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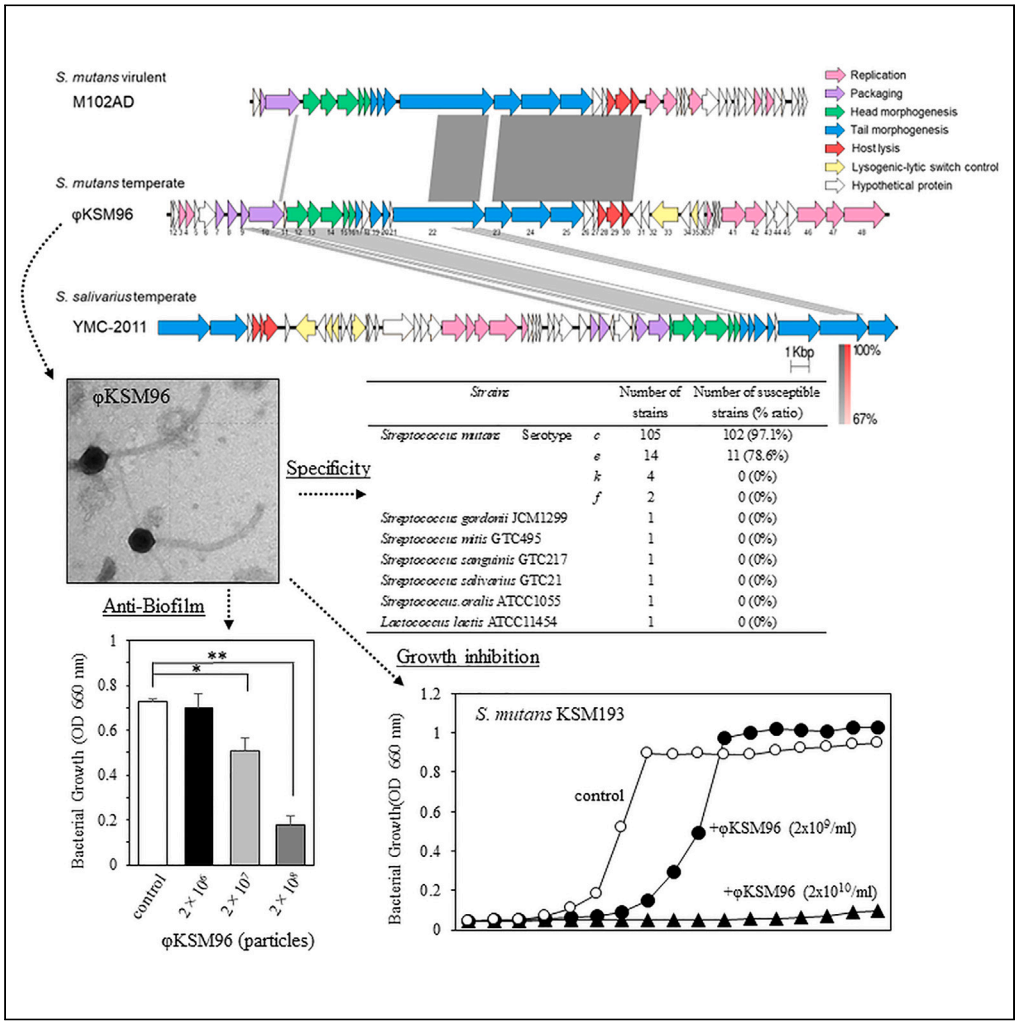

Strains			Number of strains	Number of susceptible strains (% ratio)
Streptococcus mutans	Serotype	*c*	105	102 (97.1%)
		e	14	11 (78.6%)
		k	4	0 (0%)
		f	2	0 (0%)
Streptococcus gordonii JCM1299			1	0 (0%)
Streptococcus mitis GTC495			1	0 (0%)
Streptococcus sanguinis GTC217			1	0 (0%)
Streptococcus salivarius GTC21			1	0 (0%)
Streptococcus oralis ATCC1055			1	0 (0%)
Lactococcus lactis ATCC11454			1	0 (0%)

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Highlights

Isolation and characterization of temperate bacteriophage φKSM96 from *Streptococcus mutans*

φKSM96 has a circular DNA 39,820 bp long and reveals Siphoviridae morphology

φKSM96 shows a specific activity against only *S. mutans* strains

φKSM96 strongly inhibits *S. mutans* growth and biofilm formation

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Isolation of *Streptococcus mutans* temperate bacteriophage with broad killing activity to *S. mutans* clinical isolates

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SUMMARY

Bacteriophages are expected to be therapeutic agents against infectious diseases. *Streptococcus mutans* are involved in dental plaque formation related to dental caries and periodontitis. In *S. mutans*, lytic bacteriophages have been isolated previously, but the isolation of temperate bacteriophage has not been reported although their presence in the genome has been confirmed. Here, we report the isolation of temperate bacteriophage, φKSM96, from *S. mutans*. φKSM96 has a circular DNA 39,820 bp long and reveals Siphoviridae morphology. φKSM96 shows a broad range of susceptibility against *S. mutans* strains with different serotypes. By the addition of φKSM96, *S. mutans* growth and biofilm formation were significantly inhibited. In cocultures of *S. mutans* with other bacterial species, the proportion of *S. mutans* significantly decreased in the presence of φKSM96. In summary, φKSM96 shows selective anti-*S. mutans* activity. The isolation of temperate bacteriophage is important for future genetic manipulation to create more efficient bacteriophages.

INTRODUCTION

Streptococcus mutans, which is endemic in the human oral cavity, is a major cariogenic pathogen.[1,2] The major virulence factors of *S. mutans* are water-insoluble glucan formation and acid production.[1–3] Water-insoluble glucan is a key component of dental plaque, which is considered to be a hotbed for tooth decay and periodontitis. The accumulated acids produced by *S. mutans* in dental plaques demineralize calcium phosphate, a component of teeth. Therefore, elimination of *S. mutans* from the oral cavity is thought to inhibit dental plaque formation, thereby preventing the onset of dental caries and periodontal disease. Furthermore, the relationship between oral diseases, especially periodontal diseases, and systemic diseases has recently been studied, and in particular, the associations between oral bacteria and various diseases, such as bacteremia, diabetes, arteriosclerosis, rheumatoid arthritis, Alzheimer's disease, and cancer, have been reported.[4–9] *S. mutans* has been reported to be associated with endocarditis, stroke and cognitive dysfunction.[10,11] Therefore, the elimination of *S. mutans* has been linked to the prevention of systemic diseases and may contribute to improving quality of life. To date, several approaches for plaque control, such as brushing and the use of mouthwashes, including disinfectants, have been used to prevent dental caries.[12,13] Although these approaches are not specific for *S. mutans*, such an approach is expected to be used in the development of a new method.

Currently, therapies utilizing bacteriophages are projected to be candidates for developing new antibacterial agents.[14,15] In particular, studies are underway to isolate and clinically apply bacteriophages of antibiotic-resistant bacteria such as *Acinetobacter* and *Pseudomonas aeruginosa*, which are causative agents of pulmonary infections.[16–18] Bacteriophages are viruses that specifically infect bacteria. Unlike antibiotics, bacteriophages infect bacteria by inserting their DNA to amplify the bacteriophage particles and finally disrupting the bacterial cell membranes during virus release to the outside environment. The spectrum of bacteriophage infection is narrow, as it is limited to certain species or strains.[19] Therefore, the application of bacteriophages to humans can specifically attack the target bacterium with no influence on other bacteria that are localized the same sites as the commensal bacteria. The search for bacteriophages that specifically kill pathogenic bacteria for use as new antimicrobial agents has intensified. Generally, it has been considered that lytic bacteriophage is a good candidate for the therapy due to high lytic activity and avoiding the horizontal gene transfer. However, recent advances in DNA sequencing technologies and genetic engineering techniques have enabled the application of temperate bacteriophages.[20] The advantages of focusing on temperate bacteriophage for the therapy is as follows. Compared to lytic phage, it is easier to find the bacteriophage because almost half of the

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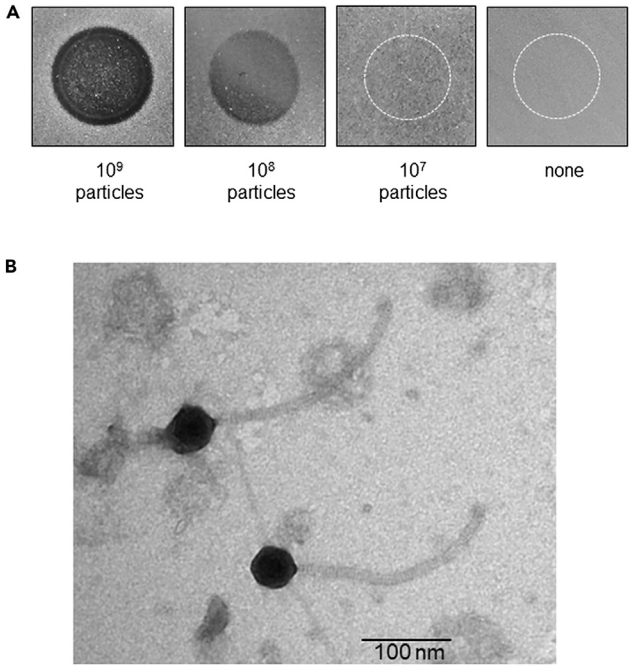

Figure 1. Isolation φKSM96

(A) Anti-*S. mutans* activity of φKSM96. Dotted line, spotted area.

(B) Electron microscopy observation of φKSM96.

sequenced bacteria were lysogens.[21] Also, it could be possible to convert temperate bacteriophage to lytic one by eliminating the genes responsible for lysogenic life cycle. Therefore, several attempts using temperate bacteriophages has been performed for therapeutic purposes.[20] Based on this background, the application of bacteriophages specific for *S. mutans* is promising for the prevention of oral diseases and systemic diseases. There have been several reports regarding oral streptococcal bacteriophages, including *S. mutans* bacteriophages. Only 6 *S mutans* lytic bacteriophages, e10,[22] f1,[22] M102,[23] M102AD,[24] ΦAPCM01,[25] and SMHBZ8,[26] have been isolated, while the isolation of temperate bacteriophage of *S. mutans* has not been reported. In this study, we firstly isolated and characterized a novel temperate bacteriophage from *S. mutans*.

RESULTS

Identification of the bacteriophage from *S. mutans* KSM96

Among 125 *S. mutans* isolates, we found the predicted entire genome of the temperate bacteriophage from the KSM96 genome data. Then, we prepared the bacteriophage particles from KSM96 induced by mitomycin C treatment (0.1 μg/mL). After purification of the bacteriophage, we found that it had lytic activity when the bacteriophage solution was spotted on trypticase soy agar (TSA) inoculated with *S. mutans* UA159 (Figure 1A).

Transmission electron microscopy revealed that the morphological features of φKSM96 consist of a head and tail, with a head diameter of approximately 50–60 nm, a long tail (1 μm), and a short tail fibrous structure at the tip, similar to the typical Siphoviridae morphology (Figure 1B).

Complete genome sequence of the bacteriophage from KSM96

The complete sequence of the strain KSM96 revealed that the bacteriophage DNA of KSM96 inserted into the chromosome at the region between *comGB* (*comYB* in *S. mutans* UA159) and *comGC* (*comYC* in *S. mutans* UA159), which encode the competence type IV pilus assembly protein ComGB and the competence type IV pilus major pillin ComGC, respectively (Figure S1).

The bacteriophage DNA of KSM96 was circular with a length of 39,820 bp and a G + C content of 35% (Figure 2). The nucleotide sequence of this bacteriophage genome was subjected to a BLASTn search, and no similar sequences were found in the database. Therefore, this bacteriophage was designated φKSM96, and its complete sequence was deposited in GenBank under accession number GeneBank: OQ627164. Forty-eight coding sequences (CDSs) were identified in the φKSM96 genome, and the predicted function of each CDS is summarized in Table S1. Fourteen of the CDSs encoded hypothetical proteins, and the remaining 34 CDSs were assigned to different functional groups, including replication, packaging, head morphogenesis, tail morphogenesis, host lysis, and lysogenic conversion. A potential *ori* region

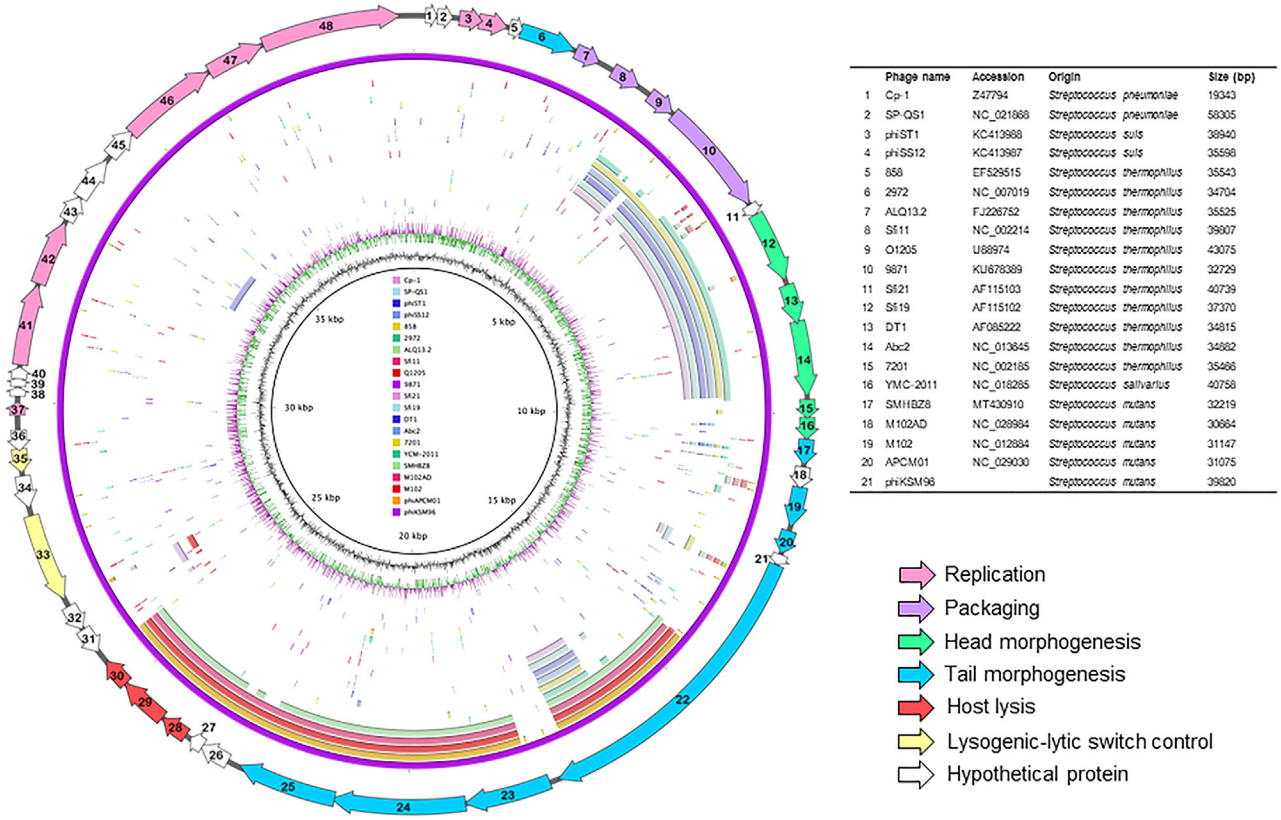

Figure 2. Genetic map of φKSM96 and its comparison with other streptococcal bacteriophages

ORFs are shown as arrows, indicating the orientation of transcription. The arrow numbers indicate the ORF number displayed in Table S1. Colors indicate the classification of gene function. The φKSM96 sequences were aligned and compared to 20 streptococcal bacteriophage genome sequences. See also Figure S1.

was identified using Ori-Finder. This region (nucleotides 39,624 to 229) is located between CDS No. 1 (encoding a hypothetical protein) and CDS No. 48 (encoding an AAA family ATPase), has an AT content of 68%, and contains 2 DnaA boxes.

Comparison of φKSM96 with other bacteriophages

Comparison of the φKSM96 genome with other streptococcal bacteriophages indicated that φKSM96 carried some CDSs for host lysis and tail morphogenesis similar to those from 4 *S. mutans* bacteriophages, M102AD, M102, SMHBZ8, and φAPCM01, while some CDSs for head morphogenesis and packaging were similar to those from *Streptococcus salivarius* bacteriophage YMC-2011 (Figure 3A). φKSM96 had less than 25% sequence identity to M102AD, a previously reported *S. mutans* virulent phage DNA (Figure 3A). However, some restricted regions, including tail morphogenesis and host lysis, showed 86% similarity with those of M102AD. Additionally, the region of head morphogenesis and packaging in φKSM96 showed similarity with those of YMC-2011, an *S. salivarius* temperate phage. Two temperate bacteriophages, φKSM96 and YMC-2011, contained genes responsible for lysogenic conversion, while virulent bacteriophage M102AD did not contain these genes. Figure 3B shows the phylogenetic tree analysis among 21 streptococcal bacteriophages, including five *S. mutans* phages (φKSM96, M102AD, M102, SMHBZ8, and phiAPCM01) and sixteen phages infecting *Streptococcus suis*, *Streptococcus salivarius*, *Streptococcus thermophilus*, and *Streptococcus pneumoniae*. The φKSM96 genome was more closely related to some *S. mutans* phages than to other *Streptococcus* species-infecting phages. Among the five *S. mutans* phages, the four virulent phages M102AD, M102, SMHBZ8, and phiAPCM01 were close together and relatively distant from the temperate phage φKSM96.

Susceptibility evaluation of various bacterial species to φKSM96

To investigate the specificity of φKSM96 against *S. mutans* strains and other bacterial species, we evaluated the antibacterial activity against each strain. Among 125 *S. mutans* isolates, 113 isolates showed susceptibility to φKSM96 (90.4%), while 12 strains (9.6%) were insensitive to φKSM96 (Table 1). *S. mutans* KSM96 showed low susceptibility to φKSM96 and retained the entire genome of φKSM96. We also investigated the relationship between the susceptibility of each isolate with different serotypes (*c*, *e*, *k*, and *f*) and found that 12 φKSM96-insensitive isolates were serotypes *c* (3/105 strains), *e* (3/11 strains) *k* (4/4 strains) and *f* (2/2 strains). Then, we investigated the susceptibility of other oral streptococci and *L. lactis* to φKSM96 and found that they were insensitive to φKSM96.

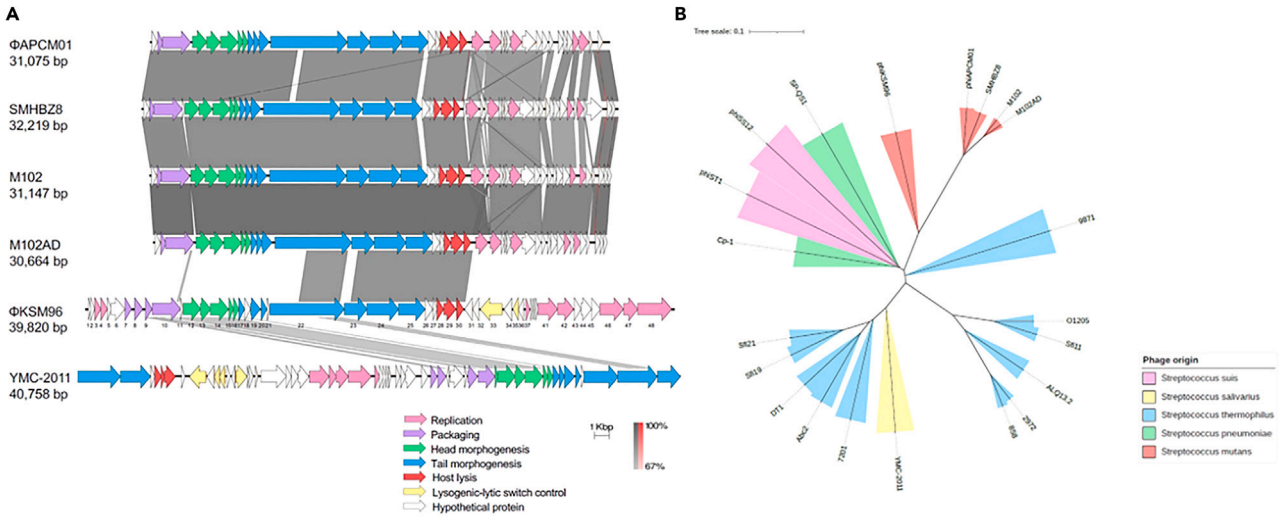

Figure 3. Comparison of φKSM96 with other bacteriophages

(A) The φKSM96 sequence was compared with the sequences of 4 *S. mutans* virulent bacteriophages (M102AD, M102, SMHBZ8, and φAPCM01) and one *S. salivarius* temperate bacteriophage (YMC-2011).

(B) A phylogenetic tree was constructed with 21 bacteriophage genome sequences, including φKSM96 determined in this study, and 20 sequences obtained from the NCBI database. See also Figures S1 and S4.

Inhibition of *S. mutans* growth by φKSM96

To investigate the effect of φKSM96 on *S. mutans* growth, various concentrations of φKSM96 were added to culture with several *S. mutans* isolates (10^7 cells/5 mL trypticase soy broth [TSB]) followed by monitoring the optical density (OD) (Figure 4A). The growth of highly susceptible strains (KSM193 and KSM17) was strongly inhibited by the addition of φKSM96 (2×10^{10} particles) and partially inhibited by 2×10^{10} particles of φKSM96, while the growth of an intermediate susceptible strain (KSM96) was partially inhibited by the addition of φKSM96 (2×10^{10} particles). The φKSM96-insensitive strain, KSM56, showed no growth inhibition by addition of φKSM96. Then, we investigated the growth inhibition by 2×10^{10} φKSM96 particles on 16 *S. mutans* isolates (Table S2). The growth of all isolates was inhibited by the addition of φKSM96, although the inhibition rate was different among strains. We also investigated the inhibitory effect of φKSM96 on growing *S. mutans* bacterial cells. φKSM96 (2×10^{10} particles) was added to KSM193 culture with OD_{660nm} = 0.2, and the OD was monitored during growth. Growth inhibition was strongly observed (Figure 4B). In addition, we performed a one-step growth curve assay and found that bacteriophage particles increased twice after 3 h compared to that of 0 time (Figure S2).

Stability against temperature, pH, and disinfectant of φKSM96

The thermal stability of φKSM96 was shown in Figure 5. Bacteriophage particles were inactivated above 50°C for 1 h. In pH stability test, bacteriophage particles treated with pH 3.0 and 9.0 for 1 h did not form lysis zone, while lytic activity of the particles in pH 4.0–6.0 and

Table 1. Susceptibility of φKSM96 against oral bacteria

Strains	Number of strains	Number of susceptible strains (% ratio)
Streptococcus mutans		
Serotype		
c	105	102 (97.1%)
e	14	11 (78.6%)
k	4	0 (0%)
f	2	0 (0%)
Streptococcus gordonii JCM1299	1	0 (0%)
Streptococcus mitis GTC495	1	0 (0%)
Streptococcus sanguinis GTC217	1	0 (0%)
Streptococcus salivarius GTC21	1	0 (0%)
Streptococcus.oralis ATCC1055	1	0 (0%)
Lactococcus lactis ATCC11454	1	0 (0%)

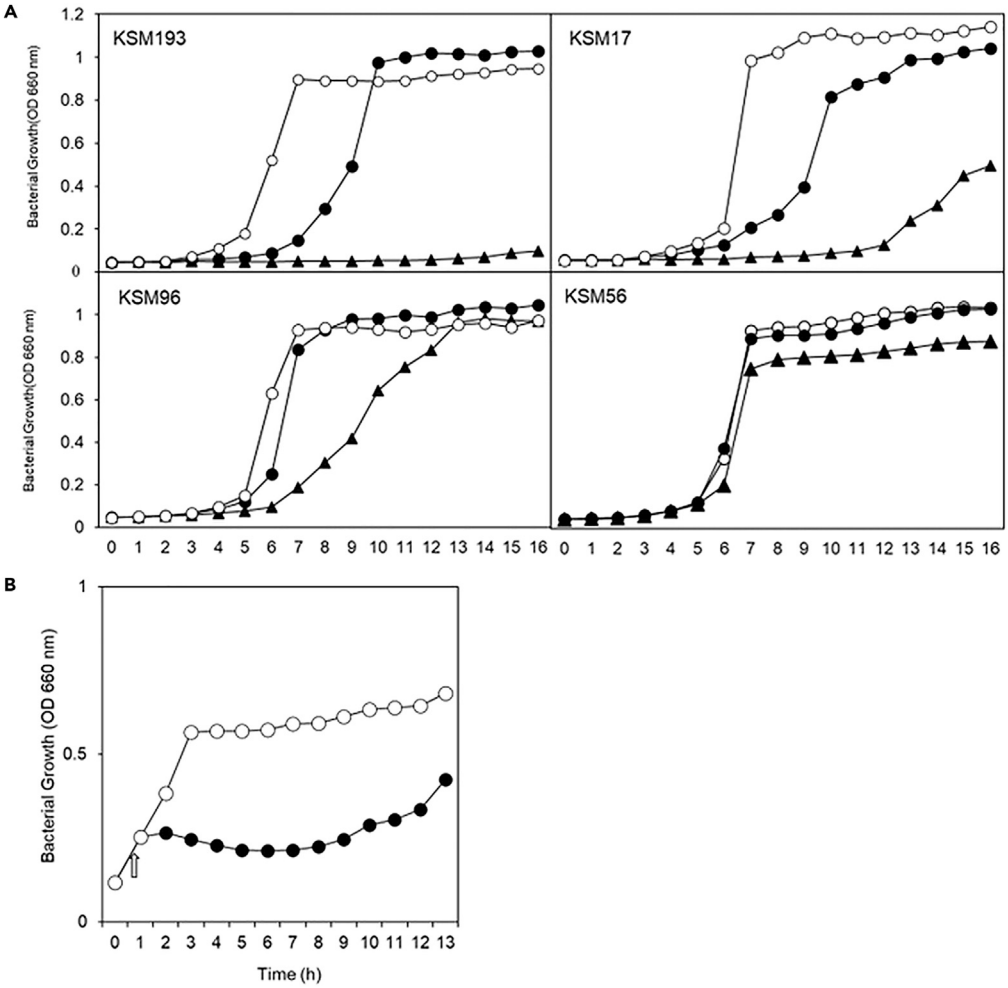

Figure 4. Effect of φKSM96 on *S mutans* growth

(A) *S. mutans* (1.0 × 10^7 CFU/mL) was incubated in TSB with φKSM96 (closed circle: 2.0×10^9 particles/ml, closed triangle: 2.0×10^{10} particles/ml) or without φKSM96 (open circle) at 37°C under 5% CO_2. The optical density at 660 nm was monitored. Three independent experiments were performed, and the mean was calculated.

(B) *S. mutans* was grown in TSB at 37°C under 5% CO_2. When *S. mutans* grew to the exponential phase (optical density at 660 nm = 0.25), φKSM96 (2.0×10^9 particles/ml) was added, and the optical density was monitored. Three independent experiments were performed, and the mean was calculated. Closed circle, φKSM96 2.0×10^{10} particles/ml; open circle, without φKSM96.

See also Figure S2.

pH 8.0 was decreased compared to that of pH 7.0. In disinfectant stability assay, φKSM96 treated with 70% ethanol did not show the lysis zone.

Inhibition of *S. mutans* biofilm by φKSM96

The inhibitory effect of φKSM96 on *S. mutans* biofilm formation was observed (Figure 6). After inoculation of *S. mutans* KSM197 (10^7 cells) into the well, biofilm formation was suppressed by the addition of φKSM96, showing 30% (2 × 10^9 particles) and 80% (2 × 10^{10} particles) suppression. After inoculation of *S. mutans* (10^5 cells) into the well, biofilm formation was suppressed by the addition of φKSM96, showing 90% (2 × 10^9 particles) and 95% (2 × 10^{10} particles) suppression. However, a biofilm inhibitory effect was not observed by the addition of 2 × 10^8 φKSM96 particles under both conditions with 8 *S. mutans* isolates. An inhibitory effect of φKSM96 was observed in all isolates (Table S3).

Effect of φKSM96 on *S. mutans* growth in cocultures with other bacterial species

We first examined the effect of φKSM96 on *S. mutans* growth when grown in coculture with another strain (Figure S3). Before incubation, equal amounts of bacterial cells (*S. mutans* and other species) were added to the medium. During coculture with *L. lactis*, the proportions of

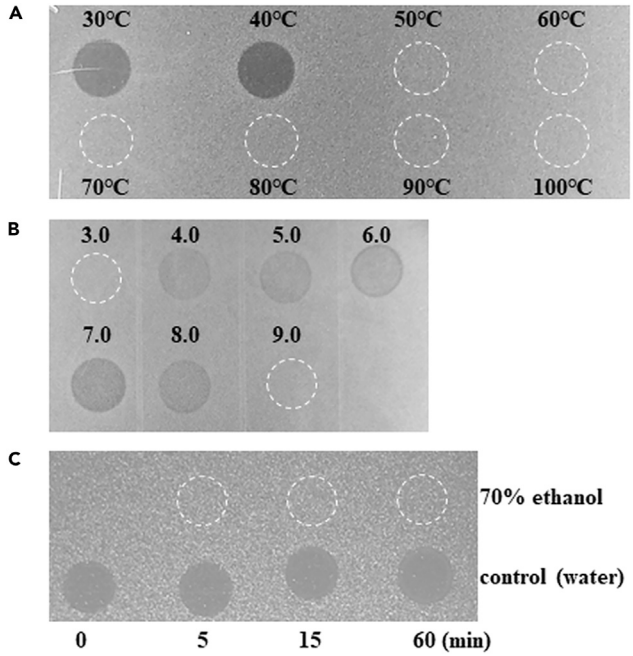

Figure 5. Temperature, pH and disinfectant stability of φKSM96

(A) For temperature stability test, 5 μL φKSM96 (1× 10^{10} particles) was mixed with 45 μL of TSB and incubated at various temperatures for 1 h. Overnight culture of KSM193 was diluted 10-fold with TSB, and 100 μL of the diluted solution was spread on TSA. Each bacteriophage solution (5 μL) was spotted on TSA and incubated overnight at 37°C with 5% CO2.

(B) For pH stability test, 5 μL φKSM96 (1× 10^{10} particles) was mixed with 45 μL of TSB at different pH and incubated at room temperature for 1 h. After neutralization, the bacteriophage solution was spotted on *S. mutans*-inoculated TSA as described previously.

(C) For disinfectant stability test, 70% ethanol was used. Five microliter of φKSM96 (1× 10^{10} particles) was mixed with 45 μL of 70% ethanol or distilled water, then incubated at room temperature for 1 h. After 10-fold dilution with distilled water, 5 μL of the bacteriophage solution was spotted on *S. mutans*-inoculated TSA as described previously.

S. mutans with or without φKSM96 were 7% and 0.5%, respectively. During coculture with *S. sanguinis*, the proportions of *S. mutans* with or without φKSM96 were 10% and 0.8%, respectively. A decrease in the *S. mutans* proportion was also observed by the addition φKSM96 in co-cultures with *S. salivarius*, *S. gordonii*, and *S. mitis*.

Then, we investigated the effect of φKSM96 on *S. mutans* growth in cocultures with 4 bacterial species (*S. mutans*, *S. sanguinis*, *S. salivarius*, and *S. mitis*) (Figure 7). The proportions of *S. mutans* KSM29 or KSM83 were significantly decreased by the addition of φKSM96, giving values

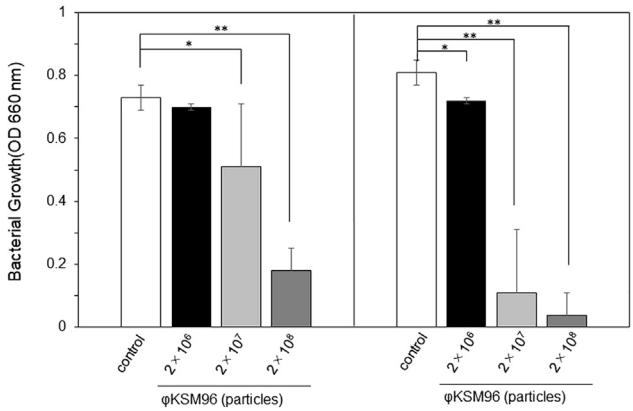

Figure 6. Effect of φKSM96 on *S mutans* biofilm formation

S. mutans (left, 10^7 cells/ml; right, 10^5 cells/ml) was grown in TSB containing 2% sucrose with or without various concentrations of φKSM96. After 24 h of incubation, biofilm cells were stained with 0.1% crystal violet. Three independent experiments were performed, and the mean ± SD was calculated. The data were analyzed for statistically significant differences compared to the control by one-way ANOVA followed by Dunnett's post hoc test. *, $p < 0$ 0.05; **, $p < 0.01$.

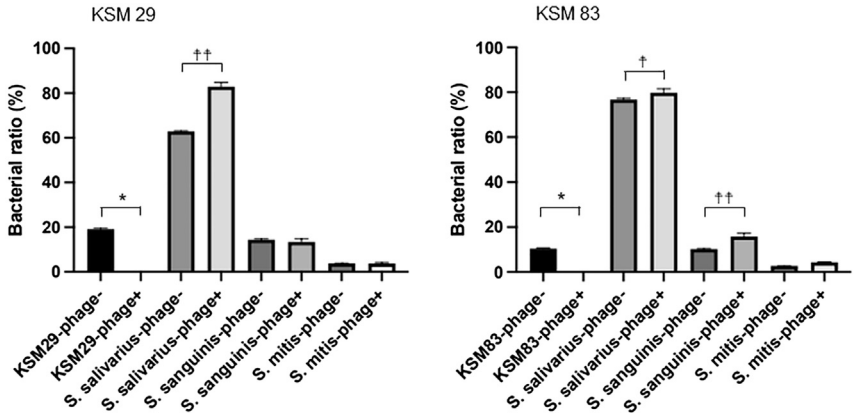

Figure 7. Effect of φKSM96 on *S mutans* proportion among 4 bacterial species co-cultured

Coculture assays were performed according to the method described in the experimental model and subject details. Three independent experiments were performed, and the mean ± SD was calculated. Post hoc multiple comparisons between the groups without and with phage in each bacterium were performed by using Tukey's test. *, $p < 0.0001$, decreasing compared to without phage; †, $p < 0.005$, increasing compared to without phage; ††, $p < 0.0001$, increasing compared to without phage. See also Figure S3.

of 19.2% or 10.4% without φKSM96 and 0.06% or 0.1% with φKSM96. In co-culturing with KSM29, the proportions of *S. sanguinis* and *S. mitis* were not changed, while the proportion of *S. salivarius* was increased in the presence of φKSM96 compared to the absence of φKSM96. In co-culturing with KSM83, the proportion of *S. mitis* was not changed, while the proportions of *S. salivarius* and *S. sanguinis* were increased in the presence of φKSM96 compared to the absence of φKSM96.

DISCUSSION

In this study, we firstly isolated a temperate bacteriophage from *S. mutans*, which showed the broad bactericidal activity against *S. mutans* isolates. To date, 6 lytic bacteriophages, M102, M102AD, φAPCM01, SMHBZ8, e10, and f1, have been isolated from human saliva,[22–26] although genome analysis of e10 and f1 has not been clarified. There are no reports regarding temperate bacteriophage isolation, although three intact prophages were identified in the *S. mutans* genome.[27] We showed that φKSM96 had a wide range of activity among different *S. mutans* strains, while other lytic bacteriophages had a narrow host range. It was demonstrated that 1 of 17, 1 of 25, and 13 of 17 the tested strains were susceptible to the bacteriophages ΦAPCM01, M102AD, and SMHBZ8, respectively.[23–26] Considering the infection capability of bacteriophages, phage-host interactions play a key role in infection. Infection with bacteriophage M102 or M102AD was reported to be specific to serotype *c* antigen.[23,24] Mutations in *rgpA*, *rgpB*, or *rgpG*, which are important for the synthesis of serotype antigens, resulted in loss of infection by phage M102.[28] SMHBZ8 also showed activity against *S. mutans* strains with serotype *c* antigen.[26] ΦAPCM01 only infected one serotype *e* strain among 17 strains, including 13 serotype *c* and 4 serotype *e* strains,[25] suggesting that bacteriophage absorption is not always defined by serotype antigen. In gram-positive bacteria, sugars of wall teichoic acids/lipoteichoic acid and capsular polysaccharide were reported as receptors for bacteriophage.[29] *S. mutans* serotype *c* and *e* strains were susceptible to φKSM96 but not serotype *k*. Therefore, it is considered that the receptor for φKSM96 might be a well-conserved sugar moiety among *S. mutans* strains with different serotypes. φKSM96 did not produce a lytic plaque similar to other virulent bacteriophages, and 100-fold higher concentrations of bacteriophage particles against *S. mutans* cells were required to inhibit *S. mutans* growth. Since virulent bacteriophage induces bacterial lysis through releasing particles after amplification, the bactericidal effect of this temperate bacteriophage, φKSM96, was not same with that of virulent bacteriophage. Also, in the growth experiment (Figure 4B), the addition of φKSM96 did not cause strong lysis. Furthermore, in one-step growth curve assay, it was demonstrated that the number of virulent phages was significantly increased (10 to 100-fold increase) for 1 h.[30,31] However, the number of φKSM96 particles was not increased significantly (1.3-fold increase for 1 h). Based on these results, it is speculated that φKSM96 causes bactericidal effects via endolysin or holin after absorption into bacterial cells.

In the growth experiment, *S. mutans* strains could grow after the suppression of growth by adding φKSM96. To investigate whether bacteriophage integration was generated, the appropriate dilutions of the re-growing culture were inoculated on TSA. Then, we pick up 16 colonies and investigated the bacteriophage integration into chromosome and the susceptibility of φKSM96 against these strains. Finally, we found these strains had no bacteriophage-integration and showed the susceptibility against φKSM96 (data not shown). Therefore, the efficiency of bacteriophage-integration seems to be low, so we consider that the resistance to this bacteriophage is unlikely to occur.

The genome size of φKSM96 is 39,820 bp with a total of 48 CDSs identified, a longer genome length than those of previously reported phage genomes: 31,147 bp for M102,[23] 30,664 bp for M102AD,[24] 31,075 bp for ΦAPCM01,[25] and 32,460 bp for SMHBZ8.[26] Since φKSM96 is a temperate phage, φKSM96 possesses the factors responsible for lysogenic-lytic conversion. Phylogenetic tree analysis revealed that φKSM96 did not show high similarity with the other 5 *S. mutans* bacteriophages. Only some CDSs responsible for host lysis and tail morphogenesis were similar to those of the other 4 *S. mutans* bacteriophages, and some CDSs responsible for packaging and head morphogenesis

were similar to those of *S. salivarius* and *S. thermophilus* bacteriophages. In the 5 *S. mutans* bacteriophage genomes, including φKSM96, two predicted genes responsible for endolysin (peptidoglycan hydrolase) were identified. Bacteriophage-related peptidoglycan hydrolases are considered to be associated with the penetration of nucleic acids into bacterial cells for infection and degradation the cell wall for bacteriophage release.[32,33] Since the peptidoglycan structure is generally conserved among bacterial species, it is reasonable that the peptidoglycan hydrolases in the 5 *S. mutans* bacteriophages showed high similarity (Figure S4). However, there are some differences in amino acid sequences among the 5 bacteriophages, suggesting that these differences may affect their enzymatic activity, which alters their infectious activity against *S. mutans*.

In this study, we clearly demonstrated the inhibitory effect this phage has on bacterial growth under both planktonic and biofilm conditions. Biofilms (dental plaque) cause tooth decay and periodontal diseases. To prevent oral diseases, inhibition of biofilm formation would be effective.[34,35] However, biofilms are formed by water-insoluble extracellular polysaccharides, causing resistance to immune factors, antibacterial agents and disinfectants. Several attempts to use bacteriophages for *S. mutans* biofilms have been investigated, and an inhibitory effect was found *in vitro*.[25,26] Therefore, bacteriophages are good candidates for the prevention of biofilm formation. In addition, the lytic efficacy of SMHBZ8 against *S. mutans* was demonstrated in a caries model using human dentin samples and extracted jaw mice.[26,36] Although the effect of *S. mutans* bacteriophages in *in vivo* model is not demonstrated, *S. mutans* bacteriophage is a possible candidate for the prevention of dental caries. Compared to virulent phage, temperate phage is easy to do genetic manipulation for making more efficient bacteriophage, because genetic modification on chromosomal DNA can be performed.[20] Several attempts using temperate bacteriophage have been conducted. In *Enterococcus faecalis* and *Listeria monocytogenes*, improving lytic activity of temperate bacteriophage by gene engineering was demonstrated.[37,38] In addition, it was demonstrated that a bacteriophage cocktail containing several temperate bacteriophages was effective against *Clostridioides difficile*.[39,40] In this point, it is very meaningful that we could isolate *S. mutans* which harbored the genes for the intact temperate bacteriophage.

We also attempted to determine the effect of φKSM96 on the proportion of *S. mutans* when cocultured with other oral *Streptococcus* species. Since *S. mutans* is colocalized with many bacterial species in the oral cavity, it is worth determining whether this phage specifically acts on *S. mutans* under coculture conditions. Even in cocultures with 4 bacterial species, φKSM96 specifically inhibited *S. mutans* growth. Therefore, φKSM96 did not adsorb to the cell surface of the other bacterial species. Such specificity implies that φKSM96 application to the oral cavity does not affect the composition of oral bacterial flora except *S. mutans*.

In conclusion, we identified an *S. mutans* temperate bacteriophage that showed broad-range susceptibility among *S. mutans* strains. Although the mechanism for the broad activity of φKSM96 remains unknown, further analysis may lead to the development of an agent for the elimination of *S. mutans* in the oral cavity.

Limitations of the study

φKSM96 isolated in this study had a broad spectrum to *S. mutans* clinical isolates but did not show antibacterial effect to some isolates with different serotypes. We have proposed the killing mechanism of this bacteriophage by endolysin and holin, yet its precise killing mechanism remains unknown. Since the high number of φKSM96 particles were required to have a killing effect compared to virulent bacteriophages, genetic modification of φKSM96 may be required to increase its killing activity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108465.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.K., M.K.-M., H.I., and K.T.; methodology, M.K.-M, M.L., K.S., J.H., and J.F.; genome studies, J.H., M.L., and M.S.; data analysis, K.P., M.K.-M, M.L., K.S., J.H., H.I., and J.F.; data interpretation, all authors; manuscript writing – original draft and figures, H.K., K.S., M.L., J.F., and M.K.-M.; manuscript review and editing, all authors. All authors have read and approved the final draft of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLES

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Streptococcus mutans UA159	Murchison et al.[41]	N/A
Streptococcus mutans KSM96	Watanabe et al.[42]	N/A
Streptococcus mutans KSM strains	Watanabe et al.[42]	N/A
Streptococcus gordonii JCM1299	Japan collection of Microorganism (JCM)	JCM
Streptococcus mitis GTC495	Gifu University Collection (GTC)	GTC
Streptococcus sanguinis GTC217	GTC	GTC
Streptococcus salivarius GTC21	GTC	GTC
Streptococcus.oralis ATCC1055	American Type Culture Collection (ATCC)	ATCC
Lactococcus lactis ATCC11454	ATCC	ATCC
Chemicals, peptides, and recombinant proteins		
trypticase soy broth	Becton, Dickinson and Company	211825
mitomycin C	Fujifilm Wako Pure Chemicals	133–15931
Triton X-100	Katayama Chemicals	30–5140
EDTA	Wako Pure Chemicals	345–01865
Tris-HCl	Nacalai tesque	35434–21
Agar for bacteria	NISSUI	05835
MgSO4	Katayama Chemicals	19–0480
NaCl	Fujifilm Wako Pure Chemicals	191–01665
gelatin	Wako Pure Chemicals	7–18
Critical commercial assays		
FS Essential DNA Green Master	Roche	215392
Phage DNA isolation kit	Norgen	46800
Deposited data		
phage genome data	This paper	GenBank: OQ627164
Oligonucleotides		
See Table S1	FASMAC	N/A
Software and algorithms		
PHASTER		https://phaster.ca/
shovill v1.0.9 pipeline	Torsten Seemann	https://github.com/tseemann/shovill
SnapGene	GSL Biotech LLC	
CSI Phylogeny 1.4 pipeline	Center for Genomic Epidemiology (Lungby, Denmark)	https://www.genomicepidemiology.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be answered by the lead contact, Hitoshi Komatsuzawa (komatsuz@hiroshima-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Sequences of φKSM96 generated in this study have been deposited to NCBI database under accession number OQ627164 and are publicly available as of the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table S4. The *S. mutans* clinical isolates were obtained previously.[42] Oral streptococcus strains were cultured in TSB (Becton, Dickinson and Company, New Jersey, USA) at 37°C under 5% CO_2. *Staphylococcus aureus* was grown aerobically in TSB at 37°C.

METHOD DETAILS

Identification of the bacteriophage genome from *S. mutans* strains

Previously, we obtained the whole genome data of 125 clinical *S. mutans* isolates.[42] Using the genome data of these 125 isolates, we searched the predicted bacteriophage genome with PHASTER (https://phaster.ca/).

Isolation of a temperate bacteriophage from *S. mutans* KSM96

A small portion (200 μL) of an overnight culture of *S. mutans* KSM96 grown in 5 mL of TSB was inoculated into 20 mL of fresh TSB and cultured for 5 h at 37°C under 5% CO_2. When the turbidity at 660 nm reached 0.2 to 0.3, mitomycin C (Fujifilm Wako Pure Chemicals) (final concentration: 0.1 μg/mL) was added to the culture medium. After 16 h of incubation, the culture medium was centrifuged (5,000 × g, 15 min, 4°C) with a centrifuge (Allegra X-30R, Beckman Coulter). The supernatant was filtered through a filter membrane (DISMIC-25CS Φ25 mm, successor: 0.45 μm, Toyo Roshi Kaisha, Ltd). Using a high-speed centrifuge (Optima (trademark) TL, Beckman Company), and the filtrate was centrifuged at 50,000 ×g for 2 h at 4°C. The precipitate was suspended in 50 μL of TSB. To determine the lytic activity of the bacteriophage, we used the following method. Overnight cultures of *S. mutans* UA159 (100 μL) were inoculated on TSA. Then, 2 μL of bacteriophage solution was spotted on TSA plates. After overnight incubation, the lysis zone was observed.

To evaluate the particle numbers in the bacteriophage solution, quantitative PCR was used. Since KSM96 carried the φKSM96 genome (one copy), we used the chromosomal DNA of KSM96 to generate a calibration curve with the number of φKSM96. Overnight culture of KSM96 was adjusted to an OD_{660nm} of 1.0 (10^9 cells/ml). One milliliter of bacterial suspension (10^9 cells) was centrifuged at 10,000 × g for 5 min, and then bacterial cells were suspended in 1 mL of lysis buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0]). After incubation at 95°C for 5 min, the bacterial suspension was centrifuged at 10,000 × g for 10 min, and the supernatant was used as the control (10^9 copies of bacteriophage genome/ml). Using various amounts of the bacteriophage genome, quantitative PCR using specific primers was performed to construct a calibration curve. Various 10-fold dilutions of bacteriophage solution (10 μL) were mixed with lysis buffer (90 μL) and heated at 95°C for 15 min. Then, quantitative PCR was performed using a LightCycler 96 system (Roche). The reagent was FS Essential DNA Green Master (Roche), and the specific primers are shown in Table S5. The Ct values calculated by quantitative PCR using each DNA dilution were used to create a calibration curve of the number of bacteria and Ct values.

Susceptibility testing of various bacterial species to φKSM96

To determine the susceptibility of *S. mutans* clinical isolates and other streptococcal species to φKSM96, overnight cultures of various bacteria were diluted 10-fold with TSB, and 100 μL of the diluted solution was spread on TSA. The bacteriophage solution (5 μL; 2 ×10^8 cells) was dropped onto TSA and incubated overnight at 37°C under the appropriate conditions. The zone of inhibition was measured.

To determine the susceptibility of *S. mutans* KSM193 to various particle numbers of φKSM96, 5 μL of bacteriophage solution with various particle numbers was spotted on TSA covered with 3.5 mL of soft agar (0.75% TSA) containing *S. mutans* cells (10^8 cells). After 24 h of incubation at 37°C, the zone of inhibition was measured.

Electron microscopy observations of φKSM96

The phage suspensions obtained was dropped onto an ester support membrane (EM Japan, U1009; https://www.em-japan.com/supportfilm.html) and adsorbed. The support membrane was washed twice with SM buffer (10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin, 50 mM Tris-HCl [pH 7.5]). After staining the support films with 2% uranyl acetate, the samples were observed with a transmission electron microscope (75 kV; Hitachi HT7700).

Determination and analysis of the whole φKSM96 genome sequence

Bacteriophage DNA was extracted from the phage particles using the Phage DNA isolation kit (Norgen). Extracted DNA was used to prepare DNA libraries for sequencing using enzymatic pretreatment reagents. Shotgun sequencing was performed using MiSeq (Illumina) and HiSeq X-Five (Illumina), and raw data (FASTQ files) were obtained. The Illumina reads were assembled using the shovill v1.0.9 pipeline

(https://github.com/tseemann/shovill) to obtain draft genome sequences (FASTA files). The FASTA files were used to search for genes using the phage analysis software PHASTER and protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Gene maps were created using SnapGene (GSL Biotech LLC). The DNA sequence of φKSM96 was subjected to homology analysis with other bacteriophage DNAs using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree analysis was performed with the CSI Phylogeny 1.4 pipeline available from the Center for Genomic Epidemiology (Lungby, Denmark) (https://www.genomicepidemiology.org/) for SNP calling and then annotated using the iTOL web-based tool (https://itol.embl.de). The tree was constructed to scale, with branch lengths of the same units as those of the evolutionary distances used to infer the phylogenetic tree. Amino acid alignments were performed by SnapGene software (GSL Biotech LLC).

One-step growth curve assay

The one-step growth curve assay of φKSM96 was performed with the method described elsewhere.[43] φKSM96 (2×10^{10} particles) was added to 2 mL of *S. mutans* KSM193 at exponential phase (OD660 nm of 0.2) and incubated for 30 min at 37°C with 5% CO_2. Then, bacterial cells were collected by the centrifugation at 10,000xg for 1 min. The bacterial cells were resuspended in 2 mL of fresh prewarmed TSB and incubated at 37°C with 5% CO_2. After 15, 30, 60, 120, 180 min, 100 μL of the culture was centrifuged and the supernatant was heated for 5 min at 95°C. Then, the number of bacteriophage particles was calculated by quantitative PCR using specific primers with the method described above. Three independent experiments were performed, and the mean value was calculated.

Temperature, pH and disinfectant stability tests

For temperature stability test, 5 μL φKSM96 (1×10^{10} particles) was mixed with 45 μL TSB and incubated at various temperature (40, 50, 60, 70, 80, 100°C) for 1 h. Overnight culture of KSM193 was diluted 10-fold with TSB, and 100 μL of the diluted solution was spread on TSA. Each bacteriophage solution (5 μL) was spotted on TSA and incubated overnight at 37°C with 5% CO_2.

For pH stability test, 5 μL φKSM96 (1×10^{10} particles) was mixed with 45 μL of TSB at different pH (3, 4, 5, 6, 7, 8, 9), which was adjusted using either 1M HCl or 1M NaOH, and incubated at room temperature for 1 h. After neutralizing with appropriate volume of 1M Tris-HCl (pH 7.5) or 3M sodium acetate (pH 5.2), the bacteriophage solution was spotted on *S. mutans*-inoculated TSA as described above.

For disinfectant stability test, 70% ethanol was used. Five microliters of φKSM96 (1×10^{10} particles) was mixed with 45 μL of 70% ethanol or distilled water, then incubated at room temperature for 1 h. Five microliter of the bacteriophage solution was spotted on *S. mutans*-inoculated TSA as described above.

Effect of φKSM96 on *S. mutans* growth

Two experiments were performed to evaluate growth inhibition by φKSM96. Overnight cultures of clinical isolates of *S. mutans* (highly susceptible strains: KSM193, KSM17, the non-susceptible strain KSM56, and the bacteriophage-producing strain KSM96) were adjusted to an OD_{660nm} of 1.0 with TSB. Ten microliters of each solution (10^7 CFU/ml) was inoculated into 80 μL of TSB. Then, 10 μL of bacteriophage (2×10^8, 2×10^9, 2×10^{10} particles) was added to the bacterial culture at the same time as inoculation of the bacteria. The optical density at 660 nm was monitored with a microplate reader (iMark (Trademark), Bio-Rad) over time during incubation (KSM193, KSM17, KSM56, KSM96). Three independent experiments were performed, and the mean value was calculated. For the other strains, the OD was measured at 6 and 12 h after inoculation, and the percentage of growth inhibition was calculated. Three independent experiments were performed, and the mean ± SD was calculated.

In another experiment, the bacteriophage was inoculated after the *S. mutans* KSM193 culture reached an OD660 nm of 0.2. Bacteriophage (2×10^{10} particles) was added to the bacterial culture at an OD660 nm of 0.2. The optical density at 660 nm was monitored over time during incubation. Three independent experiments were performed, and the mean value was calculated.

Effect of φKSM96 on *S. mutans* biofilm formation

The overnight culture was adjusted to an OD660 nm of 1.0 (10^9 cells/mL) with TSB, and the bacterial culture was diluted 100-fold (10^7 cells/mL). Bacterial cells (10^5 or 10^7 cells in 10 μL) were inoculated into 90 μL of TSB containing 2% sucrose in a 96-well plate (Nunc.). Then, 10 μL of various concentrations of bacteriophage (2×10^6, 2×10^7, 2×10^8 particles) was added to each well. After incubation for 16 h at 37°C with 5% CO_2, the medium was removed, and the contents of each well were washed three times with buffered saline. Then, 100 μL of 0.1% crystal violet solution was added to each well and allowed to settle at room temperature for 15 min. The crystal violet solution was then removed, and the contents of each well were washed three times with buffered saline. The absorbance at 595 nm was measured with a microplate reader (Bio-Rad). Three independent experiments were performed, and the mean ± SD was calculated. The data were analyzed for statistically significant differences compared to the control by two-way ANOVA followed by Dunnett's post hoc test. *$p < 0.05$ and **$p < 0.0001$.

Effect of φKSM96 during coculture of *S. mutans* with other bacterial species

Before the coculture assay, a calibration curve was constructed for each species according to the method described above. The specific primers for each bacterial species are listed in Table S5. Overnight cultures of *S. mutans* (highly susceptible strains KSM193, KSM83, KSM29) and other bacterial species were adjusted to an OD660 nm of 1.0 with TSB (10^9/mL). Then, each bacterial suspension was subjected to 100-fold dilution (10^7 cells/mL) with TSB. Ten microliters (10^5 cells) of each *S. mutans* solution and the other species (one or three species)

were added to 500 μL of TSB. Then, bacteriophage (20 μL; 2×10^{10} particles) was added to the mixed bacterial medium. After incubation at 37°C with 5% CO_2 for 8 h, bacterial cells were collected by centrifugation (5,000×g, 15 min, 4°C). DNA extraction and quantitative PCR were performed according to the method described above. The Ct value of each species was calculated by quantitative PCR using species-specific primers. Using a calibration curve, the number of bacteria in each sample was calculated, and the proportion of each bacterium was calculated. Three independent experiments were performed, and the mean ± SD was calculated. The statistically significant differences were analyzed by Tukey's multiple comparisons test. *, $p < 0.0001$, decreasing compared to without phage; †, $p < 0.005$, increasing compared to without phage; ††, $p < 0.0001$, increasing compared to without phage.

QUANTIFICATION AND STATISTICAL ANALYSIS

Two-way ANOVA followed by Dunnett's post hoc test for biofilm assay ($p < 0.05$ and $p < 0.0001$) and Tukey's multiple comparisons test for coculture assay ($p < 0.005$, $p < 0.0001$) was performed using GraphPad Prism version 10.1.0 to compare statistical significance. Three independent experiments were performed.