Selective activities of STAMPs against *Streptococcus mutans*

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Abstract. The present study aimed to design, synthesize and screen specifically targeted antimicrobial peptides (STAMPs) that can selectively kill *Streptococcus mutans* (*S. mutans*) in the biofilm, and to detect protein metabolism, in order to investigate the mechanism of the antibacterial functions of STAMPs against *S. mutans*. A series of STAMPs were synthesized, and their effects on the selective antibacterial activity of *S. mutans* on single species and multi-species biofilms under the condition of the planktonic state were studied. The total protein of *S. mutans* was extracted before and after C11H, and matrix-assisted laser adsorption ionization-time of flight mass spectrometry identification was performed. The antibacterial activity on planktonic *S. mutans* was increased 3- to 4-fold via C8H, C11H, C12H, C13H, and C14H compared with hLF1-11 (H) alone, and there was no difference between *Streptococcus gordonii* (*S. gordonii*) and *Streptococcus sanguis* (*S. sanguis*). C8H, C11H, C12H, C13H, and C14H had significant inhibitory effects on the growth of *S. mutans* biofilm, but there were no significant effects on *S. gordonii* and *S. sanguis* biofilms. The number of *S. mutans* in biofilm decreased at 4 h after C8H, C11H, C12H, C13H and C14H and C8, C11, C12, C13 and C14 had no effect on the growth of planktonic and biofilm states of *S. mutans*, *S. gordonii* and *S. sanguis* species. C11H and C12H exhibited the most obvious effects, followed by C13H and C14H, and then C8H. A total of 21 protein spots with a mean change ratio of 1.5 were identified, all of which were downregulated after C11H. A total of 19 proteins were successfully identified, including cell cycle-relative proteins, nucleic acid metabolism-related enzymes and proteins, virulence factors, protein biosynthesis and regulation, proteins involved in energy metabolism, and proteins with unknown function. In the present study, STAMPs with selective antibacterial activity against *S. mutans* grown in planktonic or biofilm states but without obvious effects on oral *Streptococci* and multi-species biofilm were successfully designed and synthesized. Differential protein expression before and after C11H was identified. The mechanism of the antibacterial function was also discussed. Results of the present study laid the foundation for application of STAMPs in the prevention and treatment of dental caries.

Introduction

Dental caries affect a large proportion of the world population. *S. mutans* is considered a major etiologic agent involved in human dental caries. In the past several decades, efforts have been made to remove *S. mutans* from the biofilm community via replacement therapy, passive or active immunization attempts, and targeted antimicrobial agents. However, most antimicrobial agents have broad spectra of activity, which can usually kill benign and pathogenic organisms indiscriminately. When the normal dental flora is disturbed by any number of factors, microbial infections at the mucosal surface may happen. *Candida albicans* and *Staphylococcus aureus* are two examples of classical opportunistic pathogens that take advantage of increased niche size after antibiotic treatment (1,2). The problems resulting from wide-spectrum antibiotic use highlight the fundamental need for new 'targeted' antibiotic therapies to combat mucosal pathogens with a minimal impact on normal microflora. In 2006, Eckert *et al* suggested the concept of specifically targeted antimicrobial peptides (STAMPs) (3). STAMPs containing the target peptide, antibacterial peptide and/or connecting regions can selectively target and kill bacteria. STAMPs independently play roles in different regions to greatly increase anti-bactericidal capacity.

At present, bacterial pheromone or signal peptide is usually used as the targeting region for STAMPs (3-7). Pheromone or signal peptide is a kind of special signaling molecule that mediates the exchange of cellular signals between gram-positive bacteria and fungi. The signal peptide-mediated quorum-sensing (QS) system indicates that *S. mutans* is a type of cariogenic bacteria in dental plaque biofilm. Deformation of the *S. mutans* QS system mainly regulates the gene expression state, bacteriocin production and biological behavior through the functions of competence-stimulating peptide (CSP) and the dual signal transduction system. CSP and its fragments

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Table I. Strains in the study.

Strains	Source
S. mutans UA 159	Institute of Stomatological Research, *Sun Yat*-sen University
S. mutans GS-5	Institute of Stomatological Research, Sun Yat-sen University
luc-ldh reporter strain of *S. mutans*	The present study, and refs, 12 and 13
S. gordonii ATCC 10558	China General Microbiological Culture Collection Management Center
S. sanguis ATCC 49295	China General Microbiological Culture Collection Management Center

Table II. Peptides in the study.

Peptides	Amino acid sequence	Molecular weight
CSPC8 (C8)	TFFRLFNR	1,100.6
CSPC11 (C11)	TFFRLFNRSFT	1,435.66
CSPC12 (C12)	LSTFFRLFN	1,144.35
CSPC13 (C13)	STFFRLFNRSFTQ	1,650.87
CSPC14 (C14)	LSTFFRLFNRSFTQ	1,764.03
hLF1-11 (H)	GRRRRSVQWCA	1,493.6
CSPC8hLF1-11 (C8H)	TFFRLFNRGRRRRSVQWCA	2,575.88
CSPC11hLF1-11 (C11H)	TFFRLFNRSFTGRRRRSVQWCA	2,912.1
CSPC12hLF1-11 (C12H)	LSTFFRLFNGRRRRSVQWCA	2,619.93
CSPC13hLF1-11 (C13H)	STFFRLFNRSFTQGRRRRSVQWCA	3,126.45
CSPC14hLF1-11 (C14H)	LSTFFRLFNRSFTQGRRRRSVQWCA	3,239.61

are the targets of the antibacterial functions of STAMPs against *S. mutans*, and the region from 6 th threonine to 13 th arginine is sufficient for targeting (8). Seven allele genes from 36 clinical isolates of *S. mutans* were isolated from three different mature CSPs, and four kinds of different amino acids were the same with 12 different CSP sequences (9). CSP had two functional regions: The C-terminal region of the CSP and the structure of the α helix core, which is located between the fourth leucine and the twentieth glycine, and constitutes the essential region of the receptor binding (10). Previous findings showed that the hydrophobic property of CSP was very important for the recognition of CSP, and that the activity of CSP was not affected by the hydrophobic leucine or phenylalanine substitution. The 9th, 12th and 13th hydrophilic residues were not affected, and 18th alanine was also important for the function of CSP (11). Thus, CSP of *S. mutans* may be used as the targeting peptide domain. However, the functional fragment of CSP and the receptor-binding region remain to be determined. Furthermore, the molecular mechanism of STAMPs against *S. mutans* has yet to be elucidated.

Thus, in the present study, a series of STAMPs were designed and chemically synthesized. The aim was to examine the possibility of different fragments of CSP to serve as the STAMP-targeting domain. In addition, the proteomic analysis with two-dimensional differential in gel electrophoresis and matrix-assisted laser adsorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) were performed to investigate the molecular mechanism of the function of STAMPs against *S. mutans*.

Materials and methods

Strains and peptides. Strains in the study are shown in Table I. In this experiment, peptide synthesis was performed by Invitrogen; Thermo Fisher Scientific (Shanghai) Co., Ltd. (Shanghai, China) and all sequences are listed in Table II.

STAMPs MICs of S. mutans, S. gordonii, and S. sanguis. The antimicrobial activities of the peptides against oral *Streptococci* strains were investigated by serial microbroth dilution. Minimal inhibitory concentrations (MICs) of the peptides were determined using the broth microdilution method. *S. mutans*. UA 159, *S. mutans* GS-5, *S. gordonii* ATCC-10558 and *S. sanguis* ATCC-49295 were cultured anaerobically overnight in brain heart infusion (BHI) broth (BD Biosciences, San Diego, CA, USA) at a temperature of 37˚C. The medium was diluted (1:20) in fresh BHI and cultured anaerobically for 2.5 h to reach the mid-logarithmic growth phase (diluted to 1×10^5-10^6 CFU/ml). Briefly, 2-fold serial dilutions of each peptide were prepared in BHI medium at a volume of 200 μl per well in 96-well round-bottom microtitre plates (Costar, Cambridge, MA, USA). Then, 20 μl per well of bacterial cell suspension with a final concentration of 1×10^5 CFU/ml for all bacterial species was transferred to the microtitre plates for cell culture. After anaerobic incubation at 37˚C for 24 h, the MIC endpoint was defined as the lowest concentration of the test agents that completely inhibited cell growth compared with the drug-free control. The MIC values were expressed as the median of at least three independent experiments.

Effect of STAMPs on the biofilm of a single species. The *S. mutans* UA 159, *S. gordonii* ATCC-10558 and *S. sanguis* ATCC-49295 were cultured anaerobically overnight in BHI liquid medium. Then culture medium was diluted (1:20) in fresh BHI and cultured anaerobically for 2.5 h to reach the mid-logarithmic growth phase. The concentration was then adjusted to 10^7 CFU/ml. *S. mutans* UA 159 BHI medium contained 0.5% sucrose, *S. gordonii* ATCC-10558 and *S. sanguis* ATCC-49295 BHI medium contained 0.5% glucose and mannose, respectively. Bacteria were transferred to 96-well flat bottom plate with 200 μl for each well. After centrifugation for 10 min at 4,390 x g, the plates were incubated at 37°C for 4 h to form a biofilm. The supernatant was discarded and 100 μl phosphate buffered saline (PBS) containing diluted peptide was added to reach the final concentration of 128 μg/ml, followed by incubation for 5 min with PBS as the control. The supernatant was discarded, and 100 μl fresh BHI culture medium containing diluted residue peptide was added, followed by centrifugation for 10 min at 4,390 x g. Subsequently, fresh medium containing sugar was added, followed by incubation at 37°C. An automatic enzyme standard instrument was used to measure OD600, once every 30 min for 5 h. Three technical and biological repeats were used for each experiment. Statistical analysis software SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used, single-factor analysis of variance was performed to analyze the differences in OD600 values among *S. mutans* UA 159, *S. gordonii* ATCC-10558, and *S. sanguis* ATCC-49295. P<0.05 was considered to indicate a statistically significant analysis.

Effect of STAMPs on the biofilm of multi species. The *luc-ldh* reporter strain of *S. mutans*, *S. gordonii* ATCC-10558, and *S. sanguis* ATCC-49295 were cultured anaerobically overnight in BHI liquid medium. After that the medium was diluted (1:20) in fresh BHI and cultured anaerobically for 2.5 h to reach the mid-logarithmic growth phase, and then the concentration was adjusted to $1x10^7$ CFU/ml. BHI medium for the *luc-ldh* reporter strain of *S. mutans* contained 0.5% sucrose, BHI medium for *S. gordonii* ATCC-10558 and *S. sanguis* ATCC-49295 contained 0.5% glucose and mannose, respectively. Bacteria were transferred to a 96-well plate with 200 μl for each well, followed by centrifugation for 2 min at 4,000 x g. Biofilm was formed after anaerobic culture was carried out for 4 h at 37°C. The supernatant was discarded, and 100 μl PBS containing diluted peptide was added to the final concentration of 128 μg/ml. The supernatant was then removed, and 200 μl fresh BHI culture medium containing diluted residue peptide was added, followed by centrifugation for 10 min at 4,390 x g. Subsequently, 200 μl fresh BHI medium containing sugar was added, followed by anaerobic cultivation at 37°C for 5,10,60 and 120 min, respectively, to measure OD600 values to detect biofilm growth. Then 50 μl of 1 mM D-luciferin (Promega Corp., Madison, WI, USA) was added, and the growth of *S. mutans* was detected using a luminous RLU detection analyzer. Technical and biological repeats were performed for each experiment. Statistical analysis software SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used, and single-factor analysis of variance was performed to analyze the difference in OD600 values. P<0.05 was considered to indicate a statistically significant analysis.

Bacterial total protein extraction. The *S. mutans* UA 159 were cultured anaerobically overnight in BHI liquid medium, and the medium was diluted (1:20) in fresh BHI and cultured anaerobically for 2.5 h to reach the mid-logarithmic growth phase. The concentration was adjusted to $1x10^6$ CFU/ml. C11H was added to the experimental group to reach 20 μg/ml, followed by anaerobic culture at 37°C for 1 h, and the same volume of sterile ultrapure water was added to the control group. Centrifugation for 15 min at 10,000 x g at 4°C was performed to collect bacteria in the experimental and control groups. The supernatant was discarded, followed by an ice bath. Then, 500 μl of lysate containing protease inhibitors (1 mm PMSF, 1 μg/ml pepstatin, 5 μg/ml aprotinin) and 1 mg/ml lysozyme were added, followed by an ice bath for 15 min. Ultrasound (5 sec on/off, with an intensity of 37% for 10 min) was performed, followed by centrifugation (DHS 4 C; 10,000 x g for 50 min) to isolate protein. The extracted protein was purified using an Amersham clean-up kit (GE Healthcare Life Sciences, Uppsala, Sweden), quantified with a GE 2-D Quant kit (Amersham Biosciences, Sweden) and fluorescence labeling was performed with CyDye DIGE Flour (minimal Dye) Labelling kit (GE Healthcare, Arlington, TX, USA).

MALDI-TOF-MS identification of protein spots. Targeted points were placed into the MALDI-TOF-MS, and the parameter was set to reflection mode, ion source accelerating voltage of 24 kV, 1 and 2 for 22 kV acceleration voltage, ion delay extract 0,000 ns, vacuum $1.4x10^{-7}$ torr, mass spectrum signal accumulation 300 times in a single scan, with standard maker peak as external standard calibration mass spectrum peak, positive ion spectrum measurement, measurement range control in 700-4,000. Peptide mass fingerprint (PMF) of the sample was obtained. Using LIFT software, four peaks with main strength of PMF were subjected to cascade mass spectrum analysis. The obtained maps were retrieved using Biotools software, with Mascot (Matrix Science, Ltd., London, UK) as the search engine. Search parameter settings were as follows: Database for NCBInr, key species of bacteria, data retrieval method for combined, maximum allowable leakage cutting locus was 1, and enzyme trypsin. The quality error range was calculated as: PMF 50 ppm, MS/MS 0.5 Da.

Results

Antimicrobial activities of STAMPs and its components against planktonic S. mutans, S. gordonii and S. sanguis. Antimicrobial activities of STAMPs and its components against *S. mutans*, *S. gordonii* and *S. sanguis* are shown in Table III. MICs of C8H, C11H, C12H, C13H and C14H were used against the *S. mutans* strains at a dose of 10.67-13.33 μg/ml, and hLF1-11 was used against the *S. mutans* strains of MICs at a dose of 37.33-48.00 μg/ml. C8H, C11H, C12H, C13H and C14H increased antibacterial activity against *S. mutans* by 3-4 times compared with hLF1-11 alone. There were no obvious differences on the effects of C8H, C11H, C12H, C13H, C14H and hLF1-11 on antimicrobial activity against *S. gordonii* and *S. sanguis*. The MICs of C8, C11, C12, and C13 and C14 against *S. mutans*, *S. gordonii* and *S. sanguis* were >256.00 g/ml. The results suggested that binding to the targeting peptide domain improved the selective

Table III. Effects of STAMPs and its components on *S. mutans*, *S. gordonii* and *S. sanguis* MICs.

	IMC ± SD (μg/ml)			
Peptides	*S. mutans* UA159	*S. mutans* GS-5	*S. gordonii* ATCC-10558	*S. sanguis* ATCC-49295
hLF 1-11	37.33±13.06	48.00±17.53	64.00±13.06	138.67±62.92
C8	>256.00	>256.00	>256.00	>256.00
C8H	10.67±4.00	12.44±4.22	49.78±16.87	99.56±33.73
C11	>256.00	>256.00	>256.00	>256.00
C11H	10.67±4.00	12.44±4.22	49.78±16.87	99.56±33.73
C12	>256.00	>256.00	>256.00	>256.00
C12H	10.67±4.00	13.33±4.00	49.78±16.87	99.56±33.73
C13	>256.00	>256.00	>256.00	>256.00
C13H	10.67±4.00	13.33±4.00	49.78±16.87	99.56±33.73
C14	>256.00	>256.00	>256.00	>256.00
C14H	10.67±4.00	13.33±4.00	49.78±16.87	99.56±33.73
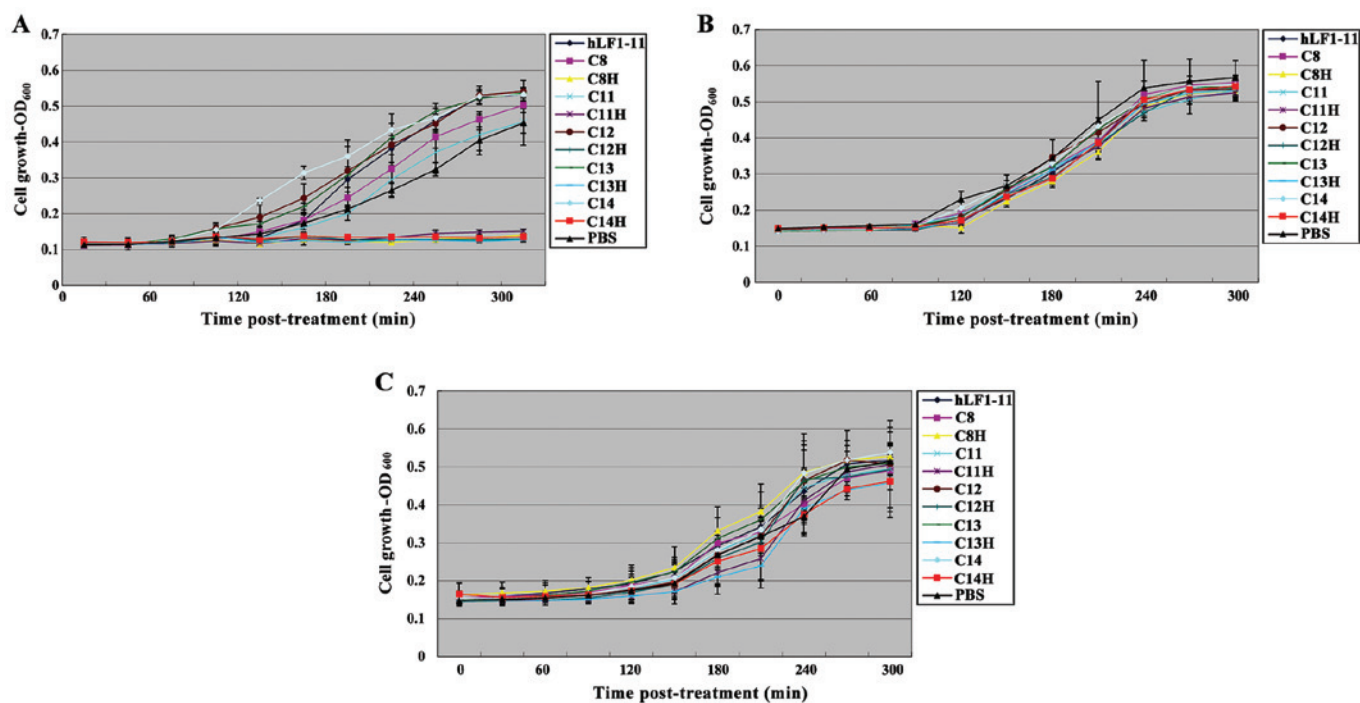
Figure 1. Effects of STAMPs and its composition on the growth of biofilm of single species. (A) *S. mutans*, (B) *S. gordonii*, and (C) *S. sanguis*. STAMPs, specifically targeted antimicrobial peptides.

antibacterial effect of hLF1-11 on *S. mutans* without affecting *S. gordonii* and *S. sanguis*.

Effects of STAMPs and its components on the single bacteria biofilm. *S. mutans* in the body usually occurs in the form of dental plaque biofilm. In order to detect the roles of STAMPs on *S. mutans* in biofilm, biofilms formed by *S. mutans*, *S. gordonii* and *S. sanguis* and after treatment with STAMPS for 5 min were used. Biofilm growth is evident in Fig. 1. After *S. mutans* was treated with C8H, C11H, C12H, C13H and C14H for 5 min, the growth of biofilm was significantly suppressed, whereas hLF1-11, C8, C11, C12, C13 and C14 did

not affect *S. mutans* biofilm growth (Fig. 1A). Biofilm growth of *S. gordonii* and *S. sanguis* was not affected by C8H, C11H, C12H, C13H, C14H, hLF1-11, C8, C11, C12, and C13 and C14 (Fig. 1B and C). The results suggested specific targeting of antimicrobial peptides C8H, C11H, C12H, C13H and C14H in a short period of time can specifically increase the antibacterial effects on bacterial biofilm of *S. mutans*.

Effects of STAMPs and its components on bacterial multi-species biofilms. The dental plaque biofilm is a complex structure comprising a variety of bacteria that simulate the *S. mutans* growth environment *in vivo*. Bacterial biofilms
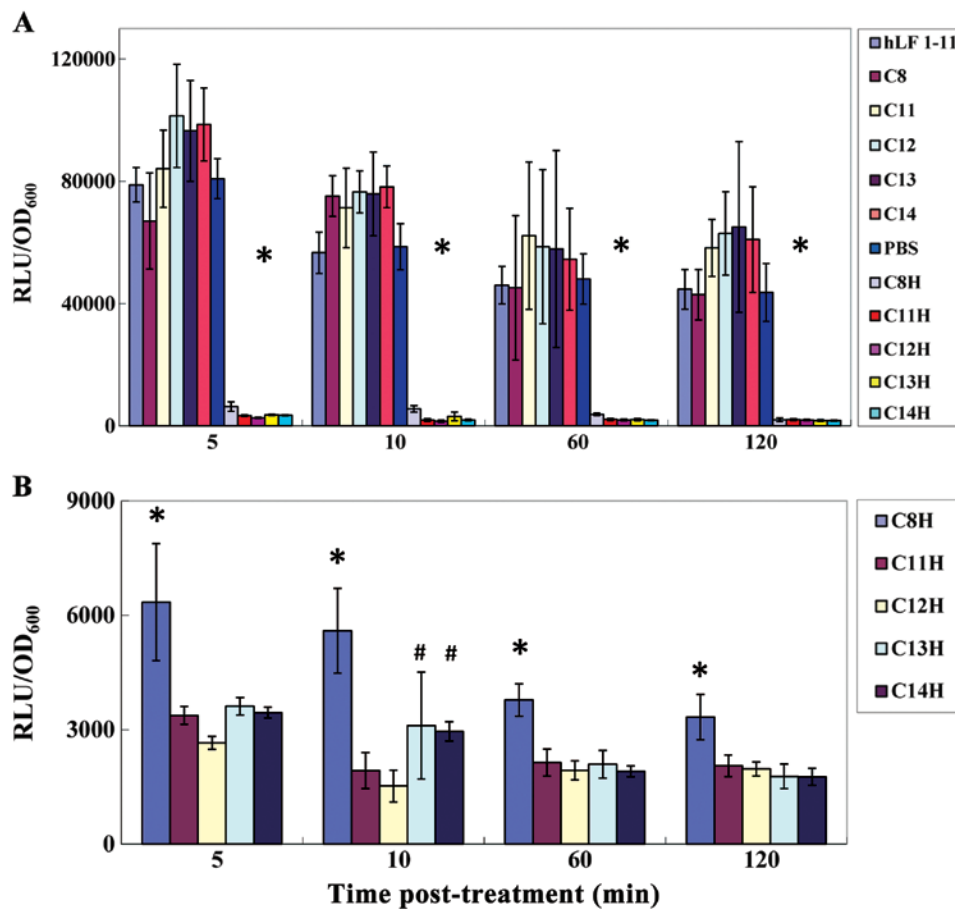
Figure 2. Effects of STAMPs and its composition on the growth of Biofilm of multi species. (A) Effect of STAMPs and different components on the *Streptococcus* species in the multi species biofilm. *C8H, C11H, C12H, C13H and C14H were significantly different from hLF1-11, C8, C11, C12, C13 and C14. (B) Effect of STAMPs and different components on the growth of *S. mutans* in the multi-species biofilm. *C8H was significantly different from C11H, C12H, C13H and C14H; #difference of C13H, C14H and C12H, C11H was statistically significant.

formed by *luc-ldh* reporter strains of *S. mutans*, *S. gordonii* and *S. sanguis* were used, and the effects of STAMPs and its components are shown in Fig. 2. RLU values indicated the number of living bacteria in the biofilm of *S. mutans*. After treatment with C8H, C11H, C12H, C13H and C14H for 5 min, RLU/OD600 was significantly decreased, resulting in the number of living bacterium decreasing significantly. RLU/OD600 remained at a low level at 5,10,60 and 120 min following removal of C8H, C11H, C12H, C13H and C14H, indicating long-term effects. hLF1-11, C8, C11, C12, and C13 and C14 showed no significant effects on growth, and RLU/OD600 values of bacterial biofilm formed by *S. mutans* (Fig. 2A). Statistical analysis revealed statistically significant effects of those factors on bacterial biofilm formed by *S. mutans* (P<0.05). As shown in Fig. 2B, statistical analysis revealed that C8H and C11H, C8H and C12H, C8H and C13H, and C8H and C14H showed statistically significant effects at 5,60 and 120 min after fresh BHI culture medium was added. C8H and C11H, C8H and C12H, C8H and C13H, C8H and C14H, C11H and C13H, C12H and C13H, C11H and C14H, and C12H and C14H showed statistically significant differences at 10 min after fresh BHI culture was added. No significant difference was found between C11H and C12H, and C13H and C14H. Thus, C11H and C12H have the strongest effects, followed by C13H and C14H, and then C8H.

Mass spectrum identification. A total of 21 protein spots with a mean change ratio of 1.5 were identified, all of which were downregulated after C11H treatment, and 19 proteins were successfully identified. The proteins included cell cycle relative proteins, nucleic acid metabolism-related enzymes and proteins, virulence factors, protein biosynthesis and regulation, proteins involved in energy metabolism and proteins with unknown function (Table IV).

Discussion

In the present study, CSP of *S. mutans* is employed as the targeting peptide domain. However, the functional fragment of CSP and receptor binding has not been clearly determined. Therefore, five targeting peptide domains were designed: i) CSPC8 (C8)8, 6-13 amino acid of CSP, TFFRLFNR; ii) CSPC11 (C11), contained the 7th, 11st, and 15th amino acid of CSP, TFFRLFNRSFT; iii) CSPC12 (C12), 4-12 amino acid of CSP, LSTFFRLFN; iv) CSPC13 (C13), four amino acids of the amino terminal and four amino acids of the carboxyl terminal of CSP were removed, STFFRLFNRSFTQ; v) CSPC14 (C14), three amino acids of the amino terminal and four amino acids of the carboxyl terminal of CSP were removed, LSTFFRLFNRSFTQ. Antibacterial peptides were selected at an early stage by the research group to demonstrate

Table IV. Differential protein expression profiles for C11H.

Cellular role	Master no.	Average ration	Protein name	Accession no.	Coverage (%)	Molecular mass Kda (theoretical)	PI (theoretical)
Cell cycle relative proteins	654	-4.75	Putative 40K cell wall protein precursor	gil24379086	16	67.474	5.66
	1327	-11.09	Putative cell division protein DivIVA	gil24379038	34	30.803	4.48
	1341	-5.58	Putative cell division protein DivIVA	gil24379038	34	30.803	4.48
	1355	-8	Putative cell division A protein DivIV	gil24379038	34	30.803	4.48
Nucleotide metabolism	824	-12.84	Inositol-5 monophosphate dehydrogenase	gil24380485	18	53.118	5.66
	2068	-5.54	Putative dTDP-4-keto-L-rhamnose reductase	gil24379857	34	22.407	4.98
	2141	-4.63	Orotate phosphoribo-syltransferase	gil24379645	27	22.802	5.37
	2334	-5.37	Single-strand DNA-binding protein	gil24380219	40	18.321	4.98
Virulence factors	261	-17.52	Hypothetical protein SMU.689	gil24379161	14	107.128	5.22
Protein biosynthesis and regulation	2035	-4.19	Elongation factor P	gil24380208	24	20.768	4.89
	2072	-4.7	Ribosome recycling factor	gil24380004	30	20.63	5. 58
	1781	-4.2	Heat shock protein GrpE (HSP-70 cofactor)	gil24378605	35	20.676	4.6
Energy metabolism amino acid metabolism	1241	-5.4	Branch ed-chain amino acid aminotransferase	gil24379628	32	37.822	5.03
	1431	-5.09	Putative cystein e sythetase A; O-acetylserine lyase	gil24378980	34	32.417	5.82
	1805	-4.51	Putative amino acid ABC transporter, ATP-binding protein	gil24379908	21	28.934	5.85
Fatty acid metabolism	1551	-4.34	Putative trans-2-enoyl-ACP reductase	gil24380114	44	33.571	5.74
Nitrogen metabolism	1628	-4.12	NAD synthetase	gil24378953	28	30.156	5.16
Glucometabolism	2008	-6.09	Putative N-acetyglucosamine-6-phosphate isomerase	gil24379109	42	25.601	5.34
Unknown function	1506	-5.93	Hypothetical protein SMU.1760c	gil24380128	30	33.119	5.12
	1576	-6.04	Hypothetical protein SMU.1789c	gil24380155	31	25.725	4.51
	2188	-6.14	Hypothetical protein SMU.1716c	gil24380090	55	19.895	5.13

the promising antimicrobial activity of hLF1-11 (12-14). STAMPs can be used to connect the targeting peptide domain to the antimicrobial peptide via a short fragment amino acid sequence, but can also be directly connected. In addition, the use of the connector is determined by use of the targeting peptide and antimicrobial peptide domains (3-5,15). He *et al*

have found that, for antimicrobial peptides rich in arginine and tryptophan, the connector did not improve the targeting of STAMPs (16). hLF1-11 contains five arginine, which may reduce the number of amino acids in STAMPs, and it was easier to be synthesized. Therefore, there was no connecting domain in the STAMPs in the present study.

To determine the specific-targeted antimicrobial activities of STAMPs against the *S.mutans*, the antimicrobial activities of the peptides against oral *Streptococci strains* were investigated by serial microbroth dilution. MICs of the peptides showed that C8H, C11H, C12H, C13H and C14H increased antimicrobial activity specifically against planktonic *S. mutans* by 3- to 4-fold compared with hLF1-11 alone. In addition, there was no significant difference of antimicrobial activities of C8H, C11H, C12H, C13H, C14H and hLF1-11 against *S. gordonii* and *S. sanguis*. C8, C11, C12, C13 and C14 did not show antimicrobial activities against *S. mutans*, *S. gordonii* and *S. sanguis*. The results suggest that antimicrobial activities and specificity against *S. mutans* of hLF1-11 were enhanced after the connection to targeted domains, but other tested oral Streptococci were not affected. *S. mutans* is present in oral biofilms (17). Additionally, the antimicrobial sensitivity of drugs is reduced 100-1,000 times in biofilm bacteria compared with bacteria in the planktonic state (18). Thus, the effects of STAMPs and its components against *S. mutans*, *S. gordonii* and *S. sanguis* in single species biofilm were tested. It was found that, 5 min after treatment with C8H, C11H, C12H, C13H and C14H, respectively, the number of *S. mutans* live bacteria in a biofilm were significantly reduced. However, 5 min after C8H, C11H, C12H, C13H, C14H, hLF1-11, C8, C11, C12, and C13 and C14, respectively, the growth of *S. gordonii* and *S. sanguis* biofilms was not affected. The results showed the specific antimicrobial activity of C8H, C11H, C12H, C13H and C14H against *S. mutans* in single species biofilm was significant even after treatment over a short period of time (5 min). *S. mutans* coexists with many bacteria in the dental plaque biofilm, a complex structure comprising a variety of bacteria. In order to mimic the growing environment of *S. mutans*, this experiment observed the roles of the STAMPs and its components in multispecies bacterial biofilms. However, 5 min after treatment with C8H, C11H, C12H, C13H and C14H, respectively, the number of *S. mutans* live bacteria in a biofilm was significantly reduced. hLF1-11, C8, C11, C12, and C13 and C14 had no effect on the growth of *S. mutans*. However, 5 min after the treatment of C8H, C11H, C12H, C13H, C14H, hLF1-11, C8, C11, C12, and C13 and C14, respectively, the growth of *S. gordonii* and *S. sanguis* in biofilm was not affected. These data suggested that STAMPs can improve the selective inhibition on the growth of *S. mutans* without affecting other bacteria in the biofilm.

Previous findings showed that hLF1-11 can penetrate the cell wall and the cell membrane without destroying the integrity of the cell membrane, and hLF1-11 has DNA binding force (14). Thus, we speculated that DNA may be the target. In order to study the mechanism underlying the effects of STAMPs on *S. mutans*, changes in the protein expression of *S. mutans* treated with C11H was examined. Additionally, 19 proteins were successfully identified, including cell cycle relative proteins, nucleic acid metabolism-related enzymes and proteins, virulence factors, protein biosynthesis and regulation, proteins involved in energy metabolism and

proteins with unknown function. We found that C11H inhibited inositol-5-monophosphate dehydrogenase (IMPDH), dTDP-4-keto-L-rhamnose reductase and orotate phosphoribosyl transferase expression. These enzymes participate in nucleic acid metabolism. IMPDH is a key enzyme involved in purine metabolism. Through the oxidation reduction reaction and hydrolysis catalyzed by phosphoric acid cyclohexyl 6, IMPDH plays an important role in controlling the number of guanine nucleotides (19). Deoxidation thymidine diphosphate-4-keto 1 is a kind of rhamnose reductase belonging to the oxidoreductase family, which was mainly involved in nucleotide sugar metabolism, streptomycin synthesis and polyketide synthesis of sugar and other metabolic reactions (20). Oral acid phosphoryl glycosyltransferase is one of the members of the phosphoribosyl transferase family and is involved in the synthesis and the recovery of the synthesis of purine nucleotides and pyrimidine nucleotides. Orotate phosphoribosyl transferase catalytic orotic acid and phosphoribosyl pyrophosphate generate inorganic pyrophosphate and 5'-phosphate acid whey acid riboside (OMP) in the presence of Mg^{2+}, while OMP generates 5'-uridine with the function of decarboxylase, and the former is the key factor in the synthesis of pyrimidine nucleotide (21,22). Single-stranded DNA binding protein (SSBP) is a single-stranded region along the replication fork that prevents the formation of new single-strand DNA to form double-stranded DNA or nucleic acid enzyme degradation of protein. In cells, a large number of SSBPs bind to single-strand DNA to stretch the DNA without causing nodules and bending to facilitate the role of single DNA to serve as a template. Thus, SSBP does not possess enzyme activity nor binds to ATP, which is an essential ingredient for DNA replication, recombination and repair (23,24). Proteomic analysis confirmed that C11H inhibited DNA-related enzymes and proteins, which is one of the mechanisms of its antibacterial effect on *S. mutans*. It also indicates that the antibacterial effect of C11H was achieved by the hLF1-11.

In conclusion, STAMPs were successfully designed and screened and the overall level of protein metabolism was explored to investigate the mechanism of antibacterial function. The major findings of this study are i) STAMPs had selective antibacterial activity against *S. mutans* grown in liquid or biofilm states but did not affect other oral Streptococci tested. ii) C11H and C12H exhibited the strongest effects, followed by C13H and C14H, and C8H. iii) C11H inhibited the expression of nucleic acid metabolism-related enzymes, which was one of the mechanisms of the inhibitory effects on *S. mutans*. Therefore, STAMPs can be used to improve antimicrobial therapies in the treatment of dental caries.

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