Spontaneous resistance to phage NPV1 confers increased daptomycin susceptibility in *Enterococcus faecalis* OG1RF

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Abstract

Enterococcus faecalis is a Gram-positive opportunistic pathogen that inhabits the 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 resistance to ϕNPV1 and their effects on *E. faecalis* susceptibilities to daptomycin and sodium chloride-induced osmotic stress. 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 loss of an extracellular polymer in strains with inactivated *epaR.* Phage-resistant *epaR* mutants exhibited increased daptomycin and osmotic stress susceptibility. Our results demonstrate that spontaneous resistance to ϕNPV1 comes at a cost in *E. faecalis* OG1RF, resulting in concomitant increased susceptibilities to daptomycin and sodium chloride-induced osmotic stress.

28 **Introduction**

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

35

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

44

45 One advantage of phage therapy is limited damage to the native microbiome because of the
46 specificity of the phage to its host (7). The first step to a successful phage infection is the
47 attachment of the phage particle to the proper receptor present on the surface of the host cell.
48 Phage receptors have been extensively studied in certain phage families, including the T series
49 phages, Mu, and λ for Gram-negative bacteria (8-11). Some phage receptors have been
50 characterized in Gram-positive bacteria, including receptors for ϕSPP-1 of *Bacillus subtilis* (12)
51 and the phage c2 group of *Lactococcus lactis* (13, 14). YueB, the ϕSPP-1 receptor, and PIP, the
52 phage c2 receptor, are orthologs and are required for irreversible phage adsorption (12).
53 Enterococcal phage receptors have not been well-characterized. Previously, we and

54 collaborators identified PIP as a receptor and potential DNA channel for the *E. faecalis* phages
55 ϕVPE25 and ϕVFW (15).
56
57 Bacteria can evolve phage resistance. Mechanisms of phage resistance include modification or
58 loss of the phage receptor (16). However, as phage receptors generally serve physiological
59 functions in the cell, the modification or loss of a receptor could come at a cost for the bacterial
60 host. Indeed, spontaneous phage-resistant mutants have altered antibiotic sensitivity in *P.*
61 *aeruginosa* (17). Phages utilizing receptors that have roles in antibiotic resistance could be
62 advantageous for re-sensitizing resistant bacteria to antibiotics.
63
64 Considering the increasingly limited treatment options for *E. faecalis* infections and the revival of
65 interest in using phage therapy to treat bacterial infections, it is crucial that we know the
66 receptor(s) of enterococcal phages since effective phage cocktails use phages targeting
67 multiple different receptors (18). Moreover, the roles of these receptors in enterococcal
68 physiology should be elucidated. In this report, we used a combination of genomic and genetic
69 approaches to identify a receptor for the tailed, virulent phage ϕNPV1 (19, 20), which infects *E.*
70 *faecalis* OG1RF by a PIP-independent mechanism.
71
72 **Results**
73 **Deletion of *epaR* alters susceptibility to ϕNPV1**. We isolated a OG1RFΔPIP (15) strain with
74 spontaneous resistance to ϕNPV1 (Figure 1). We refer to this strain as OG1RF-C. The genome
75 sequence of OG1RF-C was determined. We identified non-synonymous substitutions in *epaR,*
76 *bgsB, iolA2*, and OG1RF_10252 (Table 2). *epaR* is one of the 18 conserved genes of the *epa*
77 gene cluster (*epaA-epaR*), which codes for synthesis of the enterococcal polysaccharide
78 antigen (Epa) (20). *epaR* encodes a putative glycosyltransferase with 5 predicted
79 transmembrane domains, and its role in Epa biosynthesis has not been investigated. The

80 product of *bgsB* is a putative cytoplasmic protein catalyzing the transfer of glucose from UDP-
81 glucose to diacylglycerol (DAG) to form monoglucosyl-DAG. An additional glucose is added to
82 glucosyl-DAG by *bgsA*, forming diglucosyl-DAG. From diglucosyl-DAG, the polymerization of
83 glycerol-phosphate can occur, resulting in lipoteichoic acid (LTA) (21). *iolA2* is predicted to
84 encode a methylmalonate-semialdehyde dehydrogenase, which catalyzes the breakdown of
85 malonic semialdehyde to acetyl-CoA and CO_2 (22). OG1RF_10252 is predicted to encode a
86 Acyl-ACP_TE domain (pfam01643; e-value $7.510e^{-114}$) which catalyzes the termination of fatty
87 acyl group extension by hydrolyzing an acyl group on the fatty acid.
88
89 To begin to elucidate the roles of these genes in ϕNPV1 susceptibility, we constructed in-frame
90 deletions of *epaR* and *bgsB*, generating strains OG1RF∆*epaR* and OG1RF∆*bgsB*, respectively,
91 and a double deletion strain, OG1RF∆*epaR*∆*bgsB*. Phage susceptibility of each of these
92 mutants was assayed. Deletion of *epaR* alone was sufficient to confer phage resistance (Figure
93 1). In contrast, deletion of *bgsB* alone did not alter phage susceptibility. These results indicate
94 that variation in *epaR* is the major factor conferring resistance to ϕNPV1 in OG1RF-C. Since we
95 observed that deletion of *epaR* in OG1RF conferred phage resistance to the same extent as
96 that observed for OG1RF-C, we did not investigate the effects of *iolA2* and OG1RF_10252 on
97 phage resistance in this study.
98
99 **The OG1RF-C *epaR* allele confers ϕNPV1 resistance.** To determine whether *epaR* mutation
100 is the major contributor to ϕNPV1 resistance in OG1RF-C, we generated strain
101 OG1RF∆*epaR*_Ec, an OG1RF∆*epaR* strain complemented *in cis* with the *epaR* allele from
102 OG1RF-C. We also generated strain OG1RF∆*epaR*_Ew, an OG1RF∆*epaR* strain with a
103 reconstituted wild-type *epaR*. Complementation with the *epaR* allele of OG1RF-C conferred
104 phage resistance to OG1RF∆*epaR* (Figure 2). The wild-type *epaR* allele restored phage
105 susceptibility to OG1RF∆*epaR* (Figure 2). Because the mutated *epaR* allele from OG1RF-C

106 confers a phage resistance phenotype, as did deletion of *epaR*, we infer that the *epaR* mutation
107 in OG1RF-C confers loss of function.
108
109 ***epaR* is required for phage adsorption.** We were next interested in how *epaR* inactivation
110 protects OG1RF from ϕNPV1 infection. After 15 minutes incubation with ϕNPV1, ~95% of the
111 phage adsorbed to wild-type OG1RF (Figure 3). In contrast, under the same conditions, ~1-2%
112 of the phage adsorbed to OG1RF-C or OG1RF∆*epaR*. Consistent with the observation that
113 *bgsB* deletion alone confers no significant phage resistance, ~90% of the phage adsorbed to
114 OG1RF∆*bgsB* within the same experimental settings. These data indicate that *epaR* is required
115 for ϕNPV1 adsorption.
116
117 **Inactivation of *epaR* leads to a loss of a polymer.** Next, we sought to determine whether the
118 Epa polymer was intact in mutants defective for ϕNPV1 adsorption. The Epa polymer has been
119 extracted and visualized by different groups using different methods (20, 23). We based our
120 method on that from Teng, et al. (20). We found that solubilizing the precipitation with 50%
121 acetic acid improved visualization of the polymers. Gel electrophoresis analysis of carbohydrate
122 extracts found that OG1RF with either an *epaR* deletion or the *epaR* allele from OG1RF-C
123 exhibited loss of a band (P1) that is present in wild-type OG1RF, OG1RF∆*bgsB,* and the
124 reconstituted *epaR* strain, OG1RF∆*epaR*_Ew (Figure 4). We conclude that P1 represents an
125 *epaR*-dependent polymer. Since NPV1 cannot bind to *epaR* mutants, we hypothesized that the
126 P1 polymer is the phage receptor. However, we when pre-incubated NPV1 with crude
127 carbohydrate extract prior to infection of host cells, we did not observe a decrease in PFU for
128 any extract (Figure S1), suggesting that the polymers in the crude extract are not sufficient for
129 phage adsorption. We also observed an increase in the intensity of the P2 band in
130 OG1RF∆*bgsB* and OG1RF∆*bgsB*∆*epaR* compared to wild-type OG1RF, suggesting that the

131 product P2 is increased in these two deletion strains. Since deletion of *bgsB* in *E. faecalis*
132 results in accumulation of LTA (21), product P2 may represent LTA.
133
134 **NPV1-resistant mutants have increased susceptibility to daptomycin.** Dale et al. reported
135 increased daptomycin susceptibility in an OG1RF derivative with a deletion in *epaO* (23).
136 Moreover, we identified a *bgsB* mutation in a laboratory-evolved *E. faecium* isolate with
137 decreased daptomycin susceptibility (24). Because of these results, we investigated the
138 daptomycin MIC of our OG1RF mutants (Figure 5). We found that OG1RF-C, OG1RFΔ*epaR*,
139 and OG1RFΔ*epaR* complemented with the OG1RF-C *epaR* allele were each significantly more
140 susceptible to daptomycin than OG1RF. Interestingly, deletion of *bgsB* also conferred increased
141 daptomycin susceptibility (Figure 5). This was complemented by expression of the wild-type
142 *bgsB* allele *in cis*, but not by expression of the OG1RF-C *bgsB* allele *in cis* (Figure 5). Finally,
143 daptomycin susceptibility was substantially altered in the OG1RF∆*epaR*∆*bgsB* mutant, with 3 of
144 6 experimental trials resulting in an MIC below the level of detection of the E-test strip (<0.016
145 µg/mL; a value of 0.008 µg/mL was used for these data points in statistical analysis). Without
146 complete data regarding the MIC of OG1RF∆*epaR*∆*bgsB*, we did not quantitatively determine
147 whether there is a synergistic relationship between *bgsB* and *epaR* regarding daptomycin
148 susceptibility.
149
150 ***epaR* protects OG1RF from osmotic stress**. The *epa* gene cluster was up-regulated when *E.*
151 *faecalis* V583 was grown with 6.5% sodium chloride supplementation, indicating that the Epa
152 polymer has a role in osmotic stress response (25). As such, we investigated the effect of
153 sodium chloride on our *epaR* mutants. We tested our mutants for their tolerance for sodium
154 chloride stress using BHI agar supplemented with sodium chloride at concentrations of 0%,
155 2.5%, 5%, and 7.5%. Overnight cultures in stationary phase were serially diluted and spotted on
156 these agars. We observed fewer CFU for OG1RF-C, OG1RF∆*epaR*, and OG1RF ∆*epaR*∆*bgsB*

157 compared to the wild-type at 7.5% sodium chloride after 72 h incubation (Figure 6). When
158 sodium chloride concentrations of 5% or lower were used, no effect on growth was observed.
159 Interestingly, when cells from an overnight culture were spotted, OG1RF∆*bgsB* showed no
160 difference in CFU compared to the wild-type (Figure 6). However, when cells were cultured in
161 fresh BHI broth 1 h prior to spotting, a CFU difference was observed for OG1RF∆*bgsB* with
162 7.5% sodium chloride (Figure S2). These data suggest that both *epaR* and *bgsB* play roles in
163 sodium chloride-induced osmotic stress response.
164
165 **EpaR constitutes a major mutational pathway for ϕNPV1 resistance.** We isolated 9
166 spontaneous ϕNPV1-resistant mutants of OG1RF and sequenced the *epaR* region of these
167 mutants. All 9 mutants have non-synonymous substitutions in *epaR* (Table 3). Since we know
168 that mutations in *epaR* affect daptomycin susceptibility, we also determined the daptomycin MIC
169 of these ϕNPV1-resistant strains and found that all were more significantly more susceptible to
170 daptomycin than the wild-type (Figure S3).
171
172 Most of the EpaR sequence (amino acid positions 35-458 of 484 total) is a predicted sugar
173 transferase domain (TIGR03025; E-value: $2.45e^{-137}$). This domain consists of a conserved C-
174 terminal region responsible for the sugar transferase activity (pfam02397) and a variable N-
175 terminal region with predicted flippase activity. All *epaR* mutations in spontaneously ΦNPV1-
176 resistant OG1RF strains occur in the C-terminal region of the sugar transferase domain (Table
177 3). We identified other proteins containing the same predicted sugar transferase domain as
178 EpaR, and we determined that the altered amino acid positions in our mutants are conserved
179 across 6 of these proteins (Figure S4).
180
181
182

183 **Discussion**

184 Due to the high frequency of antibiotic resistance in *E. faecalis*, alternatives to antibiotics, such
185 as phage therapy, are of increasing interest in the United States. In this study, we investigated
186 mechanisms for spontaneous phage resistance in *E. faecalis*. We have reported here that *epaR*
187 is indispensable for ϕNPV1 adsorption to *E. faecalis* OG1RF, and that inactivating mutations in
188 *epaR* constitute a major pathway for ϕNPV1 resistance in this strain background. We also found
189 that inactivating mutations in *epaR* and *bgsB* result in increased susceptibilities to daptomycin
190 and sodium chloride stress. Our results show that resistance to ϕNPV1 comes at a cost.

191

192 Adsorption to the host is the first step to a productive phage infection. For a tailed phage particle
193 to successfully adsorb to the host, the tail apparatus on the phage must recognize the
194 corresponding receptor(s) on the host cell surface. When challenged with a high phage titer in a
195 resource-limited environment, receptor mutations in host cells are favored over use of
196 intracellular defense mechanisms (26). This preference for receptor mutations is an especially
197 important consideration in the design of phage cocktails, as using phage that recognize the
198 same receptors could result in decreased efficacy of the treatment (18). Receptors for
199 enterococcal phages have not been well studied. We and collaborators recently identified PIP
200 as a receptor for phage ϕVPE25 and ϕVFW, but PIP is not the sole player in host cell
201 recognition, as ϕVFW and ϕVPE25 can still adsorb to a PIP deletion strain (15). PIP may act as
202 a DNA channel as is implicated in studies of *L. lactis* (14).

203

204 The *epa* gene cluster is involved in the synthesis of a cell wall rhamnose polysaccharide
205 referred to as the **e**nterococcal **p**olysaccharide **a**ntigen (Epa). There is precedence for cell wall
206 rhamnose polysaccharides as phage receptors. The structure of the rhamnose polysaccharide
207 dictates phage host range in *L. lactis* and *S. mutans* (27, 28). In *E. faecalis*, the *epa* gene

208 cluster consists of 18 core genes (*epaA* to *epaR*) and a set of strain-variable genes that occur
209 downstream (20, 29). Unfortunately, the Epa structure has not been determined (30), which is a
210 critical gap in knowledge about the enterococcal cell surface.

211

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

228

229 Daptomycin is a lipopeptide antibiotic that is used to treat certain Gram-positive bacterial
230 infections (31). The mechanism of action for daptomycin in *B. subtilis* begins with daptomycin
231 binding to the cell membrane, and ultimately leads to the displacement of membrane-associated
232 proteins essential for cell wall and phospholipid biosynthesis (32). In our study, we found that

233 inactivating mutations in *epaR* and in *bgsB* lead to increased daptomycin susceptibility in *E.*
234 *faecalis*. Deletion of *bgsB* results in loss of glycolipids in the membrane, a longer chain length in
235 the LTA, and increased charge density of the membrane (21). A higher charge density might
236 contribute to daptomycin susceptibility through charge-charge interaction with the calcium-
237 bound daptomycin, but this is speculative. Dale et al. reported that deletion of *epaO* results in
238 increased daptomycin susceptibility and loss of the Epa polymer, suggesting that the complete
239 Epa polymer is important for daptomycin resistance (23). The loss of the Epa polymer also
240 results in defects in cell wall architecture (20, 33). More research on the Epa polymer is required
241 to mechanistically assess its contribution to antibiotic susceptibility in enterococci.
242
243 In summary, in this study we characterized a mechanism for spontaneous ϕNPV1 resistance in
244 *E. faecalis* OG1RF. The major advance of this study is that we determined that phage
245 resistance comes at a cost to *E. faecalis* OG1RF; specifically, increased daptomycin
246 susceptibility. *E. faecalis* OG1RF is a natively daptomycin-sensitive strain that became more
247 sensitive as a result of phage resistance. We did not assess whether ϕNPV1 treatment could
248 reverse resistance in daptomycin-resistant clinical isolates. This is an exciting area for future
249 research.
250
251 **Material and Methods**
252 **Bacterial strains, media, and bacteriophages.** A complete list of bacterial strains and
253 bacteriophage used in this study can be found in Table 1. *E. faecalis* strains were cultured in
254 brain heart infusion (BHI; Difco) at 37°C without agitation. *E. coli* strains were cultured in LB
255 broth at 37°C with shaking at 225 rpm unless otherwise stated. Plates of the appropriate media
256 were made by adding 1.5% agar to the broth prior to autoclaving. For MIC testing, Muller Hinton
257 media supplemented with 1.5% agar (MHA) was used. Phages were stored in phage buffer as
258 previously described (34). Chloramphenicol (Cm) was used at a concentration of 15 µg/mL

259 when required for selection. 5-bromo 4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was
260 used at 120 µg/mL and 40 µg/ml for *E. faecalis* and *E. coli*, respectively. The upper soft agar for
261 phage assays was M17 medium supplemented with 0.75% agar, while the lower layer was BHI
262 supplemented with 1.5% agar.
263
264 **Routine molecular techniques and DNA sequencing.** Routine PCR reactions were
265 performed using Taq polymerase (NEB) per the manufacturer's instructions. Phusion
266 polymerase (Fisher) was used for cloning procedures per the manufacturer's instructions.
267 Plasmid was purified using the GeneJET Plasmid miniprep kit (Fisher). Genomic DNA was
268 isolated using the Ultraclean microbial DNA isolation kit (MoBio). Restriction enzymes, Klenow
269 fragment, T4 polynucleotide kinase (PNK), T4 DNA ligase, and calf intestinal phosphatase (CIP)
270 from NEB were used as instructed by the manufacturer. DNA sequencing was performed at the
271 Massachusetts General Hospital DNA sequencing facility. A complete list of primers used in this
272 study can be found in Table S1.
273
274 **Phage spot assay.** 0.5 mL of an exponentially growing culture was added to 3 mL soft agar
275 and poured onto BHI agar. 10 µL of the of phage mixture to be titered was spotted onto the soft
276 agar. Plaques were counted after 16 h incubation at 37°C, unless otherwise stated.
277
278 **Phage propagation and storage.** Phage stocks were prepared by mixing 450 µL of an
279 overnight culture of *E. faecalis* OG1RF with ϕNPV1 at an M.O.I of 10^{-2}. The mixture was
280 incubated at 37°C for 15 min and subsequently added to 3 mL M17 soft agar maintained at
281 55°C. The soft agar was then poured onto BHI agar and incubated at 37°C for 18 h. 5 mL of
282 phage buffer was added to the confluently lysed plate and incubated for 20 min at 37°C with
283 shaking at 75 rpm. The lysate was then collected and centrifuged at 16.6 x g for 1 min to
284 remove cellular debris. The supernatant was filtered with a Whatman 0.2 µm filter to obtain the

285 phage stock. The phage stock was stored at 4°C in the dark. Phage titer was determined using
286 phage spot assays.
287
288 **Generation of OG1RF deletion mutants.** Gene deletion was carried out via the markerless
289 deletion procedure described by Thurlow et al. (35) with some modifications. Briefly, two 1.0 kb
290 regions flanking *epaR* were amplified with primers 1-4 from Table S1. The two amplified
291 products were ligated with an overlap PCR extension through a 21 bp complementary region
292 underlined in Table S1. The approximately 2.0 kb product was purified and digested with BamHI
293 and EcoRI. The digested product was ligated to plasmid pLT06 through restriction sites added
294 on the primers (highlighted in red in Table S1). The ligation product was then purified and
295 electroporated and propagated in *E. coli* EC1000. OG1RF was made electrocompetent using
296 the glycine method (36) (3% glycine) and transformed with 1 µg of the plasmid. OG1RF
297 transformants were screened for successful transformation and subsequently inoculated in BHI
298 supplemented with Cm at 30°C. The culture was diluted 1:100 in BHI and incubated at 30°C for
299 2 h followed by 42°C for 4 h. Dilutions of the culture were plated on BHI agar supplemented with
300 Cm and X-gal, and large blue colonies were screened for plasmid integration using primers 5
301 and 19. The positive colonies were then restruck, incubated at 42°C, and screened once again
302 for plasmid integration. Positive clones were cultured in BHI broth at 30°C for 18 h. To counter-
303 select against clones harboring the plasmid, dilutions of the culture were made on MM9YEG
304 agar, and the deletion of *epaR* was determined by colony PCR with primers 5 and 6 after 36 h
305 incubation at 37°C. Clones positive for the deletion were then restruck on BHI agar and
306 screened again using the same primers. Positive clones were verified for plasmid loss by
307 streaking on BHI agar supplemented with Cm. The *epaR* region was sequenced to confirm the
308 deletion. Deletion of *bgsB* was obtained in a similar fashion.
309

310 **Complementation.** Complementation of *epaR* in an OG1RF∆*epaR* background was obtained
311 using a similar strategy to deletion. The insert containing the *epaR* gene and flanking 500 bp
312 upstream and downstream regions was amplified from either OG1RF or OG1RF-C using
313 primers 7 and 8. pLT06 was digested with SphI and blunt-ended with Klenow fragment; the
314 blunt-end product was then treated with CIP. The insert was phosphorylated with T4 PNK and
315 blunt-end ligated to pLT06. The plasmid was purified and transformed into EC1000. Clones with
316 the correct insert size were screened, and their plasmids isolated. Subsequent steps for
317 transformation of OG1RF, integration of the plasmid and counterselection on MM9YEG were as
318 described above for the deletion process. Positive clones for the complementation were
319 confirmed with primers 5 and 6 after counter-selection on MM9YEG. The complemented *epaR*
320 allele was verified through Sanger sequencing.
321
322 **Assessment of phage resistance and sodium chloride stress tolerance.** For assessment of
323 phage resistance, 500 µL of an 8×10^9 PFU phage stock was added to 3 mL M17 soft agar. The
324 mixture was then poured onto BHI agar. Bacterial culture dilutions were spotted on the soft agar
325 and incubated at 37°C for 18 h. For assessment of osmotic stress tolerance, BHI plates were
326 supplemented with NaCl (0%, 2.5%, 5%, and 7.5%). Overnight cultures of bacteria are serially
327 diluted and spotted on NaCl-supplemented plates. Plates were imaged after 72 h incubation.
328 For some experiments, overnight cultures were subcultured into fresh BHI broth for 1 h prior to
329 serial dilution and spotting.
330
331 **Phage adsorption assay.** An overnight bacterial culture was diluted 1:5 in fresh BHI broth. The
332 culture was then equilibrated at 37°C for 20 min in a water bath. ϕNPV1 was added at an M.O.I
333 of 10^{-3}. After 15 min, a 1 mL aliquot was centrifuged at 16.6 x g for 1 min at room temperature.
334 500 µL of the supernatant was collected, and its titer is determined with the phage spot assay. A

335 medium with only phage added (no bacteria) was used as control. Percent adsorption was
336 determined as follow:

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

337

338 **Isolation of ϕNPV1 resistant mutants.** For isolation of a ϕNPV-1 resistant strain from an
339 OG1RF∆PIP background, ϕNPV-1 was used to infect OG1RF∆PIP in a soft agar overlay. The
340 confluently lysed plates were incubated until presumptive phage-resistant colonies were
341 observed. These colonies were cultured in BHI broth and used as hosts for ϕNPV1 infection to
342 confirm phage resistance. A confirmed ϕNPV-1 resistant strain, referred to in our study as
343 OG1RF-C, was stocked and used for genome sequencing.

344

345 For isolation of ϕNPV-1 resistant strains from an OG1RF background, 500 µL of an overnight
346 culture of OG1RF was infected with ϕNPV1 at an M.O.I of 10^{-1} in a soft agar overlay. 10
347 colonies that arose on the confluently lysed plate were struck on BHI plates and incubated at
348 37°C for 18 h. Single colonies from each of the plates were tested for phage resistance by
349 cross-streaking against ϕNPV1. 9 colonies that showed little to no lysis were stocked and used
350 for daptomycin susceptibility testing and *epaR* sequencing.

351

352 **Polysaccharide analysis.** Polysaccharide extraction was performed as described by Teng et
353 al. with some modifications (20). 200 mL of an overnight culture was centrifuged and
354 resuspended in 750 µL 50 mM Tris buffer, pH 7.5. Mutanolysin (0.25 U/µL) and lysozyme (5
355 mg/mL) were added to the suspension. The suspension was incubated at 37°C for 2 h.
356 Subsequently, 10 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.15 mg/mL DNase I, and 0.15 mg/mL RNase A
357 were added. After an additional 2 h incubation at 37°C, the suspension was centrifuged, and the
358 cellar debris discarded. Proteinase K (100 µg/mL) was added to the clear supernatant, and the

359 mixture was incubated for 16 h. Afterwards, the supernatant was extracted twice with
360 chloroform-phenol-isoamyl alcohol (Sigma Aldrich) and once with chloroform. Ethanol was
361 added to a final concentration of 80% to precipitate the polysaccharide. The precipitate was
362 collected by centrifugation and air-dried. The pellet was resuspended in 50% acetic acid (v/v) in
363 deionized water, and the insoluble material was removed by centrifugation. 30 µL was loaded
364 onto an 1% agarose gel and electrophoresed for 30 min at 130 V. The gel was soaked in
365 staining solution containing Stains-All (Alfa Aesar) and left overnight with gentle rocking. The
366 staining solution was 25% isopropanol, 10% formamide, 65% water, and 0.005% Stains-All.
367 After 18 h, the gel was destained under light for 40 min prior to visualization.
368
369 **Daptomycin MIC.** Daptomycin MIC was assessed using Etest strips (BioMérieux). 3-5 colonies
370 of similar sizes were resuspended in 500 µL BHI and evenly distributed over a MHA plate using
371 a sterile cotton swab. A daptomycin Etest strip was placed onto the plate, and the plate was
372 incubated for 18 h at 37°C. MIC was determined by recording the number closest to the zone of
373 inhibition. The MIC reported for each strain is the average of at least three trials. For trials in
374 which the daptomycin MIC was below the detection limit of the strip (<0.016 µg/mL), the MIC
375 was reported as 0.008 µg/mL for the purposes of statistical analysis. Data were analyzed using
376 the two-tailed unpaired Student's t test.
377
378 **Whole genome sequencing and analysis of OG1RF-C.** OG1RF-C genomic DNA was isolated
379 from overnight culture using the Ultraclean Microbial DNA Isolation kit (Mo Bio) per the
380 manufacturer's instruction. The gDNA was sequenced using MiSeq with 2 x 150 bp chemistry at
381 MR DNA (Shallowater, Texas). After sequencing, the reads were mapped to the complete
382 OG1RF reference (NC_017316.1) using CLC Genomics Workbench (Qiagen). Putative
383 mutations were detected using the basic variant detector in CLC Genomic Workbench. Variants
384 occurring at ≥50% frequency in the read assembly and resulting in non-synonymous

385 substitutions were confirmed with Sanger sequencing. BlastP and NCBI Conserved Domains
386 were used to analyze conserved domains in proteins. Amino acid alignment was performed with
387 CLUSTALW (37). Transmembrane helices were predicted with TMHMM version 2.0 (38).
388
389 **Accession numbers.** The Illumina reads for resequencing of OG1RF-C have been deposited in
390 the Sequence Read Archive under accession number PRJNA450206.
391
392 **Acknowledgments**
393 This work was supported by Public Health Service grant R01AI116610 to K.L.P. Khang Ho was
394 supported by a University of Texas at Dallas Undergraduate Research Fellowship. We gratefully
395 thank Dr. Gary Dunny for providing phage NPV1.
396
397

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522

523 **Figure legends.**

524 **Figure 1. Phage susceptibility of *E. faecalis* OG1RF and derivatives.** Overnight cultures
525 were diluted in PBS and spotted on BHI plates with or without 10^9 PFU/mL phiNPV1. Images
526 were taken after 18 h incubation at 37°C. The image shown is representative of three
527 independent trials.

528

529 **Figure 2. Phage susceptibility of complemented strains of OG1RF∆*epaR.*** Overnight
530 cultures were diluted in PBS and spotted on BHI plates with or without 10^9 PFU/mL phiNPV1.
531 Images were taken after 18 h incubation at 37°C. The image shown is representative of three
532 independent trials.

533

534 **Figure 3. Phage adsorption assays.** Overnight cultures were diluted 1:5 in fresh BHI and
535 equilibrated at 37°C. phiNPV1 was added at a M.O.I. of 10^{-2}. After 15 min incubation, 1 mL of
536 each culture was centrifuged, and the supernatant was titered with the phage spot assay. A
537 medium with only phage was used as the control. Percent adsorption was calculated as [(PFU
538 culture – PFU control)/ PFU control] *100%. Data are the average of three independent trials.

539

540 **Figure 4. Carbohydrate extract analysis.** Carbohydrate was extracted from 200 mL overnight
541 cultures and visualized with Stains-all. The image shown is representative of two independent
542 trials.

543

544 **Figure 5. Daptomycin MICs of *E. faecalis* OG1RF and derivatives.** Daptomycin MIC was
545 determined by Etest. Data are the average of at least three independent trials. For statistical
546 analysis, DAP MICs were compared to that of wild-type OG1RF. ***, $p < 0.001$; and *, $p < 0.05$.

547

548 **Figure 6. Susceptibility to sodium chloride-induced osmotic stress.** Overnight cultures
549 were diluted in PBS and spotted on BHI plates with or without sodium chloride. Images were
550 taken after 72 h incubation. The image shown is representative of three independent trials.
551
552 **Supplemental Figures**
553
554 **Figure S1: Percent PFU recovery from pre-incubation of phiNPV1 with polysaccharide**
555 **extract**. Polysaccharide extract was prepared as described above except for the final solvent
556 being phage buffer rather than 50% acetic acid. phiNPV1 was added at a PFU of 10^5 and the
557 mixture was incubated for 15 minutes at 37°C. The PFU was evaluated with phage spot assay.
558 The percent PFU recovery was calculated using a phage buffer only as the base value. The
559 experiment was performed in duplicate.
560
561 **Figure S2: Effects of osmotic pressure on *epaR* and *bgsB* deletion strains of OG1RF**. 500
562 µL of overnight cultures of indicated strains were centrifuged. The pellets were then
563 resuspended in fresh broth for 1 hour before spotting on plates with (A) or without NaCl (B). The
564 image was taken after 72 hours of incubation. The image is a representative of 3 replicates.
565
566 **Figure S3: Daptomycin MIC of 9 phiNPV1 resistant strains derived from a confluently**
567 **lysed plate**. 3-5 colonies of indicated strains were resuspended in BHI and swabbed onto a
568 MHB plate. Daptomycin strip was applied to the surface of each plate. The plates were
569 incubated for 18 hours prior to MIC determination. The data represent the average of 6 trials.
570 MIC data points below the detection limit of 0.016 µg/mL were taken to be 0.008 µg/mL for
571 statistical treatment. ***, $p < 0.001$.
572

573 **Figure S4: Clustal W alignment of EpaR and its homologues**. Conserved residues are
574 highlighted in red. Blue asterisks indicate Blue asterisks indicate where substitutions occurred in
575 phiNPV1 resistant strains of OG1RF.
576

577 Table 1. Strains and plasmid used.
578

Strains and plasmids	Description	Reference
***E. faecalis* strains**		
OG1RF	Human oral cavity isolate	(39, 40)
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*epaR* with wild-type *bgsB* complementation *in cis*	This work
OG1RFΔ*bgsB _Bc*	OG1RFΔ*epaR* 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*	(41)
Plasmids		
pLT06	Cloning vector, temperature sensitive *repA*, Cam^r	(35)
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

579
580 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

581

582 Table 3. *epaR* variations in spontaneous ϕNPV1-resistant *E. faecalis* OG1RF strains.
583

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	D362N
R6	Deletion	Deletion of 101 bp	M356fs
R2	Deletion	Deletion of 1 bp	D355fs

584
585

Figure 1

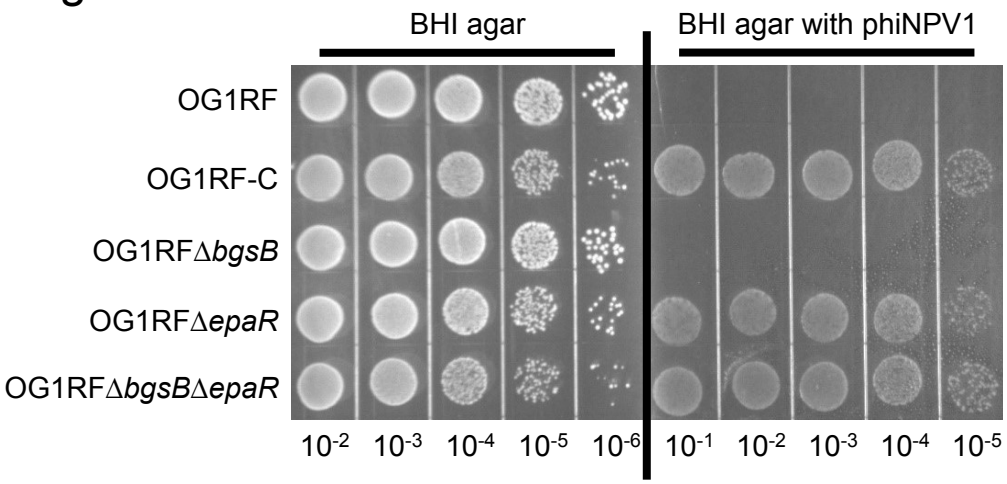

Figure 1. Phage susceptibility of *E. faecalis* OG1RF and derivatives. Overnight cultures were diluted in PBS and spotted on BHI plates with or without 10^9 PFU/mL phiNPV1. Images were taken after 18 h incubation at 37°C. The image shown is representative of three independent trials.

Figure 2

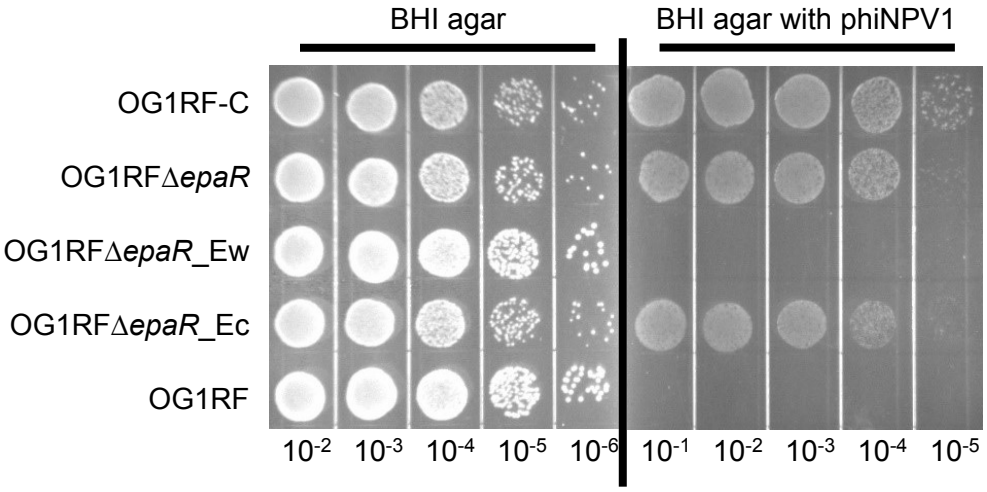

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Figure 3

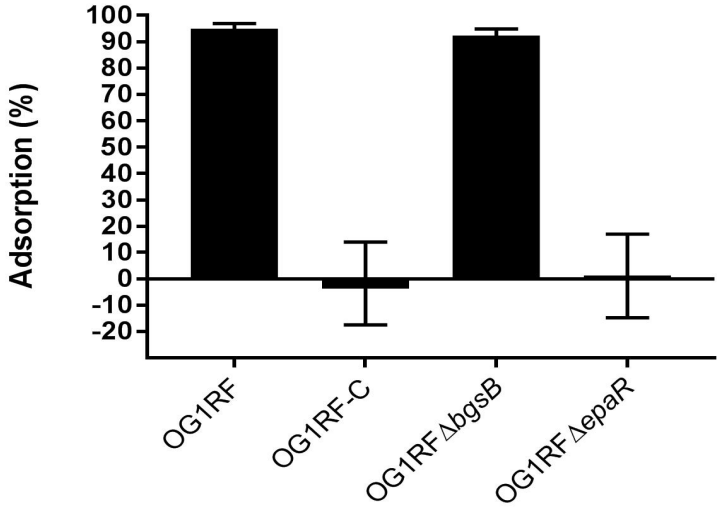

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Figure 4

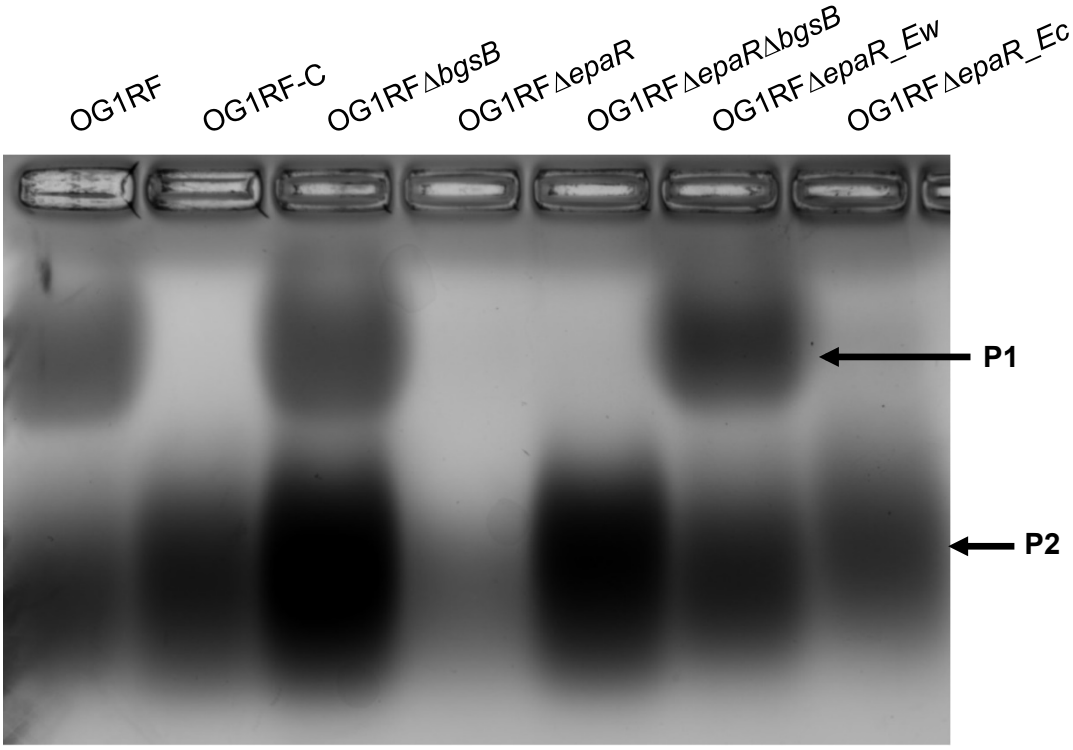

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Figure 5

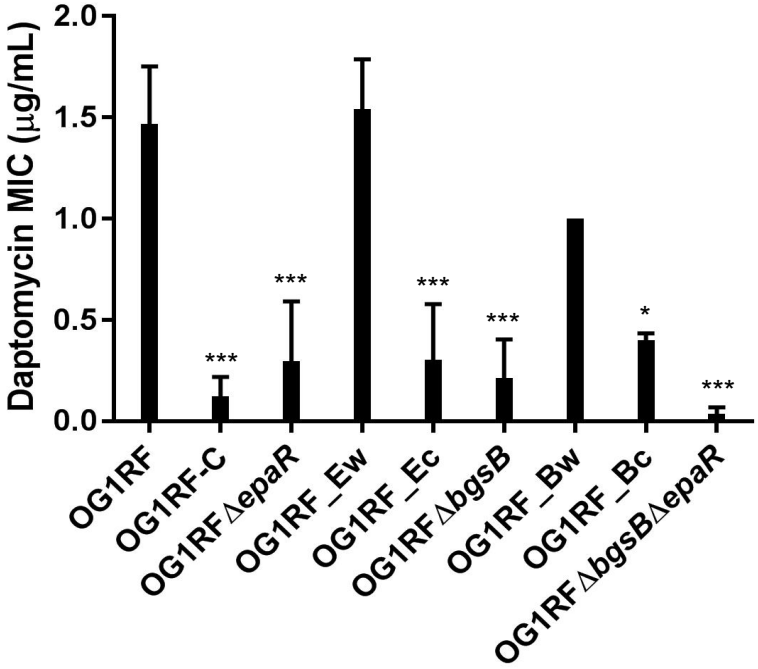

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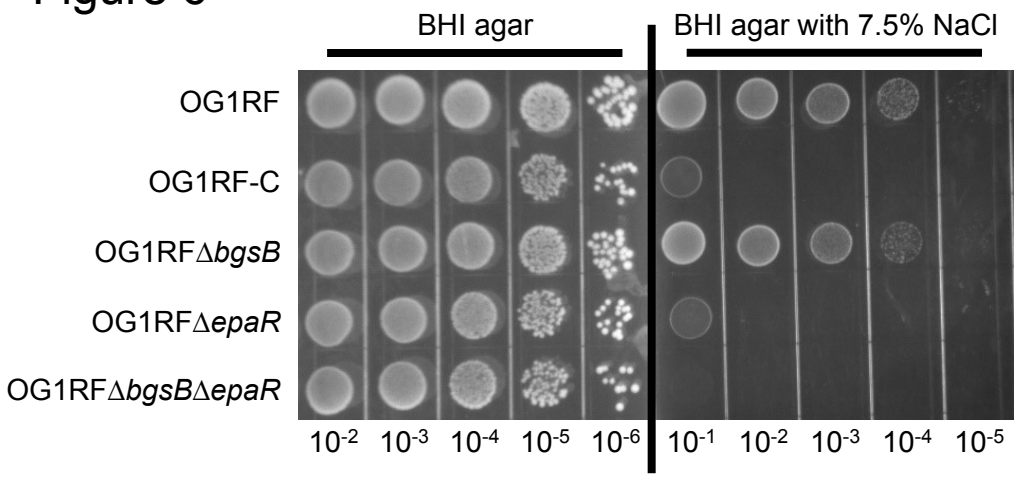
Figure 6



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