Phage infection mediates inhibition of bystander bacteria

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Abstract

Bacteriophages (phages) are being considered as potential alternative therapeutics for the treatment of multidrug resistant bacterial infections. Considering most phages have a narrow host-range, the generally accepted dogma is that therapeutic phages will have a marginal impact on bacterial strains outside of their intended target bacterium. We have discovered that lytic phage infection induces transcription of type VIIb secretion system (T7SS) genes in the pathobiont *Enterococcus faecalis*. Phage induction of T7SS genes mediates cell contact dependent antagonism of diverse Gram positive bystander bacteria. This phage induced T7SS antagonism is attributed to cell membrane damage. Phage driven T7SS antagonism of neighboring cells is abrogated by deleting *essB*, a T7SS structural component that is required for secretion of T7SS toxic substrates. Expression of a predicted immunity gene in bystander bacteria confers protection against T7SS mediated inhibition, implicating an upstream LXG domain toxin in intraspecies antagonism. Additionally, phage induction of T7SS gene expression requires IreK, a Serine/Threonine PASTA kinase. Phage induction of T7SS antimicrobial activity signals through a non-canonical IreK stress response pathway. Our findings highlight how phage infection of a target bacterium can unintentionally affect neighboring bystander bacteria. Furthermore, our work indicates that before phages become a standard of care in the clinic, we must clearly understand how bacteria respond to phage infection and what, if any, collateral effects phage therapy may have on non-target bacteria, such as the microbiota.

Introduction

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

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

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

Staphylococcus and *Streptococcus* are well characterized (25) but T7SS-mediated antibacterial antagonism has not been described in *Enterococcus*. The environmental cues and regulatory pathways that govern T7SS expression and activity are poorly understood, although recent studies indicate that exposure to serum and membrane stresses triggered by pulmonary surfactants, fatty acids and phage infection stimulate T7SS gene expression (24, 28-31). This motivated us to determine if phage induced T7SS gene expression in *E. faecalis* results in the inhibition of non-kin bacterial cells that are not phage targets (bystanders). We discovered that phage infected *E. faecalis* produces potent T7SS antibacterial activity against bystander bacteria. Expression of a T7SS antitoxin (immunity factor) gene in bystander cells confers protection against phage mediated T7SS inhibition. Additionally, we discovered that membrane stress during phage infection induces transcription of T7SS genes via a non-canonical IreK signaling pathway. To our knowledge, the enterococcal T7SS is the only example of secretion system induction during phage infection. These data shed light on how phage infection of a cognate bacterial host can influence polymicrobial interactions and raises the possibility that phages may impose unintended compositional shifts among bystander bacteria in the microbiota during phage therapy.

Results

Phage mediated induction of *E. faecalis* T7SS leads to interspecies antagonism.

A hallmark feature of phage therapy is that because phages often have a narrow host range, they will not influence the growth of non-susceptible bacteria occupying the same niche (22). We discovered that infection of *E. faecalis* OG1RF by phage VPE25, induces the expression of T7SS genes (24). The *E. faecalis* OG1RF T7SS locus is absent in the commonly studied vancomycin-resistant strain V583, despite conservation of flanking genes (Fig. 1A) (32, 33). Homologs of the *E. faecalis* T7SS gene *exsA* are found throughout three of the four *Enterococcus* species groups (34), including *Enterococcus faecium*, suggesting a wide distribution of T7SS loci in enterococci

(Fig. S1). *In silico* analyses predict that the *E. faecalis* T7SS locus encodes multiple WXG100 family effectors and LXG family polymorphic toxins (27, 35). Hence, we hypothesized that induction of T7SS genes during phage infection and consequently the heightened production of T7SS substrates would indirectly influence the growth of non-kin phage-resistant bacterial cells.

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

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

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

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

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

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

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

182 expression of OG1RF_11122, and not OG1RF_11110 or 11112, partially neutralized phage
183 induced T7SS antagonism (Fig. 3B), confirming an essential role for the OG1RF_11122 gene
184 product in immunity, and suggesting that OG1RF_11121 is at least partly responsible for T7SSb
185 mediated intra-species antagonism.
186
187 **Sub-lethal antibiotic stress promotes T7SS dependent antagonism**
188 Considering two genetically distinct phages trigger the induction of T7SS genes in *E. faecalis*
189 (24), we reasoned that T7SS induction could be a result of phage mediated cellular damage and
190 not specifically directed by a phage encoded protein. Antibiotics elicit a range of damage induced
191 stress responses in bacteria (47-49), therefore, we investigated the effects of subinhibitory
192 concentrations of antibiotics on T7SS expression in *E. faecalis*.
193 To investigate the influence of sublethal antibiotic concentrations on *E. faecalis* OG1RF T7SS
194 transcription, we determined the minimum inhibitory concentrations (MIC) of ampicillin,
195 vancomycin, and daptomycin (Fig.S6A-S6C) and monitored T7SS gene expression in *E. faecalis*
196 OG1RF cells treated with a sub-lethal dose of antibiotic (50% of the MIC). We found that bacterial
197 T7SS genes were significantly upregulated in the presence of the cell membrane targeting
198 antibiotic, daptomycin, relative to the untreated control (Fig. 4A). In contrast, the cell wall
199 biosynthesis inhibitors ampicillin and vancomycin either did not induce or had a minor impact on
200 T7SS mRNA levels, respectively (Fig. 4A). Additionally, induction of T7SS transcription occurred
201 when bacteria are challenged with a sub-inhibitory concentration of the DNA targeting antibiotics
202 ciprofloxacin and mitomycin C (Fig. 4B, Fig. S6D–S6E). Collectively, these data show that T7SS
203 induction in *E. faecalis* occurs in response to cell envelope and DNA stress.
204 We next assessed the influence of daptomycin driven T7SS induction on inter-enterococal
205 antagonism. Since T7SS expression is less robust in the presence of daptomycin compared to
206 phage infection (24), a 10:1 ratio of daptomycin treated *E. faecalis* OG1RF was required for
207 growth inhibition of *E. faecalis* Δpip_{V583} during co-culture (Fig. 4C). Consistent with our previous

208 results, daptomycin induced T7SS inhibition of *E. faecalis* Δ*pip*$_{V583}$ was contact dependent (Fig.
209 4D). To facilitate T7SS mediated contact-dependent killing of the target strain during daptomycin
210 exposure, we performed the inhibition assay on nutrient agar plates. The sub-inhibitory
211 concentration of daptomycin (2.5 µg/ml) used in liquid culture was toxic to the cells on agar plates,
212 so we lowered the daptomycin concentration to 0.5 µg/ml to prevent drug toxicity in the agar-
213 based antagonism assay. Plating T7SS producing *E. faecalis* OG1RF cells and *E. faecalis*
214 Δ*pip*$_{V583}$ bystander cells at a ratio of 10:1 resulted in ~10–fold inhibition of bystander growth (Fig.
215 4E). These data show that in addition to phages, antibiotics can be sensed by *E. faecalis* thereby
216 inducing T7SS antagonism of non-kin bacterial cells. These data also suggest that the magnitude
217 of T7SS gene expression is directly related to the potency of T7SS inhibition.
218
219 **IreK facilitates T7SS expression in phage infected *E. faecalis* OG1RF via a non-canonical**
220 **signaling pathway**
221 Having established that phage and daptomycin mediated membrane damage stimulates
222 heightened *E. faecalis* OG1RF T7SS gene expression and antagonistic activity, we next sought
223 to identify the genetic determinants that sense this damage and promote T7SS transcription. Two-
224 component systems, LiaR/S and CroS/R, and the PASTA kinase family protein IreK are well-
225 characterized modulators of enterococcal cell envelope homeostasis and antimicrobial tolerance
226 (50-52). Aberrant cardiolipin microdomain remodeling in the bacterial cell membrane in the
227 absence of the LiaR response regulator results in daptomycin hypersensitivity and virulence
228 attenuation (53). CroS/R signaling and subsequent modulation of gene expression govern cell
229 wall integrity and promote resistance to cephalosporins, glycopeptides and beta—lactam
230 antibiotics (54-56). The *ireK* encoded transmembrane Ser/Thr kinase regulates cell wall
231 homeostasis, antimicrobial resistance, and contributes to bacterial fitness during long-term
232 colonization of the intestinal tract (51, 57, 58). Recently it has been shown that direct cross-talk

between IreK and the CroS/R system positively impacts enterococcal cephalosporin resistance (59).

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

Discussion

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

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

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

interactions and enterococcal persistence in the intestine. However, the molecular mechanism by which IreK facilitates T7SS transcription remains unanswered.

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

Enterococci occupy polymicrobial infections often interacting with other bacteria (71-74). Although commensal *E. faecalis* antagonize virulent *S. aureus* through the production of superoxide (75), the two species also exhibit growth synergy via exchange of critical nutrients (76). Here, we show that phage treatment of *E. faecalis* OG1RF can indirectly impact the growth of neighboring phage-resistant bacteria, including *S. aureus*, in a T7SS–dependent manner, suggesting that phage therapy directed against enterococci driving T7SS activity could be useful for the treatment of polymicrobial infections. However, the counter argument is that phage therapy directed against enterococci could push a bacterial community toward dysbiosis, as phage induced T7SS activity could directly inhibit beneficial bystander bacteria. This raises questions about the consequences of phage mediated off-target effects on bacteria. Could phage induced T7SS activity be used to reduce phage expansion into other closely related strains as a means to dilute phages out of a population, or is it simply that phage induction of the T7SS serves as a mechanism that benefits a select few within a population to aid in their reoccupation of a niche

311 upon overcoming phage infection? Future studies aimed at exploring enterococcal T7SS
312 antagonism in polymicrobial communities should help elucidate the impact of phages on microbial
313 community composition.
314
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320 strains used in this study.
321
322 **Materials and Methods**
323 **Bacteria and bacteriophage.** Bacteria and phages used in this study are listed in Table S1.
324 Bacteria were grown with aeration in Todd-Hewitt broth (THB) or on THB agar supplemented with
325 10mM $MgSO_4$ at 37°C. The following antibiotic concentrations were added to media for the
326 selection of specific bacterial strains or species: *E. faecalis* OG1RF (25 µg/ml fusidic acid, 50
327 µg/ml rifampin), *E. faecalis* V583 Δpip_{V583} (100 µg/ml gentamycin), *S. aureus* AH2146 LAC
328 Φ11:LL29 (1 µg/ml tetracyclin), *L. monocytogenes* 10403S (100 µg/ml streptomycin), *V. cholerae*
329 C6706 int I4::TnFL63 and *S. enterica* serovar Typhimurium 140285 put::Kan (50 µg/ml
330 kanamycin). *S. agalactiae* COH1 was distinguished from *E. faecalis* on Chrome indicator Agar
331 (CHROMagar StrepB SB282). We were unable to differentially select *E. coli, S. pyogenes* and *S.*
332 *mitis* from *E. faecalis* based on antibiotic sensitivity. Therefore, colony counts of these bacteria in
333 co-culture experiments were acquired by subtracting the *E. faecalis* colony numbers on selective
334 media from total number of colonies on non-selective media. Strains harboring pLZ12A and its
335 derivatives were grown in the presence of 20 µg/ml chloramphenicol.
336

Bioinformatic analyses. Genome sequences of *E. faecalis* V583 (NC_004668.1) and OG1RF (NC_017316.1) were obtained from NCBI. Alignments were generated and visualized using EasyFig (77). OG1RF protein domains were identified using KEGG (78) and ExPASy PROSITE (79). Structure modeling of OG1RF_12414 was done with Phyre2 (80). The EsxA phylogenetic tree was constructed in MEGA version X (81) using non-redundant protein sequences obtained from NCBI BLAST (82) with OG1RF_11100 as input and was edited using the Interactive Tree Of Life browser (83). OG1RF_11109 was used as an input for the NCBI Conserved Domain Architecture Retrieval Tool (84) to identify protein domains that co-occur with LXG domains in *Enterococcus*.

Antibiotic sensitivity profiles. Antibiotic susceptibility profiles for ampicillin, vancomycin, and daptomycin were determined using a broth microdilution assay. Overnight (O/N) *E. faecalis* OG1RF cultures were diluted to 5×10^6 CFU/ml and 100 µl was added to each well of a 96-well plate to give a final cell density of 5×10^5 CFU/ml. Antibiotic stocks were added to the first column of each row, mixed thoroughly, and were serially diluted 2-fold across the rows. The last column was used as a no drug control. Cultures containing daptomycin were supplemented with 50 µg/ml $CaCl_2$. Bacterial growth was monitored by measuring absorbance (OD_{600}) using a Synergy H1 microplate reader set to 37°C with continuous shaking O/N. Growth curves are presented as the average of three biological replicates. A concentration of antibiotic just below the drug amount that inhibits bacterial growth was deemed sub-lethal and used to examine T7SS genes expression.

Co-culture bacterial antagonism assays. For inter- and intraspecies antagonism assays in liquid media, O/N cultures of different bacteria were diluted in THB containing 10mM $MgSO_4$ to an OD_{600} of 0.2 and mixed together in a 1:1 or 10:1 ratio. The mixed cell suspensions were either left untreated or treated with phage VPE25 (MOI 0.01) / daptomycin (2.5 µg/ml) and grown at

363 37°C with aeration. For antagonism experiments on agar plates, O/N cultures of different strains
364 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
365 mixed culture suspension was added to 5 ml THB + 0.35% agar at 55°C and were poured over
366 the surface of a THB agar plate in the absence or presence of daptomycin (0.5 µg/ml). The plates
367 were incubated at 37°C under static conditions for 24 hours. Cells were harvested by scraping off
368 the top agar, resuspending in 5 ml of PBS, and the cfus were obtained by plating serially diluted
369 cell suspension on appropriate selective or differentiating agar plates. Relative viability was
370 calculated from the ratio of target strain cfu in the treated versus the untreated co-culture. The
371 assays were performed in biological triplicates.
372
373 **RNA extraction and quantitative PCR.** RNA was extracted from phage treated or untreated *E.*
374 *faecalis* OG1RF cells by using an RNeasy Mini Kit (Qiagen) with the following published
375 modifications (24). cDNA was generated from 1 µg of RNA using qScript cDNA SuperMix
376 (QuantaBio) and transcript levels were analyzed by qPCR using PowerUp™ SYBR Green Master
377 Mix (Applied Biosystems). Transcript abundances were normalized to the 16S rRNA gene
378 transcripts and fold–change was calculated by comparing to untreated controls. All data are
379 represented as the average of three biological replicates.
380
381 **Bacterial growth curves.** 25 ml of 10mM $MgSO_4$ supplemented THB was inoculated with O/N
382 cultures of *E. faecalis* diluted to an OD_{600} of 0.025 and distributed to a 96-well plate in 0.1 ml
383 volumes. Cultures were incubated at 37° C with aeration. OD_{600} was measured periodically for 18
384 hours in a Synergy H1 microplate reader.
385
386 **Efficiency of plating (EOP) assays.** To investigate if phage VPE25 can infect and lyse *E.*
387 *faecalis* mutants and various other bacterial species, 10^7 PFU/ml of phage was serially diluted
388 and the phage was titered on each strain using a THB agar overlay plaque assay. EOP is

389 expressed as the percentage of phage titer from each strain relative to the wild type *E. faecalis*
390 OG1RF control. Data are presented as the average of three biological replicates.
391
392 **Construction of *E. faecalis* mutants and complementation.** Isolation of *E. faecalis* genomic
393 DNA was performed using a ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research). All PCR
394 reactions used for cloning were performed with high fidelity KOD Hot Start DNA Polymerase (EMD
395 Millipore). *E. faecalis* Δ*essB* was generated by allelic replacement by cloning an in frame *essB*
396 deletion product into pLT06 using Gibson Assembly® Master Mix (New England Biolabs),
397 integrating this construct into the chromosome, and resolving the deletion mutant by homologous
398 recombination (85-87). For ectopic expression of putative immunity proteins, coding regions of
399 OG1RF_11110, OG1RF_11112, and OG1RF_11122 were cloned downstream of the *bacA*
400 promoter (P_{bacA}) by restriction digestion and ligation into the shuttle vector pLZ12A (20). Primer
401 sequences and restriction enzymes used for cloning are listed in Table S1. Plasmids were
402 introduced into electrocompetent *E. faecalis* cells as previously described (20).
403
404 **Statistical analysis.** Statistical tests were performed using GraphPad – Prism version 8.2.1. For
405 qPCR and bacterial competition assays, unpaired Student's t-tests were used. *P* values are
406 indicated in the figure legends.
407
408 **References**

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612

613 **Figure Legends**

614

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

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

Figure 3. Identification of *E. faecalis* T7SS toxins and an immunity protein that confers protection to bystander bacteria. (A) LXG domains in OG1RF_11109 and OG1RF_11121 were identified using KEGG and ExPASy PROSITE. Other putative toxic effectors in OG1RF were identified by homology to OG1RF_11109 or OG1RF_11121. Homology diagrams were rendered in EasyFig. **(B)** *E. faecalis* OG1RF T7SS mediated growth inhibition of phage resistant *E. faecalis* Δpip_{V583} during infection is alleviated by expressing OG1RF_11122 in *E. faecalis* Δpip_{V583} but not in the presence of empty vector, or expressing OG1RF_11110, or OG1RF_11112. E, represents empty vector. C, is expression vector. Data represent three biological replicates. *$P < 0.0001$ by unpaired Student's t-test.

Figure 4. Sub-lethal antibiotic treatment enhances T7SS gene expression leading to inhibition of bystander bacteria. Altered expression of T7SS genes upon exposure to sub-inhibitory concentrations of **(A)** ampicillin (0.19 μg/ml), vancomycin (0.78 μg/ml) or daptomycin (6.25 μg/ml) and **(B)** ciprofloxacin (2 μg/ml) or mitomycin C (4 μg/ml) for 40 minutes relative to the untreated control. **(C-E)** Contact–dependent T7SS mediated inhibition of bystander bacteria in the presence of daptomycin. Relative viability of *E. faecalis* Δpip_{V583} was measured during co-culture with *E. faecalis* OG1RF or Δ*essB* antagonists in the presence and absence of daptomycin treatment in **(C)** liquid culture (2.5 μg/ml), **(D)** trans-well plates to prevent physical engagement between cells (2.5 μg/ml) and **(E)** on agar media (0.5 μg/ml). E – empty vector and C –

complementation vector. Data represent three biological replicates. *P < 0.01, **P < 0.001 to 0.0001 by unpaired Student's t-test.

Figure 5. IreK is necessary for the induction of *E. faecalis* T7SS expression and subsequent inhibition of bystander bacteria during phage infection. (A) Phage infection leads to enhanced expression of T7SS genes in the wild type *E. faecalis* OG1RF but not in an Δ*ireK* mutant strain. **(B)** Growth inhibition of *E. faecalis* Δpip_{V583} during phage infection of *E. faecalis* OG1RF is abrogated in an Δ*ireK* mutant of OG1RF. Data represent three replicates *P < 0.00001 by unpaired Student's t-test.

Figure S1. Phylogenetic tree of EsxA sequences in *Enterococcus*. Non-redundant sequences (n=96) were identified using NCBI BLAST with OG1RF EsxA (OG1RF_11100) as the input. The tree was constructed in MEGAX using the Maximum Likelihood method and JTT matrix-based model and is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree with the highest log likelihood (-3544.39) is shown. *E. faecalis* sequences are highlighted in purple, and the GenBank identifier for EsxA from OG1RF (AEA93787.1) is shown in bold red font.

Figure S2. Phage VPE25 infects wild type *E. faecalis* OG1RF and Δ*essB* with similar efficacy. The direct measurement of phage particles released from wild type *E. faeaclis* OG1RF and the Δ*essB* mutant strain following phage VPE25 infection. Data represent three biological replicates.

Figure S3. Putative orphan toxins in OG1RF are found as full-length LXG-domain proteins in other bacteria. OG1RF_11111, OG1RF_11113, and OG1RF_11123 sequences were used as input for NCBI BLAST. Alignments and homology were rendered in EasyFig.

Figure S4. A distal OG1RF locus encodes an additional LXG-domain protein. (A) Schematic showing homology between V583 (NC_004668.1, top) and OG1RF (NC_017316.1, bottom). Sequences were obtained from NCBI, and homology comparisons were rendered in EasyFig. **(B)** Cartoon depicting the LXG domain of OG1RF_12414 (identified using KEGG and ExPASy PROSITE). **(C)** Predicted structural homology between OG1RF_12414 (lilac) and the *Pseudomonas protogens* Pf-5 Tne2/Tni2 complex (PDB 6B12). Tne2 is shown in green, and Tni2 is shown in gray. Structural modeling was done using PHYRE2, and images were rendered in Pymol.

Figure S5. Domain architecture of *Enterococcus* LXG proteins. Domain architectures were identified using the NCBI Conserved Domain Architectural Retrieval Tool (DART) with OG1RF_11109 as an input. Diagrams are drawn to scale.

Figure S6. Antibiotic susceptibility of *E. faecalis* OG1RF. Growth of wild type *E. faecalis* OG1RF was monitored over 18 hours in the presence or absence of **(A)** ampicillin, **(B)** vancomycin and **(C)** daptomycin in microtiter plates. The antibiotic concentrations highlighted with a blue box were deemed sub-inhibitory and used to investigate T7SS expression levels. Early log-phase cultures of *E. faecalis* OG1RF were grown in the presence or absence of **(D)** mitomycin C (4 µg/ml) or **(E)** ciprofloxacin (2 µg/ml) to show that even though these routine concentrations used to promote a stress response in other *E. faecalis*, they do not prevent bacterial growth.

Figure S7. *E. faecalis* mutants of cell wall homeostasis show no growth defects and respond to phage VPE25 infection. (A) Optical density of wild type E. *faecalis* and isogenic mutants were monitored for 18 hours. **(B)** While all the strains were susceptible to phage VPE25 infection, the proportion of released phage particles was diminished in the *croR* and *croS*

transposon mutant background. Data represent three biological replicates. *$P < 0.001$ by unpaired Student's t-test.

Figure S8. Quantitative PCR demonstrates that LiaR/S and CroS/R two-component systems do not influence T7SS gene expression during phage infection. (A- F) mRNA transcript levels of T7SS genes are enhanced in the transposon mutants of *liaR*, *liaS*, *croR* and *croS* strains similar to wild type *E. faecalis* OG1RF during phage infection (MOI = 1) compared to untreated controls. Data represent three biological replicates. *$P < 0.01$, **$P < 0.0001$ by unpaired Student's t-test.

Figure S9. Sub-lethal ceftriaxone exposure does not influence T7SS gene expression in *E. faecalis* OG1RF. Transcription of T7SS genes are not elevated 20 minutes post ceftriaxone (128µg/ml) administration relative to the untreated controls.

Table S1. List of bacterial strains, phages, plasmids and primers used in this study.

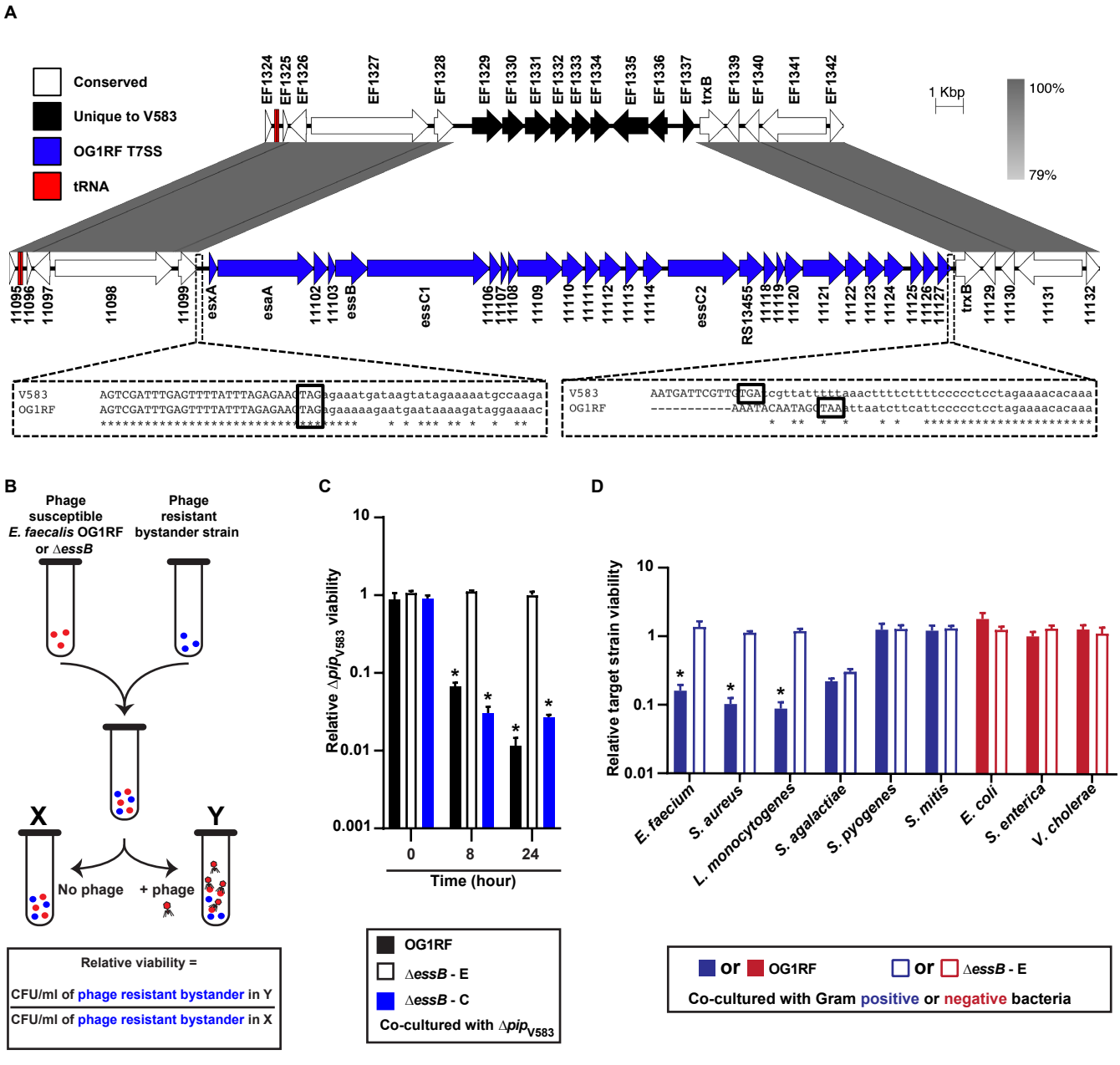
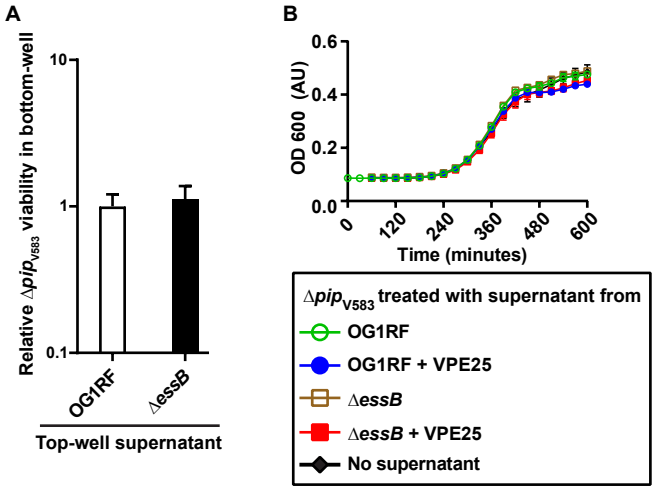


Fig. 1



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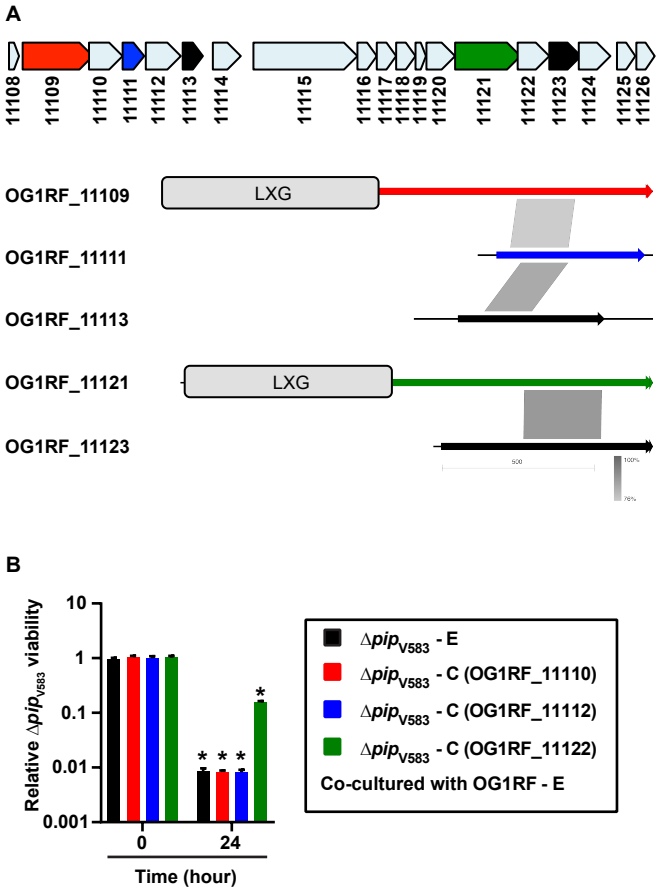

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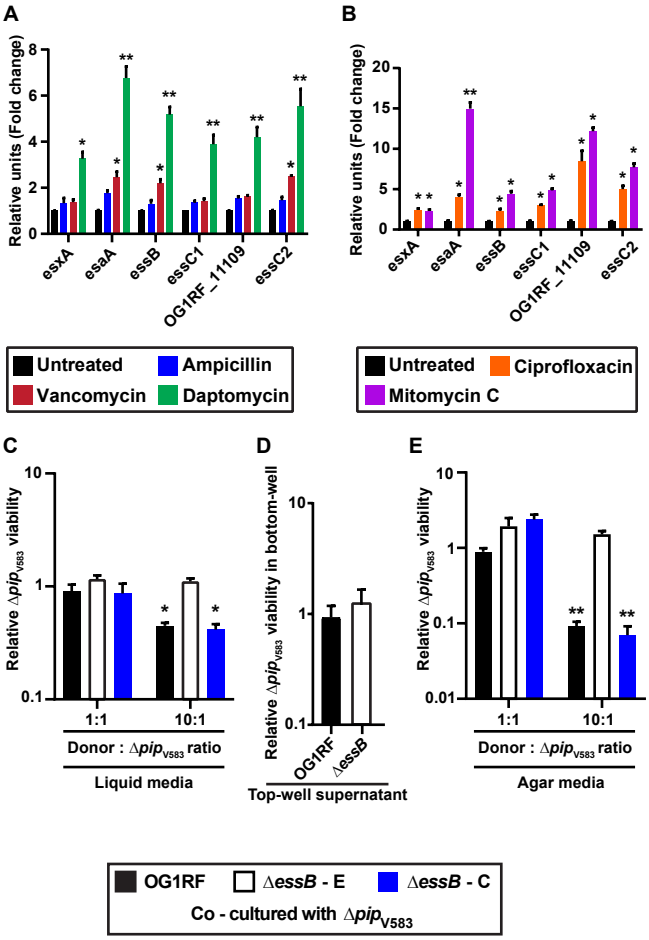


Fig. 4

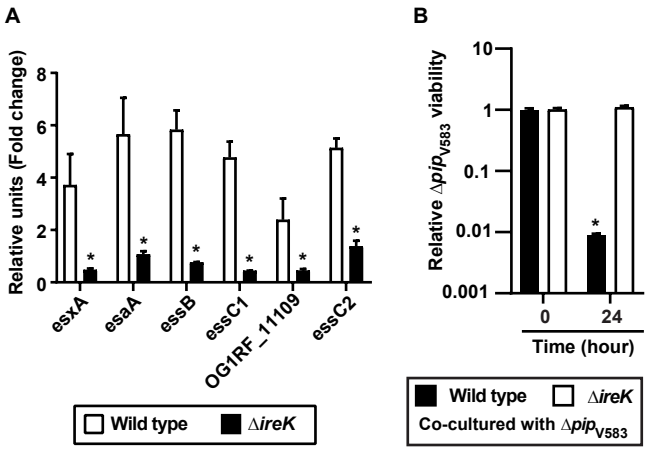


Fig. 5

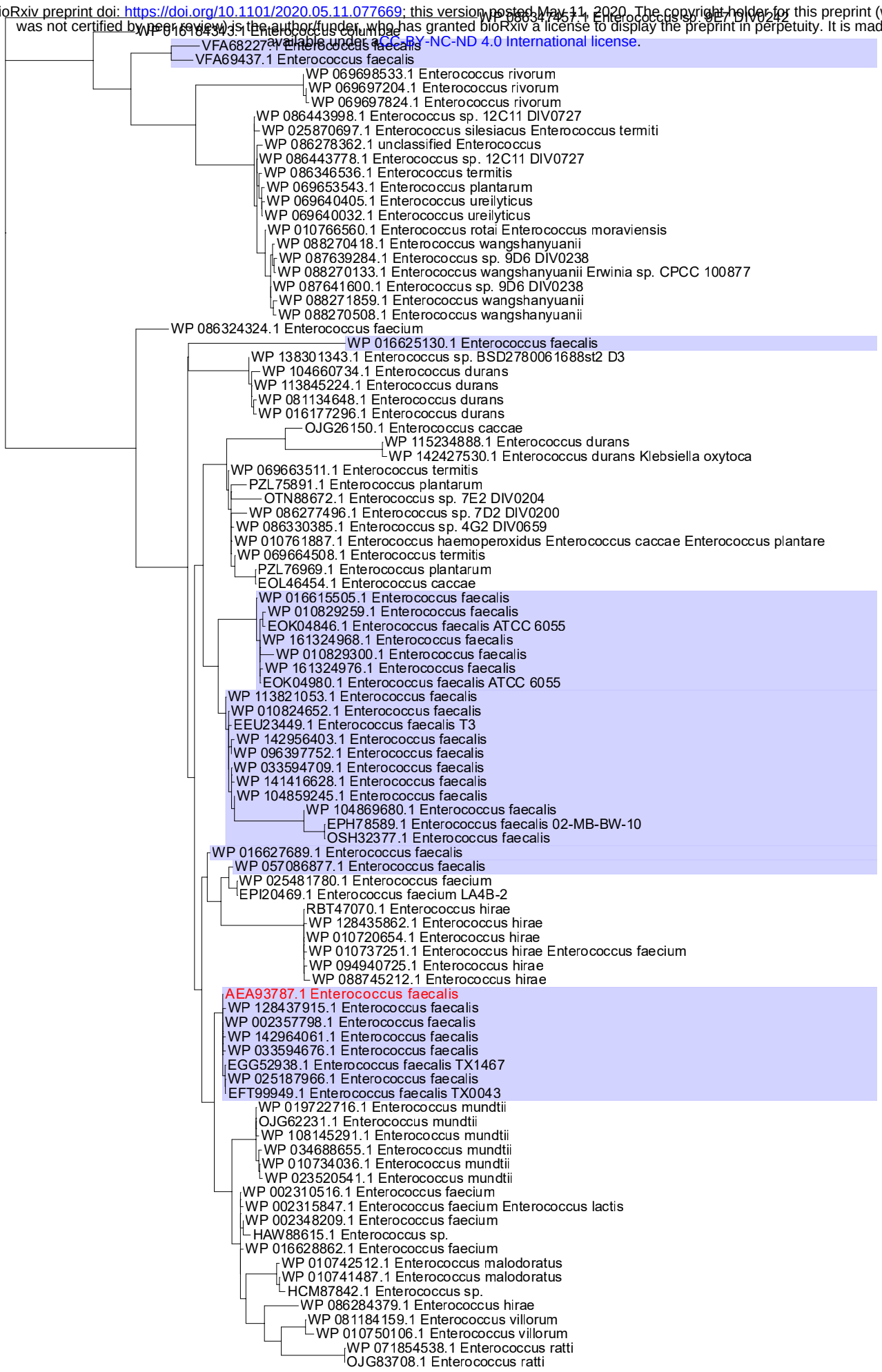


Fig. S1

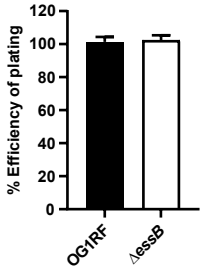


Fig. S2

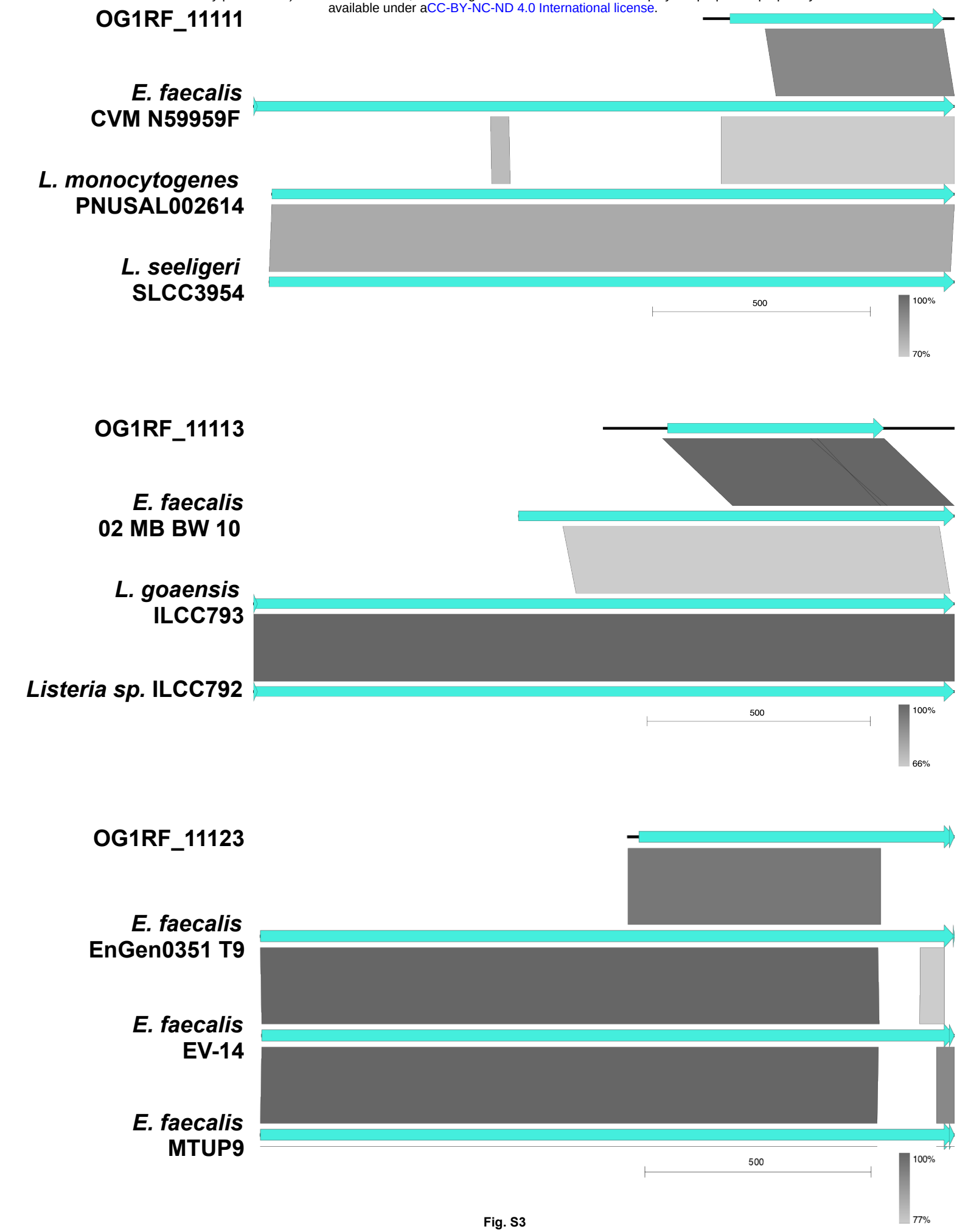
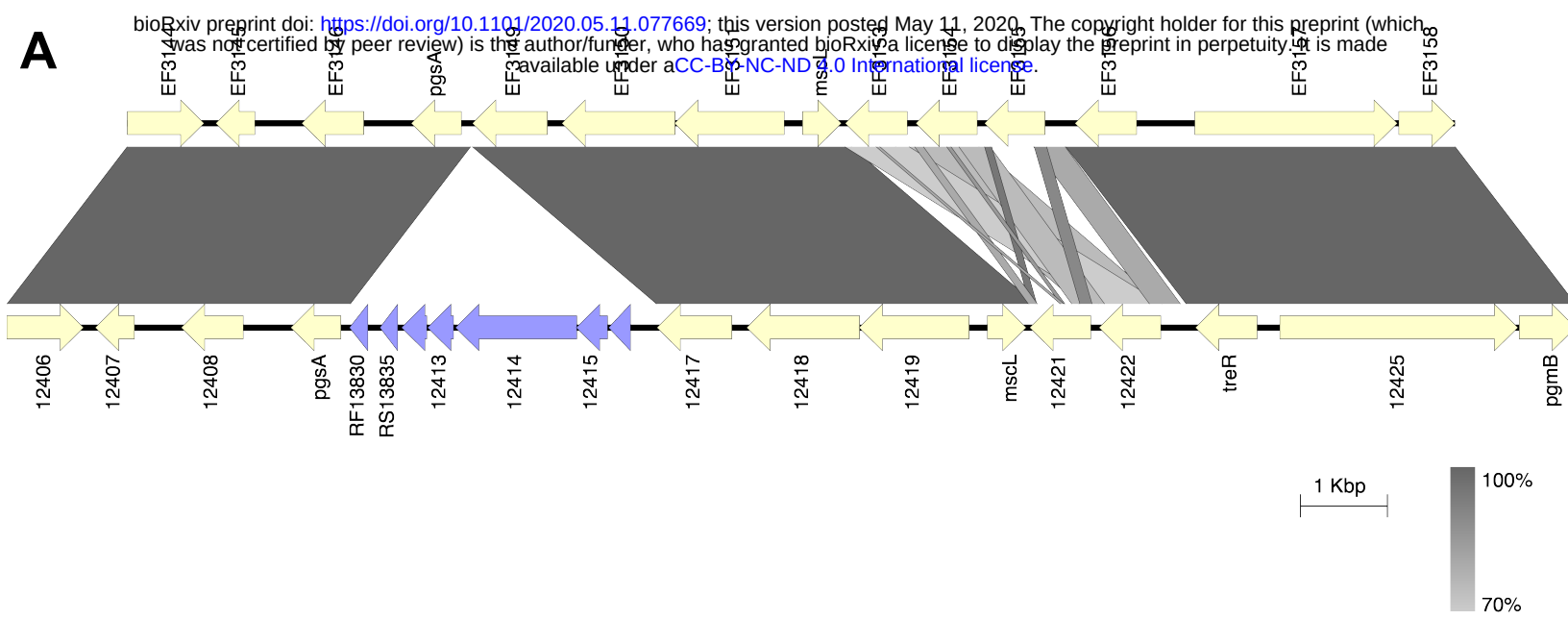
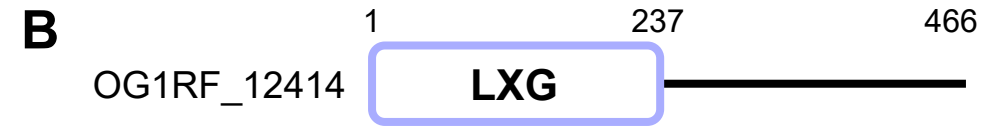


Fig. S3





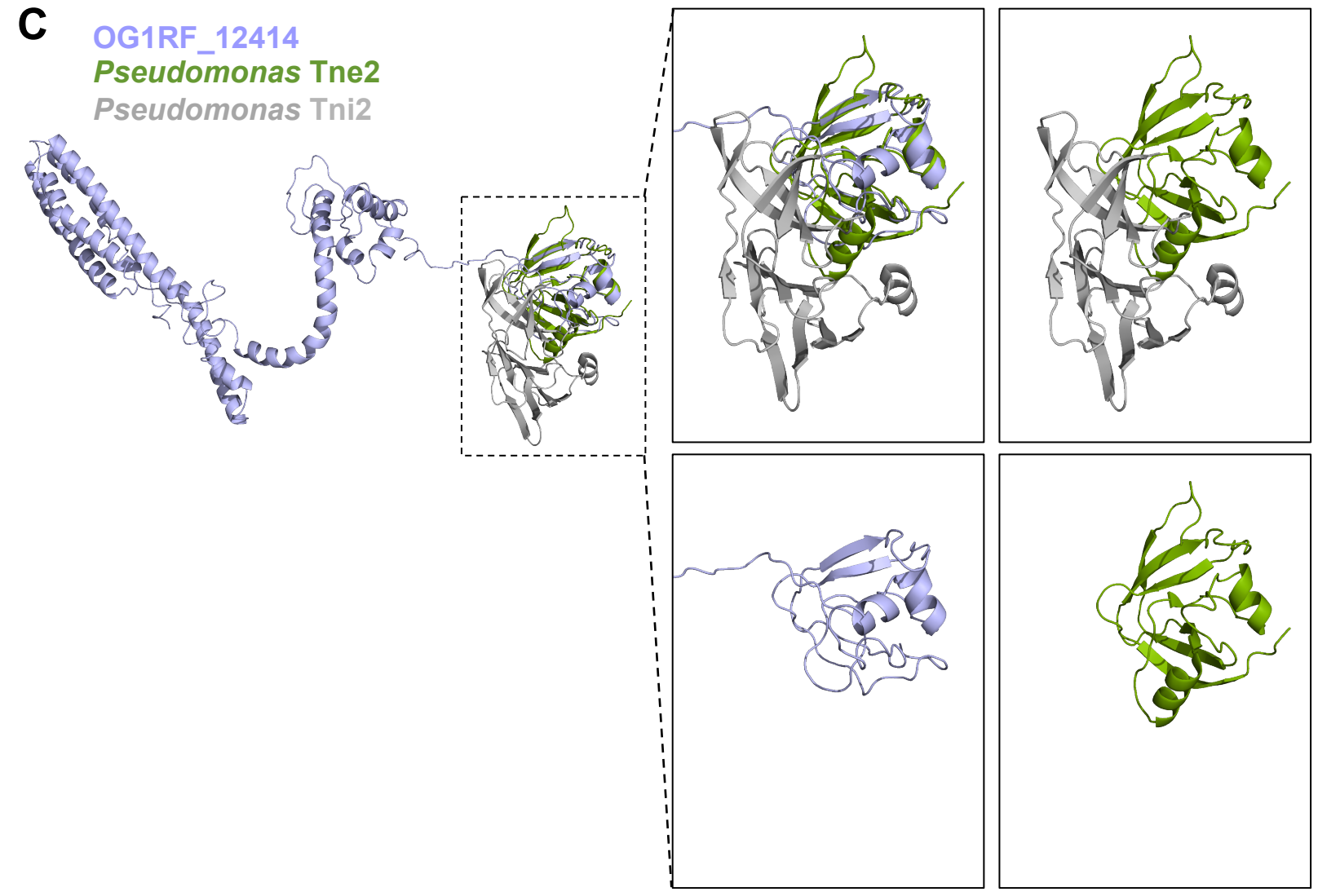


Fig. S4

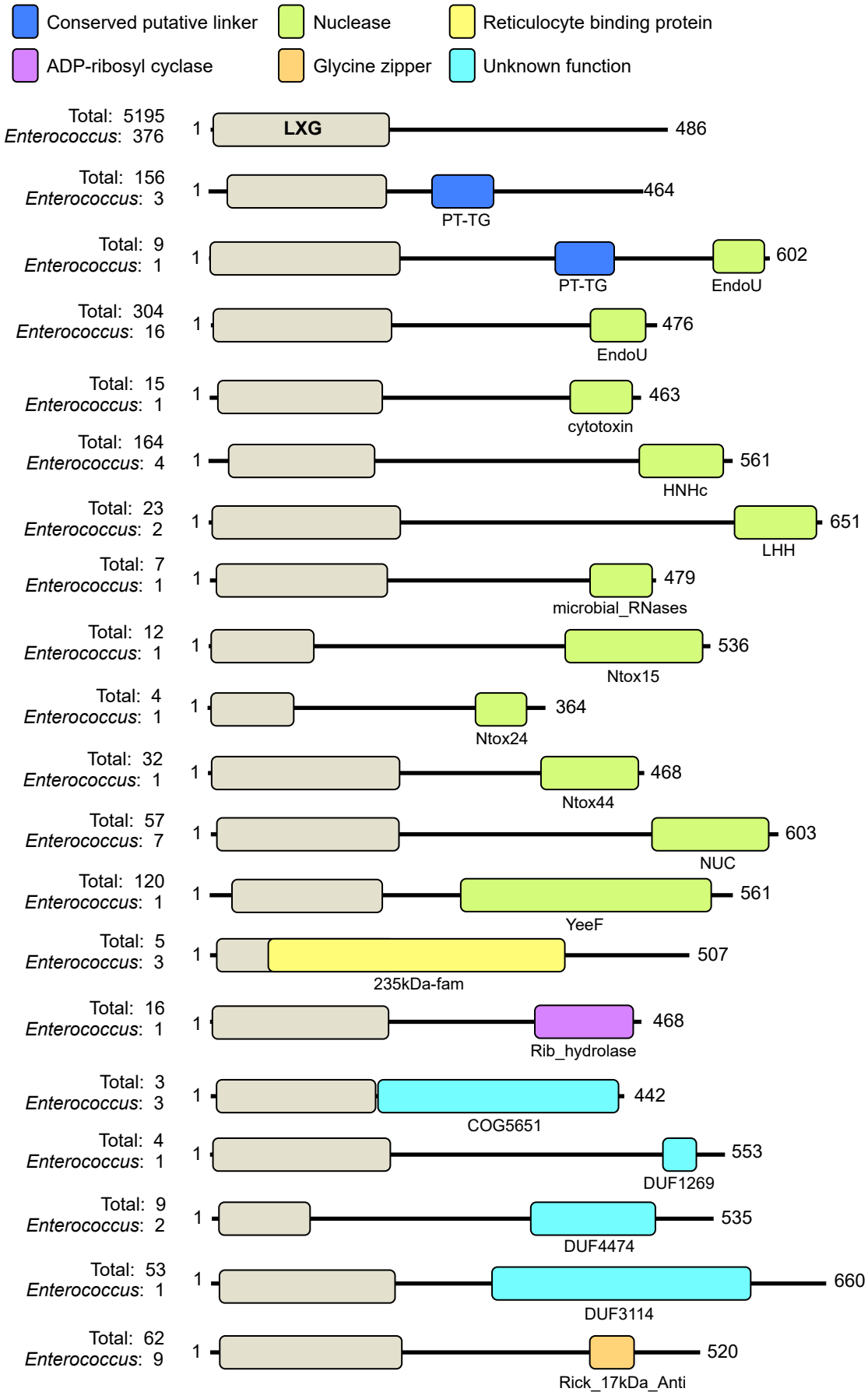


Fig. S5

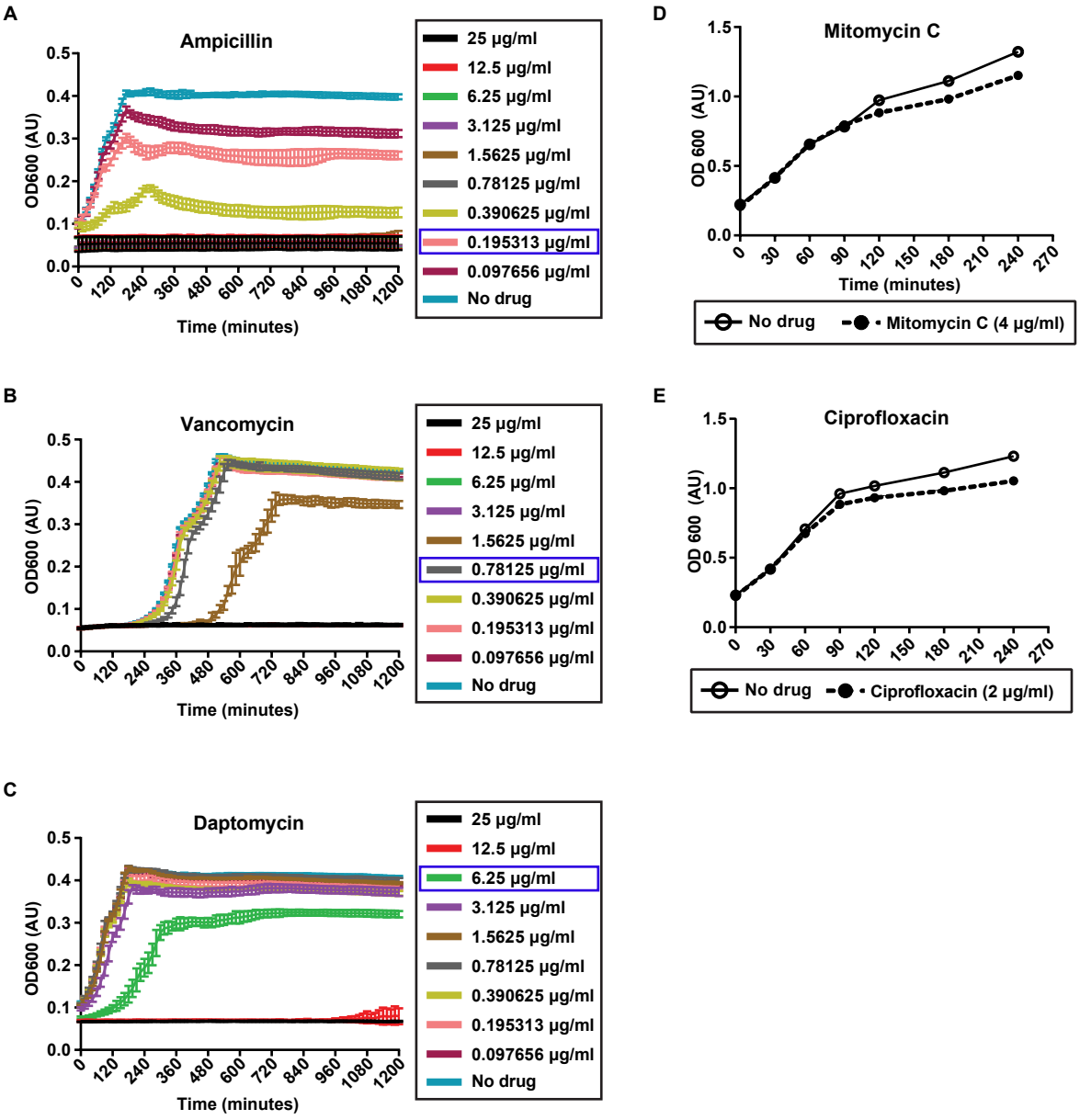


Fig. S6

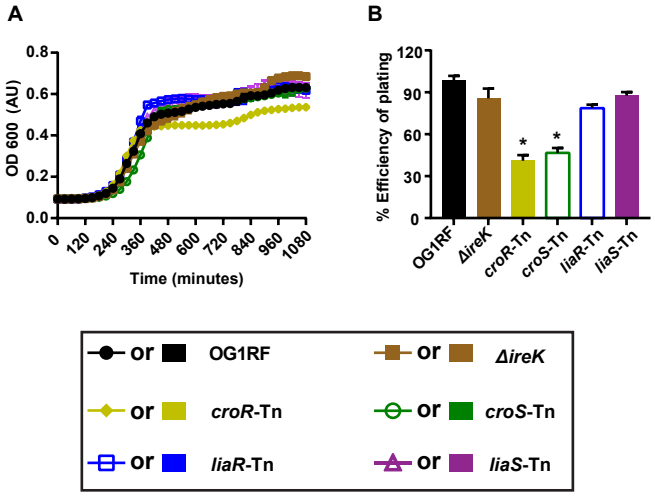


Fig. S7

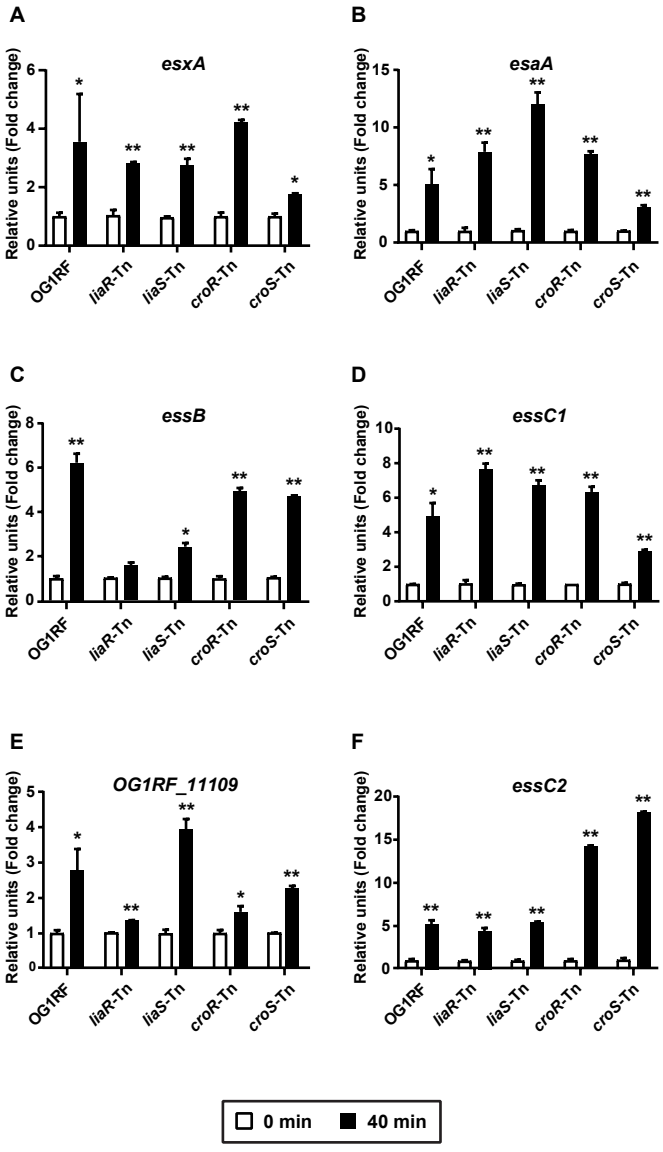


Fig. S8

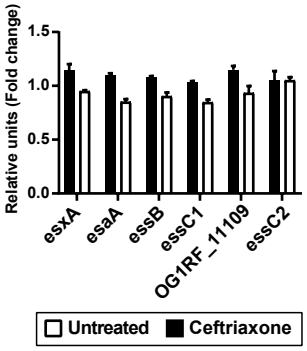


Fig. S9

Table S1. List of bacterial strains, phages, plasmids, and primers used in this study

Strains, phages, Plasmids, and primers	Characteristics and/or description	Reference /Source
Enterococcus faecalis		
OG1RF	Human oral isolate; Rf^R, Fa^R	(1)
Δ*pip*$_{V583}$	V583 background with a deletion of *pip*. Vm^R, Em^R, Gm^R	(2)
Δ*ireK*	*E. faecalis* OG1RF CK119	(3)
Δ*essB*	*E. faecalis* OG1RF markerless deletion in OG1RF_11104	This study
croR-Tn	*E. faecalis* OG1RF *croR* transposon mutant. Rf^R, Fa^R, Cm^R	(4)
croS-Tn	*E. faecalis* OG1RF *croS* transposon mutant. Rf^R, Fa^R, Cm^R	(4)
liaR-Tn	*E. faecalis* OG1RF *liaR* transposon mutant. Rf^R, Fa^R, Cm^R	(4)
liaS-Tn	*E. faecalis* OG1RF *liaS* transposon mutant. Rf^R, Fa^R, Cm^R	(4)
OG1RF -E	*E. faecalis* OG1RF carrying pLZ12A empty vector. Rf^R, Fa^R, Cm^R	This study
Δ*essB* - E	Δ*essB* strain carrying pLZ12A empty vector. Rf^R, Fa^R, Cm^R	This study
Δ*essB* - C	Δ*essB* strain carrying pLZ12A complementation vector. Rf^R, Fa^R, Cm^R	This study
Δ*pip*$_{V583}$ - E	Δ*pip*$_{V583}$ strain carrying pLZ12A empty vector. Vm^R, Em^R, Gm^R, Cm^R	(5)
Δ*pip*$_{V583}$ - C (OG1RF_11110)	Δ*pip*$_{V583}$ strain carrying pLZ12A containing coding sequence of OG1RF_11110 from P-*bacA*. Vm^R, Em^R, Gm^R, Cm^R	This study
Δ*pip*$_{V583}$ - C (OG1RF_11112)	Δ*pip*$_{V583}$ strain carrying pLZ12A containing coding sequence of OG1RF_11112 from P-*bacA*. Vm^R, Em^R, Gm^R, Cm^R	This study
Δ*pip*$_{V583}$ - C (OG1RF_11122)	Δ*pip*$_{V583}$ strain carrying pLZ12A containing coding sequence of OG1RF_11122 from P-*bacA*. Vm^R, Em^R, Gm^R	This study
Other bacteria		
S. aureus	*Staphylococcus aureus* strain LAC* φ11::LL29 *tet*. Tc^R	(6)
E. faecium	*Enterococcus faecium* strain 1,231,410. Vm^R, Em^R	(7)
L. monocytogenes	*Listeria monocytogenes* 10403S. St^R	(8)
S. agalactiae	*Streptococcus agalactiae* strain COH1.	(9)
S. pyogenes	*Streptococcus pyogenes* ATCC 12384.	ATCC

S. mitis	*Streptococcus mitis* NS5. Clinical isolate from UT Southwestern Clinical Microbiology Laboratory	
S. enterica	*Salmonella enterica* serovar Typhimurium. AV09379 *put*::Kan; Kn^R	(10)
V. cholerae	*Vibrio cholerae C6706 int I4::TnFL63*; Kn^R	(11)
Escherichia coli		
TG1	*[F' traD36 proAB lacIqZ ΔM15] supE thi-1 Δ(lac-proAB) Δ(mcrBhsdSM)5(rK - mK -)*	Lucigen
K12	*Escherichia coli* K12, ATCC 25404	ATCC
Phage		
VPE25	Siphoviridae; Wastewater isolate	(2)
Plasmids		
pLZ12A	*bacA* promoter cloned into shuttle vector pLZ12; pSH71 origin; Cm^R	(5, 12)
pLT06	*E. faecalis* allelic exchange vector; Cm^R	(13)
pBD01	Δ*essB* construct cloned into pLT06 by Gibson assembly. Cm^R	This study
pLZ12A:*essB*	*essB* complementation vector. Cloned into PstI/BamHI site. Cm^R	This study
pLZ12A: *OG1RF_11110*	pLZ12A expressing OG1RF_11110 from P_{bacA}. Cloned into PstI/BamHI site. Cm^R	This study
pLZ12A: *OG1RF_11112*	pLZ12A expressing OG1RF_11112 from P_{bacA}. Cloned into PstI/BamHI site. Cm^R	This study
pLZ12A: *OG1RF_11122*	pLZ12A expressing OG1RF_11122 from P_{bacA}. Cloned into PstI/BamHI site. Cm^R	This study
Primers		
essB-F	NNNNNNCTGCAGATGAGCGATTAAAGGATATTTCA; Forward primer to generate pLZ12A: *essB*; PstI site	This study
essB-R	NNNNNNGGATCCTTACTATTTTCGTTGTCATCC; Reverse primer to generate pLZ12A: *essB*; BamHI site	This study
OG1RF_11110-F	NNNNNNCTGCAGATGGACTTCCAAGGTGGTAAAATTAT ; Forward primer to generate pLZ12A: *OF1RF_11110*; PstI site	This study

OG1RF_11110-R	NNNNNNGGATCCTTATTCTCCGTACCATTCCTCTTTA; Reverse primer to generate pLZ12A: *OF1RF_11110*; BamHI site	This study
OG1RF_11112-F	NNNNNNCTGCAGATGAATAAAATCTTAAATAAAATATCTTTTG; Forward primer to generate pLZ12A: *OF1RF_11112*; PstI site	This study
OG1RF_11112-R	NNNNNNGGATCCCTAACTATCTTCACCATACCATTCTTG; Reverse primer to generate pLZ12A: *OF1RF_11112*; BamHI site	This study
OG1RF_11122-F	NNNNNNCTGCAGATGGTTTTCATGATAAAAAATTATGTACC; Forward primer to generate pLZ12A: *OF1RF_11122*; PstI site	This study
OG1RF_11122-R	NNNNNNGGATCCTTATTTTTTGGTTCTCTTGTTCTTC; Reverse primer to generate pLZ12A: *OF1RF_11122*; BamHI site	This study
RT-*esxA*-F	AAGGGCAAGCATTTCAAGCG; qPCR forward primer for OG1RF_11100	(14)
RT-*esxA*-R	TCTTGACGGTCACGTTCTGC; qPCR reverse primer for OG1RF_11100	(14)
RT-*esaA*-F	CCAATGGCTTGGCAACTGAC; qPCR forward primer for OG1RF_11101	(14)
RT-*esaA*-R	GCGAACGAACGTGCATTTTG; qPCR reverse primer for OG1RF_11101	(14)
RT-*essB*-F	GGGAATGGCACCCTGAAAGA; qPCR forward primer for OG1RF_11104	(14)
RT-*essB* -R	CTTCGCGCTTGGCTTTTTGA; qPCR reverse primer for OG1RF_11104	(14)
RT-*essC1*-F	TTGGAAAGGTGGCGGAATAG; qPCR forward primer for OG1RF_11105	(14)
RT-*essC1*-R	TCTGCTTTGATACTGGCTAAGG; qPCR reverse primer for OG1RF_11105	(14)
RT-*11109*-F	GCTTTGGAGAACGCTGAACG; qPCR forward primer for OG1RF_11109	(14)

RT-*11109*-R	TTTTGACAGTCTTGCGCTCG; qPCR reverse primer for OG1RF_11109	(14)
RT-*essC2*-F	CTCAACCGGATCGTGCTTATT; qPCR forward primer for OG1RF_11115	(14)
RT-*essC2*-R	CCTTGGTAGCGAATGGATCATAG; qPCR reverse primer for OG1RF_11115	(14)
RT-16S-F	CGCTTCTTTCCTCCCGAGT; qPCR forward primer 16S rRNA gene	(14)
RT-16S-F	GCCATGCGGCATAAACTG; qPCR reverse primer 16S rRNA gene	(14)

Cm^R - chloramphenicol resistant; Rf^R - rifampicin resistance; Fa^R - fusidic acid resistance; Vm^R - vancomycin resistance; Em^R - erythromycin resistance; Gm^R - Gentamicin resistance; Tc^R - tetracycline resistance; St^R = streptomycin resistance; Kn^R = Kanamycin resistance. Restriction enzyme sites are underlined.

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