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Effect of a genetically engineered bacteriophage on *Enterococcus faecalis* biofilms

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ABSTRACT

Objective: *Enterococcus faecalis* is a Gram-positive, facultative anaerobic bacterium that is associated with failed endodontic cases and nosocomial infections. *E. faecalis* can form biofilms, penetrate dentinal tubules and survive in root canals with scarce nutritional supplies. These properties can make *E. faecalis* resistant to conventional endodontic disinfection therapy. Furthermore, treatment may be complicated by the fact that many *E. faecalis* strains are resistant to antibiotics. A potential alternative to antibiotic therapy is phage therapy. ϕEf11 is a temperate phage that infects strains of *E. faecalis*. It was previously sequenced and genetically engineered to modify its properties in order to render it useful as a therapeutic agent in phage therapy. In the current study, we have further genetically modified the phage to create phage ϕEf11/ϕFL1C(Δ36)^{PnisA}. The aim of this study was to evaluate the efficacy of bacteriophage ϕEf11/ϕFL1C(Δ36)^{PnisA}, to disrupt biofilms of two *Enterococcus faecalis* strains: JH2-2 (vancomycin-sensitive) and V583 (vancomycin-resistant).

Methods: 24 h static biofilms of *E. faecalis* strains JH2-2(pMSP3535 *nis*R/K) and V583 (pMSP3535*nis*R/K), formed on cover slips, were inoculated with bacteriophage ϕEf11/ϕFL1C(Δ36)^{PnisA}. After 24 and 48 h incubation, the bacterial biomass was imaged by confocal microscopy and viable cells were quantified by colony forming unit measurement.

Results: The results showed a 10-100-fold decrease in viable cells (CFU/biofilm) after phage treatment, which was consistent with comparisons of treated and untreated biofilm images visualized as max projections of the Z-series.

Conclusion: The biomass of both vancomycin-sensitive and vancomycin-resistant *E. faecalis* biofilms is markedly reduced following infection by bacteriophage ϕEf11/ϕFL1C(Δ36)^{PnisA}.

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1. Introduction

Enterococcus faecalis is a Gram-positive, facultative anaerobic bacterium that has been found in the oral cavity in association with periodontal disease (Rams, Feik, Young, Hammond, & Slots, 1992) and endodontic infections (Molander, Reit, Dahlén, & Kvist, 1998; Peciuliene, Balciuniene, Eriksen, & Haapasalo, 2000; Pinheiro et al., 2003).

While this organism is generally related to commensal life in the gastrointestinal tract, (Facklam, 2002) it can also cause systemic opportunistic infections (Gilmore and Ferretti, 2003; Hunt, 1998; Jett, Huycke, & Gilmore, 1994; Moellering, 1992; Woodford, 1998) that can be difficult to eliminate due to its abilities to form mature biofilms (Distel, Hatton, & Gillespie, 2002; Mohamed and Huang, 2007; Rams et al., 1992) and survive in hostile environments with scarce nutritional supplies and extreme alkaline pH's (Figdor, Davies, & Sundqvist, 2003; McHugh, Zhang, Michalek, & Eleazer, 2004; Sedgley, Lennan, & Appelbe, 2005; Stevens and Grossman, 1983). In the oral cavity this organism has the ability to form biofilms in infected root canals as a monoinfection, without the support of other species (Fabricius, Dahlén, Holm, & Möller, 1982; Siren, Haapasalo, Ranta, Salmi, &

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Kerosuo, 1997). It is able to penetrate dentinal tubules and remain in the root canal wall (Hubble, Hatton, Nallapareddy, Murray, & Gillespie, 2003; Love, 2001) thereby rendering it more resistant to conventional endodontic disinfection therapies which require direct contact. *E. faecalis* has been frequently associated with failed endodontic cases (Molander et al., 1998; Peciuliene et al., 2000; Pinheiro et al., 2003; Ricucci and Siqueira, 2010; Rôças, Siqueira, & Santos, 2004) and its presence may prevent the repair process of apical periodontitis (Ricucci and Siqueira, 2010; Stuart, Schwartz, Beeson, & Owatz, 2006). Further complicating treatment of infections by this organism (and its close relative, *E. faecium*) is the emergence of multidrug-resistant (MDR) strains (Arias and Murray, 2009; Bonten, Willems, & Weinstein, 2001; Cetinkaya, Falk, & Mayhall, 2000; Gold and Moellering, 1996; Noskin, 1997; Uttley, Collins, Naidoo, & George, 1988). Multidrug resistance among pathogenic microorganisms is an important topic of global health concern nowadays, as diseases previously thought to be eradicated can once again jeopardize human life. Consequently, alternative antimicrobial strategies for oral, as well as systemic infections by MDR bacteria are urgently needed. One such alternative to antibiotic treatment, is phage therapy: The use of virulent bacterial viruses (phages/bacteriophages) to control infections by their pathogenic host cells (Burrowes, Harper, Anderson, McConville, & Enright, 2011; Sulakvelidze, Alavidze, & Morris, 2001; Summers, 2001). In this regard, we previously isolated a bacteriophage, ØEf11, that we induced from a root canal isolate of *E. faecalis* (Stevens, Porras, & Delisle, 2009). Subsequently, we sequenced and annotated the genome of this phage (Stevens, Ektefaie, & Fouts, 2011) and then genetically engineered it (Zhang, Fouts, DePew, & Stevens, 2013) to modify its properties in order to render it useful as a therapeutic agent in phage therapy. Several iterations of genetic constructs of phage ϕEf11 have been produced in the course of our genetic engineering of the phage. In the present work, we have further modified one of these ϕEf11 derivatives to produce phage ϕEf11/ϕFL1C(Δ36)^{PnisA}. This derivative features the replacement of five open reading frames (ORFs) of phage ϕEf11 by 5 ORFs of a ϕFL1C prophage, deletion of the CI repressor determinant (ORF36), and the replacement of the wild type promoter controlling lytic cycle functions with a nisin-inducible promoter. The product of these genetic modifications [phage ϕEf11/ϕFL1C(Δ36)^{PnisA}] is a phage that is incapable of lysogeny, insensitive to repression by the CI gene product, and has a much wider host range than the wild type virus (phage ϕEf11), but cannot initiate lytic infection in the absence of *nis*R, *nis*K, the determinants of the two component system that regulates the P^{nisA} nisin promoter (Bryan, Bae, Kleerebezem, & Dunny, 2000; Kuipers, Beerthuyzen, de Ruyter, Luesink, & de Vos, 1995). Since the properties of the genetically engineered virus [phage ϕEf11/ϕFL1C (Δ36)^{PnisA}] are markedly different from the wild type phage (phage ϕEf11), we wished to evaluate the efficacy of phage ϕEf11/ϕFL1C (Δ36)^{PnisA} to disrupt biofilms of two *E. faecalis* strains: JH2-2 (vancomycin sensitive) and V583 (vancomycin resistant).

2. Material and methods

2.1. Bacterial strains, and growth conditions

E. faecalis JH2-2 (fusidic acid and rifampin resistant, vancomycin sensitive) was originally isolated by Jacob & Hobbs (1974). *E. faecalis* V583 (vancomycin resistant) was originally isolated by Sahm et al. (1989). Both strains were grown in brain heart infusion (BHI) broth, and transformed with plasmid pMSP3535, *cat*, *nis*R *nis*K, using procedures previously described (Zhang et al., 2013). JH2-2[ϕEf11(Δ61-1, ϕFL1C40-44)] is a lysogenic *E. faecalis* strain harboring prophage ϕEf11(Δ61-1, ϕFL1C40-44), that was prepared previously (Zhang et al., 2013). *E. faecalis* JH2-2(pMSP3535, *cat*, *nis*R/K) was prepared by transforming strain JH2-2 with plasmid (pMSP3535, *cat*, *nis*R/K), and selecting transformants on chloramphenicol-containing media. *nis*R/K are the determinants for the two-component system for regulating the P^{nisA} nisin promotor (Bryan et al., 2000; Kuipers et al., 1995) and *cat* (chloramphenicol acetyl transferase) was used as a selection marker. *E. faecalis* V583(pMSP3535,*cat nis*R/K) was prepared in a similar manner, substituting *E. faecalis* V583 for strain JH2-2.

2.2. Construction of phage ϕEf11/ϕFL1C(Δ36)^{PnisA}

A recombinant plasmid containing a 1 kb DNA fragment upstream of ϕEF11 ORF36, a nisin promoter (P^{nisA}), plus an erythromycin selection marker (*erm*), and a 1 kb fragment downstream of phage ϕEf11P^{cro} (the wild type promoter controlling lytic cycle functions in phage ϕEf11) was constructed essentially as described previously (Zhang et al., 2013). This plasmid was electroporated into a competent strain of *E. faecalis* JH2-2[ϕEf11(Δ61-1, ϕFL1C40-44], harboring the spontaneous recombinant prophage ϕEf11(Δ61-1,ϕFL1C40-44) (Zhang et al., 2013). Following homologous recombination, transformant JH2-2 [ϕEf11(Δ61-1, ϕFL1C40-44, Δ36, *erm*, ΔP^{cro}, P^{nisA})] clones were selected on erythromycin-containing BHI agar plates. The presence of ϕEf11 ORF31, *erm*, P^{nisA}, and the absence of ORF36 and P^{cro} in these cells, were confirmed by PCR analysis. The primers and predicted amplicon sizes are shown in Table 1. These mutant lysogenic clones, which will be referred to as *E. faecalis* JH2-2 [ϕEf11/ϕFL1C(Δ36)^{PnisA}], were subsequently transformed with pMSP3535-*cat, nis*R/K, a plasmid that harbors the two-component *nis*R/K system for regulating the P^{nisA} promotor in phage ϕEf11/ ϕFL1C(Δ36)^{PnisA}, and a chloramphenicol selection marker (*cat*). Induction of *E. faecalis* JH2-2[ϕEf11/ϕFL1C(Δ36)^{PnisA}**]**(pMSP3535-*cat,nis*R/K) by the addition of nisin yielded phage ϕEf11/ϕFL1C (Δ36)^{PnisA}.

2.3. Preparation of phage suspensions

Lysogenic *E. faecalis* JH2-2[ϕEf11/ϕFL1C(Δ36)^{PnisA}**]**(pMSP3535, cat,*nis*R/K) was grown in BHI broth at 37 °C to early log phase. Nisin

Table 1
Primers used for PCR confirmation of insertion or deletion of targeted genes.

Targeted fragment	Primer name	Sequence	Product length
ORF31	EF31F	5′-AAGTTGTTTCCGTGTCAACGTGGC-3′	417bp
	EF31R	5′-GTGTCCATCATGGTCGTTTAGCAG-3′	
ORF36	EF36F	5′-TTATCAGGGTCTGGTGAATGCG-3′	480bp
	EF36R	5′-GCAACTTATGAGTGAGCGCAA-3′	
pnisA + erm	Pnis-ErmF	5′-CTGACGTCACAAAAGCGACTCATAGAATTATTTCCTCC-3′	1102bp
	Pnis-ErmR	5′-TAGTTATAAGACTAGATCTGATCCGTA-3′	

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