# Loss of function mutations in *epaR* confer resistance to phage NPV1 infection in *Enterococcus faecalis* OG1RF

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**Abstract**

*Enterococcus faecalis* is a Gram-positive opportunistic pathogen that inhabits the human gastrointestinal tract. Because of the high frequency of antibiotic resistance among *Enterococcus* clinical isolates, interest in using phage to treat enterococcal infections and to decolonize high-risk patients for antibiotic-resistant *Enterococcus* is rising. Bacteria can evolve phage resistance, but there is little published information on these mechanisms in *E. faecalis*. In this report, we identified genetic determinants of *E. faecalis* resistance to ϕNPV1. We found that loss-of-function mutations in *epaR* confer ϕNPV1 resistance by blocking phage adsorption. We attribute the inability of the phage to adsorb to the modification or loss of an extracellular polymer in strains with inactivated *epaR.* Phage-resistant *epaR* mutants exhibited increased daptomycin and osmotic stress susceptibilities. Our results demonstrate that *in vitro* spontaneous resistance to ϕNPV1 comes at a cost in *E. faecalis* OG1RF.

26 **Introduction**

27 *Enterococcus faecalis* is a Gram-positive bacterium that inhabits the human gastrointestinal
28 tract and is associated with nosocomial infections (1). Infections caused by *E. faecalis* can be
29 difficult to treat because of the high frequency of resistance to multiple antibiotics among *E.*
30 *faecalis* clinical isolates (2). The antibiotic daptomycin can be used to treat certain infections
31 caused by multidrug-resistant enterococci. Daptomycin is a lipopeptide antibiotic that interacts
32 with the enterococcal cell surface and disrupts membrane structure and function (3).

33

34 Bacteriophages (phages) are bacterial viruses and natural predators of bacteria. It is reasonable
35 to expect that phages can be employed to treat bacterial infections. However, phages have not
36 been extensively studied in the Western world in the context of therapeutic application until
37 recently due to the availability of antibiotics (4). In recent years, interest in using phages to treat
38 bacterial infections (phage therapy) has reemerged because of the emergence of multidrug-
39 resistant bacteria. For *E. faecalis*, promising studies include the use of phage to eliminate
40 biofilm, a major barrier to antibiotic treatment, and to increase survival rates in mouse models of
41 enterococcal infection (5, 6).

42

43 One advantage of phage therapy is limited damage to the native microbiome because of the
44 specificity of the phage to its host (7). Typically, lytic phage have narrow host ranges and are
45 species-specific or target a range of strains within a species. The first step to a successful
46 phage infection is the attachment of the phage particle to the proper receptor present on the
47 surface of the host cell. Phage receptors have been extensively studied in certain phage
48 families, including the T series phages, Mu, and λ for Gram-negative bacteria (8-11). Some
49 phage receptors have been characterized in Gram-positive bacteria, including receptors for
50 ϕSPP-1 of *Bacillus subtilis* (12) and the phage c2 group of *Lactococcus lactis* (13, 14). YueB,
51 the ϕSPP-1 receptor, and PIP, the phage c2 receptor, are orthologs and are required for

52 irreversible phage adsorption (12). Enterococcal phage receptors have not been well-
53 characterized. Previously, we and collaborators identified PIP as a receptor and potential DNA
54 channel for the *E. faecalis* phages ϕVPE25 and ϕVFW (15).
55
56 Bacteria can evolve phage resistance. Mechanisms of phage resistance include modification or
57 loss of the phage receptor (16). However, as phage receptors generally serve physiological
58 functions in the cell, the modification or loss of a receptor could come at a cost for the bacterial
59 host. For example, spontaneous phage-resistant mutants have altered antibiotic sensitivity in *P.*
60 *aeruginosa* (17). Phages utilizing receptors that have roles in antibiotic resistance could be
61 advantageous for re-sensitizing resistant bacteria to antibiotics.
62
63 Considering the increasingly limited treatment options for *E. faecalis* infections and the revival of
64 interest in using phage therapy to treat bacterial infections, it is crucial that we know the
65 receptor(s) of enterococcal phages since effective phage cocktails use phages targeting
66 multiple different receptors (18). Moreover, the roles of these receptors in enterococcal
67 physiology should be elucidated. The tailed, virulent phage ϕNPV1 was found in previous
68 studies to infect *E. faecalis* OG1RF (19, 20). In this study, we used a combination of genomic
69 and genetic approaches to investigate the ϕNPV1 receptor in *E. faecalis* OG1RF.
70
71 **Results**
72 **Deletion of *epaR* alters susceptibility to ϕNPV1**. We isolated a OG1RF∆PIP (15) strain with
73 spontaneous resistance to ϕNPV1 (Figure 1). We refer to this strain as OG1RF-C. The genome
74 sequence of OG1RF-C was determined. We identified non-synonymous substitutions in *epaR,*
75 *bgsB, iolA2*, and OG1RF_10252 (Table 2). *epaR* is one of the 18 conserved genes of the *epa*
76 gene cluster (*epaA-epaR*), which codes for synthesis of the enterococcal polysaccharide
77 antigen (Epa) (20). *epaR* encodes a putative glycosyltransferase with 5 predicted

78 transmembrane domains, and its role in Epa biosynthesis has not been investigated. The
79 product of *bgsB* is a putative cytoplasmic protein catalyzing the transfer of glucose from UDP-
80 glucose to diacylglycerol (DAG) to form monoglucosyl-DAG. An additional glucose is added to
81 glucosyl-DAG by *bgsA*, forming diglucosyl-DAG. From diglucosyl-DAG, the polymerization of
82 glycerol-phosphate can occur, resulting in lipoteichoic acid (LTA) (21). *iolA2* is predicted to
83 encode a methylmalonate-semialdehyde dehydrogenase, which catalyzes the breakdown of
84 malonic semialdehyde to acetyl-CoA and $CO_2$ (22). OG1RF_10252 is predicted to encode a
85 Acyl-ACP_TE domain (pfam01643; e-value $7.510e^{-114}$) which catalyzes the termination of fatty
86 acyl group extension by hydrolyzing an acyl group on the fatty acid.
87
88 To begin to elucidate the roles of these genes in ϕNPV1 susceptibility, we constructed in-frame
89 deletions of *epaR* and *bgsB*, generating strains OG1RF∆*epaR* and OG1RF∆*bgsB*, respectively,
90 and a double deletion strain, OG1RF∆*epaR*∆*bgsB*. Phage susceptibility of each of these
91 mutants was assayed. Deletion of *epaR* alone was sufficient to confer phage resistance (Figure
92 1). In contrast, deletion of *bgsB* alone did not alter phage susceptibility. These results indicate
93 that variation in *epaR* is the major factor conferring resistance to ϕNPV1 in OG1RF-C. Since we
94 observed that deletion of *epaR* in OG1RF conferred phage resistance to the same extent as
95 that observed for OG1RF-C, we did not investigate the effects of *iolA2* and OG1RF_10252 on
96 phage resistance in this study.
97
98 **The OG1RF-C *epaR* allele confers ϕNPV1 resistance.** To determine whether *epaR* mutation
99 is the major contributor to ϕNPV1 resistance in OG1RF-C, we generated strain
100 OG1RF∆*epaR*_Ec, an OG1RF∆*epaR* strain complemented *in cis* with the *epaR* allele from
101 OG1RF-C. We also generated strain OG1RF∆*epaR*_Ew, an OG1RF∆*epaR* strain with a
102 reconstituted wild-type *epaR*. Complementation with the *epaR* allele of OG1RF-C conferred
103 phage resistance to OG1RF∆*epaR* (Figure 1B). The wild-type *epaR* allele restored phage

104 susceptibility to OG1RF∆*epaR* (Figure 1B). Because the mutated *epaR* allele from OG1RF-C
105 confers a phage resistance phenotype, as did deletion of *epaR*, we infer that the *epaR* mutation
106 in OG1RF-C confers loss of function.
107
108 **Growth rates of *E. faecalis* strains.** We determined generation times for wild-type *E. faecalis*
109 OG1RF, OG1RF-C, and the *epaR* and *bgsB* deletion mutants and complemented strains
110 cultured in BHI broth (Table S1). Average generation times range from a minimum of 27.8
111 minutes for the *bgsB* deletion mutant to a maximum of 42.7 minutes for the *epaR bgsB* double
112 deletion mutant. The generation times for the wild-type and OG1RF-C strains were similar (30.4
113 versus 33.9 minutes).
114
115 ***epaR* is required for phage adsorption.** We were next interested in how *epaR* inactivation
116 protects OG1RF from ϕNPV1 infection. After 15 minutes incubation with ϕNPV1, ~95% of the
117 phage adsorbed to wild-type OG1RF (Figure 2). In contrast, under the same conditions, ~1-2%
118 of the phage adsorbed to OG1RF-C or OG1RF∆*epaR*. Consistent with the observation that
119 *bgsB* deletion alone confers no significant phage resistance, ~90% of the phage adsorbed to
120 OG1RF∆*bgsB* within the same experimental settings. These data indicate that *epaR* is required
121 for ϕNPV1 adsorption.
122
123 **Inactivation of *epaR* alters the Epa polymer.** Next, we sought to determine whether the Epa
124 polymer was altered in mutants defective for ϕNPV1 adsorption. The Epa polymer has been
125 extracted and visualized by different groups using different methods (20, 23). We based our
126 method on that from Teng, et al. (20). We found that solubilizing the precipitation with 50%
127 acetic acid improved visualization of the polymers. Gel electrophoresis analysis of carbohydrate
128 extracts found that OG1RF with either an *epaR* deletion or the *epaR* allele from OG1RF-C
129 exhibited loss of a band (P1) that is present in wild-type OG1RF, OG1RF∆*bgsB,* and the

130 reconstituted *epaR* strain, OG1RF∆*epaR*_Ew (Figure 3). We conclude that P1 represents an
131 *epaR*-dependent polymer. Note that the cationic dye Stains-All was used for polymer detection.
132 With the staining methodology used, we cannot state conclusively whether the P1 polymer fails
133 to be synthesized in *epaR* mutants, or if it is synthesized but has a different charge than in wild-
134 type OG1RF. Since NPV1 cannot bind to *epaR* mutants, we hypothesized that the P1 polymer
135 is the phage receptor. However, when we pre-incubated NPV1 with crude carbohydrate extract
136 prior to infection of host cells, we did not observe a decrease in PFU for any extract (Figure S1),
137 suggesting that the polymers in the crude extract are not sufficient for phage adsorption.
138
139 We observed an increase in the intensity of the P2 band in OG1RF∆*bgsB* and
140 OG1RF∆*bgsB*∆*epaR* compared to wild-type OG1RF, suggesting that the product P2 is
141 increased in these two deletion strains. Since deletion of *bgsB* in *E. faecalis* results in
142 accumulation of LTA (21), product P2 may represent LTA.
143
144 **NPV1-resistant mutants have increased susceptibility to daptomycin.** Dale et al. reported
145 increased daptomycin susceptibility in an OG1RF derivative with a deletion in *epaO* (23).
146 Moreover, we identified a *bgsB* mutation in a laboratory-evolved *E. faecium* isolate with
147 decreased daptomycin susceptibility (24). Because of these results, we investigated the
148 daptomycin susceptibilities of our OG1RF mutants (Figure 4). We found that OG1RF-C,
149 OG1RF∆*epaR*, and OG1RF∆*epaR* complemented with the OG1RF-C *epaR* allele were each
150 significantly more susceptible to daptomycin than OG1RF. Interestingly, deletion of *bgsB* also
151 conferred increased daptomycin susceptibility (Figure 4). This was complemented by
152 expression of the wild-type *bgsB* allele *in cis*, but not by expression of the OG1RF-C *bgsB* allele
153 *in cis* (Figure 4). Finally, daptomycin susceptibility was substantially altered in the
154 OG1RF∆*epaR*∆*bgsB* mutant, with 3 of 6 experimental trials resulting in an MIC below the level
155 of detection of the E-test strip (<0.016 µg/mL; a value of 0.008 µg/mL was used for these data

156 points in statistical analysis). Without complete data regarding the MIC of OG1RF∆*epaR*∆*bgsB*,
157 we did not quantitatively determine whether there is a synergistic relationship between *bgsB*
158 and *epaR* regarding daptomycin susceptibility.
159
160 ***epaR* mutants have increased sodium chloride stress susceptibility**. The *epa* gene cluster
161 was up-regulated when *E. faecalis* V583 was grown with 6.5% sodium chloride
162 supplementation, indicating that the Epa polymer has a role in osmotic stress response (25). As
163 such, we investigated the effect of sodium chloride on our *epaR* mutants. We tested our
164 mutants for their tolerance for sodium chloride stress using BHI agar supplemented with sodium
165 chloride at concentrations of 0%, 2.5%, 5%, and 7.5%. Overnight cultures in stationary phase
166 were serially diluted and spotted on these agars. We observed fewer CFU for OG1RF-C,
167 OG1RF∆*epaR*, and OG1RF ∆*epaR*∆*bgsB* compared to the wild-type at 7.5% sodium chloride
168 after 72 h incubation (Figure 5). When sodium chloride concentrations of 5% or lower were
169 used, no effect on growth was observed.
170
171 **Multiple different spontaneous *epaR* mutations confer ϕNPV1 resistance.** We isolated 9
172 spontaneous ϕNPV1-resistant mutants of OG1RF and sequenced the *epaR* region of these
173 mutants. All 9 mutants have non-synonymous substitutions in *epaR* (Table 3). Since we know
174 that mutations in *epaR* affect daptomycin susceptibility, we also determined the daptomycin MIC
175 of these ϕNPV1-resistant strains and found that all were more significantly more susceptible to
176 daptomycin than the wild-type (Figure S2).
177
178 Most of the EpaR sequence (amino acid positions 35-458 of 484 total) is a predicted sugar
179 transferase domain (TIGR03025; E-value: $2.45e^{-137}$). This domain consists of a conserved C-
180 terminal region responsible for the sugar transferase activity (pfam02397) and a variable N-
181 terminal region with predicted flippase activity. All *epaR* mutations in spontaneously ΦNPV1-

182 resistant OG1RF strains occur in the region encoding the sugar transferase domain (Table 3).
183 We identified other proteins containing the same predicted sugar transferase domain as EpaR,
184 and we determined that the altered amino acid positions in our mutants are conserved across
185 most of these proteins (Figure S3).
186
187 **Discussion**
188 Due to the high frequency of antibiotic resistance in *E. faecalis*, alternatives to antibiotics, such
189 as phage therapy, are of increasing interest in the United States. In this study, we investigated
190 mechanisms for spontaneous phage resistance in *E. faecalis*. We have reported here that *epaR*
191 is indispensable for ϕNPV1 adsorption to *E. faecalis* OG1RF, and that inactivating mutations in
192 *epaR* constitute a major pathway for ϕNPV1 resistance in this strain background. We also found
193 that inactivating mutations in *epaR* and *bgsB* result in increased susceptibilities to daptomycin
194 and sodium chloride stress. Our results show that resistance to ϕNPV1 comes at a cost.
195
196 Adsorption to the host is the first step to a productive phage infection. For a tailed phage particle
197 to successfully adsorb to the host, the tail apparatus on the phage must recognize the
198 corresponding receptor(s) on the host cell surface. When challenged with a high phage titer in a
199 resource-limited environment, receptor mutations in host cells are favored over use of
200 intracellular defense mechanisms (26). This preference for receptor mutations is an especially
201 important consideration in the design of phage cocktails, as using phage that recognize the
202 same receptors could result in decreased efficacy of the treatment (18). Receptors for
203 enterococcal phages have not been well studied. We and collaborators recently identified PIP
204 as a receptor for phage ϕVPE25 and ϕVFW, but PIP is not the sole player in host cell
205 recognition, as ϕVFW and ϕVPE25 can still adsorb to a PIP deletion strain (15). PIP may act as
206 a DNA channel as is implicated in studies of *L. lactis* (14).

207 The *epa* gene cluster is involved in the synthesis of a cell wall rhamnose polysaccharide
208 referred to as Epa. There is precedence for cell wall rhamnose polysaccharides as phage
209 receptors. The structure of the rhamnose polysaccharide dictates phage host range in *L. lactis*
210 and *S. mutans* (27, 28). In *E. faecalis*, the *epa* gene cluster consists of 18 core genes (*epaA* to
211 *epaR*) and a set of strain-variable genes that occur downstream (20, 29). Unfortunately, the Epa
212 structure has not been determined (30), which is a critical gap in knowledge about the
213 enterococcal cell surface.

214 The connection between the *epa* gene cluster and ϕNPV1 resistance was first investigated by
215 Teng et al, who assessed ΦNPV1 susceptibilities of *E. faecalis* OG1RF mutants with disruptions
216 in *epaA, epaB, epaE, epaM,* and *epaN* (20). No ϕNPV1 plaques were obtained for *epaB, epaE,*
217 *epaM,* and *epaN* mutants, and plaque production was reduced by 50% in the *epaA* mutant as
218 compared to the wild-type. However, when the wild-type strain and the *epaA* and *epaB* mutants
219 were assessed for ϕNPV1 adsorption, no differences were noted. Teng et al also examined the
220 polysaccharide content of their mutants and found that production of the 'P1' product was
221 absent in the *epaB, epaM, epaN* and *epaE* mutants, but a new polysaccharide product referred
222 to as 'PS12' was synthesized. For the *epaA* mutant, both P1 and P12 were produced. The
223 results from Teng et al. suggest that a complete Epa product is required for productive ϕNPV1
224 infection. Our results support this conclusion as ϕNPV1 does not absorb to our *epaR* mutants,
225 nor do *epaR* mutants synthesize the P1 (or P12) product. However, the P1 product may not be
226 the only requirement for ϕNPV1 adsorption because no significant decrease in PFU was
227 observed when ϕNPV1 was pre-incubated with crude polysaccharide extracts from OG1RF
228 strains with either wild-type or mutant *epaR* (Figure S1). Alternatively, the availability of P1 to
229 the phage may differ in whole cells versus crude extracts.
230

231 We investigated the daptomycin susceptibilities of our NPV1-resistant strains with *epaR*
232 mutations because a mutation elsewhere in the *epa* locus was previously linked to daptomycin
233 susceptibility in *E. faecalis*. Specifically, Dale et al. reported that deletion of *epaO* results in
234 increased daptomycin susceptibility (23). Daptomycin is a lipopeptide antibiotic that is used to
235 treat certain Gram-positive bacterial infections (31). The mechanism of action for daptomycin in
236 *B. subtilis* begins with daptomycin binding to the cell membrane, and ultimately leads to the
237 displacement of membrane-associated proteins essential for cell wall and phospholipid
238 biosynthesis (32). In our study, we found that inactivating mutations in *epaR* lead to increased
239 daptomycin susceptibility in *E. faecalis*. The loss of the Epa polymer results in defects in cell
240 wall architecture (20, 33), suggesting that this polymer plays a critical role in enterococcal cell
241 surface physiology. More research on the Epa polymer is required to mechanistically assess its
242 contribution to antibiotic susceptibility in enterococci. In our study, deletion of *bgsB* also resulted
243 in increased daptomycin susceptibility in *E. faecalis.* Deletion of *bgsB* results in loss of
244 glycolipids in the membrane, a longer chain length in the LTA, and increased charge density of
245 the membrane (21). A higher charge density might contribute to daptomycin susceptibility
246 through charge-charge interaction with the calcium-bound daptomycin, but this is speculative.
247 Note that a weakness of our study is that we did not evaluate whether susceptibilities to other
248 antibiotics are altered concomitantly with spontaneous ϕNPV1 resistance or as a result of *epaR*
249 or *bgsB* deletion. Therefore, we cannot comment on whether the altered antibiotic
250 susceptibilities of these strains are specific to daptomycin or are a general defect potentially
251 related to altered membrane/cell wall permeability.
252
253 In summary, in this study we characterized a mechanism for spontaneous ϕNPV1 resistance in
254 *E. faecalis* OG1RF and demonstrated that *in vitro* spontaneous ϕNPV1 resistance is
255 accompanied by fitness trade-offs including altered susceptibilities to an antibiotic and to
256 osmotic stress. Experiments for future work include determining the host range of ϕNPV1, and

257 whether other enterococcal phage use the Epa polymer as a receptor for *E. faecalis* adsorption.
258 A critical experiment in terms of possible therapeutic application of ϕNPV1 is to determine
259 whether ϕNPV1 resistance arises *in vivo* (i.e. in the gastrointestinal tract, or during experimental
260 treatment of an *E. faecalis* infection using phage therapy) by the same mechanism as *in vitro*. If
261 ϕNPV1 resistance arises *in vivo* by *epaR* or other *epa* locus inactivation, and this confers an *in*
262 *vivo* fitness cost to *E. faecalis*, then ϕNPV1 and/or other Epa-targeting phage could be of utility
263 for anti-*E. faecalis* therapies.
264

265 **Material and Methods**

266 **Bacterial strains, media, and bacteriophages.** A complete list of bacterial strains and
267 bacteriophage used in this study can be found in Table 1. *E. faecalis* strains were cultured in
268 brain heart infusion (BHI) at 37°C without agitation. *E. coli* strains were cultured in LB broth at
269 37°C with shaking at 225 rpm unless otherwise stated. Plates of the appropriate media were
270 made by adding 1.5% agar to the broth prior to autoclaving. For MIC testing, Muller Hinton
271 media supplemented with 1.5% agar (MHA) was used. Phages were stored in phage buffer as
272 previously described (34). Chloramphenicol (Cm) was used at a concentration of 15 µg/mL
273 when required for selection. 5-bromo 4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was
274 used at 120 µg/mL and 40 µg/ml for *E. faecalis* and *E. coli*, respectively. The upper soft agar for
275 phage assays was M17 medium supplemented with 0.75% agar, while the lower layer was BHI
276 supplemented with 1.5% agar.
277

278 **Routine molecular techniques and DNA sequencing.** Routine PCR reactions were
279 performed using Taq polymerase (NEB) per the manufacturer's instructions. Phusion
280 polymerase (Fisher) was used for cloning procedures per the manufacturer's instructions.
281 Plasmid was purified using the GeneJET Plasmid miniprep kit (Fisher). Genomic DNA was
282 isolated using the Ultraclean microbial DNA isolation kit (MoBio). Restriction enzymes, Klenow

283 fragment, T4 polynucleotide kinase (PNK), T4 DNA ligase, and calf intestinal phosphatase (CIP)
284 from NEB were used as instructed by the manufacturer. DNA sequencing was performed at the
285 Massachusetts General Hospital DNA sequencing facility. A complete list of primers used in this
286 study can be found in Table S2.
287
288 **Growth curves.** Growth curves were performed in triplicate with a Biotek Synergy microplate
289 reader essentially as previously described (35). Overnight broth cultures were diluted 1:1000
290 into fresh BHI broth and aliquoted into 96 well plates. The optical density at 600 nm of cultures
291 was monitored for 20 h.
292
293 **Phage spot assay.** 0.5 mL of an exponentially growing culture was added to 3 mL soft agar
294 and poured onto BHI agar. 10 µL of the of phage mixture to be titered was spotted onto the soft
295 agar. Plaques were counted after 16 h incubation at 37°C, unless otherwise stated.
296
297 **Phage propagation and storage.** Phage stocks were prepared by mixing 450 µL of an
298 overnight culture of *E. faecalis* OG1RF with ϕNPV1 at an M.O.I of $10^{-2}$. The mixture was
299 incubated at 37°C for 15 min and subsequently added to 3 mL M17 soft agar maintained at
300 55°C. The soft agar was then poured onto BHI agar and incubated at 37°C for 18 h. 5 mL of
301 phage buffer was added to the confluently lysed plate and incubated for 20 min at 37°C with
302 shaking at 75 rpm. The lysate was then collected and centrifuged at 16.6 x g for 1 min to
303 remove cellular debris. The supernatant was filtered with a Whatman 0.2 µm filter to obtain the
304 phage stock. The phage stock was stored at 4°C in the dark. Phage titer was determined using
305 phage spot assays.
306
307 **Generation of OG1RF deletion mutants.** Gene deletion was carried out via the markerless
308 deletion procedure described by Thurlow et al. (36) with some modifications. Briefly, two 1.0 kb

309 regions flanking *epaR* were amplified with primers 1-4 from Table S2. The two amplified
310 products were ligated with an overlap PCR extension through a 21 bp complementary region
311 underlined in Table S2. The approximately 2.0 kb product was purified and digested with BamHI
312 and EcoRI. The digested product was ligated to plasmid pLT06 through restriction sites added
313 on the primers (highlighted in red in Table S2). The ligation product was then purified and
314 electroporated and propagated in *E. coli* EC1000. OG1RF was made electrocompetent using
315 the glycine method (37) (3% glycine) and transformed with 1 µg of the plasmid. OG1RF
316 transformants were screened for successful transformation and subsequently inoculated in BHI
317 supplemented with Cm at 30°C. The culture was diluted 1:100 in BHI and incubated at 30°C for
318 2 h followed by 42°C for 4 h. Dilutions of the culture were plated on BHI agar supplemented with
319 Cm and X-gal, and large blue colonies were screened for plasmid integration using primers 5
320 and 19. The positive colonies were then restruck, incubated at 42°C, and screened once again
321 for plasmid integration. Positive clones were cultured in BHI broth at 30°C for 18 h. To counter-
322 select against clones harboring the plasmid, dilutions of the culture were made on MM9YEG
323 agar, and the deletion of *epaR* was determined by colony PCR with primers 5 and 6 after 36 h
324 incubation at 37°C. Clones positive for the deletion were then restruck on BHI agar and
325 screened again using the same primers. Positive clones were verified for plasmid loss by
326 streaking on BHI agar supplemented with Cm. The *epaR* region was sequenced to confirm the
327 deletion. Deletion of *bgsB* was obtained in a similar fashion.
328
329 **Complementation.** Complementation of *epaR* in an OG1RF∆*epaR* background was obtained
330 using a similar strategy to deletion. The insert containing the *epaR* gene and flanking 500 bp
331 upstream and downstream regions was amplified from either OG1RF or OG1RF-C using
332 primers 7 and 8. pLT06 was digested with SphI and blunt-ended with Klenow fragment; the
333 blunt-end product was then treated with CIP. The insert was phosphorylated with T4 PNK and
334 blunt-end ligated to pLT06. The plasmid was purified and transformed into EC1000. Clones with

335 the correct insert size were screened, and their plasmids isolated. Subsequent steps for
336 transformation of OG1RF, integration of the plasmid and counterselection on MM9YEG were as
337 described above for the deletion process. Positive clones for the complementation were
338 confirmed with primers 5 and 6 after counter-selection on MM9YEG. The complemented *epaR*
339 allele was verified through Sanger sequencing.
340
341 **Assessment of phage resistance.** For assessment of ϕNPV1 resistance, 500 µL of an $8\times10^9$
342 PFU ϕNPV1 stock was added to 3 mL M17 soft agar. The mixture was then poured onto BHI
343 agar. Bacterial culture dilutions were spotted on the soft agar and incubated at 37°C for 18 h.
344 ϕNPV1-resistant bacteria grow on ϕNPV1-containing agar. ϕNPV1-susceptible bacteria do not
345 grow on ϕNPV1-containing agar.
346
347 **Assessment of sodium chloride stress tolerance.** For assessment of osmotic stress
348 tolerance, BHI plates were supplemented with NaCl (0%, 2.5%, 5%, and 7.5%). Overnight
349 cultures of bacteria are serially diluted and spotted on NaCl-supplemented plates. Plates were
350 imaged after 72 h incubation.
351
352 **Phage adsorption assay.** An overnight bacterial culture was diluted 1:5 in fresh BHI broth. The
353 culture was then equilibrated at 37°C for 20 min in a water bath. ϕNPV1 was added at an M.O.I
354 of $10^{-3}$. After 15 min, a 1 mL aliquot was centrifuged at 16.6 x g for 1 min at room temperature.
355 500 µL of the supernatant was collected, and its titer is determined with the phage spot assay. A
356 medium with only phage added (no bacteria) was used as control. Percent adsorption was
357 determined as follow:

$$\text{Percent Adsorption}(\%) = \frac{PFU_{control} - PFU_{supernantant}}{PFU_{control}} \times 100\%$$

358

359 **Isolation of ϕNPV1 resistant mutants.** For isolation of a ϕNPV-1 resistant strain from an
360 OG1RF∆PIP background, ϕNPV-1 was used to infect OG1RF∆PIP in a soft agar overlay. The
361 confluently lysed plates were incubated until presumptive phage-resistant colonies arising in the
362 soft agar were observed. These colonies were cultured in BHI broth and used as hosts for
363 ϕNPV1 infection to confirm phage resistance. A confirmed ϕNPV-1 resistant strain, referred to in
364 our study as OG1RF-C, was stocked and used for genome sequencing.
365
366 For isolation of ϕNPV-1 resistant strains from an OG1RF background, 500 µL of an overnight
367 culture of OG1RF was infected with ϕNPV1 at an M.O.I of $10^{-1}$ in a soft agar overlay. 10
368 colonies that arose on the confluently lysed plate were struck on BHI plates and incubated at
369 37°C for 18 h. Single colonies from each of the plates were tested for phage resistance by
370 cross-streaking against ϕNPV1. 9 colonies that showed little to no lysis were stocked and used
371 for daptomycin susceptibility testing and *epaR* sequencing.
372
373 **Polysaccharide analysis.** Polysaccharide extraction was performed as described by Teng et
374 al. with some modifications (20). 200 mL of an overnight culture was centrifuged and
375 resuspended in 750 µL 50 mM Tris buffer, pH 7.5. Mutanolysin (0.25 U/µL) and lysozyme (5
376 mg/mL) were added to the suspension. The suspension was incubated at 37°C for 2 h.
377 Subsequently, 10 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.15 mg/mL DNase I, and 0.15 mg/mL RNase A
378 were added. After an additional 2 h incubation at 37°C, the suspension was centrifuged, and the
379 cellar debris discarded. Proteinase K (100 µg/mL) was added to the clear supernatant, and the
380 mixture was incubated for 16 h. Afterwards, the supernatant was extracted twice with
381 chloroform-phenol-isoamyl alcohol (Sigma Aldrich) and once with chloroform. Ethanol was
382 added to a final concentration of 80% to precipitate the polysaccharide. The precipitate was
383 collected by centrifugation and air-dried. The pellet was resuspended in 50% acetic acid (v/v) in
384 deionized water, and the insoluble material was removed by centrifugation. 30 µL was loaded

385 onto an 1% agarose gel and electrophoresed for 30 min at 130 V. The gel was soaked in
386 staining solution containing Stains-All (Alfa Aesar) and left overnight with gentle rocking. The
387 staining solution was 25% isopropanol, 10% formamide, 65% water, and 0.005% Stains-All.
388 After 18 h, the gel was destained under light for 40 min prior to visualization.
389
390 **Daptomycin MIC.** Daptomycin MIC was assessed using Etest strips (BioMérieux). 3-5 colonies
391 of similar sizes were resuspended in 500 µL BHI broth and distributed evenly over a MHA plate
392 using a sterile cotton swab. Please note that this inoculation method deviates from clinical
393 susceptibility testing criteria in that we did not determine the CFU of our inocula nor normalize
394 the density of the inocula to a McFarland standard. A daptomycin Etest strip was placed onto
395 the plate, and the plate was incubated for 18 h at 37°C. MIC was determined by recording the
396 number closest to the zone of inhibition. The MIC reported for each strain is the average of at
397 least three independent trials. For trials in which the daptomycin MIC was below the detection
398 limit of the strip (<0.016 µg/mL), the MIC was reported as 0.008 µg/mL for the purposes of
399 statistical analysis. Data were analyzed using the two-tailed unpaired Student's t test.
400
401 **Whole genome sequencing and analysis of OG1RF-C.** OG1RF-C genomic DNA was isolated
402 from overnight broth culture using the Ultraclean Microbial DNA Isolation kit (Mo Bio) per the
403 manufacturer's instruction. The gDNA was sequenced using MiSeq with 2 x 150 bp chemistry at
404 MR DNA (Shallowater, Texas). After sequencing, the reads were mapped to the complete
405 OG1RF reference (NC_017316.1) using CLC Genomics Workbench (Qiagen). Putative
406 mutations were detected using the basic variant detector in CLC Genomic Workbench. Variants
407 occurring at ≥50% frequency in the read assembly and resulting in non-synonymous
408 substitutions were confirmed with Sanger sequencing. BlastP and NCBI Conserved Domains
409 were used to analyze conserved domains in proteins. Amino acid alignment was performed with
410 CLUSTALW (38). Transmembrane helices were predicted with TMHMM version 2.0 (39).

411

412 **Accession numbers.** The Illumina reads for OG1RF-C have been deposited in the Sequence

413 Read Archive under accession number PRJNA450206.

414

415 **Acknowledgments**

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419

420

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422


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544
545

546 **Figure legends.**

547 **Figure 1. Phage susceptibility assays.** Overnight cultures were diluted in PBS and spotted on
548 BHI plates with or without $10^9$ PFU/mL phiNPV1. Images were taken after 18 h incubation at
549 37°C. The image shown is representative of three independent trials. (A) phiNPV1 susceptibility
550 of *E. faecalis* OG1RF and derivatives. (B) phiNPV1 susceptibility of complemented strains of
551 OG1RF∆*epaR.*

552

553 **Figure 2. phiNPV1 adsorption assays.** Overnight cultures were diluted 1:5 in fresh BHI and
554 equilibrated at 37°C. phiNPV1 was added at a M.O.I. of $10^{-2}$. After 15 min incubation, 1 mL of
555 each culture was centrifuged, and the supernatant was titered with the phage spot assay. A
556 medium with only phage was used as the control. Percent adsorption was calculated as [(PFU
557 culture – PFU control)/ PFU control] *100%. Data are the average of three independent trials.

558

559 **Figure 3. Carbohydrate extract analysis.** Carbohydrate was extracted from 200 mL overnight
560 cultures and visualized with Stains-all. The image shown is representative of two independent
561 trials.

562

563 **Figure 4. Daptomycin MICs of *E. faecalis* OG1RF and derivatives.** Daptomycin MIC was
564 determined by Etest. Data are the average of at least three independent trials. For statistical
565 analysis, DAP MICs were compared to that of wild-type OG1RF. ***, $p < 0.001$; and *, $p < 0.05$.
566 Note that the names of complemented strains (see Table 1) have been shortened for figure
567 clarity

568

569 **Figure 5. Susceptibility to sodium chloride-induced osmotic stress.** Overnight cultures
570 were diluted in PBS and spotted on BHI plates with or without sodium chloride. Images were
571 taken after 72 h incubation. The image shown is representative of three independent trials.

572

573 **Supplemental Figures**

574

575 **Figure S1: Percent PFU recovery from pre-incubation of phiNPV1 with polysaccharide**
576 **extract**. Polysaccharide extract was prepared as described above except for the final solvent
577 being phage buffer rather than 50% acetic acid. phiNPV1 was added at a PFU of $10^5$ and the
578 mixture was incubated for 15 minutes at 37°C. The PFU was evaluated with phage spot assay.
579 The percent PFU recovery was calculated using a phage buffer only as the base value. The
580 experiment was performed in duplicate.

581

582 **Figure S2: Daptomycin MIC of 9 phiNPV1 resistant strains derived from a confluently**
583 **lysed plate**. 3-5 colonies of indicated strains were resuspended in BHI and swabbed onto a
584 MHB plate. Daptomycin strip was applied to the surface of each plate. The plates were
585 incubated for 18 hours prior to MIC determination. The data represent the average of 6 trials.
586 MIC data points below the detection limit of 0.016 µg/mL were taken to be 0.008 µg/mL for
587 statistical treatment. ***, $p < 0.001$.

588

589 **Figure S3: Clustal W alignment of EpaR and its homologues**. Conserved residues are
590 highlighted in red. Blue asterisks indicate Blue asterisks indicate where substitutions occurred in
591 phiNPV1 resistant strains of OG1RF.

592

593 Table 1. Strains and plasmid used.
594

| Strains and plasmids | Description | Reference |
|---|---|---|
| ***E. faecalis* strains** | | |
| OG1RF | Human oral cavity isolate | (40, 41) |
| OG1RFΔPIP | PIP deletion strain | (15) |
| OG1RF-C | OG1RFΔPIP ϕNPV1-resistant strain | This work |
| OG1RFΔ*bgsB* | OG1RF *bgsB* deletion mutant | This work |
| OG1RFΔ*epaR* | OG1RF *epaR* deletion mutant | This work |
| OG1RFΔ*bgsB*Δ*epaR* | OG1RF *bgsB* and *epaR* double deletion mutant | This work |
| OG1RFΔ*epaR_Ew* | OG1RFΔ*epaR* with wild-type *epaR* complementation *in cis* | This work |
| OG1RFΔ*epaR_Ec* | OG1RFΔ*epaR* complemented with OG1RF-C *epaR* allele *in cis* | This work |
| OG1RFΔ*bgsB_Bw* | OG1RFΔ*bgsB* with wild-type *bgsB* complementation *in cis* | This work |
| OG1RFΔ*bgsB_Bc* | OG1RFΔ*bgsB* complemented with OG1RF-C *bgsB* allele *in cis* | This work |
| OG1RF_R2 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R3 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R4 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R5 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R6 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R7 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R8 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R9 | OG1RF ϕNPV1-resistant strain | This work |
| OG1RF_R10 | OG1RF ϕNPV1-resistant strain | This work |
| ***E. coli* strains** | | |
| EC1000 | Cloning host. Provides *repA in trans* | (42) |
| **Plasmids** | | |
| pLT06 | Cloning vector, temperature sensitive *repA*, $Cam^r$ | (36) |
| pLT06_Δ*bgsB* | *bgsB* deletion construct | This work |
| pLT06_Δ*epaR* | *epaR* deletion construct | This work |
| pLT06_Ew | *epaR* wild-type allele complementation construct | This work |
| pLT06_Ec | *epaR* OG1RF-C allele complementation construct | This work |
| pLT06_Bw | *bgsB* wild-type allele complementation construct | This work |
| pLT06_Bc | *bgsB* OG1RF-C allele complementation construct | This work |

595
596 Table 2. SNPs detected in OG1RF-C strain.

| **Position** | **Base pair change** | **Amino acid change** | **Annotation** |
|---|---|---|---|
| 1800903 | C→T | D361N | *epaR* |
| 1878245 | G→T | S453Y | *bgsB* |
| 2308650 | C→A | G134* | *iolA* |
| 260665 | Insertion of A | H44fs | OG1RF_10252 |

597

598 Table 3. *epaR* variations in spontaneous ϕNPV1-resistant *E. faecalis* OG1RF strains.
599

| **Strain** | **Mutation type** | **Mutation** | **Amino acid change** |
|---|---|---|---|
| R3 | Transversion | C→ A | G409V |
| R5 | | C→ A | D428Y |
| R7 | | C→ A | G409V |
| R10 | | C→ A | G409V |
| R8 | | C→ A | D428Y |
| R4 | | T→ A | K424* |
| R9 | Transition | C→ T | G378D |
| OG1RF-C | | C→ T | D361N |
| R6 | Deletion | Deletion of 101 bp | G372fs |
| R2 | Deletion | Deletion of 1 bp | D373fs |

600
601

Figure 1

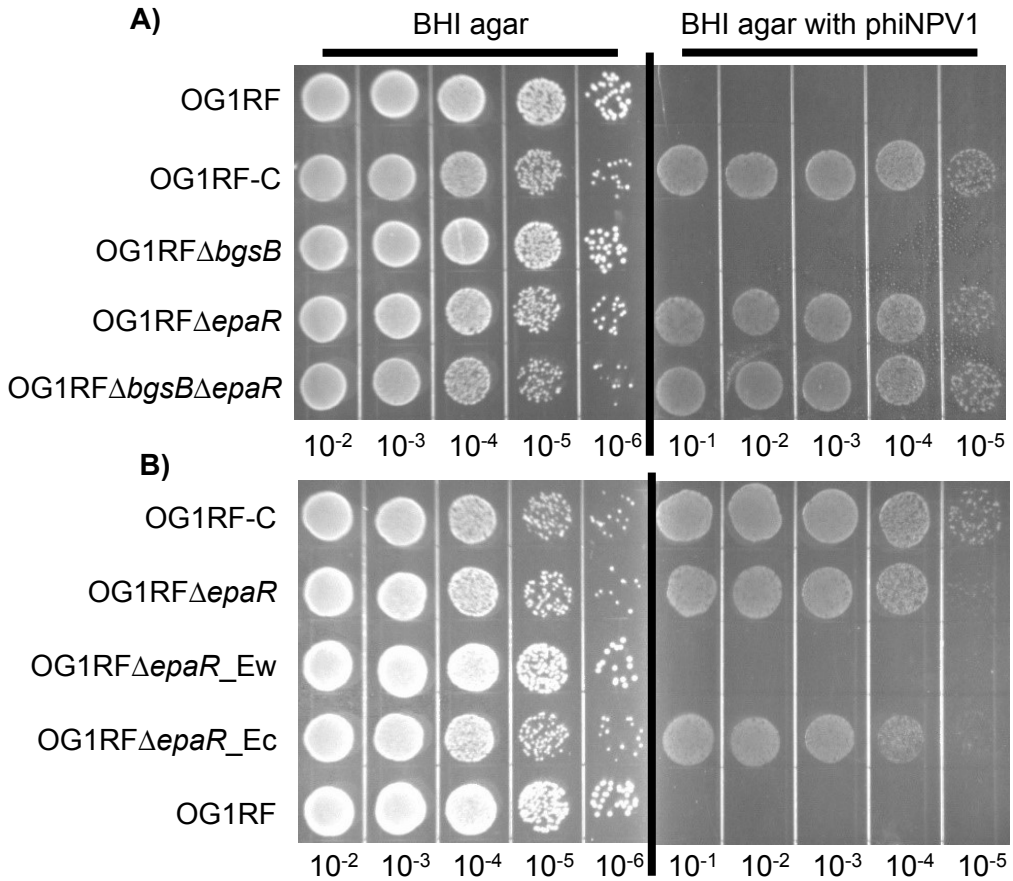
A)
BHI agar
BHI agar with phiNPV1
OG1RF
OG1RF-C
OG1RFΔ*bgsB*
OG1RFΔ*epaR*
OG1RFΔ*bgsB*Δ*epaR*
$10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$ $10^{-6}$ $10^{-1}$ $10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$
B)
OG1RF-C
OG1RFΔ*epaR*
OG1RFΔ*epaR*_Ew
OG1RFΔ*epaR*_Ec
OG1RF
$10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$ $10^{-6}$ $10^{-1}$ $10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$

Figure 2

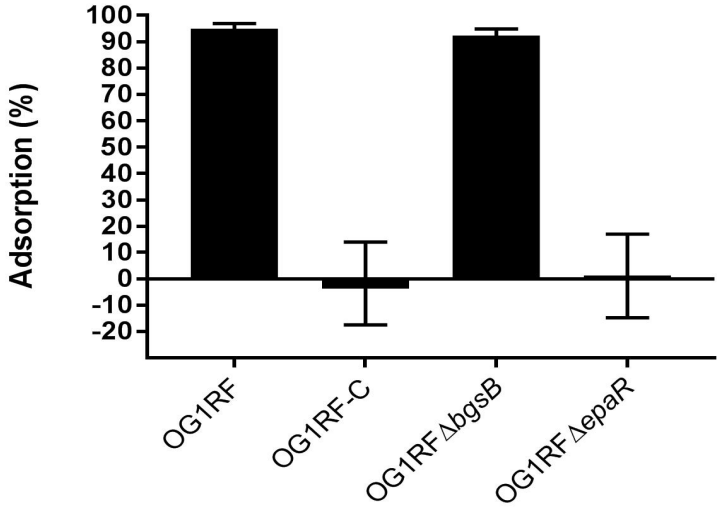
Adsorption (%)
100
90
80
70
60
50
40
30
20
10
0
-10
-20
OG1RF
OG1RF-C
OG1RFΔbgsB
OG1RFΔepaR

Figure 3

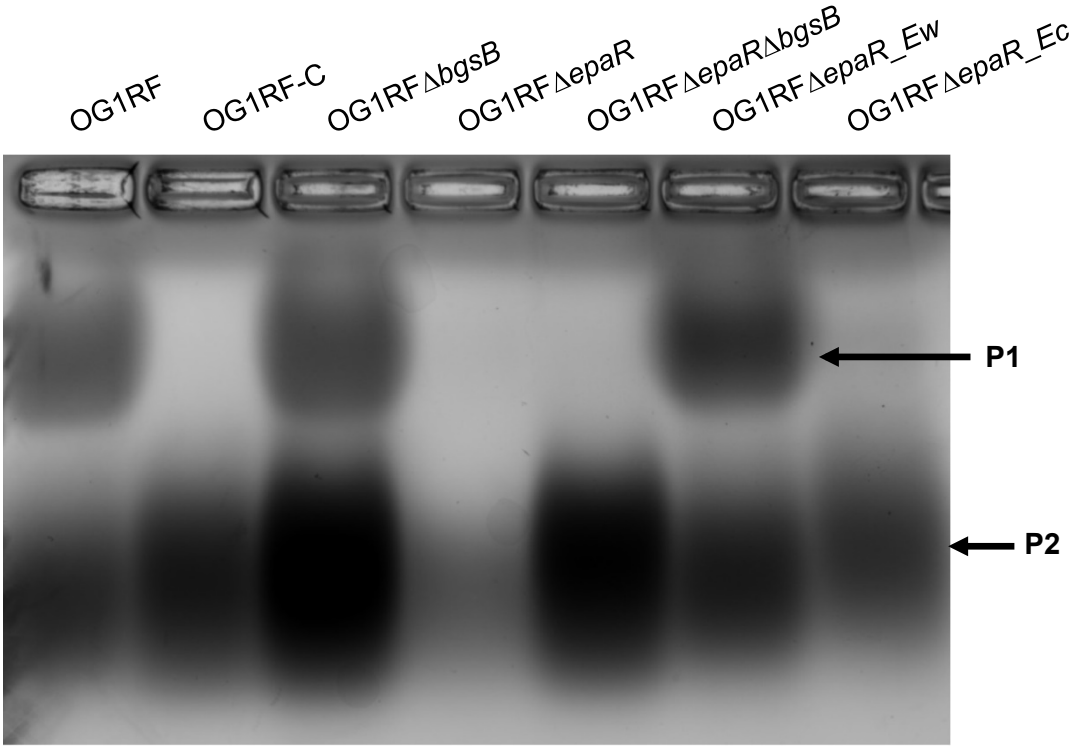
OG1RF
OG1RF-C
OG1RF Δ*bgsB*
OG1RF Δ*epaR*
OG1RF Δ*epaR*Δ*bgsB*
OG1RF Δ*epaR_Ew*
OG1RF Δ*epaR_Ec*
P1
P2

Figure 4

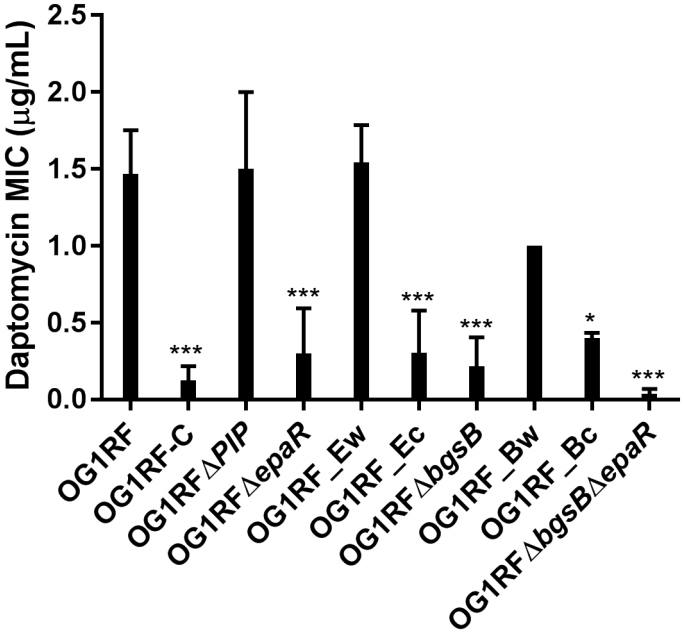
Daptomycin MIC (μg/mL)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
*
***
OG1RF
OG1RF-C
OG1RFΔPIP
OG1RFΔepaR
OG1RF_Ew
OG1RF_Ec
OG1RFΔbgsB
OG1RF_Bw
OG1RF_Bc
OG1RFΔbgsBΔepaR

Figure 5

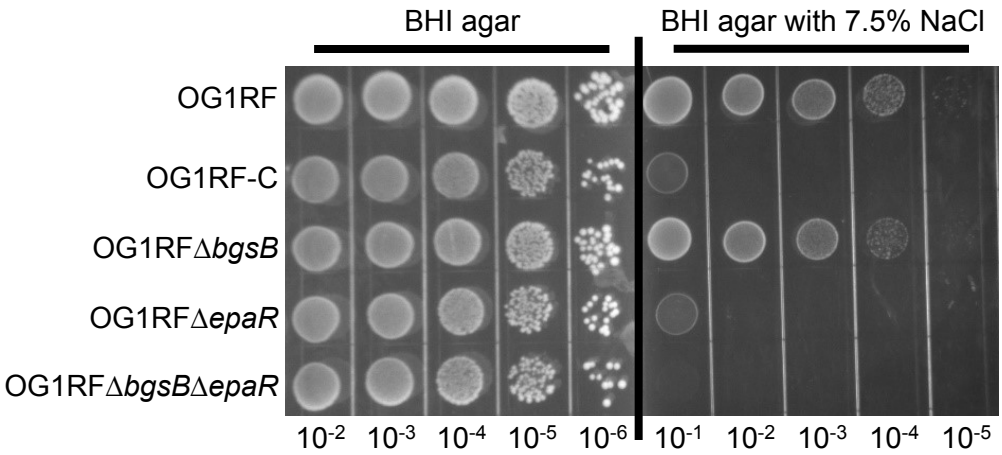
BHI agar
BHI agar with 7.5% NaCl
OG1RF
OG1RF-C
OG1RFΔ*bgsB*
OG1RFΔ*epaR*
OG1RFΔ*bgsB*Δ*epaR*
$10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$ $10^{-6}$
$10^{-1}$ $10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$

Table S1. Average generation times of *E. faecalis* strains.

| ***E. faecalis* strain** | **Average generation time (minutes)** |
|---|---|
| OG1RF | 30.4 |
| OG1RF-C | 33.9 |
| Δ*epaR* | 37.4 |
| Δ*epaR_Ew* | 34.1 |
| Δ*bgsB* | 27.8 |
| Δ*bgsB _Bw* | 39.7 |
| Δ*epaR* Δ*bgsB* | 42.7 |

Table S2. Primers used in this study.

| Number | Name | Sequence (5'-3') |
|---|---|---|
| 1 | epaR_arm1_BamHI | CGCGGATCCGCGCGATTGGTGCAACGTTGATGT |
| 2 | epaR_arm1_ovl | ACTAGCGCGGCCGCTTGCTCCTCGTCTTGCATTACTCCATTCAC |
| 3 | epaR_arm2_ovl | GGAGCAAGCGGCCGCGCTAGTGGATTGGATGAGTCTGAAGAACG |
| 4 | epaR_arm2_EcoRI | CCGGAATTCCGGTCCACTTTTCCTTTTCCTCCCT |
| 5 | EpaR_ins check for | GAAACCGCTGGGGACACATA |
| 6 | EpaR_ins check rev | TCTGGTTGGATTTCAAAATTTGAGACT |
| 7 | BgsB_arm1_BamHI | CGCGGATCCGCGGGCGTAAATAGCCATCGTATG |
| 8 | BgsB_arm1_ovl | ACTAGCGCGGCCGCTTGCTCCAGAAGTCGCTACCCCACTCA |
| 9 | BgsB_arm2_ovl | GGAGCAAGCGGCCGCGCTAGTACTTTGATCAACTTCAAATGGAAAAAG |
| 10 | BgsB_arm2_EcoRI | CCGGAATTCCGGAGGCTTTCATATAATTCTGTCGCT |
| 11 | BgsB_ins check for | ACGCTTTTATCCCTCAGAAGAA |
| 12 | BgsB_ins check rev | GGGACAAACAACACCTGTCG |
| 13 | epaR_KI for | CGCGGATCCGCGTTGGTGTCATTGGCTATCTGA |
| 14 | epaR_KI rev | CCGGAATTCCGGCGCTCATGAGTAATGTTGCCA |
| 15 | Orif | CAATAATCGCATCCGATTGCA |
| 16 | KS05 | CCTATTATACCATATTTTGGA |

1 **Supplemental Figures**

2 Figure S1

3

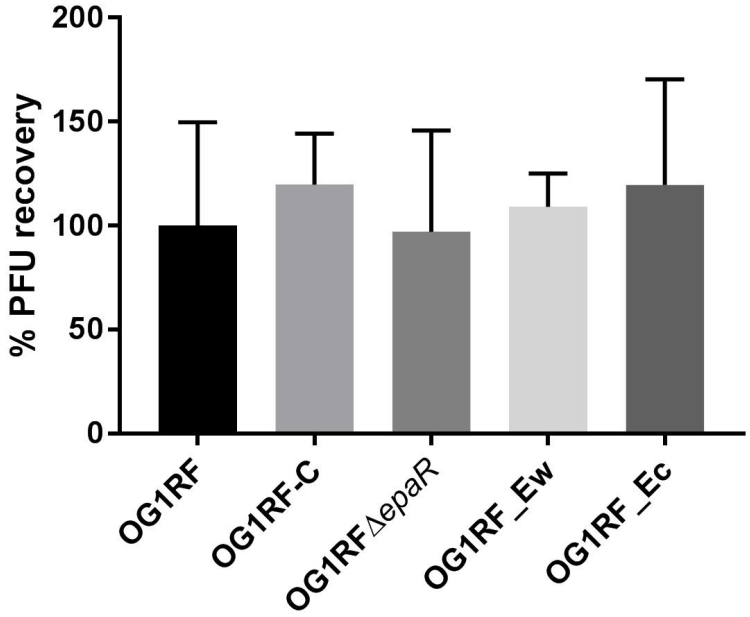


**Figure S1: Percent PFU recovery from pre-incubation of phiNPV1 with polysaccharide extract**. Polysaccharide extract was prepared as described above except for the final solvent being phage buffer rather than 50% acetic acid. phiNPV1 was added at a PFU of $10^5$ and the mixture was incubated for 15 minutes at 37°C. The PFU was evaluated with phage spot assay. The percent PFU recovery was calculated using a phage buffer only as the base value. The experiment was performed in duplicate.

4 Figure S2

5

6

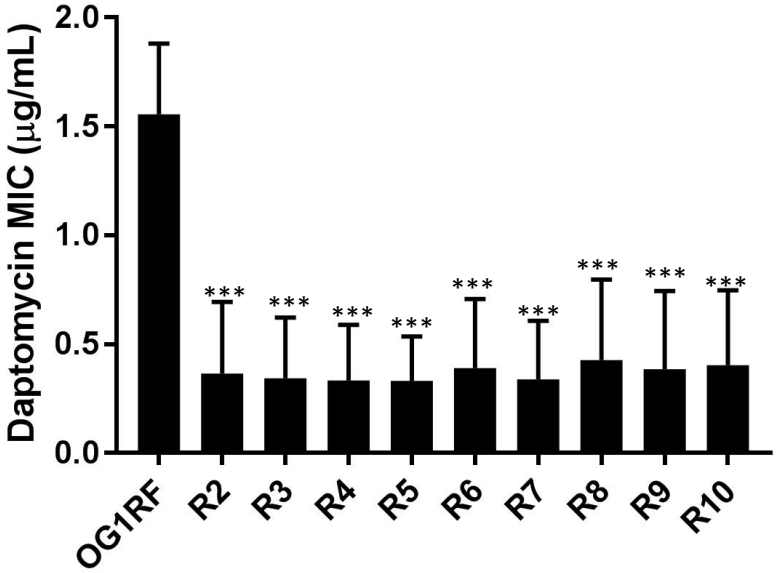


**Figure S2: Daptomycin MIC of 9 phiNPV1 resistant strains derived from a confluently lysed plate**. 3-5 colonies of indicated strains were resuspended in BHI and swabbed onto a MHB plate. Daptomycin strip was applied to the surface of each plate. The plates were incubated for 18 hours prior to MIC determination. The data represent the average of 6 trials. MIC data points below the detection limit of 0.016 μg/mL were taken to be 0.008 μg/mL for statistical treatment. ***, $p < 0.001$.

7

Figure S3

8

```
EpsB      ----QFAMQDAGNNLVVLTGPTPGVGKSFVSANLAAVIATGGKRVLLIDADMRKGYLHQY      590
EpaR      DELPQLINVIRG--DMSIVGPRPERPFFVDQFN-----------------QENPNY---Y      380
WcaJ      DELPQFINVLTG--GMSIVGPRPHAVAHNEQYR-----------------QLIEGY---M      399
GumD      DELPQIFNVLGG--SMSIVGPRPHAAQHNTHYE-----------------KLINHY---M      419
WbaP      DELPQLFNVLKG--DMSLVGPRPIVSDELERYC-----------------DDVDY-----      417
CpsE      DELPQFYNVLKG--DMSLVGTRPPTVDEYEKYN-----------------STQKR-----      389
          *                   *

EpsB      FGKDRKPGLLDLLA--GNRSIEQVVHREVVPGLDFIATGLFPHNPSELLLNPRMVELMDT      648
EpaR      LRHNVRAGITGYAQVYGKYAS-----------------------------------DY-N      404
WcaJ      LRHKVKPGITGWAQINGWRGET--------DTLE----------------------KM-E      428
GumD      QRHYVKPGITGWAQVNGFRGET--------PELR----------------------TM-K      448
WbaP      -YLMAKPGMTGLWQVSGRNDV-----------------------------------DY-D      440
CpsE      -RLSFKPGITGLWQISGRNNIT----------------------------------DF-D      413
                     *

EpsB      FRSQYDLVLVDTPPVLAVADTAILAARAGLVLLVTRFERSTLGEIRETIKQLQHANVDVR      708
EpaR      SKLNFDLLYIKNYSLL--LDMKILLQTIKILFDKVSSRGLDESEEREEL-----------      451
WcaJ      KRVEFDLEYIREWSVW--FDIKIVFLTVFKGFVNKAAY----------------------      464
GumD      KRIQYDLDYIRRWSLW--LDIRIIVLTAVRVLGQKTAY----------------------      484
WbaP      TRVYFDSWYVKNWTLW--NDIAILFKTAKVVLRRDGAY----------------------      476
CpsE      EIVKLDVQYINEWSIW--SDIKIILLTLKVVLLGTGAK----------------------      449
           *   *
```

**Figure S3: Clustal W alignment of EpaR and its homologues**. Conserved residues are highlighted in red. Blue asterisks indicate where substitutions occurred in phiNPV1 resistant strains of OG1RF.