Spontaneous resistance to phage NPV1 confers increased daptomycin susceptibility in

*Enterococcus faecalis* OG1RF

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

Department of Biological Sciences, The University of Texas at Dallas

*Information for corresponding author:

800 West Campbell Road, BSB12

Richardson, Texas 75080

(972) 883-6014

kelli.palmer@utdallas.edu
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.
Introduction

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

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

One advantage of phage therapy is limited damage to the native microbiome because of the specificity of the phage to its host (7). The first step to a successful phage infection is the attachment of the phage particle to the proper receptor present on the surface of the host cell. Phage receptors have been extensively studied in certain phage families, including the T series phages, Mu, and λ for Gram-negative bacteria (8-11). Some phage receptors have been characterized in Gram-positive bacteria, including receptors for ϕSPP-1 of Bacillus subtilis (12) and the phage c2 group of Lactococcus lactis (13, 14). YueB, the ϕSPP-1 receptor, and PIP, the phage c2 receptor, are orthologs and are required for irreversible phage adsorption (12). Enterococcal phage receptors have not been well-characterized. Previously, we and
collaborators identified PIP as a receptor and potential DNA channel for the *E. faecalis* phages \( \phi VPE25 \) and \( \phi VFW \) (15).

Bacteria can evolve phage resistance. Mechanisms of phage resistance include modification or loss of the phage receptor (16). However, as phage receptors generally serve physiological functions in the cell, the modification or loss of a receptor could come at a cost for the bacterial host. Indeed, spontaneous phage-resistant mutants have altered antibiotic sensitivity in *P. aeruginosa* (17). Phages utilizing receptors that have roles in antibiotic resistance could be advantageous for re-sensitizing resistant bacteria to antibiotics.

Considering the increasingly limited treatment options for *E. faecalis* infections and the revival of interest in using phage therapy to treat bacterial infections, it is crucial that we know the receptor(s) of enterococcal phages since effective phage cocktails use phages targeting multiple different receptors (18). Moreover, the roles of these receptors in enterococcal physiology should be elucidated. In this report, we used a combination of genomic and genetic approaches to identify a receptor for the tailed, virulent phage \( \phi NPV1 \) (19, 20), which infects *E. faecalis* OG1RF by a PIP-independent mechanism.

**Results**

Deletion of *epaR* alters susceptibility to \( \phi NPV1 \). We isolated a OG1RF\( \Delta \)PIP (15) strain with spontaneous resistance to \( \phi NPV1 \) (Figure 1). We refer to this strain as OG1RF-C. The genome sequence of OG1RF-C was determined. We identified non-synonymous substitutions in *epaR*, *bgsB*, *iolA2*, and OG1RF\_10252 (Table 2). *epaR* is one of the 18 conserved genes of the *epa* gene cluster (*epaA-epaR*), which codes for synthesis of the enterococcal polysaccharide antigen (Epa) (20). *epaR* encodes a putative glycosyltransferase with 5 predicted transmembrane domains, and its role in Epa biosynthesis has not been investigated. The
product of $bgsB$ is a putative cytoplasmic protein catalyzing the transfer of glucose from UDP-glucose to diacylglycerol (DAG) to form monoglucoyl-DAG. An additional glucose is added to glucosyl-DAG by $bgsA$, forming diglucoyl-DAG. From diglucoyl-DAG, the polymerization of glycerol-phosphate can occur, resulting in lipoteichoic acid (LTA) (21).

$iolA2$ is predicted to encode a methylmalonate-semialdehyde dehydrogenase, which catalyzes the breakdown of malonic semialdehyde to acetyl-CoA and CO$_2$ (22). $OG1RF_10252$ is predicted to encode an Acyl-ACP_TE domain (pfam01643; e-value $7.510e^{-114}$) which catalyzes the termination of fatty acyl group extension by hydrolyzing an acyl group on the fatty acid.

To begin to elucidate the roles of these genes in $\phi$NPV1 susceptibility, we constructed in-frame deletions of $epaR$ and $bgsB$, generating strains OG1RF$\Delta$$epaR$ and OG1RF$\Delta$$bgsB$, respectively, and a double deletion strain, OG1RF$\Delta$$epaR$$\Delta$$bgsB$. Phage susceptibility of each of these mutants was assayed. Deletion of $epaR$ alone was sufficient to confer phage resistance (Figure 1). In contrast, deletion of $bgsB$ alone did not alter phage susceptibility. These results indicate that variation in $epaR$ is the major factor conferring resistance to $\phi$NPV1 in OG1RF-C. Since we observed that deletion of $epaR$ in OG1RF conferred phage resistance to the same extent as that observed for OG1RF-C, we did not investigate the effects of $iolA2$ and OG1RF_10252 on phage resistance in this study.

The OG1RF-C epaR allele confers $\phi$NPV1 resistance. To determine whether $epaR$ mutation is the major contributor to $\phi$NPV1 resistance in OG1RF-C, we generated strain OG1RF$\Delta$$epaR$$_{Ec}$, an OG1RF$\Delta$$epaR$ strain complemented in cis with the $epaR$ allele from OG1RF-C. We also generated strain OG1RF$\Delta$$epaR$$_{Ew}$, an OG1RF$\Delta$$epaR$ strain with a reconstituted wild-type $epaR$. Complementation with the $epaR$ allele of OG1RF-C conferred phage resistance to OG1RF$\Delta$$epaR$ (Figure 2). The wild-type $epaR$ allele restored phage susceptibility to OG1RF$\Delta$$epaR$ (Figure 2). Because the mutated $epaR$ allele from OG1RF-C
confers a phage resistance phenotype, as did deletion of \textit{epaR}, we infer that the \textit{epaR} mutation in OG1RF-C confers loss of function.

\textbf{\textit{epaR} is required for phage adsorption.} We were next interested in how \textit{epaR} inactivation protects OG1RF from \textit{ϕNPV1} infection. After 15 minutes incubation with \textit{ϕNPV1}, ~95\% of the phage adsorbed to wild-type OG1RF (Figure 3). In contrast, under the same conditions, ~1-2\% of the phage adsorbed to OG1RF-C or OG1RF\textit{ΔepaR}. Consistent with the observation that \textit{bgsB} deletion alone confers no significant phage resistance, ~90\% of the phage adsorbed to OG1RF\textit{ΔbgsB} within the same experimental settings. These data indicate that \textit{epaR} is required for \textit{ϕNPV1} adsorption.

\textbf{Inactivation of \textit{epaR} leads to a loss of a polymer.} Next, we sought to determine whether the \textit{Epa} polymer was intact in mutants defective for \textit{ϕNPV1} adsorption. The \textit{Epa} polymer has been extracted and visualized by different groups using different methods (20, 23). We based our method on that from Teng, et al. (20). We found that solubilizing the precipitation with 50\% acetic acid improved visualization of the polymers. Gel electrophoresis analysis of carbohydrate extracts found that OG1RF with either an \textit{epaR} deletion or the \textit{epaR} allele from OG1RF-C exhibited loss of a band (P1) that is present in wild-type OG1RF, OG1RF\textit{ΔbgsB}, and the reconstituted \textit{epaR} strain, OG1RF\textit{ΔepaR}_Ew (Figure 4). We conclude that P1 represents an \textit{epaR}-dependent polymer. Since NPV1 cannot bind to \textit{epaR} mutants, we hypothesized that the P1 polymer is the phage receptor. However, we when pre-incubated NPV1 with crude carbohydrate extract prior to infection of host cells, we did not observe a decrease in PFU for any extract (Figure S1), suggesting that the polymers in the crude extract are not sufficient for phage adsorption. We also observed an increase in the intensity of the P2 band in OG1RF\textit{ΔbgsB} and OG1RF\textit{ΔbgsBΔepaR} compared to wild-type OG1RF, suggesting that the
product P2 is increased in these two deletion strains. Since deletion of \textit{bgsB} in \textit{E. faecalis}
results in accumulation of LTA (21), product P2 may represent LTA.

\textbf{NPV1-resistant mutants have increased susceptibility to daptomycin.} Dale et al. reported
increased daptomycin susceptibility in an OG1RF derivative with a deletion in \textit{epaO} (23). Moreover, we identified a \textit{bgsB} mutation in a laboratory-evolved \textit{E. faecium} isolate with
decreased daptomycin susceptibility (24). Because of these results, we investigated the
daptomycin MIC of our OG1RF mutants (Figure 5). We found that OG1RF-C, OG1RF\textit{ΔepaR},
and OG1RF\textit{ΔepaR} complemented with the OG1RF-C \textit{epaR} allele were each significantly more
susceptible to daptomycin than OG1RF. Interestingly, deletion of \textit{bgsB} also conferred increased
daptomycin susceptibility (Figure 5). This was complemented by expression of the wild-type
\textit{bgsB} allele \textit{in cis}, but not by expression of the OG1RF-C \textit{bgsB} allele \textit{in cis} (Figure 5). Finally,
daptomycin susceptibility was substantially altered in the OG1RF\textit{ΔepaRΔbgsB} mutant, with 3 of
6 experimental trials resulting in an MIC below the level of detection of the E-test strip (<0.016
µg/mL; a value of 0.008 µg/mL was used for these data points in statistical analysis). Without
complete data regarding the MIC of OG1RF\textit{ΔepaRΔbgsB}, we did not quantitatively determine
whether there is a synergistic relationship between \textit{bgsB} and \textit{epaR} regarding daptomycin
susceptibility.

\textbf{epaR protects OG1RF from osmotic stress.} The \textit{epa} gene cluster was up-regulated when \textit{E. faecalis} V583 was grown with 6.5\% sodium chloride supplementation, indicating that the Epa
polymer has a role in osmotic stress response (25). As such, we investigated the effect of
sodium chloride on our \textit{epaR} mutants. We tested our mutants for their tolerance for sodium
chloride stress using BHI agar supplemented with sodium chloride at concentrations of 0\%,
2.5\%, 5\%, and 7.5\%. Overnight cultures in stationary phase were serially diluted and spotted on
these agars. We observed fewer CFU for OG1RF-C, OG1RF\textit{ΔepaR}, and OG1RF \textit{ΔepaRΔbgsB}
compared to the wild-type at 7.5% sodium chloride after 72 h incubation (Figure 6). When sodium chloride concentrations of 5% or lower were used, no effect on growth was observed. Interestingly, when cells from an overnight culture were spotted, OG1RFΔbgsB showed no difference in CFU compared to the wild-type (Figure 6). However, when cells were cultured in fresh BHI broth 1 h prior to spotting, a CFU difference was observed for OG1RFΔbgsB with 7.5% sodium chloride (Figure S2). These data suggest that both epaR and bgsB play roles in sodium chloride-induced osmotic stress response.

**EpaR constitutes a major mutational pathway for φNPV1 resistance.** We isolated 9 spontaneous φNPV1-resistant mutants of OG1RF and sequenced the epaR region of these mutants. All 9 mutants have non-synonymous substitutions in epaR (Table 3). Since we know that mutations in epaR affect daptomycin susceptibility, we also determined the daptomycin MIC of these φNPV1-resistant strains and found that all were more significantly more susceptible to daptomycin than the wild-type (Figure S3).

Most of the EpaR sequence (amino acid positions 35-458 of 484 total) is a predicted sugar transferase domain (TIGR03025; E-value: 2.45e-137). This domain consists of a conserved C-terminal region responsible for the sugar transferase activity (pfam02397) and a variable N-terminal region with predicted flippase activity. All epaR mutations in spontaneously φNPV1-resistant OG1RF strains occur in the C-terminal region of the sugar transferase domain (Table 3). We identified other proteins containing the same predicted sugar transferase domain as EpaR, and we determined that the altered amino acid positions in our mutants are conserved across 6 of these proteins (Figure S4).
Discussion

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

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

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

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

Daptomycin is a lipopeptide antibiotic that is used to treat certain Gram-positive bacterial infections (31). The mechanism of action for daptomycin in B. subtilis begins with daptomycin binding to the cell membrane, and ultimately leads to the displacement of membrane-associated proteins essential for cell wall and phospholipid biosynthesis (32). In our study, we found that
inactivating mutations in *epaR* and in *bgsB* lead to increased daptomycin susceptibility in *E. faecalis*. Deletion of *bgsB* results in loss of glycolipids in the membrane, a longer chain length in the LTA, and increased charge density of the membrane (21). A higher charge density might contribute to daptomycin susceptibility through charge-charge interaction with the calcium-bound daptomycin, but this is speculative. Dale et al. reported that deletion of *epaO* results in increased daptomycin susceptibility and loss of the Epa polymer, suggesting that the complete Epa polymer is important for daptomycin resistance (23). The loss of the Epa polymer also results in defects in cell wall architecture (20, 33). More research on the Epa polymer is required to mechanistically assess its contribution to antibiotic susceptibility in enterococci.

In summary, in this study we characterized a mechanism for spontaneous \(\phi\)NPV1 resistance in *E. faecalis* OG1RF. The major advance of this study is that we determined that phage resistance comes at a cost to *E. faecalis* OG1RF; specifically, increased daptomycin susceptibility. *E. faecalis* OG1RF is a natively daptomycin-sensitive strain that became more sensitive as a result of phage resistance. We did not assess whether \(\phi\)NPV1 treatment could reverse resistance in daptomycin-resistant clinical isolates. This is an exciting area for future research.

**Material and Methods**

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

**Routine molecular techniques and DNA sequencing.** Routine PCR reactions were performed using Taq polymerase (NEB) per the manufacturer's instructions. Phusion polymerase (Fisher) was used for cloning procedures per the manufacturer’s instructions. Plasmid was purified using the GeneJET Plasmid miniprep kit (Fisher). Genomic DNA was isolated using the Ultraclean microbial DNA isolation kit (MoBio). Restriction enzymes, Klenow fragment, T4 polynucleotide kinase (PNK), T4 DNA ligase, and calf intestinal phosphatase (CIP) from NEB were used as instructed by the manufacturer. DNA sequencing was performed at the Massachusetts General Hospital DNA sequencing facility. A complete list of primers used in this study can be found in Table S1.

**Phage spot assay.** 0.5 mL of an exponentially growing culture was added to 3 mL soft agar and poured onto BHI agar. 10 μL of the of phage mixture to be titered was spotted onto the soft agar. Plaques were counted after 16 h incubation at 37°C, unless otherwise stated.

**Phage propagation and storage.** Phage stocks were prepared by mixing 450 μL of an overnight culture of *E. faecalis* OG1RF with ϕNPV1 at an M.O.I of 10⁻². The mixture was incubated at 37°C for 15 min and subsequently added to 3 mL M17 soft agar maintained at 55°C. The soft agar was then poured onto BHI agar and incubated at 37°C for 18 h. 5 mL of phage buffer was added to the confluenctly lysed plate and incubated for 20 min at 37°C with shaking at 75 rpm. The lysate was then collected and centrifuged at 16.6 x g for 1 min to remove cellular debris. The supernatant was filtered with a Whatman 0.2 μm filter to obtain the
phage stock. The phage stock was stored at 4°C in the dark. Phage titer was determined using phage spot assays.

**Generation of OG1RF deletion mutants.** Gene deletion was carried out via the markerless deletion procedure described by Thurlow et al. (35) with some modifications. Briefly, two 1.0 kb regions flanking *epaR* were amplified with primers 1-4 from Table S1. The two amplified products were ligated with an overlap PCR extension through a 21 bp complementary region underlined in Table S1. The approximately 2.0 kb product was purified and digested with BamHI and EcoRI. The digested product was ligated to plasmid pLT06 through restriction sites added on the primers (highlighted in red in Table S1). The ligation product was then purified and electroporated and propagated in *E. coli* EC1000. OG1RF was made electrocompetent using the glycine method (36) (3% glycine) and transformed with 1 µg of the plasmid. OG1RF transformants were screened for successful transformation and subsequently inoculated in BHI supplemented with Cm at 30°C. The culture was diluted 1:100 in BHI and incubated at 30°C for 2 h followed by 42°C for 4 h. Dilutions of the culture were plated on BHI agar supplemented with Cm and X-gal, and large blue colonies were screened for plasmid integration using primers 5 and 19. The positive colonies were then restruck, incubated at 42°C, and screened once again for plasmid integration. Positive clones were cultured in BHI broth at 30°C for 18 h. To counter-select against clones harboring the plasmid, dilutions of the culture were made on MM9YEG agar, and the deletion of *epaR* was determined by colony PCR with primers 5 and 6 after 36 h incubation at 37°C. Clones positive for the deletion were then restruck on BHI agar and screened again using the same primers. Positive clones were verified for plasmid loss by streaking on BHI agar supplemented with Cm. The *epaR* region was sequenced to confirm the deletion. Deletion of *bgsB* was obtained in a similar fashion.
Complementation. Complementation of epaR in an OG1RFΔepaR background was obtained using a similar strategy to deletion. The insert containing the epaR gene and flanking 500 bp upstream and downstream regions was amplified from either OG1RF or OG1RF-C using primers 7 and 8. pLT06 was digested with SphI and blunt-ended with Klenow fragment; the blunt-end product was then treated with CIP. The insert was phosphorylated with T4 PNK and blunt-end ligated to pLT06. The plasmid was purified and transformed into EC1000. Clones with the correct insert size were screened, and their plasmids isolated. Subsequent steps for transformation of OG1RF, integration of the plasmid and counterselection on MM9YEG were as described above for the deletion process. Positive clones for the complementation were confirmed with primers 5 and 6 after counter-selection on MM9YEG. The complemented epaR allele was verified through Sanger sequencing.

Assessment of phage resistance and sodium chloride stress tolerance. For assessment of phage resistance, 500 µL of an 8×10^9 PFU phage stock was added to 3 mL M17 soft agar. The mixture was then poured onto BHI agar. Bacterial culture dilutions were spotted on the soft agar and incubated at 37°C for 18 h. For assessment of osmotic stress tolerance, BHI plates were supplemented with NaCl (0%, 2.5%, 5%, and 7.5%). Overnight cultures of bacteria are serially diluted and spotted on NaCl-supplemented plates. Plates were imaged after 72 h incubation. For some experiments, overnight cultures were subcultured into fresh BHI broth for 1 h prior to serial dilution and spotting.

Phage adsorption assay. An overnight bacterial culture was diluted 1:5 in fresh BHI broth. The culture was then equilibrated at 37°C for 20 min in a water bath. φNPV1 was added at an M.O.I of 10^{-3}. After 15 min, a 1 mL aliquot was centrifuged at 16.6 x g for 1 min at room temperature. 500 µL of the supernatant was collected, and its titer is determined with the phage spot assay. A
medium with only phage added (no bacteria) was used as control. Percent adsorption was
determined as follow:

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

**Isolation of ϕNPV1 resistant mutants.** For isolation of a ϕNPV-1 resistant strain from an
OG1RFΔPIP background, ϕNPV-1 was used to infect OG1RFΔPIP in a soft agar overlay. The
confluently lysed plates were incubated until presumptive phage-resistant colonies were
observed. These colonies were cultured in BHI broth and used as hosts for ϕNPV1 infection to
confirm phage resistance. A confirmed ϕNPV-1 resistant strain, referred to in our study as
OG1RF-C, was stocked and used for genome sequencing.

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

**Polysaccharide analysis.** Polysaccharide extraction was performed as described by Teng et
al. with some modifications (20). 200 mL of an overnight culture was centrifuged and
resuspended in 750 µL 50 mM Tris buffer, pH 7.5. Mutanolysin (0.25 U/µL) and lysozyme (5
mg/mL) were added to the suspension. The suspension was incubated at 37°C for 2 h.
Subsequently, 10 mM MgSO₄, 2.5 mM CaCl₂, 0.15 mg/mL DNase I, and 0.15 mg/mL RNase A
were added. After an additional 2 h incubation at 37°C, the suspension was centrifuged, and the
cellar debris discarded. Proteinase K (100 µg/mL) was added to the clear supernatant, and the
mixture was incubated for 16 h. Afterwards, the supernatant was extracted twice with chloroform-phenol-isooamyl alcohol (Sigma Aldrich) and once with chloroform. Ethanol was added to a final concentration of 80% to precipitate the polysaccharide. The precipitate was collected by centrifugation and air-dried. The pellet was resuspended in 50% acetic acid (v/v) in deionized water, and the insoluble material was removed by centrifugation. 30 µL was loaded onto an 1% agarose gel and electrophoresed for 30 min at 130 V. The gel was soaked in staining solution containing Stains-All (Alfa Aesar) and left overnight with gentle rocking. The staining solution was 25% isopropanol, 10% formamide, 65% water, and 0.005% Stains-All. After 18 h, the gel was destained under light for 40 min prior to visualization.

**Daptomycin MIC.** Daptomycin MIC was assessed using Etest strips (BioMérieux). 3-5 colonies of similar sizes were resuspended in 500 µL BHI and evenly distributed over a MHA plate using a sterile cotton swab. A daptomycin Etest strip was placed onto the plate, and the plate was incubated for 18 h at 37°C. MIC was determined by recording the number closest to the zone of inhibition. The MIC reported for each strain is the average of at least three trials. For trials in which the daptomycin MIC was below the detection limit of the strip (<0.016 µg/mL), the MIC was reported as 0.008 µg/mL for the purposes of statistical analysis. Data were analyzed using the two-tailed unpaired Student's t test.

**Whole genome sequencing and analysis of OG1RF-C.** OG1RF-C genomic DNA was isolated from overnight culture using the Ultraclean Microbial DNA Isolation kit (Mo Bio) per the manufacturer's instruction. The gDNA was sequenced using MiSeq with 2 x 150 bp chemistry at MR DNA (Shallowater, Texas). After sequencing, the reads were mapped to the complete OG1RF reference (NC_017316.1) using CLC Genomics Workbench (Qiagen). Putative mutations were detected using the basic variant detector in CLC Genomic Workbench. Variants occurring at ≥50% frequency in the read assembly and resulting in non-synonymous
substitutions were confirmed with Sanger sequencing. BlastP and NCBI Conserved Domains were used to analyze conserved domains in proteins. Amino acid alignment was performed with CLUSTALW (37). Transmembrane helices were predicted with TMHMM version 2.0 (38).

Accession numbers. The Illumina reads for resequencing of OG1RF-C have been deposited in the Sequence Read Archive under accession number PRJNA450206.

Acknowledgments
This work was supported by Public Health Service grant R01AI116610 to K.L.P. Khang Ho was supported by a University of Texas at Dallas Undergraduate Research Fellowship. We gratefully thank Dr. Gary Dunny for providing phage NPV1.
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Figure legends.

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. Phage susceptibility of complemented strains of OG1RF$\Delta$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. 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 $[(\text{PFU culture} - \text{PFU control})/ \text{PFU control}] \times 100\%$. Data are the average of three independent trials.

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. 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. 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.

Supplemental Figures

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.

Figure S2: Effects of osmotic pressure on epaR and bgsB deletion strains of OG1RF. 500 µL of overnight cultures of indicated strains were centrifuged. The pellets were then resuspended in fresh broth for 1 hour before spotting on plates with (A) or without NaCl (B). The image was taken after 72 hours of incubation. The image is a representative of 3 replicates.

Figure S3: 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.
**Figure S4: 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.
Table 1. Strains and plasmid used.

| Strains and plasmids       | Description                                                                 | Reference  |
|----------------------------|-----------------------------------------------------------------------------|------------|
| **E. faecalis strains**    |                                                                             |            |
| OG1RF                      | Human oral cavity isolate                                                   | (39, 40)   |
| OG1RF∆PIP                  | PIP deletion strain                                                         | (15)       |
| OG1RF-C                    | OG1RF∆PIP φNPV1-resistant strain                                            | This work  |
| OG1RF∆bgsB                 | OG1RF bgsB deletion mutant                                                  | This work  |
| OG1RF∆epaR                 | OG1RF epaR deletion mutant                                                  | This work  |
| OG1RF∆bgsB∆epaR            | OG1RF bgsB and epaR double deletion mutant                                   | This work  |
| OG1RF∆epaR_Ew              | OG1RF∆epaR with wild-type epaR complementation in cis                       | This work  |
| OG1RF∆epaR_Ec              | OG1RF∆epaR complemented with OG1RF-C epaR allele in cis                     | This work  |
| OG1RF∆bgsB _Bw             | OG1RF∆epaR with wild-type bgsB complementation in cis                       | This work  |
| OG1RF∆bgsB _Bc             | OG1RF∆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’                           | (35)       |
| pLT06_∆bgsB                | bgsB deletion construct                                                     | This work  |
| pLT06_∆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  |
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 |
| Strain | Mutation type | Mutation | Amino acid change |
|--------|--------------|----------|------------------|
| R3     | Transversion | C→A      | G409V            |
| R5     | Transversion | C→A      | D428Y            |
| R7     | Transversion | C→A      | G409V            |
| R10    | Transversion | C→A      | G409V            |
| R8     | Transversion | C→A      | D428Y            |
| R4     | Transition   | T→A      | K424*            |
| R9     | Transition   | C→T      | G378D            |
| OG1RF-C| Deletion     | C→T      | D362N            |
| R6     | Deletion     | Deletion of 101 bp | M356fs |
| R2     | Deletion     | Deletion of 1 bp | D355fs |
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