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

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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-methylbenzyldiamine (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.
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 (specinomycin resistant; constructed from UA140) (6), Streptococcus gordoni Chassl (DLL), 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% N2, 10% CO2, 10% H2) (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 (Aapsec, 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 dichloromethane (DMC) (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). Peptides were purified from the DCM and NMP wash cycles with a 50% methanol—1% aqueous acetic acid elution profile. Peptide purity was 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 (38). Briefly, a 4 molar excess of 5,6-carboxyfluorescein was added to peptide solutions and the resultant peptide solution was precipitated in methyl tert-butyl ether. The peptides were then resuspended 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 NaHCO3, 20 mM HEPES, 150 mM NaCl, 1 mM MgCl2, 0.1% cetyltrimethylammonium bromide (CTAB), pH 7.4) containing 25 μM peptide—1% sucrose and 100 μl of fresh TH broth (without sucrose). Plates were incubated at 37°C under anaerobic conditions, and the bacterial recovery was monitored by recording A630 values after 4 h of incubation. An unpaired Student t test was utilized for statistical analysis.

**Activity against multisppecies 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 × 105 CFU/well) and Streptococcus oralis, S. gordoni, S. sanguinis, S. mitis, and S. salivarius (grown overnight and adjusted to 2 × 105 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 quantify 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 [FIKDFIER]) and Sm4 (previously S3L1-5 [WWYNWQD W]) (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 ([AAKHAHRA]), 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
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)Sm6, 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

<table>
<thead>
<tr>
<th>Targeting peptide (name)</th>
<th>Linker (name)</th>
<th>Antimicrobial peptide (name)</th>
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<tbody>
<tr>
<td>WWHSWWSTSW (Sm1)</td>
<td>GGG (L1)</td>
<td>FHFHLHF (PL-135)</td>
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<tr>
<td>WWSYWWTQOW (Sm2)</td>
<td>SAT (L3)</td>
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<td>WWKDWWERW (Sm3)</td>
<td>ASASA (L5)</td>
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<td>PYP (L7)</td>
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<td>WWQDWWNEW (Sm5)</td>
<td>PSGSP (L8)</td>
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<td>FIKHFHHRF (Sm6)</td>
<td>PSPSP (L9)</td>
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<td>LIKHILHLRL (Sm7)</td>
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<th>Targeting peptide (name)</th>
<th>Linker (name)</th>
<th>Antimicrobial peptide (name)</th>
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<td>SGG (L6)</td>
<td>LQLKQQLKQLQOF (α-7)</td>
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<td></td>
<td>NH(CH2)_7CO(LC)</td>
<td>IKOLLHFQRF (B-38)</td>
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<td></td>
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<td>FKKFWRFWRFF (B-33)</td>
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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.
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 indicated 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), FKFFWKWF RRF (B-33), IKQLLHHFQRF (B-38), RWRRLLKKHLLH (α-11), and LQLLKQLLKQF (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 against *S. mutans*. Additionally, MICs were improved 2- to 8-fold compared with active PL-135-containing constructs. Within library 2, little diff-

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**TABLE 2. MICs of active library 1 and library 2 STAMPs**

<table>
<thead>
<tr>
<th>Peptide Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC (μg/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>S. mu</th>
<th>S. sob</th>
<th>S. san</th>
<th>S. mit</th>
<th>S. gor</th>
<th>MRSA</th>
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<tr>
<td>Sm1 WWWWSSSWTW</td>
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<td>Sm4 WWWNNQWQ</td>
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<td>Sm5 WWODWWNEW</td>
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<tr>
<td>Sm6 FIJKFIHRF</td>
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<td>Bc1 AAKHAHAARHA</td>
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<sup>a</sup>Active refers to a MIC of <100 μg/ml against *S. mutans*.

<sup>b</sup>All amidated C termini.

<sup>c</sup>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.

<sup>d</sup>Replotted from reference 12.

<sup>e</sup>nt, not tested.
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...
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

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**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).

**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).
were treated with the peptide for 1 min at 25°C and other oral streptococci were grown in saliva and treated 

selectivity and activity, mature biofilms consisting of S. mutans and other oral streptococci 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 G10KHe and G10KHn (oriented as target domain-killing domain and killing domain-target domain, respectively) both bind specifically to the target bacterium surface, but only G10KHe 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 effect 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 stalling 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 $1 \times 10^9$ to $1 \times 10^{12}$ CFU/ml of bacteria in the mouth, of which as many as $1 \times 10^7$/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.

ACKNOWLEDGMENTS

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