1   Spontaneous resistance to phage NPV1 confers increased daptomycin susceptibility in

2   *Enterococcus faecalis* OG1RF

3

4   Khang Ho, Wenwen Huo, Savannah Pas, and Kelli L. Palmer*

5   Department of Biological Sciences, The University of Texas at Dallas

6

7   *Information for corresponding author:

8   800 West Campbell Road, BSB12

9   Richardson, Texas 75080

10   (972) 883-6014

11   kelli.palmer@utdallas.edu

12

13 **Abstract**

14 *Enterococcus faecalis* is a Gram-positive opportunistic pathogen that inhabits the

15 gastrointestinal tract. Because of the high frequency of antibiotic resistance among

16 *Enterococcus* clinical isolates, interest in using phage to treat enterococcal infections and to

17 decolonize high-risk patients for antibiotic-resistant *Enterococcus* is rising. Bacteria can evolve

18 phage resistance, but there is little published information on these mechanisms in *E. faecalis*. In

19 this report, we identified genetic determinants of resistance to ɸNPV1 and their effects on *E.*

20 *faecalis* susceptibilities to daptomycin and sodium chloride-induced osmotic stress. We found

21 that loss-of-function mutations in *epaR* confer ɸNPV1 resistance by blocking phage adsorption.

22 We attribute the inability of the phage to adsorb to the loss of an extracellular polymer in strains

23 with inactivated *epaR.* Phage-resistant *epaR* mutants exhibited increased daptomycin and

24 osmotic stress susceptibility. Our results demonstrate that spontaneous resistance to ɸNPV1

25 comes at a cost in *E. faecalis* OG1RF, resulting in concomitant increased susceptibilities to

26 daptomycin and sodium chloride-induced osmotic stress.

27

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 ɸSPP-1 of *Bacillus subtilis* (12)

51 and the phage c2 group of *Lactococcus lactis* (13, 14). YueB, the ɸSPP-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   ɸVPE25 and ɸVFW (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 ɸNPV1 (19, 20), which infects *E.*

70   *faecalis* OG1RF by a PIP-independent mechanism.

71

72   **Results**

73   **Deletion of *epaR* alters susceptibility to ɸNPV1**. We isolated a OG1RFΔPIP (15) strain with

74   spontaneous resistance to ɸNPV1 (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

4

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 ɸNPV1 susceptibility, we constructed in-frame

90   deletions of *epaR* and *bgsB*, generating strains OG1RFΔ*epaR* and OG1RFΔ*bgsB*, respectively,

91   and a double deletion strain, OG1RFΔ*epaR*Δ*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 ɸNPV1 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 ɸNPV1 resistance.** To determine whether *epaR* mutation

100   is the major contributor to ɸNPV1 resistance in OG1RF-C, we generated strain

101   OG1RFΔ*epaR*_Ec, an OG1RFΔ*epaR* strain complemented *in cis* with the *epaR* allele from

102   OG1RF-C. We also generated strain OG1RFΔ*epaR*_Ew, an OG1RFΔ*epaR* strain with a

103   reconstituted wild-type *epaR*. Complementation with the *epaR* allele of OG1RF-C conferred

104   phage resistance to OG1RFΔ*epaR* (Figure 2). The wild-type *epaR* allele restored phage

105   susceptibility to OG1RFΔ*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 OG1RFΔ*epaR*. Consistent with the observation that

113   *bgsB* deletion alone confers no significant phage resistance, ~90% of the phage adsorbed to

114   OG1RFΔ*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, OG1RFΔ*bgsB,* and the

124   reconstituted *epaR* strain, OG1RFΔ*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   OG1RFΔ*bgsB* and OG1RFΔ*bgsB*Δ*epaR* compared to wild-type OG1RF, suggesting that the

6

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, OG1RF∆*epaR*,

139   and OG1RF∆*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*

7

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, OG1RFΔ*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 OG1RFΔ*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

8

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

9

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

10

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

11

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 ɸNPV1 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 OG1RFΔ*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 ɸNPV1 resistant mutants.** For isolation of a ɸNPV-1 resistant strain from an

339   OG1RFΔPIP background, ɸNPV-1 was used to infect OG1RFΔPIP 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 ɸNPV1 infection to

342   confirm phage resistance. A confirmed ɸNPV-1 resistant strain, referred to in our study as

343   OG1RF-C, was stocked and used for genome sequencing.

344

345   For isolation of ɸNPV-1 resistant strains from an OG1RF background, 500 µL of an overnight

346   culture of OG1RF was infected with ɸNPV1 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 ɸNPV1. 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

15

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

16

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

398 **References Cited**
399

400 1. **Lebreton F, Willems RJL, Gilmore MS.** 2014. Enterococcus Diversity, Origins in

401 Nature, and Gut Colonization. *In* Gilmore MS, Clewell DB, Ike Y, Shankar N (ed.),

402 Enterococci: From Commensals to Leading Causes of Drug Resistant Infection, Boston.

403 2. **Kristich CJ, Rice LB, Arias CA.** 2014. Enterococcal Infection-Treatment and Antibiotic

404 Resistance. *In* Gilmore MS, Clewell DB, Ike Y, Shankar N (ed.), Enterococci: From

405 Commensals to Leading Causes of Drug Resistant Infection, Boston.

406 3. **Tran TT, Munita JM, Arias CA.** 2015. Mechanisms of drug resistance: daptomycin

407 resistance. Ann N Y Acad Sci **1354:**32-53.

408 4. **Deresinski S.** 2009. Bacteriophage therapy: exploiting smaller fleas. Clinical infectious

409 diseases : an official publication of the Infectious Diseases Society of America **48:**1096-

410 1101.

411 5. **Khalifa L, Brosh Y, Gelman D, Coppenhagen-Glazer S, Beyth S, Poradosu-Cohen**

412 **R, Que YA, Beyth N, Hazan R.** 2015. Targeting *Enterococcus faecalis* biofilms with

413 phage therapy. Applied and environmental microbiology **81:**2696-2705.

414 6. **Zhang W, Mi Z, Yin X, Fan H, An X, Zhang Z, Chen J, Tong Y.** 2013. Characterization

415 of *Enterococcus faecalis* phage IME-EF1 and its endolysin. PloS one **8:**e80435.

416 7. **Loc-Carrillo C, Abedon ST.** 2011. Pros and cons of phage therapy. Bacteriophage

417 **1:**111-114.

418 8. **Langenscheid J, Killmann H, Braun V.** 2004. A FhuA mutant of *Escherichia coli* is

419 infected by phage T1-independent of TonB. FEMS microbiology letters **234:**133-137.

420 9. **Yu F, Mizushima S.** 1982. Roles of lipopolysaccharide and outer membrane protein

421 OmpC of *Escherichia coli* K-12 in the receptor function for bacteriophage T4. Journal of

422 bacteriology **151:**718-722.

423   10.   **Sandulache R, Prehm P, Kamp D.** 1984. Cell wall receptor for bacteriophage Mu G(+).

424   Journal of bacteriology **160:**299-303.

425   11.   **Marchal C, Perrin D, Hedgpeth J, Hofnung M.** 1980. Synthesis and maturation of

426   lambda receptor in *Escherichia coli* K-12: *in vivo* and *in vitro* expression of gene *lamB*

427   under *lac* promoter control. Proceedings of the National Academy of Sciences of the

428   United States of America **77:**1491-1495.

429   12.   **Baptista C, Santos MA, Sao-Jose C.** 2008. Phage SPP1 reversible adsorption to

430   *Bacillus subtilis* cell wall teichoic acids accelerates virus recognition of membrane

431   receptor YueB. Journal of bacteriology **190:**4989-4996.

432   13.   **Tremblay DM, Tegoni M, Spinelli S, Campanacci V, Blangy S, Huyghe C, Desmyter**

433   **A, Labrie S, Moineau S, Cambillau C.** 2006. Receptor-binding protein of *Lactococcus*

434   *lactis* phages: identification and characterization of the saccharide receptor-binding site.

435   Journal of bacteriology **188:**2400-2410.

436   14.   **Monteville MR, Ardestani B, Geller BL.** 1994. Lactococcal bacteriophages require a

437   host cell wall carbohydrate and a plasma membrane protein for adsorption and ejection

438   of DNA. Applied and environmental microbiology **60:**3204-3211.

439   15.   **Duerkop BA, Huo W, Bhardwaj P, Palmer KL, Hooper LV.** 2016. Molecular Basis for

440   Lytic Bacteriophage Resistance in Enterococci. MBio **7**.

441   16.   **Labrie SJ, Samson JE, Moineau S.** 2010. Bacteriophage resistance mechanisms.

442   Nature reviews. Microbiology **8:**317-327.

443   17.   **Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE.** 2016. Phage

444   selection restores antibiotic sensitivity in MDR *Pseudomonas aeruginosa*. Scientific

445   reports **6:**26717.

446   18.   **Chan BK, Abedon ST, Loc-Carrillo C.** 2013. Phage cocktails and the future of phage

447   therapy. Future microbiology **8:**769-783.

448   19.   **Trotter KM, Dunny GM.** 1990. Mutants of *Enterococcus faecalis* deficient as recipients

449   in mating with donors carrying pheromone-inducible plasmids. Plasmid **24:**57-67.

450   20.   **Teng F, Singh KV, Bourgogne A, Zeng J, Murray BE.** 2009. Further characterization

451   of the *epa* gene cluster and Epa polysaccharides of *Enterococcus faecalis*. Infect Immun

452   **77:**3759-3767.

453   21.   **Theilacker C, Sava I, Sanchez-Carballo P, Bao Y, Kropec A, Grohmann E, Holst O,**

454   **Huebner J.** 2011. Deletion of the glycosyltransferase *bgsB* of *Enterococcus faecalis*

455   leads to a complete loss of glycolipids from the cell membrane and to impaired biofilm

456   formation. BMC microbiology **11:**67.

457   22.   **Yoshida K, Yamaguchi M, Morinaga T, Kinehara M, Ikeuchi M, Ashida H, Fujita Y.**

458   2008. *myo*-Inositol catabolism in *Bacillus subtilis*. The Journal of biological chemistry

459   **283:**10415-10424.

460   23.   **Dale JL, Cagnazzo J, Phan CQ, Barnes AM, Dunny GM.** 2015. Multiple roles for

461   *Enterococcus faecalis* glycosyltransferases in biofilm-associated antibiotic resistance,

462   cell envelope integrity, and conjugative transfer. Antimicrobial agents and chemotherapy

463   **59:**4094-4105.

464   24.   **Bhardwaj P, Hans A, Ruikar K, Guan Z, Palmer KL.** 2018. Reduced Chlorhexidine

465   and Daptomycin Susceptibility in Vancomycin-Resistant *Enterococcus faecium* after

466   Serial Chlorhexidine Exposure. Antimicrobial agents and chemotherapy **62**.

467   25.   **Solheim M, La Rosa SL, Mathisen T, Snipen LG, Nes IF, Brede DA.** 2014.

468   Transcriptomic and functional analysis of NaCl-induced stress in *Enterococcus faecalis*.

469   PloS one **9:**e94571.

470   26.   **Westra ER, van Houte S, Oyesiku-Blakemore S, Makin B, Broniewski JM, Best A,**

471   **Bondy-Denomy J, Davidson A, Boots M, Buckling A.** 2015. Parasite Exposure Drives

472   Selective Evolution of Constitutive versus Inducible Defense. Current biology : CB

473   **25:**1043-1049.

474 27. **Ainsworth S, Sadovskaya I, Vinogradov E, Courtin P, Guerardel Y, Mahony J,**

475 **Grard T, Cambillau C, Chapot-Chartier MP, van Sinderen D.** 2014. Differences in

476 lactococcal cell wall polysaccharide structure are major determining factors in

477 bacteriophage sensitivity. MBio **5:**e00880-00814.

478 28. **Shibata Y, Yamashita Y, van der Ploeg JR.** 2009. The serotype-specific glucose side

479 chain of rhamnose-glucose polysaccharides is essential for adsorption of bacteriophage

480 M102 to *Streptococcus mutans*. FEMS microbiology letters **294:**68-73.

481 29. **Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G,**

482 **Gevers D, Walker S, Wortman J, Feldgarden M, Haas B, Birren B, Gilmore MS.**

483 2012. Comparative genomics of enterococci: variation in *Enterococcus faecalis*, clade

484 structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*.

485 MBio **3:**e00318-00311.

486 30. **Mistou MY, Sutcliffe IC, van Sorge NM.** 2016. Bacterial glycobiology: rhamnose-

487 containing cell wall polysaccharides in Gram-positive bacteria. FEMS microbiology

488 reviews **40:**464-479.

489 31. **Humphries RM, Pollett S, Sakoulas G.** 2013. A current perspective on daptomycin for

490 the clinical microbiologist. Clin Microbiol Rev **26:**759-780.

491 32. **Muller A, Wenzel M, Strahl H, Grein F, Saaki TN, Kohl B, Siersma T, Bandow JE,**

492 **Sahl HG, Schneider T, Hamoen LW.** 2016. Daptomycin inhibits cell envelope synthesis

493 by interfering with fluid membrane microdomains. Proceedings of the National Academy

494 of Sciences of the United States of America.

495 33. **Dale JL, Nilson JL, Barnes AMT, Dunny GM.** 2017. Restructuring of *Enterococcus*

496 *faecalis* biofilm architecture in response to antibiotic-induced stress. NPJ Biofilms

497 Microbiomes **3:**15.

498 34. **Kutter E, Sulakvelidze A.** 2005. Bacteriophages : biology and applications. CRC Press,

499 Boca Raton, FL.

500   35.   **Thurlow LR, Thomas VC, Hancock LE.** 2009. Capsular polysaccharide production in

501   *Enterococcus faecalis* and contribution of CpsF to capsule serospecificity. Journal of

502   bacteriology **191:**6203-6210.

503   36.   **Cruz-Rodz AL, Gilmore MS.** 1990. High efficiency introduction of plasmid DNA into

504   glycine treated *Enterococcus faecalis* by electroporation. Molecular & general genetics :

505   MGG **224:**152-154.

506   37.   **Thompson JD, Higgins DG, Gibson TJ.** 1994. CLUSTAL W: improving the sensitivity

507   of progressive multiple sequence alignment through sequence weighting, position-

508   specific gap penalties and weight matrix choice. Nucleic acids research **22:**4673-4680.

509   38.   **Krogh A, Larsson B, von Heijne G, Sonnhammer EL.** 2001. Predicting

510   transmembrane protein topology with a hidden Markov model: application to complete

511   genomes. Journal of molecular biology **305:**567-580.

512   39.   **Gold OG, Jordan HV, van Houte J.** 1975. The prevalence of enterococci in the human

513   mouth and their pathogenicity in animal models. Archives of oral biology **20:**473-477.

514   40.   **Dunny GM, Brown BL, Clewell DB.** 1978. Induced cell aggregation and mating in

515   *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proceedings of the

516   National Academy of Sciences of the United States of America **75:**3479-3483.

517   41.   **Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M,**

518   **Venema G, Kok J.** 1996. A general system for generating unlabelled gene

519   replacements in bacterial chromosomes. Molecular & general genetics : MGG **253:**217-

520   224.

521

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 OG1RFΔ*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

23

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

24

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

26

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
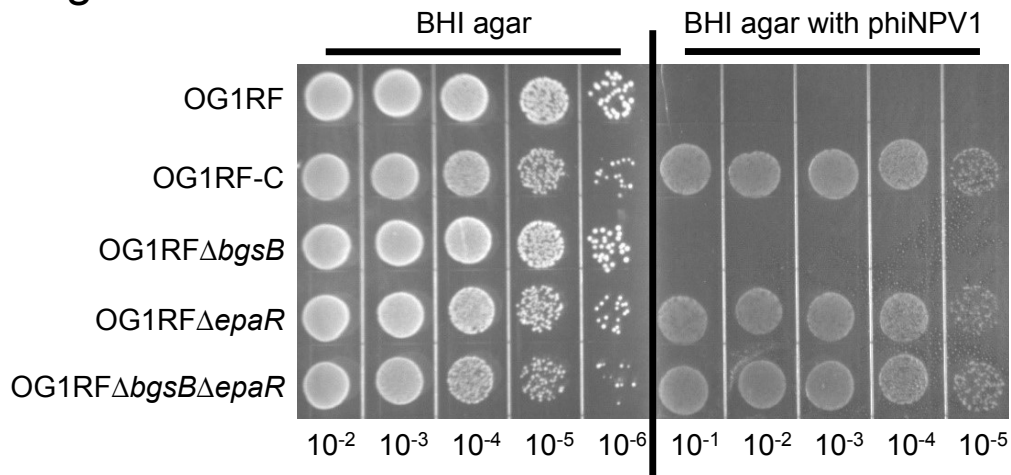
584
585

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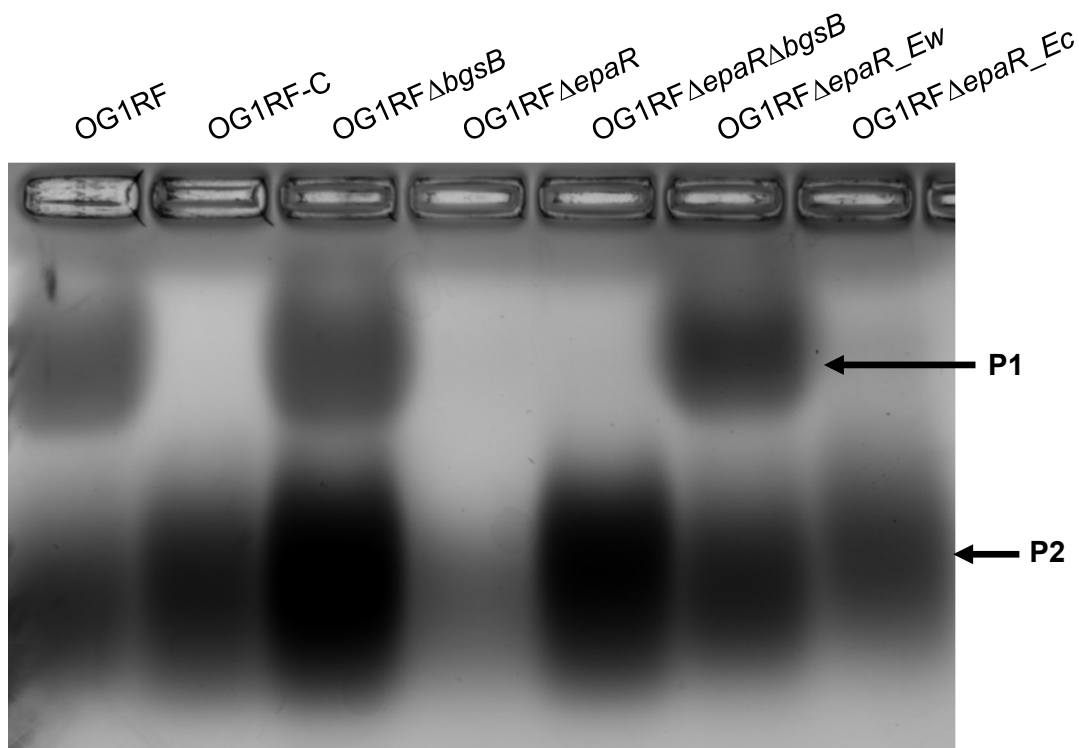
Figure 2
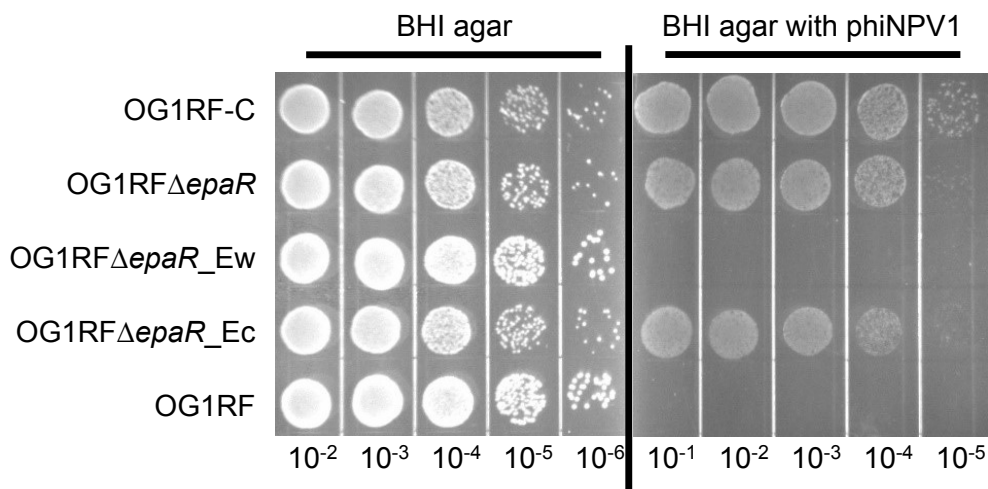
Figure 2. Phage susceptibility of complemented strains of OG1RF∆*epaR*. Overnight cultures were diluted in PBS and spotted on BHI plates with or without 10^9 PFU/mL phiNPV1. Images were taken after 18 h incubation at 37°C. The image shown is representative of three independent trials.

Figure 3
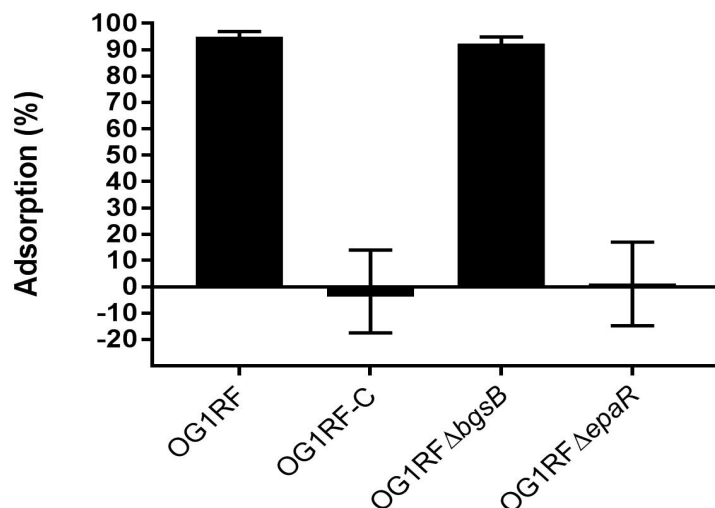
Figure 3. Phage adsorption assays. Overnight cultures were diluted 1:5 in fresh BHI and equilibrated at 37°C. phiNPV1 was added at a M.O.I. of 10^{-2}. After 15 min incubation, 1 mL of each culture was centrifuged, and the supernatant was titered with the phage spot assay. A medium with only phage was used as the control. Percent adsorption was calculated as [(PFU culture − PFU control)/ PFU control] *100%. Data are the average of three independent trials.
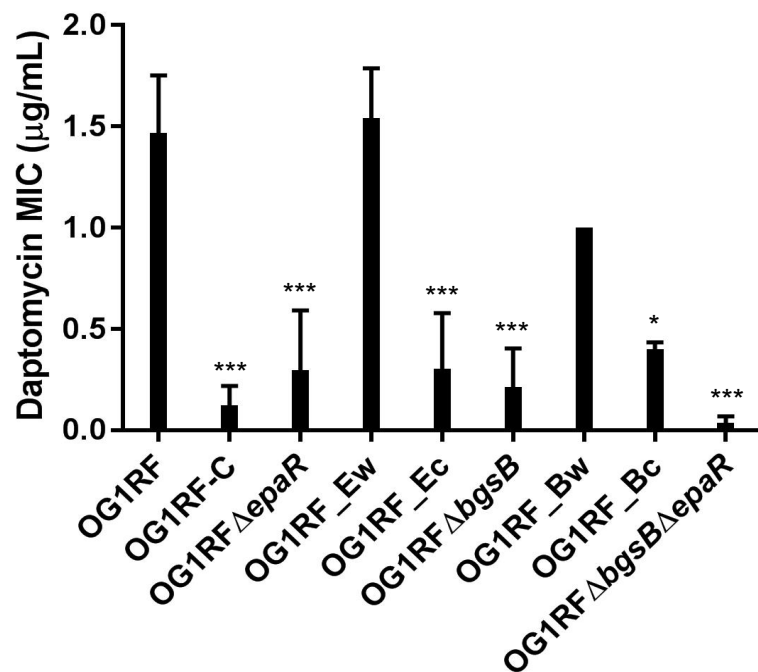
Figure 4

Figure 4. Carbohydrate extract analysis. Carbohydrate was extracted from 200 mL overnight cultures and visualized with Stains-all. The image shown is representative of two independent trials.
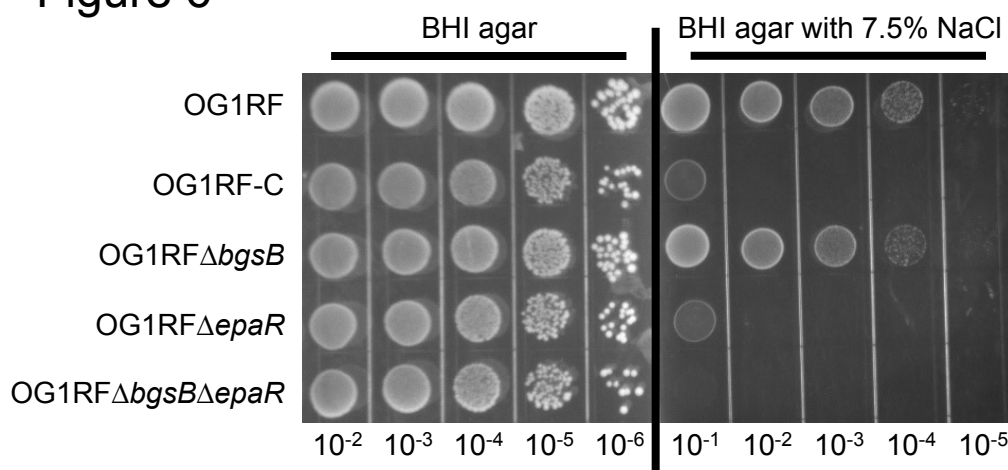
Figure 5

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

Figure 6

Figure 6. Susceptibility to sodium chloride-induced osmotic stress. Overnight cultures were diluted in PBS and spotted on BHI plates with or without sodium chloride. Images were taken after 72 h incubation. The image shown is representative of three independent trials.