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Streptococcus mutans strains recovered from caries-active or caries-free individuals differ in sensitivity to host anti-microbial peptides

Ekarat Phattarataratip[1,2], **Bonny Olson**[1], **Barbara Broffitt**[1], **Fang Qian**[1], **Kim A. Brogden**[1], **David R. Drake**[1], **Steven M. Levy**[1], and **Jeffrey A. Banas**[1,*]

[1] University of Iowa College of Dentistry, Iowa City, Iowa 52242, United States

SUMMARY

Antimicrobial peptides (AMPs) are among the repertoire of host innate immune defenses. In the oral cavity, several AMPs are present in saliva and have antimicrobial activities against oral bacteria, including *Streptococcus mutans*, a primary etiologic agent of dental caries. In this study, we hypothesized that unique *S. mutans* strains as determined by DNA fingerprinting from sixty 13 year-old subjects with or without caries experience would have different susceptibilities to α-defensins-1-3 (HNP-1-3), β-defensins-2-3 (HBD-2-3) and LL-37. The salivary levels of these peptides in subjects also were measured by enzyme-linked immunosorbent assays (ELISA). We found that *S. mutans* strains from caries-active subjects showed greater resistance to salivary HNP-1-2, HBD-2-3 and LL-37 at varying concentrations than those from caries-free subjects. In addition, combinations of these peptides increased their antimicrobial activity against *S. mutans* either additively or synergistically. The salivary levels of these peptides were highly variable among subjects with no correlation to host caries experience. However, the levels of a number of these peptides in saliva appeared to be positively correlated within an individual. Our findings suggest that the relative ability of *S. mutans* to resist host salivary AMPs may be considered a potential virulence factor for this species such that *S. mutans* strains that are more resistant to these peptides may have an ecological advantage to preferentially colonize within dental plaque and increase the risk of dental caries.

Keywords

Dental caries; defensins; *S. mutans*; innate immunity; saliva

INTRODUCTION

The oral cavity represents a unique environment in that the wide array of microbial species inhabiting the dental plaque biofilm interacts closely with host saliva. The protective attributes of saliva, such as its physical cleansing and buffering capacities, as well as a number of host innate and adaptive immune components, function collectively to maintain the balance between health and disease. The significance of saliva in the prevention of dental caries is well-recognized. The potential roles of a number of salivary proteins in the dental caries process have been a subject of interest in dental research, including a variety of salivary cationic antimicrobial peptides (AMPs), primarily the human neutrophil peptide α-defensins (HNP-1-3), human β-defensins (HBD-1-3), LL-37 and histatins. These peptides

[*]Correspondence: Dr. Jeff Banas, Dows Institute for Dental Research, College of Dentistry, University of Iowa, N436 Dental, Iowa City, Iowa 52242, USA. Tel: (+1)319-335-9911; Fax (+1)319-335-8895; Jeffrey-banas@uiowa.edu.
[2]Current Address: Chulalongkorn University, Department of Oral Pathology, Bangkok, Thailand

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are expressed from various sources, including salivary gland acini and ducts, oral epithelium and neutrophils. These peptides possess broad spectrum antimicrobial activity against various microorganisms. In the oral cavity, the defensins and LL-37 are potent against a number of bacterial species, while histatins are predominantly antifungal (Joly *et al.*, 2004, Sugimoto *et al.*, 2006, Bartie *et al.*, 2008, Nishimura *et al.*, 2004a).

The roles of AMPs in a range of other bacterial infections have been intensely investigated. Several studies suggested that virulence potentials of several bacterial species were determined by their abilities to resist host AMPs (Nizet *et al., 2001*, Groisman *et al., 1992*). In addition, it was shown in murine models that AMPs were required for host resistance against certain infections and that differential expression of selected peptides could determine host susceptibility to diseases (Wu *et al.*, 2009a, Wu *et al.*, 2009b).

Evidence for a role of AMPs in dental caries has been reported. The level of HNP-1-3 was shown to be higher in saliva from caries-free children, compared to that from caries-active children, whereas no significant correlations between host caries experience and levels of HBD-3 or LL-37 were observed (Tao *et al.*, 2005). These findings suggest a role for HNP-1-3 in dental caries protection and also raise the possibility of using salivary HNP-1-3 levels as a predictor of caries risk.

A handful of studies have investigated the susceptibility of *S. mutans*, primarily laboratory strains, to host AMPs in saliva (Maisetta *et al.*, 2003, Ouhara *et al.*, 2005, Joly et al., 2004, Nishimura *et al.*, 2004b). The susceptibility patterns appeared to be strain-specific. However, the potential contribution and association of the susceptibility profiles of this cariogenic bacterium with host caries experience has not been defined.

In this study, we hypothesized that various strains of *S. mutans* possess different inherent susceptibility/resistance profiles to host salivary AMPs and that host-specific quantities of these peptides may influence plaque colonization by particular *S. mutans* strains. We showed that *S. mutans* strains from caries-free subjects were more susceptible to host AMPs than those from caries-active subjects. The differences in strain susceptibility to these peptides may influence the colonization of *S. mutans* strains in biofilms and potentially contribute to an individual's relative risk for dental caries. Examination of salivary concentrations of these peptides showed that their levels were highly variable among individuals and not associated with caries experience in this subject group.

METHODS

Study sample and sample collection

Saliva and plaque samples were obtained from sixty children enrolled in the longitudinal Iowa Fluoride Study (Levy *et al.*, 2001). For these analyses, equal numbers of subjects were randomly selected from among caries-free and caries-active groups. All children were 13 years old at the time of sampling. Subjects were designated high-caries when they had 3 or more carious or filled surfaces ($D_{2\text{-}3}F$). Oral examination and sample collection were performed by trained and calibrated investigators using a standardized protocol (Warren *et al.*, 2001). Subjects were constrained from eating, drinking (other than plain water), chewing gum or brushing teeth for 60 minutes before sampling. Plaque samples were taken from pits and fissures of occlusal surfaces of second molars using a sterile CytoSoft® Brush. Stimulated saliva was collected using Parafilm® wax. Samples were frozen at −80°C for later analysis. Investigators were blinded from demographic data and caries status of subjects while assays were performed.

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S. mutans isolation and genotyping

Plaque samples were diluted in Trypticase Soy Broth supplemented with Yeast Extract (TSBYE) and plated on blood agar and Mitis-Salivarius-Kanamycin-Bacitracin (MSKB) agar for determination of total bacterial count and MS count, respectively. Ten colonies of presumed *S. mutans* per subject were selected and isolates were identified as *S. mutans* when they were positive for fermentation of mannitol, raffinose, salicin, sorbitol and negative for arginine hydrolysis and further confirmed by Gram stain and negative catalase tests. Ten *S. mutans* isolates per subject were frozen in TSBYE with 10% glycerol and kept at −80°C.

DNA extractions from *S. mutans* isolates were performed using the MasterPure™ Gram Positive DNA extraction kit (Epicentre Biotechnologies, Madison, WI). Genotypic characterization of *S. mutans* isolates were performed by arbitrarily primed polymerase chain reaction (AP-PCR) using 2 oligonucleotide primers, OPA-2 (5′-TGCCGAGCTG) and OPA-13 (5′-CAGCACCCAC). PCR reactions were performed in a volume of 25 μl containing 2.5 μl of 10X reaction buffer, 7 mM $MgCl_2$, 1.25 units of Taq DNA Polymerase (Applied Biosystem, Foster City, CA), 200 mM of dNTP mix (Invitrogen, Calsbad, CA), 100 pmole of primer and 50 ng of template DNA. AP-PCR reactions were run for 45 cycles of 1 minute at 94°C, 1 minute at 36°C, and 2 minute at 72°C. Template DNA from *S. mutans* laboratory strain ATCC25175 was used as a control in all reactions. A negative control reaction in which template DNA was omitted was used to exclude the possibility of DNA contaminations. All PCR amplitypes were run on 1.5% agarose gel electrophoresis and subsequently stained with ethidium bromide.

AP-PCR fingerprinting patterns of *S. mutans* amplitypes from all subjects were analyzed using the GelCompar II program (Applied Maths Inc., Austin, TX). AP-PCR gel images were digitalized into the program database and normalized according to manufacturer's instruction. Similarity coefficients were calculated by curve-based Pearson product-moment correlation. The unweighted pair group method using arithmetic averages (UPGMA) was used for clustering analysis of all strains. Dendrodrams were generated from the composite data set of both OPA2 and OPA13 fingerprinting profiles.

Antimicrobial susceptibility tests

Unique *S. mutans* strains from AP-PCR genotyping were subjected to antimicrobial susceptibility tests with a panel of AMPs, which included HNP-1-3, HBD-2-3 and LL-37. The selection of AMPs was based upon literature that reported their presence in saliva and their commercial availability. Additionally, the antimicrobial activity of three other peptides, HBD-1, lysozyme and histatin-5, against 16 *S. mutans* strains was also assessed in preliminary experiments. Data revealed no susceptibility of *S. mutans* strains to these peptides at concentrations 20 μg/ml or below; therefore, these three peptides were omitted from future experiments.

Antimicrobial susceptibility tests were performed using the alamarBlue® assay. In previous studies, as well as in our preliminary data, alamarBlue® assays and viable count assays for colony forming units (CFU) were comparable for evaluating bacterial viability (Vanitha & Paramasivan, 2004, Pettit *et al.*, 2005, Montoro *et al.*, 2005, Martin *et al.*, 2005, Martin *et al.*, 2003, Tenover *et al.*, 1995, Yajko *et al.*, 1995, DeForge *et al.*, 2000). *S. mutans* strains were grown overnight in THBYE, centrifuged, and resuspended to 10^6 CFU/ml in Mueller-Hinton broth (MHB) for the HNP-1-3 tests or resuspended to 10^8 CFU/ml in 0.01 M sodium phosphate buffer (pH 7.4) for the HBD-2-3 and LL-37 tests. The choice to resuspend in MHB or sodium phosphate buffer and the selection of bacterial concentrations were based on conditions that optimized the activity of each particular peptide.

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All peptides were tested at 3 different concentrations, 5 μg/ml, 1.5 μg/ml and 0.5 μg/ml, by incubating 90 μl of bacterial suspension with 10 μl of 10X stock of each peptide in BD Falcon™ black with clear bottom 96-well Microtest™ Optilux™ Plates. A growth control reaction was also set up using peptide diluent in place of each peptide. Following 2 hours incubation at 37°C, 10 μl of alamarBlue® reagent was added to each reaction and incubation continued for 4 hours. Fluorescent intensity was then measured at 560 nm and 590 nm using a fluorescent microplate reader (SpectraMax® M2e, Molecular Devices, Sunnyvale, CA). Assays were performed in duplicate. Percent viabilities of bacteria in each reaction were calculated using the following formula: Percent viability = (relative fluorescent units (RFU) of test peptide/relative fluorescent units of growth control) × 100.

AMP combination analysis

To examine the combined effect of these AMPs against *S. mutans*, the alamarBlue® assays were performed using serial dilutions of two peptides in a checkerboard assay format (Krogstad, 1986). In addition, the alamarBlue® assays were modified to permit the combination analysis of all peptide pairs. *S. mutans* clinical strains were grown as previously described, centrifuged and resuspended to 10^6 CFU/ml in 0.01 M sodium phosphate buffer (pH 7.4). The peptide concentrations used ranged from approximately twice the concentration that produced 50% killing of bacteria (EC50) to five serial two-fold dilutions below this concentration. The bacterial suspension was incubated with specified concentrations of peptides for 2 hrs at 37°C. Following incubation, 100 μl of MHB and 10 μl of alamarBlue® reagent were added to each reaction and incubation continued for 4 hrs. Plates were read using the fluorescent microplate reader as previously described.

Fractional inhibitory concentration (FIC) of each peptide was calculated as the concentration that gave 50% killing (EC50) when used in combination divided by the concentration that showed the same effect when used alone (Singh *et al.*, 2000, Krogstad, 1986). The interaction of two peptides was also assessed using the FIC index, calculated by adding up their individual FIC values from the most effective combination. An FIC index approximately equal to 1 indicates an additive interaction. An FIC index of less than 1 reveals synergistic interaction, whereas an index of more than 1 is indicative of an antagonistic interaction. Peptide interactions were also evaluated by plotting an isobologram using the FIC values of different peptide combinations. If the peptide combination is additive, synergistic or antagonistic, the isobol will be straight, concave or convex, respectively. The degree of concavity or convexity also corresponds to the degree of synergy or antagonism (Tallarida, 2001, Tallarida, 2006).

Salivary AMP analysis

Saliva samples were thawed, cleared by centrifugation twice at 15,000 rpm for 10 minutes and used to measure the AMPs levels by enzyme-linked immunosorbent assays (ELISA). The assays for HNP-1-3 and LL-37 (Hycult Biotechnology, Uden, The Netherlands) were performed according to the manufacturer's instructions, with the exception that, for the LL-37 assay, $MgCl_2$ was added to the wash/dilution buffer to a final concentration of 300 nM. Sandwich ELISA assays for HBD-2 and HBD-3 were developed using recombinant peptide standards, capture and detection antibodies from Peprotech (Rocky Hill, NJ). Briefly, Nunc Maxisorp™ 96-well plates were coated with 100 μl of capture antibody overnight. The capture antibodies used were 2 μg/ml goat anti-HBD-2 and 3 μg/ml of rabbit anti-HBD-3 for HBD-2 and HBD-3 assays, respectively. Plates were blocked with 300 μl of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hr and incubated with recombinant peptide standards and saliva samples for 1 hr. Standards and saliva samples were diluted in the sample diluent (0.05% Tween-20, 0.1% BSA, 300 mM $MgCl_2$ in PBS) and assayed in triplicate. Divalent cations were added to sample diluents to

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overcome the salivary masking effect of these peptides. $MgCl_2$ (300 nM) improved peptide recovery to an optimal level and therefore was used in this study. Following sample incubation, plates were sequentially incubated with 100 μl of detection antibodies for 1 hr, avidin-horse radish peroxidase (HRP) conjugate for 30 minutes and 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) liquid substrate (Sigma-Aldrich, St. Louis, MO). Optical density (OD) readings were read at wavelengths of 405–650 nm. Detection antibodies used were biotinylated goat anti-HBD-2 and biotinylated rabbit anti-HBD-3 for HBD-2 and HBD-3 assays, respectively. Total protein concentrations in saliva samples were measured using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using a two-sample t-test. A non-parametric Wilcoxon rank-sum test was used when the assumption of normally distributed data became invalid by the Shapiro-Wilks test. Correlation analyses were performed using a Pearson correlation test and Spearman rank correlation test. For all tests, the criterion for statistical significance was a p-value ≤0.05. SAS for Windows (v9.1, SAS Institute Inc, Cary, NC, USA) was used for the data analysis.

RESULTS

Subject demographics

Of the 60 subjects participating in this study, 38 subjects were females and 22 were males. The caries-active group consisted of 20 girls/10 boys and caries-free group consisted of 18 girls/12 boys. 58 subjects were Caucasian and one each were Hispanic and African-American. All subjects except two were in their permanent dentitions. Subjects were healthy with no current health conditions. One subject reported current antibiotics use. The majority of subjects (87%) brushed their teeth at least daily.

Mutans streptococci (MS) in dental plaque

To examine the relationship between the presence of MS in dental plaque and the caries experience of subjects, levels of MS and total cultivable bacteria from plaque samples were enumerated. Caries-active subjects showed significantly greater MS counts (p=0.04) and ratios of MS to total plaque bacteria (p=0.02) than caries-free subjects. The average level of MS recovered from caries-active subjects was 1.56×10^6 CFU/ml, whereas caries-free subjects harbored an average of 5.42×10^5 CFU/ml. Likewise, the average ratio of MS to total bacteria from dental plaque of caries-active subjects was 0.096, also significantly greater than the average value of 0.056 for caries-free subjects.

S. mutans genotyping

Ten *S. mutans* isolates were collected from plaque samples of each subject, giving a total of 600 isolates equally divided between caries-free and caries-active subjects. AP-PCR patterns generated from the DNA of these *S. mutans* isolates contained approximately 8–15 bands from each primer, representing fragments ranging from 0.3 to more than 1.5 kilobases in size. Overall, we found 74 distinct *S. mutans* amplitypes from 60 subjects (Table 1). A single distinct amplitype was observed in 47 subjects (78.33%). Twelve subjects (20%) harbored two distinct amplitypes and one subject (1.67%) harbored three amplitypes. Of the 13 subjects harboring more than one *S. mutans* amplitype, only 2 subjects (15.38%) were caries-free, whereas 11 subjects (84.62%) belonged to the caries-active group. Based on the nonparametric Wilcoxon rank-sum test, the number of *S. mutans* amplitypes was significantly greater in subjects with caries experience than in caries-free subjects (p<0.01).

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Overall, 42 genotypically distinct *S. mutans* strains were recovered from the caries-active group and 32 isolates were recovered from the caries-free group.

A dendrogram analysis demonstrated the genotypic variation among the 74 *S. mutans* strains obtained from all subjects (Fig. S1). The percentage similarities between each strain-pair based on the Pearson product-moment correlation ranged from 47.0% to 98.6%, indicating a large amount of genetic heterogeneity among the strains. Strains from 9 out of 13 subjects that harboured 2 or more amplitypes showed only a few band differences in the AP-PCR profiles compared to other genotypes from the same subject. The similarity values of these strains were more than 90% and tended to cluster closely together in the dendrogram (Fig. S2).

Susceptibility of *S. mutans* clinical strains to salivary AMPs and host caries experience

The 74 *S. mutans* clinical strains isolated were analyzed for their susceptibilities to HNP-1-3, HBD-2-3, and LL-37. Of all peptides tested, HBD-3 and LL-37 showed the most potent bactericidal activity against *S. mutans* strains. Among the α-defensins, *S. mutans* strains were most susceptible to HNP-1, followed by HNP-2 and HNP-3, respectively.

A two-sample t-test was used to compare the susceptibility profiles of *S. mutans* strains from caries-active and caries-free subjects. Since some subjects possessed more than one distinct strain and the proportion of each strain was variable in these subjects, we calculated the average susceptibilities of *S. mutans* from each clinical group using two methods: the first used the percentage viability of the predominant *S. mutans* strains from each subject to ensure equal weighting per subject, and the second method averaged the percentage viabilities of all distinct strains to account for all strains that were isolated. Results from analyses of predominant strains and all strains are shown in Fig. 1 and S3, respectively.

Comparison of the susceptibilities to HNP-1 showed that strains from the two groups differed in their average percent viabilities for the predominant strains at all three HNP-1 concentrations. *S. mutans* strains from caries-free subjects showed significantly greater susceptibility to HNP-1 than those from children with caries experience at 5 μg/ml ($p \leq 0.01$), 1.5 μg/ml ($p=0.04$), and 0.5 μg/ml ($p=0.03$) (Fig. 1A). Analyzing the average percent viability of all strains resulted in significant differences at HNP-1 concentrations of 5 μg/ml ($p=0.01$) and 0.5 μg/ml ($p=0.03$) (Fig. S3A). A similar trend was also observed at 1.5 μg/ml HNP-1; however, this did not reach statistical significance ($p=0.08$).

S. mutans strains from caries-active and caries-free subjects also showed significant differences in their susceptibility to HNP-2. Analyses of both the predominant strains and all strains revealed that the average percentage viability of *S. mutans* exposed to 5 μg/ml HNP-2 was significantly greater when isolated from subjects with caries experience than when isolated from children with no caries experience ($p=0.01$ and 0.02, respectively) (Fig. 1B and S3B).

In contrast to results with HNP-1 and HNP-2, *S. mutans* isolates showed nearly 50% less susceptibility to HNP-3. At all three concentrations tested, no statistically significant differences in susceptibility to HNP-3 were detected between *S. mutans* strains from both groups (Fig. 1C and S3C).

Susceptibility tests with two β-defensins, HBD-2 and HBD-3, also showed significant differences between groups. *S. mutans* strains from caries-free subjects showed significantly greater susceptibility to HBD-2 at 1.5 μg/ml and 0.5 μg/ml and HBD-3 at 0.5 μg/ml, whether analyzing the predominant strains or all strains ($p<0.05$) (Fig. 1D–E and S3D–E).

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Lastly, the tests of *S. mutans* strains against LL-37 also showed that *S. mutans* strains from caries-free subjects were more susceptible to LL-37 at 1.5 μg/ml and 0.5 μg/ml, compared to those from caries-active subjects. Though the difference did not reach statistical significance, the same trend was also noted at 5 μ/ml LL-37 for both the analyses of predominant strains (p=0.06) as well as all strains (p=0.06) (Fig. 1F and S3F).

Relationships between susceptibility profiles of *S. mutans* to AMPs

Our findings showed that all AMPs tested were active against *S. mutans* strains in a dose-dependent manner. These peptides share several common properties. They are small, cationic and are believed to exert their antimicrobial activities on the bacterial cytoplasmic membrane. It was of interest to examine whether *S. mutans* strains showed similar susceptibility patterns to these peptides. To determine this, percent viabilities of *S. mutans* exposed to different peptides at 5 μg/ml were correlated using Pearson correlation analysis. The data showed significant correlations between percentage viabilities to HNP-1 and HNP-2 (r=0.94, p<0.01), HNP-1 and HNP-3 (r=0.69, p<0.01), HNP-2 and HNP-3 (r=0.78, p<0.01), HBD-2 and HBD-3 (r=0.56, p<0.01), HBD-2 and LL-37 (r=0.60, p<0.01), and HBD-3 and LL-37 (r=0.53, p<0.01 (Table S1) Analyses between the pairs of three α-defensins and the β-defensins or LL-37 revealed no significant correlations.

Combined effects of salivary AMPs

The effects of AMPs in combination were examined. Pairwise combinations of these AMPs appear to enhance their antimicrobial activities either additively or synergistically. None of the combinations tested demonstrated antagonistic activity (Fig. S4).

Salivary AMP levels and host caries experience

Salivary analysis showed that AMP levels were highly variable among subjects despite normalization with total salivary protein concentrations. Mean total protein concentration in saliva was 935.2±273.4 μg/ml. Mean concentrations of HNP-1-3, LL-37, HBD-3 and HBD-2 were 1,913±1,157 ng/ml, 15.81±10.43 ng/ml, 2.233±2.183 ng/ml and 0.734±0.811 ng/ml, respectively (Table 2).

To assess the relationships between the levels of AMPs in saliva and caries experience in children, two-sample t-tests were used to compare mean salivary levels of each peptide, with or without normalization to total salivary protein, for caries-free and caries-active subjects. For all peptides tested, we found no significant differences in salivary levels between the two groups: HNP1-3 (p=0.37), HNP1-3 relative to total protein (p=0.84); LL-37 (p=0.94), LL-37 relative to total protein (p=0.44); HBD-3 (p=0.81), HBD-3 relative to total protein (p=0.54); HBD-2 (p=0.23), and HBD-2 relative to total protein (p=0.54). Interestingly, our data showed that the mean total salivary protein level was significantly greater in children with caries experience than that observed in caries-free children (p=0.02). Mean total salivary protein concentrations were 1,018±306.3 μg/ml (range from 574.8 to 1,661 μg/ml) in caries-active subjects and 852.2±209.7 μg/ml (range from 385.6 to 1,241 μg/ml) in caries-free subjects (Table 3).

Relationships between the salivary AMP levels

Since these AMPs to some extent play overlapping and/or collaborative roles in host defense against oral pathogens, we next evaluated the correlations between AMP levels in saliva within individuals. Results are shown in Figure S5. Pearson correlation analyses showed statistically significant correlations between the levels of LL-37 and HNP1-3 (r=0.66, p<0.01) and between the levels of LL-37 relative to total protein and HNP1-3 relative to total protein (r=0.65, p<0.01). In addition, statistically significant correlations were found

between HBD-2 and HBD-3 levels (r=0.50, p<0.01) and between HBD-2 relative to total protein and HBD-3 relative to total protein (r=0.67, p<0.01). We also found significant correlations between HNP1-3 and HBD-2 concentrations in saliva (r=0.37, p<0.01) and between the levels of HBD-3 relative to total protein and LL-37 relative to total protein (r=0.26, p<0.05). No significant correlations were observed between other variables.

Relationships between the salivary AMP levels and MS or total bacteria in dental plaque

We further examined the relationships between the salivary levels of AMPs and MS levels within dental plaque. Analyses showed no significant correlations between the ratio of MS relative to total bacteria in dental plaque and salivary levels of any AMPs tested with or without normalization to total salivary protein. However, we found a significant positive relationship between the plaque MS count and HNP-1-3 concentration relative to total protein (r=0.28, p=0.03) (Fig. S6). The correlation analysis between MS count and the non-normalized salivary concentration of HNP-1-3 also revealed a similar trend but did not reach statistical significance (p=0.07). No significant correlations were found between MS counts and salivary concentrations of other peptides.

The relationships between salivary levels of AMPs and total cultivable bacteria in dental plaque were also examined. Spearman rank correlation tests showed no statistically significant correlations between salivary levels of each peptide, with or without normalization, and total plaque bacteria. However, it should be noted that there was a trend towards an increasing relationship between total plaque bacteria and salivary concentration of HBD-3 (p=0.06) and salivary concentration of HBD-3 relative to total protein (p=0.09), particularly when considering caries-active subjects only (data not shown).

Relationships between the salivary AMP levels and the susceptibility profiles of *S. mutans*

We next asked if there were relationships between salivary AMP levels and susceptibility to the corresponding peptide of *S. mutans* isolated from the same individual. It was expected that *S. mutans* strains would be most resistant to the AMPs that were represented at the highest levels in saliva. Pearson correlation analyses showed a significant positive relationship between the salivary level of HNP-1-3 relative to total protein and the percentage viability of *S. mutans* strains to 5 μg/ml HNP-1 (r=0.26, p=0.04) (Fig. S7).

DISCUSSION

In this study, several findings were demonstrated. For the first time, it was shown that *S. mutans* strains from individuals with caries experience showed greater resistance to salivary AMPs, compared to those obtained from caries-free individuals, suggesting that the ability of *S. mutans* to resist these peptides may be another factor that can help define the relative virulence of this bacterium. Significant differences were observed with HNP-1-2, HBD-2-3 and LL-37 at varying concentrations. We deliberately selected physiological concentrations of AMPs to assay. Although the susceptibility profiles of *S. mutans* strains to the three HNPs were highly correlated, significant differences between the susceptibilities to HNP-3 between both groups were not observed at the three concentrations tested, potentially due to the overall lower antimicrobial activity of this peptide. The susceptibility profiles of *S. mutans* strains to HBD-2, HBD-3 and LL-37 were also correlated, suggesting that these peptides may act by similar mechanisms, yet possess varying potentials for combating pathogens.

Based on our data, we speculate that *S. mutans* strains that are more resistant to host AMPs may have an ecological advantage over the more susceptible strains for surviving within dental plaque. It has been observed that *S. mutans* compete relatively poorly with other non-

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pathogenic microorganisms at neutral pH (Bradshaw *et al.*, 1996). *S. mutans* appears to be more susceptible to host AMPs than oral streptococci that constitute the major proportion in normal non-pathogenic plaque, such as *S. sanguinis, S. mitis* and *S. oralis* (Bartie *et al.*, 2008, Joly *et al*., 2004, Nishimura *et al.*, 2004a, Ouhara *et al.*, 2005). This suggests that salivary AMPs may play a role in maintaining the oral health by limiting the growth of potentially pathogenic organisms such as *S. mutans* and allowing the more resistant non-pathogenic bacteria to establish colonization and dominate. Therefore, differences in susceptibility profiles of *S. mutans* strains may be significant in providing the resistant strains with a competitive advantage over susceptible strains. In particular, under conditions favorable for growth, such as the increased presence of fermentable sugar and low pH, resistant *S. mutans* strains may better survive and populate, leading to greater cariogenic potential.

However, we did not find statistically significant differences between salivary levels of HNP-1-3, LL-37, HBD-3 and HBD-2 between caries-free and caries-active subjects. These results partially differed from those of a previous study (Tao *et al*., 2005), which showed significantly higher HNP-1-3 levels in saliva of caries-free subjects compared to that of caries-active subjects but no significant differences in LL-37 and HBD-3 levels between groups. This difference between studies may be the result of several factors. In this study, we used stimulated saliva as a source for AMP detection, whereas unstimulated whole saliva samples were used in the Tao *et al*. study. Differences between the concentrations of specific AMPs in stimulated versus unstimulated saliva have not been reported to date. However, it has been noted that salivary protein composition can differ, depending on whether saliva was collected under stimulated or unstimulated conditions (Oberg *et al.*, 1982, Rantonen & Meurman, 2000). In addition, one notable difference is the subject ethnicity. While the overwhelming majority of our subjects were Caucasians, subjects in their study were mostly Hispanic. Several studies have observed not only differences in caries prevalence among people with diverse racial/ethnic backgrounds, but also variation in salivary protein composition (Everhart *et al.*, 1973, Johnson, 2005, Sivakumar *et al.*, 2009, Zakhary *et al.*, 2007). However, no racial/ethnic differences in AMP expression or genetic polymorphisms have yet been confirmed. Future studies may potentially help clarify the effect, if any, of race/ethnicity on AMP expression.

Our results do not support the measurement of salivary AMP levels as a reliable tool to predict caries risk, at least not for stimulated saliva. As noted earlier, increased HNPs levels in saliva have been observed in patients with various oral inflammatory conditions (Mizukawa *et al.*, 1998, Mizukawa *et al.*, 1999a, Mizukawa *et al.*, 1999b). The infiltration of neutrophils within these lesions is believed to be responsible for the increased defensin levels (Abiko & Saitoh, 2007). These findings support the role of salivary AMPs as a first line of host immune response in the oral cavity. However, their levels of expression appear to be associated with a wide range of inflammatory conditions in a rather non-specific manner. Any degree of simultaneous gingival or oral mucosal inflammation can contribute to the levels of these peptides in saliva irrespective of host caries activity.

It is of note that salivary HNP-1-3 levels detected in this study were well within the range of their antimicrobial activity, supporting a physiologically relevant role in oral immune defense. LL-37 and HBDs, however, were found within the ng/ml range in saliva in this subject group. These salivary concentrations are generally lower than their effective range *in vitro*, though the actual physiological concentrations of these peptides at different sites in the oral cavity are not known. The HBDs are expressed from oral keratinocytes as well as salivary duct cells, and their expression is particularly strong at regions close to mucosal surfaces such as at gingival margins around the tooth, allowing for close contact with supragingival plaque (Dale & Fredericks, 2005). Similarly, LL-37 is secreted from multiple

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sources, including salivary ducts, oral epithelium and neutrophils. The levels of HNP-1-3 and LL-37 are concentrated in gingival crevicular fluid, in close proximity to tooth surfaces. This could potentially increase the concentrations of these peptides substantially within the environment surrounding tooth structure and, thus, closely influence microbial ecology within dental plaque. These AMPs can work additively or synergistically to enhance their antimicrobial activity. Furthermore, the α-defensins, β-defensins, and LL-37, have other immunomodulatory roles, chemoattractant activity, and can stimulate the adaptive immune response, including enhancing the production of IgA or IgG (Yang *et al.*, 2004). Therefore, the effect of saliva must be considered in its entirety to explain completely its contribution to dental health.

Unexpectedly, we found a significantly greater level of total salivary protein in subjects with caries experience than in caries-free subjects. Since protein concentrations in saliva are also dependent on salivary flow rate, it is possible that these caries-active subjects may have had lower salivary flow rates than caries-free subjects, which led to more concentrated protein in saliva and increased susceptibility to dental caries. Another explanation is that caries-active subjects may have higher concentrations of specific protein components in saliva that potentially facilitate dental caries formation. Several studies have suggested a role for specific salivary proteins in the adhesion of bacteria onto oral surfaces by forming adherent biofilms, or pellicles (Scannapieco, 1994, Bradway *et al.*, 1989). A recent study also reported a positive correlation between the total protein and glycoprotein contents in saliva and saliva-promoted *S. mutans* adhesion to hydroxyapatite (Shimotoyodome *et al*., 2007). A number of salivary components, when adsorbed to oral surfaces, were described to mediate molecular interactions with oral bacteria, including mucins (Stinson *et al.*, 1982), α-amylase (Scannapieco *et al.*, 1995, Scannapieco *et al.*, 1993), fibronectin (Dawson & Ellen, 1990) and PrPs (Russell & Mansson-Rahemtulla, 1989, Gibbons & Hay, 1989). Therefore, this pellicle-mediated bacterial adhesion could provide the basis for the robust formation of dental plaque populated with sufficient proportions of cariogens to increase risk of dental caries. Moreover, the role of salivary proteins as a source of nutrients to oral bacteria has also been suggested. During dental plaque formation, plaque bacterial colonizers may require specific salivary proteins that they can degrade to provide nutrients for their metabolism, allowing further growth, multiplication and aggregation to occur (Scannapieco, 1994, Bowden & Li, 1997).

Interestingly, we found that the salivary levels of several AMPs were correlated within an individual. The correlations between the salivary levels of HNP-1-3 and LL-37, and between HBD-2 and HBD-3, are consistent with the natural sources of these peptides, since the majority of HNP-1-3 and LL-37 are produced from neutrophils, and both HBD-2 and HBD-3 are secreted from oral epithelial cells and salivary duct cells. In addition, we also observed increasing relationships between salivary levels of HNP-1-3 and HBD-2 and between LL-37 and HBD-3. Collectively, it appears that these AMPs tend to be produced together in saliva, probably due to the similar types of inflammatory stimuli present as a part of the innate immune response. This may provide an appropriate setting for these peptides to work additively or synergistically to increase their antimicrobial activity against pathogens.

Also examined were the relationships between the salivary AMP levels and the MS levels in dental plaque. We found a significant positive correlation between the HNP-1-3 relative to total protein and the MS count. Likewise, the correlation analyses between total bacteria in dental plaque and salivary levels of HBD-3 or HBD-2 showed trends toward increasing relationships between these variables. These results are consistent with previous findings that the expression of HBD-3 and HBD-2 in oral epithelial cells is inducible upon bacterial contact or in response to pro-inflammatory stimuli, including IL-1β, TNF-α or IFN-γ (Chung & Dale, 2004, Joly *et al.*, 2005, Krisanaprakornkit *et al.*, 2000, Mathews *et al.*, 1999,

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Taguchi & Imai, 2006). Their expression, however, varies with different bacterial species (Ji *et al.*, 2007, Kimball *et al.*, 2006, Krisanaprakornkit *et al*., 2000, Vankeerberghen *et al.*, 2005). A recent study demonstrated that a *S. mitis* biofilm significantly upregulated HBD-2 gene expression in gingival epithelial cells, whereas *S. mutans* biofilm was a poor inducer (Eberhard *et al.*, 2009). It is possible that the more resistant early colonizers in dental plaque may induce the expression of AMPs, which in turn limits the colonization and survival of more sensitive, potentially pathogenic microorganisms, such as *S. mutans* (Dale & Fredericks, 2005, Weinberg *et al.*, 1998).

Lastly, we examined the relationship between levels of salivary AMPs and the susceptibility/resistance profiles of *S. mutans* isolated from the same individuals. A significant correlation was observed between the salivary level of HNP-1-3 relative to total protein and the percentage viability of *S. mutans* strains to 5 μg/ml HNP-1 (p=0.04), suggesting that individuals with higher levels of HNP-1-3 in saliva tend to harbor *S. mutans* strains that are more resistant to HNP-1. This finding is in the line with a previous study, which found that, in a series of selection experiments, microbial agents are able to evolve *in vitro* in response to a gradual increase of AMP concentrations such that, over several hundred generations, there is natural selection for organisms that are more resistant to the tested peptide (Perron *et al.*, 2006). These data support our findings that increasing resistance to salivary AMPs is one mechanism that promotes *S. mutans* survival in the oral environment, potentially elevating the risk of dental caries.

In conclusion, our findings support the roles of host salivary AMPs in shaping *S. mutans* ecology by restricting the overall growth of this cariogenic bacterium. The presence of these peptides in saliva and dental plaque may be a factor in the selection of more resistant strains that better populate the plaque, thereby increasing the likelihood of initiating or propagating the dental caries process, particularly when the surrounding environment favors their growth. In other words, the relative ability of *S. mutans* to resist the antimicrobial activity of these peptides may constitute a virulence factor for this organism. However, the expression of AMPs in saliva is associated with the presence of local inflammatory stimuli, such as the dental plaque microflora, and is not specific to host dental caries experience.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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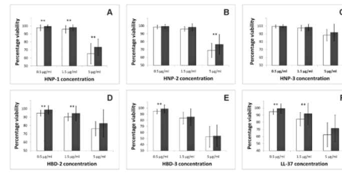

Fig. 1. Susceptibility of *S. mutans* from caries-active versus caries-free subjects: analysis of predominant strains from subjects

(A) HNP-1; (B) HNP-2; (C) HNP-3; (D) HBD-2; (E) HBD-3; (F) LL-37. White bars represent caries-free subjects. Black bars represent caries-active subjects. Asterisks indicate statistically significant differences in viabilities between both groups ($p<0.05$).

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Table 1

Numbers of subjects harboring 1-3 distinct *S. mutans* amplitypes.

Subjects	Sex	Numbers of distinct *S. mutans* amplitypes per subjects		
		1	**2**	**3**
All subjects	Girls (38)	30	7	1
	Boys (22)	17	5	0
	All (60)	47	12	1
Caries-active subjects	Girls (20)	13	6	1
	Boys (10)	6	4	0
	All (30)	19	10	1
Caries-free subjects	Girls (18)	17	1	0
	Boys (12)	11	1	0
	All (30)	28	2	0

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Table 2

Levels of AMP in saliva.

	Total salivary protein (µg/ml)	Salivary concentration (ng/ml)				Salivary concentration relative to total protein (ng/mg)			
		HNP-1-3	LL-37	HBD-3	HBD-2	HNP-1-3	LL-37	HBD-3	HBD-2
Mean	935.19	1913.56	15.80	2.23	0.73	2034.33	17.48	2.57	0.78
SD	273.36	1157.25	10.44	2.18	0.81	1069.12	11.37	3.07	0.81
Median	933.76	1443.36	14.20	1.67	0.39	1704.90	15.52	1.88	0.44
Maximum	1661.19	5213.06	71.02	11.56	3.89	5545.39	77.21	19.36	3.35
Minimum	385.57	548.63	3.93	0.15	0.08	631.38	4.02	0.11	0.08

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Table 3

Mean salivary AMP levels between caries-active and caries-free subjects.

Salivary concentrations	Caries-active	Caries-free	t-test p-values
HNP-1-3 (ng/ml)	2047.44±1254.76	1779.68±1055.03	p=0.37
HNP-1-3 relative to total protein (ng/mg)	2006.42±1125.17	2062.24±1028.52	p=0.84
LL-37 (ng/ml)	15.92±7.50	15.70±12.86	p=0.94
LL-37 relative to total protein (ng/mg)	16.33±8.29	18.64±13.84	p=0.44
HBD-3 (ng/ml)	2.16±1.91	2.30±2.46	p=0.80
HBD-3 relative to total protein (ng/mg)	2.33±2.39	2.82±3.65	p=0.54
HBD-2 (ng/ml)	0.86±0.90	0.85±0.83	p=0.23
HBD-2 relative to total protein (ng/mg)	0.61±0.71	0.72±0.81	p=0.54
Total protein (μg/ml)	1,018.19±306.33	852.19±209.69	p=0.02*