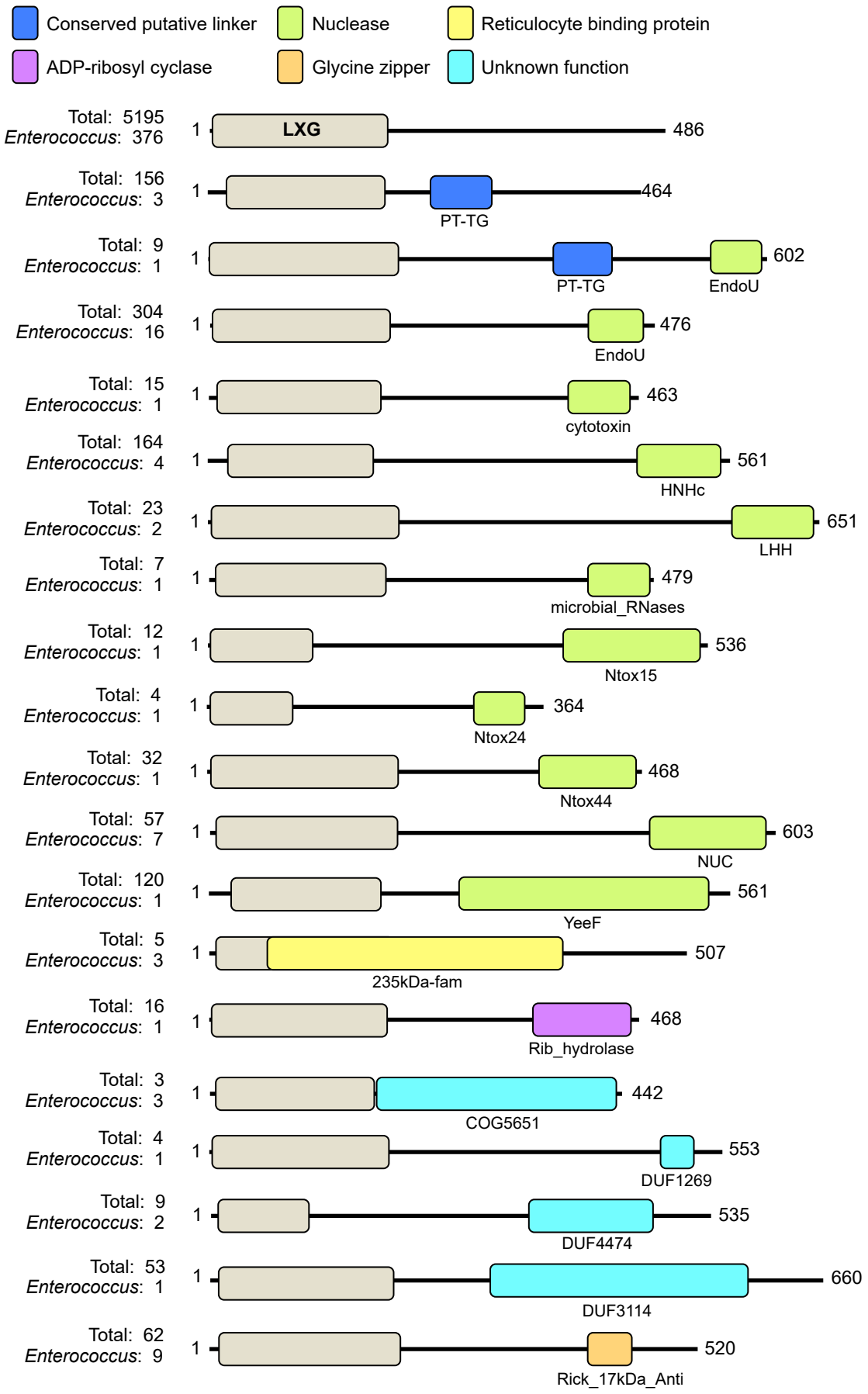
Conserved putative linker
Nuclease
Reticulocyte binding protein
ADP-ribosyl cyclase
Glycine zipper
Unknown function
Total: 5195
Enterococcus: 376
LXG
486
Total: 156
Enterococcus: 3
PT-TG
464
Total: 9
Enterococcus: 1
PT-TG
EndoU
602
Total: 304
Enterococcus: 16
EndoU
476
Total: 15
Enterococcus: 1
cytotoxin
463
Total: 164
Enterococcus: 4
HNHc
561
Total: 23
Enterococcus: 2
LHH
651
Total: 7
Enterococcus: 1
microbial_RNases
479
Total: 12
Enterococcus: 1
Ntox15
536
Total: 4
Enterococcus: 1
Ntox24
364
Total: 32
Enterococcus: 1
Ntox44
468
Total: 57
Enterococcus: 7
NUC
603
Total: 120
Enterococcus: 1
YeeF
561
Total: 5
Enterococcus: 3
235kDa-fam
507
Total: 16
Enterococcus: 1
Rib_hydrolase
468
Total: 3
Enterococcus: 3
COG5651
442
Total: 4
Enterococcus: 1
DUF1269
553
Total: 9
Enterococcus: 2
DUF4474
535
Total: 53
Enterococcus: 1
DUF3114
660
Total: 62
Enterococcus: 9
Rick_17kDa_Anti
520

Fig. S5

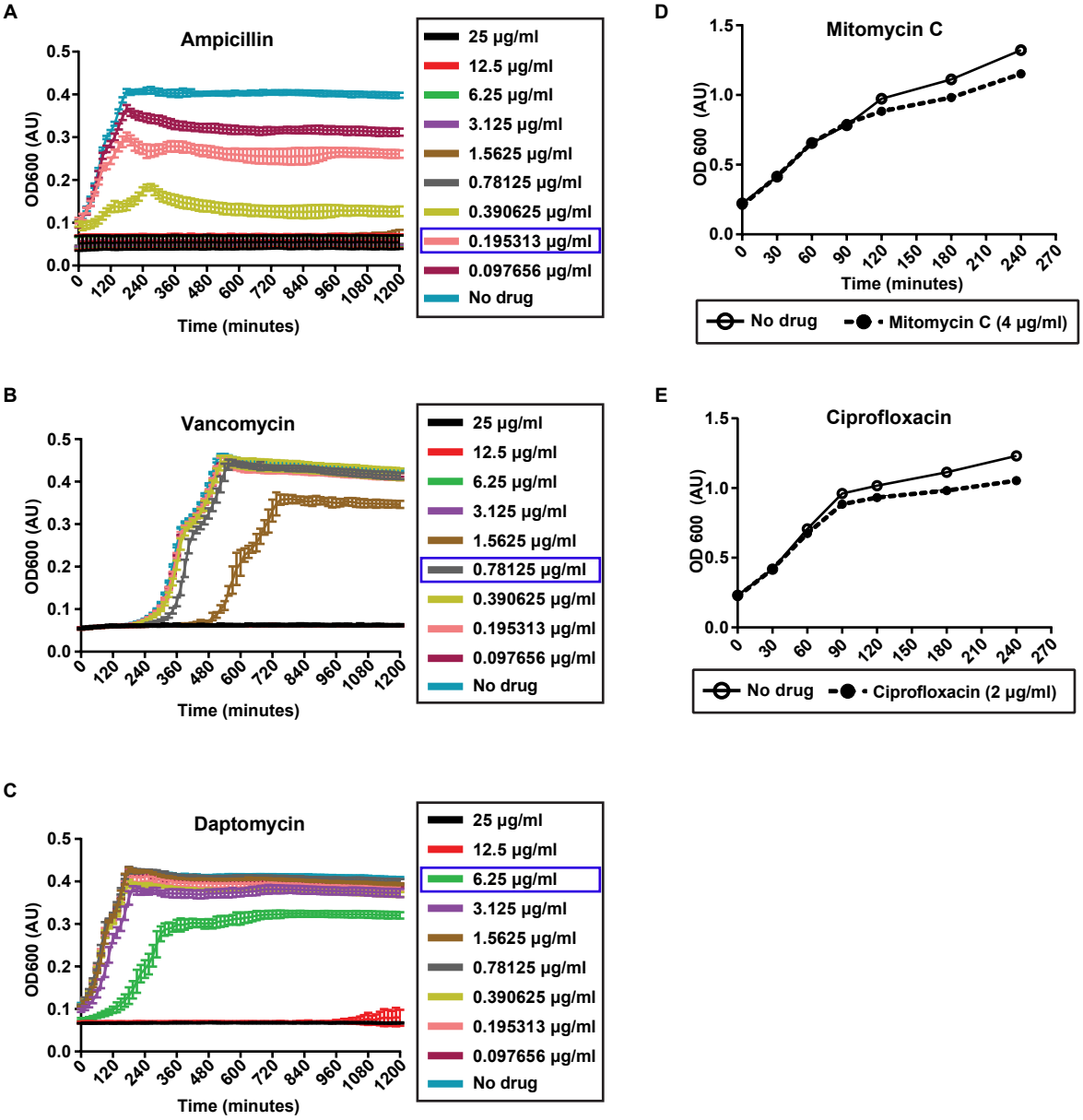
A
Ampicillin
OD600 (AU)
Time (minutes)
25 µg/ml
12.5 µg/ml
6.25 µg/ml
3.125 µg/ml
1.5625 µg/ml
0.78125 µg/ml
0.390625 µg/ml
0.195313 µg/ml
0.097656 µg/ml
No drug
B
Vancomycin
OD600 (AU)
Time (minutes)
25 µg/ml
12.5 µg/ml
6.25 µg/ml
3.125 µg/ml
1.5625 µg/ml
0.78125 µg/ml
0.390625 µg/ml
0.195313 µg/ml
0.097656 µg/ml
No drug
C
Daptomycin
OD600 (AU)
Time (minutes)
25 µg/ml
12.5 µg/ml
6.25 µg/ml
3.125 µg/ml
1.5625 µg/ml
0.78125 µg/ml
0.390625 µg/ml
0.195313 µg/ml
0.097656 µg/ml
No drug
D
Mitomycin C
OD 600 (AU)
Time (minutes)
No drug
Mitomycin C (4 µg/ml)
E
Ciprofloxacin
OD 600 (AU)
No drug
Ciprofloxacin (2 µg/ml)

Fig. S6

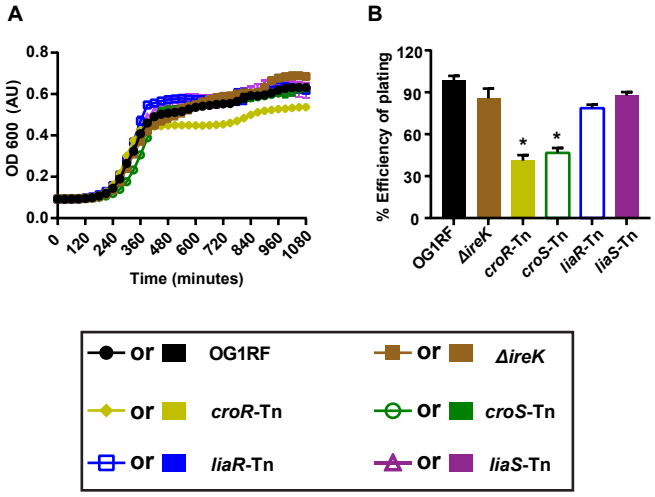
A
B
OD 600 (AU)
0.8
0.6
0.4
0.2
0.0
0
120
240
360
480
600
720
840
960
1080
Time (minutes)
% Efficiency of plating
120
90
60
30
0
*
*
OG1RF
ΔireK
croR-Tn
croS-Tn
liaR-Tn
liaS-Tn
or OG1RF
or ΔireK
or croR-Tn
or croS-Tn
or liaR-Tn
or liaS-Tn

Fig. S7

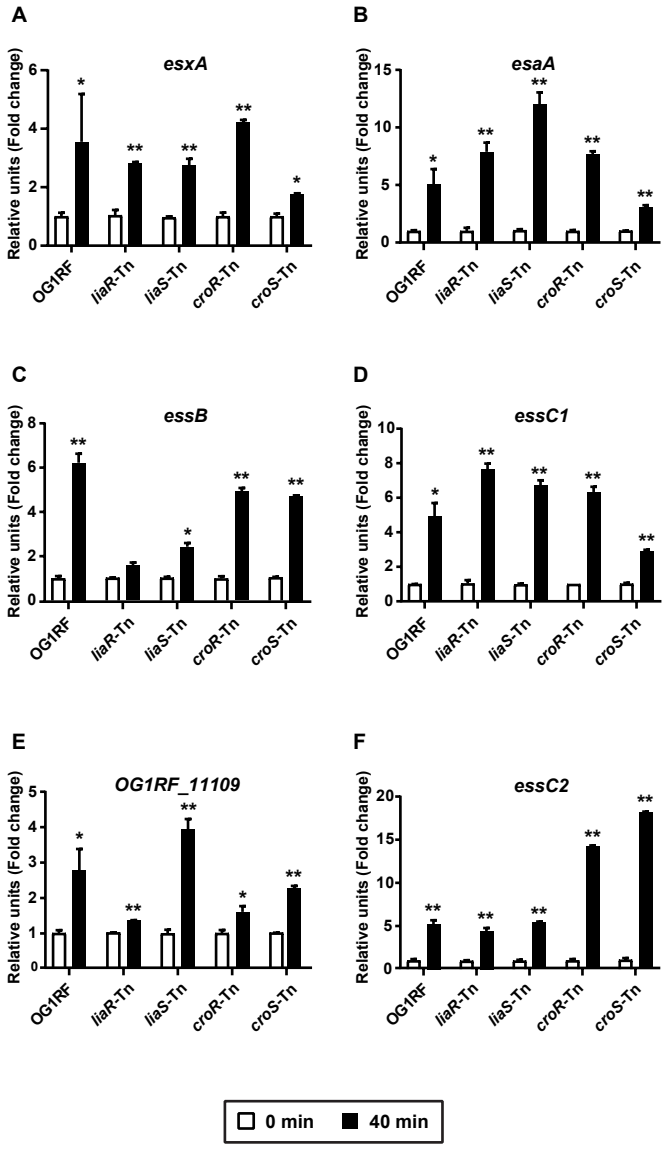
A
esxA
Relative units (Fold change)
OG1RF
liaR-Tn
liaS-Tn
croR-Tn
croS-Tn
B
esaA
C
essB
D
essC1
E
OG1RF_11109
F
essC2
0 min
40 min

Fig. S8

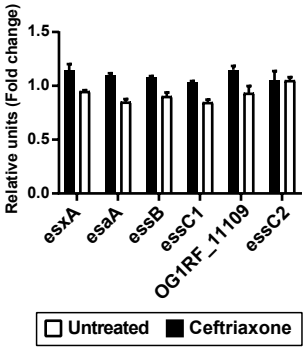
Relative units (Fold change)
1.5
1.0
0.5
0.0
esxA
esaA
essB
essC1
OG1RF_11109
essC2
Untreated
Ceftriaxone

Fig. S9