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Genotypic diversity and virulence traits of *Streptococcus mutans* in caries-free and caries-active individuals

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The present study evaluated the relationship between clonal diversity and some virulence traits of *Streptococcus mutans* isolated from eight caries-free and eight caries-active subjects. A total of 155 *S. mutans* isolates from caries-free subjects and 144 isolates from caries-active subjects were obtained from samples of saliva, dental plaque and tongue surface and identified by PCR. The isolates were submitted to arbitrarily primed (AP)-PCR (OPA-2 and OPA-13) and multilocus enzyme electrophoresis (MLEE) to establish the genotypic diversity. Production of water-insoluble glucan (WIG) (monitored by SDS-PAGE), final pH of cultures and the ability of bacterial cells to adhere to smooth glass in the presence of sucrose were measured. High and comparable abilities of MLEE and AP-PCR were found to distinguish *S. mutans* genotypes, using Simpson's index of discrimination (0·971 and 0·968, respectively). The results showed a significant difference ($P < 0{\cdot}01$) in the number of genotypes when caries-free and caries-active groups were compared by both fingerprinting methods used. Final pH ($P = 0{\cdot}32$) and the percentage of adherence to a glass surface ($P = 0{\cdot}62$) did not show differences between the two groups; however, the intensities of WIG bands from the caries-active group were greater than those from the caries-free group ($P < 0{\cdot}01$). In addition, WIG was positively correlated with the ability of *S. mutans* to adhere to a glass surface ($r = 0{\cdot}34$, $P = 0{\cdot}02$) from caries-active subjects. These data showed that AP-PCR analysis and MLEE are both effective methods for assessing the genetic relatedness of *S. mutans*. Using these techniques, it was found that there is a larger number of genotypes of *S. mutans* with increased ability to synthesize WIG in caries-active individuals.

INTRODUCTION

The mutans streptococci are generally considered to be the principal aetiological agent of dental caries (Hamada & Slade, 1980; Loesche, 1986); however, they are widely distributed not only in populations with moderate or high caries prevalence (Beighton *et al.*, 1987; Alaluusua *et al.*, 1987) but also in populations having no or low caries experience (Carlsson *et al.*, 1985; Matee *et al.*, 1993). One possible explanation for their presence in subjects with low caries experience is that the virulence factors and dependence on caries-promoting factors of the mutans streptococci can differ between populations with contrasting caries prevalence (Emilson *et al.*, 1987).

Several studies have showed genetic heterogeneity among *Streptococcus mutans* strains (Saarela *et al.*, 1996; Li & Caufield 1998; Grönroos & Alaluusua, 2000); however, the relationship between caries activity and the genetic diversity of *S. mutans* still controversial. Alaluusua *et al.* (1996) suggested that caries-active children with high sucrose consumption carried greater ribotype diversity of mutans streptococci compared with caries-free children. On the other hand, Kreulen *et al.* (1997) showed a negative correlation between caries activity and genotypic diversity.

One important characteristic of *S. mutans* in promoting caries development is the ability to adhere firmly to the tooth surface in the presence of sucrose (Alaluusua *et al.*, 1997), and this adherence is mediated mainly by the enzymic action of the glucosyltransferase (GTF) enzymes (Loesche, 1986; Kuramitsu, 1993). These enzymes are considered fundamental for the virulence of *S. mutans* in the pathogenesis of dental caries (Yamashita *et al.*, 1993).

The aims of the present study were to evaluate the genotypic

Abbreviations: AP-PCR, arbitrarily primed PCR; GTF, glucosyltransferase; MLEE, multilocus enzyme electrophoresis; WIG, water-insoluble glucan.

diversity of *S. mutans* in caries-free and caries-active subjects and to compare some virulence traits between strains isolated from these two groups.

METHODS

Subjects. The study group consisted of 16 young adults aged between 18 and 29 years (23·5 ± 3·91). Each group, of caries-free and caries-active individuals [DMF (decayed, missing, filled) = 12·0 ± 3·07], contained eight subjects. One examiner, who was trained, examined all the volunteers. Only a mouth mirror and light were used. The teeth were cleaned and dried with a cotton-wool roll and all surfaces were examined visually for dental caries. Written informed consent was obtained from all individuals and the experimental procedures were approved by the Institutional Ethical Committee of Faculty of Dentistry of Piracicaba, University of Campinas.

Sampling. Volunteers were instructed not to brush their teeth during the preceding 12 h and not to drink or eat anything for 2 h before sampling. Non-stimulated saliva samples were collected in graduated tubes. Pooled samples of dental plaque were taken with sterile dental probes from several surfaces of the anterior and posterior teeth and a sample from the tongue surface was collected with a sterile swab. The samples were dispersed in a sterile 0·15 M NaCl solution and serially diluted.

Culture. In order to detect mutans streptococci, 10 µl undiluted samples and 10^{-1} to 10^{-3} dilutions were cultured on mitis salivarius agar (Difco) supplemented with 20 % sucrose (Synth) and 0·2 U bacitracin ml^{-1} (Sigma) (MSB agar; Gold *et al.*, 1973). Plates were incubated at 37 °C for 48 h in an atmosphere of 10 % CO_2 (Cole Palmer Instruments).

Isolation of mutans streptococci and strain identification. After growth on MSB agar plates, 45 isolates representing morphological types from each sample were taken and inoculated in brain heart infusion (BHI) broth (Difco) and incubated as described above. All isolates were submitted to PCR to identify *S. mutans*.

Extraction of chromosomal DNA. DNA from strains was extracted using a simple DNA preparation, modified from Welsh & McClelland (1990) and Saarela *et al.* (1996), in which the cells from an overnight culture are washed and boiled for 10 min with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0), the debris was pelleted and the supernatant was used for identification by PCR and genotyping by arbitrarily primed (AP)-PCR.

PCR identification. DNA samples from mutans streptococcus isolates were identified as *S. mutans* by PCR using primers designed by Oho *et al.* (2000) to amplify a 517 bp sequence of the glucosyltransferase B gene (*gtfB*). The sequences of these primers were 5′-ACTACACTTTCG GGTGGCTTGG-3′ and 5′-CAGTATAAGCGCCAGTTTCATC-3′.

The PCR was processed in a 25 µl mixture containing 1× reaction buffer (10 mM Tris/HCl, 50 mM KCl, pH 8·3), 1·5 mM $MgCl_2$, 0·1 mM dNTPs, 0·2 µM of each primer, 1·5 U *Taq* DNA polymerase (Invitrogen) and 2·5 µl DNA sample. Purified genomic DNA from *S. mutans* ATCC 25175^T and distilled water were respectively used as positive and negative controls.

PCR amplification was performed using a GeneAmp PCR System 2400 (Perkin Elmer) under the following conditions: a denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 1 min and a final elongation step at 72 °C for 10 min (Oho *et al.*, 2000). PCR products were analysed by electrophoresis in 1·5 % agarose gel using TBE buffer (pH 8·0). A 100 bp DNA ladder was included in each gel. The DNA was stained with 0·5 µg ethidium bromide ml^{-1} and visualized under UV illumination.

AP-PCR typing. Strains identified as *S. mutans* were genotyped. AP-PCR fingerprinting was performed with two random primers, OPA-02 (5′-TGCCGAGCTG-3′) and OPA-13 (5′-CAGCACCCAC-3′) (Saarela *et al.*, 1996).

AP-PCR was processed in 25 µl mixtures containing 1× reaction buffer, 3·5 mM $MgCl_2$, 0·2 mM dNTPs, 0·4 mM primers, 2·5 U *Taq* DNA polymerase and 2·5 µl DNA sample. The temperature profile in a thermocycler was 35 cycles of 94 °C for 1 min, 36 °C for 2 min and 72 °C for 2 min, with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 5 min. Amplification products were analysed electrophoretically in 1·5 % agarose gels using TBE buffer (pH 8·0).

Ethidium bromide-stained gel images were captured with a high-resolution imaging system (Image Master; LISCAP). A 100 bp DNA ladder served as a molecular-size marker in each gel. Individual AP-PCR amplicons were marked and the individual bands were analysed by using the Dice coefficient (> 95 %) and UPGMA cluster analysis. Molecular masses for each band or amplicon were computed and analysed by the Sigma Gel software program. This was performed for all DNA patterns produced by the AP-PCR method. To assure reproducibility, some PCRs were carried out in at least two independent amplifications, using different DNA preparations. In every AP-PCR, *S. mutans* ATCC 25175^T chromosomal DNA was used as a positive control.

Multilocus enzyme electrophoresis (MLEE) typing. After growth in BHI broth as described above, cell pellets were harvested by centrifugation and washed three times with 40 mM PBS (pH 7) and an approximately equal volume of 0·55 mm glass beads and 1 ml PBS were added. Tubes were inserted in a Mini-Bead Beater cell disruptor (Biospec Inc.) that was programmed for 4500 r.p.m. spins, in two cycles of 1 minute each. After centrifugation at 5000 ***g***, supernatants were absorbed in five 12 mm Whatman-3 paper wicks that were placed at −70 °C until required for use (Selander *et al.*, 1986).

Electrophoresis was carried out in 13 % hydrolysed starch supports in buffer solutions A [Tris/citrate, pH 8 (tank) and 1 : 30 Tris/citrate, pH 8 (gel)], B [Tris/citrate, pH 6·3 (tank) and Tris/citrate, pH 6·7 (gel)], C [borate, pH 8·2 (tank) and Tris/citrate, pH 8·7 (gel)] and D [lithium hydroxide, pH 8·1 (tank) and 1 : 9 lithium hydroxide/Tris/citrate, pH 8·3 (gel)] (Selander *et al.*, 1986).

After running, gels were sliced in 1·2 mm thicknesses. Gel slices were used for detection of enzymic activity of protein bands corresponding to aconitase (ACO), alcohol dehydrogenase (ADH), α-amylase (α-AM), aspartate dehydrogenase (ASD), malic enzyme (ME), α-esterase (α-EST), β-esterase (β-EST), glucose dehydrogenase (GDH), glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), mannitol dehydrogenase (MADH), mannitol-1-phosphate dehydrogenase (M1P), mannose-phosphate isomerase (MPI), nucleoside phosphorylase (NSP), peroxidase (PO), phenylalanyl-leucine peptidase (PLP), sorbitol dehydrogenase (SDH), superoxide dismutase (SOD) and glutamate-oxaloacetate transaminase (GOT). After their appearance, bands were scored according to their respective relative mobilities as proposed by Selander *et al.* (1986).

Final pH analysis of *S. mutans* isolates. The lowest pH at which acidogenesis by genotypes of *S. mutans* was completely inhibited was tested (van Houte *et al.*, 1996). For this purpose, 100 µl aliquots of 20-h cultures were transferred to test tubes containing 5 ml phenol red dextrose broth (Difco) with a final concentration of 1 % glucose and incubated at 37 °C in an atmosphere of 10 % CO_2 for 3 days. The final

pH was measured with a standardized pH meter (Nova Técnica). Uninoculated control tubes were also incubated under the same conditions. The experiment was performed in triplicate.

Water-insoluble glucan (WIG) synthesis. One strain representative of each genotype identified from each group was examined for its ability to synthesize WIG according to Mattos-Graner *et al.* (2000). Isolates from frozen stocks were grown in BHI broth at 37 °C in an atmosphere of 10 % CO_2 for 20 h. Aliquots of 100 µl culture were then transferred to fresh BHI broth and grown under the same conditions for 20 h. Cultures were then centrifuged at 4 °C (5000 ***g***) and supernatants were neutralized and dialysed against Tris/HCl (pH 6·8), first at 10 mM and then at 1·5 mM, at 4 °C and then concentrated 100-fold by freeze-drying. Equal volumes of protein samples obtained from cultures of the same numbers of cells (OD_{550} 0·8–1·0) were then separated in duplicate SDS-PAGE 6 % gels in a Mini Protean slab gel (Bio-Rad), according to Laemmli (1970). Molecular-mass standards (Sigma) were included in all gels. *S. mutans* components migrating to positions corresponding to 150–160 kDa were detected after being stained with Coomassie blue G (Serva Blue). After electrophoresis, duplicate SDS-PAGE gels were incubated at 37 °C overnight with a solution of 1 % Triton X-100, 5 % sucrose in 0·2 M sodium phosphate buffer (pH 6·5), 0·2 % dextran T70 (Bio-Rad) and 0·02 % sodium azide. Opaque white bands indicated synthesis of water-insoluble polysaccharides. The principal glucan band observed in *S. mutans* culture supernatants was associated with the slower-migrating, approximately 160 kDa, GTF component. This band was interpreted as being synthesized by GTF-B, since this isoenzyme is the larger of the two *S. mutans* GTF isoenzymes synthesizing WIG (Shiroza *et al.*, 1987). Glucan gels were photographed against a black surface. Intensities of the Coomassie-blue-stained *S. mutans* GTF-B bands and the corresponding WIG bands were measured by scanning densitometry (Bio-Rad GS 700) (Hazlett *et al.*, 1998). To eliminate differences in WIG production due to variations in the amount of GTF production, we divided the normalized intensities of the glucan bands by the normalized intensities obtained for the corresponding GTF bands observed in stained gels. The values obtained were defined as the GTF activity ratios.

Adherence analysis. Sucrose-dependent adherence of resting cells of genotypes of *S. mutans* was determined turbidimetrically as follows (Hamada & Torii, 1978). Isolates from frozen stocks were grown in BHI broth at 37 °C in an anaerobic atmosphere for 20 h. Aliquots of 100 µl cells (OD_{550} 0·8–1·0) were then transferred to fresh BHI broth containing 1 % sucrose and grown under the same conditions at an angle of 30° for 24 h. After incubation, culture tubes were vigorously mixed in a vortex mixer for 5 s and non-adhering cells were transferred to fresh tubes. Aliquots of 3 ml potassium phosphate buffer (0·05 M, pH 7·0) were added to the first tube and agitated for 5 s: the released cells were transferred to a third tube. The second and third tubes were centrifuged for 5 min at 5000 ***g*** and the pellets were resuspended in the same buffer. All tests tubes were then sonicated at 10 % amplitude for 30 s (Sonics & Materials Inc.), followed by spectrophotometric measurement of suspended cells at 550 nm (Spectronic 20; Genesys). We calculated the percentage of adhered cells by dividing the cell density of adherent cells by the values of total cell density. Adherence tests were performed in triplicate for all tested strains.

Statistics. The Newman–Keuls test was used to test differences between the number of genotypes by the two techniques (AP-PCR and MLEE) in caries-free and caries-active individuals. To test the discriminatory index of each technique, Simpson's index of diversity was used (Hunter & Gaston, 1988). The Mann–Whitney U test was used to analyse caries-active and caries-free individuals for differences in final pH, percentage of adherent cells, GTF and WIG band intensities. Associations between variables were tested by Spearman's rank correlation analysis. Association between the number of genotypes found by both techniques and the number of strains used was studied by Pearson's correlation coefficient.

RESULTS

AP-PCR analyses

All 299 *S. mutans* strains isolated in this study were analysed with two individual primers by the AP-PCR. The test was performed with OPA-02 and OPA-13, and each of these primers generated a different spectrum of amplicons, indicative of genetic polymorphism; when the results obtained with the two primers were combined, the strains were classified into 24 distinct genotypes in the caries-free group (one to four per subject) and into 44 distinct genotypes from the caries-active group (two to eight per subject) (Table 1). In comparative analyses, only high-intensity bands were used to discriminate strains. *S. mutans* from unrelated individuals displayed distinctive DNA fingerprints, and comparison of the similarity indices showed greater diversity among isolates from different individuals.

Table 1. Numbers of genotypes of *S. mutans* isolated from caries-free and caries-active individuals, assessed by MLEE and AP-PCR typing

Numbers of genotypes found in caries-active subjects by both techniques were statistically significantly larger (Newman–Keuls, $P < 0·01$). No differences were observed between the number of genotypes found by MLEE and AP-PCR in either group.

Subject	Isolates (*n*)	Clonal types (*n*)	
		MLEE	AP-PCR
Caries-free			
A	27	4	4
C	8	4	3
D	25	3	3
E	17	2	3
G	19	4	3
I	18	3	1
O	23	3	4
R	18	4	3
Total	155	27	24
Caries-active			
C1	26	5	2
C2	19	11	8
C3	19	7	6
C4	15	6	6
C5	11	5	4
C7	20	8	7
C8	16	5	4
C9	18	4	7
Total	144	51	44

MLEE analyses

Optimal zymographic conditions for each enzyme for a given bacterial species are determined by testing several conditions systems. We tested four different buffers with variable pH; however, among the enzyme systems analysed, the majority of dehydrogenases (ACO, ADH, ASD, GDH, G6PD, IDH, LDH, MDH, MADH, ME, SDH), as well as α-AM, α-EST, β-EST and PO showed no activity for any *S. mutans* strain. It is possible that the conditions used were not suitable for detection of activity of the *S. mutans* enzymes. Alternatively, such enzymes were not produced or were produced in quantities too small to be detected by the applied method. The six enzymes that showed good discriminatory ability for *S. mutans* were LAP, M1P, MPI, NSP, PLP and GOT. In MLEE analyses, the results obtained with all six enzymes combined revealed 27 different genotypes from the caries-free group (two to four per subject) and 51 genotypes from the caries-active group (four to 11 per subject).

The numbers of genotypes obtained in each group are shown in Table 1. Differences in the mean number of genotypes between groups were statistically significant (Newman–Keuls, $P < 0{\cdot}01$) for both techniques; however, no statistical difference was found between the techniques when the same group was compared (Newman–Keuls, $P > 0{\cdot}05$). No association between the number of strains used and the number of genotypes found was observed (Pearson's correlation; $P > 0{\cdot}05$). These data show the strong ability of both techniques to distinguish *S. mutans* genotypes; using Simpson's index of discrimination, MLEE and AP-PCR respectively showed values of discrimination of 0·971 and 0·968.

Final pH

Final pH ranged from 4·15 to 4·64 among the genotypes of *S. mutans* strains isolated from caries-free individuals (mean $4{\cdot}27 \pm 0{\cdot}16$) and from 4·03 to 4·32 among genotypes of *S. mutans* strains isolated from caries-active individuals (mean $4{\cdot}21 \pm 0{\cdot}09$). No significant difference was detected between the means of the two groups.

WIG synthesis and adherence to glass surfaces

The WIG band intensities obtained by scanning densitometry ranged from 0·26 to 0·87 in caries-free subjects (mean $0{\cdot}48 \pm 0{\cdot}11$) and from 0·50 to 0·95 in caries-active subjects (mean $0{\cdot}66 \pm 0{\cdot}08$). There was a statistical difference between the WIG synthesis of caries-free and caries-active subjects (Mann–Whitney; $P < 0{\cdot}01$) (Table 2). We observed a statistically significant association between the WIG intensity values and percentages of growing cells adhering to glass surfaces in the presence of sucrose (Spearman; $r = 0{\cdot}34$; $P = 0{\cdot}02$) among the genotypes isolated from caries-active volunteers. On the other hand, in caries-free subjects, these variables were not associated (Spearman; $r = -0{\cdot}03$; $P = 0{\cdot}86$) (Fig. 1). No significant differences were found in GTF band intensity between the two groups ($P > 0{\cdot}05$) and no association between GTF production and WIG synthesis was found in either group (Spearman; $P > 0{\cdot}05$).

DISCUSSION

Some previous work has shown genetic variability of *S. mutans* in nursing-bottle caries and healthy children (Alaluusua *et al.*, 1996; Kreulen *et al.*, 1997). Different genotypes isolated from the mouth of caries-active children were the same in terms of GTF production (Alaluusua *et al.*, 1997) but significantly different in WIG synthesis (Mattos-Graner *et al.*, 2000) from those that had colonized caries-free children. In our study, strains isolated from young adults were used to analyse whether there is an association between the number of genotypes and caries activity. We also aimed to compare virulence factors of *S. mutans* between genotypes obtained from caries-free and caries-active subjects.

To assess the genotypic identity of the strains, we used two different techniques, MLEE and AP-PCR. AP-PCR has been used extensively for *S. mutans* genotyping (Saarela *et al.*, 1996; Alaluusua *et al.*, 1996; Li & Caufield, 1998; Grönroos & Alaluusua, 2000; Redmo Emanuelsson *et al.*, 2003). Gilmour

Table 2. GTF activity, WIG synthesis, percentages of cells adhering to glass surfaces in the presence of sucrose and final pH values for *S. mutans* strains

GTF production was measured as the intensity of protein-stained GTF bands and WIG synthesis as the intensity of WIG bands measured by scanning densitometry; the GTF activity ratio is the intensity of WIG bands normalized against the intensity of the corresponding protein-stained GTF bands. Adherence is the proportion of spectrophotometric measurement (at 550 nm) of cells adhering to glass as a percentage of total cell density. Values are means ± SD.

Virulence trait	Caries-free ($n = 8$)	Caries-active ($n = 8$)	Statistical significance
GTF production	$0{\cdot}36 \pm 0{\cdot}06$	$0{\cdot}41 \pm 0{\cdot}06$	$P = 0{\cdot}13$ (ANOVA)
WIG synthesis	$0{\cdot}48 \pm 0{\cdot}11$	$0{\cdot}66 \pm 0{\cdot}08$	$P < 0{\cdot}01$ (Mann–Whitney)
GTF activity ratio	$1{\cdot}47 \pm 0{\cdot}44$	$1{\cdot}75 \pm 0{\cdot}36$	$P = 0{\cdot}18$ (ANOVA)
Adherence (%)	$20{\cdot}02 \pm 7{\cdot}05$	$23{\cdot}68 \pm 8{\cdot}03$	$P = 0{\cdot}62$ (ANOVA)
Final pH	$4{\cdot}27 \pm 0{\cdot}16$	$4{\cdot}21 \pm 0{\cdot}09$	$P = 0{\cdot}32$ (ANOVA)

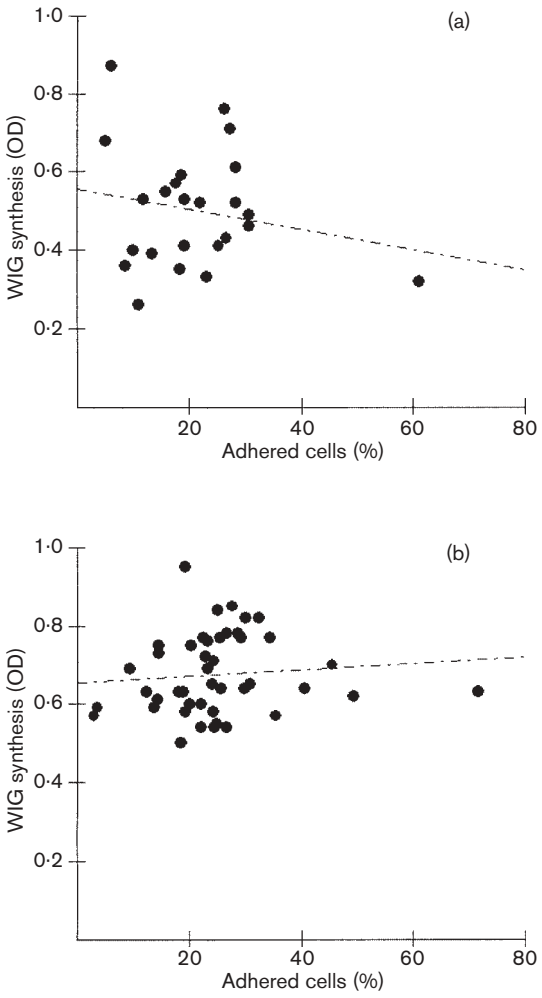

Fig. 1. Relationship between WIG synthesis and sucrose-dependent adherence of growing cells to glass surfaces in clinical isolates of *S. mutans*. Values are expressed as means of triplicate data. No statistical association was found in strains of the caries-free group (a), but a statistical association was found in strains of the caries-active group (b) (see Results).

et al. (1987) carried out a study in which the use of MLEE was proposed for clustering related species of oral streptococci in some groups as mutans streptococci. Despite the fact that such methodology may segregate strains not related within a certain species, it has never been used before for differentiation of *S. mutans* specimens. In our study, we found that MLEE could be applied as a discriminating tool for *S. mutans*. In spite of the fact that diversity analysis by MLEE was based in only six enzyme loci, its discrimination ability proved to be a useful tool for establishing the genetic diversity of *S. mutans* strains.

Previous studies with bacteria and fungi have established concordance between results obtained by AP-PCR and MLEE (Tibayrenc *et al.*, 1993; Pujol *et al.*, 1997). In our study, no significant differences were observed between the number of genotypes found by MLEE and AP-PCR in either group, showing that both techniques are suitable for *S. mutans* typing. AP-PCR proved to be faster, but showed low reproducibility for low-intensity bands (Tibayrenc *et al.*, 1993). MLEE, although highly reproducible, is more time-consuming (Boerlin, 1997). In our study, both techniques were highly discriminatory. According to Hunter & Gaston (1988), the acceptable level of discrimination depends on a number of factors, but an index of greater than 0·90 would seem to be desirable if the typing results are to be interpreted with confidence. The results of discrimination obtained from MLEE and AP-PCR in our study were 0·971 and 0·968, respectively.

The findings that caries-active subjects have more genotypes than caries-free is contrary to the results obtained from Kreulen *et al.* (1997), who showed a negative relationship between caries activity and genotype diversity. However, our results are in agreement with earlier reports (Hirose *et al.*, 1993; Alaluusua *et al.*, 1996) that also found that caries-active subjects have a larger number of genotypes of *S. mutans*. In a recent study of young adults (mean age of 25·2 years), Redmo Emanuelsson *et al.* (2003) found a maximum of seven genotypes in subjects who had previous caries experience, which is in agreement with our results, where we found a maximum of eight genotypes in caries-active subjects using AP-PCR. Heavy colonization and growth of multiple genotypes is likely to be a consequence of frequent consumption of fermentable carbohydrates, and it is possible that the simultaneous action of several strains with possibly differing cariogenic potential further increases the risk of caries (Alaluusua *et al.*, 1996). Redmo Emanuelsson *et al.* (2003) suggested that the larger number of genotypes found could be because of the larger number of isolates analysed, which increases the possibility of detecting different genotypes; however, the differences in the number of strains used from each volunteer in this study showed no relationship with the number of genotypes detected in either group.

One of the virulence factors studied, final pH, showed no difference between the two groups. No relationship was observed between values of final pH with caries activity, in agreement with studies showing no direct correlation between acid production and caries scores in fresh isolates of *S. mutans* from adults (Köhler *et al.*, 1995) and from children (Mattos-Graner *et al.*, 2000). Such findings might be explained by the multifactorial nature of dental caries. Alternatively, the measurement of final pH after 72 h could hide a significant difference between the groups at earlier times (de Soet *et al.*, 1989).

Although adherence to a glass surface in the presence of sucrose was not significantly different between strains of *S. mutans* from caries-free and caries-active subjects, we have observed a statistically significant positive association between the level of synthesis of WIG by *S. mutans* clinical isolates and the proportion of adherent cells in the presence of sucrose in caries-active subjects, but not in caries-free subjects. These findings are in accordance with earlier reports

(Mattos-Graner *et al.*, 2000) and may suggest that isolates from subjects with high caries activity are better able to colonize and accumulate on teeth and consequently to induce caries. However, the different numbers of genotypes tested in the caries-free and caries-active groups may have accounted for this difference. For our experiments, just a single representative strain of each genotype was selected for phenotypic analysis. The caries-free subjects had smaller number of strains tested, since they showed lower genotypic diversity when compared with caries-active individuals. In addition to the smaller number of genotypes tested in the caries-free group, a single genotype showed low synthesis of WIG but high adherence. Another reason for the lack of association between WIG and adherence to glass surfaces might be that other proteins may influence adherence, such as glucan-binding proteins produced by *S. mutans* (Mattos-Graner *et al.*, 2001).

These differences in the synthesis of WIG between genotypes could be associated with different levels of virulence. This is important, since it has been demonstrated that WIG produced from sucrose modifies the physico-chemical properties of dental plaque, including a low inorganic concentration of calcium, phosphorus and fluoride (Cury *et al.*, 1997) and increased porosity of the dental plaque matrix (Zero *et al.*, 1992), making it more cariogenic.

It has been demonstrated that adherence is mediated by enzymic action of GTFs from *S. mutans* (Loesche, 1986; Kuramitsu, 1993). In a previous study (Alaluusua *et al.*, 1997), no relationship of GTF production between isolates of *S. mutans* from caries-free and caries-active children was apparent. We demonstrated no association between WIG synthesis and the intensity of GTF bands obtained from Coomassie-blue-stained gels. This would suggest that differences in the amount of WIG produced may be independent of the amount of GTF expressed. Variations in enzymic activity could be related to a polymorphism in *gtf* genes, as described for clinical isolates of *S. mutans* (Chia *et al.*, 1991).

In summary, this study revealed a larger number of genotypes in caries-active subjects and the occurrence of genotypes with greater abilities to produce WIG in caries-active individuals when compared with genotypes isolated from caries-free subjects.

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REFERENCES

Alaluusua, S., Kleemola-Kujala, E., Nyström, M., Evälahti, M. & Grönroos, L. (1987). Caries in the primary teeth and salivary *Streptococcus mutans* and lactobacillus levels as indicators of caries in permanent teeth. *Pediatr Dent* **9**, 126–130.

Alaluusua, S., Mattö, J., Grönroos, L., Innila, S., Torkko, H., Asikainem, S., Jousimies-Somer, H. & Saarela, M. (1996). Oral colonization by more than one clonal type of mutans streptococcus in children with nursing-bottle dental caries. *Arch Oral Biol* **41**, 167–173.

Alaluusua, S., Grönroos, L., Zhu, X., Saarela, M., Mattö, J., Asikainem, S. & Fukushima, K. (1997). Production of glucosyltransferases by clinical mutans streptococcal isolates as determined by semiquantitative cross-dot assay. *Arch Oral Biol* **42**, 417–422.

Beighton, D., Rippon, H. R. & Thomas, H. E. C. (1987). The distribution of *Streptococcus mutans* serotypes and dental caries in a group of 5- to 8-year-old Hampshire schoolchildren. *Br Dent J* **162**, 103–106.

Boerlin, P. (1997). Applications of multilocus enzyme electrophoresis in medical microbiology. *J Microbiol Methods* **28**, 221–231.

Carlsson, P., Olsson, B. & Bratthall, D. (1985). The relationship between the bacterium *Streptococcus mutans* in the saliva and dental caries in children in Mozambique. *Arch Oral Biol* **30**, 265–268.

Chia, J. S., Hsu, T. Y., Teng, L. J., Chen, J. Y., Hahn, L. J. & Yang, C. S. (1991). Glucosyltransferase gene polymorphism among *Streptococcus mutans* strains. *Infect Immun* **59**, 1656–1660.

Cury, J. A., Rebello, M. A. B. & Del Bel Cury, A. A. (1997). In situ relationship between sucrose exposure and the composition of dental plaque. *Caries Res* **31**, 356–360.

de Soet, J. J., Toors, F. A. & de Graaff, J. (1989). Acidogenesis by oral streptococci at different pH values. *Caries Res* **23**, 14–17.

Emilson, C. G., Carlsson, P. & Bratthall, D. (1987). Strains of mutans streptococci isolated in a population with extremely low caries prevalence are cariogenic in the hamster model. *Oral Microbiol Immunol* **2**, 183–186.

Gilmour, M. N., Whittam, T. S., Kilian, M. & Selander, R. K. (1987). Genetic relationships among the oral streptococci. *J Bacteriol* **169**, 5247–5257.

Gold, O. G., Jordan, H. V. & van Houte, J. (1973). A selective medium for *Streptococcus mutans*. *Arch Oral Biol* **18**, 1357–1364.

Grönroos, L. & Alaluusua, S. (2000). Site-specific oral colonization of mutans streptococci detected by arbitrarily primed PCR fingerprinting. *Caries Res* **34**, 474–480.

Hamada, S. & Slade, H. D. (1980). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* **44**, 331–384.

Hamada, S. & Torii, M. (1978). Effect of sucrose in culture media on the location of glucosyltransferase of *Streptococcus mutans* and cell adherence to glass surfaces. *Infect Immun* **20**, 592–599.

Hazlett, K. R. O., Michalek, S. M. & Banas, J. A. (1998). Inactivation of the *gbpA* gene of *Streptococcus mutans* increases virulence and promotes *in vivo* accumulation of recombination between the glucosyltransferase B and C genes. *Infect Immun* **66**, 2180–2185.

Hirose, H., Hirose, K., Isogai, E., Miura, H. & Ueda, I. (1993). Close association between *Streptococcus sobrinus* in the saliva of young children and smooth-surface caries increment. *Caries Res* **27**, 292–297.

Hunter, P. R. & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* **26**, 2465–2466.

Köhler, B., Birkhed, D. & Olsson, S. (1995). Acid production by human strains of *Streptococcus mutans* and *Streptococcus sobrinus*. *Caries Res* **29**, 402–406.

Kreulen, C. M., de Soet, H. J., Hogeveen, R. & Veerkamp, J. S. (1997). *Streptococcus mutans* in children using nursing bottles. *ASDC J Dent Child* **64**, 107–111.

Kuramitsu, H. K. (1993). Virulence factors of mutans streptococci: role of molecular genetics. *Crit Rev Oral Biol Med* **4**, 159–176.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Li, Y. & Caufield, P. W. (1998). Arbitrarily primed polymerase chain reaction fingerprinting for the genotypic identification of mutans streptococci from humans. *Oral Microbiol Immunol* **13**, 17–22.

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* **50**, 353–380.

Matee, M. I. N., Mikx, F. H. M., de Soet, J. S., Maselle, S. Y., de Graff, J. & van Palenstein Helderman, W. H. (1993). Mutans streptococci in caries-active and caries-free infants in Tanzania. *Oral Microbiol Immunol* **8**, 322–324.

Mattos-Graner, R. O., Smith, D. J., King, W. F. & Mayer, M. P. (2000). Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children. *J Dent Res* **79**, 1371–1377.

Mattos-Graner, R. O., Jin, S., King, W. F., Chen, T., Smith, D. J. & Duncan, M. J. (2001). Cloning of the *Streptococcus mutans* gene encoding glucan binding protein B and analysis of genetic diversity and protein production in clinical isolates. *Infect Immun* **69**, 6931–6941.

Oho, T., Yamashita, Y., Shimazaki, Y., Kushiyama, M. & Koga, T. (2000). Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction. *Oral Microbiol Immunol* **15**, 258–262.

Pujol, C., Joly, S., Lockhart, S. R., Noel, S., Tybayrenc, M. & Soll, D. R. (1997). Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive DNA probe Ca3 for fingerprinting *Candida albicans*. *J Clin Microbiol* **35**, 2348–2358.

Redmo Emanuelsson, I. M., Carlsson, P., Hamberg, K. & Bratthall, D. (2003). Tracing genotypes of mutans streptococci on tooth sites by random amplified polymorphic DNA (RAPD) analysis. *Oral Microbiol Immunol* **18**, 24–29.

Saarela, M., Hannula, J., Mattö, J., Asikainen, S. & Alaluusua, S. (1996). Typing of mutans streptococci by arbitrarily primed polymerase chain reaction. *Arch Oral Biol* **41**, 821–826.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.

Shiroza, T., Ueda, S., Kuramitsu, H. K. (1987). Sequence analysis of the *gtf*B gene from *Streptococcus mutans*. *J Bacteriol* **169**, 4263–4270.

Tibayrenc, M., Neubauer, K., Barnabé, C., Guerrini, F., Skarecky, D. & Ayala, F. J. (1993). Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proc Natl Acad Sci U S A* **90**, 1335–1339.

van Houte, J., Lopman, J. & Kent, R. (1996). The final pH of bacteria comprising the predominant flora on sound and carious human root and enamel surfaces. *J Dent Res* **75**, 1008–1014.

Welsh, J. & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213–7218.

Yamashita, Y., Bowen, W. H., Burne, R. A. & Kuramitsu, H. K. (1993). Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. *Infect Immun* **61**, 3811–3817.

Zero, D. T., Fu, J., Anne, K. M., Cassata, S., McCormack, S. M. & Gwinner, L. M. (1992). An improved intra-oral enamel demineralization test model for the study of dental caries. *J Dent Res* **71** (Special Issue), 871–878.