Targeted Killing of *Streptococcus mutans* by a Pheromone-Guided “Smart” Antimicrobial Peptide

Randal Eckert,¹ Jian He,² Daniel K. Yarbrough,² Fengxia Qi,²
Maxwell H. Anderson,³ and Wenyan Shi^{1,2*}

Department of Microbiology, Immunology, and Molecular Genetics¹ and School of Dentistry,² University of California, Los Angeles, California 90095, and C3 Jian, Incorporated, Sequim, Washington 98382³

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Within the repertoire of antibiotics available to a prescribing clinician, the majority affect a broad range of microorganisms, including the normal flora. The ecological disruption resulting from antibiotic treatment frequently results in secondary infections or other negative clinical consequences. To address this problem, our laboratory has recently developed a new class of pathogen-selective molecules, called specifically (or selectively) *targeted antimicrobial peptides* (STAMPs), based on the fusion of a species-specific targeting peptide domain with a wide-spectrum antimicrobial peptide domain. In the current study, we focused on achieving targeted killing of *Streptococcus mutans*, a cavity-causing bacterium that resides in a multispecies microbial community (dental plaque). In particular, we explored the possibility of utilizing a pheromone produced by *S. mutans*, namely, the competence stimulating peptide (CSP), as a STAMP targeting domain to mediate *S. mutans*-specific delivery of an antimicrobial peptide domain. We discovered that STAMPs constructed with peptides derived from CSP were potent against *S. mutans* grown in liquid or biofilm states but did not affect other oral streptococci tested. Further studies showed that an 8-amino-acid region within the CSP sequence is sufficient for targeted delivery of the antimicrobial peptide domain to *S. mutans*. The STAMPs presented here are capable of eliminating *S. mutans* from multispecies biofilms without affecting closely related noncariogenic oral streptococci, indicating the potential of these molecules to be developed into “probiotic” antibiotics which could selectively eliminate pathogens while preserving the protective benefits of a healthy normal flora.

The indigenous microflora found at human mucosal surfaces is critical for acquiring nutrients and providing protective colonization against pathogenic microorganisms (9, 35, 36, 40, 41, 47). When the normal flora is disrupted by any number of factors, the result is often microbial infections at the mucosal surface, many of which affect populations worldwide (32, 44). The lack of a robust immune response at mucosal surfaces has limited the prescribing clinician to conventional antibiotics or antimicrobials for treatment of mucosal infections. Unfortunately for the normal flora, most small-molecule antibiotics have broad spectra of activity, killing benign and pathogenic organisms indiscriminately. This effect often leads to severe antibiotic-associated infections due to the vacated niche available for pathogen colonization. *Clostridium difficile*, *Candida albicans*, and *Staphylococcus aureus* are examples of classical opportunistic pathogens that take advantage of increased niche size after antibiotic treatment (19, 28, 42). The problems resulting from wide-spectrum antibiotic use, combined with the emergence of drug-resistant strains, highlight the fundamental need for new “targeted” antibiotic therapies to combat mucosal pathogens with a minimal impact on normal microflora.

Previous efforts toward achieving target-specific antimicrobial therapy consisted of conjugating antibiotics to monoclonal antibodies (W. Shi, S. L. Morrison, K. Trinh, L. Wims, L. Chen, M. H. Anderson, and F. Qi, U.S. patent application

20030143234) or constructing large fusion proteins with bactericidal and bacterial recognition domains (38, 39). To date, neither method has resulted in functional, effective therapeutics; this is due to the low efficiency of chemical conjugation, instability of large proteins, or high cost of production.

Recently, we developed a new class of targeted antimicrobials, called specifically (or selectively) *targeted antimicrobial peptides* (STAMPs). Constructed from short peptides that can be chemically synthesized with high yields in vitro, STAMPs contain a targeting peptide domain fused to an antimicrobial peptide domain; despite being conjoined, these domains remain functionally independent. As a result, STAMPs have increased killing potency, selectivity, and kinetics against targeted bacteria (15). In our previous report, we used a de novo rationally designed targeting peptide for STAMP construction. In this study, we explored the possibility of co-opting a natural bacterial pheromone (competence stimulating peptide [CSP]) to serve as the targeting peptide domain in a STAMP designed for an oral bacterial pathogen, *Streptococcus mutans* (22, 23, 30).

S. mutans has been implicated as a primary pathogen involved in the formation of dental caries (7, 12, 24–26), one of the most prevalent and costly diseases associated with oral mucosal surface infections (4, 5). Despite the presence of over 500 species of indigenous oral flora (1), dental caries result from the overgrowth of a handful of cariogenic pathogens, including *S. mutans*. In order to eliminate or reduce dental caries, numerous studies have focused, through various means, on the prevention of *S. mutans* colonization. However, efforts such as vaccination have yet to yield effective results, and not

* Corresponding author. Mailing address: UCLA School of Dentistry, 10833 Le Conte Avenue, Los Angeles, CA 90095-1668. Phone: (310) 825-8356. Fax: (310) 794-7109. E-mail: wenyan@ucla.edu.

only is the outright sterilization of the oral cavity impossible but efforts to achieve such sterilization have also been associated with secondary infections and resistance evolution (19, 28, 45). We reasoned that the STAMP technology described above could selectively eliminate *S. mutans* without compromising the protective colonization provided by the indigenous flora, thereby preventing *S. mutans* recolonization and caries progression. Here we report the successful construction and characterization of a set of CSP-derived STAMPs against *S. mutans*.

MATERIALS AND METHODS

Strains and growth conditions. All *S. mutans*, *S. gordonii* Challis (DL1), and *S. sanguinis* NY101 strains were grown in Todd-Hewitt (TH) broth medium (Fisher) at 37°C under anaerobic conditions (80% N₂, 10% CO₂, 10% H₂). *S. mutans* strains UA159 (2), ATCC 25175, and T8 (43) are wild-type clinical isolates, while *comD* is a knockout mutant that was constructed previously from the wild-type UA140 background (37). Luciferase-expressing *S. mutans* strain JM11 was constructed from strain UA140 as described previously (29).

Synthesis and purification of peptides. All peptides listed in Table 1 (also see Table 3) were synthesized using double-coupling cycles by standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis methods (431A peptide synthesizer [Applied Biosciences] or Apex396 [Advanced Chemtech]) as described previously (15). Completed peptides were cleaved from the resin with 95% trifluoroacetic acid with appropriate scavengers and purified by reverse-phase high-performance liquid chromatography (ACTA purifier; Amersham) to 90% to 95% purity. Peptide molecular mass was determined by matrix-assisted laser desorption/ionization mass spectrometry. Peptides C16G2, G2, and M8G2 were synthesized using amidated C termini and Fmoc-Tyr(tBu)-Rink Amide MBHA resin (Anaspec). All other peptides were synthesized with the appropriately substituted Wang resins.

Fluorescent labeling of peptides and fluorescence microscopy. Aliquots of CSP_{C16}, CSP-fragment peptides (see Table 3), and C16G2 were labeled with carboxyfluorescein (Sigma) as described previously (15, 16). After peptide cleavage but prior to the bacterial labeling assay, fluorescence intensity values per micromole peptide were checked fluorimetrically (VersaFluor, Bio-Rad) (λ_{ex} = 488 nm, λ_{em} = 520 nm) and found to be relatively similar (data not shown). To evaluate the level of peptide binding to bacteria, streptococci from an overnight culture (optical density at 600 nanometers [OD₆₀₀] of 0.7 to 1.0) were washed with phosphate-buffered saline (1× PBS), diluted 1:2 into 1× PBS, and exposed to peptide (16 μ M) for 5 min at 25°C. After incubation with peptide, unbound agent was removed from the bacteria by three cycles of centrifugation (5 min, 16,000 × g) and resuspension in 1× PBS. Labeling of oral streptococci was evaluated using bright-field and fluorescence microscopy (Nikon E400) at 40× magnification. The digital images utilized for the semiquantitative binding assessment were acquired with the factory-supplied software (SPOT; Diagnostics).

Determination of antimicrobial activity. The general antimicrobial activity of peptides against planktonic bacteria was determined by a MIC assay in TH broth (all oral streptococci) (37). Briefly, exponentially growing bacterial cells were diluted to $\sim 1 \times 10^5$ CFU/ml in TH broth and placed into 96-well plates (Fisher). Peptides were then serially diluted and added to the bacteria. MICs were determined by identifying the concentration of peptide that completely inhibited bacterial growth after approximately 24 h of incubation.

Determination of bactericidal kinetics. To determine the short-term killing rate and selectivity of C16G2 and G2 we performed time-kill experiments, essentially as described previously (15). *S. mutans* UA159, *S. gordonii*, or *S. sanguinis* bacteria were grown to log phase and diluted to $\sim 1 \times 10^5$ CFU/ml in growth medium. Under aerobic conditions, 25 μ M G2 or C16G2 was added to the cell suspension and incubated at 25°C. At 1 min, 10 μ l of cell suspension was removed, rescued by dilution into growth medium (1:50), and kept on ice. For plating, 20 to 500 μ l of rescued cells was spread on growth medium agar plates and colonies were counted after overnight incubation at 37°C under anaerobic conditions. We considered 60 CFU/ml to be the detection limit for this assay. Values of surviving CFU per milliliter were expressed as the ratio of survivors from C16G2-treated cultures to the CFU per milliliter from samples exposed to G2.

Examination of antimicrobial activity against single-species biofilms. To initiate biofilm formation, $\sim 1 \times 10^7$ bacteria per well (from overnight cultures) were seeded in TH medium (100 μ l) to a 96-well flat-bottom plate. For all streptococci except *S. mutans*, the medium was supplemented with 0.5% (wt/vol)

TABLE 1. Peptide sequences (single-letter amino acid code) of CSP, CSP_{C16}-containing STAMPs, and STAMP components

Peptide	Amino acid sequence ^a	Molecular wt (observed)
CSP	SGSLSTFFRLFNRSFTQALGK	2,364.9
CSP _{C16}	TFFRLFNRSFTQALGK	1,933.3
G2	KNLRIIRKGIHIKKY ^b	1,993.5
C16G2	TFFRLFNRSFTQALGKG ^b GK ^b NLRIIRKGIHIKKY ^b	4,079.0
CSP _{M8}	TFFRLFN	1,100.6
M8G2	TFFRLFN ^b GGGK ^b NLRIIRKGIHIKKY ^b	3,246.9
S6L3-33	FKKFWKWFRRF	1,677.5
C16-33	TRRRLFNRSFTQALGKSGGGF ^b KKFWKWFRRF	3,849.0
M8-33	TFFRLFN ^b SGGGF ^b KKFWKWFRRF	3,016.9

^a Linker regions between targeting and killing peptides are underlined.

^b Peptide C-terminal amidation.

mannose and glucose. *S. mutans* UA159 biofilms were grown with 0.5% (wt/vol) sucrose. Plates were then centrifuged briefly to pellet the cells, and bacteria were incubated for 3 to 4 h at 37°C for biofilm formation. After incubation, the supernatant was carefully removed and biofilms were treated with 25 μ M peptide in 1× PBS or 1× PBS alone for 1 min. The peptide solution was then removed, and 100 μ l TH medium was added to further dilute any remaining peptide. To minimize biofilm loss, cells were briefly centrifuged after TH medium addition, after which the supernatants were removed and fresh medium plus appropriate sugars were returned. Cells were then incubated anaerobically at 37°C, and biofilm growth was monitored over time by measuring absorbance at OD₆₀₀ with a microplate spectrophotometer (Benchmark Plus; Bio-Rad).

Evaluation of antimicrobial activity against bacterial biofilm in saliva. For these experiments, we employed methods similar to those previously described (8). A day prior to the assay, saliva was collected and pooled from five adult volunteers in the laboratory, diluted 1:4 in TH broth, and centrifuged at 2,000 × g for 10 min. The supernatant was then filter sterilized (Nunc) (0.2 μ m filter) and stored at 4°C. A portion of pooled saliva was also diluted 1:2 in 1× PBS and processed as before. On the day of the assay, overnight cultures of JM11 and other oral streptococci were normalized to an OD₆₀₀ of 1.0 and $\sim 3 \times 10^6$ CFU/ml of each species were added to 10 ml of the TH medium-diluted saliva. Sucrose, mannose, and glucose (1% wt/vol each) were then added, and the solution was mixed. Aliquots (500 μ l) of the saliva and bacteria mixture were then placed into 1.5 ml Eppendorf tubes (Fisher). After a brief centrifugation (4,000 × g, 2 min), the tubes were incubated for 3 to 4 h at 37°C to form multispecies biofilms. The supernatants were then removed and the spent media replaced with 100 μ l PBS-diluted saliva (1:2) plus 25 μ M (freshly added) peptide. After biofilms were exposed to the agent for 5 min, the PBS-saliva was removed, cells were briefly centrifuged, and 500 μ l fresh TH medium-saliva with sugars was returned. At each time point, total biofilm growth was measured by reading absorbance at OD₆₀₀ and the health of *S. mutans* within the population was examined by relative luciferase expression (relative light unit [RLU] production) as described previously (29). Briefly, biofilms were resuspended by vortex and aspiration and 100 μ l of each sample was transferred to a new Eppendorf tube with 25 μ l 1 mM D-luciferin (Sigma) solution suspended in 0.1 M citrate buffer, pH 6.0. For the 2 h time point, biofilms were stimulated after resuspension by the addition of 1% sucrose 30 min prior to recording luciferase activity. RLU production was measured using a TD 20/20 luminometer (Turner Biosystems), and reported values were obtained from the average results for three independent samples. The data were plotted as the RLU/OD₆₀₀ over time.

RESULTS

Design and construction of C16G2, a CSP-derived STAMP against *S. mutans*. The initial CSP-derived STAMP was constructed by synthesizing full-length *S. mutans*-specific CSP (21 amino acids) with the antimicrobial peptide G2 (16 amino acids, derived from the wide-spectrum antimicrobial peptide novispirin G10) (15) at either the C terminus or the N terminus. Biological testing of these STAMPs did not reveal any

TABLE 2. MICs of G2-containing STAMPs and STAMP components against bacteria

Strain	MIC (μM) ^a					
	CSP	CSP _{C16}	G2	C16G2	CSP _{M8}	M8G2
<i>S. mutans</i>						
UA159	50.8 $\pm$ 9.3	>60	12.1 $\pm$ 4.5	3.0 $\pm$ 1.6	>60	3.25 $\pm$ 1.9
25175	>60	>60	14.8 $\pm$ 2.0	3.8 $\pm$ 0.3	>60	3.5 $\pm$ 0.5
T8	>60	>60	14.2 $\pm$ 1.5	3.7 $\pm$ 0.2	>60	NT ^b
<i>comD</i>	>60	>60	15.3 $\pm$ 4.2	5.1 $\pm$ 2.4	>60	4.0 $\pm$ 2.0
Non- <i>S. mutans</i>						
<i>S. gordonii</i>	>60	>60	41.3 $\pm$ 14.0	23.5 $\pm$ 7.8	>60	20 $\pm$ 5.0
<i>S. sanguinis</i>	>60	>60	33.6 $\pm$ 7.5	19.1 $\pm$ 4.0	>60	15 $\pm$ 2.5

^a MICs represent averages of at least 3 independent experiments with standard deviations.

^b NT, not tested.

antimicrobial activity (data not shown). We reasoned that steric hindrance caused by the full-length CSP might be inhibiting G2 antimicrobial function. Therefore, since a shorter targeting domain could be advantageous, we theorized that the C-terminal 16 amino acids of CSP, called CSP_{C16} (which in previous studies was shown to still have pheromone activity) (37), could be used as a substitute for CSP. Peptides containing CSP_{C16} at either the N or C terminus of G2, with different linker regions of flexible amino acids in between, were then synthesized and screened for their antimicrobial activities (data not shown). From among the potential STAMPs, C16G2, which consisted of (from the N to C terminus) CSP_{C16}, a short peptide linker (GGG), and G2 (Table 1), was selected for further study due to its improved MIC, greatly enhanced killing kinetics, and selectivity against *S. mutans* (compared to that of G2 alone), as described below.

C16G2 has enhanced antimicrobial activity and specificity against planktonic *S. mutans* cells. To evaluate the antimicrobial activity and general specificity of C16G2, MIC tests were performed against a panel of bacterial species, including various strains of *S. mutans* and closely related oral streptococci (18). As shown in Table 2, the MICs for C16G2 ranged from 3 to 5 μM for all *S. mutans* strains tested, a four- to fivefold increase in antimicrobial activity over that seen with the parental antimicrobial peptide G2 (12 to 20 μM). In comparison, we observed little (twofold or less) difference in susceptibility between G2 and C16G2 against *S. gordonii* and *S. sanguinis*.

Previously, we showed that an anti-*Pseudomonas* STAMP did not show much improvement in MIC after 24 h incubation but displayed greatly enhanced killing kinetics and specificity (compared to the untargeted parental antimicrobial peptide) against the targeted bacteria during a short time exposure (15). Therefore, we performed comparative experiments to examine the killing ability of C16G2 and G2 for its targeted and untargeted bacteria after a short time exposure. As shown in Fig. 1, with 1 min of exposure, C16G2 was over 20-fold more active against its targeted bacterium, *S. mutans*, in comparison to G2, whereas it exhibited a level of activity similar to that seen with G2 against other oral streptococci tested. These findings provided the first indications that the addition of the CSP_{C16} targeting domain to G2 had resulted in an antimicrobial with selective activity against *S. mutans* but against not other closely related oral streptococci.

C16G2 is also active against biofilm cells. *S. mutans* bacteria predominantly exist in a biofilm growth state in vivo. As it is well known that biofilm-associated cells are 100- to 1,000-fold more resistant to antibiotics (14, 17, 46), we tested whether C16G2 still has activity against *S. mutans* biofilms in vitro. For these experiments, biofilm-associated *S. mutans*, *S. gordonii*, or *S. sanguinis* bacteria were treated with 25 μM C16G2, G2, CSP, CSP_{C16}, or 1 $\times$ PBS for 1 min and washed, and their regrowth was monitored over time. As shown in Fig. 2, *S. gordonii* or *S. sanguinis* biofilms exposed to any of the peptides tested grew similarly to untreated biofilms after peptide addition and removal (Fig. 2A and B). In contrast, *S. mutans* strain UA159 (Fig. 2C), as well as strains T8 and 25175 (data not shown), was severely inhibited by treatment with C16G2 but was unaffected by treatment with the other peptides. These results indicate that C16G2 can function as an anti-*S. mutans* STAMP in a biofilm environment with only a short period of exposure (1 min), a time period which is relevant for clinical treatments of the oral cavity (6, 27, 48).

C16G2 can selectively eliminate *S. mutans* from a mixed species biofilm. In addition to growing as biofilm in vivo, *S. mutans* bacteria are also constantly bathed in saliva as they adhere to the tooth surface. Therefore, we examined whether C16G2 could selectively kill *S. mutans* under these conditions.

FIG. 1. Selective killing activity of C16G2 against *S. mutans*. *S. mutans*, *S. sanguinis*, and *S. gordonii* planktonic cells were exposed to 25 μM of the STAMP C16G2, or its untargeted parent antimicrobial peptide G2, for 1 min. Surviving CFU per milliliter were detected and compared. Data represent averages of the results of at least three independent experiments.

FIG. 2. Inhibitory activity of G2 and C16G2 against single-species biofilms. *S. gordonii* (A), *S. sanguinis* (B), and *S. mutans* (C) monoculture biofilms were grown and then exposed for 1 min to a 25 μ M concentration of STAMP or a STAMP component (as indicated in the figure), washed, and regrown with fresh medium. Biofilm recovery was monitored over time by OD_{600} . Data represent averages of the results from three independent experiments.

In these experiments, two species of noncariogenic oral streptococci (*S. gordonii* and *S. sanguinis*) were mixed with *S. mutans* JM11, a strain harboring a transcriptional fusion between luciferase (*luc*) and the promoter for the constitutively active gene lactate dehydrogenase (*ldh*), which has the same susceptibility to C16G2 as the wild-type UA159 (data not shown). JM11 has been previously utilized to measure the fitness of *S. mutans* populations, and decreasing RLU production was shown to strongly correlate with reduced cell viability (29). The mixed-species biofilms were formed with saliva, and then peptides (25 μ M) suspended in saliva were added for 5 min and removed and the posttreatment growth of the biofilm was further monitored. The number of viable *S. mutans* cells within the biofilm was quantified in parallel by luciferase expression. We found that C16G2 was able to dramatically reduce the *S. mutans* population within the mixture (reflected in the low level of luciferase activity) after 5 min of exposure compared to the results seen with CSP_{C16} and G2 (Fig. 3). Interestingly, even after 120 min posttreatment, the total number of *S. mu-*

FIG. 3. C16G2 activity against *S. mutans* within a multispecies biofilm. Mixed cultures of *S. mutans*, *S. sanguinis*, and *S. gordonii* were allowed to form a biofilm in saliva and were then exposed to 25 μ M C16G2, CSP_{C16}, or G2. After being washed, the biofilms were allowed to recover in fresh medium-saliva. The regrowth of the biofilm over time was monitored by measuring absorbance at OD_{600} , while the health of the *S. mutans* within the biofilm was measured by luciferase activity (RLU production). The data were plotted as RLU/ OD_{600} and represent averages of the results of at least three independent experiments.

tans within the mixture remained low (Fig. 3). Taken together, these results indicate that a short exposure of C16G2 is capable of selectively inhibiting the growth of *S. mutans* within a multispecies biofilm and in the presence of saliva for a minimum of 2 h without harming bystander bacteria or affecting the overall health of the biofilm.

Enhanced antimicrobial activity of C16G2 is related to targeted ComD-independent binding of CSP_{C16} to *S. mutans*. To further explore the mechanism of C16G2-enhanced activity against *S. mutans*, we fluorescently labeled CSP_{C16} and C16G2 and tested their ability to bind to *S. mutans* and other streptococci. Consistent with observed killing activity, we found that CSP_{C16} and C16G2 could specifically bind to *S. mutans* with a very short time exposure (1 to 2 min) but not to other oral streptococci (data not shown).

We also tested the effect of C16G2 on the *comD* mutant, as previous genetic studies suggested that CSP may interact with ComD to activate DNA competence in *S. mutans* (23). To our surprise, similar MICs were observed for UA159 and the *comD* strain (Table 2). Consistent with this observation, we also found that fluorescently labeled CSP_{C16} (Table 3), and C16G2 (data not shown) bound to UA159 and the *comD* mutant in a similar manner, indicating that the specific binding ability of CSP to *S. mutans* is independent of the presence of ComD.

An 8-amino-acid sequence within C16 is required for species-specific recognition. To determine whether there was a region within the CSP_{C16} sequence that was responsible for *S. mutans*-specific binding, we synthesized a series of fluorescently labeled CSP_{C16} fragments and analyzed their ability to bind to *S. mutans*. The following strategies were utilized in dissecting the CSP_{C16} sequence (Table 3). First, a series of fragments were constructed by generating deletions of three or four amino acids, from the N to C terminus, across the CSP_{C16} sequence (C16-1 to C16-5). Peptides lacking larger portions of the C or N terminus of CSP_{C16} (C16-6 to C16-12) were also synthesized. Additionally, peptides with Arg to Asn substitu-

TABLE 3. Binding of CSP-fragment peptides to *S. mutans*

Peptide	Amino acid sequence ^a	Relative <i>S. mutans</i> binding ^b
C16	TFFRLFNRSFTQALGK	+++
3- to 4-amino-acid internal deletions		
C16-1	--RLFNRSFTQALGK	—
C16-2	TFF---NRSFTQALGK	—
C16-3	TFFRLF---TQALGK	++
C16-4	TFFRLFNRS---ALGK	+++
C16-5	TFFRLFNRSFTQ---K	+++
Terminal deletions		
C16-6	TFFRLFNRS-----	+++
C16-7	---RLFNRSFTQA---	—
C16-8	-----RSFTQALGK	—
C16-9	TFF-----	—
C16-10	TFFR-----	—
C16-11	TFFRL-----	+
C16-12 (CSP _{M8})	TFFRLFN-----	+++
Substitutions		
C16-13	TGGRLGNRS G TQALGK	—
C16-14	TFF N LFN N SFTQALGK	—
Alanine scanning		
C16-15	AAAA LFNRSFTQALGK	—
C16-16	TFFR AAAA SFTQALGK	+
C16-17	TFFRLFN AAAA ALGK	++
C16-18	TFFRLFNRSFTQ AAAA	+++

^a Substituted amino acids are shown in boldface.^b Reported relative binding data represent results obtained with both UA159 and *comD*. +++, majority of cells brightly stained; ++, low-intensity fluorescent signal; +, very weak/diffuse staining; —, no fluorescent signal.

tions (a positive to negative change in charge) (C16-4) or Phe to Gly substitutions (for a general decrease in hydrophobicity) (C16-3), as well as peptides representing a four-residue Ala scan of the C16 sequence (C16-15 to C16-18), were constructed. Binding assays were performed as described previously (15); the results are summarized in Table 3. CSP_{C16} and any peptides containing Thr6 through Arg13 (TFFRLFN) of CSP were detected as bound to *S. mutans* UA159 or *comD* cells, while any interruption to this region via deletion, substitution, or Ala scanning reduced the detected fluorescent binding compared to CSP_{C16} results. Some peptides, such as C16-3, -11, -16, and -17, which contained only Thr6-Phe11 and Phe7-Phe11, showed binding but at a weaker intensity than CSP_{C16} or any other peptides with the complete Thr6-Arg13 region. Additionally, we observed that Arg to Asn or Phe to Gly substitutions were deleterious to cell binding, suggesting that these residues within TFFRLFN (called CSP_{M8}) are required for binding to *S. mutans*. Interestingly, CSP_{M8} exhibited little or no binding to the other oral streptococci listed in Table 2 (data not shown), indicating that CSP_{M8} may also be capable of specifically binding to *S. mutans* surfaces.

M8G2 has anti-*S. mutans* activity similar to that of C16G2. Based on the data presented above, we further hypothesized that CSP_{M8} would be sufficient to function as an alternative targeting domain for an anti-*S. mutans* STAMP. To test this hypothesis, CSP_{M8} and G2 were synthesized together to form the STAMP M8G2 (Table 1). As shown in Table 2, M8G2

displayed MICs against *S. mutans* and other oral streptococci similar to those seen with C16G2. Furthermore, single-species biofilm inhibition assays showed that M8G2, like C16G2, was capable of inhibiting the recovery of *S. mutans* biofilms (Fig. 4A), but not *S. sanguinis* biofilms (Fig. 4B), after 1 min of exposure. Since the CSP_{M8} domain is much smaller than that of CSP_{C16} and consequently easier to chemically synthesize, these results provide a basis for a future design of shorter anti-*S. mutans* STAMPs based on CSP_{M8}.

CSP_{C16}/CSP_{M8}-guided STAMPs are functional with an alternative killing domain. Since the targeting and antimicrobial components of a STAMP are functionally independent, despite being synthesized as one peptide (15), we reasoned that a combination of CSP_{C16} or CSP_{M8} with a different general antimicrobial peptide could also result in increased killing activity and selectivity towards *S. mutans* compared with the results obtained with the untargeted killing peptide alone. Therefore, we conjugated both targeting peptides to S6L3-33, a model wide-spectrum antimicrobial peptide developed in our laboratory (J. He, R. Eckert, T. Pharm, M. D. Simanian, C. Hu, D. K. Yarbrough, F. Qi, M. H. Anderson, and W. Shi, unpublished data), in an arrangement similar to those of C16G2 and M8G2, to yield the STAMPs C16-33 and M8-33 (Table 1). As shown in Table 4, we observed a two- to threefold difference in MICs between S6L3-33 and the derived STAMPs against *S. mutans* and the other oral streptococci tested. However, when single-species biofilm studies were conducted (the results are shown in Fig. 5), the *S. mutans*-selective activity of

FIG. 4. Activity of M8G2 against oral bacteria in biofilms. *S. mutans* (A) or *S. sanguinis* (B) single-species biofilms were mock treated or exposed to 25 μ M M8G2 (as specified in the figure). After removal of the STAMP and the addition of fresh medium, biofilm recovery was monitored over time by monitoring absorbance at OD₆₀₀. The data represent the averages of the results of three independent experiments.

TABLE 4. MICs of STAMPs constructed with the S6L3-33 antimicrobial region

Peptide	MIC $\pm$ SD (μ M) for indicated strain ^a			
	UA159	<i>comD</i>	<i>S. sanguinis</i>	<i>S. gordonii</i>
S6L3-33	7.0 $\pm$ 3.0	6.5 $\pm$ 2.5	40 $\pm$ 7.5	20 $\pm$ 5.0
C16-33	2.5 $\pm$ 2.1	2.2 $\pm$ 0.5	13.3 $\pm$ 5.8	14.6 $\pm$ 5.0
M8-33	2.5 $\pm$ 2.0	2.5 $\pm$ 2.0	20 $\pm$ 2.0	10 $\pm$ 2.5

^a MICs represent averages $\pm$ standard deviations of the results of at least three independent experiments.

the STAMPs was readily apparent: both C16-33 and M8-33 were capable of retarding *S. mutans* biofilm growth after a short exposure (Fig. 5A), while cultures of *S. sanguinis* were not affected by STAMP administration (Fig. 5B). These results indicate a clear enhancement of STAMP activity selective for *S. mutans* biofilms.

DISCUSSION

In this study we successfully synthesized and evaluated a series of STAMPs which exhibited specificity for *S. mutans* and not other oral streptococci. The STAMPs were designed for *S. mutans*-selective activity by incorporating portions of a natural pheromone produced by these cariogenic bacteria (CSP) as the targeting domain within the linear STAMP peptide. By exclusively utilizing short (<3 kDa) linear peptides for the targeting and antimicrobial regions, we were able to rapidly synthesize and isolate the complete STAMP molecule in one piece via solid-phase chemical methods, a distinct advantage over the recombinant expression and difficult purification routes necessary to construct the large (>70 kDa) protein-based targeted antimicrobials that have been described previously (38, 39). Additionally, the flexibility provided by synthetic routes enabled us to easily increase STAMP diversity by switching between different combinations of targeting domains (CSP_{M8} and CSP_{C16}) and killing domains (G2 and S6L3-33) when constructing STAMPs for use against *S. mutans*, a task that would otherwise require tedious cloning procedures.

Our data suggest that CSP_{C16}-*S. mutans* binding is species specific but is not dependent on the ComD surface receptor. Furthermore, the CSP_{M8} sequence within CSP appears to be sufficient for selective *S. mutans* binding. These results suggest that a natural *S. mutans*-specific targeting sequence is present within this pheromone that may bind to an alternative receptor (lipid, carbohydrate, or protein) on the bacterial surface prior to interaction with ComD. Biologically, the CSP_{M8} sequence may function to “sequester” CSP molecules on the *S. mutans* surface, thereby aiding in acquisition of a communication signal for recognition by sensor kinases. Further studies are under way to determine whether minimal binding sequences exist in other species-specific pheromones and whether these sequences are themselves capable of stimulating sensor kinases. Interestingly, CSP_{M8} alone appears unable to regulate competence (R. Eckert, F. Qi, and W. Shi, unpublished data).

As shown in Fig. 5, we were able to synthesize CSP_{C16} and CSP_{M8} in combination with an alternative antimicrobial peptide (S6L3-33) without the loss of *S. mutans*-selective killing ability. This finding further validates the notion that the

STAMP targeting and antimicrobial domains function independently and are capable of being linked in different combinations without the loss of activity. This suggests that future STAMP construction will be an unlimited “tunable” process whereby a myriad of combinations of antimicrobial, linker, and targeting domains can be synthesized in order to select a STAMP with the best specific activity. Furthermore, bacterial STAMP resistance (should it evolve) (34) could be easily overcome by switching to alternative, functionally analogous STAMP components, as was done with G2 and S6L3-33 in this study. Additionally, peptide pheromones are widely utilized by pathogenic bacteria (13, 21), especially gram-positive organisms (11, 33), and therefore represent a large and growing pool from which future targeting peptides could be selected for STAMP construction.

C16G2, M8G2, C16-33, and M8-33 displayed robust specific activity against targeted *S. mutans* bacteria in planktonic cultures and in biofilms with both single and multispecies, suggesting that we were able to construct a set of functional STAMPs that can discriminate between *S. mutans* and other noncariogenic oral streptococci. This selective activity, combined with the low cytotoxicity of these peptides (R. Eckert, I. McHardy, J. He, and W. Shi, unpublished data), indicates that they may be useful for anticaries therapeutic development. Currently, treatments for *S. mutans* infection include abstinence from dietary sugars, mechanical removal of the dental plaque, and general biocide mouthwashes. While all are tem-

FIG. 5. Biofilm-inhibitory activity of S6L3-33 and S6L3-33-containing STAMPs. Single-species biofilms of *S. mutans* (A) or *S. sanguinis* (B) were treated with M8-33, C16-33, or S6L3-33 alone (as specified in the figure) for 1 min. After agent removal and stringent washing, the regrowth of the biofilms was tracked over 4 h by measuring absorbance at OD₆₀₀ after the addition of fresh medium. The data represent average values obtained from the results of at least three independent assays.

porarily effective to various degrees, the unavoidable loss of normal flora that occurs with mechanical removal or general antibiotic treatment allows *S. mutans* to reestablish a niche in the oral cavity without difficulty (10, 20, 31). Therefore, a STAMP with *S. mutans*-selective killing ability would be an ideal solution and could allow the normal flora to outgrow affected *S. mutans* populations. Such an "antibiotic-probiotic" therapeutic could help prevent dental caries progression and the high health care costs associated with this disease (3).

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REFERENCES

- Aas, J. A., B. J. Paster, L. N. Stokes, I. Olsen, and F. E. Dewhirst. 2005. Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* **43**:5721–5732.
- Ajdic, D., W. M. McShan, R. E. McLaughlin, G. Savic, J. Chang, M. B. Carson, C. Primeaux, R. Tian, S. Kenton, H. Jia, S. Lin, Y. Qian, S. Li, H. Zhu, F. Najjar, H. Lai, J. White, B. A. Roe, and J. J. Ferretti. 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc. Natl. Acad. Sci. USA* **99**:14434–14439.
- Anderson, M. H., and W. Shi. 2006. A probiotic approach to caries management. *Pediatr. Dent.* **28**:151–153.
- Anonymous. 2002. Infectious diseases—dental caries. Washington State Department of Health, Olympia, Wash.
- Anonymous. 2000. Oral health in America: a report of the Surgeon General. Department of Health and Human Services, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Md.
- Axelsson, P., and J. Lindhe. 1987. Efficacy of mouthrinses in inhibiting dental plaque and gingivitis in man. *J. Clin. Periodontol.* **14**:205–212.
- Beighton, D. 2005. The complex oral microflora of high-risk individuals and groups and its role in the caries process. *Community Dent. Oral Epidemiol.* **33**:248–255.
- Bleher, D. S., R. J. Palmer, Jr., J. B. Xavier, J. S. Almeida, and P. E. Kolenbrander. 2003. Autoinducer 2 production by *Streptococcus gordonii* DL1 and the biofilm phenotype of a *luxS* mutant are influenced by nutritional conditions. *J. Bacteriol.* **185**:4851–4860.
- Boman, H. G. 2000. Innate immunity and the normal microflora. *Immunol. Rev.* **173**:5–16.
- Caufield, P. W., A. P. Dasanayake, Y. Li, Y. Pan, J. Hsu, and J. M. Hardin. 2000. Natural history of *Streptococcus sanguinis* in the oral cavity of infants: evidence for a discrete window of infectivity. *Infect. Immun.* **68**:4018–4023.
- Chandler, J. R., and G. M. Dunny. 2004. Enterococcal peptide sex pheromones: synthesis and control of biological activity. *Peptides* **25**:1377–1388.
- Corby, P. M., J. Lyons-Weiler, W. A. Bretz, T. C. Hart, J. A. Aas, T. Boumenna, J. Goss, A. L. Corby, H. M. Junior, R. J. Weyant, and B. J. Paster. 2005. Microbial risk indicators of early childhood caries. *J. Clin. Microbiol.* **43**:5753–5759.
- Dirix, G., P. Monsieurs, B. Dombrecht, R. Daniels, K. Marchal, J. Vanderleyden, and J. Michiels. 2004. Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. *Peptides* **25**:1425–1440.
- Donlan, R. M., and J. W. Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15**:167–193.
- Eckert, R., F. Qi, D. K. Yarbrough, J. He, M. H. Anderson, and W. Shi. 2006. Adding selectivity to antimicrobial peptides: rational design of a multidomain peptide against *Pseudomonas* spp. *Antimicrob. Agents Chemother.* **50**:1480–1488.
- Fischer, R., O. Mader, G. Jung, and R. Brock. 2003. Extending the applicability of carboxyfluorescein in solid-phase synthesis. *Bioconj. Chem.* **14**:653–660.
- Fux, C. A., P. Stoodley, L. Hall-Stoodley, and J. W. Costerton. 2003. Bacterial biofilms: a diagnostic and therapeutic challenge. *Expert Rev. Anti-Infect. Ther.* **1**:667–683.
- Gilmour, M. N., T. S. Whittam, M. Kilian, and R. K. Selander. 1987. Genetic relationships among the oral streptococci. *J. Bacteriol.* **169**:5247–5257.
- Huang, M. Y., and J. H. Wang. 2003. Impact of antibiotic use on fungus colonization in patients hospitalized due to fever. *J. Microbiol. Immunol. Infect.* **36**:123–128.
- Keene, H. J., and I. L. Shklar. 1974. Relationship of *Streptococcus mutans* carrier status to the development of carious lesions in initially caries-free recruits. *J. Dent. Res.* **53**:1295.
- Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and W. M. de Vos. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol. Microbiol.* **24**:895–904.
- Kreth, J., J. Merritt, W. Shi, and F. Qi. 2005. Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Mol. Microbiol.* **57**:392–404.
- Li, Y. H., P. C. Lau, J. H. Lee, R. P. Ellen, and D. G. Cvitkovitch. 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J. Bacteriol.* **183**:897–908.
- Loesche, W. J. 1986. The identification of bacteria associated with periodontal disease and dental caries by enzymatic methods. *Oral Microbiol. Immunol.* **1**:65–72.
- Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* **50**:353–380.
- Loesche, W. J., A. Walenga, and P. Loos. 1973. Recovery of *Streptococcus mutans* and *Streptococcus sanguis* from a dental explorer after clinical examination of single human teeth. *Arch. Oral Biol.* **18**:571–575.
- Mandel, I. D. 1988. Chemotherapeutic agents for controlling plaque and gingivitis. *J. Clin. Periodontol.* **15**:488–498.
- Marsh, P., and M. V. Martin. 1999. Oral microbiology, 4th ed. Wright, Oxford, United Kingdom.
- Merritt, J., J. Kreth, F. Qi, R. Sullivan, and W. Shi. 2005. Non-disruptive, real-time analyses of the metabolic status and viability of *Streptococcus mutans* cells in response to antimicrobial treatments. *J. Microbiol. Methods* **61**:161–170.
- Merritt, J., F. Qi, and W. Shi. 2005. A unique nine-gene comY operon in *Streptococcus mutans*. *Microbiology* **151**:157–166.
- Mikx, F. H., J. S. Van Der Hoeven, A. J. Plasschaert, and K. G. Konig. 1975. Effect of *Actinomyces viscosus* on the establishment and symbiosis of *Streptococcus mutans* and *Streptococcus sanguis* in SPF rats on different sucrose diets. *Caries Res.* **9**:1–20.
- Mitchell, T. J. 2003. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat. Rev. Microbiol.* **1**:219–230.
- Otto, M. 2001. *Staphylococcus aureus* and *Staphylococcus epidermidis* peptide pheromones produced by the accessory gene regulator agr system. *Peptides* **22**:1603–1608.
- Perron, G. G., M. Zasloff, and G. Bell. 2006. Experimental evolution of resistance to an antimicrobial peptide. *Proc. Biol. Sci.* **273**:251–256.
- Pickard, K. M., A. R. Bremner, J. N. Gordon, and T. T. MacDonald. 2004. Microbial-gut interactions in health and disease. Immune responses. *Best Pract. Res. Clin. Gastroenterol.* **18**:271–285.
- Pultz, N. J., C. K. Høyen, and C. J. Donskey. 2004. Inhibition of methicillin-resistant *Staphylococcus aureus* by an in vitro continuous-flow culture containing human stool microflora. *FEMS Microbiol. Lett.* **241**:201–205.
- Qi, F., J. Kreth, C. M. Levesque, O. Kay, R. W. Mair, W. Shi, D. G. Cvitkovitch, and S. D. Goodman. 2005. Peptide pheromone induced cell death of *Streptococcus mutans*. *FEMS Microbiol. Lett.* **251**:321–326.
- Qiu, X. Q., J. Zhang, H. Wang, and G. Y. Wu. 2005. A novel engineered peptide, a narrow-spectrum antibiotic, is effective against vancomycin-resistant *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* **49**:1184–1189.
- Qiu, X. Q., H. Wang, X. F. Lu, J. Zhang, S. F. Li, G. Cheng, L. Wan, L. Yang, J. Y. Zuo, Y. Q. Zhou, H. Y. Wang, X. Cheng, S. H. Zhang, Z. R. Ou, Z. C. Zhong, J. Q. Cheng, Y. P. Li, and G. Y. Wu. 2003. An engineered multidomain bactericidal peptide as a model for targeted antibiotics against specific bacteria. *Nat. Biotechnol.* **21**:1480–1485.
- Rastall, R. A. 2004. Bacteria in the gut: friends and foes and how to alter the balance. *J. Nutr.* **134**:2022S–2026S.
- Rhee, K. J., P. Sethupathi, A. Driks, D. K. Lanning, and K. L. Knight. 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J. Immunol.* **172**:1118–1124.
- Rhee, K. Y., R. Soave, and C. Maltz. 2004. Methicillin-resistant *Staphylococcus aureus* as a cause of antibiotic-associated diarrhea. *J. Clin. Gastroenterol.* **38**:299–300.
- Rogers, A. H. 1975. Bacteriocin types of *Streptococcus mutans* in human mouths. *Arch. Oral Biol.* **20**:853–858.
- Sansonetti, P. J. 2004. War and peace at mucosal surfaces. *Nat. Rev. Immunol.* **4**:953–964.
- Stewart, P. S. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol.* **292**:107–113.
- Stewart, P. S., and J. William Costerton. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet* **358**:135–138.
- Taskalova-Hogenova, H., R. Stepankova, T. Hudcovic, L. Tuckova, B. Cukrowska, R. Lodinova-Zadnikova, H. Kozakova, P. Rossmann, J. Bartova, D. Sokol, D. P. Funda, D. Borovska, Z. Rehakova, J. Sinkora, J. Hofman, P. Drastich, and A. Kokesova. 2004. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol. Lett.* **93**:97–108.
- Walker, C. B. 1988. Microbiological effects of mouthrinses containing antimicrobials. *J. Clin. Periodontol.* **15**:499–505.