Isolation of a Novel Aggregatibacter actinomycetemcomitans Serotype b Bacteriophage Capable of Lysing Bacteria within a Biofilm

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A bacteriophage specific for Aggregatibacter actinomycetemcomitans serotype b, able to kill the bacterium within a biofilm, was isolated. Random mutagenesis of this phage rendered a bacteriophage able to kill 99% of the bacteria within a biofilm. This is the first report of a biocontrol experiment against A. actinomycetemcomitans.

Periodontitis is an infection of the supporting tissues of the tooth caused by bacteria or bacterial groups embedded in a biofilm (14). Aggregatibacter actinomycetemcomitans (formerly, Actinobacillus actinomycetemcomitans) is a capnophilic, nonmotile Gram-negative bacterium (11, 22) related to the aggressive form of periodontitis (6–10, 18, 23); its isolates are classified into seven serotypes, a to g (12, 24), with serotype b frequently associated with disease (2, 13, 20) and serotype c with oral health (19). Periodontitis caused by A. actinomycetemcomitans often requires antibiotic therapy besides mechanical treatment due to the bacterium’s ability to form a biofilm in the periodontal pocket and on all mucous membrane surfaces in the oral cavity (6). Biofilms are organized in highly efficient and stable ecosystems (15), and it has been proposed that bacterial susceptibility to antibiotics is reduced within this structure (4), making it virtually impossible to completely remove bacteria from biofilms with antibiotics only (6). These features make periodontitis a complex disease and motivate the search for novel antimicrobial therapies such as oral microbiota modification and phage therapy (1, 16). Phage therapy has been attempted for systemic diseases (1) and to control infections in the oral cavity (17). Our aim was to isolate a bacteriophage for A. actinomycetemcomitans and evaluate its effec-

FIG. 1. Infection of an A. actinomycetemcomitans biofilm with phages Aab001 and Aab001-1. A biofilm of A. actinomycetemcomitans cultured during 24 h under capnophilic conditions was infected with Aab001 or Aab001-1 at different MOIs. The results are expressed as percentages of CFU/ml recovered 24 h postinfection compared with the CFU/ml recovered before infection (a) or from quantified biofilm at 595 nm (b). As a control, we used a biofilm of A. actinomycetemcomitans without infection (Aa). Bars depict the average values from three independent experiments. An asterisk represents significance in relation to the control (P < 0.05).

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were propagated by adding 2 ml of the sample to 20 ml of from saliva and wastewater from dental chair drainages. They mental material. Seventeen clinical samples were obtained strains used in this work are listed in Table S1 in the supple-
ctinomycetemcomitans 600 nm (OD600) of 0.15 to 0.2. After 24 h of incubation at 37°C, supernatants were spot tested over lawns of PAA005 grown on 
brain heart infusion (BHI) agar. One sample generated a 
diameter (see Fig. S1 in the supplemental material); this could 
result from a 0.01% crystal violet solution for 15 min and quantified by 
solubilizing the crystal violet with ethanol and measuring its 
absorbance at 595 nm (5, 21). Additionally a CFU count was 
performed by plating dilutions on BHI agar. To challenge this 
biofilm with phages, the culture medium was removed, and 
fresh culture medium containing phage at different multiplic-
ities of infection (MOIs) was added. At 24 h postinfection, the 
CFU and the amount of biofilm were measured. Antimicrobial 
activity of phages on an 
A. actinomycetemcomitans 
serotype b strains, and 
serotype c strains, and 
A. actinomycetemcomitans 
biofilm, showing this ability only with the phage Aabψ01-1. Furthermore, both phages were able to propagate in 
a restricted host range. To test if PAA005 has an inducible 
host range, other bacteria were tested, including 
A. actinomycetemcomitans 
serotype c, and 
serotype b; this would not affect the indigenous microbiota. In addition, they 
were not susceptible to Aabψ01, indicating that this phage has a 
restricted host range. To test if PAA005 has an inducible 
infected viral particle in its genome, a culture of PAA005 was 
induced by UV light at 302 nm for 40 s (3), and the supernatant 
was tested as described above. No bacterial lysis was observed 
(data not shown), indicating that Aabψ01 was not a product of a 
lysogen induction.

Infection of A. actinomycetemcomitans by Aabψ01. Infection 
of PAA005 liquid cultures at early exponential phase (OD600 
of 0.1 to 0.2) with Aabψ01 caused the exponential phase to be 
interrupted at an OD600 of 0.5, the point at which a massive 
cell lysis, faster than the bacterial generation, started, causing 
a continuous decrease in the OD600 until a stable reading of 
0.22, indicating that the phage did not kill the bacterial culture 
completely. To try and improve the lysis, we performed a 
mutagenesis on phage Aabψ01 by exposing a suspension of 
phage to UV light (260 nm) from a 15-W germicidal lamp at a 
dose rate corresponding to about 15 min (26). After UV treat-
ment, serial dilutions were made from the lysate and spotted 
on the surface of a lawn of A. actinomycetemcomitans. Clear 
lysis plaques were selected and propagated, and one new lysate 
was chosen for further studies; it was designated Aabψ01-1. When a PAA005 culture at early exponential phase was in-
fected with Aabψ01-1, the exponential phase reached an 
OD600 of 0.35, and we observed an improvement of the cell 
lysis with a decrease in the OD600 to 0.1.

Antimicrobial activity of phages Aabψ01 and Aabψ01-1 on 
an A. actinomycetemcomitans biofilm. An A. actinomycetem-
comitans biofilm was allowed to form on polystyrene flat-bot-
tom 96-well plates (Cellstar Greiner Bio One, Germany) inoc-
ulated with 200 µl of a 1/100 dilution of a PAA005 overnight 
culture incubated in a capnophilic (CO2-rich) environment at 
37°C. The biofilm present on each well was stained with 200 
µl of a 0.01% crystal violet solution for 15 min and quantified by 
solubilizing the crystal violet with ethanol and measuring its 
absorbance at 595 nm (5, 21). Additionally a CFU count was 
performed by plating dilutions on BHI agar. To challenge this 
biofilm with phages, the culture medium was removed, and 
fresh culture medium containing phage at different multiplic-
ities of infection (MOIs) was added. At 24 h postinfection, the 
CFU and the amount of biofilm were measured. Antimicrobial 
activity of phages on an A. actinomycetemcomitans biofilm was 
analyzed by comparing the CFU/ml of bacteria recovered from 
24-h biofilms treated with the phage lysate at different MOIs 
and those recovered from untreated biofilm. The minimum 
necessary MOI to achieve the best decrease of the A. actino-
mycetemcomitans counts for the Aabψ01 phage was 0.1, while 
for the Aabψ01-1 phage, it was 0.01 (Fig. 1a). Interestingly, 
phages were not necessarily able to reduce the amount of 
biofilm, showing this ability only with the phage Aabψ01-1 at a 
high MOI (Fig. 1b). We characterized the cell viability within 
the biofilm by confocal microscopy. Biofilms formed on cov-
erslips were treated with phages as described above and stained 
with Syto9 and propidium iodide (LIVE/DEAD BacLight bacterial viability kit L13152; Molecular Probes, Invitrogen), dem-
strating that both bacteriophages were able to penetrate the 
biofilm and to produce the lysis of serotype b but not serotype 
c (Fig. 2). Furthermore, both phages were able to propagate in 
the infected biofilms (not shown).

Aabψ01 and Aabψ01-1 specifically infect A. actinomycetem-
comitans; therefore, a possible clinical use of these phages 
would not affect the indigenous microbiota. In addition, they 
are highly selective in infection of serotype b but not serotype 
c. Serotype b is frequently associated with aggressive periodont-
titis (2, 13, 20), although differences in serotype distribution 
have been shown within the world population (10, 25), so the 
use of Aabψ01 and Aabψ01-1 against aggressive periodontitis, 
although possible, would not be universal. Despite this disad-
antage, Aabψ01 could be used as a rapid laboratory method 
to distinguish between serotype b and serotype c.
Our findings constitute the first report of an in vitro evaluation of a phage for A. actinomycetemcomitans to develop a therapy against the microorganism within its biofilm. Our results, then, show the identification of a novel bacteriophage for A. actinomycetemcomitans with the ability to eliminate the bacteria within its biofilm, a promising result of our search for a therapy helpful for the elimination of bacteria resistant to antibiotics and bacteria present in infectious biofilms. More studies involving phage therapy in animals and humans are necessary to determine the effectiveness of this alternative against aggressive periodontitis. Our laboratory is currently working on a comprehensive characterization of both Aab601 and Aab601-I to reach a better understanding of their potentials as biocidal agents.

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