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Genetic modifications to temperate *Enterococcus faecalis* phage ϕEf11 that abolish the establishment of lysogeny and sensitivity to repressor, and increase host range and productivity of lytic infection

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ϕEf11 is a temperate bacteriophage originally isolated by induction from a lysogenic *Enterococcus faecalis* strain recovered from an infected root canal, and the ϕEf11 prophage is widely disseminated among strains of *E. faecalis*. Because *E. faecalis* has emerged as a significant opportunistic human pathogen, we were interested in examining the genes and regulatory sequences predicted to be critical in the establishment/maintenance of lysogeny by ϕEf11 as a first step in the construction of the genome of a virulent, highly lytic phage that could be used in treating serious *E. faecalis* infections. Passage of ϕEf11 in *E. faecalis* JH2-2 yielded a variant that produced large, extensively spreading plaques in lawns of indicator cells, and elevated phage titres in broth cultures. Genetic analysis of the cloned virus producing the large plaques revealed that the variant was a recombinant between ϕEf11 and a defective ϕFL1C-like prophage located in the *E. faecalis* JH2-2 chromosome. The recombinant possessed five ORFs of the defective ϕFL1C-like prophage in place of six ORFs of the ϕEf11 genome. Deletion of the putative lysogeny gene module (ORFs 31–36) and replacement of the putative *cro* promoter from the recombinant phage genome with a nisin-inducible promoter resulted in no loss of virus infectivity. The genetic construct incorporating all the aforementioned ϕEf11 genomic modifications resulted in the generation of a variant that was incapable of lysogeny and insensitive to repressor, rendering it virulent and highly lytic, with a notably extended host range.

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INTRODUCTION

ϕEf11 is a temperate bacteriophage that was induced from a lysogenic root canal isolate of *Enterococcus faecalis* (Stevens *et al.*, 2009). It is a member of the family *Siphoviridae*, with a long (130 nm) non-contractile tail and a small (41 nm diameter) spherical/icosohedral head. The phage produces small, turbid plaques in lawns of *E. faecalis* JH2-2. The ϕEf11 DNA has been sequenced and annotated, disclosing a genome of 42 822 bp encoding 65 ORFs (Stevens *et al.*, 2011). Furthermore, our previous studies disclosed that the ϕEf11 DNA restriction pattern produced with certain restriction enzymes, such as *Nsi*I, produced several fragments in submolar amounts (Stevens *et al.* 2009). This would be expected to occur in the case of a circularly permuted genome due to headful packaging of a concatemeric phage DNA during viral maturation.

The ϕEf11 host species, *E. faecalis*, and closely related species, such as *Enterococcus faecium*, have emerged as significant human pathogens, being major aetiological agents of infectious endocarditis, nosocomial infections, burn infections, urinary tract infections, meningitis and surgical wound infections (Lewis & Zervos, 1990; Moellering, 1992; Megran, 1992; Emori & Gaynes, 1993; Jett *et al.*, 1994; Edgeworth *et al.*, 1999; Richards *et al.*, 2000; National Nosocomial Infections Surveillance System, 2004; Biedenbach *et al.*, 2004; Linden, 2007). In terms of oral disease, *E. faecalis* is the most commonly isolated species from infected root canals of teeth that fail to heal following root canal therapy (Sundqvist *et al.*, 1998; Peciuliene *et al.*, 2000; Pinheiro *et al.*, 2003; Siqueira & Rôças, 2004; Stuart *et al.*, 2006; Zoletti *et al.*, 2006).

One supplementary table and five supplementary figures are available with the online version of this paper.

Complicating management of these infections is the development of resistance among many enterococcal strains against many of the available, previously effective antibiotics, including vancomycin (Havard et al., 1959; Murray & Mederski-Samaroj, 1983; Uttley *et al.*, 1988; Grayson *et al.*, 1991; Bonten *et al.*, 2001; Tenover & McDonald, 2005). Although a modest number of new antibiotics, such as linezolid and daptomycin, have been developed to provide treatment alternatives in cases of infection by organisms that are resistant to all previously available antibiotics, there have been numerous reports of resistance by *E. faecalis* and *E. faecium* strains to these antibiotics as well (Eliopoulos *et al.*, 1998; Prystowsky *et al.*, 2001; Gonzales et al., 2001; Herrero *et al.*, 2002; Johnson *et al.*, 2004; Munoz-Price *et al.*, 2005; Kanafani *et al.*, 2007; Hidron *et al.*, 2008; Marshall *et al.*, 2009; Kelesidis *et al.*, 2011; Ross *et al.*, 2011; Ntokou *et al.*, 2012). Therefore, alternative approaches to manage these infections should be explored.

We have been exploring the possibility of engineering variants of phage ϕEf11 that might be effective in controlling these infections. ϕEf11 possesses several characteristics making it a favourable candidate virus to be used in phage therapy: there are no toxin-related genes detected in the ϕEf11 genome, and it encodes several (4–6) genes encoding proteins with lysis-associated functions (Stevens *et al.*, 2011). However, as a temperate virus that is difficult to propagate, wild-type ϕEf11 would certainly not be suitable as a potential therapeutic agent. It would not be expected to infect strains of *E. faecalis* lysogens due to repressor-mediated superinfection immunity, nor would it be expected to be highly lytic by virtue of its ability to enter into a lysogenic life cycle. Consequently, in its wild-type state, it would have an unacceptably limited host range. Therefore, we sought to determine whether appropriate genetic modifications of ϕEf11 would result in a virulent, highly lytic variant that was insensitive to repression and incapable of lysogeny. These studies shed light on some of the regulatory mechanisms that function in controlling the life cycle of this bacterial virus, and generated a variant phage that had a notably broader host range. Here, we present the identification of the phage virulence determinants, and the development of a virulent version of the temperate *E. faecalis* bacteriophage ϕEf11.

METHODS

Bacterial strains and growth conditions. TUSoD11 is a lysogenic *E. faecalis* strain, harbouring a ϕEf11 prophage, which was previously isolated from an infected root canal (Stevens *et al.*, 2009). Following curing, the non-lysogenic variant of this strain was designated *E. faecalis* TUSoD11 ($\Delta\phi$Ef11). JH2-2 is a Fus^r, Rif^r mutant of a clinical *E. faecalis* isolate (Jacob & Hobbs, 1974) that was generously provided to us by Dr Nathan Shankar. In the course of this study, it was found that this strain harboured a ϕFL1C-type prophage element (Yasmin *et al.*, 2010), indicating that this strain was a lysogen with a defective prophage. Other *E. faecalis* strains used in this study are listed in Table 1. All strains were grown in brain–heart infusion (BHI) broth (or on BHI agar, with appropriate antibiotics). *Escherichia coli* One Shot Mach-T1 (Invitrogen) was used in cloning plasmids as described below. Cells were grown in Luria–Bertani (LB) medium supplemented with the appropriate antibiotics. Additional bacterial species used as negative controls in PCR experiments are also listed in Table 1.

Construction of recombinant plasmids. The nisin promoter (P^{nisA}) cassette containing an erythromycin selection marker (*erm*) was PCR-amplified using the AccuPrime DNA *Taq* Polymerase High Fidelity kit (Invitrogen) with primer set PNISaF/PNISR (see Table 2 for primer specifications) from plasmid pMSP3535 (Bryan *et al.*, 2000), a kind gift from Dr B. Buttaro. PCRs were performed in 30 µl reaction mixtures containing 2 µl template DNA, 2 µl (20 pmol) forward primer, 2 µl (20 pmol) reverse primer, 21.5 µl distilled H_2O, 2 µl buffer (provided by the manufacturer) and 0.5 µl AccuPrime DNA *Taq* polymerase. The PCR programme used was: 95 °C for 2 min, followed by 35 cycles of (i) 95 °C for 45 s, (ii) 55 °C for 45 s and (iii) 72 °C for 2 min. This was followed by an additional 5 min extension at 72 °C. Following PCR, the amplicons were detected by agarose gel electrophoresis and ethidium bromide staining. The amplicons generated by this procedure were cloned into pCR8/GW/TOPO vector (Invitrogen) to create pErm-PnisA (Fig. S1, available in *Microbiology* Online). The two-component nisin sensor system (*nisR*/*nisK*) which controls the activation of P^{nisA} by nisin was also amplified from pMSP3535 by PCR, using primer set RKnpF/RKaxR, and cloned into pCR8/GW/TOPO to create pRK. The P^{nisA} fragment plus the erythromycin selection marker was digested from pErm-PnisA with *Aat*II and *Sph*I, and inserted into pRK to create pRK-Erm-PnisA. A fragment (pre31) of 1088 bp from nucleotide coordinates 24 585 to 25 672 of ϕEf11 (upstream of ORF31, the first gene of the putative lysogeny module) and a fragment (post36) of 1090 bp from 28 588 to 29 577 of ϕEf11 (immediately upstream of the putative *cro* gene, ORF37) were PCR-amplified using primer sets EF31UF/EF31UR and EF37DF/EF37DR, respectively, and cloned into pCR8/GW/TOPO to create pPre31 and pPost36. The post36 fragment was cut out from the pPost 36 with *Bam*HI and *Sph*I and inserted into pRK-Erm-PnisA, to create pPost36-RK-PnisA. The pPre31 was first digested with *Eco*RI and blunt-ended with the Klenow fragment of DNA polymerase I (Promega), then digested with *Pst*I. Following this, the digested pre31 fragment was cloned into pPost36-RK-PnisA to create the allelic exchange plasmid pΔ31-36 P^{nisA}.

Isolation of spontaneous phage ϕEf11/ϕFL1C-like recombinant [ϕEf11(Δ61-1, ϕFL1C40-44)] and the creation of a lysogen harbouring the recombinant prophage. An exponential phase BHI broth culture of *E. faecalis* JH2-2 was inoculated with phage ϕEf11. After incubation at 37 °C for 1 h, the culture was centrifuged (17 000 ***g*** for 3 min) and the supernatant was filtered (0.45 µm) before being plaque-assayed. After overnight incubation at 37 °C, the plates were examined, and several large, extensively spreading plaques were noted among a background of small, turbid plaques. These large plaques were picked, and the virus in these large plaques was cloned by successive plaque purifications. The genomic DNA from the cloned virus was sequenced by Sanger di-deoxy sequencing reactions as described previously (Stevens *et al.*, 2011).

To create a lysogen harbouring a ϕEf11(Δ61-1, ϕFL1C40-44) prophage, JH2-2 cells from surviving colonies in the centre of the large plaques produced by this virus were cloned and screened for the presence of the recombinant phage genome. This was done by PCR using primers (EF60F/FL1A35R) that recognized ϕEf11 ORF 60 at the 5′ end and ϕFL1C ORF 40 at the 3′ end (see Table 2 for primer specifications). The lysogen harbouring this recombinant prophage was designated *E. faecalis* JH2-2[ϕEf11(Δ61-1, ϕFL1C40-44); (Fig. 1). In addition, virus spontaneously released from this lysogen was detected by plaque assay, and also confirmed to be recombinant by PCR analysis.

Table 1. Bacterial strains used in this study

Strain	Characteristics	Source*
E. faecalis		
TUSoD11	Lysogenic root canal isolate harbouring ϕEf11 prophage	1
JH2-2	Rif^r Fus^r, clinical isolate harbouring defective ϕFL1C prophage	2, 3
OG1RF	Rif^r Fus^r	3, 4
MMH594	Gen^r	3
OG1SSp	Str^r Spc^r	5
ER3/2s, ER5/1	Root canal isolates	6, 7
E1, E2, E3, E4, E5, E6, E7, E8, E10, E11	Oral isolates	6, 8
GS1, GS2, GS3, GS4, GS5, GS6, GS7, GS8, GS9, GS10, GS12, GS13, GS14, GS15, GS16, GS17, GS18, GS19, GS21, GS22, GS23, GS24, GS25, GS26, GS27, GS28, GS29, GS30, GS31, GS32, GS33	Root canal isolates	9
GS34	Tongue	7, 6
OS25	Oral isolate	7, 10
AA-OR3, AA-OR4, AA-OR26, AA-OR34	Oral isolates	7, 11
AA-T4, AA-T26	Tongue	7, 11
V583	Van^r, clinical isolate	7, 12
OS16	Oral isolate	7, 10
TUSoD1, TUSoD2, TUSoD3	Lysogenic root canal isolates	1
TUSoD9, TUSoD10, TUSoD12, TUSoD15, TUSoD17, TUSoD18	Root canal isolates	1
Non-enterococcal spp.		
Streptococcus mutans 10449	Grown in BHI broth	13
Streptococcus sanguis 43055	Grown in BHI broth	13
Fingoldia (*Peptostreptococcus*) *magna* (*magnus*)	Grown in chopped meat broth	13
Clostridium perfringens 13124	Grown in modified PY broth	13
Actinomyces israelii 10049	Grown in BHI broth	13
Eubacterium lentum 43033	Grown in chopped meat broth	13

*1, Stevens *et al.* (2009); 2, Jacob & Hobbs (1974); 3, Dr Nathan Shankar; 4, Dunny *et al.* (1979); 5, Dr Gary Dunny; 6, Johnson *et al.* (2006); 7, Dr Christine Sedgley; 8, Sedgley *et al.* (2004); 9, Sedgley *et al.* (2005a); 10, Sedgley *et al.* (2005b); 11, Sedgley *et al.* (2006); 12, Sahm *et al.* (1989); 13, ATCC.

Deletion of the lysogeny module and replacement of *cro* promoter with P^{nisA} by allelic exchange. Cells of *E. faecalis* lysogen JH2-2[ϕEf11(Δ61-1, ϕFL1C40-44)] were made competent using the procedures described by Shepard & Gilmore (1995). Briefly, the cells were grown in SGM17 medium (37.25 g M17 l^{-1}, 0.5 M sucrose and 8 % glycine) for 48 h at 37 °C. The cells were then harvested by centrifugation, washed twice with EB buffer (0.5 M sucrose and 10 % glycerol), and finally resuspended in EB buffer. Plasmid pΔ31-36 P^{nisA} was linearized with *Xho*I and then electroporated into the competent JH2-2 lysogens using the Bio-Rad MicroPulser System. Following electroporation, 1 ml SGM17MC medium (SGM17 plus 10 mM $MgCl_2$ and 10 mM $CaCl_2$) was added to the electroporation cuvette, which was then incubated for 2 h. Transformants were selected on BHI agar containing erythromycin (30 μg ml^{-1}). Presumptive transformant colonies were screened for deletion of the lysogeny module genes (ϕEf11 ORFs 31–36) and replacement of P^{cro} by P^{nisA} by PCR using primers EF31UUF/RK5R, PNIS3F/37DDR, EF31MF/EF31MR and EF36MF/EF36MR. In addition, control of lytic functions in the prophage by P^{nisA} was demonstrated by measuring phage induction in the presence or absence of nisin (40 ng ml^{-1}). The phage recovered from the induced lysogens lacking ORFs 31–36 and P^{cro}, but containing the P^{nisA} promoter, was designated ϕEf11(*vir*)^{PnisA}.

Screening for the presence of ϕEf11 prophages in *E. faecalis* strains. Primers specific to ϕEf11 were designed from ϕEf11 ORF 43 (GenBank accession number GQ452243). This sequence (ORF 43) of the ϕEf11 genome was chosen as searches of all available databases failed to disclose any homologous sequences to this gene. The forward (ϕEf11F) and reverse (ϕEf11R) primers for amplification of a 165 bp amplicon of this gene are specified in Table 2. Template DNA was prepared as follows: 10 ml broth cultures of each strain to be screened were pelleted by centrifugation, washed in 4 ml wash solution [20 mM Tris/HCl (pH 8.5), 0.85 % NaCl], resuspended in 2 ml lysis buffer [1 % Triton X-100, 20 mM Tris/HCl (pH 8.5), 2 mM EDTA], and heated to 95–100 °C for 10 min. The suspension was then centrifuged and the supernatants were collected and frozen at −80 °C until used in PCR assays (Goncharoff *et al.*, 1993). Extracts from *E. faecalis* TUSoD11 (lysogenic for ϕEf11) were used as positive controls, and extracts from *E. faecalis* JH2-2 (non-lysogenic for ϕEf11) and

Table 2. PCR primers used in this study

Primer	Sequence (5′→3′)	Use
EF31UF	GATAGTTCTTGTTTCGACAAATCAC	Amplify upstream of ϕEf11 ORF 31
EF31UR	CTGTCGACGTTCCTGCAGAGCTCTAAATAAATATGGCAAGTA	Amplify upstream of ϕEf11 ORF 31
EF37DF	CTGGATCCATGTGCTATGATTACTCAAAATTAGCAG	Amplify downstream of ϕEf11 ORF 36
EF37DR	CTGCATGCCCTTTACCAGTAATTTTCGGCGT	Amplify downstream of ϕEf11 ORF 36
RKnpF	CTCCATGGTCTCTCCTGCAGATAGAATTCTCATGTTTGACAGCTTATCA	Amplify *nisR* and *nisK*
RKaxR	CTGCATGCTCTCTCGACGTCGCCAGTTAATAGTTTGCCGAA	Amplify *nisR* and *nisK*
PNISaF	CTGACGTCACAAAAGCGACTCATAGAATTATTTCCTCC	Amplify Erm-P^{nisA}
PNISR	GCTTATCGAAATTAATACGACTCACTATAGG	Amplify Erm-P^{nisA}
EF31UUF	AAGAGCACCTCAAATTCCAGT	Detection of ϕEf11 ΔORFs 31–36 (upstream)
RK5R	TGATAAGCTGTCAAACATGAGAATTCT	Detection of ϕEf11 ΔORFs 31–36 (upstream)
37DDR	TGTGATTTGCATGTAGACATCTCCT	Detection of ϕEf11 ΔORFs 31–36 (downstream)
PNIS3F	TTGTAAAACAGGAGACTCTGCATG	Detection of ϕEf11 ΔORFs 31–36 (downstream)
EF31MF	AAGTTGTTTCCGTGTCAACGTGGC	Detection of ϕEf11 ORF 31 deletion
EF31MR	GTGTCCATCATGGTCGTTTAGCAG	Detection of ϕEf11 ORF 31 deletion
EF36MF	TTATCAGGGTCTGGTGAATGCG	Detection of ϕEf11 ORF 36 deletion
EF36MR	GCAACTTATGAGTGAGCGCAA	Detection of ϕEf11 ORF 36 deletion
ϕEf11F	GAGAGTGGAAGTGGATTCAATG	Detection of ϕEf11 ORF 43
ϕEf11R	GCACTTTCATCTAAACTCTCG	Detection of ϕEf11 ORF 43
EF44F	ACCAAGATTTGACGCAGAAGTTGCC	Detection of ϕEf11 ORF 44
EF44R	TGGCCATCGTCGTCTTTATCTGCT	Detection of ϕEf11 ORF 44
EF60F	AGACGTTTGGACCGAATAGCTGGT	Detection of ϕEf11 ORF 60
EF60R	TGCGGTAAGCTTCTGCGAATTCAA	Detection of ϕEf11 ORF 60
Fl1A35F	GGGAACTAGCAGTTGAAGAATCGC	Detection of ϕFL1C gp40
Fl1A35R	TTCCTTTGTACTATCTTGATCTCCA	Detection of ϕFL1C gp40
Fl1A37F	GAGCGTTTAGATAAGTCGGATTGG	Detection of ϕFL1C gp44
Fl1A38R	CCAAGTTTCTTTAGCCTGGTCACG	Detection of ϕFL1C gp44

numerous unrelated species (see Table 1) were used as negative controls. Reaction mixtures (total 40 µl) for PCR contained 5 µl template DNA, 5 µl (50 pmol) forward primer, 5 µl (50 pmol) reverse primer, 5 µl distilled H_2O and 20 µl 2× Go *Taq* green PCR master mix (Promega). The PCR programme used was 97 °C for 1 min, followed by 26 cycles of (i) 97 °C for 1 min, (ii) 50 °C for 45 s and (iii) 72 °C for 1 min. This was followed by an additional 4 min at 72 °C. Following PCR, amplification products were detected by agarose (2 %) gel electrophoresis and ethidium bromide staining.

Preparation of cured *E. faecalis* TUSoD11. Cells of *E. faecalis* TUSoD11 were made competent for electroporation as described above. After electroporation with the allelic exchange vector pΔ31-36 PnisA, erythromycin-resistant colonies were screened for homologous recombination-mediated deletion of the lysogeny module genes (ORFs 31–36) in the genome of *E. faecalis* TUSoD11. Strains exhibiting deletion of ORFs 31–36 were further tested by PCR for the presence of ϕEf11 genes outside of the lysogeny module. In addition to clones containing ϕEf11 genes other than ORFs 31–36, a few rare clones were identified that lacked any of the ϕEf11 genes. Such clones could not be induced, but could now be infected by phage ϕEf11. These cured clones were designated *E. faecalis* TUSoD11(ΔϕEf11).

Testing adsorption of ϕEf11 and ϕEf11(Δ61-1, ϕFL1C40-44) to lysogenic and non-lysogenic *E. faecalis* strains. *E. faecalis* strains JH2-2, TUSoD11 and the cured strain, TUSoD11 (ΔϕEf11), were grown in BHI medium to exponential phase. Then, 100 µl of ϕEf11 or ϕEf11(Δ61-1, ϕFL1C40-44) preparations were added to 1 ml *E. faecalis* strains. After incubation at 37 °C for 10 min the mixtures were centrifuged at 17 000 ***g*** for 3 min, the supernatants were filtered through 0.45 µm filters and filtrates containing any unadsorbed phage were plaque-assayed, using JH2-2 indicator cells, to determine residual phage titres.

One-step growth curve. Cells of an exponential phase BHI broth culture (2 ml) of *E. faecalis* JH2-2 were collected by centrifugation, resuspended in 1 ml BHI broth, and inoculated with 100 µl of a stock culture of either phage ϕEf11, ϕEf11(Δ61-1, ϕFL1C40-44) or ϕEf11 $(vir)^{PnisA}$. After incubation for 30 min to allow phage adsorption, the cells were recovered by centrifugation, washed three times in BHI broth and finally resuspended in 10 ml BHI broth. Aliquots (500 µl) of the suspension were made, and each was incubated at 37 °C. At various time points, an aliquot was centrifuged to remove the cells, and the supernatant was plaque-assayed, using fresh JH2-2 indicator cells, for phage titre.

Host range determination for ϕEf11, ϕEf11(Δ61-1, ϕFL1C40-44) and ϕEf11$(vir)^{PnisA}$. Plaque assays and spot tests were conducted with wild-type phage ϕEf11 and recombinant phages ϕEf11(Δ61-1, ϕFL1C40-44) and ϕEf11$(vir)^{PnisA}$ using the panel of 67 *E. faecalis* strains listed in Table 1 as indicators. The *E. faecalis* panel included both lysogenic and non-lysogenic strains. Lytic infection by each phage was detected by plaque assay with each *E. faecalis* indicator strain.

RESULTS

Isolation of spontaneous ϕEf11/ϕFL1C recombinant

Following repeated propagation and plaque assay of phage ϕEf11 on host strain *E. faecalis* JH2-2, it became evident

that variants of the wild-type virus were being generated. Whereas wild-type φEf11 produced small, turbid plaques in lawns of JH2-2, approximately 0.02 % of the plaques appeared as large, extensively spreading, somewhat clearer zones of lysis (Fig. 2). Interestingly, incubation of plaque assays of clones obtained by plaque purification of the virus producing these larger plaques resulted in continued expansion of the plaques to the extent that virtually the entire JH2-2 lawn was lysed (Fig. 2). In contrast, wild-type plaques typically disappeared after extended incubation, presumably due to growth of surviving lysogens within the plaques (Fig. 2). Agarose gel electrophoresis analysis of the *Nde*I restriction fragments of the DNA from the virus producing these large plaques revealed that it was missing one of the fragments (fragment 6, 2.79 kbp) that was present in the *Nde*I DNA digestion of the original φEf11 isolate (Fig. S2). In addition, it was also noted that another of the *Nde*I fragments (fragment 2, approx. 9.4 kbp) from the DNA of the virus producing the large plaques had increased in size (compared with *Nde*I fragment 2 from the original φEf11 DNA) by an amount approximately equal to the size of the missing *Nde*I fragment 6 (Fig. S2). Close inspection of the φEf11 *Nde*I restriction map (Fig. S3) and the φEf11 *Nde*I restriction digest summary (Table S1) revealed that *Nde*I fragment 6 was composed of the two extreme ends of the genome (fragment coordinates 0–1036 plus 41 068–42 822), and that in a circularly permuted genome, this fragment is immediately adjacent to *Nde*I fragment 2 (coordinates 33 692–41 068; Fig. S3). Sequencing this region of the genome disclosed that ORFs 60–65 and 1 (coordinates 39 671–42 822 and 1–336) were replaced by ORFs 40–44 (coordinates 14 600–17 336) of *E. faecalis* phage φFL1C (Fig. 1). Furthermore, the *Nde*I restriction site at co-ordinate 41 068, which divides *Nde*I fragment 2 from *Nde*I fragment 6 in the φEf11 DNA, is absent in the φFL1C DNA and consequently in the DNA of the recombinant virus (Fig. 1). PCR, restriction fragment analysis and partial sequencing of the recombinant DNA failed to detect any other modifications to the φEf11 genome. Consequently, this φEf11/φFL1C recombinant was designated phage φEf11(Δ61-1, φFL1C40-44). Because the JH2-2 genome was the only possible source of the φFL1C genes, we decided to screen *E. faecalis* JH2-2 for the φFL1C prophage. φFL1C (ORFs 40–44)-specific primers (Table 2) were used in PCR with JH2-2 extracts, prepared as described previously. As seen in Fig. S4, φFL1C-specific amplicons were generated from the JH2-2 templates and the φFL1C-specific primers, confirming the presence of (at least a portion of) a φFL1C prophage in the JH2-2 chromosome. PCR, using JH2-2 template DNA and primers specific for regions of the φFL1C genome other than ORFs 40–44, failed to produce any amplicons (data not shown).

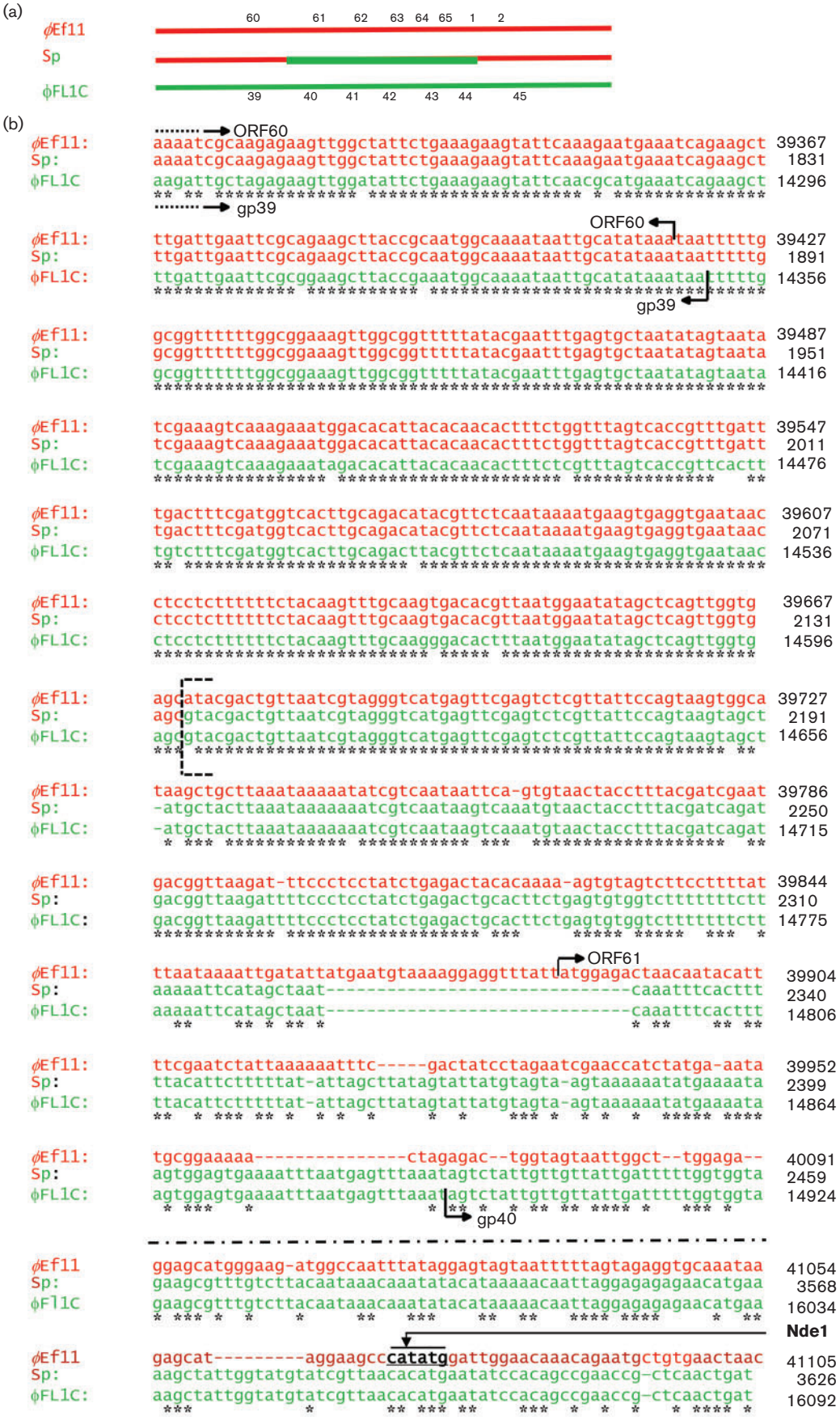
Deletion of the lysogeny module and replacement of *cro* promoter in φEf11(Δ61-1, φFL1C40-44) by allelic exchange

Although the φEf11(Δ61-1, φFL1C40-44) recombinant exhibited enhanced lytic activity (compared with wild-type virus) as judged by the extensively enlarged plaques it forms in lawns of host cells (Fig. 2), and the elevated titres it achieved in productive infection (Fig. 3), these variants of phage φEf11 would still be expected to be subject to repression due to superinfection immunity, and be limited in lytic infection due to the possibility of entering into lysogeny, rather than generating a productive infection. Consequently, we sought to delete all lysogeny-related genes and render regulatory genetic elements insensitive to repressor control. Clones of JH2-2[φEf11(Δ61-1, φFL1C40-44)] transformed with plasmid pΔ31-36 PnisA were selected on erythromycin-containing media. PCR analysis and sequencing of these erythromycin-resistant JH2-2[φEf11(Δ31-36, ΔP^{CRO},P^{nisA}, *erm*, *nisR*/*K*, Δ61-1, φFL1C40-44)] clones demonstrated that they lacked φEf11 ORFs 31–36, and the φEf11 *cro* promoter, but contained the nisin promoter (P^{nisA}) and *nisR*/*nisK* (Figs 4 and S5). Furthermore, exposure of a population of this lysogenic clone, harbouring a mutant prophage containing the nisin promoter (P^{nisA}) in place of the wild-type *cro* promoter/operator (P^{CRO}), to nisin (40 ng ml^{-1}) resulted in the induction of phage, yielding a mean (±SD) titre of 6.82×10^7 p.f.u. ml^{-1} ($\pm 0.31 \times 10^7$). In the absence of nisin, a similar population of these lysogens spontaneously released phage, producing a titre of 5.57×10^5 p.f.u. ml^{-1} ($\pm 0.31 \times 10^5$). In contrast, phage induction from lysogens {JH2-2[φEf11(Δ61-1, φFL1C40-44)]} containing a prophage with the wild-type *cro* promoter/operator did not appear to be affected by the presence of nisin: in the presence of nisin (40 ng ml^{-1}), these cells produced a phage titre of 3.36×10^5 p.f.u. ml^{-1} ($\pm 0.25 \times 10^5$), whereas the same cells produced a titre of 3.31×10^5 p.f.u. ml^{-1} ($\pm 0.38 \times 10^5$) in the absence of nisin. These data suggest that productive infection was now under control of the nisin-sensitive promoter (P^{nisA}), albeit this being somewhat leaky.

The virus obtained, phage [φEf11(Δ31-36,ΔP^{CRO}, P^{nisA}, *erm*, *nisR*/*K*,Δ61-1, φFL1C40-44)], by nisin induction of the JH2-2[φEf11(Δ31-36, ΔP^{CRO}, P^{nisA}, *erm*, *nisR*/*K*, Δ61-1, φFL1C40-44)] lysogens produced large, clear plaques (Fig. 2), and was designated φEf11(*vir*)^{PnisA}. As shown below, this derivative of temperate phage φEf11 had all the characteristics of a virulent virus.

Isolation of cured *E. faecalis* TUSoD11

After electroporation of *E. faecalis* TUSoD11 with the gene exchange vector pΔ31-36 PnisA, erythromycin-resistant colonies were screened by PCR for deletion of ORFs 31–36. Unexpectedly, a few colonies were found with deletions of not only the intended ORF 31–ORF 36 lysogeny module,


(a)
ϕEf11
Sp
ϕFL1C
60 61 62 63 64 65 1 2
39 40 41 42 43 44 45
(b)
ORF60
gp39
ORF60
gp39
ORF61
gp40
Nde1
catatg

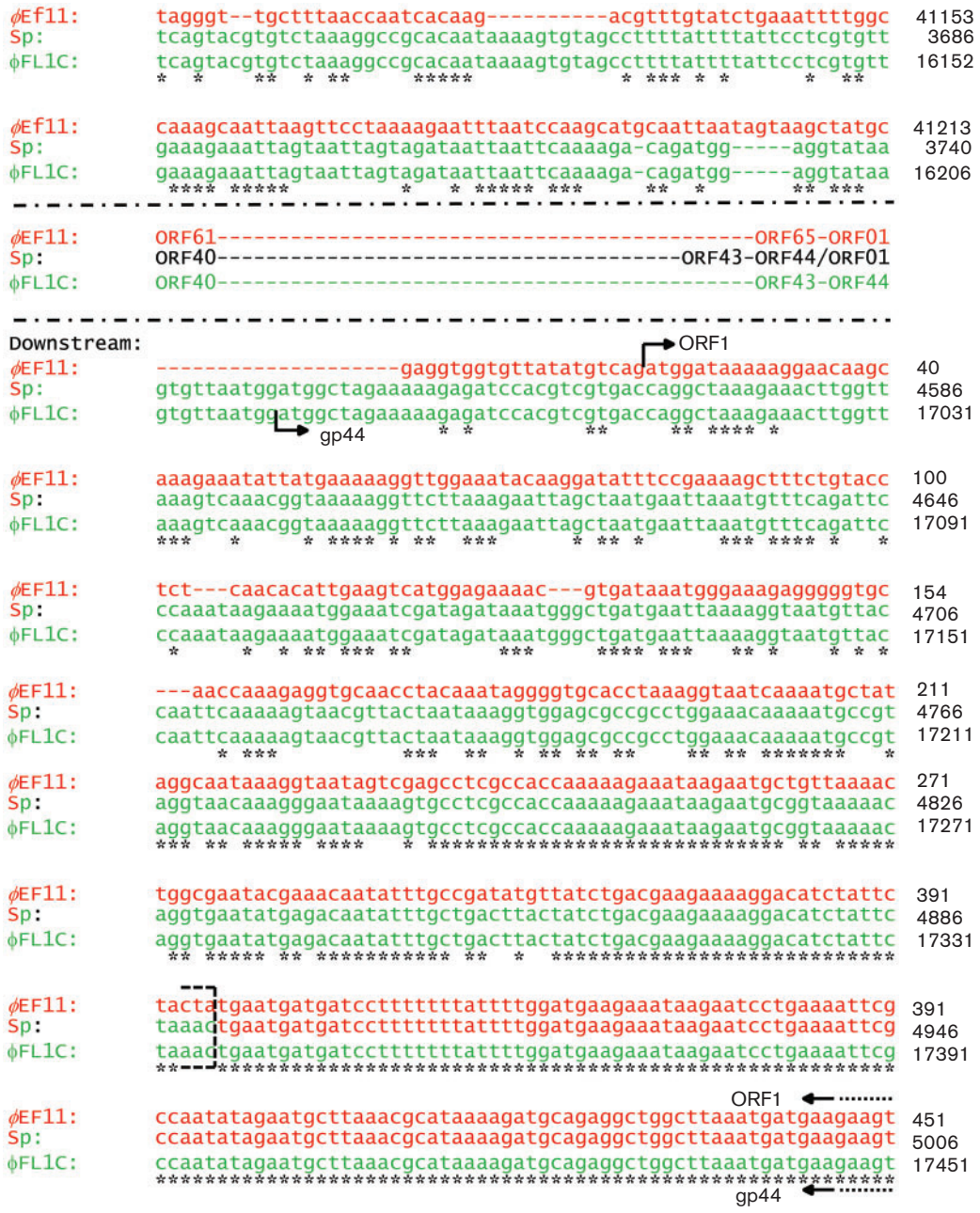


Fig. 1. Sequence alignment of phages ϕEf11, ϕFL1C and spontaneous recombinant phage [ϕEf11(Δ61-1, ϕFL1C 40-44)] in the region of recombination (ϕEf11 ORFs 60/61-1 and ϕFL1C ORFs 39/40–44). Red indicates ϕEf11 sequences, green indicates ϕFL1C sequences. Sp, Spontaneous recombinant. Genomic coordinates are indicated to the right of each row of sequence. Sites of sequence identity between ϕEf11 and ϕEf11(Δ61-1, ϕFL1C 40-44) are indicated by asterisks. (a) Overview of the regions of ϕEf11 and ϕFL1C that recombined to yield recombinant ϕEf11(Δ61-1, ϕFL1C 40-44). (b) ϕEf11 sequence from 39 307 (within ORF 60) to 451 (within ORF 1), and ϕFL1C sequence from 14 236 (within ORF 39) to 17 451 (within ORF 44). The segment of the ϕEf11 sequence that has been replaced by the ϕFL1C sequence to form the ϕEf11(Δ61-1, ϕFL1C 40-44) recombinant is indicated by [ ]. *Nde*I restriction site in ϕEf11 sequence is indicated.

but also all other phage genes outside this region. These clones may have been generated by the homologous recombination between the gene exchange vector and a permutated and terminally redundant prophage DNA that may have positioned the ORF 30 and ORF 37 regions at either end of the ϕEf11 prophage within the host *E. faecalis* TUSoD11 chromosome. These *E. faecalis* clones, lacking any detectable ϕEf11 genes, were designated TUSoD11 (ΔϕEf11), and were further tested for phage induction. No phage could be induced from these cells.

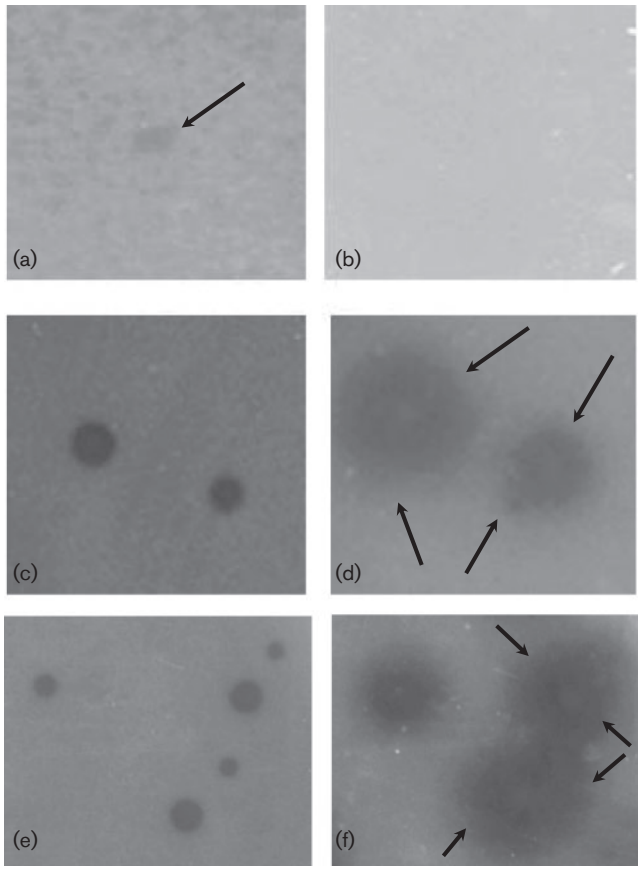


Fig. 2. Plaque assay of ϕEf11 wild-type (wt), spontaneous recombinant [(ϕEf11(Δ61-1, ϕFL1C40-44)] and virulent mutant [ϕEf11(*vir*)^{PnisA}]. (a) wt after incubation for 1 day, (b) wt after incubation for 2 days, (c) spontaneous recombinant after incubation for 1 day, (d) spontaneous recombinant after incubation for 4 days, (e) virulent mutant after incubation for 1 day, (f) virulent mutant after incubation for 4 days.

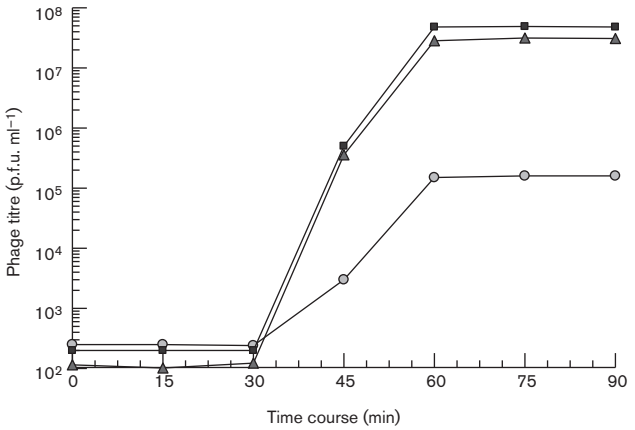


Fig. 3. One-step growth curve for phages ϕEf11 (wild-type), ϕEf11(Δ61-1, ϕFL1C40-44) (spontaneous recombinant) and ϕEf11(*vir*)^{PnisA} (virulent variant). Exponential phase broth cultures of *E. faecalis* JH2-2 were infected with a phage stock. After adsorption for 30 min, the cells were collected by centrifugation, washed and incubated at 37 °C. At various time points aliquots of the suspension were centrifuged to remove the cells, and the supernatants were plaque assayed for phage titre using JH2-2 indicator cells. (●) ϕEf11 titre (p.f.u. ml^{-1}), (■) ϕEf11(Δ61-1, ϕFL1C40-44) titre, (▲) ϕEf11(*vir*)^{PnisA} titre.

Restoration of adsorption of ϕEf11 and ϕEf11(Δ61-1, ϕFL1C40-44) by a cured *E. faecalis* strain

Although it is not surprising that neither ϕEf11 nor ϕEf11(Δ61-1, ϕFL1C40-44) could produce a viable infection on the lysogenic TUSoD11 strain due to superinfection immunity, it was interesting that incubation of either ϕEf11 or ϕEf11(Δ61-1, ϕFL1C40-44) with a cell suspension of lysogenic *E. faecalis* strain TUSoD11 failed to result in phage adsorption to the cells. In contrast, cell suspensions of either strain JH2-2 (non-lysogenic with respect to ϕEf11) or TUSoD11(ΔϕEf11), a cured *E. faecalis* strain, effectively adsorbed both virus strains (Table 3).

Host range of ϕEf11(*vir*)^{PnisA}

The ability of ϕEf11(*vir*)^{PnisA}, in comparison with wt ϕEf11, to generate a productive infection in strains of *E. faecalis* is shown in Table 4. Whereas wild-type ϕEf11 productively infected only four (6 %) of the 67 *E. faecalis* strains tested, productive infection occurred in 33 (49 %) of these strains inoculated with phage ϕEf11(*vir*)^{PnisA}. The panel of *E. faecalis* strains was also screened by PCR for the presence of a prophage, using ϕEf11-specific primers. Among the strains tested, 14 were found to be ϕEf11 lysogens (data not shown). Of these 14 ϕEf11 lysogens, none was susceptible to wild-type ϕEf11, although four of these lysogenic strains (strains GS2, GS8, GS22 and GS25) could be productively infected by ϕEf11(*vir*)^{PnisA}. Furthermore, the presence of the ϕEf11 repressor gene (*cI*, ORF 36) was confirmed in these ϕEf11(*vir*)^{PnisA} -susceptible lysogenic strains by PCR (data not shown).

DISCUSSION

The outcome between the competing, alternative life cycles of lysogeny and productive infection in temperate bacterial viruses is largely determined by the presence or absence of functional early expression genes, such as those coding for the repressor and the integrase proteins, and the related early gene promoters (Ptashne, 2004). In the case of bacteriophage ϕEf11, a genomic module of six contiguous putative early expression genes with lysogeny-related functions was identified by sequencing and homology comparison with known lysogeny-related genes of other bacterial viruses (Stevens *et al.*, 2011). In the same study, a region of the ϕEf11 genome with marked similarity to the

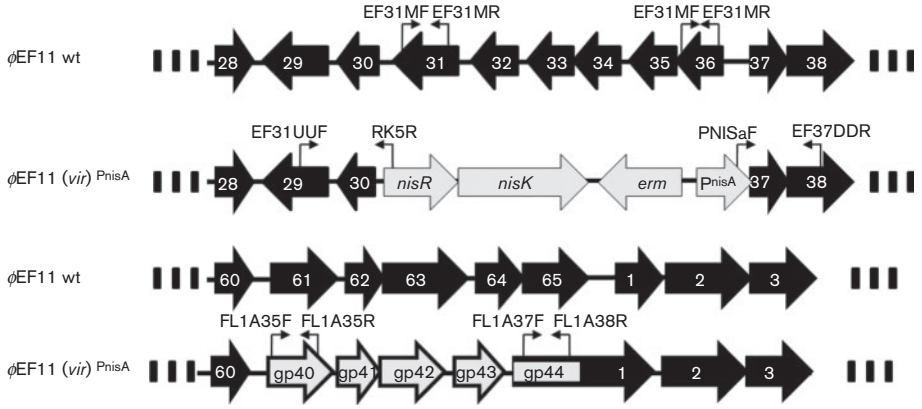


Fig. 4. ϕEF11 (wild-type) and ϕEF11(*vir*)^{PnisA} sequence comparison. Note that in the virulent mutant, ϕEF11(*vir*)^{PnisA}, ORFs ORFs 31–36 as well as the *cro* promoter were allelically exchanged for the nisin promoter cassette, and ORFs 61–1 were allelically exchanged with gp40-gp44 of ϕFL1C. Primer binding sites are indicated by ➡ and ⬅

P_R and P_L early promoter region of the temperate lactococcal bacteriophage TP901-1 (Madsen & Hammer, 1998) was also detected, suggesting a similar regulatory function in ϕEf11. To confirm the predicted functions of these regions of the ϕEf11 genome, and to develop a potentially useful agent for phage therapy, we wished to determine whether deletion or replacement of these sites in phage ϕEf11 would result in a derivative virus with virulent rather than temperate properties. There is substantial precedent for temperate to virulent conversion of phage by modification of these genomic sites (Bailone & Devoret, 1978; Flashman, 1978; Donnelly-Wu *et al.*, 1993; Bruttin & Brüssow, 1996; Ford *et al.*, 1998).

Our previous analysis of the phage ϕEf11 genome concluded that a module of six contiguous genes (ORFs 31–36) was responsible for functions related to the establishment of lysogeny (Stevens *et al.*, 2011). This module included a putative integrase (ORF 31) and a putative *cI* repressor (ORF 36). In the present investigation, we found that these six genes are completely dispensable for lytic cycle function, as deletion of these genes did not prevent productive infection by the virus. Infection of lawns of host cells by the mutant virus lacking these genes produced clear plaques. Furthermore, we were unable to recover surviving (presumptive lysogenic) cells from the plaques produced by the mutant virus lacking ORFs 31–36. Therefore, these data suggest that the predicted lysogeny-related functions of these genes were correct, and that by deleting these genes from the viral genome, we have generated a ϕEf11 mutant that is incapable of lysogeny. It should be noted, however, that complementation studies to confirm the function of these (putative lysogeny-related) genes could not be carried out because the ϕEf11*(vir)*^{PnisA} genome includes a nisin promoter, in place of the wild-type *cro* promoter, which is not sensitive to repression by the *cI* gene product (see below).

Similarly, we previously identified a stem–loop structure surrounded by P_L and P_R promoter sequences in the ϕEf11 genome lying between a putative *cI* repressor gene and a putative *cro* gene, which was highly similar to the P_R/P_L promoter/operator region of *Lactococcus* bacteriophage TP901-1 (Stevens *et al.*, 2011; Madsen & Hammer 1998). In deleting this sequence, and replacing it with a nisin-inducible promoter, we have generated a virus that was

Table 3. Phage adsorption by lysogenic and non-lysogenic *E. faecalis* strains

Phage suspensions were incubated with each of the indicated *E. faecalis* strains for 10 min, whereupon the cultures were centrifuged and filtered to remove the cells along with all adsorbed phage. The cell-free filtrates were then assayed for residual phage titre. Values represent the mean of triplicate assays ± SD.

Phage	Phage titre before adsorption	Residual phage titre after adsorption with:		
		Lysogen TUSoD11	Non-lysogen JH2-2	Non-lysogen cured TUSoD11
ϕEf11	1.2×10^5	$1.17 \times 10^5 \pm 0.16 \times 10^5$	$3.2 \times 10^2 \pm 0.25 \times 10^2$	$2.74 \times 10^2 \pm 0.16 \times 10^2$
ϕEf11(Δ61-1, ϕFL1C40-44)	5.58×10^7	$5.23 \times 10^7 \pm 0.23 \times 10^7$	$4.79 \times 10^2 \pm 0.23 \times 1^2$	$3.82 \times 10^2 \pm 0.17 \times 10^2$

Table 4. Host range of *E. faecalis* phages

E. faecalis strain	Phage		
	φEf11 (wild type)	**φEf11(Δ61-1, φFL1C39-44) (spontaneous recombinant)**	**φEf11(Δ31-36, ΔP^{CRO}, P^{nisA}, Δ61-1, φFL1C39-44) (virulent mutant)**
OG1RF	–	–	–
ER3/2s	–	–	–
ER5/1	–	–	+
E1	+	+	+*
E2†	–	–	–
E3†	–	–	–
E4†	–	–	–
E5†	–	–	–
E6	–	–	–
E7†	–	–	–
E8	–	–	+
E10	–	–	+
E11	–	–	+
GS1	–	–	–
GS2†	–	–	+
GS3	–	–	+
GS4	–	–	–
GS5	–	–	–
GS6	–	–	+
GS7	–	–	+
GS8†	–	–	+
GS9†	–	–	–
GS10	–	–	–
GS12	–	–	–
GS13	–	–	+
GS14	–	–	+*
GS15	–	–	+
GS16	–	–	+
GS17	–	–	–
GS18	–	–	–
GS19	–	–	+
GS21	–	–	–
GS22†	–	–	+
GS23†	–	–	–
GS24	–	–	+
GS25†	–	–	+
GS26	–	–	+
GS27	–	–	+
GS28	–	–	–
GS29†	–	–	–
GS30	–	–	+*
GS31	–	–	–
GS32	–	–	–
GS33†	–	–	–
GS34	–	–	–
OS25	–	–	+
AA-OR3	–	–	+
AA-OR4	–	–	+
AA-OR26	–	–	+*
AA-OR34†	–	–	–
AA-T4	–	–	+
AA-26	–	–	+*
V583	–	–	+
OS16	–	–	+
TUSoD1	+*	+*	+
TUSoD2	–	–	–
TUSoD3	–	–	–
TUSoD9	–	–	+
TUSoD10	–	–	–
TUSoD12	–	–	–
TUSoD15	–	–	–
TUSoD17	–	–	–
TUSoD18	+*	–	–
MMH594	–	–	–
OG1SSP	–	–	+
DG16	–	–	–
JH2-2	+	+	+
Cumulative	6.0%	4.5%	49.3%

*Sensitive to phage (spot test).
†Lysogenic *E. faecalis* strain containing φEf11 prophage.
+, Sensitive to phage (plaque assay); –, not sensitive to phage.

capable of productively infecting *E. faecalis* (φEf11) lysogens, in the presence of the φEf11 *cI* repressor protein. Therefore, these data support our computationally based prediction that this region of the φEf11 genome (i.e. between ORFs 36 and 37) is a regulatory sequence that determines the outcome of phage infection between lysogeny and a productive infective cycle. Thus, it appears that the φEf11(*vir*)^{pnisA} variant resulting from replacing the wild-type P_R/P_L promoter/operator sequence with a nisin-inducible promoter, P^{nisA}, is indeed insensitive to *cI* repression.

Surprisingly, spontaneous recombinational replacement of five genes (ORFs 61–65) of the DNA replication/modification module and one gene (ORF 1/terminase A) of the packaging module by five genes (ORFs 40–44) of *E. faecalis* phage φFL1C also had an effect on the virulence properties of the virus. While this genetic recombination had no effect upon host range, it did markedly alter the lytic properties observed during infection of either broth cultures or soft agar overlay lawns of susceptible host cells. Broth cultures rapidly and more thoroughly cleared, after infection by the recombinant phage φEf11(Δ61-1, φFL1C40-44), as compared with infection by the wild-type φEf11 virus. Similarly, plaques produced by the recombinant phage φEf11(Δ61-1, φFL1C40-44) appeared as large, extensively spreading lytic zones with a clearer centre, compared with

those formed by the wild-type ϕEf11 virus. As the predicted function of the replaced genes involved either DNA replication/modification (ORFs 61–65), or packaging (ORF 1), we hypothesize that the replacement (ϕFL1C) genes contributed to a more robust, more productive lytic infection by increasing the efficiency of either phage DNA synthesis or packaging, or both.

The results of one-step growth experiments for wild-type ϕEf11 and recombinant ϕEf11(Δ61-1, ϕFL1C40-44) phages appear to bear out the above hypothesis that recombination of ϕEf11 with the ϕFL1C genes results in a greatly (>100-fold) enhanced production of progeny virus (Fig. 3). As both the wild-type (ϕEf11) and the recombinant ϕEf11(Δ61-1, ϕFL1C40-44) phages are temperate, and we do not know what proportion of infected cells undergoes productive infection and what proportion becomes lysogens, we cannot accurately determine a burst size for either strain (although the latent period of both strains, approx. 30 min, appears to be similar). That is, it is not possible to determine from these data whether the difference in titre observed between the two phage strains is due to a greater burst size produced by the recombinant or a higher proportion of cells in the population that undergoes lytic infection (or both). Nevertheless, these data do demonstrate that a much greater titre of virus is produced in *E. faecalis* populations infected by the ϕEf11(Δ61-1, ϕFL1C40-44) recombinant as compared with the wild-type virus.

In addition, the source of the ϕFL1C genes (i.e., the *E. faecalis* JH2-2 chromosome) was unexpected, as previous studies reported that this *E. faecalis* strain was susceptible to ϕFL1C infection, and in fact could form ϕFL1C lysogens following ϕFL1C infection, suggesting that this strain did not initially harbour a ϕFL1C prophage (Yasmin *et al.*, 2010). This conundrum was solved when we used PCR to attempt to detect other regions of the ϕFL1C genome in JH2-2. No other regions of the ϕFL1C genome could be detected in JH2-2, suggesting that the ϕFL1C sequence that we detected was part of a defective (incomplete) prophage, or was the only ϕFL1C-like portion of a complete prophage. Under such circumstances, it would not be surprising for JH2-2 cells (lacking the ϕFL1C immunity functions) to be susceptible to ϕFL1C infection, while being the source of a limited number of ϕFL1C genes.

The lack of adsorption of either the wild-type or the spontaneous recombinant phage ϕEf11(Δ61-1, ϕFL1C40-44) by lysogenic *E. faecalis* strain TUSoD11 suggests either that this lysogen lacked the phage receptor, or that the phage receptor had been modified, rendering it incapable of binding the phage ligand. The fact that curing TUSoD11 rendered it capable of adsorbing ϕEf11 supports the notion that lysogeny either altered or eliminated the phage receptor on the cell surface. Prophage-mediated modification of phage receptors has been long known and well documented for several other phage/host systems (Uetake *et al.*, 1958; Holloway & Cooper, 1962; Losick & Robbins, 1967; Castillo & Bartell, 1974; Gemski *et al.*, 1975; Kuzio & Kropinski, 1983; Tomás & Kay, 1984). Enzymes specified by these phages catalyse modifications of phage receptor sites (e.g. *O*-acetylation of the lipopolysaccharide side chains, changes in the bonding between the lipopolysaccharide trisaccharide units from α1⟶4 to β1⟶4) on the host cell, resulting in the inability of the cell to adsorb additional phage. Although the previous examples of phage receptor modification involve Gram-negative bacteria, it is possible that a similar phenomenon may occur in *E. faecalis* as the phage receptors in most Gram-positive, low G+C bacteria are cell surface polysaccharides (Vidaver & Brock, 1966; Douglas & Wolin, 1971; Cleary *et al.*, 1977; Yokokura 1977; Keogh & Pettingill, 1983; Valyasevi *et al.*, 1990; Schäfer *et al.*, 1991). If this is the case, then evolutionarily speaking, it may be that originally *E. faecalis* TUSoD11 was non-lysogenic and possessed cell-surface phage receptors. Upon infection and lysogenization by a temperate phage ϕEf11 at some time in the past, the cell's phage receptors were lost or modified, resulting in a non-phage-adsorbable cell surface. However, once cured of the phage, the cell surface changed back to its original form that could again adsorb the phage and support virus infection. However, note that we found some lysogenic *E. faecalis* strains to be sensitive to ϕEf11(*vir*)$^{\mathrm{PnisA}}$, indicating that they must be able to adsorb the virus. Why some lysogens (e.g. *E. faecalis* GS8) should be able to adsorb the phage while others (e.g. TUSoD11) do not remains to be determined.

In summary, we have developed a virulent variant of temperate *E. faecalis* phage ϕEf11 that is incapable of lysogeny, is insensitive to *cI* repression, is highly lytic and has a greatly extended host range compared with the wild-type virus. In the course of constructing this virus we have increased our understanding of the genome of phage ϕEf11 by confirming the predicted function of several of the genomic loci. These include regions responsible for establishing and maintaining lysogeny. In addition, we have determined that incorporating allelic alternatives in a region of the genome responsible for DNA replication/modification and packaging results in enhancement of productive infection in the host cell population, thereby capturing phage mozaicism happening in real-time.

We recognize that further genetic modifications will be necessary to develop a phage that may be clinically useful in managing enterococcal infections. Replacing the repressor-sensitive *cro* promoter with the repressor-insensitive, nisin-inducible promoter system to drive phage lytic infection functions proved to be a very effective and useful strategy in making genetic modifications in the virus. However, because the inducing agent, nisin, is a toxic protein, this approach would not be suitable in an agent designed for therapeutic application. Consequently, we are exploring alternative, constitutive promoters to replace the nisin-inducible promoter in variants of the ϕEf11(*vir*)$^{\mathrm{PnisA}}$ strain. Similarly, we used antibiotic (erythromycin) resistance to select transformant lysogen clones containing prophages

with the desired genotype. It would be necessary to delete this determinant prior to using this agent clinically. Finally, while the virulent variant phage that we have constructed possesses an eminently greater host range than the wild-type virus, there are still *E. faecalis* strains that do not appear to be sensitive to this virus. We are exploring additional strategies aimed at further expanding the host range of the ϕEf11(*vir*)^{PnisA} virulent variant that we have constructed.

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