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Systematic Approach to Optimizing Specifically Targeted Antimicrobial Peptides against *Streptococcus mutans*▽

Jian He,[1] Daniel K. Yarbrough,[2] Jens Kreth,[3] Maxwell H. Anderson,[2] Wenyuan Shi,[1]* and Randal Eckert[2]*

School of Dentistry, University of California, Los Angeles, California 90095[1]; C3 Jian Inc., Inglewood, California 90301[2]; and Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104[3]

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Previously we reported a novel strategy of "targeted killing" through the design of narrow-spectrum molecules known as specifically targeted antimicrobial peptides (STAMPs) (R. Eckert et al., Antimicrob. Agents Chemother. 50:3651–3657, 2006; R. Eckert et al., Antimicrob. Agents Chemother. 50:1480–1488, 2006). Construction of these molecules requires the identification and the subsequent utilization of two conjoined yet functionally independent peptide components: the targeting and killing regions. In this study, we sought to design and synthesize a large number of STAMPs targeting *Streptococcus mutans*, the primary etiologic agent of human dental caries, in order to identify candidate peptides with increased killing speed and selectivity compared with their unmodified precursor antimicrobial peptides (AMPs). We hypothesized that a combinatorial approach, utilizing a set number of AMP, targeting, and linker regions, would be an effective method for the identification of STAMPs with the desired level of activity. STAMPs composed of the Sm6 *S. mutans* binding peptide and the PL-135 AMP displayed selectivity at MICs after incubation for 18 to 24 h. A STAMP where PL-135 was replaced by the B-33 killing domain exhibited both selectivity and rapid killing within 1 min of exposure and displayed activity against multispecies biofilms grown in the presence of saliva. These results suggest that potent and selective STAMP molecules can be designed and improved via a tunable "building-block" approach.

Pathogenic microorganisms have been a continuous source of human suffering and mortality throughout the course of human history and have spurred the clinical development of novel therapeutics. Even today, the overall burden of infectious disease remains high, constituting a leading (and rising) cause of death worldwide (16, 18). The conventional medical response to bacterial infections, administration of small-molecule antibiotics, has become less effective against emerging pathogens due to the evolution of drug resistance stemming in part from the misuse of antibiotics (13). Additionally, antibiotics and oral antiseptics currently in use to treat mucosal infections eliminate pathogens and bystander bacteria alike, an outcome that can be associated with negative clinical consequences (15, 17). Therefore, there is an unmet medical need to develop novel, narrow-spectrum therapeutics capable of maintaining the protective benefits of the normal microflora during treatment.

Our strategy for creating novel, selective antibacterial agents is based on the addition of a targeting peptide to an existing broad-spectrum antimicrobial peptide (AMP), thereby generating a specifically targeted antimicrobial peptide (STAMP) selective for a particular bacterial species or strain. A completed STAMP consists of conjoined but functionally independent targeting and killing regions, separated by a small flexible linker, all within a linear peptide sequence. The STAMP targeting region drives enhancement of antimicrobial activity by increasing binding to the surface of a targeted pathogen, utilizing specific determinants such as overall membrane hydrophobicity, charge, and/or pheromone receptors, which in turn leads to increased selective accumulation of the killing moiety (6, 7).

As both the killing and targeting regions of the STAMP are linear peptides, we approached the design process using a tunable combinatorial methodology where, for example, the targeting peptide component is held constant, while a number of killing peptides are conjoined utilizing a variety of linker molecules, or vice versa, in order to generate a library of related STAMPs. Previously, we successfully demonstrated a pilot version of this approach when constructing G10KHc (6), a STAMP with *Pseudomonas*-selective activity, and when designing C16G2 (7), a STAMP specific for *Streptococcus mutans*, the leading causative agent of human tooth decay.

In this study, synthetic targeting and antimicrobial peptide libraries were utilized as building blocks to generate a number of novel STAMPs with *S. mutans*-selective activity. STAMPs designed by these methods were then improved through tuning the linker and killing peptides present to yield completed lead STAMP molecules that demonstrated activity against *S. mutans* biofilms.

MATERIALS AND METHODS

Reagents. Wang resin, Rink–4-methylbenzhydrylamine (MBHA) resin, 9-fluorenylmethoxycarbonyl (Fmoc) amino acids, *N*-hydroxybenzotriazole hydrate (HOBT), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Anaspec (San Jose, CA). All other solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA) and were of high-pressure liquid chromatography (HPLC) or peptide synthesis grade.

* Corresponding author. Mailing address for R. Eckert: C3 Jian Inc., 423 Hindry Ave., Ste. D, Inglewood, CA 90301. Phone: (310) 665-2928. Fax: (310) 665-2963. E-mail: reckert@c3-jian.com. E-mail address for W. Shi: wenyuan@ucla.edu.

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PerioGard (chlorhexidine gluconate oral rinse; Colgate-Palmolive, New York, NY) was utilized as 0.12% chlorhexidine where noted.

Bacterial growth. *S. mutans* wild-type UA159 (1) and JM11 (spectinomycin resistant; constructed from UA140) (6), *Streptococcus gordonii* Challis (DL1), *Streptococcus sobrinus* ATCC 33478, *Streptococcus mitis* ATCC 903, and *Streptococcus sanguinis* NY101 strains were grown in brain heart infusion (BHI) or Todd-Hewitt (TH) medium at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) (6). *Pseudomonas aeruginosa* (PAK) (22) and *Escherichia coli* W3110 (25) strains were cultured in Luria-Bertani (LB) medium in an aerobic atmosphere at 37°C. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) were grown in BHI medium under aerobic conditions at 37°C (4).

Peptide synthesis and purification. Peptides were synthesized using standard solid-phase (Fmoc) chemistry with an Apex 396 peptide synthesizer (Aapptec, Louisville, KY) at a 0.01 mM scale. N-terminal deblocking was conducted with 0.6 ml of 25% (vol/vol) piperidine in dimethylformamide (DMF), followed by agitation for 27 min and wash cycles with dichlormethane (DCM) (1 ml; one wash cycle) and *N*-methylpyrrolidone (NMP) (0.8 ml; seven wash cycles). Subsequent amino acid coupling cycles were conducted with a mixture of Fmoc-protected amino acid (5 eq), HOBT (5 eq), HBTU (5 eq), *N,N*-diisopropylethylamine (DIEA; 10 eq)–DMF (0.1 ml), and NMP (0.2 ml) with agitation for 45 min. The washing cycle was repeated before the next round of deprotection and coupling. After synthesis, peptides were washed in methanol and dried for 24 h. Protected peptides were cleaved with 1 ml of trifluoroacetic acid (TFA)–thioanisole–water–1,2-ethanedithiol (10 ml:0.5 ml:0.5 ml:0.25 ml) for 3 h at room temperature and the resultant peptide solution was precipitated in methyl tert-butyl ether.

Analytical and preparative HPLC was conducted as described previously (5, 11) to refine each peptide to 80 to 90% purity. Correct peptide mass values were confirmed by matrix-assisted laser desorption ionization (MALDI) (Voyager 4219 workstation; Applied Biosystems, Foster City, CA) or electrospray ionization (ESI) mass spectroscopy (Waters 3100 mass detector; Waters, Milford, MA) as described previously (11). Measurements were made in linear, positive ion mode with an α-cyano-4-hydroxycinnamic acid matrix where appropriate (data not shown). Fluorescent labels were added to the peptide N terminus as described previously (7). Briefly, a 4 molar excess of 5,6-carboxyfluorescein was added to the resin in 10-eq DIEA for 2 h after assembly of the linear sequence prior to cleavage.

Binding of targeting peptides to biofilms. The binding of targeting peptides and STAMPs was assessed by fluorescence microscopy. *S. mutans* UA159 was grown overnight and diluted at 1:5,000 in fresh TH medium with 1% sucrose before 400 μl was seeded to 48-well flat-bottom plates (Costar, Lowell, MA). Biofilms were grown for 24 h, and the spent medium was replaced with buffer (10 mM $NaHCO_3$, 20 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% cetyltrimethylammonium bromide [CTAB], pH 7.4) containing 25 μM peptide. After 3 min of incubation at room temperature, the supernatants were removed and the biofilms washed twice with buffer prior to the acquisition of bright-field and green fluorescence images (Nikon E400 microscope) (green channel exposure, 350 ms). Digital images were collected and analyzed with the software supplied by the manufacturer (SPOT; Diagnostics) (6). To quantitate binding, biofilm-associated green pixels were selected from each image by the use of Gimp software (http://www.gimp.org) and expressed as binding intensity units, as described previously (14).

MICs of peptides. Peptide MICs were determined by broth microdilution (6, 20). Briefly, 2-fold serial dilutions of each peptide were prepared with 50% BHI medium–50% sterile water (for oral streptococci; all other bacteria were diluted in 1× Mueller-Hinton broth) at a volume of 100 μl per well in 96-well flat-bottom microtiter plates. The concentrations of peptides for the first test round ranged from 500 to 0.97 μg/ml. If activity was detected below 62.5 μg/ml, a second round of MIC tests with concentrations of 64 to 0.5 μg/ml was conducted in some cases, and the mode of the results from the second round was reported. In either case, the microtiter plate was inoculated with a bacterial cell suspension at a final concentration of $\sim 1 \times 10^5$ CFU/ml and incubated at 37°C for 16 to 20 h under the appropriate conditions. After incubation, absorbance at 600 nm (A_{600}) was measured using a microplate UV-Vis spectrophotometer (model 3550; Bio-Rad, Hercules, CA) to assess cell growth. The MIC endpoint was calculated as the lowest concentration of antibacterial agent that completely inhibited growth or that produced an at least 90% reduction in turbidity compared with that of a peptide-free control. At least 3 independent tests were conducted per peptide. For peptides insoluble in aqueous solutions, stock solutions were prepared in 50% methanol and appropriate solvent controls were utilized. Cell growth was not affected by 5% (vol/vol) methanol, as described previously (12).

Peptide killing kinetics. To determine antimicrobial kinetics and specificity, assays similar to traditional time-kill experiments were performed, as described previously (6, 7). Briefly, overnight bacterial cultures were diluted in BHI medium to an A_{600} of 0.08 and peptides were added as indicated. Aliquots were then removed at various intervals and diluted at 1:50 in BHI medium and kept on ice until being plated on appropriate growth medium. After 24 h of incubation, colonies were counted and the numbers of surviving colony-forming units per milliliter determined. All assays were repeated at least three times; the average numbers of recovered colony-forming units per milliliter and standard deviations were determined. Statistical analysis was conducted utilizing an unpaired Student *t* test.

Activity against monoculture *S. mutans* biofilms. STAMPs were tested for antibiofilm activity as described previously (6). Briefly, overnight cultures of *S. mutans* were diluted 1:50 in TH broth medium supplemented with 0.5% (wt/vol) sucrose and 100 μl of bacterial suspension was added to each well of a 96-well microtiter plate. After centrifugation, bacteria were then incubated under anaerobic conditions at 37°C for 4 h. Supernatants were then removed and replaced with 25 μM peptide–1× phosphate-buffered saline (PBS) for 30 s to 1 min, followed by removal, washing, and replacement with 100 μl of fresh TH broth (without sucrose). Plates were then incubated at 37°C under anaerobic conditions, and the bacterial recovery was monitored by recording A_{600} values after 4 h of incubation. An unpaired Student *t* test was utilized for statistical analysis.

Activity against multispecies biofilms. Defined mixed-species biofilms were grown in 48-well flat-bottom plates (400 μl per well) in TH medium supplemented with 50% (vol/vol) filter-sterilized human saliva (pooled from healthy volunteers) and 1% sucrose. Biofilms were inoculated with *S. mutans* JM11 (grown overnight and seeded at a final concentration of 1×10^6 CFU/well) and *Streptococcus oralis*, *S. gordonii*, *S. sanguinis*, *S. mitis*, and *S. salivarius* (grown overnight and adjusted to 2×10^5 CFU/well each). Biofilms were incubated 24 h at 37°C under anaerobic conditions. After growth, biofilms were washed once with 1× PBS to remove loose aggregates and treated with 50 μM peptide–200 μl of 1× PBS (or with a commercial agent) for 10 min. Posttreatment, biofilms were mechanically detached and disrupted by scraping and agitation followed by resuspension in 100 μl of 1× PBS. Suspensions were serially diluted and plated on TH agar (to measure total numbers of surviving oral streptococci) and on TH agar supplemented with 800 μg/ml spectinomycin (to quantitate numbers of *S. mutans* JM11 colony-forming units per milliliter). The detection level of the assay was 100 CFU/ml. The antimicrobial effects of peptide against total biofilm or *S. mutans* populations and the ratio of surviving *S. mutans* bacteria to total numbers of oral streptococci were then calculated.

RESULTS

STAMPs consist of 3 regions: one targeting region and one antimicrobial region, connected via a flexible linker region. For this report, we conjoined examples of each to construct a pool of initial STAMP candidates. These peptides were then evaluated for anti-*S. mutans* activity and selectivity, their design was improved, and the activity of the resultant STAMPs against *S. mutans* and mixed-species biofilms was evaluated.

Selection of components and STAMP library 1 design. As described elsewhere, we generated several novel *S. mutans*-specific binding peptides, including Sm8 (previously S3L1-10 [FIKDFIERF]) and Sm4 (previously S3L1-5 [WWYNWWQDW]) (7). In order to generate additional potential *S. mutans* targeting domains, residues differing in hydrophobicity and/or charge were replaced at defined positions with respect to these base sequences to yield a series of related peptides that were then evaluated for binding to *S. mutans* biofilms (a list is presented in Table 1). As shown in Fig. 1, several of the variants were found to retain biofilm binding, whereas Bc1 ([AAKHAAHRA]), a control peptide not related to Sm4 or Sm8, failed to bind to *S. mutans*. Therefore, peptides Sm1, Sm2, Sm3, Sm5, Sm6, and Sm7, as well as peptide Sm4, were regarded in the present study as the pool of *S. mutans* targeting vectors for library 1 STAMP construction. For the antimicrobial component, we selected PL-135, a short peptide based on an AMP isolated from tunicates (24), for the initial round of design. We hypothesized that linker regions and attachment

TABLE 1. STAMP constituent regions utilized in STAMP libraries 1 and 2

Targeting peptide (name)	Linker (name)	Antimicrobial peptide (name)
Library 1		
WWHSWWSTW (Sm1)	GGG (L1)	FHFHLHF (PL-135)
WWSYWWTQW (Sm2)	SAT (L3)	
WWKDWWERW (Sm3)	ASASA (L5)	
WWYNWWQDW (Sm4)	PYP (L7)	
WWQDWWNEW (Sm5)	PSGSP (L8)	
FIKHFIHRF (Sm6)	PSPSP (L9)	
LIKHILHRL (Sm7)		
Library 2		
FIKHFIHRF (Sm6)	GGG (L1)	RWWRWF (2C-4)
	SAT (L3)	RWRRLLKKLHHLLH (α-11)
	SGG (L6)	LQLLKQLLIKLLKQF (α-7)
	$NH(CH_2)_7CO$(LC)	IKQLLHFFQRF (B-38)
		FKKFWKWFRRF (B-33)

orientations would exert an influence on STAMP activity. Therefore, we conjugated each potential targeting peptide to the N or C terminus of PL-135 through six different linkers (GGG [designated L1], SAT [L3], ASASA [L5], PYP [L7], PSGSP [L8], and PSPSP [L9]), as shown in Table 1, leading to the synthesis of 84 STAMPs.

Activity of STAMP library 1. To roughly gauge STAMP antimicrobial activity and *S. mutans* selectivity, MIC assays were conducted with *S. mutans* and a panel of bacteria, including two oral *Streptococcus* species, *S. sanguinis* and *S. sobrinus* (Table 2). Of the 84 molecules, STAMPs containing Sm6 conjoined to the C terminus of PL-135 [PL(L1)Sm6, PL(L3)Sma6, PL(L5)Sm6, PL(L7)Sm6, PL(L8)Sm6, and PL(L9)Sm6] or Sm7 conjoined to the N terminus of PL-135 [Sm7(L1)PL] were found to be active against *S. mutans* at concentrations lower than 100 μg/ml. These peptides were more active (two to four 2-fold-dilution steps) against *S. mutans* than against the other oral streptococci or the nonoral organisms tested. In

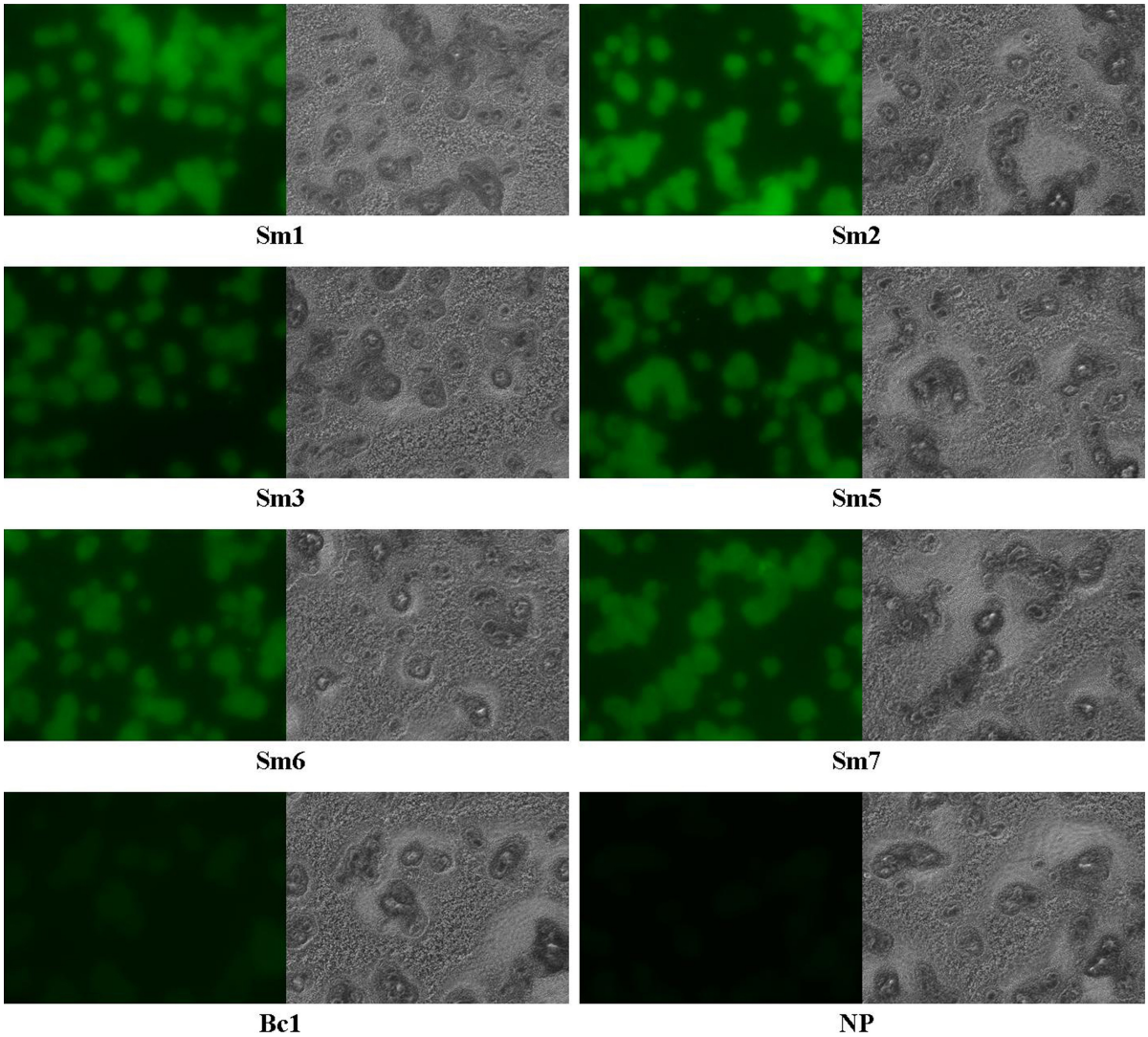

FIG. 1. Binding of targeting peptides to *S. mutans* biofilms. Fluorescently labeled peptides were evaluated for binding to *S. mutans* biofilms. After incubation with 25 μM peptide, biofilms were washed repeatedly prior to acquisition of bright-field (right panels) and fluorescence (left panels) images from the same field (×20 magnification). NP, no peptide.

TABLE 2. MICs of active[a] library 1 and library 2 STAMPs

Peptide	Sequence[b]	MIC (μg/ml)[c]								
		S. mu	S. sob	S. san	S. mit	S. gor	MRSA	EF	PA	*E. coli*
Targeting										
Sm1	WWHSWWSTW	125	125	125	125	125	>125	>125	>125	125
Sm2	WWSYWWTQW	125	125	125	125	125	>125	>125	>125	125
Sm3	WWKDWWERW	>500	>500	>500	>500	>500	>500	>500	>500	>500
Sm4	WWYNWWQDW	>500	>500	>500	>500	>500	>500	>500	>500	>500
Sm5	WWQDWWNEW	>500	>500	>500	>500	>500	>500	>500	>500	>500
Sm6	FIKHFIHRF	125	125	125	>500	>500	>500	>500	>500	>500
Sm7	LIKHILHRL	125	>500	>500	>500	>500	>500	>500	>500	>500
Bc1	AAKHAAHRA	>500	>500	>500	>500	>500	>500	>500	>500	>500
Library 1										
PL(L7)Sm6	FHFHLHFPYPFIKHFIHRF	32	62.5	62.5	nt[e]	nt	>500	250	125	125
PL(L3)Sm6	FHFHLHFSATFIKHFIHRF	16	250	62.5	nt	nt	125	62.5	125	125
PL(L1)Sm6	FHFHLHFGGGFIKHFIHRF	32	62.5	62.5	nt	nt	125	62.5	62.5	62.5
PL(L8)Sm6	FHFHLHFPSGSPFIKHFIHRF	62.5	62.5	62.5	nt	nt	>500	250	>500	125
PL(L9)Sm6	FHFHLHFPSPSPFIKHFIHRF	32	125	125	nt	nt	>500	250	125	62.5
PL(L5)Sm6	FHFHLHFASASAFIKHFIHRF	16	32	32	nt	nt	62.5	62.5	125	62.5
Sm7(L1)PL	LIKHILHRLGGGFHFHLHF	16	32	62.5	nt	nt	62.5	32	250	250
PL-135	FHFHLHF	8	nt	16	nt	nt	32	16	16	8
Library 2										
2C-4[d]	RWRWRWF	4–8	nt	32	32	32	32	nt	32	nt
Sm6(L1)2C	FIKHFIHRFGGGRWRWRWF	8	nt	32	32	32	32	nt	62.5	nt
Sm6(L6)2C	FIKHFIHRFSGGRWRWRWF	8	nt	32	32	32	32	nt	62.5	nt
Sm6(L3)2C	FIKHFIHRFSATRWRWRWF	8	nt	32	16	32	125	nt	62.5	nt
Sm6(LC)2C	FIKHFIHRF-[NH(CH_2)$_7$CO]-RWRWRWF	8	nt	32	16	32	32	nt	62.5	nt
2C(L1)Sm6	RWRWRWFGGGFIKHFIHRF	8	nt	32	32	32	32	nt	62.5	nt
2C(L6)Sm6	RWRWRWFSGGFIKHFIHRF	8	nt	32	32	32	32	nt	62.5	nt
2C(L3)Sm6	RWRWRWFSATFIKHFIHRF	8	nt	32	16	62.5	62.5	nt	62.5	nt
2C(LC)Sm6	RWRWRWF-[NH(CH_2)$_7$CO]-FIKHFIHRF	32	nt	32	32	62.5	62.5	nt	125	nt
α-11[d]	RWRRLLKKLHHLLH	8	nt	16	32	16	16	nt	8	nt
Sm6(LC)α11	FIKHFIHRF-[NH(CH_2)$_7$CO]-RWRRLLKKLHHLLH	16	nt	32	32	32	62.5	nt	62.5	nt
Sm6(L3)α11	FIKHFIHRFSATRWRRLLKKLHHLLH	16	nt	32	32	32	16	nt	32	nt
Sm6(L6)α11	FIKHFIHRFSGGRWRRLLKKLHHLLH	16	nt	16	62.5	32	32	nt	32	nt
α11(LC)Sm6	RWRRLLKKLHHLLH-[NH(CH_2)$_7$CO]-FIKHFIHRF	8	nt	16	32	16	62.5	nt	32	nt
α11(L3)Sm6	RWRRLLKKLHHLLHSATFIKHFIHRF	8	nt	16	16	16	32	nt	32	nt
α11(L6)Sm6	RWRRLLKKLHHLLHSGGFIKHFIHRF	8	nt	32	32	16	32	nt	32	nt
α-7[d]	LQLLKQLLKLLKQF	8	nt	8	16	8	16	nt	8	nt
Sm6(LC)α7	FIKHFIHRF-[NH(CH_2)$_7$CO]-LQLLKQLLKLLKQF	16	nt	32	62.5	32	62.5	nt	62.5	nt
Sm6(L3)α7	FIKHFIHRFSATLQLLKQLLKLLKQF	16	nt	16	32	8	62.5	nt	32	nt
Sm6(L6)α7	FIKHFIHRFSGGLQLLKQLLKLLKQF	16	nt	32	62.5	8	32	nt	32	nt
α7(LC)Sm6	LQLLKQLLKLLKQF-[NH(CH_2)$_7$CO]-FIKHFIHRF	8	nt	32	32	16	62.5	nt	32	nt
α7(L3)Sm6	LQLLKQLLKLLKQFSATFIKHFIHRF	8	nt	16	32	16	32	nt	32	nt
α7(L6)Sm6	LQLLKQLLKLLKQFSGGFIKHFIHRF	8	nt	16	32	16	32	nt	32	nt
B-38[d]	IKQLLHFFQRF	24	nt	62.5	62.5	32	62.5	nt	62.5	nt
B38(L1)Sm6	IKQLLHFFQRFGGGFIKHFIHRF	8	nt	32	16	32	62.5	nt	125	nt
Sm6(L1)B38	FIKHFIHRFGGGIKQLLHFFQRF	8	nt	32	16	32	62.5	nt	125	nt
B-33[d]	FKKFWKWFRRF	8–24	nt	32	16	16	16	nt	32	nt
Sm6(L1)B33	FIKHFIHRFGGGFKKFWKWFRRF	4	nt	16	32	62.5	16	nt	62.5	nt
B33(L1)Sm6	FKKFWKWFRRFGGGFIKHFIHRF	8	nt	32	62.5	125	32	nt	125	nt

[a] "Active" refers to a MIC of <100 μg/ml against *S. mutans*.
[b] All amidated C termini.
[c] MICs represent the modes of the results obtained with at least three independent experiments. Strains: S. mu, *S. mutans* UA159; S. gor, *S. gordonii*; S. san, *S. sanguinis*; S. mit, *S. mitis*; S. sob, *S. sobrinus*; EF, *Enterococcus faecalis*; PA, *P. aeruginosa*.
[d] Replotted from reference 12.
[e] nt, not tested.

contrast, native PL-135 had similar MICs for all strains examined (Table 2).

Further potential of PL-135-based STAMPs. Antiseptic oral rinses, such as chlorhexidine or Listerine (Johnson and Johnson, New Brunswick, NJ), are rapid-acting nonselective bactericidal agents that can inactivate bacteria within seconds of contact (3). In order for STAMPs to be useful oral rinse ingredients, the antimicrobial kinetics must approach this scale. Therefore, the killing kinetics of the lead library 1 STAMPs from Table 2 were evaluated (data not shown). The results indicate that these PL-135-containing STAMPs, although selective for *S. mutans* when measured by MIC, are not rapid killers of this bacterium *in vitro*, requiring several hours of exposure for observable antimicrobial activity. Therefore, we sought to improve our STAMP pool by substituting alternative AMP domains for *S. mutans* STAMP construction.

Library 2: tuning the design of Sm6-containing STAMPs. We conjugated Sm6 with RWRWRWF(2C-4), FKKFWKWF RRF(B-33), IKQLLHFFQRF(B-38), RWRRLLKKLHHLLH (α-11), and LQLLKQLLKLLKQF(α-7) (attached at the C or N terminus), five AMPs selected from our previous studies (12), to construct library 2. The linkers selected to make a total of 40 STAMPs were L1, SGG (L6), L3, and LC (8-amino caprylic acid) (Table 1).

As shown in Table 2, over half the library 2 STAMPs (n = 24) had MICs under 100 μg/ml for *S. mutans* (unlike library 1). Additionally, MICs were improved 2- to 8-fold compared with active PL-135-containing constructs. Within library 2, little dif-

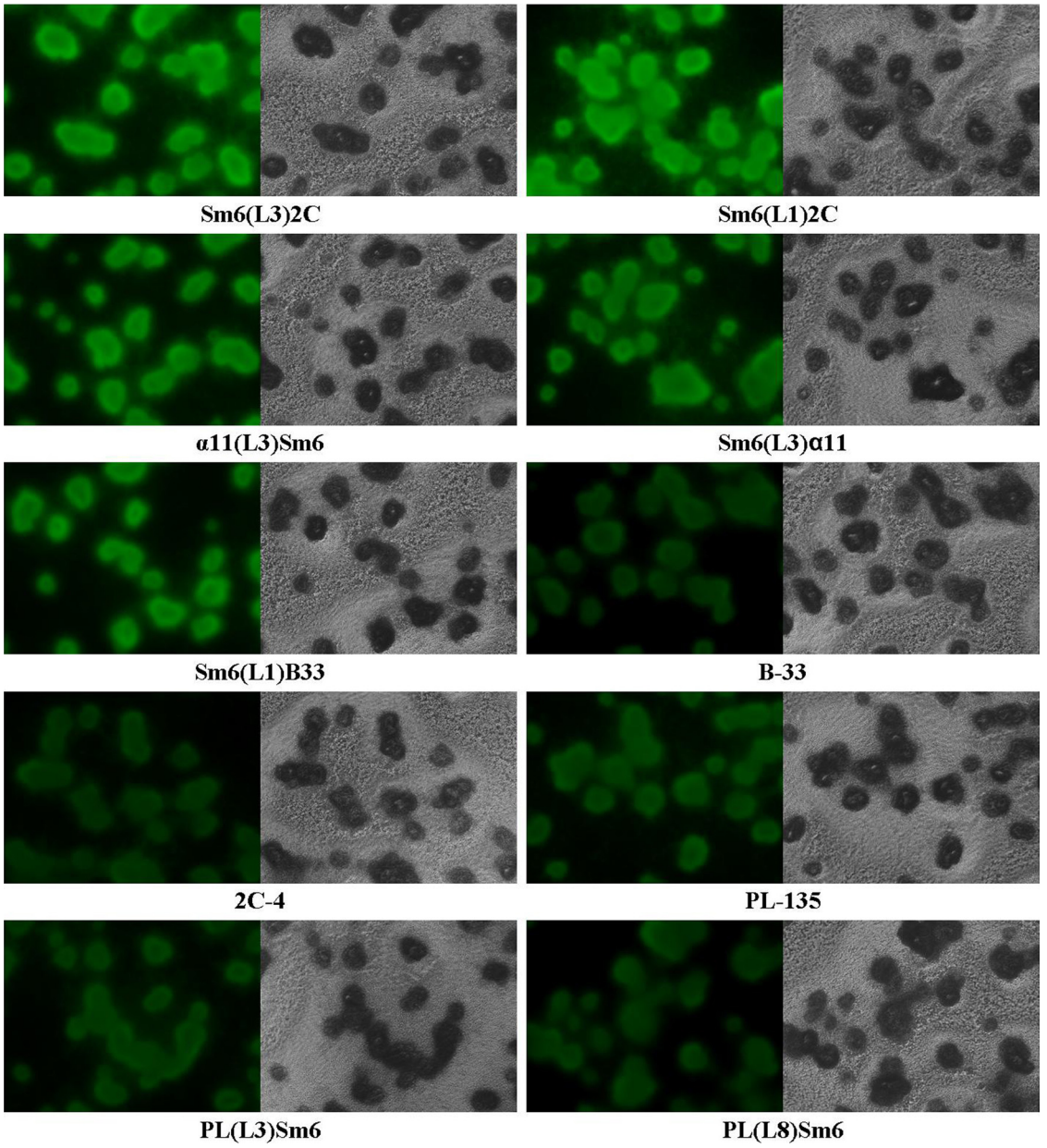

FIG. 2. Binding of STAMPs and AMPs to *S. mutans* biofilms. *S. mutans* biofilms were grown for 24 h and exposed to 25 μM STAMP (or untargeted AMP B-33 or 2C-4) for 3 min. Postincubation, biofilms were washed repeatedly and bright-field (right panels) and fluorescence (left panels) images were acquired from the same field (×20 magnification).

ference in activity was observed between constructs where the targeting peptide was attached to the N or C terminus of the AMP region, and little MIC change between linkers employed was noted. It was also apparent that, in nearly all cases, these STAMPs were more active against *S. mutans* than against the other oral and nonoral bacteria tested. Peptide Sm6(L1)B33 demonstrated the lowest MIC mode at 4 μg/ml, which was an improvement over the MIC for the killing peptide alone (12).

Due to increased potency of library 2 versus library 1 constructs, we investigated the function of the Sm6 targeting region within each set of STAMPs by examining binding to *S. mutans* biofilms. As shown in Fig. 2 and 3, STAMPs from library 1 were found to have significantly lower binding intensities than library 2 constructs (Student's *t* test; $P < 0.01$). In addition, there was an obvious increase in library 2 STAMP binding to *S. mutans* biofilms versus parent AMP results [Fig. 2 and 3; compare B-33 or 2C-4 alone to Sm6(L1)2C, Sm6(L3)2C, or Sm6(L1)B33], suggesting that the targeting peptide was functioning as hypothesized. Interestingly, the biofilm labeling intensities for library 2 STAMPs where Sm6 was attached at the N or C terminus of the AMP region were similar [the results for Sm6(L3)α11 and α11(L3)Sm6 are shown as examples].

Taken together, these data suggest that library 2 STAMPs

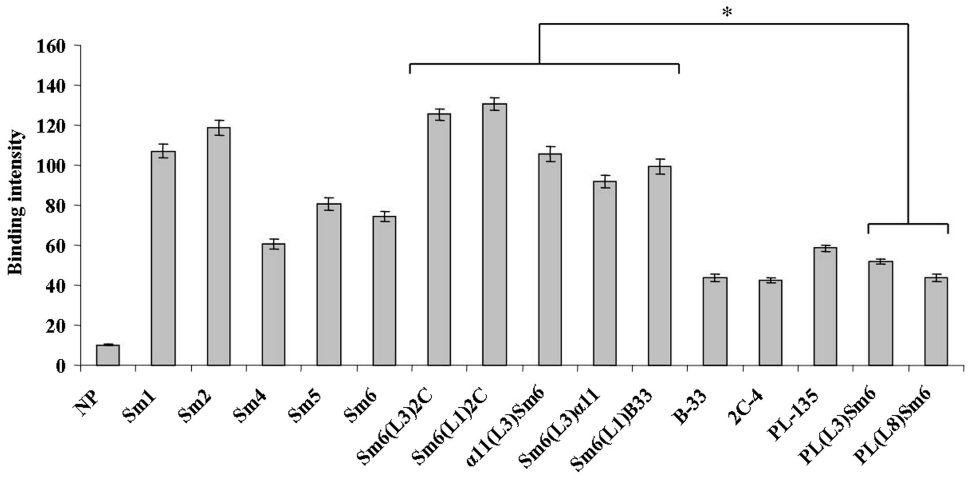

FIG. 3. Quantitative analysis of peptide binding to *S. mutans* biofilms. As described for Fig. 1 and 2, the binding of 25 μM targeting peptide, AMP, or STAMP to mature *S. mutans* biofilms was analyzed by fluorescence microscopy and units of bound peptide (representing levels of binding intensity) were quantified. Data represent at least three independent experiments with standard deviations. *, significant statistical difference between library 1 and library 2 STAMP results ($P < 0.01$; Student's *t* test).

can effectively inhibit the growth of *S. mutans* at generally improved potencies compared to the PL-135-containing STAMPs in library 1 and that Sm6-dependent biofilm binding is retained in potent STAMP constructs.

STAMP killing kinetics against oral bacteria. Since the MIC assay measures antimicrobial activity after overnight incubation, large differences in killing rates between STAMPs and parental AMPs may be obscured in this assay, especially when

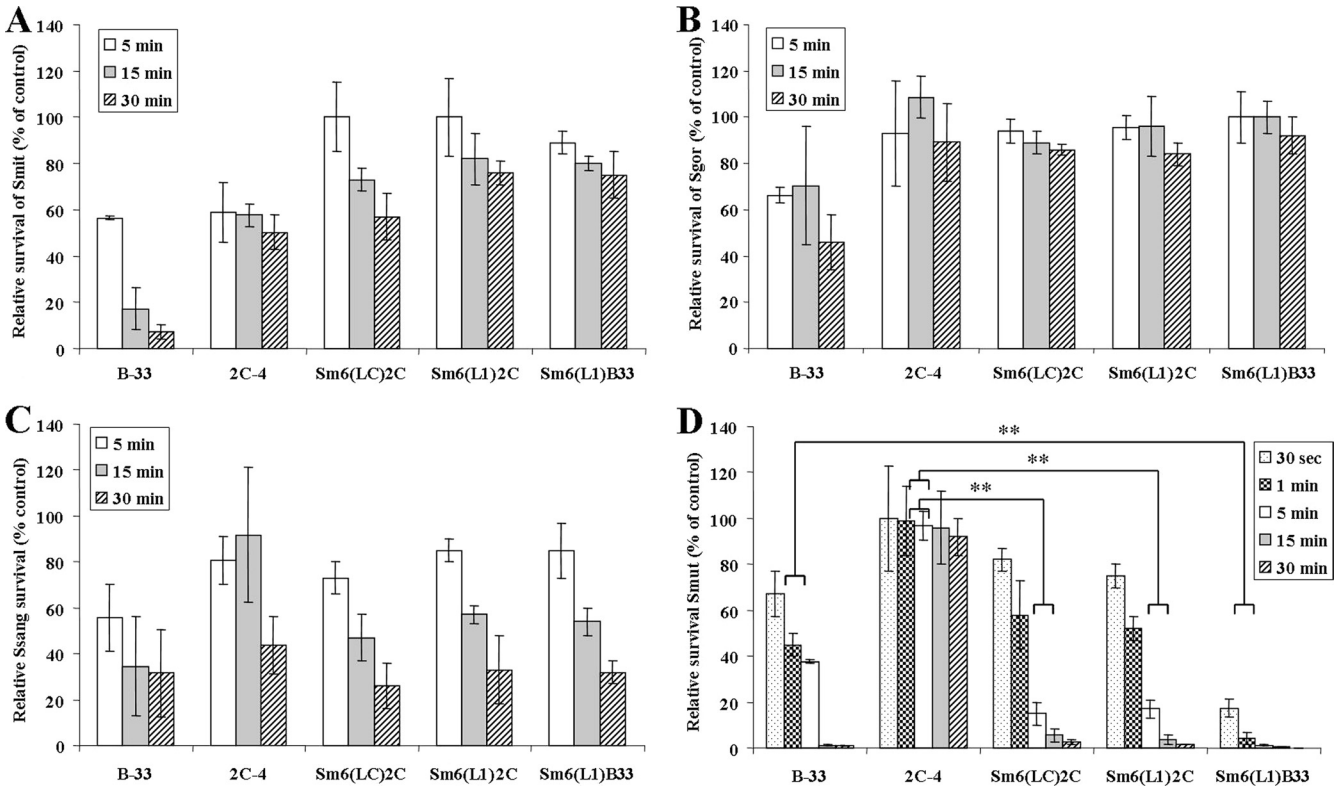

FIG. 4. Killing kinetics of selected peptides against oral streptococci. All tested bacteria, including *S. mitis* (Smit) (A), *S. gordonii* (Sgor) (B), *S. sanguinis* (Ssang) (C), and *S. mutans* UA159 (Smut) (D), were treated with peptide solutions at 25 μg/ml for 30 s to 2 h, and survivors were plated. Data represent averages of the results obtained with three independent experiments. **, significant statistical difference between STAMP- and parental AMP-treated sample results ($P < 0.001$; Student's *t* test).

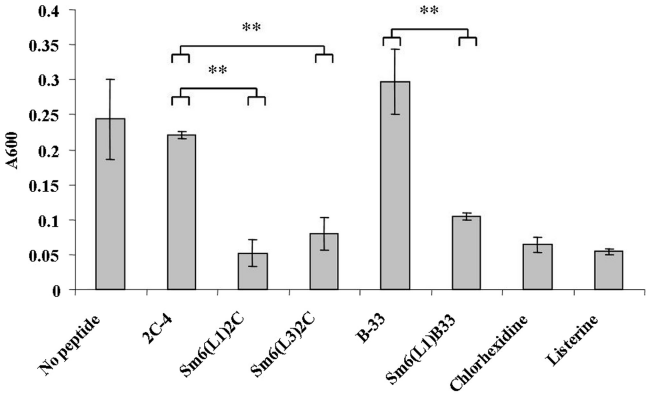

FIG. 5. Inhibitory activity of STAMPs against monoculture biofilms. *S. mutans* biofilms were grown and exposed to 25 μg/ml of STAMP, unmodified parental AMP, or oral antiseptic (for 1 min), washed, and replenished with fresh medium. Levels of bacterial recovery were monitored after 4 h and are quantified as A_{600} values. Data represent averages of the results obtained with three experiments. **, significant statistical difference between parental AMP- and STAMP-treated biofilm results ($P < 0.001$; Student's *t* test).

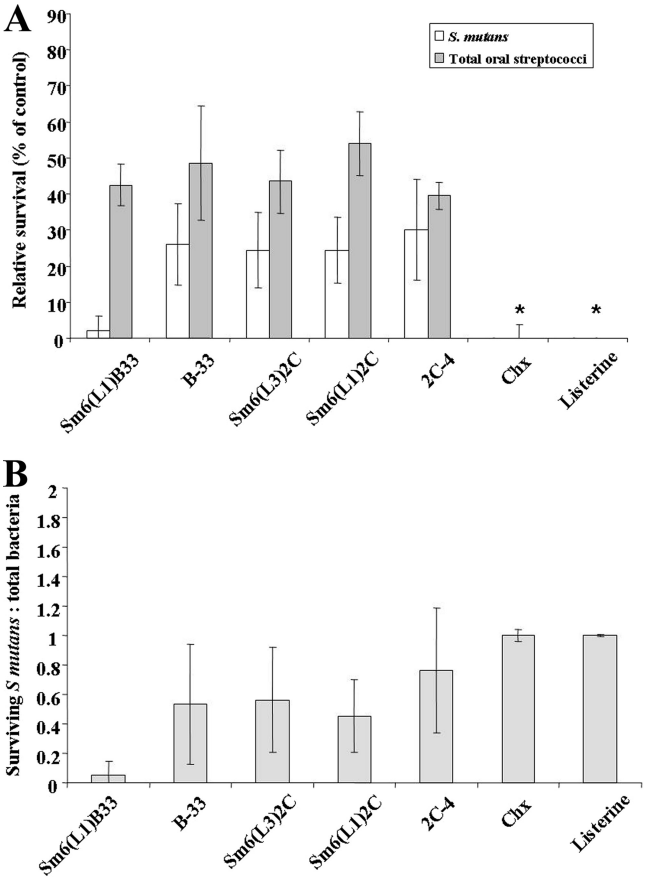

FIG. 6. Activity and selectivity of STAMPs against multispecies biofilms. Defined mixed-species biofilms of *S. mutans* and other oral streptococci were grown in 50% saliva prior to treatment with STAMP, parental AMP, or a commercial product. (A) Antimicrobial activity was calculated as the percentage of untreated *S. mutans* colony-forming units per milliliter or of total oral streptococci colony-forming units per milliliter recovered after treatment. (B) Selectivity was assessed as the ratio of surviving *S. mutans* bacteria to total oral bacteria. Data represent the averages of the results obtained with at least three independent experiments. *, fewer than 100 oral streptococci or *S. mutans* colony-forming units per milliliter detected.

the target organism is susceptible to the AMP (7, 11). To further assess any significant selectivity and short-term antimicrobial activity of the library 2 STAMPs, time-kill assays were performed using a variety of oral bacteria. Against the targeted bacterium *S. mutans* (examples shown in Fig. 4D), the STAMPs acted significantly faster than the killing peptide alone within 5 min of treatment [$P < 0.001$, comparing B-33 alone to Sm6(L1)B33 at 1 min or 2C-4 versus either 2C-4-containing STAMP at 5 min]. In contrast, other oral streptococci, such as *S. mitis* and *S. gordonii*, were less affected by STAMP treatments (Fig. 4A to C). Peptide Sm6(L1)B33 exhibited the fastest killing kinetics and best selectivity: killing was observed even when cells were treated for as little as 30s, a timescale more appropriate for oral cavity therapeutic applications. As expected from their wide spectra of activities (12), parental AMPs 2C-4 and B-33 had similar levels of activity against the strains examined.

Activity against monoculture *S. mutans* biofilms. Although rapid killing of *S. mutans* planktonic monocultures was apparent from the data shown in Fig. 4, it remained unclear whether these STAMPs would make suitable antimicrobial agents in the oral cavity, where dental plaque biofilms predominate (21, 23). To investigate, *S. mutans* biofilms were treated with STAMP, Listerine, or 0.12% chlorhexidine and the postantibiotic effect was observed after 4 h. As shown in Fig. 5, STAMPs Sm6(L1)2C, Sm6(L3)2C, and Sm6(L1)B33 were found to significantly ($P < 0.001$) inhibit the viability of biofilms when cells were treated with the peptide for 1 min at 25 μg/ml, compared to the viability seen with mock-treated biofilms or biofilms treated with untargeted AMP. Similar antimicrobial effects were observed for Listerine and chlorhexidine. These results suggest that STAMP treatment results in a level of *S. mutans* biofilm killing similar to that observed with established wide-spectrum oral antiseptics.

Activity against mixed-species biofilms. To fully evaluate selectivity and activity, mature biofilms consisting of *S. mutans* and other oral streptococci were grown in saliva and treated with a STAMP, a parental AMP, or an oral antiseptic, and the surviving numbers of colony-forming units per milliliter were quantitated. As shown in Fig. 6A, compared with untreated biofilms, >95% of both *S. mutans* and non-*S. mutans* oral streptococci (as measured in numbers of colony-forming units per milliliter) were eliminated after chlorhexidine or Listerine treatment, which is consistent with the nonspecific activity of these agents. In contrast, Sm6(L1)B33 treatment resulted in a strong decrease in recoverable *S. mutans* numbers, whereas the population of non-*S. mutans* oral streptococci was less affected. This resulted in a ratio of surviving *S. mutans* to oral streptococci of <0.1 for these samples (Fig. 6B). The remaining STAMPs tested, as well as the parental B-33 and 2C-4 AMPs, were largely ineffective, though B-33, Sm6(L1)2C, and Sm6(L3)2C showed a modest degree of antimicrobial selectivity toward *S. mutans*. Overall, these results suggest that Sm6(L1)B33 retains robust activity and selectivity for *S. mutans* in a mixed-species biofilm system.

DISCUSSION

In this report, we present a novel strategy for the design and synthesis of STAMPs with activity against the oral pathogen *S. mutans*. Successful design was achieved through a tunable, building-block approach that utilized various combinations of antimicrobial, targeting, and linker regions. Our results demonstrate that less-efficacious STAMPs can be improved when alternative killing regions are substituted in the design. This process resulted in Sm6(L1)B33, a STAMP that displayed killing kinetics consistent with oral therapeutic applications and selectivity for *S. mutans* in multispecies biofilms.

The data presented suggest that the activity of the PL-135 AMP may be inhibited by conjugation to other peptide subunits, as unmodified PL-135 displayed MIC activity against *S. mutans* that was 2- to 8-fold better than that of progeny STAMPs, as shown in Table 2. Furthermore, library 1 STAMPs exhibited significantly reduced biofilm binding compared to library 2 conjugates with identical targeting regions, suggesting PL-135 interference in Sm6 activity as well. The unusually small size of PL-135 may impose a severe restriction on amino acid additions, especially when the mode of antimicrobial action depends on sequence-dependent self-association on the cell membrane or on binding to a discrete intracellular bacterial target (2). It remains unclear why PL-135 should inhibit Sm6 targeting peptide function.

Our results suggest that the optimal arrangement of STAMP domains is likely AMP specific and depends on which of the domains least affects, or even enhances, the antimicrobial mechanism. For example, the *Pseudomonas*-specific STAMPs G10KHc and G10KHn (oriented as target domain-killing domain and killing domain-target domain, respectively) both bind specifically to the target bacterium surface, but only G10KHc shows significant membrane disruption activity (5, 7). Further biochemical studies of pilot STAMP libraries of greater diversity are being conducted to fully evaluate whether correct pairings can be more accurately predicted.

Interestingly, Sm6 and Sm7 containing library 1 STAMPs were active against *S. mutans*, whereas the constructs with any one of the other targeting peptides listed in Table 1 were not. Targeting peptides Sm1 through Sm5 are strongly hydrophobic compared with Sm6 and Sm7 (8), and it may be possible that this characteristic limits the dissociation of these molecules from the hydrophobic components of the *S. mutans* cell wall, resulting in their inhibitory affect on AMPs when conjugated, in similarity to the results seen with some strong lipopolysaccharide (LPS)-binding AMPs (19). However, the systematic design strategy employed here allowed us to generate a diverse array of STAMPs, including useful compounds such as Sm6(L1)B33, despite these stumbling blocks.

It remains to be seen whether the selectivity observed with the STAMPs described in this report can be maintained in the oral cavity during treatment. Typically, oral-care antimicrobials are applied at high doses, suggesting that any selectivity "window" would be overwhelmed by nonspecific STAMP activity at higher concentrations. However, there are up to a total of $1 \times 10^{8-9}$ CFU/ml of bacteria in the mouth, of which as many as 1×10^{7}/ml can be *S. mutans* (9, 10). These bacterial burden levels are 10 to 100 times higher than those employed in the assays reported here, which suggests that typical oral therapeutic concentrations are necessary for activity and selectivity. Additionally, the typical 30 s to 2 min of treatment duration for oral rinse formulations may limit STAMP antimicrobial activity to targeted organisms, as seen in Fig. 4.

In conclusion, this report details the rational design of *S. mutans*-selective STAMPs with enhanced antimicrobial killing kinetics and selectivity compared to untargeted AMPs. The *S. mutans*-selective STAMPs were constructed using a tunable, combinatorial approach that generated a diverse number of STAMP sequences for antimicrobial evaluation and improvement, a process that may serve as an example for the systematic development of novel selective antimicrobial agents. We propose that these STAMPs could be useful in the design of therapeutics against oral or other mucosal pathogens, where the high diversity of "probiotic" beneficial microflora limits the effectiveness of broad-spectrum antimicrobial agents.

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