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Article

Anti-*Streptococcus mutans* phage a possible treatment against caries inducing pathogen

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Abstract: *Streptococcus mutans* is a key bacterium in dental caries- one of the most prevalent chronic infectious diseases. Conventional treatment both fails to specifically target the pathogenic bacteria and attempts to eradicate commensal bacteria as well. Thus, caries remains one of most common and challenging diseases. The use of bacterial viruses as anti-bacterial agents, is gaining interest worldwide. Hardly any phages were described against *S. mutans*.

The objective of this study was to isolate anti-*S. mutans* phages and to characterize their antimicrobial properties.

Human saliva samples were filtered and screened for potential phages. Standard double-layered agar method was used for isolation. Whole genome sequence analysis and morphology visualization by TEM, were used for anti-*S. mutans* phage identification. Antibacterial properties were evaluated using clinical strains and ATCC strains of *S. mutans* in various states. Antibacterial effect was also tested on human cariogenic dentin.

One phage against *S. mutans* was isolated and termed SMHBZ8. This phage showed effective lytic activity in vitro against both planktonic and biofilm *S. mutans* cultures. Moreover, the phage showed antibacterial effect when used on cariogenic dentin.

The isolation and characterization of SMHBZ8 may be the first step in developing a potential phage therapy for dental caries.

Keywords: *S. mutans*; Phage therapy; Dental caries; *S. mutans* phage; Bacteriophage; Biofilm.

1. Introduction

Dental caries (tooth decay), is the most common infectious disease worldwide (1-3).

One of the main pathogens that has an important part in the development of dental caries is *Streptococcus mutans* (4-6). *S. mutans* is an acidogenic and aciduric Gram-positive bacterium that naturally inhabits the oral cavity (7). *S. mutans* is classified into serotypes c, e, f and k based on the chemical composition of its serotype-specific polysaccharides, while 70-80% of the strains found in the oral cavity are classified as serotype c, followed by e (~ 20%), and f and k (less than 5% each) (8, 9).

Despite the remarkable progress in reducing caries prevalence by means of fluoridation, improved oral hygiene, and increased access to dental care, dental caries remains one of the most common chronic diseases (10). Nearly 20% of children between the ages of 2-4 have detectable caries, and by the age of 17 almost 80% of adolescents will have a cavity (10). Study of the global burden of this disease reveals that untreated caries is ranked as the most prevalent health condition in the last decade, affecting the permanent dentition of 2.4 billion people (11, 12). Untreated caries can cause severe pain and mouth

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infection and affects not only the mastication function but also the individual's quality of life: smiling, speech, school attendance in children, and work productivity in adults (12, 13).

Dental caries is directly linked with the ability of bacteria to form biofilm (14). At this stage, the bacteria become inaccessible to antibacterial agents and the body's immune system (15, 16). Cariogenic bacteria can readily form biofilms on the tooth surface, rapidly producing lactic acid and consequently causing dental decay (4, 14, 17).

Currently, the incidence of caries is continuing to rise. During the past fifty years, there have not been any innovations in the prevention of dental caries. Current therapies lack sensitivity; they are not species-specific and kill pathogenic species as well as commensal species, which can protect against the formation of pathogenic biofilms. Therefore, there is a need to re-establish and develop new therapeutic strategies that prevent or eliminate biofilm formation in more precise ways, selectively targeting cariogenic bacterial biofilms and specifically geared towards preventing and treating dental caries in clinical practices (3, 11, 15, 17-22).

A promising alternative approach is bacteriophage (phages) therapy. Phages are bacterial viruses that invade bacterial cells, disrupt their metabolism, and cause the bacterium to lyse (23-25). Due to the increase in the prevalence of antibiotic resistance, phage therapy has been regaining interest in the western world (15, 24-27). The key benefits of phage therapy include: high strain specificity with low impact on the commensal microbiome; ability to multiply at the infection site and to disappear concurrently with the target pathogen; phages are natural products devoid of apparent toxicity; they are relatively easy to isolate and to genetically engineer; phages can co-evolve with their bacterial host to kill multi-drug resistant (MDR) bacteria and finally phages can efficiently destroy biofilms that conventional antibiotics usually fail to do (6, 15, 24, 25, 27-32).

Recently, phage therapy treatments were conducted mainly in Emergency Investigational New Drug (eIND) applications (33) including by our group (34). To date, to the best of our knowledge, no phage therapy treatments were applied against caries.

Despite the established significance of *S. mutans* in dental caries and the basic understanding of the importance of phages in general, there is very little data and research about *S. mutans* phages specifically and their place regarding the environment of *S. mutans* in the oral cavity (6, 15, 35, 36). It should be noted that anti-*S. mutans* phage therapy is relatively difficult (35) and several attempts in the past few years ended without success (37, 38). So far only three bacteriophages infecting Mutans streptococci have been isolated from the oral cavity and their genomes have been sequenced: M102 (39), M102AD (40) and ϕAPCM01 (41).

In the present study we describe the isolation, and the characterization of a new anti-*S. mutans* phage. Additionally, its efficacy against *S. mutans*, as the first step towards the development anti-cariogenic phage therapy is evaluated.

2. Results

2.1 Isolation of SMHBZ8 and testing its efficiency against S. mutans in logarithmic phase

Out of 254 tested samples, only one sample resulted in an anti- *S. mutans* phage, termed SMHBZ8. Initially, clear and small plaques on a double layer of BHI agar lawn of *S. mutans* were detected (Figure 1A). SMHBZ8 showed complete lysis of *S. mutans* following 24 hours of incubation (Figure 1B). Bacterial growth analysis showed effective killing of *S. mutans*, by SMHBZ8 even at an MOI of 0.1 in a dose dependent manner. No re-growth was observed for 70 hours. The final OD reading of the MOI = 0.1 sample displayed a four fold reduction compared to the untreated sample. At MOI > 0.1, SMHBZ8 demonstrated almost complete inhibition of bacterial growth. SMHBZ8 MOIs 0.1-1 inhibited growth of the *S. mutans* culture to a lesser extent during 14-20 hours, followed by rapid lysis. SMHBZ8 at MOI = 10^2, almost completely inhibited the growth of *S. mutans* (Figure 1C).

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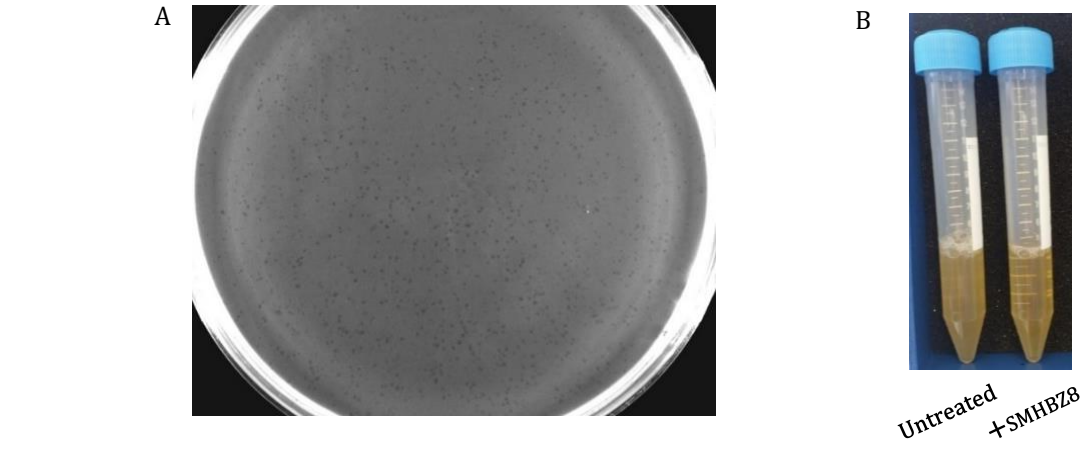

Figure 1. SMHBZ8 is a lytic phage which kills *S. mutans*
(A) SMHBZ8 displays clear plaques on a *S. mutans* lawn, after 24 hours of incubation, depicting lysed bacteria. (B) An overnight culture of *S. mutans* incubated for 24 hours in the presence of SMHBZ8 and control. Samples treated with phage were clear, as compared to the control. (C) Quantitative analysis of SMHBZ8 against a logarithmic phase *S. mutans* culture in a dose-dependent manner. The red graph shows an overnight standard growth curve of *S. mutans* in solution, the negative control. After 5 repeated experiments with similar results, a representative graph of one of those experiments was chosen. The results presented are the averages of three independent wells.

2.2. *Visualization,* whole genome sequencing and phylogeny of SMHBZ8

TEM images showed that the hexagonal head diameter of SMHBZ8 is estimated to be ~56 nm, a long non-contractile tail length is estimated to be ~244 nm long, and the tail width is ~10.9 nm (Figure 2A). The specified features of SMHBZ1, fit the *Siphoviridae* family of the *Caudovirales* order with B1 morphology (42-44).

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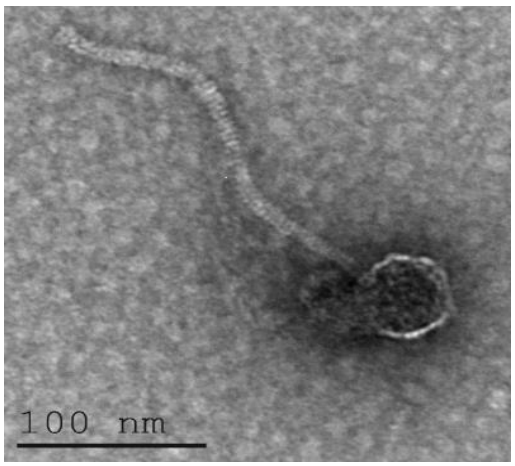
Full genome sequencing of the phage revealed that SMHBZ8 has a closed circular DNA with a genome length of 32,460 bp with a G+C content of 38.8% (Figure 2B). The accession number for its genome in the NCBI GeneBank is MT430910. DNA sequence analysis showed that SMHBZ8 appears to be closely related to other sequenced *S. mutans* phages (M102, M102AD, and ϕAPCM01). The query cover of SMHBZ8 genome is 88% to M102 and out of the similar region there is 86.15% identity, 88% to M102AD with 86.02% identity in the related region and 85% to ϕAPCM01 with 86.59% identity in the related region. However, it was found that while they share many similar genes, there are multiple point mutations and SMHBZ8 clearly contains various unique areas that identify it as a novel *S. mutans* phage which is phylogenetically distant (Figure 2C). There are only a few hypothetical genes in these areas and accordingly, their role remains elusive. The conserved region between all four phages, suggests they all evolved from a common ancestor despite being found in different countries (Figure 2D).

A



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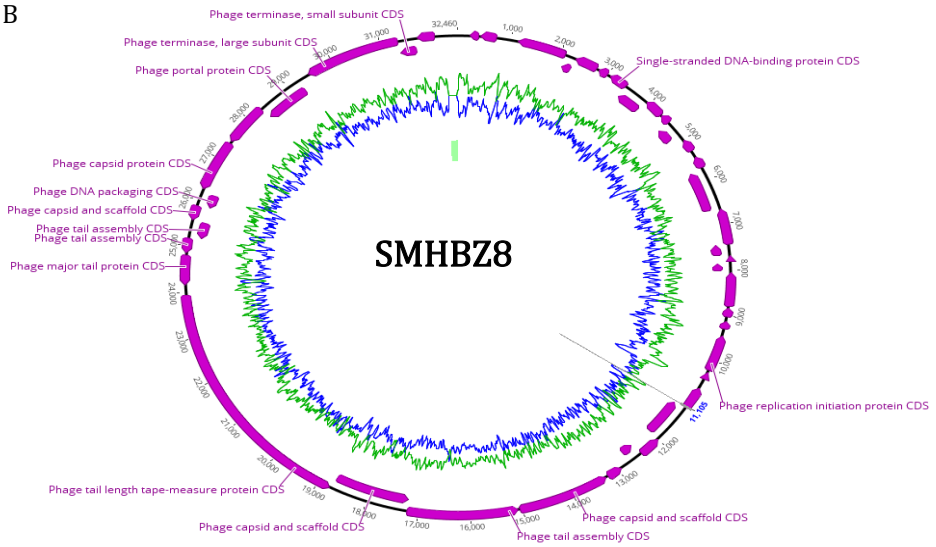

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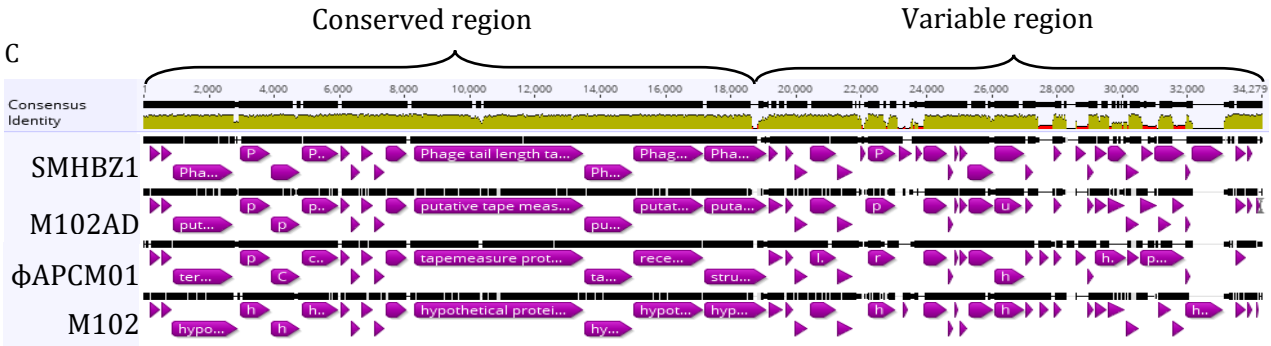

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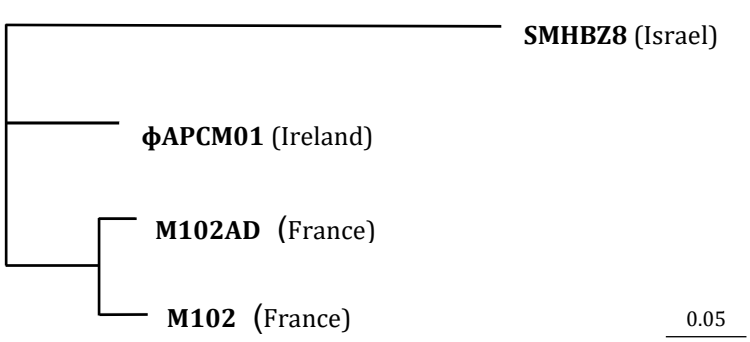

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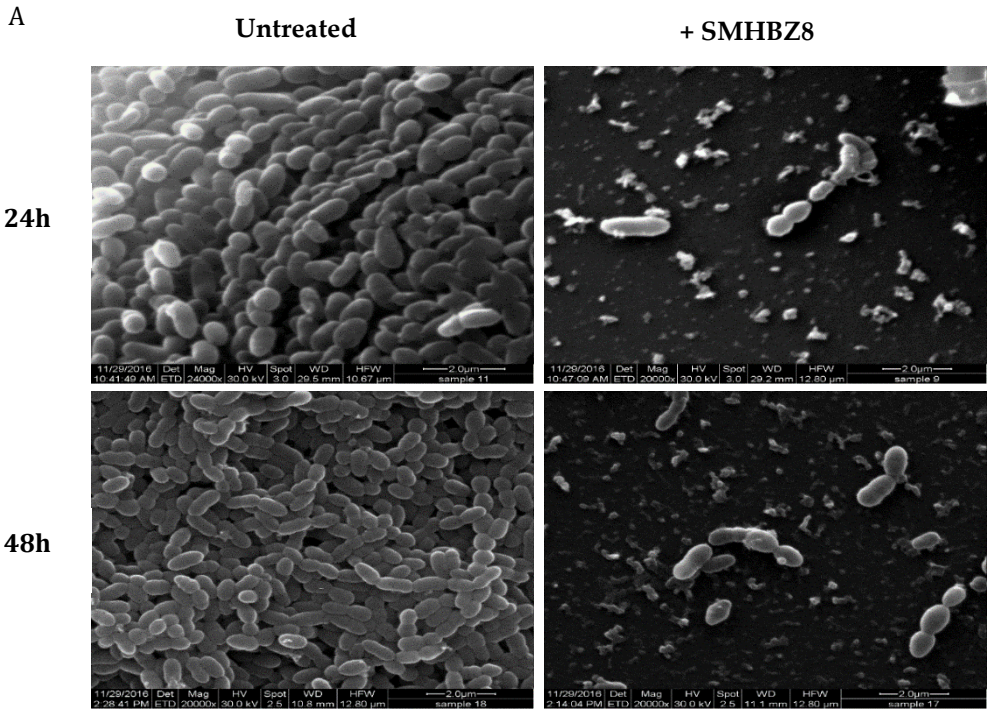
Figure 2. SMHBZ8 is a new anti *S. mutans* phage
(A) Characterization by TEM images of isolated SMHBZ8 from the collected samples. TEM images demonstrate the morphology of SMHBZ8, which appears to have a classic *Siphoviral* virion head and a long tail. (B) Schematic representation of the SMHBZ8 DNA sequence. Whole genome sequencing of SMHBZ8 identified a genome with a length of 32,460 bp. (C) Comparison of SMHBZ8 to genomes of the other three existing *S. mutans* phages showed that, while SMHBZ8 shares common genes with them, it also has new genes that make it unique and novel. The first half of the genome suggests that it is conserved, demonstrated by the yellow continuous level section, indicating the consensus sequences. However, the second half contains many areas where the identity is decreased and is no longer conserved in all four phages, indicating a variable region. (D) Phylogenetic tree of SMHBZ8 in relation to the other known *S. mutans* phages with fully sequenced genomes, suggesting they all share a common ancestor. However, SMHBZ8 seems to be the most different and phylogenetically distant from the others. Phylogenetic distance scale is 0.05 %.

2.3. SMHBZ8 activity on S. mutans biofilm formation

SEM images showed SMHBZ8 almost completely reduced an existing biofilm of 24 and 48 hours within 24 hours, as compared to the untreated biofilm which looks stable and undisrupted (Figure 3A). Using crystal violet staining, we evaluated the biofilm biomass and we also show here that SMHBZ8 almost entirely inhibited the formation of biofilm up to 72 hours. The final OD reading of 24 hours in the treated sample exhibited a three fold reduction compared to the control, for 48 hours the results in the treated sample displayed a 4.8 fold decrease vs the control and for 72 hours a reduction of 6.8 fold was observed in the treated sample vs the control (Figure 3B). In addition, a significant elimination of existing biofilm of 24 hours, 48 hours, and 11 days was observed in the treated samples; more than half the biomass was eliminated after the addition of SMHBZ8, when compared to the untreated sample, which remained stable with no reduction observed. The final OD reading of a 24 hours old biofilm treated with phage for 24 hours sample displayed a reduction of two fold compared to the control, for a 48 hour old biofilm which received 24 hours of phage, a reduction of 3.7 fold was observed compere to the control, the 24 hours old biofilm with 48 hours of phage treatment exhibited a reduction of 1.9 fold vs the control and, finally for 11 day old biofilm treated for 72 hours with phage resulted in a decrease of 1.8 fold compare to the control (Figure 3C). These results suggest that SMHBZ8 is a lytic phage capable of penetrating, controlling, and inhibiting the growth of *S. mutans* biofilm.



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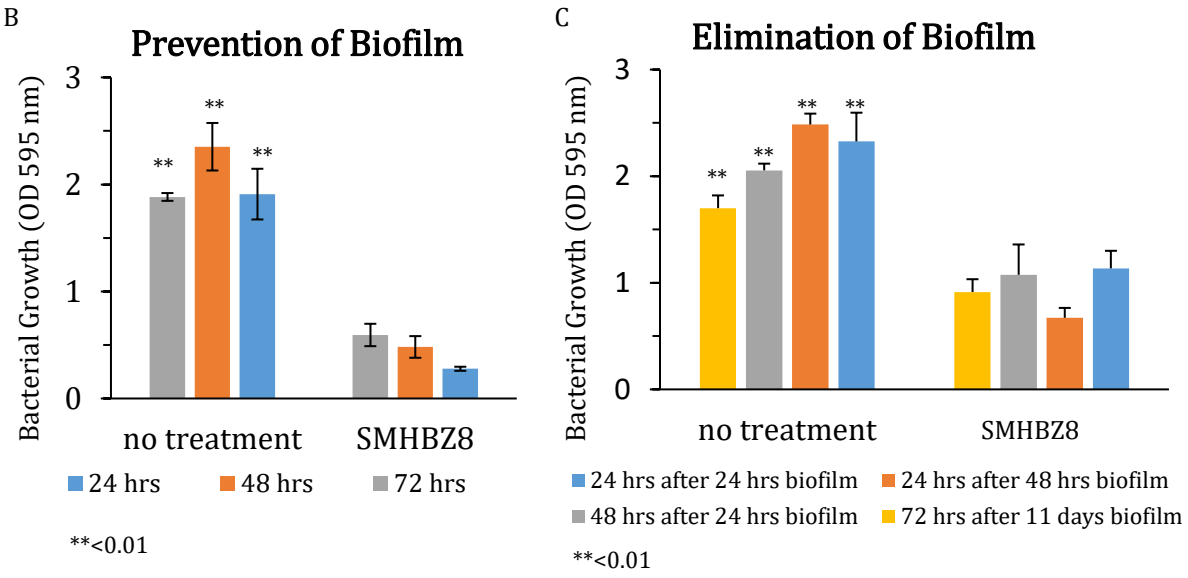

Figure 3. SMHBZ8 is efficient in penetrating and eliminating *S. mutans* biofilm. (A) SEM images showed that after SMHBZ8 was added to 24 hours or 48 hours old *S. mutans* biofilm, it was capable of eliminating it almost completely. The bacteria in the treated biofilm look scattered and disconnected, as compared to the control samples which didn't show any significant change. Using crystal violet (CV) staining: Since the experiments gave similar results, a representative graph of those experiments was chosen. (B) This graph demonstrates there is a significant inhibition of *S. mutans* biofilm formation up to 24, 48 and 72 hours almost entirely, as compared to the control. (C) SMHBZ8 was added to different *S. mutans* static biofilms at different times: to a 24 hours old biofilm, SMHBZ8 was added for either 24 or 48 hours of incubation; to a 48 hours old biofilm, it was added for 24 hours of incubation; and to an 11 day old biofilm, it was added for 72 hours of incubation. In all the experiments, pronounced reduction and destruction of mature biofilms were observed, as compared to the control experiments. These results also validate that the phage reduces the biofilm formation. All the tests were performed in triplicate. Error bars represent the standard deviations. All the treated samples were significantly different from the control samples ($P<0.01$).

2.4. SMHBZ8 specificity tests

Ten *S. mutans* clinical isolates and the 3 ATCC strains (Table 1A) were tested for plaques, after spotting of SMHBZ8 on *S. mutans* lawn. All of them were found to be sensitive to the phage and plaques were visible as demonstrated in Table 1A. SMHBZ8 was able to lyse all the clinical strains tested as well as the ATCC strains.

PCR with oligos for the different serotypes of *S. mutans* showed that the clinically isolated strains of *S. mutans* all belong to serotype c. LM7, MT703, OMZ175 and MT6219 strains were found to be resistant to SMHBZ8 (Table 1A). SMHBZ8 was found to be specifically effective against *S. mutans* serotype c.

In addition, SMHBZ8 specificity was assessed against a range of 10 types of gram-positive, gram-negative, anaerobic, and aerobic bacteria. Table 1B demonstrates the details of the tested bacteria. Bacteria other than *S. mutans* showed complete resistance to SMHBZ8. SMHBZ8 was found to be host specific, infecting only *S. mutans*.

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Table 1A

Bacterial strain	Origin	SMHBZ1	Serotype
S. mutans strains			
S. mutans (700610)	ATCC	S	c
S. mutans (27351)	ATCC	S	c
S. mutans (ES1)	Clinically isolated from saliva	S	c
S. mutans (ES2)	Clinically isolated from saliva	S	c
S. mutans (ES3)	Clinically isolated from saliva	S	c
S. mutans (ES4)	Clinically isolated from saliva	S	c
S. mutans (ES5)	Clinically isolated from saliva	S	c
S. mutans (ES6)	Clinically isolated from saliva	S	c
S. mutans (ES7)	Clinically isolated from saliva	S	c
S. mutans (ES8)	Clinically isolated from saliva	S	c
S. mutans (ES9)	Clinically isolated from saliva	S	c
S. mutans (ES10)	Clinically isolated from saliva	S	c
S. mutans (MT8148)	Kyushu University	S	c
S. mutans (MT703)	Kyushu University	R	e
S. mutans (LM7)	Kyushu University	R	e
S. mutans (OMZ175)	Kyushu University	R	f
S. mutans (M76219)	Kyushu University	R	f

Table 1B

Bacterial strain	Origin	SMHBZ1
Streptococcus strains		
Streptococcus sobrinus (lsb013)	ATCC	R
Streptococcus salivarius	ATCC	R
Streptococcus gordonii	ATCC	R
Other strains:		
E. faecalis V583	ATCC	R
Pseudomonas aeruginosa PA14 R	ATCC	R
Klebsiella pneumonia (bkp016) R	ATCC	R
Actinomyces viscosus	Clinically isolated	R
Fusobacterium nucleatum (fs014)	ATCC	R
Staphylococcus aureus	Clinically isolated	R
Escherichia coli	ATCC	R
Streptococcus salivarius	ATCC	R
Streptococcus gordonii	ATCC	R
Other strains:		
E. faecalis V583	ATCC	R

Table 1A+B.

Bacterial strains and their sensitivity or resistance to SMHBZ8.

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The indication of phage sensitivity or resistance was assessed by the presence of single plaques. Results show that all the clinical strains and the three ATCC strains that were tested were sensitive to SMHBZ8, while all the other strains that were tested were resistant to SMHBZ8.

2.5. Phage pH properties

Caries lesions occur in the presence of sucrose and low pH and thus we tested the phage efficacy in various pH values.

An overnight *S. mutans* growth culture had a pH of 5.1, while overnight *S. mutans* growth culture with phage had a pH of 6. When *S. mutans* was grown overnight in the presence of sucrose the pH was 3.5, but when we added phage to the bacteria and sucrose solution, the pH was 6 after 24 hours (Table 2A). In the next step, when we used the *S. mutans* that grew with sucrose from the previous trial (pH 3.5), we saw that the pH of the solution of *S. mutans* with sucrose solution was 4, but the addition of the phage caused an elevation of the pH to 6 (Table 2B).

We continued testing the phage in different pH of BHI medium and agar plates. In different pH BHI plates, the PFU was 10^6 PFU/ml on pH 5 and 7. No PFU were seen on pH 3, 9 and 10 plates (Table 2C and Figure 4). In different pH BHI broth, the PFU was 10^6 PFU/ml, and was the clearest on pH 5 plate and a bit less clear on pH 4 and 7 plates. On pH 3 and 9 plates the PFU was very opaque, and on pH 1 and 12 plates no PFU were seen (Table 2D and Figure 4).

Table 2A

pH	Solution
5.1	Bacteria
6	Bacteria+Phage
3.5	Bacteria+Sucrose
6	Bacteria+Sucrose+Phage

Table 2B

pH	Solution
4	BacteriaS+Sucrose
6	BacteriaS+Sucrose+Phage

Table 2C

pH	Phage activity
3	-
5	++
7	++
9	-

Table 2D

pH	Phage activity
1	-
3	-+
4	+
5	++
7	+
9	-+
12	-

Table 2. SMHBZ8 pH properties
(A) pH results after adding sucrose and phage. (B) pH results after adding sucrose and phage- second step. (C) Phage biofilm on BHI plates with different pH - the phage plaques were the clearest in pH 5 and 7. D) Phage in BHI medium with different pH- the phage plaques were the clearest in pH 5.

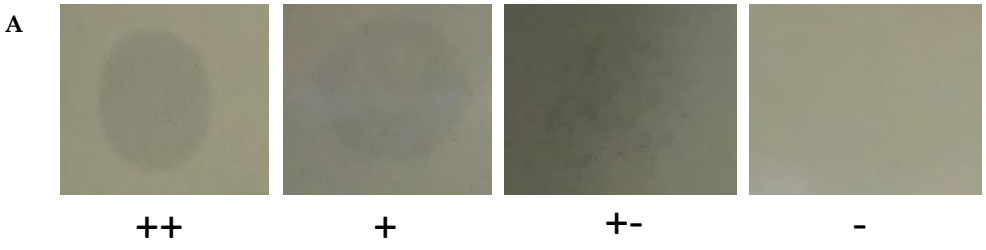

Figure 4. Phage biofilm assay
Different PFU performance. From left to right the PFU become less clear until no PFU is shown.

2.6. SMHBZ8 effect on cariogenic dentin

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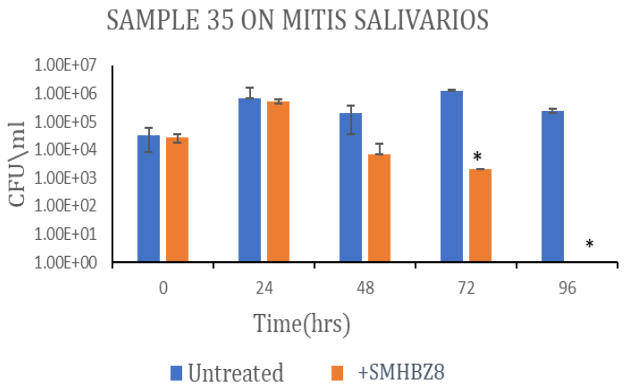
Up to 6 logs in CFU/ml reduction of bacterial counts was observed after 24-96 hours in the phage treated samples as opposed to the untreated samples (Figure 5B). The reduction was significant after 48, 72 and 96 hours of incubation- p=0.01, p=0.05 and p=0.001 respectively (Figure 5C). SEM images of the biofilm showed a reduction in the biofilm's mass within 24 hours in the phage treated samples compared to the control group which looked stable and undisrupted. This phenomenon continued during the following 3 days (Figure 5D). Furthermore, CSLM showed an increase in red staining (dead cells) after 4 days in the phage treated samples compared to the untreated group (Figure 5E).

A

B



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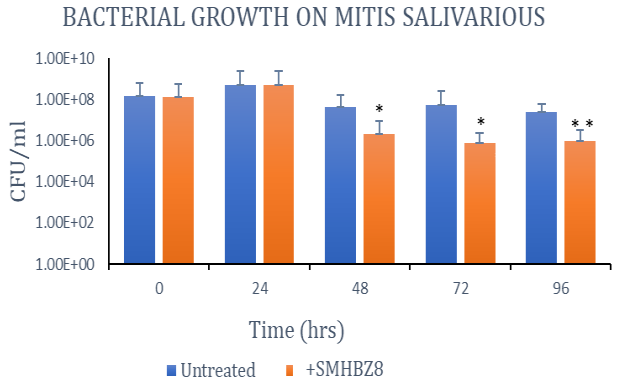

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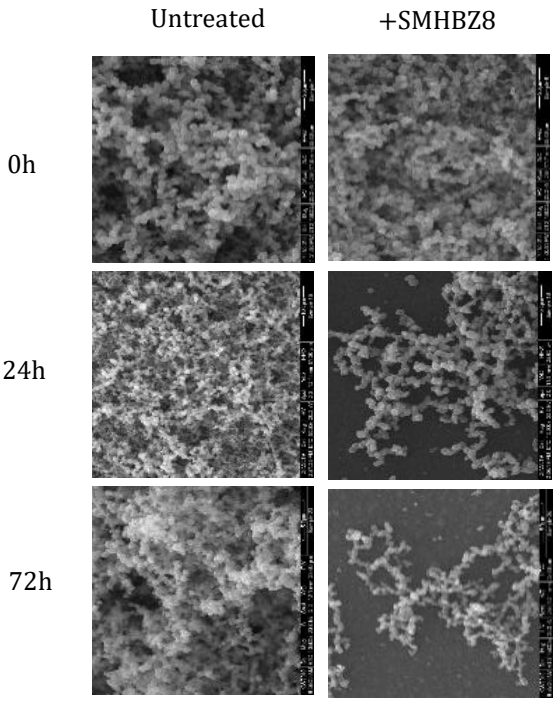

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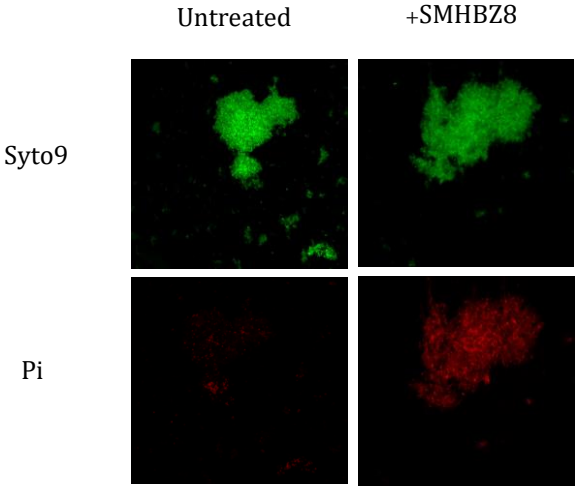

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Figure 5. SMHBZ8 reduces bacterial load in a dentin model
(A) Study chart- Dentin was isolated from patients. Phages were added to several tubes from each sample. CFU, SEM confocal, crystal violet, and optical density were performed on each of the samples. (B) Decrease in CFU/mL of a representative dentin sample treated with phages over time. (C) CFU of all samples on mitis salivarius plates over time. *p<0.05**p<0.005. D) SEM images show that the phage reduced the biofilm mass compared to the control. (E) CSLM showed an increase in dead cells as indicated by red staining

In both phage and antibiotics groups we observed a decrease in bacterial growth compared to the control group, but the reduction was more substantial in the phage group which showed a reduction of up to 3 logs. The difference between phage and antibiotic groups was significant, p=0.05 (Figure 6A). In addition, the biofilm became significantly smaller in the phage treated group, p=0.04, measured by crystal violet staining (Figure 6B). The final OD reading of 96 hours was 0.3 in the phage samples, while the reading in the antibiotics samples and the control samples were twice and four times, respectively. A difference in biofilm size and thickness can also be observed in the SEM images after 96 hours (Figure 6C). In addition, CSLM of 96 hours indicates an increase in red staining, suggesting more dead cells, in the phage treated group (Figure 6D).

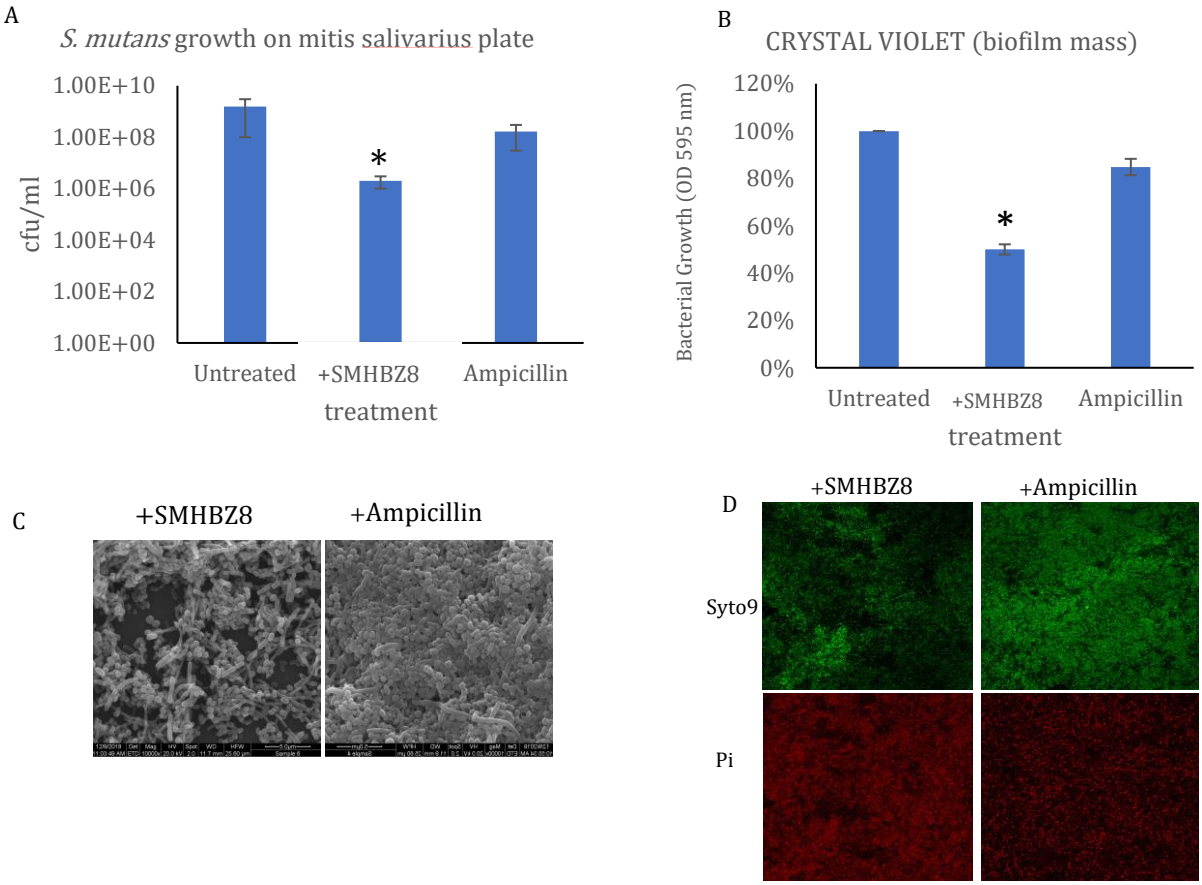

Figure 6. Phage vs. ampicillin
(A) Crystal violet staining of the biofilm showing phages reduced the biofilm size more significantly than the ampicillin, after 96 hours. (B) CFU results after 96 hours. *p<0.05 **p<0.005. (C) SEM pictures of phage treated sample. The phage reduced the biofilm mass. (D) CSLM showed an increase in dead cells as indicated by red staining.

3. Discussion

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Despite the importance of *S. mutans* in dental caries, there are only three *S. mutans* phage genomes that have been sequenced: M102 (39), M102AD (40) and ϕAPCM01 (41). Coinciding with previous reports (37, 38), we also experienced difficulty in isolating *S. mutans* phages. In the current study we examined 254 samples from various sources and tested these samples against 12 different strains of *S. mutans* and only then succeeded in isolating the active anti- *S. mutans* phage, SMHBZ8. This achievement may be attributed to the large number of samples. Alternatively, the range of methods and the variety of processing steps could also have contributed to the successful isolation of this phage. Consequently, it can be speculated that the search and isolation of new *S. mutans* phages requires not only saliva samples, but also a broad selection of samples, for example: dental plaques, teeth, and dental clinic sewage.

We characterized the anti-*S. mutans* phage, SMHBZ8, a lytic phage that effectively infects and kills planktonic and biofilm cultures of *S. mutans in vitro* and in an experimental model of cariogenic dentin. Our results confirm SMHBZ8 to be highly lytic and robust compared to the other recently isolated phages. ϕAPCM01 was able to lyse only one strain of *S. mutans* out of 17 that were tested (41), and M102AD was able to lyse only one strain out of 25 that were tested (40). Only M102 succeeded to target more *S. mutans* strains, but its efficiency was never tested against biofilms. TEM image analysis revealed that the measurements of the SMHBZ8 capsid and tail are compatible with those of the other *S. mutans* phages.

According to its genome sequence, SMHBZ8 has a genome length of 32,460 bp, longer than the others; M102 is 31,147 bp in length (39), M102AD is 30,664 bp in length (40), and ϕAPCM01 is 31,075 bp in length (41). While SMHBZ8 appears to be closely related to the other *S. mutans* phages, the differences identified in the SMHBZ8 genome compared to the other sequenced *S. mutans* phages suggests that it is indeed a unique and new phage. The unique areas in its genome are probably due to point mutations, gene rearrangements, and acquisition or deletion of genes that occur over the years during evolution, indicating that although they evolved from the same ancestor, each phage evolved independently in different countries; however, since there are only four available *S. mutans* phage genomes, it is hard to determine the timeline of those events.

Biofilm formations are distinct from planktonic culture in the mode of growth; in biofilms, there is a matrix of extracellular polymeric substances (EPS) encasing the individual bacteria. Throughout the biofilm, in the interior of the micro-colonies, there is restrictive access to oxygen and nutrients, thus the rate of cell growth may be lower; however, in the cells located on the periphery of the micro-colony, there is more access to these resources and the bacteria are more metabolically active. The process of phage infection mainly depends on their hosts, especially on their intracellular resources, which relies on their physiological status; therefore, it is expected that phages will more effectively infect planktonic culture than biofilms (45, 46).

Since oral biofilm elimination is one of the main targets for caries prevention, it was our main goal to test SMHBZ8 efficiency in eliminating *S. mutans* biofilm. As visualized in the SEM images, and further shown using CV stain (Figure 3, 5 and 6), SMHBZ8 can effectively prevent biofilms and reduce existing biofilm. Notably, despite the differences in growth and behavior of bacteria in planktonic state versus biofilm, SMHBZ8 was found to effectively kill *S. mutans* in both types of culture. Moreover, these results display one of the most important advantages of phage therapy over conventional antibiotics: the capability to efficiently destroy the entire biofilm formation. Phage therapy is a great complementary strategy to other applications used today in dentistry or as a new alternative on its own.

As demonstrated in Table 1A and 1B SMHBZ8's host range is limited to *S. mutans*. All the tested *S. mutans* ATCC strains, which demonstrated sensitivity to SMHBZ8 (UA195, 25175, 27351) belonged to the c serotype as mentioned before (Table 1A). Bacteria other than *S. mutans* showed complete resistance to SMHBZ8, thus indicating that it is indeed a lytic phage with a narrow host range that can infect only *S. mutans* type c. Like

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the other known *S. mutans* phages, SMHBZ8 displays high host specificity, which is a major advantage compared to antibiotics as the phage does not harm the beneficial bacteria.

Few results support the concept of using phage therapy against caries: As demonstrated from pH tests, when SMHBZ8 was added to a sucrose supplemented *S. mutans* media, the media became less acidic. Additionally, SMHBZ8's ability to kill its target bacterium is significantly diminished in a Ph<4 or pH>9. In the future, when phages are prescribed to patient, these results should be taken into consideration in order to use a phage solution that will get the best results in the very acidic environment of oral caries lesions (47).

By using the human cariogenic dentin model, we showed that SMHBZ8 was able to reduce *S. mutans* load in cariogenic dentin. This model is very important in a few aspects: The CFU/ml performed on BHI plates and on the mitis salivarius plates at time 0 gave almost the same values (Figure 5B, 5C), which strengthens the claim that *S. mutans* is indeed one of the main bacteria in human cariogenic lesions. In addition, there is no other model that represents caries better, due to the difficulty in creating caries in petri dishes and in animal's models. Review of the literature hasn't come up with a proper model for cariogenic dentin yet, and since *S. mutans* infects the dentin it is highly important to have this kind of model for research purposes. Moreover, this model can help us test more agents and materials against caries before trying it *in vitro* and *in vivo* models.

As mentioned before, the oral cavity is a very complicated area and the fact that the phage was able to reduce the bacterial load in the dentin, more significantly than the antibiotic, may suggest that SMHBZ8 can be a possible candidate for caries phage therapy.

Future experiments planned using this cariogenic dentin model are developing phages against other bacteria in caries lesions, like lactobacillus (48). This will enable the creation of phage cocktails covering a range of bacteria.

To our knowledge, using *S. mutans* phages against dental caries has not yet been clinically applied (49). Therefore, similarly to other medical fields that are now exploring the possibilities of phage therapy, dentistry can also progress to new horizons, in research as well as in therapeutics (48). Moreover, further efforts to find and isolate more *S. mutans* phages should be carried out in the future, thus increasing pharmacological diversity and increasing options for the creation of phage cocktails (50).

4. Materials and Methods

Bacterial growth conditions

S. mutans strains were grown in brain heart infusion (BHI) broth (Difco, Detroit, MI) or on BHI agar plates overnight at 37°C in 95% air and 5% CO_2 (v/v). Bacterial strains used for screening are listed in Table 3.

Table 3. List of bacterial strains and their growth conditions

Bacterial strain	Broth	Incubation Conditions
Streptococcus strains		
Streptococcus sobrinus (lsb013)	BHI	37°C
Streptococcus salivarius	BHI	37°C
Streptococcus gordonii	BHI	37°C
Other strains:		
E. faecalis V583	BHI	37°C, 200 rpm shaking
Pseudomonas aeruginosa PA14 R	LB	37°C, 200 rpm shaking
Klebsiella pneumonia (bkp016) R	LB	37°C, 200 rpm shaking
Actinomyces viscosus	BHI	37°C
Fusobacterium nucleatum (fs014)	LB	37°C, anaerobic

Isolation of clinical strains of S. mutans from saliva samples

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Ten saliva samples were collected from random healthy volunteers. The samples were streaked on mitis salivarius agar plates selective for *S. mutans* (51). After 24-48 hours of incubation at 37°C in 95% air 5% CO_2 (v/v), single colonies were picked into BHI broth (Difco, Detroit, MI) for propagation. The isolated bacteria were verified to be *S. mutans* by light microscopy (100X) and by specific *S. mutans* PCR primers (Left primer: TTGACTATTGCTGCCTTGGC, right primer: TTGTGCACTTTGAGGCGAAA, designed using Primer 3 version 4.0.0 http://primer3.ut.ee/). The oligonucleotides were confirmed by PCR on *S. mutans* strain ATCC UA159, with *E. faecalis* as a negative control. Ten strains of *S. mutans* were positively identified in this way.

S. mutans strains used in this study are listed in table 1A. The ATCC strain of *S. mutans*- 25175 UA159- are c serotype (52, 53). Five *S. mutans* strains, were generously provided by Kyushu University in Japan including, 1 of c serotype (MT8148), 2 of e serotype (LM7, MT703) and 2 of f serotype (OMZ175, MT6219). The serotypes of the bacteria isolated from saliva samples was determined by PCR with primers specific to the different serotypes (c serotype primers: Left primer: TTGACTATTGCTGCCTTGGC, right primer: TTGTGCACTTTGAGGCGAAA, designed by Primer 3 version 4.0.0 http://primer3.ut.ee/) (9).

Sample collection

Saliva samples, extracted teeth, and dental biofilm (dental plaque) from healthy volunteers were collected in the dental clinics of Hadassah Medical Center, Jerusalem (approved by the Hebrew University-Hadassah Institutional Ethics Committee, protocol no. MD-0351-16-HMO). Dental sewage was collected at the same clinic and regular sewage samples were taken from the decontamination facility in West Jerusalem.

Samples of cariogenic dentin were also collected in the dental clinics in Hadassah Ein Karem Campus of the Hebrew University in Jerusalem from healthy volunteers who had caries lesions (approved by the Hebrew University-Hadassah Institutional Ethics Committee, protocol no. 0680-17-HMO).

Phage isolation and propagation

Two hundred and fifty- four samples of saliva, teeth, dental plaques, dental sewage, and regular sewage were tested for anti-*S. mutans* phages. Each sample collected was centrifuged (centrifuge 5430R, rotor FA-45-24-11HS; Eppendorf, Germany) at 7,800 rpm for 10 min. The samples were first filtered through 0.45 μm filters (Merck Millipore Ltd, Ireland), then, to filter out smaller particles, 0.22 μm filters (Merck Millipore Ltd, Ireland) were used. Samples and filtrates were kept at 4°C.

Filtered samples were tested for lytic behavior using agarose spot testing as previously described (54). Briefly, 200 μl of overnight cultures of *S. mutans* (10^8 CFU/ml) were mixed with 3.5 ml pre-warmed 0.5% agarose and then overlaid on BHI agar plates. After the agarose solidified, multiple 10 μl spots from the filtered sample were placed on it. For the negative control, BHI broth was spotted on the same plate. Plates were incubated for 24-72 hours at 37°C, under 95% air 5% CO_2 (v/v) conditions, or until plaques were observed. When detected, plaque morphologies were examined, and the clearest and most lytic ones were carved out from the agar and transferred using a sterile pipette tip into a tube of BHI broth. The phages were propagated using an overnight culture of *S. mutans* and were purified by centrifugation at 7,800 × g for 10 min followed by filtration through 0.22 μm filters (27). To determine the concentration of plaque forming units (PFU), the modified double-layered agarose method was used as previously described (54). Briefly, phages were serially diluted by 10-fold, and drops of 5 μl from each dilution were spotted on BHI agar plates on which was spread 0.2 ml of an overnight culture of *S. mutans* (10^8 CFU/ml) mixed with 3.5 ml of pre-warmed 0.5% agarose (LifeGene, Israel). The plates were incubated for 24 hours at 37°C under 95% air 5% CO_2 (v/v) conditions. The number of plaques was counted the following day and the initial concentration, PFU/ml, was calculated.

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TEM analysis of the phage

To visualize the structure and morphology of the isolated phage, transmission electron microscopy (TEM) was used following the Gill method (55). One ml of isolated phage (10^8 PFU/ml) was centrifuged at 19,283 rpm (centrifuge 5430R, rotor FA-45-24-11HS; Eppendorf) for 2 hours. The supernatant was discarded and the pellet was suspended in 200 µl of 5 mM $MgSO_4$, and then incubated for 24 hours at 4°C, allowing the pellet to disperse. 10 µl of the phage were mixed with 30 µl of 5 mM $MgSO_4$ and spotted onto a strip of Parafilm laid on top of a paper towel. 30 ml of 2% uranyl acetate was added on each of the grids to prepare them. Grids were carefully placed using forceps over the drops of the phage, with the carbon side facing up. After approximately 1 min, the grids were placed on drops of 2% uranyl acetate stain, for another 10-15 sec. After the grids dried, they were stored until future use. For the images, a transmission electron microscope (Joel, TEM 1400 plus) with a charge-coupled device camera (Gatan Orius 600) was used.

DNA isolation and sequencing

The phage's DNA isolation was performed as previously described (27) using the Norgen Biotek Phage DNA Isolation Kit (Cat. # 46800). At 37°C, 1 ml of a phage lysate (10^8 PFU) was treated with RNase (50 mg/l) and DNase (100 mg/l) for 30 min in order to eradicate any bacterial nucleic acids. In order to digest both phage DNase and capsid, sodium dodecyl sulfate (20%) and Proteinase K (100 mg/l) were added, and the mixture was incubated at 52°C for 1 hour. Sequencing was performed in the Core Research Facility at the Hebrew University, Hadassah Campus as previously described (56), using a Nextera XT DNA kit (Illumina, San Diego, CA), to prepare libraries. To purify and amplify the DNA of the phage, using a phage DNA isolation kit (Norgen Biotek), AMPure XP beads and a limited-cycle PCR were used. By using the Illumina MiSeq platform, the DNA libraries were tagged, pooled, and normalized in a prevalent flow cell at 2X250 base-paired-end reads. FastQC (57) was used to control the quality of the reads, which were then filtered and trimmed by FASTX-Toolkit (58). Using Geneious Prime (Biomatters) software, the *de novo* assembly with end-trimmed reads was performed. Analysis of the open reading frames (ORFs), whole-phage genomes, phylogenetic tree generation, and comparisons to other known *S. mutans* phages were performed and analyzed using Geneious Prime® 2020.0.4 (Biomatters) software and its plugins. Annotation was performed using RAST (https://rast.nmpdr.org/rast.cgi)

Determination of the phage lytic activity in planktonic culture

Planktonic *S. mutans* growth kinetics was analyzed using a 96-well plate reader (Synergy; BioTek, Winooski, VT). The plate was prepared as previously described (59) with the following modification: the outer wells of a sterile 96 well plate were filled with powder from a CO_2 gen sachet (Thermo). To prevent the powder from spreading inside the 96 well plate, the powder filled wells were covered with autoclave tape. The empty wells were inoculated with 160 µl of BHI, 20 µl of an overnight *S. mutans* (10^8 CFU/ml) cultures, and 20 µl of phages at various multiplicities of infection (MOI) 0.001, 0.01, 1, 100, and 1000 in triplicate. Wells to which BHI was added instead of the phage, served as a negative control. Before inserting the plate to the plate reader, small holes were carefully poked into the autoclave tape covering the outer wells to allow the CO_2 to diffuse out. The plate was then covered and further sealed with tape. The reader was set to record the OD of each well at 600 nm every 20 min following 5 sec of orbital shaking while incubating at 37°C.

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Determination of phage activity on biofilm formation

S. mutans biofilms were grown in 24/96-well plates at 37°C under 95% air 5% CO_2 (v/v) conditions.

To determine the ability of the phage to prevent biofilm formation, an overnight *S. mutans* culture (10^8 CFU/ml) was mixed with phages (10^8 PFU/well), cultivated in BHI medium supplemented with 4% sucrose for 24, 48, or 72 hours.

To determine the ability of the phage to eliminate existing biofilm formations, an overnight *S. mutans* culture (10^8 CFU/ml) was cultivated in BHI medium supplemented with 4% sucrose. After 24 or 48 hours of incubation, phages were added (10^8 PFU/well) to the wells, and the incubation was continued for 24 or 48 hours additional.

The phages ability to penetrate biofilms and their efficiency against biofilms was evaluated by several methods:

In order to quantify the biomass, crystal violet staining was used as previously described (60). Briefly, the wells containing the biofilm, as mentioned above, were washed with phosphate-buffered saline (PBS), and fixed with 200 µl of methanol for 20 min. For staining, 200 µl of crystal violet (1%) were added and incubated for 20 min at room temperature. After the wells were washed with water, 200 µl of citric acid (37%) were added. The quantity of the biomass was read by the 96-well plate reader (Synergy; BioTek, Winooski, VT) at 595 nm. Each test was performed in triplicate, and the whole experiment was repeated three times.

To prepare the biofilm samples for SEM, the samples were fixed with Karnovsky's fixative (2% PFA, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH=7.4) for 4 hours at room temperature in a 24-well culture plate, followed by ½ diluted Karnovsky's fixative overnight at 4°C. Then, the biofilm samples were post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 2 hours, dehydrated in a graded series of alcohols, followed by Critical Point Drying (CPD). After sputter coating with Pd/Au, biofilm samples were observed by SEM (FEI, Quanta 200; CPD – Quorum Technologies, K850 Critical Point Drier; Sputtering – Quorum Technologies, SC7620 Spatter coater;). Each well was treated and visualized in duplicate.

Host range specificity tests

The lytic activity of the phage was screened against several *S. mutans* clinical strains, ATCC *S. mutans* strains (Table 1A) and against other oral bacterial species from our lab collection (Table 1B). For this purpose, each bacterial strain was grown overnight in their respective media and growth conditions as shown below in Table 3.

Five hundred microliters of an overnight bacterial culture, grown as specified in Table 3, were poured on the agar plates until the plate was entirely covered. After allowing the plate to dry, at least 5 spots of 10 µl each from the phage sample were added. The plates were incubated at 37°C for 24-72 hours or until plaques were visible enough to examine the differences between the strains and to test whether they were resistant or susceptible to SMHBZ8. Each experiment was repeated twice.

Effect of pH on phage activity

S. mutans culture (10^7 CFU/ml) was grown overnight with phage (10^6 PFU/ml) and 4% sucrose solution. Four test tubes were prepared in triplicate. The first contained 1 ml of an overnight *S. mutans* brought of 10^7 CFU/ml and 4 ml BHI medium. The second contained 1 ml of the same bacteria, 1 ml phages at 10^6 PFU/ml and 3 ml BHI medium. The third contained 1 ml bacteria, 0.5 ml sucrose (4% solution) and 3.5 ml BHI medium, and the fourth contained 1 ml bacteria, 1 ml phage, 0.5 ml sucrose and 2.5 ml BHI. The tubes were incubated overnight at 37°C in 95 % air 5 % CO_2 (v/v) PFU, CFU and pH were calculated after 24 hours. The PH was measured by using universal pH indicator stripes.

The bacteria from the third group served for the following assay for which two tubes were prepared: the first tube consisted of 1 ml bacteria, 0.5 ml sucrose and 3.5 ml BHI, and the second contained 1 ml bacteria, 1 ml phage, 0.5 ml of sucrose and 2.5 ml BHI. The

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tubes were kept overnight at 37°C in 95 % air 5 % CO_2 (v/v). PFU and CFU and pH were calculated after 24 hours.

Additionally, BHI agar plates were prepared with different pH: 10, 9, 7, 5, 3, using HCL and KOH acid solutions. PFU of phages at 10^6 PFU/ml was performed on these plates.

After that, BHI broth were prepared in different pH- 12, 9, 7, 5, 4, 3, 1- using HCL and KOH acid solutions. Using a 96-well plate reader (Synergy; BioTek, Winooski, VT), we diluted 10 µl of phage 10^6 PFU/ml in 180 µl of each of the different BHI, and then we performed PFU on regular pH (=7) plates of BHI.

SMHBZ8 effect on cariogenic dentin

Seventy-six dentin samples were collected from healthy volunteers who have caries lesions in the dental clinics at Hadassah Ein Kerem Campus of the Hebrew University in Jerusalem (Figure 5A). Six samples were collected from each patient (n=3 test and n=3 control). The dentin samples were collected with 20102 Carisolv excavator number 4 and transferred into an Eppendorf tube containing 1.5 ml BHI. One hundred µl of phage at 10^6 PFU/ml were added to each test tube, and 100 µl of BHI were added to the control tubes.

The cariogenic dentin sample tubes were incubated at 37°C under, 95 % air 5 % CO_2 (v/v) conditions. CFU/ml bacterial count was performed on BHI and mitis salivarius agar plates (51) (61). The CFU/ml was assessed at 0 12, 24, 48, and 72 hours.

In addition, bacterial growth of the samples was recorded by optical density (OD) change in a 96 well plate reader (Synergy; BioTek, Winooski, VT) at 600 nm. Twenty microliters of each sample were added to 3 wells with 180 µl BHI. Three wells containing 200 µl of BHI served as control. The OD was assessed at 0, 12, 24, 48, and 72 hours.

The dentin samples were prepared and viewed under SEM at 0, 24, and 72 hours. Sample preparation for SEM was done as mentioned above.

After 72-96 hours of incubation 200 µl of the dentin samples were washed gently with PBS and then stained with a live/dead cell viability kit (Life Technologies. Waltham, MA). The samples were centrifuged at 12000 RPM for 1 min, washed with 100 µl of PBS solution followed by centrifugation. Then 100 µl of the stained sample were incubated at room temperature for 20 min, centrifuged, and washed with 100 µl PBS solution followed by another centrifugation. The stained sample was fixed with 4% paraformaldehyde for 5 min followed by another PBS washing. The fluorescence emissions of the samples were detected using a Zeiss LSM 410 confocal laser microscope (Carl Zeiss). Red fluorescence was measured at 630 nm, and green fluorescence was measured at 520 nm. Horizontal plane optical sections were made at 5-µm intervals from the surface outward, and the images were displayed individually. The microscopy slices were combined to form a 3D image using Bioformats and UCSD plugins (ImageJ 1.49G) (http://imagej.nih.gov/ij/). The properties were the same for all the samples.

Three additional cariogenic dentin samples were collected from 10 patient in order to compare between phage application and conventional antibiotics application. Ampicillin was chosen due to the high susceptibility of *S. mutans* strains to it as indicated by Baker et al (62). In order to determine the Minimal Inhibitory Concentration (MIC) for ampicillin, a stock solution of 100 µg/ml ampicillin was prepared in DDW. Serial dilutions with DDW were made in 96-well microtiter plate (Thermo Fisher Scientific, Roskilde, Denmark) of 10, 1, 0.1 and 0.01 mg/ml, in a final volume of 10 ml using DDW. Five hundred microliter bacteria (10^6 CFU/ml) were added to each tube and the culture growth was recorded immediately after treatment for 24 hours at 37°C in a CO_2 5% incubator. The MIC was defined as the lowest concentration of antimicrobial that inhibited the bacterial growth. To the test tubes we added half of the tested MIC. One hundred microliter ampicillin were added to each of the tested samples in the antibiotics group.

The dentin samples which contained ampicillin were also analyzed by crystal violet staining as mentioned before.

Statistical Analysis

The statistical significance of all experiments described here was determined using Student t-test.

5. Conclusions

To conclude, our results indicate that SMHBZ8 is a promising new lytic phage against *S. mutans*. The effectiveness of SMHBZ8 makes it suitable for future *in vivo* experiments of phage therapy against *S. mutans*. Therefore, in this study, bacteriophage therapy, a new and more selective therapeutic strategy for caries prevention and management is proposed as a potential clinical alternative. These clinical options can be applied as new alternatives to toothpaste, slow-release mouthwashes, or as complementary topical application to existing methods.

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