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Genotypic and phenotypic analysis of *Streptococcus mutans* from different oral cavity sites of caries-free and caries-active children

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Lembo FL, Longo PL, Ota-Tsuzuki C, Rodrigues CRMD, Mayer MPA. Genotypic and phenotypic analysis of Streptococcus mutans *from different oral cavity sites of caries-free and caries-active children.*
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Introduction: *Streptococcus mutans* exhibits extensive genotypic diversity, but the role of this variation is poorly understood. This study aimed to determine the number and distribution of genotypes of *S. mutans* isolated from caries-active and caries-free children and to evaluate some of their phenotypic traits.
Methods: Stimulated saliva, tongue surface and biofilms over sound and carious teeth surfaces were sampled from 10 caries-free and 11 caries-active children aged 5–8 years. A total of 339 isolates of *S. mutans* were genotyped by arbitrarily primed polymerase chain reaction using OPA*2* primer. One isolate from each genotype was tested for its acid susceptibility and its ability to form a biofilm.
Results: Fifty-one distinct genotypes were determined, one to three genotypes in each oral sample. A single genotype was detected in seven children, whereas the remaining 14 children exhibited two to seven genotypes. There were no significant differences in the number of genotypes detected in caries-free and caries-active children. No correlation was observed between the number of genotypes and the mutans streptococci salivary levels. Five of the six high biofilm-forming genotypes were obtained from caries-active children, although the differences in biofilm formation between isolates from caries-free and caries-active children were not statistically significant. Genotypes with low susceptibility to acid challenge were statistically more frequent among isolates from caries-active children than among those from caries-free children.
Conclusion: The present data suggested that there were differences in the distribution of genotypes of *S. mutans* according to the oral site and that *S. mutans* populations differ in their acid susceptibility and ability to form biofilms, factors allowing their colonization of sucrose-rich environments.

Key words: genotypes; phenotypic traits; *Streptococcus mutans*

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Streptococcus mutans are gram-positive cocci found in the mouths of approximately 90% of humans and are associated with the etiology of dental caries. This species, as well as *Streptococcus sobrinus*, is resident in the oral cavity of humans, whereas the remaining species of the mutans streptococci (MS) are part of the oral microflora in animals (21).

S. mutans exhibits extensive diversity, which may affect the pathogenic potential of the clone. The activity and amount of glucosyltransferase vary among isolates (25, 26, 29). Other factors such as biofilm formation *in vitro* (25, 36), mutacin production (14, 22), and cariogenic potential *in vitro* (9) and *in vivo* (8, 17) also exhibit diversity within the species.

The human mouth can harbor one or more genotypes of *S. mutans*, which are persistent for several years (1, 14, 19, 32) even after use of chlorexidine gel (13). A high number of clones of *S. mutans* was correlated with increased caries prevalence, possibly as the result of the simultaneous activity of several genotypes with different cariogenic potentials in a site (2). The persistence and proportion of a given clone of *S. mutans* in a site may be associated with its ability to colonize and resist environmental stress (7). It was proposed that the colonization of the oral cavity by *S. mutans* was site-specific (12, 16). Thus, factors such as the ability to form biofilm (25) and to resist acid challenge (6, 38) may account for the survival of a certain genotype.

Little is known of the correlation between the phenotypic traits and the genotypes of *S. mutans*, so this study aimed to analyze the number of genotypes of *S. mutans* in oral sites of children with different caries experience and to determine the adhesive properties and the ability to survive acidic stress of these genotypes.

Materials and methods

Study design

Genotypic diversity and phenotypical traits were evaluated for *S. mutans* isolates obtained from 11 caries-active and 10 caries-free children aged 5–8 years. Forty-four caries-free or caries-active children had been previously selected. Saliva samples and biofilm samples from tongue surface and from caries-active and caries-free sites on enamel surfaces were obtained and cultivated for *S. mutans* isolation. Only isolates from 21 subjects were analyzed further. To estimate genotypic diversity, *S. mutans* isolates were genotyped by arbitrarily primed polymerase chain reaction (AP-PCR). The main exclusion parameter for a patient was the lack of at least three *S. mutans* isolates obtained from each sampled site. A total of 339 strains of *S. mutans* was obtained from the 21 subjects, comprising at least nine strains for each caries-free subject and 12 for each caries-active subject, which were randomly selected for genotyping. Then, one strain from each detected genotype was selected for the phenotypical evaluation of acid susceptibility and biofilm formation.

Patient selection

All the experimental procedures were approved by the Ethical Committee for research in humans of the Institute of Biomedical Sciences, University of São Paulo and written informed consent was obtained from the parents of all children before sample collection. Children were selected from those seeking treatment at the Pediatric Dentistry Clinic, School of Dentistry, University of São Paulo. Antibiotic therapy, topical fluoride application and use of mouth antiseptic rinses in the previous 3 months were considered exclusion parameters for this study. A calibrated examiner performed the clinical examination in all children using a mouth mirror and light as well as interproximal X-rays from the posterior teeth. Previous examination data of the 44 children supported their classification as caries-free or caries-active. Dental plaque samples, either from sound or carious surfaces, were obtained based on the initial clinical examination, then the tooth surfaces were cleaned, dried and examined visually a second time to ensure the initial diagnosis. Children whose initial diagnosis for caries was not confirmed after plaque removal were not considered and these samples were not included in the study. Caries-active children presented at least one manifest or restored caries lesion (decayed/missing/filled teeth ≥1) and at least one incipient lesion on a buccal surface with clinical characteristics of activity, i.e. white chalky rough surface. Caries-free children presented no history of caries, with no evidence, either clinical or radiographic, of manifest or restored surfaces and no incipient active enamel lesion.

Sampling

Biofilms over a smooth sound enamel surface and over an active white spot lesion on the buccal surfaces were obtained with an interdental brush (Sanifill, São Paulo, Brazil) from each child (the latter only from children in the caries-active group). The microbial sample from the tongue surface was obtained with the aid of a sterile wooden spatula. Stimulated saliva samples were collected after paraffin chewing for 5 min. Tongue surface and biofilm samples were transferred to PRAS VMGAII transport medium, and saliva samples were transferred to PRAS VMGAII medium containing charcoal (28).

S. mutans isolation and identification

All samples were taken to the laboratory and manipulated on the same day. Samples were diluted in peptone water and aliquots were inoculated on selective mitis salivarius bacitracin agar plates (Difco, Sparks, MD) (11). After incubation in 5% CO_2 at 37°C for 48 h, colonies characteristic of MS were counted and the salivary levels of MS were calculated. About 15 colonies resembling *S. mutans* (rough appearance, adherent to the agar and with glistening drops of liquid on or around the colony) from each sample were transferred to brain–heart infusion (BHI) broth (Difco) and incubated as above; the cells were kept frozen at −80°C in 20% glycerol.

The isolates were subcultured on BHI agar from frozen stocks and DNA was extracted according to Alam et al. (4), with some modifications. Briefly, the colonies were suspended with a calibrated loop into 300 μl sterile water. Cell lysis was obtained by boiling the suspension for 10 min, followed by centrifugation at 10,000 ***g*** for 10 s (Eppendorf, Hamburg, Germany). The supernatant containing DNA was transferred to another tube and stored in a freezer at −20°C until use.

The isolates were identified as *S. mutans* by PCR using primers homologous to *gtfB* described by Oho et al. (30). Each reaction consisted of 5 μl template DNA, 1 μM of each primer (5′-ACT ACA CTT TCG GGT GGC TTG G-3′ and 5′-CAG TAT AAG CGC CAG TTT CAT C-3′), 200 μM of each DNTP, 5 μl 1x PCR buffer, 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Invitrogen, São Paulo, Brazil) in a total volume of 50 μl. The amplification reaction was performed in a Perkin Elmer thermocycler (Perkin Elmer, Foster, CA) in 30 cycles as follows: denaturation 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min. As a negative control, no template DNA was added. DNA of strain GS-5 was used as template DNA in positive control reactions.

The resulting amplicons were submitted to electrophoresis in 1% agarose gel with TAE buffer (Tris–acetate 40 mM, EDTA 2 mM, pH 8.5), stained with ethidium bromide, visualized under ultraviolet light, documented by the Photo system PC3100Z and analyzed using the program SYNGENE-GENE-TOOLS ANALYSIS Software version 3.03.03 (Cambridge, UK).

The 517-base-pair amplicon was considered specific for *S. mutans*.

Genotyping

Genotyping was performed by AP-PCR using OPA*2* primer (5′-TGC CGA GCT G-3′) (20). The reaction was processed in 50 µl mixtures containing 1x PCR buffer, 2.5 U of *Taq* DNA polymerase (Invitrogen), 200 µM of each DNTP, 50 pmol primer, 7 mM $MgCl_2$ and 5 µl template DNA. Reactions were performed with the following conditions: one initial cycle of denaturation at 94°C for 5 min, followed by 45 cycles of 94° for 30 s, 36°for 30 s and 72°C for 1 min, and a final extension of 72°C for 5 min.

The amplicons were separated by electrophoresis in 2% agarose gels using TAE buffer and were stained with ethidium bromide. Images were captured with the Photo system PC3100Z and the amplicons were evaluated visually with the aid of the SYNGENE-GENE-TOOLS ANALYSIS Software version 3.03.03. To assure reproducibility, the reactions were performed twice in at least two independent amplifications. In every set of reactions, DNA from *S. mutans* GS-5 and *Streptococcus sanguinis* ATCC 10556 were included as controls.

Biofilm formation

To evaluate the ability to form biofilm, one isolate that was representative of each genotype from each child was tested using the methodology proposed by Mattos-Granner et al. (25). A total of 51 genotypes and strain GS-5 were tested. Cells were grown for 18 h in Todd Hewitt broth (Acumedia, Baltimore, MD) adjusted to an absorbance at 550 nm ($A_{550\ nm}$) of 0.3, diluted to 1 : 100 into fresh media and transferred to wells in microtiter plates. Todd Hewitt broth contains trace amounts of sucrose so it provides a substrate for Gtf activity. After incubation for 18 h at 37°C under anaerobiosis (85% N_2, 10% H_2 and 5% CO_2) in an anaerobic chamber (Plaslabs, Lansing, MI), the wells in the microtiter plates were washed and the biofilms were stained with 1% crystal violet. The absorbances of crystal violet dissolved in ethanol of the stained biofilms were measured at $A_{575\ nm}$. The absorbances of planktonic cultures grown under the same conditions were also measured ($A_{550\ nm}$). All experiments were performed in triplicate. The biofilm rate index was the ratio between the absorbance value of crystal violet eluted from the biofilm ($A_{575\ nm}$) and the absorbance value obtained from the planktonic growth ($A_{550\ nm}$).

Acid susceptibility assay

The ability to withstand acid stress was evaluated using the method described by Belli and Marquis (6), with slight modifications. Fifty-one genotypes and strain GS-5 were tested. To avoid subculturing, cells from frozen stocks with only two transfers in culture medium were inoculated in BHI broth and grown for 24 h. Then these cells were inoculated in BHI broth and grown to $A_{600\ nm} = 0.3$, harvested, and washed once with 0.1 M glycine buffer (pH 7.0). Acid stress was performed by resuspending the cell pellets in 0.1 M glycine buffer (pH 2.8) followed by incubation for 5 min. Cells resuspended in 0.1 M glycine buffer (pH 7.0) acted as positive controls. After incubation, the number of surviving cells was estimated by inoculating diluted aliquots from each tube at pH 7.0 and pH 2.8 on the surface of BHI plates. After growth in 5% CO_2 at 37°C for 48 h, the number of colony-forming units (CFU) was determined for each pH condition. The experiments were performed in triplicate. Acid susceptibility was considered as the percentage of non-viable cells at pH 2.8 in relation to the total number of viable cells at pH 7.0.

Statistical analyses

Statistical analysis was performed using the program STATGRAPHICS (Herndon, VA). Average comparison was used to compare the number of genotypes of *S. mutans* and the MS salivary levels in the caries-active and caries-free groups. Average comparison was also used to compare MS salivary levels in subjects harboring one or more genotypes of *S. mutans*. The Pearson rank correlation index was used to assess whether the number of genotypes was correlated with the number of studied strains. Fisher's exact test was used to analyze if there were differences in MS levels in children in relation to the number of detected genotypes of *S. mutans*, and to estimate if the distributions of genotypes exhibiting low acid susceptibility or high biofilm formation ability were similar among caries-active and caries-free children. Analysis of variance was used to assess differences in acid susceptibility among genotypes according to isolation site. Values of $P \leq 0.05$ were considered significant.

Results

From the 44 children that were initially selected, 21 were included in the study based on clinical data and microbiological analyses. Clinical examination revealed that the 11 selected caries-active children had a mean of 5.54 ± 3.29 restored or cavitated surfaces in primary teeth [decayed/missing/filled surfaces (DMFS) ranged from three to 32] and a mean of 0.91 ± 1.44 restored or cavitated surfaces in permanent teeth (DMFS 0–4). The 10 children in the caries-free group presented with one DMFS, no DMFS or no incipient active lesions.

MS salivary levels

Mutans streptococci salivary levels were determined in children from the caries-free and caries-active groups. In the caries-free group ($n = 10$), two children exhibited MS levels lower than 10^4 CFU/ml saliva, five showed levels between 10^4 and 10^5 CFU/ml saliva and three exhibited levels between 10^5 and 10^6 CFU/ml saliva. In the caries-active group ($n = 11$), five children showed MS levels between 10^4 and 10^5 CFU/ml saliva and six subjects had MS levels between 10^5 and 10^6 CFU/ml saliva. The average MS level was $1.5 \times 10^5 \pm 1.7 \times 10^5$ CFU/ml saliva among the 21 studied subjects. There were no statistically significant differences in average values of MS in saliva between caries-free ($1.1 \times 10^5 \pm 1.4 \times 10^5$ CFU/ml) and caries-active children ($1.8 \times 10^5 \pm 1.9 \times 10^5$ CFU/ml). This was the result of the exclusion from the study of children with low MS levels (from whom at least three *S. mutans* isolates from each site could not be obtained).

Genotyping

A total of 339 *S. mutans* isolates were genotyped (141 from caries-free children and 198 from caries-active children), three to five from each oral site from each child. Fifty-one genotypes were found among the 339 isolates. Each genotype was unique to the subject. The bands profiles of the AP-PCR data obtained for each genotype are shown in Fig. 1. The distribution of genotypes according to subject and oral site of isolation is shown in Table 1.

Despite the higher number of studied sites and isolates in the caries-active group,

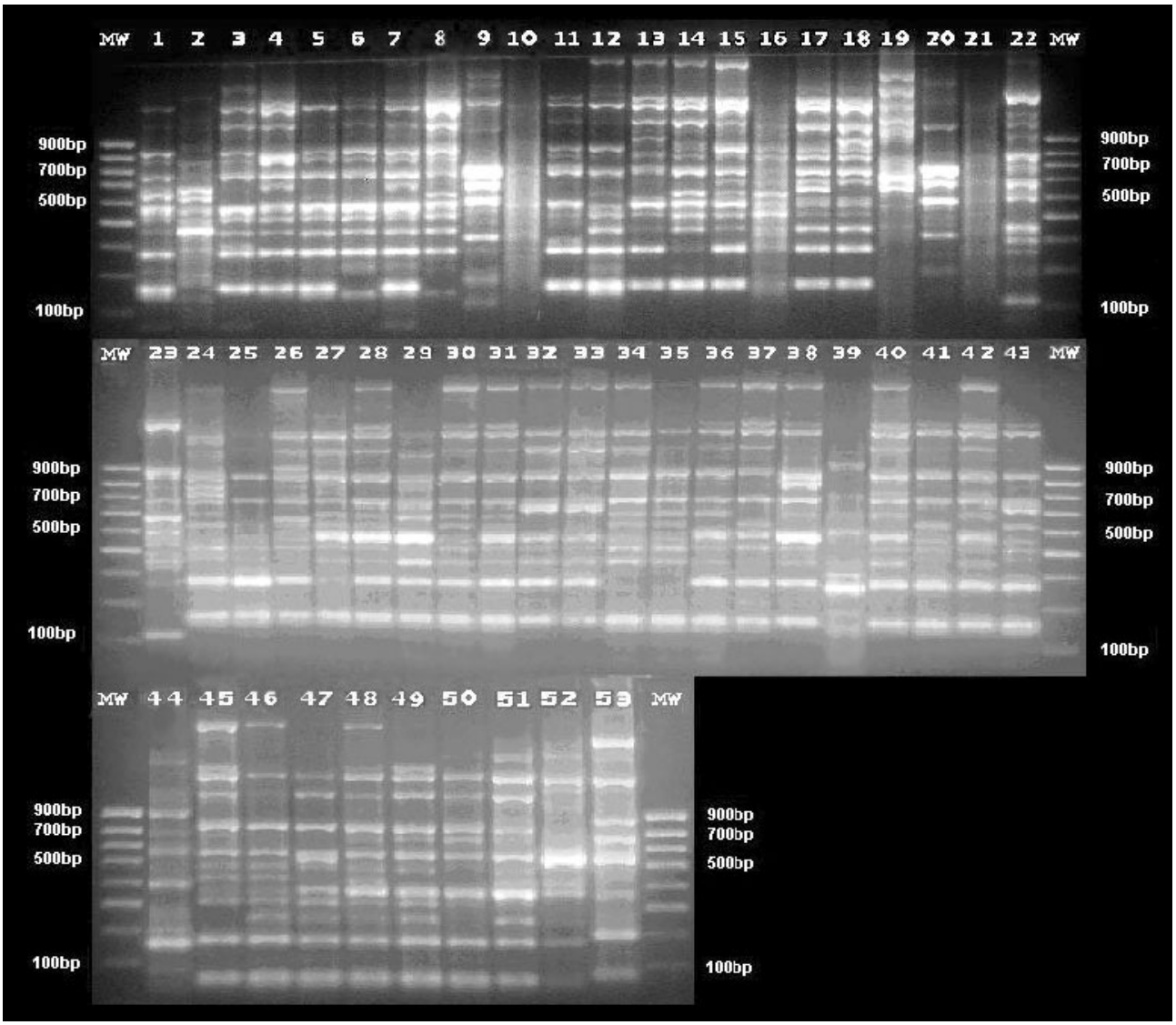

Fig. 1. AP-PCR products after electrophoresis in 2% agarose gel stained with ethidium bromide. Reactions using template DNA obtained from *Streptococcus mutans* isolates from caries-active (A) and caries-free (F) children. Lane 1, GS-5; lane 2, *Streptococcus sanguinis* ATCC 10556; lanes 3 and 4, A1; lanes 5–7, A2; lanes 8 and 9, A3; lanes 10–12, A4; lanes 13–16, A5; lanes 17–23, A6; lanes 24–27, A7; lane 28, A8; lane 29, A9; lane 30, A10; lane 31, A11; lanes 32 and 33, F1; lanes 34 and 35, F2; lanes 36–39, F3; lanes 40–43, F4; lanes 44 and 45, F5; lane 46, F6; lane 47, F7; lanes 48 and 49, F8; lane 50, F9; lanes 51–53, F10; MW lane, 100-bp DNA ladder.

the Pearson Rank correlation index indicated an absence of correlation between number of studied sites and number of detected genotypes ($R = 0.103$, $P < 0.05$). Seven subjects presented only one genotype (four caries-active and three caries-free). Fourteen children presented more than one genotype, seven from each group. A mean of 2.2 ± 1.14 genotypes were detected in caries-free subjects, whereas the mean number of genotypes in the caries-active group was 2.64 ± 1.86. There were no statistically significant differences in the number of genotypes between the groups. Salivary levels of MS in children harboring one genotype of *S. mutans* were $0.8 \times 10^5 \pm 1.5 \times 10^5$ CFU/ml saliva (range 6.0×10^3 to 4.4×10^5 CFU/ml), whereas in multicolonized children this value was $1.8 \times 10^5 \pm 1.7 \times 10^5$ (range 8.7×10^3 to 5.4×10^5 CFU/ml). Only one of seven subjects with one genotype had MS salivary levels $>10^5$ CFU/ml, whereas six of the 14 multicolonized subjects exhibited these MS levels. However, there was no statistically significant difference in mean MS salivary level between children with one or more genotypes of *S. mutans*. In addition, Fisher's exact test revealed that the distribution of children who were highly colonized by MS did not differ between children colonized by one or more genotypes ($P > 0.05$).

Thirty-one of the 51 genotypes (62.7%) were detected in saliva samples. Twenty-five genotypes detected in the biofilm (over sound surface or white spot lesion) (49%) were also detected in saliva. Six genotypes (11.7%) were only found over the tongue surface, whereas 13 genotypes were only detected in biofilm (eight genotypes in biofilm over sound dental surface and five over white spot lesions) among 20 genotypes from the biofilm that were not detected in saliva. Three genotypes were

Table 1. Number and location of *Streptococcus mutans* genotypes in caries-free and caries-active children

Child (*n*)	Site Saliva	Tongue	Biofilm over sound surface	Biofilm over incipient lesion[1]
F1 (2)	T1	T1; U2	T1; U2	
F2 (2)	T3; U4	T3; U4	T3; U4	
F3 (4)	T5; U6	T5; V7; X8	T5; X8	
F4 (4)	T9	T 9; U10; V11	T9; U10; X12	
F5 (2)	T13	U14	U14	
F6 (1)	T15	T15	T15	
F7 (1)	T16	T16	T16	
F8 (2)	T17; U18	T17; U18	T17; U18	
F9 (1)	T19	T19	T19	
F10 (3)	T20; U21	T20	T20; V22	
A1 (2)	T23	T23	T23	T23; U24
A2 (3)	T25; U26	T25; V27	T25; U26	T25; U26
A3 (2)	T28	T28	T28	T28; U29
A4 (3)	T30; U31; V32	T30; U31; V32	T30; U31; V32	V32
A5 (4)	T33; U34	T33; V35	T33; V35	U34; V35; X36
A6 (7)	T37; U38; V39	U38; X40; Z41	W42; Q43	U38
A7 (4)	T44; U45	T44; V46; X47	T44	T44; X47
A8(1)	T48	T48	T48	T48
A9 (1)	T49	T49	T49	T49
A10 (1)	T50	T50	T50	T50
A11 (1)	T51	T51	T51	T51

For each child, unique genotypes were named by a letter and number.
F = caries-free; A = caries-active; *n* = number of clones per child.
[1]Sampled only in the caries-active group.

found only in biofilms over white spot lesions, but not over the sound surface in the same subject.

Biofilm formation

Isolates (one isolate from each genotype, $n = 51$) were further submitted to assays to estimate their abilities to form biofilm and to withstand acid challenge.

The biofilm assay was based on the number of cells adhering to the well after vigorous washings in a sucrose environment. Mann–Whitney *U*-test revealed that the mean values for biofilm rate index in genotypes from caries-active patients (0.956 ± 3.41) did not differ from the genotypes from caries-free subjects (0.359 ± 0.382). Six of the 51 studied genotypes were considered highly efficient at biofilm formation (biofilm rate index > 0.8). Five of 29 genotypes from caries-active children were considered high biofilm producers whereas only one of the 22 genotypes from caries-free children exhibited this ability. Fisher's exact text revealed that the difference in distribution of genotypes that were highly efficient in biofilm formation between caries-active and caries-free children was not statistically significant ($P > 0.05$).

Acid susceptibility

Acid susceptibility was determined by the estimation of non-viable cells after the acid challenge in comparison to total viable counts at pH 7.0. Mean values of cells that lost viability after the acid challenge were $97.01 \pm 6.61\%$ among caries-free genotypes and $90.63 \pm 14.58\%$ among caries-active children. Of the 51 tested genotypes (29 from caries-free and 21 from caries-active children), 10 genotypes from seven caries-active children and only one genotype from a caries-free subject were considered more resistant to acids (<90% non-viable cells after acid challenge). Fisher's exact text revealed that the difference in distribution of more acid-resistant genotypes between caries-active and caries-free children was statistically significant ($P < 0.05$). Acid susceptibility data were also analyzed according to the oral site where the genotype was found. Analysis of variance revealed no statistically significant differences in acid susceptibility among genotypes according to the isolation site ($P > 0.05$). The distribution of genotypes from caries-free and caries-active children according to biofilm formation and susceptibility to acids is shown in Fig. 2.

Discussion

S. mutans presents diversity in both genotypical and phenotypical virulence factors. Despite the relation between caries prevalence and MS salivary levels, this organism can be detected in high levels in high sucrose consuming individuals without the development of disease (23, 27). In addition to MS, other organisms termed 'low-pH non-mutans streptococci' are able to produce large amounts of acids and may also play a significant role in the caries process (37).

To verify if *S. mutans* genotypes were equally detected in different oral sites, and if saliva isolates were representative of the genotypes colonizing other oral sites, isolates from saliva, as well as from the tongue surface, from biofilm over white spot lesions (caries-active group) and from biofilm over sound surface were genotyped by AP-PCR. Even with the analysis

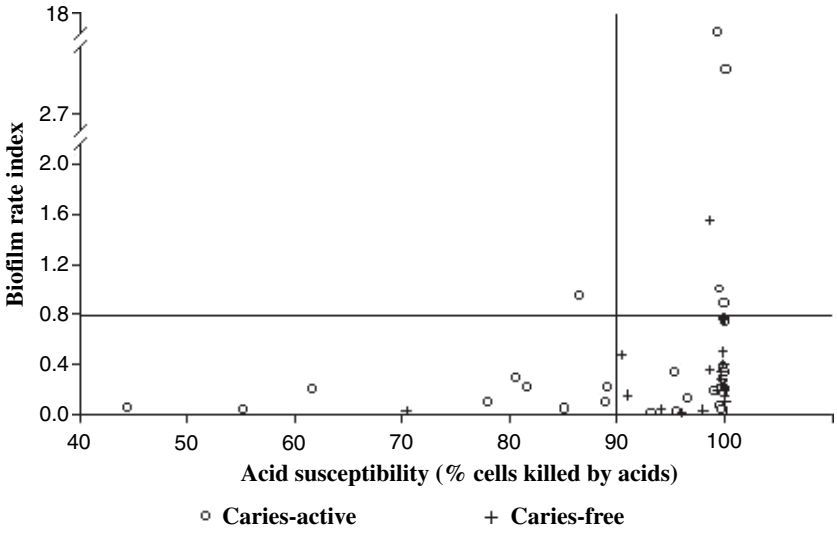

Fig. 2. Distribution of genotypes from caries-free and caries-active children according to acid susceptibility and biofilm formation. Biofilm rate index was the reason between the absorbance value of crystal violet eluted from the biofilm ($A_{575\ nm}$) and the absorbance value obtained from the planktonic growth ($A_{550\ nm}$).

of multiple isolates from different sites, a single genotype was found in four caries-active and three caries-free children. The remaining subjects had two to four detectable genotypes and only one caries-active patient harbored seven genotypes. These results diverge from data reported by Alaluusua et al. (2), who described an association between multiple genotypes and high caries activity status in infants. This association between caries and number of genotypes was also shown among 5- to 18-year-old patients and young adults (29, 31), although both studies had reported caries-active subjects colonized by a single genotype of *S. mutans*.

Other studies have also reported a lack of association between caries experience and number of genotypes of *S. mutans*. In a Swedish study, two of seven caries-active children harbored a single clone of *S. mutans* (12) and an inverse relation between the number of clones and caries experience was demonstrated in Dutch children (18). The number of studied isolates of *S. mutans* per subject and the number of sampling sites in the present study are higher than those reported in most studies so these variables cannot be regarded as the reason for the lower genetic diversity observed here among caries-active children than in other studies. The differences in results among studies could be the result of disease intensity (rampant caries or just caries-active children) and the age of the studied populations.

The degree of MS infection in the oral cavity can be estimated by MS salivary levels, and a relation between MS levels and number of genotypes of *S. mutans* has been suggested (31). However, this hypothesis was not confirmed by other studies (2, 12), including the present data. Despite only one of seven children with one genotype exhibiting high MS levels ($>10^5$ CFU/ml saliva), there was not a significant association between number of genotypes and MS levels in saliva.

In the present study, one to three genotypes were detected in each oral sampling site, which is similar to data obtained by others in adults (29, 31). However, the representative role of saliva as an indicator of oral cavity colonization was lower than previously reported (12). The observed differences in distribution of genotypes according to the oral site corroborate the hypothesis suggested by Kelstrup et al. (16) and Grönroos et al. (12) that the distribution of *S. mutans* genotypes in the oral cavity is site-specific.

The factors influencing the colonization of a site or a subject by a certain genotype of *S. mutans* are still not known. The consumption of fermentable carbohydrates (mainly sucrose) could favor the establishment of certain genotypes that are more able to survive in a sucrose-rich environment (2, 29). Several studies have suggested that diversity within *S. mutans* isolates would be associated with the ability to colonize or express factors that could induce the emergence of lesions by certain genotypes (10, 15, 17). The present study showed that *S. mutans* isolates exhibited variability in their ability to form biofilm *in vitro* in a sucrose medium, and five of the six high biofilm-forming isolates were obtained from caries-active children, although these differences were not significant. Previous studies have reported that *S. mutans* isolated from caries-free children presented a lower cariogenic potential in animal models than isolates from caries-active subjects (17). In addition, subjects with high caries activity are often infected by strains that produce significantly higher amounts of glucan compared to strains infecting caries-free children (26) and adults (29, 35). These *S. mutans* isolates producing increased amounts of glucans have high biofilm-forming ability (24), which is associated with a higher expression of *GtfC* and, to a lesser extent, *GtfB* when compared to isolates with low biofilm-forming ability (25).

Major factors contributing to the virulence of *S. mutans* also include acidogenesis and aciduricity (5) so diversity in these characteristics might help to explain the differences in virulence among clones. Previous studies reported no correlation between caries activity with acid production (17) or final pH *in vitro* (26, 29) among isolates of *S. mutans*. The unique biological nature of *S. mutans* allows it to ferment a wide range of dietary carbohydrates and, within minutes after sugar consumption, to lower the surrounding plaque pH to <4.0. This rapid acidification is followed by expression of numerous proteins, including chaperones and membrane proteins, in a process, known as the acid-tolerance response, that is necessary for cellular viability (34, 39). Growth of biofilm samples at pH 5.0 revealed that members of the genus *Streptococcus* are the dominant group in the biofilm, and mutans streptococci accounted for less than half of the streptococcal viable count (33). In the present study, the cell viability of *S. mutans* genotypes was tested after exposure to pH 2.8. A variation in acid susceptibility was observed among isolates, either from caries-active or caries-free children, suggesting that acid tolerance is a strain-specific trait. A higher prevalence of isolates able to survive after acid shock was observed among genotypes from caries-active children compared to genotypes from the caries-free group. Other studies have previously revealed that MS were highly variable with respect to acid tolerance, and biofilm from both carious and healthy sites harbored significant numbers of MS that were not acid-tolerant (33). A variation in the ability to survive and grow in an acidic environment was also reported in other species from the dental plaque, such as *Streptococcus oralis* (3).

Heavy colonization by *S. mutans* is likely to be a consequence of frequent consumption of fermentable carbohydrates, and the simultaneous action of several strains with different cariogenic potential would possibly increase the risk of caries (2). In the caries process, the frequent intake of carbohydrates, mainly sucrose, leads to a microbial succession in the biofilm towards increased numbers of bacteria with greater acidogenic and aciduric properties. The findings that *S. mutans* genotypes differ in their ability to form biofilm in sucrose media and to withstand acid challenge, and the observation that *S. mutans* genotypes highly resistant to acid shock are more prevalent in caries-active children than in caries-free children may help to explain the differences in caries activity observed among *S. mutans*-infected children.

The present data suggest that there are differences in the distribution of genotypes of *S. mutans* according to the oral site and that *S. mutans* populations differ in the intensity of factors allowing their colonization of sucrose-rich environments.

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