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Biotechnological Applications of Two Novel Lytic Bacteriophages of *Streptococcus mutans* in Tooth Decay Bio-Controlling

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ABSTRACT

Recently the prevention of bacterial infections using their specific viruses, bacteriophages, is challenging. In this research two water samples of Caspian sea were gathered and filtered through 0.45 μm membrane filters. The filtrates were added to *Streptococcus mutans* culture media in their logarithmic phase, cultivated on brain heart infusion agar and incubated at 37°C and 5% CO₂ until bacteriophage plaques were appeared. Transmission electron microscopy of purified plaques revealed two types of lytic bacteriophages. The first phage had hexagonal head measured 74 nm and was most probably related to Cystoviridae family of bacteriophages. The second lytic bacteriophage contained a bottle-shaped mantle measuring 55×125 nm that was most probably related to Ampullaviridae family of bacteriophages. The microtiter plate assay showed the addition of bacteriophages to *Streptococcus mutans* after 0, 6, 12 and 18 h of culturing the host strain in microtiter plates prevented the biofilm formation up to 99, 69, 41 and 26%, respectively. In conclusion this is the first report of two novel Cystovirus and Ampullavirus that had lytic effects on *Streptococcus mutans*. These lytic bacteriophages could be considered as specific agents for phage therapy of tooth decay.

Key words: Bacteriophage, dental plaque, medical biotechnology, oral microbiology, phage therapy, *Streptococcus mutans*

INTRODUCTION

Dental caries is a widespread infectious disease resulted from proliferative action of oral microflora living on teeth surfaces. These bacteria consume several dietary carbohydrates specially sucrose and produce lactic acid. The presence of organic acids initiate decalcification of enamel calcium phosphate crystals and consequently would be resulted in the permeability of dental soft tissues such as dentin and pulp to the degenerative enzymes of oral pathogens and ultimately tooth decay (Loesche, 1986; Van der Ploeg, 2007). Starting with a freshly cleaned tooth, the first event is specific attachment of salivary glycoproteins to the teeth surfaces. These glycoproteins are recognized by capsular polysaccharides of first teeth colonizers, oral Streptococci (Tanzer *et al*., 2001; Schaechter, 2004). According to last molecular classification methods, these microorganisms are comprised of 12 species (Picard *et al*., 2004; Schaechter, 2004; Beheshti Maal *et al*., 2010).
While some of oral Streptococci such as mutans Streptococci, *Streptococcus mutans* and *Streptococcus sobrinus*, are responsible for dental diseases (Milnes et al., 1993; Smith et al., 1993; Jacques, 1998; Van der Ploeg, 2008) others like *Streptococcus salivarius*, *Streptococcus sanguis*, *Streptococcus oralis* and *Streptococcus gordonii* are the first bacteria that colonize the teeth (Nyvad and Kilian, 1990; Tanzer et al., 2001; Van der Ploeg, 2008). There are nonspecific strategies like tooth brushing and rinsing with biocides for reduction of *Streptococcus mutans* from dental plaque. While active or passive immunization methods and replacement of *Streptococcus mutans* with non-cariogenic bacteria are also available for prevention of caries (Samaranayake, 2002) but these are still not practical approaches (Van der Ploeg, 2007). There is a report indicating production of therapeutic vaccine against *Streptococcus sobrinus* induced caries but not in human (Dinis et al., 2004). The use of bacteriophages for controlling bacterial infectious diseases has its root in exploratory studies of these specific bacterial viruses by Twort in 1915 and D’Herelle in 1917 (Marks and Sharp, 2000; Chanishvili et al., 2001). While emerging of antibiotics in 1930s resulted in a cease of phage therapy, but development of resistant bacterial strains against antibiotics encouraged new studies on applications of phage therapy in controlling several infectious diseases (Drozdova et al., 1988; Chanishvili et al., 2001; Fischetti, 2005). Although there are few reports on isolation of lytic phages of *Streptococcus mutans* and *Streptococcus sobrinus* from dental plaque samples (Armau et al., 1988; Delisle and Rostkowski, 1993), phages of *Streptococcus salivarius* from water resources (Beheshti Maal et al., 2010) and bacteriophages of *Streptococcus sobrinus* from sea (Beheshti Maal et al., 2012), other studies on isolation of bacteriophages of oral Streptococci as well as other oral pathogens had various consequences (Tylenda et al., 1985; Bachrach et al., 2003; Hitch et al., 2004). The aims of this research were isolation and identification of *Streptococcus mutans* from dental plaque samples, isolation and identification of lytic bacteriophages of the same microorganism from water resources such as Caspian sea. Ultimately the biotechnological applications of isolated bacteriophages to prevent biofilm formation by *Streptococcus mutans* were investigated.

**MATERIALS AND METHODS**

**Bacterial strains, culture media and chemicals:** The standard bacterial strain of *Streptococcus mutans* PTCC1683 (ATCC35668) provided from Iranian Research Organization for Science and Technology (IROST), Karaj, Iran was used. The main culture media that we used were Mitis-Salivarius Agar (sucrose (50 g L<sup>-1</sup>), agar (15 g L<sup>-1</sup>), enzymatic digest of protein (10 g L<sup>-1</sup>), proteose peptone (10 g L<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (4 g L<sup>-1</sup>), dextrose (1 g L<sup>-1</sup>), trypan blue (0.08 g L<sup>-1</sup>), crystal violet (0.8 g L<sup>-1</sup>), Na<sub>2</sub>TeO<sub>3</sub> solution (1 mL), distilled water (1000 mL) (Atlas, 2004) and blood agar base medium from Himedia, India, Brain Heart Infusion (BHI) Broth, Brain Heart Infusion Agar (BHIA) from Scharlau, Spain, NaCl, glycine, CaCl<sub>2</sub>, anaerobic class A gas pack, from Merck and 0.45 μm membrane filter (Millipore, white gridded).

**Collection of oral cavity samples:** The germ samples were collected from patients with mild gingivitis and periodontitis as well as healthy volunteers using sterile explorers from the upper right first molars in all individuals that had not applied antibacterial rinse and routine brushing before sampling. The samples were taken in accordance with ethical guidelines and regulations prepared by Department of Operative Dentistry, Faculty of Dentistry, Isfahan University of Medical Sciences, which have been authorized by Iranian Ministry of Health and Medical Education.
Characterization of streptococci isolated from oral cavity samples: The samples were cultured in BHI and then incubated at 37°C in 5% CO₂ for 24 h. After bacterial enrichment, the turbid broth media were cultured in Mitis-Salivarius Agar (MSA) using streak plate method and incubated at 37°C in 5% CO₂ for 48 h. The blue, shiny and mucoid colonies were examined for catalase reaction using hydrogen peroxide. The purified colonies were characterized using macroscopic and microscopic examinations.

DNA extraction of isolated Streptococcus: For extraction of DNA from isolated oral Streptococcus, a modified protocol of previous published method was followed (Hoshino et al., 2004). So the organisms were grown in 4 mL BHI at 37°C in 5% CO₂ for 24 h. Half and one milliliters of BHI was transferred to a sterile eppendorf tube and centrifuged at 11000 g for 5 min. The supernatant was discarded and 500 μL of ultrapure distilled water was added to pellet. The suspension was boiled for 12 min using a microwave oven at 500 W. The suspension was being pipetted while boiling for homogenizing the extracted DNA and then centrifuged at 11000 g for 5 min to precipitate the cell debris. The supernatant containing the DNA was transferred to another sterile eppendorf and stored at -20°C for further processing.

PCR identification of isolated Streptococcus using gtfD gene: The Primers used for molecular identification of Streptococcus mutans were the same as a previously published data for PCR identification of oral Streptococci (Hoshino et al., 2004). These primers were related to glucosyltransferase enzyme gene, gtfD, of S. mutans and were as follow: forward primer: 5’GGCACCACAACATTGGGAAGCTCAGTT3’ and reverse primer: 5’GGAATGGCCGCTAAGTCAA CAGGAT3’. Polymerase chain reaction was performed in 20 μL of a reaction mixture containing 1 μL (<10 ng μL⁻¹) of DNA template, 0.5 μM of each forward and reverse primers, 1.5 mM of MgCl₂, 0.5 mM of dNTP mixture, 0.5 U of Smar-Taq DNA polymerase and 4.2 mM of 10x PCR buffer. Amplification was done using a gradient thermal cycler (Eppendorf Mastercycler) with the following program, 30 cycles of denaturing step at 98°C for 1 min, primer annealing and extension steps at 70°C for 2 min. The PCR final products were run in electrophoresis in 80 V, 40 mA, for 35-45 min using 1% agarose gel.

Collection, enrichment and purification of bacteriophages: Two zones of Caspian sea, north of Iran, were selected for water sampling. The first zone was shorelines of Anzali Swamp at Guilan province and the second zone was the Nashtaroud bay, Mazandaran province. The water samples were gathered using sterile bottles for five minutes from the depth of 50 cm inframarine surface of the Caspian sea at mentioned areas. Then the Caspian Sea water samples were transferred to the laboratory in a portable refrigerator. Fifty milliliters of each well shaked Caspian Sea water sample was centrifuged at 3000 g for 20 min at 4°C. The supernatant was filtered through 0.45 μm millipore membrane filter using sterile Millipore filtration system and the filtrate was stored at 4°C for next use. The identified bacterial isolate from dental plaque and Streptococcus mutans ATCC35668 were cultured in 10 mL of BHI separately and incubated at 37°C for 24 h in order to be activated. After growth and obtaining appropriate turbidity, the bacterial inoculums were cultured in 100 mL BHI and incubated in a shaker incubator at 37°C and 120 rpm of shaking speed till their logarithmic phase. Ten milliliters of each Caspian sea sample filtrate were added to activated broth media, vortexed briefly and incubated for 20 min using same conditions. One milliliter of BHI was flooded on the BHA culture medium and remained for 5 min to be
absorbed on BHA. The additional BHI broth was removed from BHA and incubated at 37°C for 4-12 h, the time that phage plaques were appeared. The well isolated bacteriophage plaques, were cut aseptically and washed with 1.25% glycine and purification procedures were repeated for 3 times. Glycine filtrates from different BHAs with obvious phage plaques were collected for TEM.

**Preparation of purified bacteriophages for transmission electron microscopy:** Two drops of purified filtrated bacteriophage suspension were transferred on the two form var coated grids (EM standard, 3.2 mm diameter). The additional suspension was removed by drying paper. One of the grids was then stained using uranyl acetate solution (uranyl acetate, 2 g; methanol 35%, 10 mL) and another grid was stained using 2% phosphotungstic acid (pH: 7.2 regulated with KCl, 0.5 M) and dried following the same method and then observed through Transmission Electron Microscope (Philips, CM 10, Netherlands) at 73 K magnification.

**Inhibitory effects of isolated lytic phages on Streptococcus mutans biofilm formation using microtiter plate assay:** *Streptococcus mutans* was cultured in BHI and incubated at 37°C and 5% CO₂ for 16 h. Two hundreds microliters of host strain was added to each well of a polystyrene tissue culture plate 96 well (Biofil TCP011096) from A to H and 1-5. The column 6 was left as first negative control (without host strain and phage) and column 1 (1 -A-1-H) was considered as second negative control (host strain without phage). Column 2 (2 -A-2-H) was infected with 200 μL of isolated phages immediately. The plate was capped and incubated at 37°C and 5% CO₂. Column 3 (3 -A-3 -H) after 6 h, column 4 (4- A-4 -H) after 12 h and column 5 (5 -A-5 -H) after 18 h of incubation were infected with 200 μL of phages per well. After total of 48 h incubation, the plate was assessed for biofilm formation using microtiter plate assay as follow: Plate was emptied and each well washed for 3 times using sterile physiological saline. Then each well was stained with 200 μL of 2% crystal violet (Merck, Germany) for 5 min. Wells were washed 5 times with sterile physiological saline. For assessing the biofilm formation quantitatively, 200 μL of 33% acetic acid was added to each well and the optical density of stain was measured at 450 nm using an ELISA reader (STAT FAX 2100).

**RESULTS**

**Molecular identification of streptococci isolated from oral cavity samples:** The dental plaque bacteria were increased in BHI at 37°C and 5% CO₂ for 48 h. The cultivation of these enriched bacteria on MSA after 24-48 h incubation at 37°C and 5% CO₂ resulted in emerging of colonies that were pale blue to bluish in color. Further examinations of colonies using stereomicroscope revealed that they were smooth, convex and intermediate with 0.8-0.9 mm in diameter. Microscopic observations showed large gram positive Streptococci and finally the negative catalase test indicated that the isolates were related to Streptococcaceae family. The macroscopic and microscopic characterization of isolated oral streptococci indicated that they were members of the genus *Streptococcus* (Table 1). The expected size of gtfD gene PCR product was ~433 bp and regarding this; the species specific amplified DNA of *Streptococcus mutans* was observed in agarose gel after staining with ethidium bromide. The specific bond of *S. mutans* glucosyltransferase D gene on agarose gel was located between 400 and 500 bp bonds of ladder. Regarding its molecular weight that was suitably fitted with the expected size, 433 bp, the species of isolated oral *Streptococcus* was confirmed as *S. mutans* (Fig. 1).
Fig. 1: Gel electrophoresis of *Streptococcus mutans* gtfD gene amplification. The wells 1-6 were included the products of gtf gene PCR of *S. mutans*, *S. sobrinus*, *S. sanguis*, *S. gordonii*, *S. salivarius* and *S. oralis*, respectively. The well 7 is negative control and 8 is positive control, *Streptococcus mutans* ATCC35668

| Traits               | Characterizations on mitis-salivarius agar                                      |
|----------------------|---------------------------------------------------------------------------------|
| Macroscopic          | Pale blue to bluish, smooth, mucoid with shiny reflection, convex, very small colonies with 0.4-0.6 mm in diameter |
| Microscopic          | Medium Coeci, Long chains of Streptococi, a few diplococci, Gram Positive       |

### Isolation and purification of specific lytic bacteriophages of *Streptococcus mutans*

The addition of two Caspian sea filtrates to the isolated *Streptococcus mutans* from dental plaque and the standard strain of *Streptococcus mutans* ATCC35668 in BHI growth medium resulted in complete clearance of BHI after 12 h shaking incubation at 37°C. The cultivation of *S. mutans* Caspian sea (Guilan sample) filtrate inoculums after 20 min incubation, on BHA plates showed the bacteriophage plaques after 4 h incubation at 37°C (Fig. 2). Also cultivation of *S. mutans* Caspian Sea (Mazandaran sample) filtrate inoculums after 20 min incubation on BHA plates showed the bacteriophage plaques after 4 h incubation at 37°C (Fig. 3). The continuous contamination of new logarithmic growth of *Streptococcus mutans* by bacteriophages obtained from individual plaques resulted in more purification of them. The results showed that these specific bacteriophages were lytic phages.

### Identification of lytic bacteriophages of *Streptococcus mutans*

Transmission Electron Microscopy of purified bacteriophage suspension indicated two kinds of specific bacteriophages. The first isolated lytic bacteriophage of *Streptococcus mutans* from Anzali Swamp, Guilan province, Iran, was hexagonal with an envelope measuring 74 nm in diameter (Fig. 4). According to its morphology and size we suggested that the first specific bacteriophage of *Streptococcus mutans* isolated from Anzali Swamp in Caspian sea was most probably related to family Cystoviridae of bacteriophages. The second isolated *Streptococcus mutans* bacteriophage from Nashtaroud
Fig. 2(a-b): Plaques of *Streptococcus mutans* lytic bacteriophage isolated from Caspian sea, (a) Guilan state and (b) Mazandaran state on brain heart infusion agar after 4 h incubation at 37°C.

Fig. 3: Transmission electron micrograph of *Streptococcus mutans* lytic bacteriophage isolated from Caspian sea at Anzali Swamp, Guilan state, Iran with 74 nm diameter (Bar = 35 nm).

Shorelines, Mazandaran province, Iran contained a bottle-shaped mantle and a cone-shaped inner body. Its approximate length and width were measured 125 and 55 nm, respectively (Fig. 5). These results suggested that the second specific bacteriophage of *Streptococcus mutans* isolated from Caspian sea at Nashtaroud bay, Mazandaran province, was most probably related to family Ampullaviridae of bacteriophages.

**Inhibitory assessment of lytic bacteriophages on biofilm formation by *Streptococcus mutans***: In microtiter plate assay after staining wells with crystal violet 2% biofilms were disclosed as purple rings on the sides of each biofilm included well. Using ELISA reader a significant
Fig. 4: Transmission electron micrograph of *Streptococcus mutans* lytic bacteriophage isolated from Caspian Sea at Nashtaroud shoreline, Mazandaran state, Iran with 55 nm diameter and 125 nm length (Bar = 35 nm)

Table 2: Inhibitory effects of the isolated phages on the biofilm formation of *Streptococcus mutans* using microtiter plate assay

| Wells | Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 |
|-------|----------|----------|----------|----------|----------|----------|
| A     | 0.331    | 0.008    | 0.089    | 0.192    | 0.231    | 0.002    |
| B     | 0.320    | 0.010    | 0.109    | 0.183    | 0.242    | 0.004    |
| C     | 0.312    | 0.007    | 0.099    | 0.165    | 0.231    | 0.002    |
| D     | 0.318    | 0.009    | 0.098    | 0.193    | 0.222    | 0.004    |
| E     | 0.341    | 0.012    | 0.101    | 0.178    | 0.234    | 0.005    |
| F     | 0.316    | 0.009    | 0.109    | 0.201    | 0.241    | 0.008    |
| G     | 0.298    | 0.008    | 0.101    | 0.189    | 0.243    | 0.003    |
| H     | 0.324    | 0.015    | 0.097    | 0.204    | 0.252    | 0.003    |

Inhibitory effects of isolated phages on biofilm formation were observed when the phages were immediately added to the host strain in tissue culture plates. The measured Optical Densities (OD) of stained wells have been shown in Table 2. The average OD of Column 1-5 after subtracting of the OD of column 6 as negative control were 0.317, 0.006, 0.097, 0.185 and 0.234, respectively. These experiments showed that the addition of isolated lytic bacteriophages of *Streptococcus mutans* could prevent the formation of biofilm up to 99% (Fig. 5).

**DISCUSSION**

The Presence of lysogenic bacteriophages of *Streptococcus mutans* after induction of bacterial strains using UV exposure or mitomycin treatment have been reported but there are no evidences about purification, characterization and relationship of these prophages to known families of bacteriophages according to ICTV, International Committee of Taxonomy of Viruses (Greer *et al.*, 1971; Higuchi *et al.*, 1977). Higuchi *et al.* (1982) reported a lysogenic bacteriophage, PK1, in *Streptococcus mutans* PK 1 mucoid strain that they obtained using mitomycin C induction. They
Fig. 5: Inhibitory effects of lytic bacteriophages on the biofilm formation of *Streptococcus mutans* using microtiter plate assay. Negative control includes host strain without adding bacteriophages and its biofilm formation has been considered as 100%, 1: 0 h, 2: 6 h, 3: 12 h, 4: 18 h, 5: Negative control

have indicated that most phage particle had hexagonal heads with 95 nm in diameter and a tail with 10 nm in diameter and 150 nm in length. Armou *et al.* (1988) examined so many dental plaque samples and isolated 16 lytic bacteriophages of *Streptococcus mutans* and *Streptococcus sobrinus*. Delisle and Rostkowski (1993) studied further on aforementioned isolated bacteriophages by Armou *et al.* (1988) and confirmed that they attacked to *Streptococcus mutans* and *Streptococcus sobrinus*. They showed that those phages were strictly lytic and 3 phages of them related to Siphoviruses as tailed phages. Each of those Siphoviruses, M102, e10 and f1 had a 67-68 nm icosahedral head and a flexible, noncontractile tail with 8.3 nm in diameter and 283-287 nm in length. Also they mentioned that the existence of phages for *Streptococcus mutans* could concern the role of them in oral microbial ecology and caries formation. Van der Ploeg (2007) has resolved the complete genome sequence of two previously isolated bacteriophages of *Streptococcus mutans*, M102 and M101. In that study it has been clarified that the genome sequence of M102 had high resemblance to the bacteriophage of *Streptococcus thermophilus* in the morphogenesis aspects. While Bachrach *et al.* (2003) have tried to isolate bacteriophages for gram positive oral pathogens such as *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus salivarius* from human saliva, but they detected the lytic bacteriophages of *Enterococcus faecalis*. Although their TEM micrograph showed spherical, enveloped and spiked particles with 70 nm thickness they did not propose any known family for their phages. In a similar attempt, Hitch *et al.* (2004) have tried to isolate lytic bacteriophages for oral pathogens from human saliva. While they examined the presence of probable bacteriophages of human oral cavity against 12 species of oral Streptococci include *Streptococcus mutans*, they detected phage particles infecting *Proteus mirabilis* as a non-oral microorganism. They concluded that the oral microflora were not affected by their specific bacteriophages. In this study, we isolated and identified two novel lytic bacteriophages of *Streptococcus mutans* from Caspian sea, the greatest lake in the world, located at the north of Iran. The first bacteriophage that was isolated from Caspian Sea at Anzali Swamp, Guilan state, Iran, had a hexagonal head without tail but with a lipid envelope measuring 74 nm in diameter. According to latest bacteriophage classification of ICTV (Ackermann, 2007, 2009), its morphology and the size of isolated virion, we suggested that the first lytic phage of *Streptococcus mutans* was most probably related to Cystoviridae family of bacteriophages. The second bacteriophage of *Streptococcus mutans* we isolated from Caspian sea at the shoreline area of Nashtaroud, Mazandaran state, Iran, had a very characteristic and unique structure consist of a bottle-shaped mantle and a cone-shaped inner body measuring 125 nm in length and 55 nm in diameter.
According to latest bacteriophage classification of ICTV (Ackermann, 2009), its unique shape and size, we suggested that the second lytic phage of *Streptococcus mutans* was most probably related to Ampullaviridae family of bacteriophages. *Cystoviruses* have an envelope with a capsid size of 75-80 nm and have been reported only for *Pseudomonas* genus of bacteria (Ackermann, 2007, 2009). At the other hand the total bacteriophages found for Streptococci were 290, all of them are tailed phages, among which 2 have been related to Myoviridae, 276 were related to Siphoviridae and 12 of them were from Podoviridae family of bacteriophages (Ackermann, 2007). So far there is no report of a *Cystovirus* for *Streptococcus mutans* as a gram positive oral pathogen. Ampullaviruses are from pleomorphic phages and have been detected in hot springs and are active against *Acidianus* as a member of Archaea (Haring *et al*., 2005; Ackermann, 2009). So far there is no report indicating an *Ampullavirus* attacks to an eubacterium such as *Streptococcus mutans*. The microtiter plate assay using ELISA measurements showed that the addition of bacteriophages to *Streptococcus mutans* immediately after moving the host strain to microtiter plates prevented the biofilm formation of the host strain up to 99%. Also the experiments confirmed that the addition of specific phage to the host strain after 6, 12 and 18 h of incubation in microtiter plates inhibited the biofilm formation by *Streptococcus mutans* up to 69, 41 and 26%, respectively. We suggested that the descending inhibitory effects from 0-18 h indicated that the phages attacked their hosts effectively on their logarithmic phase of growth curve.

**CONCLUSION**

In conclusion this is the first report of isolation and identification of two novel bacteriophages related to Cystoviridae and Ampullaviridae families of bacteriophages that had lytic effects on *Streptococcus mutans* as a main agent of dental diseases. Streptococci are very sensitive gram positive bacteria and there is no evidence of their colonization in environments other than animal and human hosts like free water bodies, oceans and seas, so another impact of this study was isolation of their bacteriophages from Caspian sea, a restricted lake located in the north of Iran, with general conditions as a sea resource. While further characterization of isolated bacteriophages is challenging, they could be applied as a potential drug for preventing the dental plaque and tooth decay or for treatment of these tremendous dental disorders in the modern pharmaceutical and medical biotechnology. The lytic bacteriophages might be applicable for prophylaxis purposes in the form of mouth rinsing solutions, chewing gums or even in dentifrices.

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