HHS Public Access

Author manuscript

Arch Oral Biol. Author manuscript; available in PMC 2017 November 01.

Published in final edited form as:

Arch Oral Biol. 2016 November ; 71: 80–86. doi:10.1016/j.archoralbio.2016.07.001.

Effect of a Genetically Engineered Bacteriophage on *Enterococcus faecalis* Biofilms

Justine Monnerat Tinoco[a,*], **Bettina Buttaro**[b], **Hongming Zhang**[c], **Nadia Liss**[c], **Luciana Sassone**[a], and **Roy Stevens**

[a] Department of Endodontology, School of Dentistry, State University of Rio de Janeiro, Rio de Janeiro, RJ 20551-030, Brazil

[b] Department of Microbiology & Immunology, School of Medicine, Temple University, Philadelphia, PA 19140, USA

[c] Department of Endodontology, School of Dentistry, Temple University, Philadelphia, PA 19140, USA

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 hour 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 hours incubation, the bacterial biomass was imaged by confocal microscopy and viable cells were quantified by colony forming unit measurement.

[*]Corresponding author at: Universidade do Estado do Rio de Janeiro. Boulevard 28 de Setembro, 157 - 2° andar - sala 10 - Pavilhão Mario Franco Barrozo, CEP 20551-030 Vila Isabel, Rio de Janeiro, RJ, Brazil. Phone number; (021) 2868-8282, justinemonnerat@yahoo.com.br (J.M. Tinoco).

Competing interests: None declared.

Ethical approval: Not required.

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}.

Keywords

Antimicrobial; Bacteriophage therapy; *Enterococcus faecalis*; biofilm; resistance; Public Health

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 & Ferretti, 2003; Hunt, 1998; Jett, Huycke, & Gilmore, 1994; Moellering Jr, 1992; Woodford, 1998) that can be difficult to eliminate due to its abilities to form mature biofilms (Distel, Hatton, & Gillespie, 2002; Mohamed & 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 & 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, & 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 & Siqueira, 2010; Rôças, Siqueira, & Santos, 2004) and its presence may prevent the repair process of apical periodontitis (Ricucci & 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 & Murray, 2009; Bonten, Willems, & Weinstein, 2001; Cetinkaya, Falk, & Mayhall, 2000; Gold & Moellering Jr, 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 & 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 (Jacob & Hobbs, 1974). *E. faecalis* V583 (vancomycin resistant) was originally isolated by Sahm et al. (Sahm, Kissinger, Gilmore, Murray, Mulder, Solliday & Clarke,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 1kb DNA fragment upstream of ϕEF11 ORF36, a nisin promoter (P^{nisA}), plus an erythromycin selection marker (*erm*), and a 1kb fragment downstream of phage ϕEf11 P^{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 (40ng/mL) was then added to the culture to induce the phage, and incubation was continued for an additional 8 hours. The resulting lysate was clarified by centrifugation (10,000 × g × 10 min) and the phage was then collected by centrifugation (100,000 × g × 90 min). The resulting phage pellet was resuspended in SM buffer (0.58% NaCl, 0.2% MgSO4 • 7 H_2O, 0.05M Tris-HCl pH 7.5, 0.01% gelatin) (Sambrook, Fritsch, & Maniatis, 1989) containing nisin (40ng/mL), and filtered through a sterile 0.45 μ filter. The phage suspension was then plaque-assayed in the presence of nisin (40ng/mL) using *E. faecalis* JH2-2(pMSP3535, *cat*, *nis*R/K) as an indicator strain. The titer of this phage suspension was 5.8×10^9pfu/mL.

2.4. Static Biofilm Study

Sterile circular glass coverslips (12-mm diameter) were placed on the bottom of wells of 24-well round-bottom tissue culture plates (Techno Plastic Products, Switzerland) and covered with 2 ml of a 1:10 dilution of a BHI broth culture of *E. faecalis* [either JH2-2(pMSP3535, *cat*, *nis*R/K) or V583(pMSP3535,*cat nis*R/K)], prepared from an overnight culture grown at 37° C. After incubation at 37° C for an additional 48 hours to permit the formation of a biofilm, the medium (and planktonic cells) in each well was removed, and the adherent biofilm was gently rinsed three times with phosphate-buffered saline (PBS). Following the final rinse, the biofilm was covered with 200 μL of SM buffer (controls) or SM buffer containing phage ϕEf11/ϕFL1C(Δ36)^{PnisA} (5.8×10^9pfu/mL). The cultures (both controls and phage-inoculated) were then incubated at 37° C for 1 hour to allow phage adsorption. 1.8 ml of BHI (supplemented with nisin and Chloramphenicol) was added to each well, and the plates were maintained at 37° C for an additional 24 or 48 hours, at which time the supernatant was removed, the biofilms were gently washed two times with PBS, and the biofilms were stained using live/dead dyes (Syto 9/Propidium Iodide/PBS) and imaged by confocal microscopy. Parallel cultures were also used to determine residual biomass by measuring *E. faecalis* colony forming units.

2.5. Confocal laser scanning microscopy

Confocal microscopy was performed on *E. faecalis* biofilms grown on the glass coverslips. After being washed three times with 1 ml of PBS, the coverslips were mounted onto

microscope slides (face down) containing a drop of staining mixture (300 μl of PBS, 1 μl of Syto9, 1 μl of Propidium Iodine), and sealed with clear nail polish. Biofilms were visualized using a confocal microscope (Leica DM IRE2 Confocal Microscope with Sp5 data analysis, Leica Microsystems, Danaher, Wetzlar, Germany). The biofilms were imaged with Z-stack series taken every 0.5 μm through the biofilm and images were visualized as max projections of the Z-series.

2.6. Bacterial viability quantitation

The coverslips, containing the biofilms, were gently washed two times with PBS, then they were taken from the wells with sterile tweezers, and placed in 3 ml PBS in a 15-ml, centrifuge tube, which was kept on ice. A sonicator (Sonic Dismembrator, model 550; Fisher Scientific, Pittsburgh, US) was used for 20 seconds at a voltage amplitude of approximately 4.5 to disperse bacteria from the biofilm. Following sonication, the resulting 3 ml bacterial suspension from each biofilm was serially diluted 10-fold, and 20μl aliquots from each dilution were placed on modified thallous acetate (TA) agar plates (proteose peptone 1%, yeast extract 1 %, glucose 1 %, TA 0.2 %, triphenyl tetrazolium chloride 0.01 %, agar 1.3 %), which is selective for enterococci (Holt J. G. and Krieg H. R., 1994). Plates were incubated overnight at 37° C, and the colony forming unit titer was determined from colony counts.

3. Results

3.1. Bacteriophage Ef11/ϕFL1C(Δ36)^{PnisA}

Confirmation of the presence of the ϕEf11/ϕFL1C(Δ36)^{PnisA} prophage in *E. faecalis* JH2-2[Ef11/ϕFL1C(Δ36)^{PnisA}] lysogens was made by PCR analysis. ORF36-specific primers failed to produce an amplicon using *E. faecalis* JH2-2[ϕEf11/ϕFL1C(Δ36)^{PnisA}] DNA as a template, however, an amplicon of the predicted size (1,102 bp) was produced using nisin promoter-specific primers (Fig 1). Furthermore, in the absence of nisin, no phage could be detected in cultures of *E. faecalis* JH2-2[ϕEf11/ϕFL1C(Δ36)^{PnisA}](pMSP3535, *cat*, *nis*R *nis*K), whereas with the addition of nisin (40ng/mL), phage was induced, producing a titer of 2.4×10^7 (Table 2).

3.2. Effect of bacteriophage on *E. faecalis* biofilms

The effect of phage ϕEf11/ϕFL1C(Δ36,)^{PnisA} on biofilms of *E. faecalis* strains JH2-2(pMSP3535, *cat*, *nis*R *nis*K) and V583(pMSP3535, *cat*, *nis*R *nis*K) can be seen in Fig 2. Confocal Microscopic analysis revealed a major reduction of the bacterial population of both JH2-2 (pMSP3535, *cat*, *nis*R *nis*K) and V583 (pMSP3535, *cat*, *nis*R *nis*K) biofilms following phage infection, compared with the untreated control cultures. Dead cells (in red) and voids are much more abundant in phage-treated biofilms, while live cells (green) predominate in untreated biofilms, as shown in the Confocal images. Furthermore, dead cells can be seen throughout the depth of the biofilm layers in both the JH2-2 (pMSP3535, *cat*, *nis*R *nis*K) and V583 (pMSP3535, *cat*, *nis*R *nis*K) phage-treated cultures (Fig. 2 D, H).

A quantitative comparison was made between the control and the phage-treated biofilms. It can be seen (Table 3) that approximately 10-100-fold fewer viable cells were recovered from the phage treated biofilms, as compared to the untreated *E. faecalis* biofilms.

4. Discussion

The proliferation of antibiotic resistant strains among pathogenic bacteria has emphasized the urgent need for the development of new, alternative strategies for the management of bacterial infections. One such alternative that was considered nearly a century ago is that of phage therapy, or the use of bacterial viruses of pathogenic bacteria to infect and kill their host cells. The early successful implementation of antibiotics since the late 1940's blunted the initial interest in phage therapy in western medicine. However, with the growing emergence of antibiotic resistance, the therapeutic potential of phage therapy is now being reconsidered.

For a bacteriophage to be useful for phage therapy, it should feature certain biological properties. It should: (1) Be virulent, not temperate (i.e., incapable of lysogeny), (2) have a broad host range of strains within the targeted bacterial species, and (3) not contain genes for any toxic or pathogenic functions within its genome. We have previously described a successful three-step strategy for genetically engineering bacteriophages with these (and other desired) characteristics (Zhang et al., 2013). It consists of:

1) Transformation and allelic exchange in a lysogen to delete all lysogeny-related genes from the prophage and to replace the operator/promoter controlling the initiation of the lytic infection program with an inducible promoter. (Lysogens containing this recombinant prophage can be easily selected by inclusion of an appropriate antibiotic resistance marker in the allelic exchange that deletes the lysogeny-related genes. Furthermore, such a recombinant prophage will not be induced in the absence of the promoter's inducer).

2) Adding/replacing genes in the prophage to achieve the desired phenotypic characteristics. This may include the addition of genes that can alter host range specificity, or the deletion of genes that may be of pathogenic concern. Again, recombinant lysogens can be directly selected by the inclusion of an appropriate antibiotic resistance marker within the recombining DNA.

3) Replacing the inducible promoter (controlling phage lytic cycle functions) with a constitutive promoter found in the host cell genome. Upon replacement of the inducible promoter by the constitutive promoter, the phage productive infection cycle will be initiated resulting in the production of (mutant) progeny virions and lysis of the host cell. These (now virulent) progeny phage will be incapable of lysogeny (lacking lysogeny-related genes) and insensitive to repressor (lacking a repressor-sensitive lytic cycle promoter). In addition, they may display altered phenotypic characteristics (e.g., altered/broader host range) depending on what new genes have been introduced into the phage genome.

The bacteriophage used in this study was constructed using a portion of the above bioengineering strategy. The phage was engineered from a recombinant virus {phage

[ϕEf11(Δ61-1, ϕFL1C40-44)]} that resulted from a spontaneous recombination event that occurred between bacteriophage ϕEf11 and a defective ϕFL1C prophage that was present in the *E. faecalis* JH2-2 chromosome (Zhang et al., 2013). An *E. faecalis* JH2-2[ϕEf11(Δ61-1,ϕFL1C40-44)](pMSP3535,*cat*,*nis*R,*nis*K) lysogen containing the ϕEf11(Δ61-1, ϕFL1C40-44) prophage and plasmid pMSP3535,*cat*,*nis*R,*nis*K, was transformed with a plasmid containing a 1kb DNA fragment upstream of ϕEF11 ORF36, the nisin promoter (P^{nisA}), an erythromycin selection marker (*erm*), and a 1kb fragment downstream of phage ϕEf11 P^{cro}, the wild type lytic cycle promoter. Following homologous recombination, transformants were selected on erythromycin-containing agar plates. Broth cultures of these transformant clones were induced by the addition of nisin (40ng/mL). The resulting phage [ϕEf11/ϕFL1C(Δ36)^{PnisA}] is no longer capable of lysogeny, due to the deletion of ORF 36 (the gene for the phage repressor), and is no longer sensitive to repression, due to the replacement of the wild type lytic cycle promoter (P^{cro}) by the inducible nisin promoter (P^{nisA}). The activation of this promoter (P^{nisA}) is dependent upon the presence of nisin and the products of the genes (*nis*R and *nis*K) of the two component nisin-sensory system (Bryan et al., 2000; Kuipers et al., 1995). In this nisin-controlled expression system, *nis*R and *nis*K are constitutively expressed via the *nis*R promoter. The *nis*K gene product is a histidine kinase, which, in the presence of nisin, autophosphorylates, and subsequently transfers the phosphate group to the *nis*R gene product, a response regulator. The phosphorylated NisR protein functions as a transcription factor that activates transcription from the *nis*A promoter (P^{nisA}). Open reading frames immediately downstream from P^{nisA} will then be transcribed (de Ruyter, Kuipers, Beerthuqzen, van Alen-Boerrigter & de Vos 1996). We are using the *nis*A promoter (P^{nisA}) to drive transcription of the genes of the bacteriophage lytic cycle. In this study, we provide the *nis*R and *nis*K genes *in trans*, from plasmid pMSP3535, *cat*, *nis*R *nis*K, in the host cells of the phage.

We recognize that in its current form, the phage engineered in the present study would not be clinically useful-additional genetic modifications will be required such as, replacing the inducible P^{nisA} nisin promoter with a constitutive promoter and deleting the antibiotic resistance markers. In this regard, we are continuing to develop this (and similar) phage constructs with additional desirable properties. However, as an intermediate step in the development of an agent for use in phage therapy, we wished to examine the phage variant engineered in the present study for its ability to disrupt *E. faecalis* biofilms.

The images of the phage-treated *E. faecalis* biofilms (both vancomycin sensitive and resistant strains) are dramatically altered from the non-treated control biofilms, suggesting a gross disruption/reduction of the biofilm mass by the phage. This is true for both the surface projection images as well as the images through the depth of the biofilms. Considering the inherent resistance of biofilms to antimicrobial agents (Costerton, 2007), it is of some significance that we see cell death and biofilm disruption throughout the depth of the biofilms. The quantitative reduction (10^{-1}-10^{-2}) of viable cells in the treated, compared to the non-phage treated biofilms, while consistent with these observations, is not as impressive as the confocal biofilm images of the phage-disrupted biofilms. The sparsity of cells, most of which are stained red, seen in the imaged phage-treated biofilms, is consistent with the loss of biomass and preponderance of dead cells. The residual viable cells recovered from the phage-treated biofilms may be due to altered lytic infection kinetics of the genetically-

modified phage. It is possible that the nisin promoter, previously installed in the phage genome to facilitate genetic modification of the prophage, is not as transcriptionally-efficient as the naturally occurring wild type promoter controlling the phage lytic cycle functions. This could result in a less robust lytic infection and a more modest reduction of the targeted bacterial population. Comparison of the lytic effects on *E. faecalis* populations of infections produced by phage with nisin promoters versus wild type promoters tends to support this contention (Zhang and Stevens, unpublished observations). If this is the case, we expect that replacement of the inducible nisin promoter by a strong, constitutive promoter to regulate phage lytic cycle functions will result in a greater lytic effect on the targeted host cell population.

Biofilms consist of a community of cells embedded in an extracellular polymeric matrix (EPM) (Costerton, 2007). In the case of *E. faecalis*, the EPM is thought to include extracellular DNA and dextran (Thomas, Thurlow, Boyle, & Hancock, 2008; Thomas, Hiromasa, Harms, Thurlow, Tomich & Hancock, 2009; Li,, Liu, & Xu, 2012). It has been suggested that in order to effectively remove biofilms, it would be necessary to degrade the EPM as well as lyse/devitalize the cells. In this regard, it has been shown that *E. faecalis* biofilm formation can be reduced with treatment by dextranase and/or DNAase I (Li et al 2012). Furthermore, Lu and Collins (2007) demonstrated that bacteriophage delivery of an EPM-degrading enzyme (dispersin B) to an *Escherichia coli* biofilm reduced biofilm cell counts by approx. 4.5 orders of magnitude. Similarly, it is conceivable that the expression of a dextranase gene, cloned into the ϕEf11 bacteriophage genome, would further enhance the efficacy of the virus in removing *E. faecalis* biofilms. The genetic engineering strategy that we have devised using bacteriophage ϕEf11 provides a convenient platform upon which additional modifications, such as the insertion of a dextranse gene, can be made.

Bacteriophages that infect strains of *E. faecalis* have been known for more than fifty years (Evans 1934; Rogers & Sarles 1963). The lytic/antimicrobial activities of many of these *E. faecalis* phages have been previously reported (Khalifa Brosh, Gelman, Coppenhagen-Glazer, Beyth, Poradosu-Cohen & Hazan., 2015; Paisano, Spira, Cai, & Bombana, 2004; Parasion, Kwiatek, Mizak, Gryko, Bartoszcze & Kocik., 2012; Uchiyama, Rashel, Maeda, Takemura, Sugihara, Akechi & Matsuzaki, 2008; Zhang et al., 2013). All of these were naturally-occurring bacteriophages, and none appear to be closely related (genetically) to ϕEf11/ϕFL1C(Δ36,)^{PnisA}.

5. Conclusion

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

Acknowledgments

Funding: Support for this work came from National Institute of Dental and Craniofacial Research Public Health Service grant 1R15DE021016, and a grant from the Janssen Research and Development Company.

References

Arias CA, Murray BE. Antibiotic-resistant bugs in the 21st century--a clinical super-challenge. The New England Journal of Medicine. 2009; 360:439–443. [PubMed: 19179312]

Bonten MJ, Willems R, Weinstein RA. Vancomycin-resistant enterococci: Why are they here, and where do they come from? Lancet Infectious Diseases. 2001; 1:314–25. [PubMed: 11871804]

Bryan EM, Bae T, Kleerebezem M, Dunny GM. Improved vectors for nisin-controlled expression in gram-positive bacteria. Plasmid. 2000; 44:183–190. [PubMed: 10964628]

Burrowes B, Harper DR, Anderson J, McConville M, Enright MC. Bacteriophage therapy: potential uses in the control of antibiotic-resistant pathogens. Expert Review of Anti-Infective Therapy. 2011; 9:775–85. [PubMed: 21905786]

Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant enterococci. Clinical Microbiology Reviews. 2000; 13:686–707. [PubMed: 11023964]

Costerton, WJ. The Biofilm Primer | Springer. 8th ed.. Springer-Verlag; Berlin Heidelberg: 2007.

de Ruyter PGGA, Kuipers OP, Beerthuyzen MM, van Alen-Boerrigter I, de Vos WM. Functional analysis of promoters in the nisin gene cluster of Lactococcus lactis. Journal of Bacteriology. 1996; 178:3434–3439. [PubMed: 8655538]

Distel JW, Hatton JF, Gillespie MJ. Biofilm formation in medicated root canals. Journal of Endodontics. 2002; 28:689–693. [PubMed: 12398165]

Evans AC. The prevalence of streptococcus bacteriophage. Science. 1934; 80:40–41. [PubMed: 17818639]

Fabricius L, Dahlén G, Holm SE, Möller AJ. Influence of combinations of oral bacteria on periapical tissues of monkeys. Scandinavian Journal of Dental Research. 1982; 90:200–206. [PubMed: 7051261]

Facklam R. What happened to the streptococci: Overview of taxonomic and nomenclature changes. Clinical Microbiology Reviews. 2002; 15:613–630. [PubMed: 12364372]

Figdor D, Davies JK, Sundqvist G. Starvation survival, growth and recovery of Enterococcus faecalis in human serum. Oral Microbiology and Immunology. 2003; 18:234–239. [PubMed: 12823799]

Gilmore MS, Ferretti JJ. Microbiology. The thin line between gut commensal and pathogen. Science (New York, N.Y.). 2003; 299:1999–2002.

Gold HS, Moellering RC Jr. Antimicrobial-drug resistance. The New England Journal of Medicine. 1996; 335:1445–53. [PubMed: 8875923]

Holt, JG.; Krieg, HR. Enrichment and Isolation.. In: Gerhardt P., E.; Murray, RGE.; Wood, WA.; Krieg, NR., editors. Methods for General Microbiology. Washington, D.C.: 1994. p. 179-215.

Hubble TS, Hatton JF, Nallapareddy SR, Murray BE, Gillespie MJ. Influence of Enterococcus faecalis proteases and the collagen-binding protein, Ace, on adhesion to dentin. Oral Microbiology and Immunology. 2003; 18:121–6. [PubMed: 12654103]

Hunt CP. The emergence of enterococci as a cause of nosocomial infection. British Journal of Biomedical Science. 1998; 55:149–156. [PubMed: 10198473]

Jacob AE, Hobbs SJ. Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. Journal of Bacteriology. 1974; 117:360–372. [PubMed: 4204433]

Jett BD, Huycke MM, Gilmore MS. Virulence of enterococci. Clinical Microbiology Reviews. 1994; 7:462–78. [PubMed: 7834601]

Khalifa L, Brosh Y, Gelman D, Coppenhagen-Glazer S, Beyth S, Poradosu-Cohen R, Hazan R. Targeting Enterococcus faecalis biofilms with phage therapy. Applied and Environmental Microbiology. 2015; 81:2696–705. [PubMed: 25662974]

Kuipers OP, Beerthuyzen MM, de Ruyter PGGA, Luesink EJ, de Vos WM. Autoregulation of nisin biosynthesis in Lactococcus lactis by signal transduction. Journal of Biological Chemistry. 1995; 270:27299–27304. [PubMed: 7592991]

Li W, Liu H, Xu Q. Extracellular dextran and DNA affect the formation of Enterococcus faecalis biofilms and their susceptibility to 2% chlorhexidine. Journal of Endodontics. 2012; 38:894–898. [PubMed: 22703650]

Love RM. Enterococcus faecalis - A mechanism for its role in endodontic failure. International Endodontic Journal. 2001; 34:399–405. [PubMed: 11482724]

Lu KT, Collins JJ. Dispersing biofilms with engineered enzymatic bacteriophage. Proceedings of the National Academy of Science. 2007; 104:11, 197–11, 202.

McHugh CP, Zhang P, Michalek S, Eleazer PD. pH required to kill Enterococcus faecalis in vitro. Journal of Endodontics. 2004; 30:218–9. [PubMed: 15085049]

Moellering Jr RC. Emergence of enterococcus as a significant pathogen. Clincal Infectious Diseases. 1992; 14:1173–1178.

Mohamed JA, Huang DB. Biofilm formation by enterococci. Journal of Medical Microbiology. 2007; 56:1581–8. [PubMed: 18033823]

Molander A, Reit C, Dahlén G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. International Endodontic Journal. 1998; 31:1–7. [PubMed: 9823122]

Noskin GA. Vancomycin-resistant enterococci: clinical, microbiologic, and epidemiologic features. The Journal of Laboratory and Clinical Medicine. 1997; 130:14–20. [PubMed: 9242362]

Paisano AF, Spira B, Cai S, Bombana a. C. In vitro antimicrobial effect of bacteriophages on human dentin infected with Enterococcus faecalis ATCC 29212. Oral Microbiology and Immunology. 2004; 19:327–330. [PubMed: 15327646]

Parasion S, Kwiatek M, Mizak L, Gryko R, Bartoszcze M, Kocik J. Isolation and characterization of a novel bacteriophage ϕ4D lytic against Enterococcus faecalis strains. Current Microbiology. 2012; 65:284–289. [PubMed: 22669253]

Peciuliene V, Balciuniene I, Eriksen HM, Haapasalo M. Isolation of Enterococcus faecalis in previously root-filled canals in a Lithuanian population. Journal of Endodontics. 2000; 26:593–5. [PubMed: 11199800]

Pinheiro ET, Gomes BPFA, Ferraz CCR, Sousa ELR, Teixeira FB, Souza-Filho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. International Endodontic Journal. 2003; 36:1–11. [PubMed: 12656508]

Rams TE, Feik D, Young V, Hammond BF, Slots J. Enterococci in human periodontitis. Oral Microbiology and Immunology. 1992; 7:249–252. [PubMed: 1408361]

Ricucci D, Siqueira JF. Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. Journal of Endodontics. 2010; 36:1277–88. [PubMed: 20647081]

Rôças IN, Siqueira JF, Santos KRN. Association of Enterococcus faecalis with different forms of periradicular diseases. Journal of Endodontics. 2004; 30:315–20. [PubMed: 15107642]

Rogers CG, Sarles WB. Characterization of entercoccus bacteriophages from the small intestine of the rat. Journal of Bacteriology. 1963; 83:1378–1385. [PubMed: 14047233]

Sahm DF, Kissinger J, Gilmore MS, Murray PR, Mulder R, Solliday J, Clarke B. In vitro susceptibility studies of vancomycin-resistant Enterococcus faecalis. Antimicrobial Agents and Chemotherapy. 1989; 33:1588–1591. [PubMed: 2554802]

Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor laboratory press; New York: 1989.

Sedgley CM, Lennan SL, Appelbe OK. Survival of Enterococcus faecalis in root canals ex vivo. International Endodontic Journal. 2005; 38:735–742. [PubMed: 16164688]

Siren EK, Haapasalo MP, Ranta K, Salmi P, Kerosuo EN. Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. International Endodontic Journal. 1997; 30:91–95. [PubMed: 10332242]

Stevens RH, Ektefaie MR, Fouts DE. The annotated complete DNA sequence of Enterococcus faecalis bacteriophage ϕEf11 and its comparison with all available phage and predicted prophage genomes. FEMS Microbiology Letters. 2011; 317:9–26. [PubMed: 21204936]

Stevens RH, Grossman LI. Evaluation of the antimicrobial potential of calcium hydroxide as an intracanal medicament. Journal of Endodontics. 1983; 9:372–374. [PubMed: 6415201]

Stevens RH, Porras OD, Delisle a. L. Bacteriophages induced from lysogenic root canal isolates of Enterococcus faecalis. Oral Microbiology and Immunology. 2009; 24:278–284. [PubMed: 19572888]

Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis : Its Role in Root Canal Treatment Failure and Current Concepts in Retreatment. Journal of Endodontics. 2006; 32:93–98. [PubMed: 16427453]

Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage therapy. Antimicrobial Agents and Chemotherapy. 2001; 45:649–59. [PubMed: 11181338]

Summers WC. Bacteriophage therapy. Annual Review of Microbiology. 2001; 55:437–51.

Thomas VC, Thurlow LR, Boyle D, Hancock LE. Regulation of autolysis-dependent extracellular DNA release by Enterococcus faecalis extracellular proteases influences biofilm development. Journal of Bacteriology. 2008; 190:5690–5698. [PubMed: 18556793]

Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE. A fratricidal mechanism is respnisble for eDNA release and contributes to biofilm development of Enterococcus faecalis. Molecular Microbiology. 2009; 72:1022–1036. [PubMed: 19400795]

Uchiyama J, Rashel M, Maeda Y, Takemura I, Sugihara S, Akechi K, Matsuzaki S. Isolation and characterization of a novel Enterococcus faecalis bacteriophage EF24C as a therapeutic candidate. FEMS Microbiology Letters. 2008; 278:200–206. [PubMed: 18096017]

Uttley AC, Collins CH, Naidoo J, George RC. Vancomycin-resistant enterococci. The Lancet. 1988; 331:57–58.

Woodford N. Glycopeptide-resistant enterococci: A decade of experience. Journal of Medical Microbiology. 1998; 47:849–862. [PubMed: 9788808]

Zhang H, Fouts DE, DePew J, Stevens RH. 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. Microbiology (United Kingdom). 2013; 159:1023–1035.

Zhang W, Mi Z, Yin X, Fan H, An X, Zhang Z, Tong Y. Characterization of Enterococcus faecalis phage IME-EF1 and its endolysin. PLoS ONE. 2013; 8:e80435. [PubMed: 24236180]

HIGHLIGHTS

- An exogenous inducible promoter controls the phage lytic cycle functions
- Biofilms of both *E. faecalis* strains were disrupted by infection with bacteriophage
- Confocal Microscopic analysis showed reduction of biomass in phage-treated biofilms
- Dead cells could be seen throughout the depth of the phage-treated biofilm layers
- A 10-100-fold decrease in viable cells was observed after bacteriophage treatment

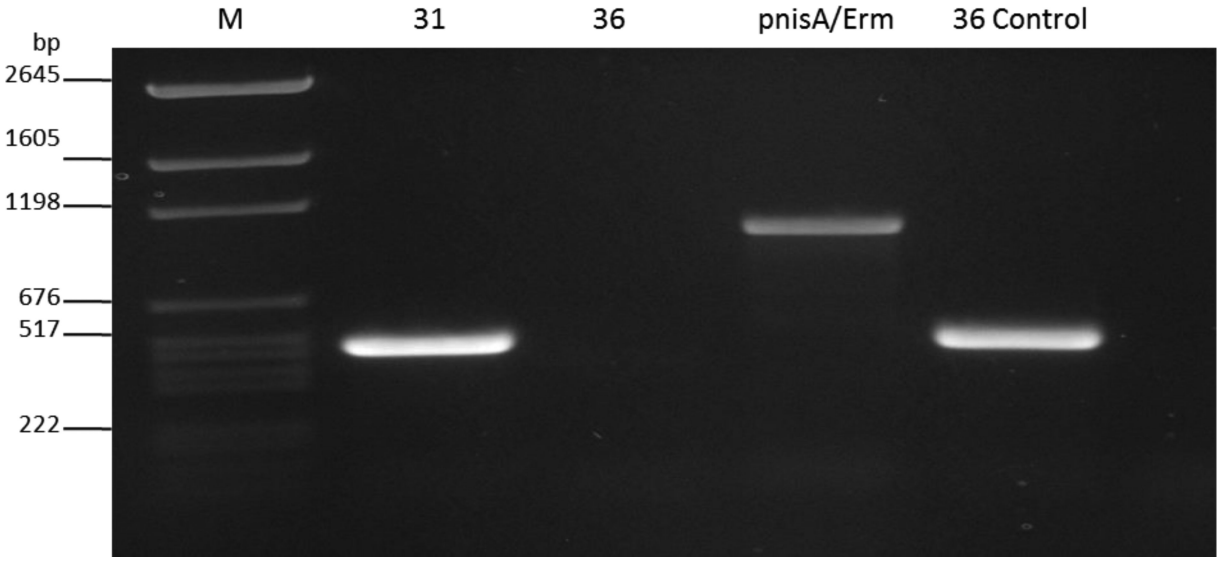

Fig.1.

PCR analysis of *E. faecalis* JH2-2[Ef11/ϕFL1C(Δ36)^{PnisA}] lysogens. M: DNA MW marker; 31: phage ϕEf11 Orf31-specific primers, 36: phage ϕEf11 Orf36-specific primers; pnisA/Erm: primers specific for fragments flanking PnisA and Erm; 36 control: phage ϕEf11 Orf36-specific primers (positive control). Template DNA: 31, 36, and PnisA/Erm= *E. faecalis* JH2-2[Ef11/ϕFL1C(Δ36)^{PnisA}], 36 Control= *E. faecalis* JH2-2[ϕEf11(Δ61-1, ϕFL1C40-44].

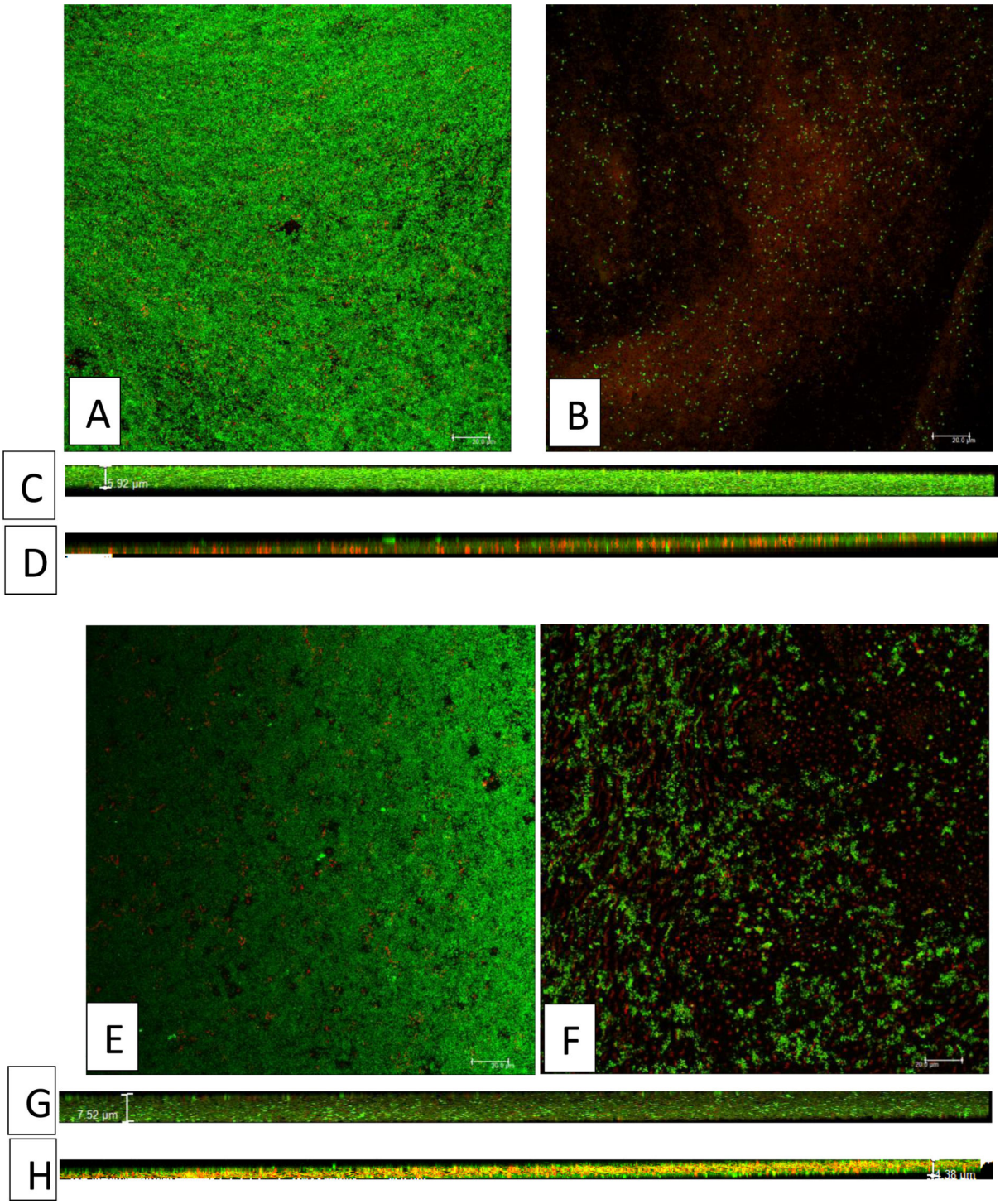

Fig 2.

Effect of phage ϕEf11/ϕFL1C(Δ36,)^{PnisA} on 2-day-old biofilms grown on glass coverslips. Biofilms of JH2-2(pMSP3535, *cat*, *nis*R *nis*K) and V583(pMSP3535, *cat*, *nis*R *nis*K) and were grown on glass coverslips in BHI medium for 48 hours. Live bacteria appear green, while dead cells and eDNA are red. (A) untreated JH2-2(pMSP3535, *cat*, *nis*R *nis*K); (B) phage-treated JH2-2(pMSP3535, *cat*, *nis*R *nis*K); (C) untreated JH2-2(pMSP3535, *cat*, *nis*R

*nis*K) through depth of the biofilm; (D) phage-treated JH2-2(pMSP3535, *cat*, *nis*R *nis*K) through depth of the biofilm; (E) untreated V583(pMSP3535, *cat*, *nis*R *nis*K); (F) phage-treated V583(pMSP3535, *cat*, *nis*R *nis*K); (G) untreated V583(pMSP3535, *cat*, *nis*R *nis*K) through the depth of the biofilm; (H) phage-treated V583(pMSP3535, *cat*, *nis*R *nis*K) through the depth of the biofilm. These images are representative of three independent experiments.

Table 1

Primers used for PCR confirmation of insertion or deletion of targeted genes

Targeted fragment	Primer name	Sequence	Product length
ORF31	EF31F EF31R	5'-AAGTTGTTTCCGTGTCAACGTGGC-3' 5'-GTGTCCATCATGGTCGTTTAGCAG-3'	417bp
ORF36	EF36F EF36R	5'-TTATCAGGGTCTGGTGAATGCG-3' 5'-GCAACTTATGAGTGAGCGCAA-3'	480bp
pnisA + erm	Pnis-ErmF Pnis-ErmR	5'-CTGACGTCACAAAAGCGACTCATAGAATTATTTCCTCC-3' 5'-TAGTTATAAGACTAGATCTGATCCGTA-3'	1,102bp

Table 2

Nisin-mediated phage induction from *E. faecalis* JH2-2[ϕEf11/ϕFL1C(Δ36)^{PnisA}] (pMSP3535, *cat*, *nis*R *nis*K). Phage titer produced from E. *faecalis* JH2- [ϕEf11/ϕFL1C(Δ36)^{PnisA}] (pMSP3535, *cat*, *nis*R *nis*K) in the presence or absence of nisin

Nisin (ng/mL)	Titer (pfu/mL)
0	0
40	2.39×10^{7}

Table 3

CFU recovery from phage-treated and untreated *E. faecalis* biofilms. CFUs were recovered from phage-treated and untreated *E.faecalis* biofilms. Values are the means of two independent experiments ± SD

Biofilm	Biomass (CFU/Biofilm)
JH2-2(pMSP3535, *cat*, *nis*R *nis*K)	2.25×10^8 (± 0,63)
JH2-2(pMSP3535, *cat*, *nis*R *nis*K)+ phage	3.60×10^6 (± 3,81)
V583(pMSP3535, *cat*, *nis*R *nis*K)	3.35×10^8 (± 0,07)
V583(pMSP3535, *cat*, *nis*R *nis*K)+ phage	4.20×10^7 (± 5,49)