AEM Accepts, published online ahead of print on 17 September 2010
Appl. Environ. Microbiol. doi:10.1128/AEM.01135-10

Isolation of a novel and specific bacteriophage for the periodontal pathogen *Fusobacterium nucleatum*

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Running title: Bacteriophage for *F. nucleatum*

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Fusobacterium nucleatum **is a periodontal pathogen that has been directly associated with the development and progression of periodontal disease, a widespread pathology that affects the support tissues of the tooth. We isolated a new bacteriophage (FnpΦ02) that specifically infects this bacterium. Transmission electron microscopy showed that the virion is composed of an icosahedral head and a segmented tail. The size of the phage genome was estimated to be approximately 59 kbp of double-stranded DNA. The morphological features and the genetic characteristics suggest that FnpΦ02 is part of the *Siphoviridae* family. Using one-step growth and adsorption experiments, the latent period, burst size and adsorption rate was estimated to be 15 h, 100 infections unit per cell and 7.5 x 10^{-10} mL min^{-1} respectively. A small fragment of phage DNA was cloned and sequenced showing a 93 % of nucleotide identity with the phage PA6 of *Propionibacterium acnes* and amino acid identity with fragments of two proteins (Gp3 and Gp4) of this phage. To our knowledge, FnpΦ02 is the first phage described infecting *Fusobacterium nucleatum* and provides the base for future exploration of phages in the control of periodontal disease.**

Introduction

The term "periodontal disease" refers to a wide set of pathologic alterations of the periodontal tissue. The most common clinical manifestations are known as gingivitis and periodontitis, and both are widely distributed around the world (18). Periodontitis is a multifactorial inflammatory-based infection of the supporting tissues of the tooth. It is essentially characterized by the progressive destruction of the periodontal ligament and the alveolar bone, leading to the loss of the affected tooth (2). Periodontitis is caused by bacteria or bacterial groups embedded in a biofilm or dental plaque that protects them against antimicrobial agents (18). The bacterial species involved in periodontal disease are predominantly anaerobic Gram negative and, although they are isolated generally from affected patients, they are also isolated from healthy individuals, in less proportion and frequency (26).

Fusobacterium nucleatum, is an anaerobic Gram-negative long bacillus, member of the microflora in the oral cavity. *F. nucleatum* is considered a periodontal pathogen because it is frequently isolated from lesions, produces a high number of tissue irritants and has the ability to form coaggregates with other periodontal pathogens, acting as a bridge between early and late colonizers in the surface of the enamel (4). Three different subspecies of *F. nucleatum* have been related to the pathology of periodontal disease, *F. nucleatum* subsp. *nucleatum*, subsp. *polymorphum* and subsp. *vincentii*, where all of them have been associated to lesions of periodontitis, but also have been isolated in high numbers from successfully treated patients (9).

Bacteriophages are viruses that can only infect and kill bacteria and have been used for many years as powerful tools for the study of bacterial genetics and, given its specificity, used in the identification and characterization of microorganisms (phage typing). Nevertheless, phages were originally described as therapeutic elements to treat human and animal infections (34). This application known as phage therapy has regained interest in the past years, particularly in an era where the antibiotic resistance and biofilm-based infections are permanent issues (25). Bacteriophages are denominated temperate when their genetic material is integrated within the bacterial genome with no immediate lysis of the bacterium, until, under certain conditions, the expression of the viral genome is induced and the production of new virus particles lyses the host cell; and are called lytic or virulent when immediately after the infection they redirect the bacterial metabolism to the production of new phages, which are released during the bacterial lysis (22, 36). There are many examples of the use of

bacteriophages at a clinical (14, 32) and commercial level (20). Specifically in the dentistry area, several bacteriophages have been isolated for diverse oral bacteria from saliva and dental plaque (12, 13, 23, 37).

Although *F. nucleatum* is an important periodontal pathogen, reports of bacteriophages for this microorganism do not exist. In this work we isolated and characterized a new bacteriophage for *F. nucleatum* from a saliva sample, designated FnpΦ02, and to our knowledge this is the first bacteriophage for this bacterium.

Materials and Methods

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. The *F. nucleatum* subsp. *polymorphum* clinical isolate strain used as the host for the isolation, dilution and propagation of the phage FnpΦ02 was called Fnp. *F. nucleatum* strains were cultured anaerobically in Brain-Heart Infusion broth (Merck, USA) or BHI agar at 37 °C for three days. All bacteria from the oral flora were incubated in the same conditions except black pigmented bacteria that were cultivated in BHI with sheep blood (5 %) supplemented with 10 μg/mL hemin-menadione (BBL, BD Ltd., USA) for at least 5 days at the same temperature. All aerobically grown bacteria used in the study was incubated in Luria-Bertani broth (10 g/L Tryptone, 5 g/L yeast extract and 5 g/L NaCl) or LB agar at 37 °C for 24-48 h. Chloramphenicol (20 μg/mL) was added when necessary. Bacterial growth was monitored by measuring optical density at 600nm (OD_{600}).

Isolation of *F. nucleatum* strains. Samples from saliva and tongue from healthy individuals and patients with periodontal disease were obtained from the Dental Clinic of the Universidad Andres Bello, Chile. All samples were grown in a selective medium for *F. nucleatum* called CVE (10 g/L Soy trypticase, 5 g/L yeast extract, 5 g/L NaCl, 0.2% glucose, 0.02% tryptophan, 5% sheep blood, 5 μg/mL crystal violet and 4 μg/mL erythromycin) (35) and incubated in anaerobic conditions for four days at 37ºC. Then, the blue colonies were picked and cultured for their identification. Initially all clinical isolates were confirmed as *F. nucleatum* by PCR (5) and RAPID32A (BioMérieux®, France). To determine which subspecies we where working with, we used specific PCR primers described in literature (17, 29) to identify subsp. *vincentii* and subsp. *nucleatum*, and designed PCR primers based on a reported sequence to identify subsp.

polymorphum (FnpF 5'-ccaggaggaatagggtagg-3'; FnpR 5'-gccatttcagcttcaactcc-3'). The PCR program used for the determination of subsp. *nucleatum*, *polymorphum* and *vicentii* was as follows: initial denaturation for 5 min at 94 ºC; 35 cycles of 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 30 min; and a final extension at 72 ºC for 10 min. A 100 µL reaction mixture contained: 0.5 U Taq polymerase (Invitrogen, CA, USA), 1.5 mM $MgCl_2$, 2 mM each dNTP, 1 µM each primer and 0.1 to 0.5 µg of template DNA. All PCRs were performed in a Multigene termocycler (Labnet Inc.).

Isolation of bacteriophages. Saliva samples from 25 healthy individuals, 85 periodontally affected patients which had not received antibiotics within the previous three months and drainage sample from dental chairs were used for the bacteriophage screening. One milliliter of each saliva sample was cleared of debris and bacteria by centrifugation at 15,000 x *g* for 10 min. Supernatant fluids were collected and kept at 4 ºC until used. To enrich bacteriophages in saliva samples, we inoculated 100 µL of the clear supernatant on a mid-log culture of *Fusobacterium nucleatum* Fnp strain (Public Health Institute, Santiago, Chile) in BHI broth (Oxoid, Basingstoke, UK) and incubated it anaerobically for 48 h. After this period, bacteria were harvested (15,000 x *g* for 3 min) and the supernatant was recovered and filtered (0.45 µm Millipore filter). Five microliters of this enriched saliva sample were spotted onto double layered plates containing 100 µL of a stationary phase culture of *F. nucleatum* mixed with 7 mL of top agar (agar 0.7 %). Plates were incubated anaerobically for 4 days at 37 ºC. Where the enriched samples inhibited bacterial growth, the clear zone was picked and propagated in a new culture. This lysate was serially diluted, spotted onto double layered plates and incubated as described above. Two lysates designated FnpΦ02 and FnpΦ13 were obtained. FnpΦ02 was selected for further studies.

Determination of the phage host range. For the determination of the phage host range, 68 different strains were tested against the bacteriophage. These strains are listed in Table 1 and were grown as detailed above. Five microliters of the phage suspension (1 x 10^7 PFU/mL) were spotted onto the plate, which had previously been inoculated with the specific bacteria and incubated for 24 to 48 h for aerobic bacteria and 7 to 10 days for anaerobic bacteria. Bacterial sensitivity to the bacteriophage was established by bacterial lysis at the place where the phage was spotted.

Electron microscopy of bacteriophages. To perform this study 50 mL of a lysate (6.75 x 10^7 PFU/mL) were centrifuged at 30,000 x g for 3 h at 4ºC in a Sorvall RC90 ultracentrifuge (AH-629/17 rotor) and the bacteriophage pellet was resuspended in 10 µL of distilled water. This phage suspension was dropped onto the 300-by 300-mesh grid, which had been treated by coating with one drop of 0.1 % bacitracin. After 3 min, the phage particles were stained with 2 % uranyl acetate for 10 s and then examined under a JEM-1200 EX II electron microscope (JEOL, Peabody, Mass) at an operating voltage of 120 kV. The virion size was estimated from the negatively stained images. To observe the phage along with bacteria, exponentially growing cells of *F. nucleatum* Fnp (100 mL) were collected by centrifugation (15,000 x *g*, 10 min). The pellet was resuspended in 1 mL of BHI medium and then inoculated with 100 µL of phage lysate (1 x 10^7 PFU/mL). The sample were treated and ultrathin sections were stained with 2 % uranyl acetate for 10 s and then examined under then same conditions that the lysate.

Growth experiments.

First, exponentially growing cultures of *F. nucleatum* Fnp (2 x 10^7 CFU/mL) were inoculated with the virus (1.25 x 10^7 PFU/mL) at MOIs of 1, 0.1, 0.01 and 0.001. The mixtures were incubated, and the changes in the bacterial culture were monitored over time by measuring optical density (OD_{600}). The same procedure was done with the *F. nuc. nucleatum* ATCC 25586 strain and the *F. necrophorum* ATCC 25286 strain as a negative control of infection.

One Step Growth. To determine the latent period, eclipse period, rise period and burst size we used the procedure described by Sillankorva et al. (30) with some modifications. Briefly, 10 mL of a exponential phase culture of *F. nucleatum* were harvested by centrifugation (15,000 x *g* for 5 min) and resuspended in fresh BHI medium to an OD_{600} of 0.1 (ca. 1 x 10^7 CFU/mL). A 5 mL aliquot of this suspension was taken and 5 µL of phage suspension (~ca. 1.25 x 10^7 PFU/mL) were added to an MOI of 0.01; phage was allowed to adsorb for 5 min at room temperature. The mixture was then centrifuged as describe above and the pellet was resuspended in 10 mL of fresh BHI medium and maintained in anaerobic conditions. Two samples were taken every 1 h over a period of 45 h. The first sample was plated immediately without any treatment and the second set of samples was plated after treatment with 1 % (vol/vol) chloroform to release intracellular phages. The number of viral particles (PFU) was

determined by spotting serial dilutions on double layer BHI plates containing *F. nucleatum* Fnp. Phage plaques were counted after the incubation time at 37 ºC.

Adsorption rate. The determinations were done with the procedure described by Sillankorva et al. (30) with small modifications. Briefly, bacteria in a exponential phase culture of *F. nucleatum* were diluted in BHI medium to an optical density (OD_{600}) of 0.1(1 x 10^7 PFU/mL). Then, 10 mL of the bacterial suspension and 100 µL of the phage suspention (1.25 x 10^7 PFU/mL) were mixed at an MOI of 0.1. The mixture was incubated at room temperature and samples were collected every minute during a total period of 10 min. Samples were treated with chloroform, diluted and plated on BHI plates. Phage plaques were counted after the incubation time at 37 ºC in anaerobic conditions.

Purification of bacteriophage DNA and restriction analysis. Phage DNA was isolated from a 50 mL lysate (6.75 x 10^7 PFU/mL) using the Qiagen® Lambda Midi Kit (Promega, Madison, USA) according to the manufacturers instructions. We tested the susceptibility of the nucleic acid to 17 restriction enzymes: BamHI, HindIII, KpnI, PstL (Invitrogen, USA) and BstEII, BfuCI, DraI, DpnI, EcoRI, EcoRV , HpaII, HaeIII, NotI, Sau3AI, SpeI, XbaI and PvuI (Promega, Madison, USA). The DNA digestion mixtures were analyzed by electrophoresis at 50 V for 3.5 h in a 1.5 % TAE agarose gel stained with ethidium bromide using a 1 kb DNA ladder (New England biolabs, USA) and Lambda Mix Marker (New England biolabs, USA) as molecular size markers. The genome size was determined using the same molecular size markers and the restriction pattern obtained with HindIII.

Sequencing of a fragment of phage genome. Phage DNA digested with HindIII was used for cloning into the pSU19 vector (Cam^R $LacZ^+$). The ligation mix was used to transform *E. coli* DH5α competent cells by electroporation and the clones were selected in Luria-Bertani plates supplemented with Chloramphenicol (20 µg/mL) and X-Gal (40 µg/mL). The DNA fragment was amplified with primer M13-F: 5'-cgccagggttttcccagtcacgac-3' and M13-R: 5'-tcacacaggaaacagctatgac-3'. The PCR product was purified from an agarose (0.8 %) gel using the "Wizard^R SV Gel and PCR clean Up System" (Promega, USA) and was sent for sequencing to Macrogene (Seul,

Korea). Sequence was analyzed with the bioinformatic tool NCBI BlastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the nucleotide analysis and NCBI ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) for the identification of open reading frames (ORF). Similarity analyses of the ORF sequences were performed using BlastP (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW2 (www.ebi.ac.uk/clustalw).

RESULTS

Isolation of a new bacteriophage for *F. nucleatum*. We analyzed saliva samples from 25 healthy individuals and 85 patients with periodontal disease from the Dental Clinic at the Universidad Andres Bello, in June 2009. Two samples, enriched as described in the methods section, showed a halo of growth inhibition of *F. nucleatum* sub. *polymorphum* Fnp in a double layered BHI plate (Fig. 1A). Lysates were obtained from the inhibition halos as described in the methods section; the first lysate was called FnpΦ02 and came from a sample from a healthy 24 years old man; while the second was called FnpΦ13 and was obtained from a sample of hydraulic dental chair drainage (Model Sagi 0.1, DentoLabs, Mexico). Only FnpΦ02 was selected for further studies. To confirm that we found a bacteriophage, the sample was propagated and diluted in order to obtain isolated lysis plaques (Fig. 1B). The lysis plaques showed heterogeneous morphology with diameters of approximately 1-2 mm, because of this, 15 of them were chosen for a new propagation. Every time this procedure was repeated we observed a variety of plaque morphologies suggesting that we isolated a single phage with no characteristic plaque morphology. Finally, the clearest plaque was picked, propagated and a lysate was obtained and preserved a 4 ºC for further studies.

Host specificity of FnpΦ02. Of all the bacterial strains tested, FnpΦ02 was only able to infect and lyse *Fusobacterium nucleatum* including our clinical isolates. Interestingly, we observed some differences in the phage effectiveness to lyse some strains, with *F. nucleatum* subsp. *polymorphum* being more sensitive to the infection than subsp. *nucleatum* and *vincentii*.

Physical properties of FnpΦ02. The transmission electron microscopy presented in Fig. 2A revealed that FnpΦ02 possessed an icosahedral head with a diameter of

approximately 75 nm and a flexible tail that was 260 nm long. At the same time, we saw *F. nucleatum* cells after the phage infection, which revealed the propagation of virus-like particles (Fig. 2B and 2C). In particular, we observed bacterial lysis and liberation of phage particles from the bacterium ends.

Infection of *F. nucleatum* with FnpΦ02. The effect of FnpΦ02 infection on *F. nuc. polymorphum* was observed through the inoculation of a bacterial culture in early exponential phase (OD_{600} at 0.15) at different MOIs (2, 1, 0.1, 0.01 and 0.001). In all the infections we saw a slow lysis of the bacterial culture and for all the MOIs tested, the phage was unable to cause a complete bacterial lysis after 80 h of incubation, so the culture reached an OD_{600} of 0.1 when the highest MOI was used (Fig. 3). The lysis of *F. nuc. nucleatum* by FnpΦ02 was lower than the lysis of *F. nuc. polymorphum* at the same MOI; as a negative control FnpΦ02 was incubated with *F. necrophorum* causing no lysis (data not shown).

One Step Growth. This analysis was performed to identify the different phases of the phage infection process. After infection of *F. nucleatum* with FnpΦ02, the phage growth parameters -latent period, eclipse period, rise period, and burst size- were determined comparing free and total phages. The system showed that the latency and eclipse period of FnpΦ02 were of 15 and 7 h, respectively. FnpΦ02 showed a burst size of ~100 phage per infected cell, measured along the 10 h rise period at 37 ºC (Fig. 4 and Table 2).

Adsorption efficiency. In the adsorption analysis of FnpΦ02 to *F. nuc. polymorphum* we observed the rapid phage-bacteria interaction, with an 87 % of phage adsorption in only 3 min, as is shown in Fig. 5. The adsorption process seems to be one rapid step, keeping the number of free phage constant until 5 min. The adsorption rate represents the affinity level between the phage and bacteria and was determined according to Barry and Walter (3) in a 3 min period resulting in an adsorption constant of 7.5×10^{-10} mL min^{-} for phage FnpΦ02[1] (Table 2).

Genome analysis of FnpΦ02. The phage genetic material was treated with restriction enzymes BamHI, HindIII, KpnI, PstI, BstEII, BfuCI, DraI, DpnI, EcoRI, EcoRV, HpaII,

HaeIII, NotI, PvuI, Sau3AI, SpeI and XbaI, of which HindIII and DraI were able to cut the viral genome, generating 8 fragments and over ten fragments, respectively (Fig. 6). The genome size of FnpΦ02 was determined from an electrophoretic digestion pattern with HindIII. Thus, the sum of the size of all fragments gave us a genome size of ~59 kbp (Fig. 6A and 6B). A small fragment (~500 bp) of the digestion with HindIII was cloned into pSU19 vector and sequenced. The sequence obtained (Accession HQ014662), showed in Fig. 7A, presented a high identity (approximately 90.5 %) with the *Propionibacterium acnes* phage PA6, a member of an unclassified genus within the *Siphoviridae* family (Fig. 7A). Neither identity nor homology was found with any other reported phage sequence. Southern hybridization, using the Lambda c1 gene as a probe, revealed no orthologous gene in FnpΦ02 (data not shown), all of these results suggest that our phage could be considered a member of the unclassified group within the *Siphoviridae* family. The FnpΦ02 genome section we analyzed contains 2 small fragments that align to open reading frames (ORFs) that have a consecutive disposition in the genomic context of phage PA6 (Fig. 7B). We found that both fragments had an amino acid similarity with two proteins from phage PA6. As it is shown in Fig. 7C, the first ORF fragment encodes a small peptide of 54 amino acids that has a 98% identity with a segment of Gp3, a 441 amino acid protein that has a putative structural function. The second ORF fragment encodes for a 72 amino acids polypeptide that shows an 84% identity and a 94% similarity with the Gp4 protein, a 251 amino acids with a putative terminase function.

DISCUSSION

Periodontal disease is a chronic pathology that does not have an effective or definitive treatment. Currently, the dental therapy involves the mechanical removal of the dental plaque and the use of antimicrobial agents, trying in both cases, to reduce the bacterial biofilm associated to tissue destruction. This treatment controls the progression of the disease for a limited time period, but the tissue damage is irreversible and may start again with the rapid recolonization by pathogenic bacteria. This deficient therapy together with the increasing concern about drug-resistant pathogenic bacteria has rekindled interest in alternative treatment of bacterial infections (16, 24). Phage therapy is a rediscovered option that uses bacteriophages to kill and otherwise control the bacterial populations in the infected hosts. Renewed interest in phage therapy has arised

from recent theoretical studies on phages and also experimental work using phage associated to bacteria with clinical relevance (14, 22, 20, 32). One of these impressive studies was done by Paisano et al. (23) where a specific phage for *Enterococcus faecalis* was able to completely eradicate a bacterial infection from human dentine.

Although several bacteriophages have been isolated for a number of oral bacteria (8, 10, 12, 13, 23, 33), there has been no reports on the isolation of a phage for Fusobacteria until now. *F. nucleatum* together with a bacterial consortium generates the well-known dental plaque, a biofilm conformation that eventually leads to the onset of periodontal disease. The interaction that occurs between bacteria in these biofilms has been described in several studies (6, 31), but the effect of oral phages in the ecology of the dental plaque is not well understood.

As a first approach to explore the possibility for phage therapy as an alternative treatment for periodontal disease, we isolated a specific phage for *Fusobacterium nucleatum*, a key bacterium in the development of periodontal disease.

FnpΦ02 was isolated from a saliva sample from a healthy individual confirming that oral bacteriophages can be isolated from samples of the oral cavity of people with and without periodontal disease (6, 8, 12, 13, 33, 37). This is because in a bacteria-phage system, the phage could be found wherever its target bacterium is found, and oral pathogens can be found in both healthy and periodontally diseased individuals (26). In the analysis of host specificity of FnpΦ02, we observed that the phage has a narrow range, evidenced by a difference between the sensitivity seen for the subspecies *F. nuc. nucleatum* and, *F. nuc. vincentii* compared to *F. nuc. polymorphum*. This different efficiency suggests that the phage receptor presents structural differences between subspecies, causing a decreased affinity and a less efficient adsorption phase (34). Based in these results, we can define an FnpΦ02 as a phage specific for the species *F. nucleatum*. The narrow host range of FnpΦ02 is an interesting feature because this phage would be harmless to the rest of oral flora in the mouth, suggesting that a specific phage therapy for the periodontal disease could be developed.

The genetic material of FnpΦ02 was found to be double-stranded DNA, since it shows susceptibility to restriction enzymes. FnpΦ02 viral DNA could only be digested by 2 of the 17 enzymes tested, which showed us that the phage DNA was highly

resistant to restriction enzymes, a well characterized feature in the genetic material of phages, which usually contain methylated bases and methylated DNA, as well as modified nucleotides (19). This result suggests that the FnpΦ02 genome has a high level of methylation that protects the DNA. The genome size (~59 kbp) is within the normal range of *Siphoviridae* family, which has a size average of 50 kbp (1) and a range of 22-121 kbp (21). These features, along with the FnpΦ02 virion structure allowed us to classify FnpΦ02 within the *Siphoviridae* family. Complete sequencing of the bacteriophage genome is currently under way and in the future we may determine if FnpΦ02 is a lytic o temperate phage. In the latter case, analysis of the lysogeny activity of FnpΦ02 will be the focus of a future study, especially due to the absence of genetic tools for the study of this bacterium today.

The *Siphoviridae* family includes a temperate virus such as the "Lambda-like" and lytic viruses such as "T1-like" and "T5-like", in addition to other viruses that are not yet classified (1). The features of plaque lysis and lysis efficiency of FnpΦ02, did not allow us to assess whether FnpΦ02 is a lytic or a temperate phage, because the lysis plaques show heterogeneous turbidity and size. This may occur if the phage infect cells at different times during the bacterial growth phase or due to the absence of certain culture conditions under which this specific phage could produce more homogenous plaque characteristics (27) On the other hand, the bacteriophage was unable to reach complete lysis of the culture of *F. nuc polymorphum* and a variable recovery of CFU from lysates at different MOIs were obtained. These observations could be indicative of a temperate phage or the fact that the optimal conditions for infection were not present. This issue can only be resolved through the analysis of the complete sequence of the phage genome.

About the life cycle parameters of FnpΦ02, we observed a difference between the phases of latent time, eclipse time and rise period versus the burst size of the phage. The first three features have higher values than the traditionally found in the *Siphoviridae* family (21); this is not surprising, since it has been shown that these parameters are highly influenced by the metabolic rate of the bacterial host. In this context, *F. nucleatum* has a long generation time of approximately 5.6 h, affecting the length of the phage life cycle. On the other hand, the burst size of FnpΦ02 (~100 phages per infected cell) is found within the normal range of the *Siphoviridae* family (3, 21).

Finally, when a small fragment of the genome of Fnpϕ02 was analyzed, we found nucleotide and amino acid identity with phage PA6. This phage as Fnpϕ02 phage belongs to the *Siphoviridae* family, and it is member of an unclassified genus. PA6 genome is smaller than the calculated FnpΦ02 genome, approximately 30 kbp and no evident genes for a lysogenic cycle have been found in it (10). The genome size of Fnpϕ02 compared with PA6 phage suggests that the genes for a lysogenic cycle could be present.

In summary, FnpΦ02 is a bacteriophage that specifically attacks the periodontal pathogen *Fusobacterium nucleatum* and seems to belong to the *Siphoviridae* family. Our laboratory is currently studying the potential use of this new bacteriophage as a biological control agent to reduce proliferation of *F. nucleatum* in pathogenic oral biofilms as a first step to explore the development of phage therapy to improve the current treatment of periodontal disease.

ACKNOWLEDGEMENTS

This work was supported by FONDECYT grant 11060181 and Universidad Andres Bello grants DI 17/06R and DI 48/09R (to M.B.).

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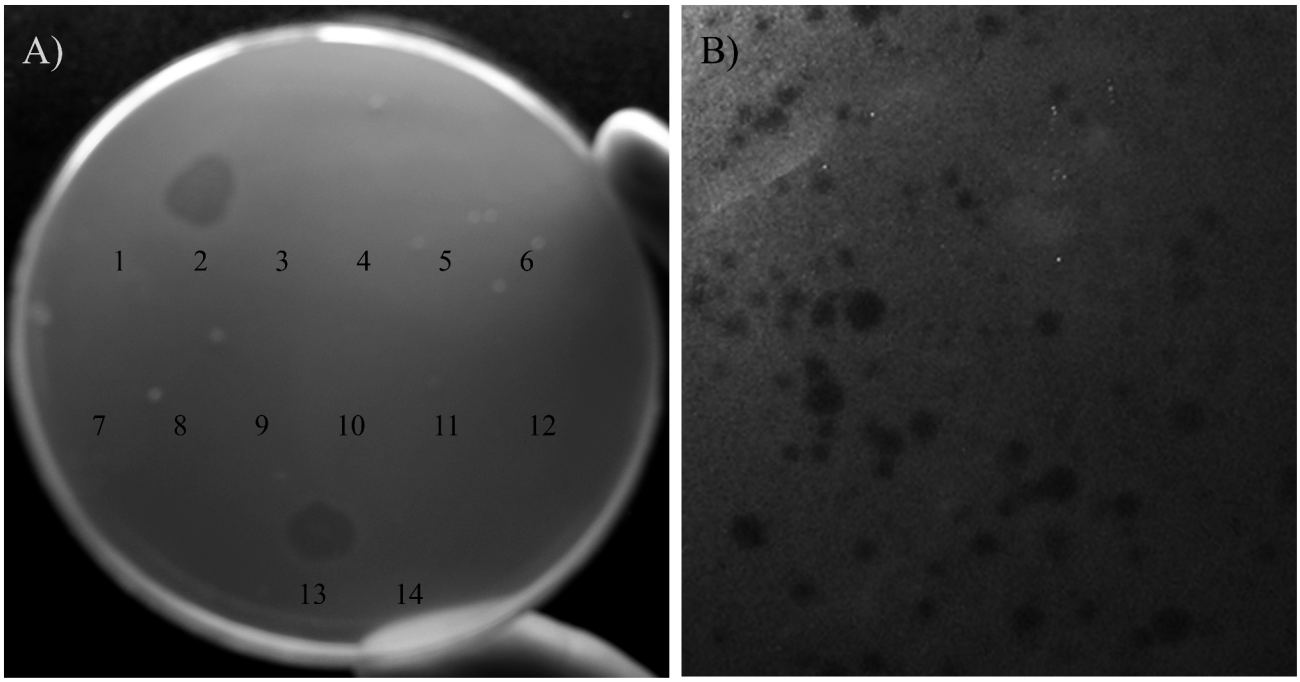
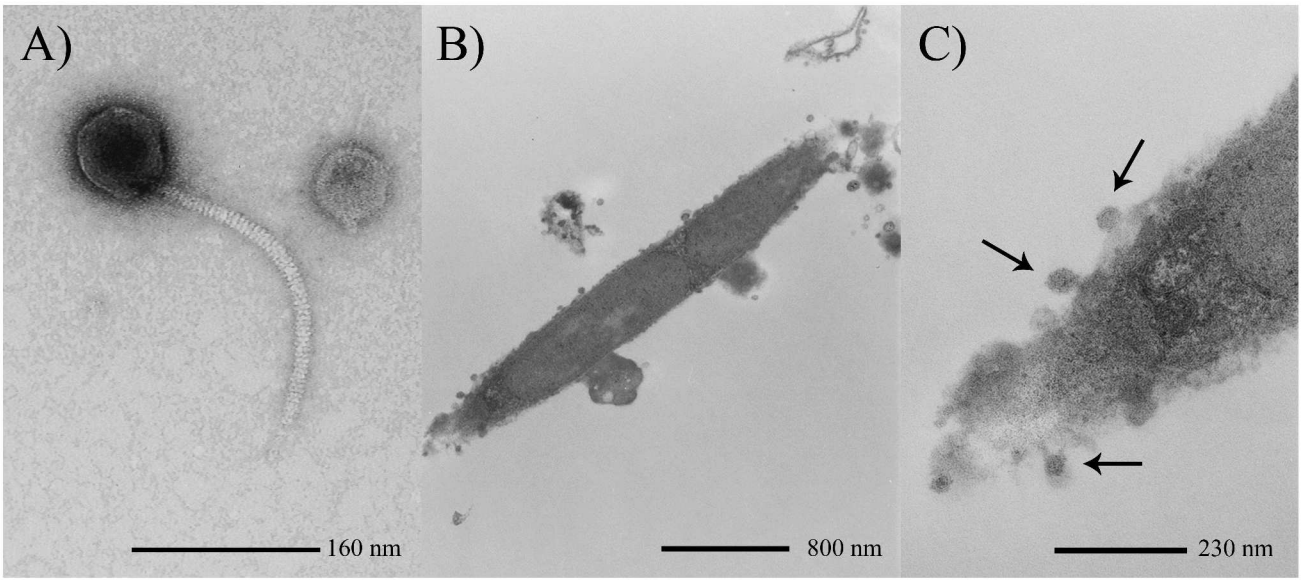
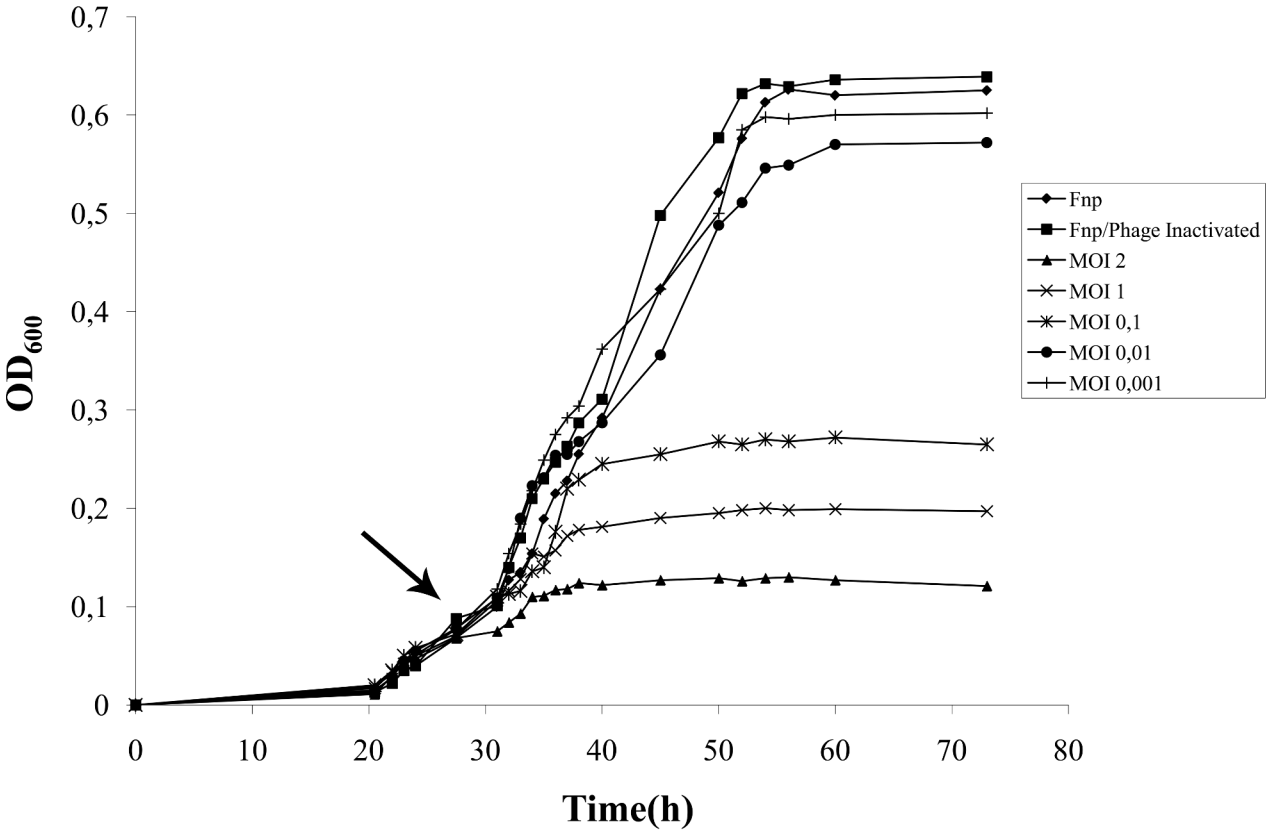
Table 1. Host range of phage FnpΦ02

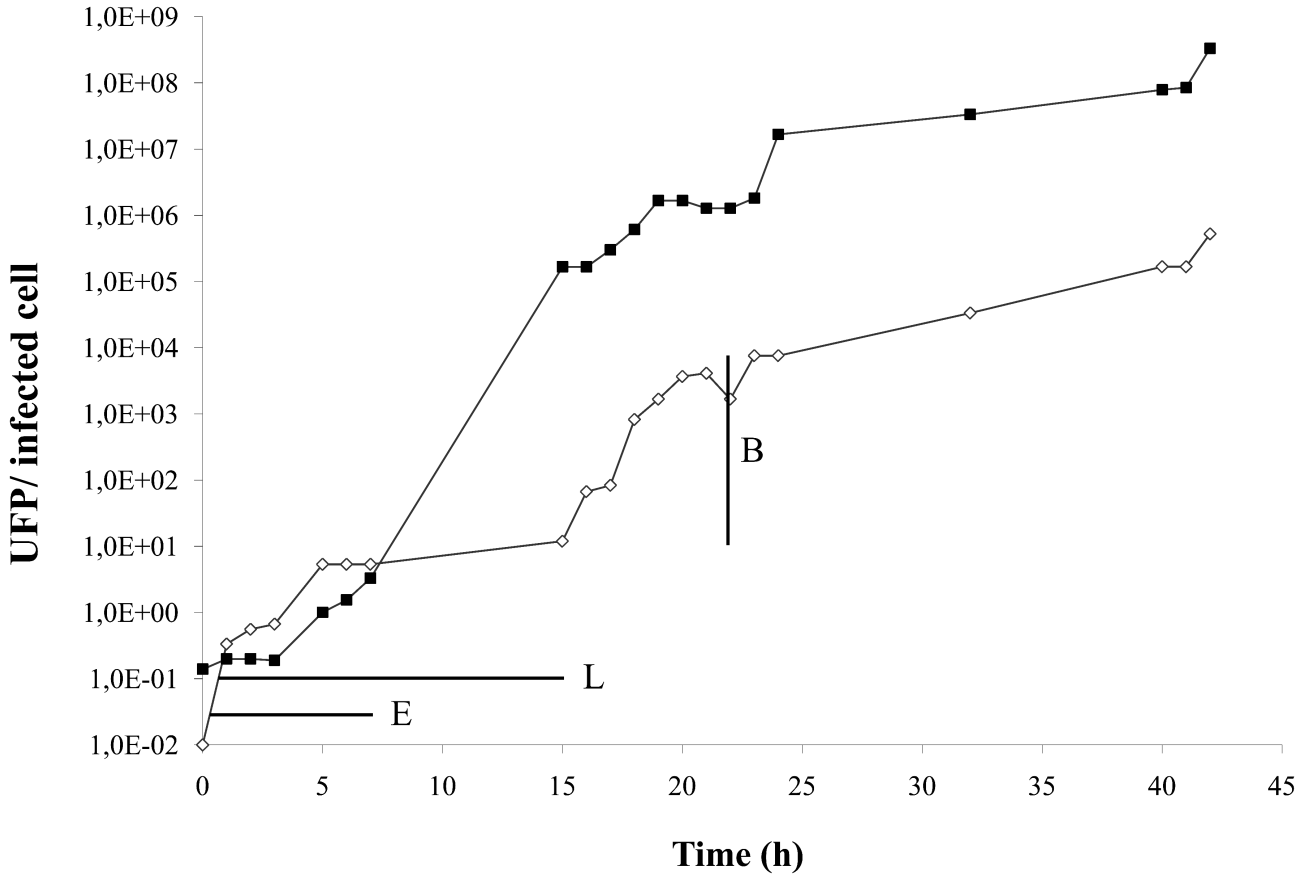
Bacteria	Reference or source	Phage Sensitivity
Fusobacteria		
**Fusobacterium nucleatum subsp. polymorphum (*Fnp)	ISP	++
Fusobacterium nucleatum subsp. polymorphum (3)	Clinical isolates, this work	++
Fusobacterium nucleatum subsp. nucleatum ATCC 25586	ATCC	+
Fusobacterium nucleatum subsp. nucleatum (3)	Clinical isolates, this work	+
Fusobacterium nucleatum subsp. vincentii (3)	Clinical isolates, this work	+
Fusobacterium necrophorum ATCC 25286	ATCC	-
[ε]Fusobacterium spp. (8)	Clinical isolates, this work	-
Oral cavity		
Gram positive		
Propionibacterium acnes (4)	Clinical isolates, this work	-
Actinomyces naeslundii (2)	Clinical isolates, this work	-
Bacteroides urealyticus (2)	Clinical isolates, this work	-
Bacteroides vulgatus ATCC8482	ATCC	-
Eubacterium limosun (4)	Clinical isolates, this work	-
Eubacterium lentum (4)	Clinical isolates, this work	-
Gram negative		
Aggregatibacter actinomycetencomitans (5)	Clinical isolates, this work	-
Porphyromonas gingivalis ATCC 33277	ATCC	-
Porphyromonas endodontalis(2)	Clinical isolate, this work	-
Prevotella nigrecens (4)	Clinical isolate, this work	-
Prevotella intermedia (3)	Clinical isolate, this work	-
Peptostreptococcus anaerobius	Clinical isolates, this work	-
Streptococcus mutans ATCC 25175	ATCC	-
Streptococcus sanguinis(2)	Clinical isolates, this work	-
Other Gram negatives		
Escherichia coli DH5α	Laboratory Stock	-
Pseudomonas aeruginosa	ISP	-
Pseudomonas oxytoca	ISP	-
Proteus mirabilis	ISP	-
Proteus vulgaris	ISP	-
Klebsiella pneumoniae	ISP	-
Salmonella enterica sv Typhimurium ATCC14028	ATCC	-
Salmonella enterica sv Typhi TY2	ISP	-
Other Gram positives		
Staphylococcus aureus ATCC43330	ATCC	-
Staphylococcus epidermidis ATCC14990	ATCC	-
Bacillus cereus	ISP	-
Streptococcus pyogenes	ISP	-

* Strain used for the isolation of FnpΦ02; [ε] identified as *Fusobacterium* by Rapid32A, but no match for species; (n): number of strain used; ISP: Public Health Institute, Santiago, Chile. ++= Sensitive, += low sensitive and -= insensitive.









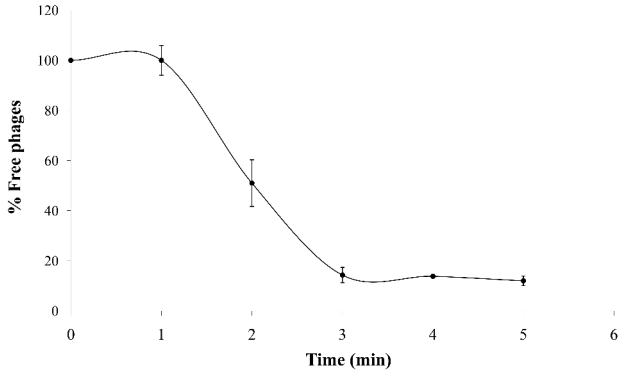
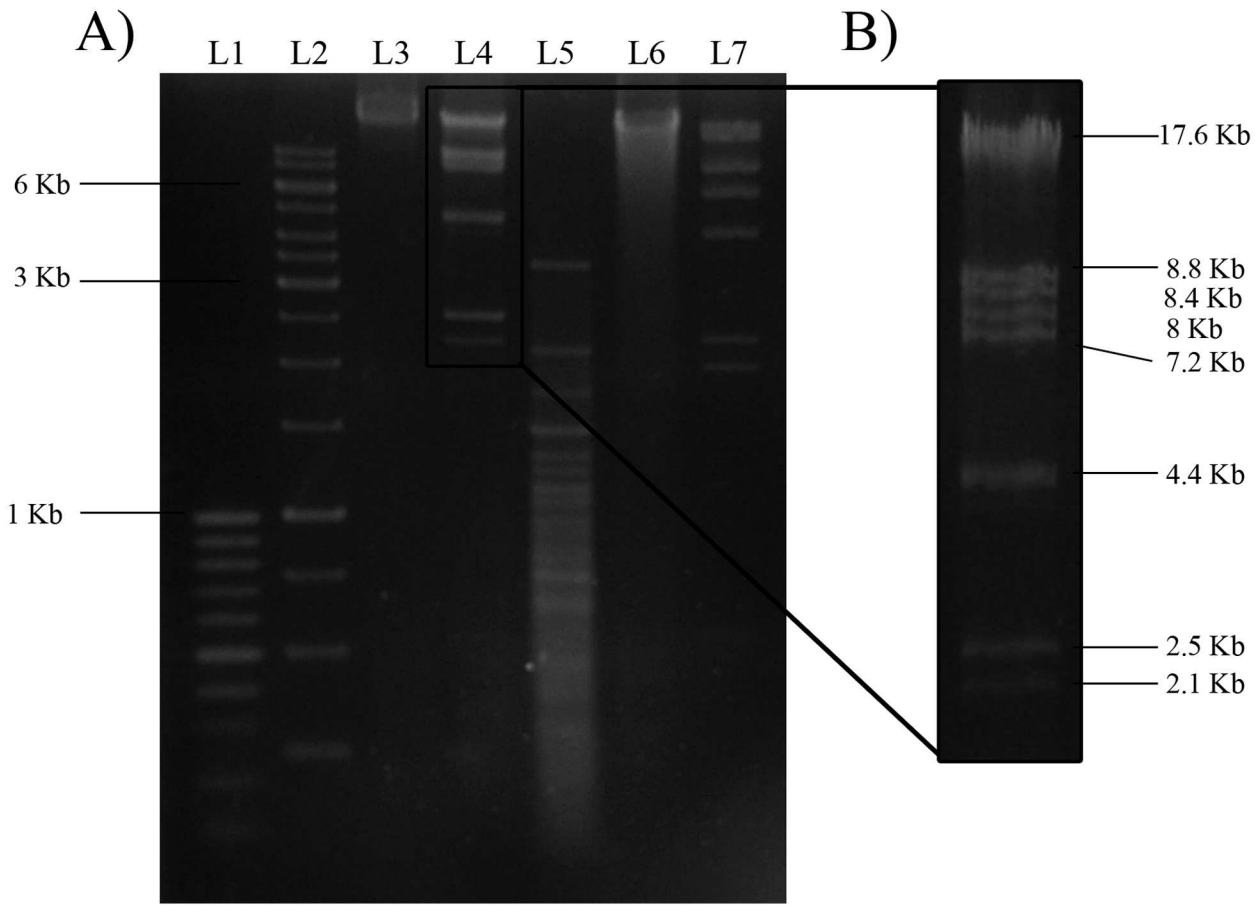


Table 2. Phage FnpΦ02 plaque features, life cycle parameters and morphological characteristics.

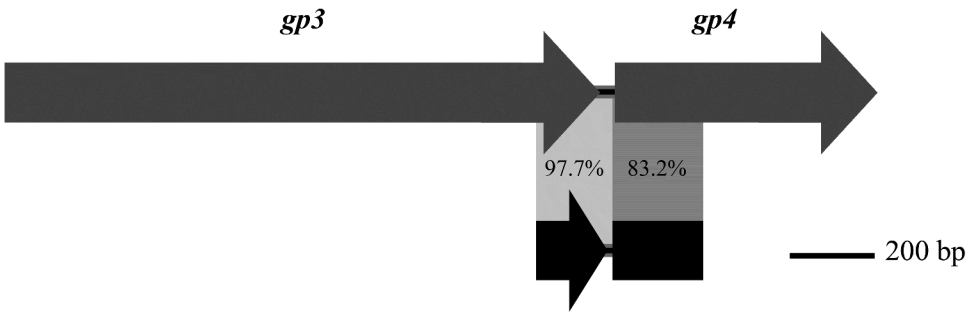
Plaque	Morphology	Life Cycle
Diameter: 1-2 mm	Head size: 83 nm	Latent period: 15 h
Heterogeneous plaques	Tail size: 211 nm	Adsorption rate: 7,5 x 10^{-10} ml min^{-1} (3 min, 87 %)
	Total size: 325 nm	Eclipse period: 7 h
		Rise period: 10 h
		Burst size: ~100 phage per infected cell



A)

```
PA6             AAGCTTGTTGGTGCCGGTATTTTGCCTGCTGATTCTCGTACGGTGTTGGAGATGTTGGGG 3106
FnpΦ02          AAGCTTGTTGGTGCCGGTATTTTGCCTGCTGATTCTCGTACGGTGTTGGAGATGTTGGGG 60
                ************************************************************

PA6             CTTGATGATGTGCAGGTTGAGGCTGTGATGCGTCATCGTGCTGAGTCGTCTGACCCGTTG 3166
FnpΦ02          CTTGATGATGTGCAGGTTGAGGCTGTGATGCGTCATCGTGCTGAGTCGTCTGACCCGTTG 20
                ************************************************************

PA6             GCGGTGCTTGCTGGGGCTATATCGCGTCAAACTAACGAGGTATGATAGGCGATGGCTTCG 3226
FnpΦ02          GCGGCACTGGCTGGGGCTATATCGCGTCAAACTAACGAGGTTTGATAGGCGATGGCTTCG 180
                ****  ** ******************************** ******************

PA6             GGGGTTGAGGCGAGGCTTGCGGCGACTGAGTATCAGCGTGAGGCGGTCAGGTTTGCTGGG 3298
FnpΦ02          GGTGCTATGTCGAGGCTTGCGGTGACTGAGTATCAGCGGCAGGCGATTCGTTTTGCCGGG 240
                ** * *  * ************ ***************  ***** *  * ***** ***

PA6             AAGTATGCGGGCTATTATTCTGAGCTTGGTCGTTTGTGGCGTGCCGGCAGGATGAGTGAC 3346
FnpΦ02          AAATACGCTGGGTATTATTCTGAGCTTGGTCGTTTGTGGCGTGCCGGGAAGATGAGTGAC 300
                ** ** ** ** ************************************ * **********

PA6             ACGCAGTATGTGCGTTTGTGTGTGGAGTTGGAGCGTGCCGGCCATGATGGTTCGGCATCG 3406
FnpΦ02          ACGCAGTATGTGCGTTTGTGTGTGGAGTTGGAGCGTGCCGGCCATGATGGTTCCGCGACT 360
                ************************************************** **  *

PA6             TTGGCTGCCAGGTTTGTGTCGGATTTTCG 3435
FnpΦ02          ATGGCGGCCAAATTCGTTTCAAAATTTCG 389
                 **** ****  ** ** **  * *****
```

B)



C)

gp3

```
PA6             KLVGAGILPADSRTVLEMLGLDDVQVEAVMRHRAESSDPLAVLAGAISRQTNEV 441
                KLVGAGILPADSRTVLEMLGLDDVQVEAVMRHRAESSDPLA LAGAISRQTNEV
FnpΦ02          KLVGAGILPADSRTVLEMLGLDDVQVEAVMRHRAESSDPLAALAGAISRQTNEV 54
```

gp4

```
PA6             MASGVEARLAATEYQREAVRFAGKYAGYYSELGRLWRAGRMSDTQYVRLCVELERAGHDG 60
                MASG  +RLA TEYQR+A+RFAGKYAGYYSELGRLWRAG+MSDTQYVRLCVELERAGHDG
FnpΦ02          MASGAMSRLAVTEYQRQAIRFAGKYAGYYSELGRLWRAGKMSDTQYVRLCVELERAGHDG 60

PA6             SASLAARFVSDF 72
                SA++AA+FVS F
FnpΦ02          SATMAAKFVSKF 72
```

Table 1. Host range of phage FnpΦ02.

* Strain used for the isolation of FnpΦ02; [£] identified as *Fusobacterium* by Rapid32A, but no match for species; (n): number of strain used; ISP: Public Health Institute, Santiago, Chile. ++= Sensitive, += low sensitive and -= insensitive.

Figure 1. **Bacteriophage isolation.** A) Double-layered plate showing growth inhibition of *F. nucleatum* by a saliva sample from a healthy 24 years old man (nº2) and a drainage sample (nº13). B) Fragment of a double-layered plate with Fnp showing the plaque morphology of phage FnpΦ02.

Figure 2. Transmission electron micrographs. A) Morphology of phage FnpΦ02. B) Fnp cell after inoculation with FnpΦ02. C) Higher magnification of the Fnp cell lysis and liberation of new phage particles. The arrows indicate liberation of new phage particles.

Figure 3. Growth curve in the presence or absence of FnpΦ02. The graph shows a representative of three separate growth curves. The arrow indicates the infection with FnpΦ02. Phage was added at different MOIs to Fnp in the early exponential growth phase ($OD_{600=}$0.1) as described in Methods. As a control, an Fnp culture was inoculated with an autoclaved phage suspension.

Figure 4. One-step growth curve of phage FnpΦ02 infection with Fnp at MOI 0.001. Shown are the PFU per infected cell in treated cultures (■) and in chloroform-treated cultures (◇). The phage growth parameters are indicated in the figure and correspond to: E-eclipse period; L-latent period and B-burst size. Representive curve of three independent assays.

Figure 5. Adsorption of FnpΦ02 to Fnp. Representative adsorption curve of three separate experiments. Adsorption was carried out at MOI of 0.01, and the supernatant was titrated at various time points to determine the amount of phage unadsorbed.

Figure 6. Restriction digest patterns electrophoresed on 1.5% agarose gel and stained with ethidium bromide. A) FnpΦ02 genomic DNA digested with restriction enzymes. Lane 1, 100 bp DNA ladder marker (Fermentas Inc., Canada); lane 2, 1 kb ladder marker (Fermentas Inc., Canada); lane 3, DNA undigested; lane 4, DNA/HindIII; lane 5, DNA/DraI; lane 6, XbaI; and lane 7, Lambda DNA/HindIII. B) Higher magnification of the bands of the digestion of FnpΦ02 with HindIII used for genome size determination. Selected sizes of the marker are indicated in the left panel.

Table 2. Phage FnpΦ02 plaque features, life cycle parameters and morphological characteristics.

Figure 7. Amino acid and nucleotide analysis of a short sequence of FnpΦ02. A) ClustalW analysis showing the nucleotide identity of Fnp**Φ**02 with PA6 phage. * show nucleotide identity. The stop codon of the *gp3* gene is in boldface and the start codon of the *gp4* gene is underlined. B) Schematic disposition of nucleotide identity of PA6 and FnpΦ02 phage and percentages of identity of the nucleotide alignments. C) Blastx comparing the amino acid identities of PA6 Gp3 and Gp4 proteins against a fragment of FnpΦ02 protein sequence. The numbers indicate the positions of the amino acid

sequences in the proteins and the DNA fragment, respectively. The middle line is the consensus sequence, where + correspond to amino acids of the same family and the empty spaces represent absence of identity.