ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2007, p. 1351–1358 Vol. 51, No. 4
0066-4804/07/$08.00+0 doi:10.1128/AAC.01270-06

Novel Synthetic Antimicrobial Peptides against *Streptococcus mutans*▽

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Received 11 October 2006/Returned for modification 7 December 2006/Accepted 1 February 2007

***Streptococcus mutans*, a common oral pathogen and the causative agent of dental caries, has persisted and even thrived on the tooth surface despite constant removal and eradication efforts. In this study, we generated a number of synthetic antimicrobial peptides against this bacterium via construction and screening of several structurally diverse peptide libraries where the hydrophobicity and charge within each library was varied incrementally in order to generate a collection of peptides with different biochemical characteristics. From these libraries, we identified multiple peptides with robust killing activity against *S. mutans*. To further improve their effectiveness, the most bactericidal peptides from each library were synthesized together as one molecule, in various combinations, with and without a flexible peptide linker between each antimicrobial region. Many of these "fusion" peptides had enhanced killing activities in comparison with those of the original nonconjoined molecules. The results presented here illustrate that small libraries of biochemically constrained peptides can be used to generate antimicrobial peptides against *S. mutans*, several of which may be likely candidates for the development of anticaries agents.**

Recently, antimicrobial peptides (AMPs) have come to the forefront as potential antibiotic surrogates due to their robust killing activity against a wide spectrum of bacterial species, including drug-resistant strains. AMPs are genetically common molecules of innate immunity that have been discovered in single-cell and multicellular forms of life (7, 11, 25, 42). Although they can differ dramatically by peptide sequence and posttranslational modification (linear, circular, etc.), the majority of AMPs appear to kill bacteria by the disruption of lipid membranes, although the details of this mechanism appear to vary widely (6, 37). Previous observations have indicated the critical role of the general hydrophobic and cationic character of AMP function, including the significant contribution of aromatic Trp and cationic Arg residues found in many AMPs (8, 44). Despite their small size (most are less than 50 amino acids), secondary structure also appears to play an important role in activity: certain linear AMPs can adopt an α-helical or β-strand confirmation upon interaction with hydrophobic environments (such as detergents or lipid vesicles) that mimic bacterial membranes, suggesting that these conformational changes are necessary for antimicrobial function (22, 44, 45). Additionally, the formation of a membrane-active α-helix (and other structures) appears to require an amphipathic spatial arrangement of residues, i.e., a gradient of hydrophobicity across the surface of the peptide (22, 24, 37).

Previously, the rational design of AMPs has focused mainly on variation of the existing natural sequences or development of novel peptides from large combinatorial libraries (4, 19, 22, 35). These efforts have yielded valuable information on AMP structure-activity relationships and design requirements for constructing both wide-spectrum AMPs and peptides selective for fungi and gram-negative or gram-positive organisms.

In this report, we aimed to develop AMPs against *Streptococcus mutans*. This often overlooked pathogen of the oral cavity is a major causative agent responsible for dental caries, one of the most prevalent and costly bacterial infections worldwide (2, 27, 31, 43). Additionally, new therapeutics against this organism are sorely needed, as *S. mutans* is a persistent colonizer of the tooth surface in the presence of dietary sugars and can remain in the oral microflora (known as dental plaque) despite dedicated mechanical removal (tooth brushing) and general antiseptic efforts (21).

As we wished to avoid the synthesis of large combinatorial libraries of random peptides, we chose instead to identify peptides with anti-*S. mutans* activity by rationally designing several small peptide libraries. Each library was limited to peptides of a defined size and structural framework, while the sequences within each library differed in aromatic/hydrophobic residue content, net positive charge, and predicted amphipathic, α-helix-forming character. Furthermore, we conjoined the active sequences to construct new "fusion" peptides to further enhance activity. Here we report the successful utilization of these approaches to construct AMPs active against *S. mutans*.

MATERIALS AND METHODS

Bacterial strains. *S. mutans* clinical isolates UA140 (32), UA159 (1), T8 (33), ATCC 25175, GS5 (23), and all gram-positive strains listed below (see Table 4) were grown under anaerobic conditions in brain heart infusion or Todd-Hewitt (TH) broth (Difco) overnight at 37°C prior to use (14). *Veillonella atypica* PK1910 was grown in *Veillonella* medium (16). All strains were grown in an anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% H_2.

Peptide syntheses and purification. Peptides were synthesized using 9-fluorenylmethoxylcarbonyl (Fmoc) solid-phase methods on an Apex 396 peptide synthesizer (AAPPTec, Louisville, KY). All amino acids, appropriately substituted resins (Anaspec), and reagents (Fisher) were purchased at peptide synthesis grade. The general synthesis of linear peptides involved the following procedure: 0.6 ml of 25% piperidine in dimethylformamide (DMF) was added to the resin that had been loaded with the first amino acid, followed by agitation for 27 min

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▽ Published ahead of print on 12 February 2007.

and wash cycles of dichloromethane (one wash with 1 ml), and *N*-methylpyrrolidone (seven washes with 0.8 ml each time). For coupling, a 5 M excess of Fmoc-protected amino acid, *N*-hydroxybenzotrazole, HBTU (*O*-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluoro-phosphate), and diisopropyl ethylamine (10 M excess) in DMF (0.1 ml) and *N*-methylpyrrolidone (0.2 ml) was added, and the reaction mixture was agitated for 45 min. Following the coupling of the last amino acid to the resin, the protected peptide was cleaved from the resin with 1 ml of trifluoroacetic acid–thioanisole–water–1,2-ethanedithiol (10 ml:0.5 ml:0.5 ml:0.25 ml) for 2 h at room temperature and washed sequentially with DMF, methanol, and dichloromethane and dried overnight under vacuum.

Analytical and preparative reverse-phase high-performance liquid chromatographies (ACTA purifier; Amersham) was conducted with a Source 15RPC column eluted with H_2O and CH_3CN with 0.1% trifluoroacetic acid in a linear gradient as described previously (13). All peptides were purified to >90% (data not shown). Peptide mass was confirmed by matrix-assisted laser desorption ionization–mass spectrometry and conducted with samples dissolved in a 1:2 mixture of H_2O-CH_3CN. Measurements were made in the linear mode, with an α-cyano-4-hydroxycinnamic acid matrix (Voyager system; ABI). The mass observed corresponded in all cases with the calculated value (data not shown).

Estimation of peptide characteristics. Peptide average hydrophobicity per residue (<*H*>) was calculated using the scale of Fauchere and Pliska (17), available at http://us.expasy.org/tools/pscale/Hphob.Fauchere.html. This method was selected for calculating the hydrophobicity of AMPs due to its agreement with experimental evidence describing the membrane affinity of individual amino acids in a host-guest system (41). The average α-helix-forming propensity per residue (<*Helix*>) was estimated utilizing the scale developed by Liu and Deber that defines the helical propensities of individual amino acids in nonpolar membrane environments, such as those encountered by AMPs at the surface of the target cell (26).

MIC assays. Antibacterial growth inhibition assays were performed using sterile 96-well plates in a final volume of 100 μl TH or *Veillonella* medium as described previously (32). Briefly, bacterial cells were grown overnight to an optical density at 600 nm of 0.75 to 0.8 (corresponding to 1×10^8 CFU/ml) and then diluted to $\sim 1 \times 10^5$ CFU/ml in broth and aliquoted onto plates. An appropriate volume of peptide stock solution (5 to 20 mg/ml, in water or methanol, depending on solubility) was then added to the first column of the plate to give 500 μg/ml or 512 μg/ml, followed by serial 1:2 dilutions across the plate to give wells containing peptides ranging from 500 to 1.95 μg/ml or 512 to 2 μg/ml. Methanol alone was also added to control for the effects of the solvent. The plates were then incubated at 37°C under anaerobic conditions for 16 to 20 h without shaking, and the MIC was determined as the concentration of peptide present in the last clear well after visual inspection. Up to 5% (vol/vol) methanol was found not to be antimicrobial (data not shown). MICs were determined in triplicate for all bacteria.

Assessment of antimicrobial kinetics. The determination of killing kinetics for fusion and parental peptides was conducted essentially as described previously (15). Briefly, medium-diluted overnight UA159 cultures ($\sim 0.5 \times 10^6$ to 1×10^6 CFU/ml) were challenged with peptide at a concentration of 25 μg/ml, and at the indicated time intervals ("0 min" indicates untreated samples), a 10-μl aliquot was removed and the surviving CFU were rescued by dilution (1:50) into growth medium and then spread on TH agar plates for quantitation. Where initial experiments suggested few survivors (<3,000 CFU/ml), entire 1-ml samples were plated without aliquots. Kinetics were determined by recording the average number of surviving CFU/ml versus incubation time in the presence of peptide (all assays were independently repeated three to five times). For Table 3, time to bactericidal killing (T_C) values were defined as the time required for the level of surviving CFU/ml from peptide-treated cultures to fall 3 $\log_{10}$ below the levels of recovered *S. mutans* CFU/ml from untreated samples.

RESULTS

Design of constrained peptide libraries. We hypothesized that a series of small peptide libraries which contained gradients of incrementally varied hydrophobic and cationic character, within constrained predicted conformational frameworks, would contain AMPs with activity against *S. mutans*. Three libraries were developed for this study: the binary, α-helix, and RW libraries. Peptides within the binary and α-helix libraries were designed from an amphipathic, α-helical sequence arrangement framework ($HCCHHCHHCC_n$, where H is a hydrophobic residue and C is a charged residue) that was developed from several published AMP amphipathic sequence templates (4, 46) and was validated using helical-wheel projections (36) and the average helical propensity <*Helix*> (Table 1). Figure 1 shows two representative projections from each library. The lengths of the peptides within the binary and α-helix libraries were limited to 11 and 14 amino acids, respectively, near the estimated number of minimal residues required to form membrane-spanning pores (38). From within this structural framework, the binary library was constructed by reducing, in a stepwise manner, the hydrophobic/aromatic and cationic components within a baseline sequence, FKKFWKW FRRF (B-33), via the substitution of less-hydrophobic/aromatic or positively charged residues (B-33 has been published with alternative nomenclature, S6L3-33 [14]). The resulting gradient of charge and hydrophobicity (<*H*>) within this library of 32 peptides is shown in Table 1. The α-helix library was designed to be longer than the binary library (14 versus 11 amino acids), and in a manner similar to that of the binary library, the hydrophobicity and charge were varied incrementally throughout the α-helix library (Table 1).

Many naturally occurring AMPs contain numerous Arg and Trp residues (8), and previous reports have shown that a predominance of these amino acids appear in hexameric AMPs generated via synthetic combinatorial, or similar, methods (3, 8). Therefore, we constructed the RW library to contain Arg- and Trp-rich (R and W) heptamers that were varied in order of basic (Arg or Lys) and Trp residues and in ratio of basic to hydrophobic residues, as shown in Table 1. These sequences were not designed to form conventional α-helix conformations, as reflected in their low <*Helix*> values.

Activity of binary library peptides. The binary library was evaluated for *S. mutans* activity against a collection of clinical isolates by an MIC assay (Table 1). Two peptides that showed the lowest MICs (range, 8 to 24 μg/ml), B-33 and B-34, were also the most hydrophobic and cationic in the binary library. Similarly, B-37, -38, -41, -53, -54, -57, and -58, peptides that were active but to a lesser degree than B-33 or B-34, possessed net charges of at least +3 and an <*H*> of >0.55. In contrast, peptides with hydrophobicity values below 0.55 were not antimicrobial, with two exceptions: B-61 and B-62 (MIC range, 16 to 32 μg/ml). Notably, these two peptides each had only one aromatic residue but a net charge of +6 that may contribute to their sustained activity.

The MIC results are consistent with the notion that net positive charge and some hydrophobic content are critical parameters for designing active peptides (8, 9). Accordingly, the addition of negatively charged residues, especially to the central area of the peptide, appears to abolish AMP activity (for example, B-43), likely due to the interruption of the positive charge necessary for peptide attraction to the bacterial surface. The data indicate two interesting exceptions, B-49 and B-50, which are weakly active despite the lack of strong cationic character. The exceptionally high <*H*> values (and therefore likely rapid partition into host membranes) for these peptides may explain this discrepancy. B-33, -34, and -38, three of the more active peptides from this library, were selected for further enhancement. Interestingly, as was the case throughout this study, no strain of *S. mutans* appeared more susceptible or resistant to the AMPs tested (data not shown).

TABLE 1. Sequences and antimicrobial activities of binary, α-helix, and RW library peptides against clinical isolates of *S. mutans*

Peptide	Sequence[a]	MIC[b] (μg/ml)	Net charge[c]	<*H*>	<*Helix*>
Binary library					
B-33	FKKFWKWFRRF*	8–24	+6	0.61	0.71
B-34	LKRFLKWFKRF*	8–24	+6	0.55	0.94
B-35	KLFKRWKHLFR	31.25–125	+5	0.18	0.85
B-36	RLLKRFKHLFK	31.25–125	+5	0.35	1.05
B-37	FKTFLKWLHRF*	24	+4	0.77	1.06
B-38	IKQLLHFFQRF*	24	+3	0.75	1.21
B-39	KLLQTFKQIFR	>250	+3	0.50	1.11
B-40	RILKELKNLFK	>250	+3	0.32	1.23
B-41	LKQFVHFIHRF*	32	+3	0.74	1.20
B-42	VKTLLHIFQRF*	31.25–125	+3	0.56	1.32
B-43	KLVEQLKEIFR	>250	+1	0.16	1.10
B-44	RVLQEIKQILK	>250	+2	0.43	1.21
B-45	VKNLAELVHRF*	>250	+2	0.35	1.25
B-46	ATHLLHALQRF*	>250	+2	0.62	1.31
B-47	KLAENVKEILR	>250	+1	0.25	1.12
B-48	RALHEAKEALK	>250	+1	0.02	1.02
B-49	FHYFWHWFHRF*	125	+2	1.09	0.79
B-50	LYHFLHWFQRF*	125	+2	1.00	1.05
B-51	YLFQTWQHLFR	>125	+1	0.83	0.95
B-52	YLLTEFQHLFK	>125	0	0.74	1.13
B-53	FKTFLQWLHRF*	16–64	+3	0.84	1.06
B-54	IKTLLHFFQRF*	32–62.5	+3	0.79	1.26
B-55	KLLQTFNQIFR	>125	+2	0.54	1.10
B-56	TILQSLKNIFK	>125	+2	0.56	1.24
B-57	LKQFVKFIHRF*	24	+4	0.64	1.18
B-58	VKQLLKIFNRF*	32–62.5	+4	0.56	1.25
B-59	KLVQQLKNIFR	>125	+3	0.38	1.11
B-60	RVLNQVKQILK	>125	+3	0.33	1.17
B-61	VKKLAKLVRRF*	16–32	+6	0.27	1.21
B-62	AKRLLKVLKRF*	16–32	+6	0.31	1.25
B-63	KLAQKVKRVLR	>125	+5	0.18	1.10
B-64	RALKRIKHVLK	>125	+5	0.06	1.15
α-Helix library					
α-4	AQAAHQAAHAAHQF*	>125	+1	0.26	1.13
α-5	KLKKLLKKLKKLLK	8	+8	0.16	1.13
α-6	LKLLKKLLKLLKKF*	8	+7	0.55	1.42
α-7	LQLLKQLLKLLKQF*	8	+4	0.72	1.42
α-8	AQAAKQAAKAAKQF*	>125	+4	0.026	1.09
α-9	RWRRWWRHFHHFFH*	8	+5	0.61	0.55
α-10	KLKKLLKRWRRWWR	8	+8	0.28	0.68
α-11	RWRRLLKKLHHLLH*	8	+6	0.44	1.02
α-12	KLKKLLKHLHHLLH*	8	+5	0.48	1.18
RW library					
1C-1	RRRRWWW	16	+4	0.39	0.24
1C-2	RRWWRRW	16	+4	0.39	0.24
1C-3	RRRWWWR	32	+4	0.39	0.24
1C-4	RWRWRWR	32	+4	0.39	0.24
2C-1	RRRFWWR	31.25–125	+4	0.32	0.41
2C-2	RRWWRRF*	12–24	+5	0.32	0.41
2C-3	RRRWWWF*	4–8	+4	0.79	0.46
2C-4	RWRWRWF*	4–8	+4	0.79	0.46
3C-1	RRRRWWK	125	+5	−0.076	0.19
3C-2	RRWWRRK	125	+5	−0.076	0.19
3C-3	RRRWWWK	31.25–125	+4	0.39	0.24
3C-4	RWRWRWK	32	+4	0.39	0.24
4C-1	RRRKWWK	250	+5	−0.073	0.19
4C-2	RRWKRRK	500	+6	−0.39	0.13
4C-3	RRRKWWK	125	+5	−0.51	0.19
4C-4	RWRKRWK	125–500	+5	−0.51	0.19

[a] An asterisk indicates an amidated C terminus.
[b] MIC range or value from all *S. mutans* isolates tested.
[c] Charge at pH 7.0, calculated as described previously (47).

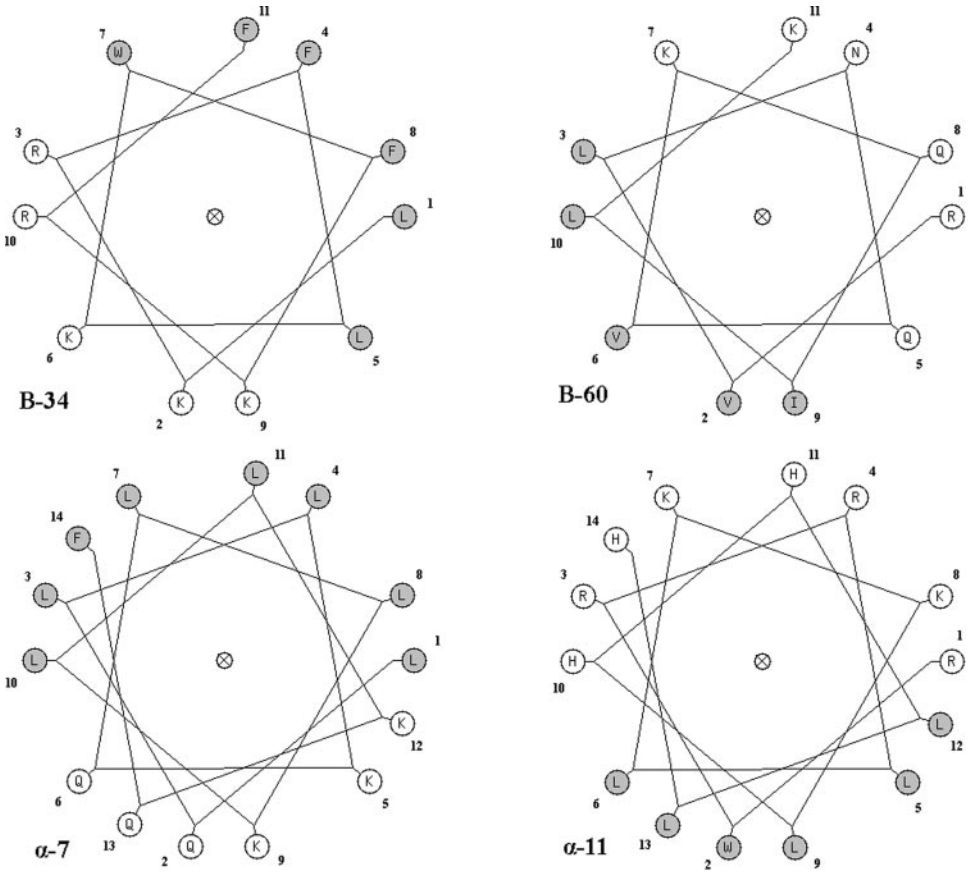

FIG. 1. α-Helix wheel projections for representative peptides from the binary and α-helix libraries. Residues are numbered consecutively from the N terminus to the C terminus, with hydrophobic residues shaded.

Activity of the α-helix library peptides. As shown in Table 1, many of the α-helix library peptides had robust activities against *S. mutans*, as evaluated by MIC: only two peptides with low net positive charges or <*H*> values (α-4 and α-8, respectively) were inactive. Though many α-helix library peptides showed equally robust killing activities against *S. mutans*, α-11 alone was selected for further modification.

Activity of RW library peptides. As shown in Table 1, we observed that 2C-3 and 2C-4 were the most active peptides against *S. mutans* (MIC range, 4 to 8 μg/ml) from the RW (Arg- and Trp-rich) library. These data indicate that a near 1:1 ratio of hydrophobic to charged amino acids (including the amidated C-terminal Phe as a charged residue) is required for robust antimicrobial activity, similar to the reported ratio of 4:3 observed by Blondelle and others examining Trp/Arg-rich AMPs (5, 28). RW library peptides with a lower ratio of hydrophobic to charged amino acids (reflected in lower <*H*> values) were not as active as 2C-3 or 2C-4. Due to their low MICs, these peptides were selected for further enhancement.

Design of fusion AMPs. Previous studies have shown that synthesizing AMPs as conjoined linear dimers or pentameric bundles, in various arrangements, can increase peptide activity compared to that of the single constituent AMP alone (10, 34). In a similar manner, we hypothesized that the activity of the *S. mutans*-active sequences shown in Table 1 could be improved by synthesizing two AMPs together, head to tail, as a single fusion linear peptide molecule. It was noted in our previous studies that a linker region between peptide domains has an impact on antimicrobial activity (15). Therefore, in the hopes of generating peptides with greater activity against *S. mutans*, we synthesized a library of fusion peptides (Table 2) that differed in the arrangement of constituent AMPs and the sequence of linker regions (or with no linker) between each.

In the binary-to-α-helix set, B-33 and B-38 were synthesized with α-11 at the N or C terminus. A tri-Gly linker, which has been shown previously to be effective in separating functionally independent peptide regions within a linear sequence (15) and may be critical for peptide secondary-structure transition in model membranes (40), was employed to separate the two AMP domains. The RW-to-RW set of fusion peptides contained 2C-3 and 2C-4 as linear homodimers with or without various linker regions in between. In the last group shown in Table 2 (binary-to-RW set), 2C-4 was synthesized with B-33, -34, or -38 at the N or C terminus, again separated by a Gly linker region. These arrangements allowed us to investigate the importance of linker composition (and presence) as well as AMP subunit arrangement (N or C terminus within the fusion peptide) on anti-*S. mutans* activity.

Antimicrobial activity of fusion AMPs. MICs revealed that fusion AMPs were largely equally or less active than their parent AMPs against *S. mutans* (Table 2). Exceptions included FBα-20, which had a reduced MIC range compared to either

TABLE 2. Sequence and activity of fusion library peptides

Peptide	Sequence[a]	MIC (μg/ml)[b]
Binary-to-α-helical linker		
FBα-12	FKKFWKWFRRF-GGG-RWRRLLKKLHHLLH*	16
FBα-13	IKQLLHFFQRF-GGG-RWRRLLKKLHHLLH*	16
FBα-20	RWRRLLKKLHHLLH-GGG-FKKFWKWFRRF*	4–8
FBα-21	RWRRLLKKLHHLLH-GGG-IKQLLHFFQRF*	16
RW-to-RW linker		
FRW-2	RRRWWWFRRRWWWF*	16
FRW-8	RWRWRWFRWRWRWF*	16
FRW-3	RRRWWWF-ASASA-RRRWWWF*	32
FRW-4	RRRWWWF-PSGSP-RRRWWWF*	32
FRW-5	RRRWWWF-GGG-RRRWWWF*	16
FRW-9	RWRWRWF-ASASA-RWRWRWF*	16
FRW-10	RWRWRWF-PSGSP-RWRWRWF*	32
FRW-11	RWRWRWF-GGG-RWRWRWF*	16
Binary-to-RW linker		
FBRW-14	FKKFWKWFRRF-GGG-RWRWRWF*	4–16
FBRW-15	IKQLLHFFQRF-GGG-RWRWRWF*	4–16
FBRW-16	LKRFLKWFKRF-GGG-RWRWRWF*	4–16
FBRW-22	RWRWRWF-GGG-FKKFWKWFRRF*	4–8
FBRW-23	RWRWRWF-GGG-IKQLLHFFQWRF*	8–16
FBRW-24	RWRWRWF-GGG-LKRFLKWFKRF*	8

[a] An asterisk denotes an amidated C terminus.
[b] MIC range or value from all *S. mutans* isolates tested, with a minimum of three independent trials.

B-33 or α-11, and FBRW-22, which had a slightly reduced MIC range compared with the parent peptide B-33. Overall, these data indicate that fusing AMPs results, at best, in only modest improvements in MIC. However, MIC assays can obscure dramatic differences in killing speed between comparable peptides (15), suggesting that a more detailed analysis of peptide killing kinetics was necessary to fully evaluate fusion AMPs.

The killing kinetics of fusion AMPs against *S. mutans* were analyzed by time-kill assays. As shown in Fig. 2A, the fusion peptide FBα-21 had an obvious increase in killing kinetics compared to either B-34 or α-11: more than 2 $\log_{10}$ fewer CFU/ml were recovered from fusion peptide-treated cultures than from parental peptide-treated samples after 30 min. This trend was representative of all the FBα peptides (data not shown).

An increase in killing kinetics for all binary-RW library conjugates (with the exception of FBRW-14 and -23) was also conspicuous (Fig. 2B): fusion peptides with the binary library AMP at the N or C terminus relative to 2C-4 were equally effective in improving the level of *S. mutans* killing more than 3 $\log_{10}$ after 30 min of treatment, compared with samples treated with either parent peptide. The results indicate that the conjoined AMP domains may be exerting an enhanced killing effect against *S. mutans* that is best observable after short periods of peptide exposure.

As shown in Table 3, we also observed an increase in killing kinetics for some RW-to-RW fusion peptides. In samples treated with the 2C-4 homodimer without a linker region (FRW-8), the surviving number of *S. mutans* CFU/ml dropped 3 $\log_{10}$ below that of untreated samples (defined as time to bactericidal activity, or T_C) by 50 min, while 2C-4 had a T_C of 220 min. This trend, to a lesser extent, was also present when samples exposed to the 2C-3 homodimer without a linker

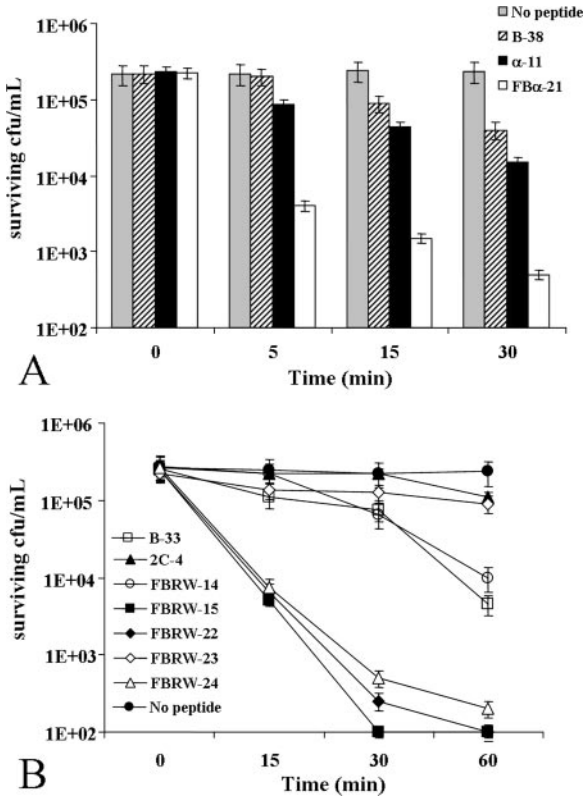

FIG. 2. Comparative killing kinetics of binary-to-α-helix and binary-to-RW fusion peptides. *S. mutans* was challenged with 25 μg/ml binary-to-α-helix (A) or binary-to-RW (B) fusion peptides or parent AMPs, and the surviving CFU were plated at various time points postaddition. Samples at 0 min were plated prior to peptide treatment. All data points represent the average of results of at least three independent experiments ± standard deviations.

TABLE 3. Bactericidal kinetics of parent RW and fusion FRW library peptides

Peptide	Linker composition	T_C (min)[a]
Parental AMPs		
2C-3		220
2C-4		230
2C-3 fusions		
FRW-2	No linker	60
FRW-3	ASASA	480
FRW-4	PSGSP	420
FRW-5	GGG	270
2C-4 fusions		
FRW-8	No linker	50
FRW-9	ASASA	45
FRW-10	PSGSP	270
FRW-11	GGG	90

[a] T_C, time required to kill 3 $\log_{10}$ of recoverable CFU/ml.

TABLE 4. MICs of selected peptides against non-*S. mutans* oral isolates

Peptide	MIC (μg/ml)				
	S. gordonii Challis DL1	*S. mitis* ATCC 903	*S. sanguinis* NY101	*L. casei* ATCC 4646	*V. atypica* PK1910
B-33	32	32	32	32	16
B-34	32	64	16	16	32
B-38	32	64	64	32	64
α-11	16	32	16	16	4
2C-3	32	32	16	16	64
2C-4	32	32	32	32	64
FBα-20	16	32	16	8	8
FBRW-14	32	64	16	8	8
FBRW-15	32	64	16	16	8
FBRW-16	64	64	16	16	8
FBRW-22	16	64	8	8	8
FBRW-24	32	64	16	8	16

(FRW-2) were compared to samples treated with its parent peptide (2C-3). Interestingly, the addition of a linker region of flexible amino acids (either PSGSP, ASASA, or GGG) between 2C-3 domains did not result in an improvement in killing kinetics (T_C of >250 min for FRW-3 to -5), while 2C-4 homodimers FRW-9 and -11 had increased bactericidal speed relative to that of the parent 2C-4 AMP despite the presence of ASASA and GGG linker regions, respectively (Table 3). These results suggest that RW-based peptide activity against *S. mutans* can be enhanced by synthesizing peptides together without a linker region, but when a linker region is utilized, the data indicate that increased kinetics are likely sequence and therefore secondary structure dependent, as 2C-4 could be improved by conjugations with linker regions of flexible amino acids (except PSGSP) and 2C-3 could not.

Peptide activity against other oral bacteria. A set of peptides with the best anti-*S. mutans* activity (as measured by MIC) were selected to investigate the activity of these AMPs against several normally commensal oral streptococcal strains and *Lactobacillus casei*, as well as the oral gram-negative organism *V. atypica* (Table 4). The MIC results indicate that these sequences were active, but to a lesser degree than seen with *S. mutans*, against *Streptococcus gordonii*, *L. casei*, and *Streptococcus sanguinis*. All peptides examined were typically only weakly active against *Streptococcus mitis*. *V. atypica* appeared more susceptible to α-11 than the other bacteria tested but was more resistant to the RW library peptides 2C-3 and 2C-4.

DISCUSSION

In the United States, the high financial burden associated with treating dental caries, especially among underprivileged and minority populations (2, 31, 43), could be alleviated by dental hygiene regimens supplemented with more-effective anti-*S. mutans* therapeutics. Accordingly, the results presented here suggest that peptides with robust activity against *S. mutans* can be identified by screening small rationally designed libraries. Interestingly, the most-active peptides derived in this study appear to have some activity against other gram-positive oral bacteria, although more data are required to fully evaluate whether the hydrophobic and amphipathic characteristics of these sequences are indicative of general anti-gram-positive bacterial activity. As *S. mutans* normally grows in a biofilm state in vivo, we were encouraged to also find that all the peptides in Table 4 had activity against in vitro *S. mutans* biofilms grown on glass slides in the presence of sucrose (data not shown).

For the binary and α-helix libraries, the results indicate that maximal *S. mutans* activity occurred in peptides with both high relative hydrophobicity and more than +3 net positive charge. Intermediate levels of activity were observed for peptides whose properties were dominated by either single trait. These results are consistent with what is known of most synthetic and natural AMPs with cationic amphipathic character and suggest that B-33, α-7, and the other active peptide sequences in these libraries may behave in a manner similar to that of other AMPs within this structural class; i.e., the positive charge of the peptide is thought to be required for the attraction of AMP to anionic bacterial membranes, while the amphipathicity is necessary for membrane interaction, helix transition, and cell killing (20, 38). These results also indicate that a high <*Helix*> value, on its own, may not correlate with increased anti-*S. mutans* activity for cationic amphipathic AMPs.

For the RW library, as was the case with the binary and α-helix libraries, the most-hydrophobic and -cationic sequences were the most active (2C-3 and -4). Despite the differences in length and periodicity compared with peptides in the other libraries, RW peptides may also be capable of gaining enhanced activity from improvements in initial peptide binding or membrane insertion. This idea is supported by evidence suggesting that Arg- and Trp-rich peptides, despite their low <*Helix*> values, form stable helix-like amphipathic arrangements upon membrane interaction which are stabilized by electrostatic bonds between the Trp Π elections and the Arg functional group (20, 29). Thus, the unique sequence properties of the RW library peptides may allow them to obtain secondary structures that are highly conducive to antimicrobial activity.

Interestingly, our results indicate that fusion peptides, AMP dimers synthesized as single linear molecules, often have in-

creased killing kinetics compared to their parental peptides. Some fusion peptides may function by increasing the number of helix-forming units per molecule at the membrane surface, as has been described for bundled AMPs (35). The data are unclear regarding the impact of linker regions between AMP segments, though it does appear that linker regions may affect individual peptides differently (see results for FRW-3 and FRW-9 in Tables 2 and 3). Studies are under way to directly investigate the impact of linker composition on AMP activity. Furthermore, our results suggest that the effect of constituent arrangement (which fusion peptide subunit goes at the C or N terminus) is also difficult to predict (compare the MICs of FBα-12 and FBα-20), though other work has demonstrated that amphipathic and putative helix-forming AMPs appear to be more tolerant of N-terminal additions for reasons that remain unclear (15, 39).

In conclusion, our results indicate that *S. mutans* appears to be susceptible to peptides with relatively high hydrophobicity and cationic charge, which were readily isolated from our constrained libraries. Additionally, fusion peptides constructed from conjoining active sequences from within and between these libraries improved the killing kinetics of these peptides.

ACKNOWLEDGMENTS

This work was supported by grants from NIH MD01831 to M.H.A. and W.S. and from C3 Jian, Inc.

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