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Differentiation of *Streptococcus mutans* and *Streptococcus sobrinus* via genotypic and phenotypic profiles from three different populations

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Li Y, Caufield PW, Redmo Emanuelsson I, Thornqvist E. Differentiation of Streptococcus mutans *and* Streptococcus sobrinus *via genotypic and phenotypic profiles from three different populations.*
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Routine identification of *Streptococcus mutans* and *Streptococcus sobrinus* is generally based upon growth on various selective media, colony morphology and biochemical characteristics. We examined various approaches of differentiating these two species through a combination of the conventional phenotypic methodology with chromosomal DNA fingerprint (CDF) and arbitrarily primed polymerase chain reaction (AP-PCR) methods. Initially, ten ATCC type strains and 20 randomly selected clinical isolates of mutans streptococci (MS) were characterized and grouped into two major types based on patterns generated by the CDF using *Hae*III digestion. The CDF's patterns with restriction fragments equal to or greater than 6.6 kb were defined as the CDF-1 group. The CDF's patterns with restriction fragments less than 6.6 kb were defined as the CDF-2 group. Both groups were then examined for biotype, serotype, and composition of DNA via thermal denaturation. AP-PCR was applied and evaluated for the capability of delineating *S. mutans* from *S. sobrinus* strains. Results of this study showed that all CDF-1 strains fit within a G+C range of 36.2% to 42.2%, whereas the CDF-2 strains had a G+C range of 45.8% to 47.0%. The serotyping assay exhibited 100% sensitivity, 90% specificity and 86.7% agreement with the CDF. The biotyping assay presented the poorest specificity (38.5%), indicating the highest variability. The capability of AP-PCR in differentiation of *S. mutans* from *S. sobrinus* was comparable to the CDF method, suggesting that either of these two approaches can and may serve as a viable alternative method to serotyping or biotyping of MS.

Key words: DNA fingerprint; AP-PCR; *S. mutans*; *S. sobrinus*

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The most common cariogenic bacteria associated with human dental caries are *Streptococcus mutans* and *Streptococcus sobrinus*. Studies showed that *S. mutans* accounts for 74% to 94% of the mutans streptococci (MS) and is overwhelmingly associated with coronal caries development in diverse population (3, 22). *S. sobrinus* is less prevalent and has been correlated with smooth-surface caries (14, 23). The distinction in isolation frequency between *S. mutans* and *S. sobrinus* may be important because the two organisms display differences in initial colonization (13) and virulence mechanisms (22).

Originally, these two microorganisms were classified on the basis of taxonomic characteristics such as colony morphology, carbohydrate antigens, serological and genetic heterogeneity (4, 7, 25), biochemical fermentation of certain substrates (27) and immunological characterization (9). In the past, these techniques sufficed as practical and widely used methods of speciation. Unfortunately, a potential problem in-

herent in the use of phenotyping and biotyping taxonomy is that not all strains within a given species present a positive result for a common trait. As a result, the testing systems are not sufficiently precise or definitive in many instances. In recent years, classification of different microorganisms has depended largely on genetic relatedness, including guanine+cytosine (G+C) content determination, DNA-DNA hybridization, and species-specific probe analyses. One major drawback of applying those techniques is they are labor- and time-intensive. Investigation into the epidemiology of *S. mutans* and *S. sobrinus* was hindered by the lack of simple tests to distinguish between the two species and, more importantly, by the absence of an effective typing method that would enable fine discrimination between strains within the same species.

Previously, we used a chromosomal DNA fingerprinting method for showing homology of strains within family members (18). We noticed that there were two predominate chromosomal DNA fingerprinting patterns among most of the clinical isolates examined. Chromosomal DNA fingerprinting of MS serotypes c/e/f strains presented higher molecular fragments than did serotypes d/g strains. The objective of this study was to determine whether these two chromosomal DNA fingerprinting profiles corresponded to a different serotype and biotype. As means for comparison, we also analyzed the mol percentage content of guanine+cytosine (mol% G+C) of standard serotype of MS strains via thermal denaturation. In addition, we evaluated the discriminative power of arbitrarily primed polymerase chain reaction (AP-PCR) in differentiating *S. mutans* from *S. sobrinus*.

Material and methods

Defining chromosomal DNA fingerprint patterns (CDF)

In our initial multicenter study of the transmission and acquisition of MS within mother-infant pairs of three populations (12, 18, 21), a total of 1328 MS isolates were included for studying the fidelity of MS transmission between mother-child pairs, 204 MS isolates from Birmingham, 720 from Beijing and 404 from Malmö. A small-scale chromosomal DNA isolation procedure was used and has been described previously (6, 18). Chromosomal DNA fingerprints of those MS were generated from *Hae*III restriction enzyme digestion. As shown in Fig. 1, there were two main profiles in restriction fragment patterns among those MS strains. To further study the difference in MS genotypic characterization, we divided the chromosomal DNA fingerprint patterns into two groups. Chromosomal DNA fingerprint type 1 (CDF-1) contained restriction fragments equal to and greater than 6.6 Kb. Chromosomal DNA fingerprint type 2 (CDF-2) contained fragments less than 6.6 Kb.

Bacterial strains and DNA preparation

A total of 30 bacterial strains were included in the study. Ten reference strains of *S. mutans* and *S. sobrinus* were selected from various sources and listed in Table 1. Chromosomal DNAs from the 30 strains were purified by a large-scale DNA extraction procedure as described previously (6). Briefly, the cells were treated with lysozyme, mutanolysin and proteinase K and then lysed with SDS (sodium dodecyl sulfate). The chromosomal DNA was purified in a CsCl-ethidium bromide gradient by ultracentrifugation performed twice. All the DNAs were removed from the gradient, dialyzed overnight and stored in TE (10 mM Tris-HCI, 1 mM EDTA, pH 8.0) buffer at 4°C.

The serotypes of the 10 reference strains were known and, therefore, were used as the "gold standard" for this study. Based upon serological and biochemical criteria, along with CDF profile, MS strains of serotype c (10449, KPSK2, Ingbritt, UA159), e (LM7) and f (OMZ175) were in the CDF-1 category. Likewise, serotype d strains (B13, 6715-15, OMZ176) and serotype g strain (OMZ65) were in the CDF-2 category.

Twenty clinical MS isolates were randomly selected from the multicenter study. Among the 20 clinical isolates of MS, 8 were from the Birmingham, Alabama cohort, 6 were from Beijing, China and 6 were from Malmö, Sweden. These isolates represented different chromosomal DNA fingerprint patterns, 10 for the CDF-1 and 10 for the CDF-2. They were serotyped with an immunofluorescence antisera assay (5) at the University of Malmö. Serotyping was performed without knowing the chromosomal DNA fingerprint categories of the isolates.

Biochemical differentiation test

All 30 strains were examined simultaneously for their biochemical reaction with selected reagents using the Minitek Differentiation System (BBL Minitek, BBL Microbiology Systems, Cockeysville, MD). The testing procedure was modified according to the company's manual and a previous investigation (15). Briefly, the bacterial strains were cultivated on Todd-Hewitt plates at 37°C in an anaerobic chamber (85% N_2, 10% CO_2, and 5% H_2) for 48 h. Colonies were transferred into thioglycolate broth and adjusted to the same density as the McFarland 0.5 standard. Fermentation of mannitol, sorbitol, raffinose or melibiose and cleavage of arginine was included as the key test for *S. mutans* and *S. sobrinus* differentiation (15, 16). The Minitek testing trays containing the bacterial samples, and the discs were incubated anaerobically for 48 h.

Guanine plus cytosine content determination

To test the hypothesis that DNA fingerprint profiles reflect the G+C content of strains of MS, determination of the denaturation temperature, T_m, which reflects the guanine plus cytosine mol percentage (G+C mol%), was performed on all 30 strains with a spectrophotometer and temperature controller (DU-7400 UV/Vis, Peltier, Beckman, Fullerton, CA). Each DNA sample, including the control DNA from salmon sperm, was adjusted to 10 μg/ml in 0.1×SSC buffer. The temperature ranged from 25°C to 90°C, with a ramp rate of 1°C/min. The same control DNA from salmon sperm was tested at each experimental run to ensure accuracy. After the T_m for each given strains was obtained, the base composition (mol% G+C) was then calculated according to the formula 0.1×SSC mol% G+C=(T_m−53.9)×2.4 (24).

Arbitrarily primed PCR (AP-PCR)

The AP-PCR amplification was performed in a total of 50 μl reaction volume (GeneAmp PCR System 2400, PE Applied Biosystems, Foster City, CA) as described previously (19). PCR reaction was run for 45 cycles of 94°C for 30 s, 36°C for 30 s and 72°C for 1 min, followed by an extension of incomplete amplification for 5 min at 72°C.

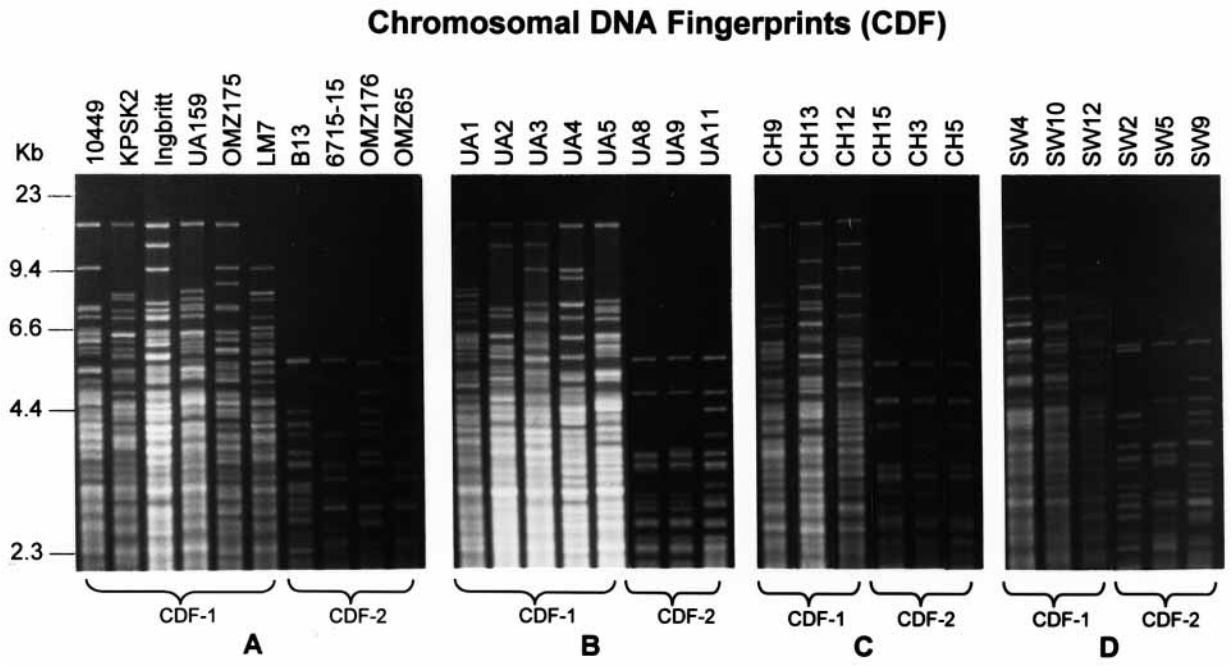

Fig. 1. Chromosomal DNA fingerprint (CDF) of the 30 MS strains. Panel A included all reference strains. Panel B, C and D consisted of strains from the Birmingham Alabama, Beijing China, and Malmö Sweden, respectively. Difference in the CDF pattern are evident among these strains. Those that contained DNA fragments greater than 6.6 Kb were defined as CDF-1 group. Those that contained DNA fragments smaller than 6.6 Kb were defined as CDF-2 group.

Each reaction mixture contained 5 µl of 10× buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3); 200 µM each of dATP, dCTP, dGTP, and dTTP; 3.5 mM $MgCl_2$; 1.25 units of *Taq* DNA polymerase, 50 pmol of 10-mer-primer OPA02, (5′-TGCCGAGCTG, Operon Technologies, Alameda, CA), and 20 ng DNA template. The primer OPA02 was selected from a total of 40 based on criteria discussed previously (19). All other AP-PCR reagents were obtained from the PE Biosystems. AP-PCR product was separated by electrophoresis on a 1.2% agarose gel in Tris-Borate-EDTA running buffer and was stained in ethidium bromide (1 µg/ml) solution. The photographic images of the results were captured digitalized with a digital imaging system (Alpha IS-1000. Alpha Innotech Corp., San Leandro, CA) and saved as Tagged Image File Format (TIFF) for visual comparison and for similarity profiles.

Statistical analysis

The five typing methods – serotype, biotype, G+C mol%, CDF, and AP-PCR fingerprint – were compared for the differentiation of *S. mutans* from *S. sobrinus*. The kappa statistic [$\kappa=(Po-Pe)/(1-Pe)$] was applied to compare the agreement in identification ability among those methods. The T_m and mol% G+C values for each MS strain were collected and summarized according to different CDF groups. The mean value for each group was evaluated by ANOVA for parametric and nonparametric analyses. A P value ≤ 0.05 was considered a significant difference. All the analyses were conducted with SPSS 10.0 software (28).

All AP-PCR fingerprint images were analyzed with the Diversity Database Software (BioRad Laboratories, Hercules, CA). To compare the similarity of fingerprint results generated by the AP-PCR assay, the similarity matrix was calculated as $d_{ku}=(T_i d_{ki}+T_j d_{kj})/(T_i+T_j)$. The unweighted pair group method using arithmetic averages (UPGMA) (29) was used for cluster analysis among those strains tested.

Results

As shown in Table 1, each MS strains was tested for serotype, biotype, T_m and mol% G+C. The medians and mean values of mol% G+C for each strain are listed in this table. To verify the accuracy of our experimental procedure, the same control DNA from salmon sperm was tested with each test run. Fig. 2 and Table 2 show that CDF-1 strains including both reference and clinical isolates presented a significantly lower mol% G+C (median=37.4) than did CDF-2 strains (median=45.8), with a corresponding left-shifted "S" curve in the thermal denaturation profiles. The differences between the two groups were significant as shown by parametric

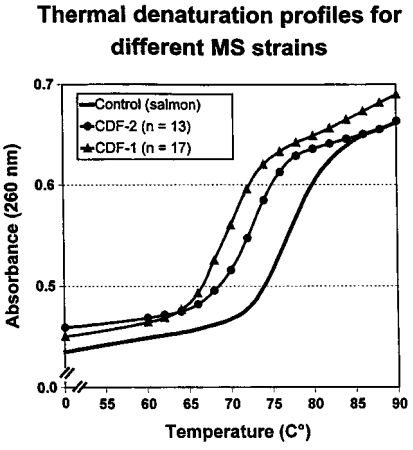

Fig. 2. Thermal denaturation profiles for different MS strains. The mean T_m value for CDF-1 group was 69.6±1.2. The mean T_m value for CDF-2 group was 73.1±0.3. This difference was statistically significant ($P<0.001$).

Table 1. Sources of *S. mutans* and *S. sobrinus* type strains and clinical isolates used and their test results

	Strain	Source	CDF[a]	Serotype[b]	Minitek[c] MN	SO	RA	ARG	ME	mol% G+C[d] from T_m
1	NCTC10449	ATCC25175	1	c	+	+	+	−	+	37.4
2	KPSK2	Bratthall	1	c	+	+	+	−	+	37.4
3	Ingbritt	Bo Krasse	1	c	+	+	+	−	+	36.2
4	UA159	Caufield (ATCC700610)	1	c	+	+	+	−	+	39.8
5	OMZ 175	Guggenheim	1	f	+	+	+	−	+	42.2
6	LM7	Gibbons	1	e	+	v	+	−	−	38.6
7	B13	Bratthall	2	d	+	v	−	−	−	45.8
8	6715-15	ATCC27352	2	d/g	+	v	v	−	−	45.8
9	OMZ 176	Guggenheim	2	d	+	+	v	−	−	47.0
10	OMZ 65	Guggenheim	2	g	+	v	−	−	−	45.8
	Clinical isolates									
11	UA1	Birmingham, Alabama	1	c/e/f	+	+	+	−	+	33.8
12	UA2		1	c/e/f	+	+	+	−	+	33.8
13	UA3		1	c/e/f	+	+	+	−	+	35.0
14	UA4		1	c/e/f	+	+	+	−	+	39.8
15	UA5		1	c/e/f	+	+	+	−	+	36.2
16	UA8		2	d/g	+	+	−	−	−	45.8
17	UA9		2	d/g	+	+	−	−	−	44.6
18	UA11		2	c/e/f	+	+	−	−	−	45.8
19	CH3	Beijing, China	2	indefinite	v	+	−	−	−	47.0
20	CH5		2	indefinite	−	−	−	−	−	42.2
21	CH9		1	c/e/f	+	+	+	−	+	38.6
22	CH12		1	c/e/f	+	+	+	−	−	37.4
23	CH13		1	c/e/f	+	+	+	−	+	36.2
24	CH15		2	d/g	+	+	−	−	−	45.8
25	SW2	Malmö, Sweden	2	d/g	+	−	−	−	−	47.0
26	SW4		1	c/e/f	+	+	+	−	+	35.0
27	SW5		2	d/g	+	+	+	−	−	47.0
28	SW9		2	indefinite	+	+	−	−	−	47.0
29	SW10		1	c/e/f	+	+	+	−	+	36.2
30	SW12		1	c/e/f	+	+	+	−	+	37.4

[a] Chromosomal DNA fingerprint patterns generated by *Hae*III enzyme digestion.
[b] Serotype for clinical isolates was determined with immunofluorescence antisera assay by the microbiologic laboratory at the Faculty of Odontology, Malmö, Sweden.
[c] Minitek™ system. MN=mannitol; SO=sorbitol; RA=raffinose; ARG=arginine; ME=melibiose; +=positive; −=negative; v=variable results.
[d] The mol% G+C value was transformed from DNA melting points (T_m), see methods.

Table 2. Comparison of DNA G+C mol% for MS strains CDF-1 *vs* CDF-2

	mol% G+C		
	Median	Mean±SD	*P*
Fingerprint			
Reference strains			
CDF1 (n=6)	38.0	38.6±2.1	<0.01[a]
CDF2 (n=4)	45.8	46.1±0.6	
Clinical isolates			
CDF1 (n=11)	36.2	36.3±1.9	<0.001[a,b]
CDF2 (n=9)	45.8	45.8±1.6	
Serotype			
Reference isolates			
c/e/f (n=6)	38.0	38.6±2.1	<0.001[a,b]
d/g (n=4)	45.8	46.1±0.6	
Clinical isolates			
c/e/f (n=18)	37.4	37.6±2.9	<0.01[c]
d/g (n=9)	45.8	46.1±0.8	
UD (n=3)	47.0	45.5±2.8	

[a] Nonparametric Mann-Whitney *U*-test for two independent samples.
[b] Student's *t*-test for two independent samples
[c] Nonparametric Mann-Whitney *U*-test for two independent samples. The UD group was not included.

(*t*-test, $P<0.001$) and nonparametric statistical analyses (*U*-test, $P<0.001$). No statistically significant difference in mol% G+C was found within each CDF group.

Another hypothesis the present study tested was the reliability of chromosomal DNA fingerprint method in differentiating *S. mutans* from *S. sobrinus*. To do this, we compared the sensitivity and specificity of serotyping and biotyping in differentiating serotypes c/e/f from serotypes d/g with that of chromosomal DNA fingerprinting. Table 3 lists the summary results for each comparison. With chromosomal DNA fingerprint as a gold standard, the serotyping assay demonstrated 100% sensitivity, 90% specificity and 86.7% agreement, with a kappa statistic of 0.92, compared with chromosomal DNA fingerprint. In addition, three of the 30

Table 3. Comparison of different methods in identifying *S. mutans* and *S. sobrinus* strains

	Chromosomal DNA fingerprint			
	CDF-1 (*c/e/f*)	CDF-2 (*d/g*)	Total	Statistics
Serotype				
c/e/f	17	1	18	Sensitivity $^{\text{serotype vs CDF}}$=100%
d/g	–	9	9	Specificity $^{\text{serotype vs CDF}}$=90%
indefinite	–	3	3	Agreement=86.7%
Total	17	13	30	
Biotype				
c/e/f	15	–	15	Sensitivity $^{\text{biotype vs CDF}}$=88.2%
d/g	–	5	5	Specificity $^{\text{biotype vs CDF}}$=38.5%
indefinite	2	8	10	Agreement=66.7%
Total	17	13	30	

strains could not be clearly assigned to any serotype by means of immunofluorescence. The biotyping method, on the other hand, indicated more variability. The fermentation tests of mannitol, sorbitol, raffinose and melibiose and hydrolysis of arginine resulted in poor specificity (38.5%) and low agreement (66.7%) compared with the CDF method. *S. mutans* strains could not always be distinguished from *S. sobrinus* strains by biochemical tests alone.

Previously, we demonstrated that AP-PCR could discern both homogeneity and heterogeneity of MS genotypes among mother and child pairs (19). The same approach has been applied to this study. Among the serotypes c/e/f strains, AP-PCR fingerprints were unique for each individual strain (Fig. 3). Interestingly, this uniqueness was not observed among all the serotypes d/g strains. Comparing AP-PCR with chromosomal DNA fingerprint, this study showed 100% agreement between the two methods in delineating these two species.

This result was further revealed by using the cluster analysis (UPGMA) as described by Swofford et al. (29). The basic data for conducting the cluster analysis were similarity metrics generated by the Diversity Database Software (Bio-Rad, Laboratories, Catalog No. 170–7561). A dendrogram of relationships derived from a similarity matrix of the AP-PCR fingerprint profiles revealed two main clusters at a distance of about 0.25. Cluster-1 consisted of all CDF-1 strains, and cluster-2 consisted of all CDF-2 strains (Fig. 4). MS strains from Beijing, China (CH3, CH5, CH15) were clustered into one subbranch with MS strains from Malmö, Sweden (SW2, SW5, SW9). CDF-2 strains from Birmingham, Alabama (UA8, UA9, UA11) had the highest similarity indices among the total strains. UA9 and UA11 were identical to each other. Comparison of the similarity indices demonstrated greater diversity among CDF-1 strains than CDF-2 strains. The clinical isolates were also more closely clustered than the reference strains. In addition, the study had another interesting observation. Fig. 5 illustrates that all CDF-1 strains consisted of an amplified fragment of 782 bp, while all of the CDF-2 strains displayed a fragment of 1070 bp. These two fragments were unique to each CDF group.

Discussion

Using the genotyping method with *Hae*III as a restriction endonuclease, we first reported that *S. mutans* serotypes c/e/f strains uniformly displayed larger restriction fragments than *S. sobrinus* serotypes d/g strains in their chromosomal DNA fingerprint in 1995 (20). This study supports our previous findings that MS strains from unrelated

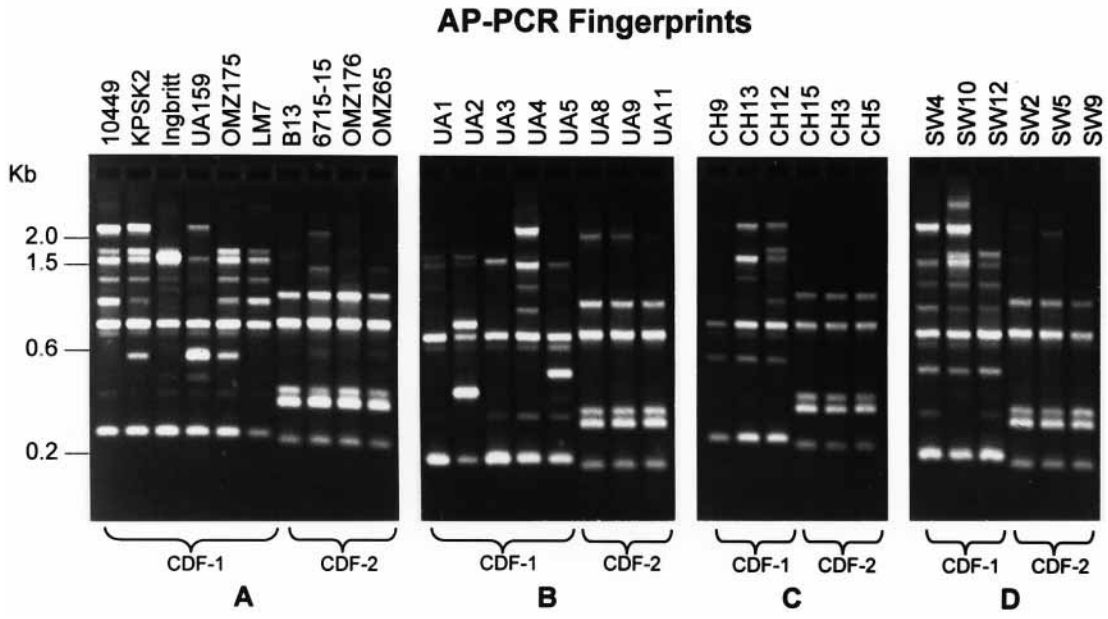

Fig. 3. AP-PCR fingerprint for the 30 MS strains. A greater number of amplicons and more diversity were displayed among the CDF-1 strains than among CDF-2 strains. The AP-PCR profile for CDF-2 strains shows more within-group similarities. The result also shows that AP-PCR has the similar discriminating power for *S. mutans* among different individuals compared to chromosomal DNA fingerprint.

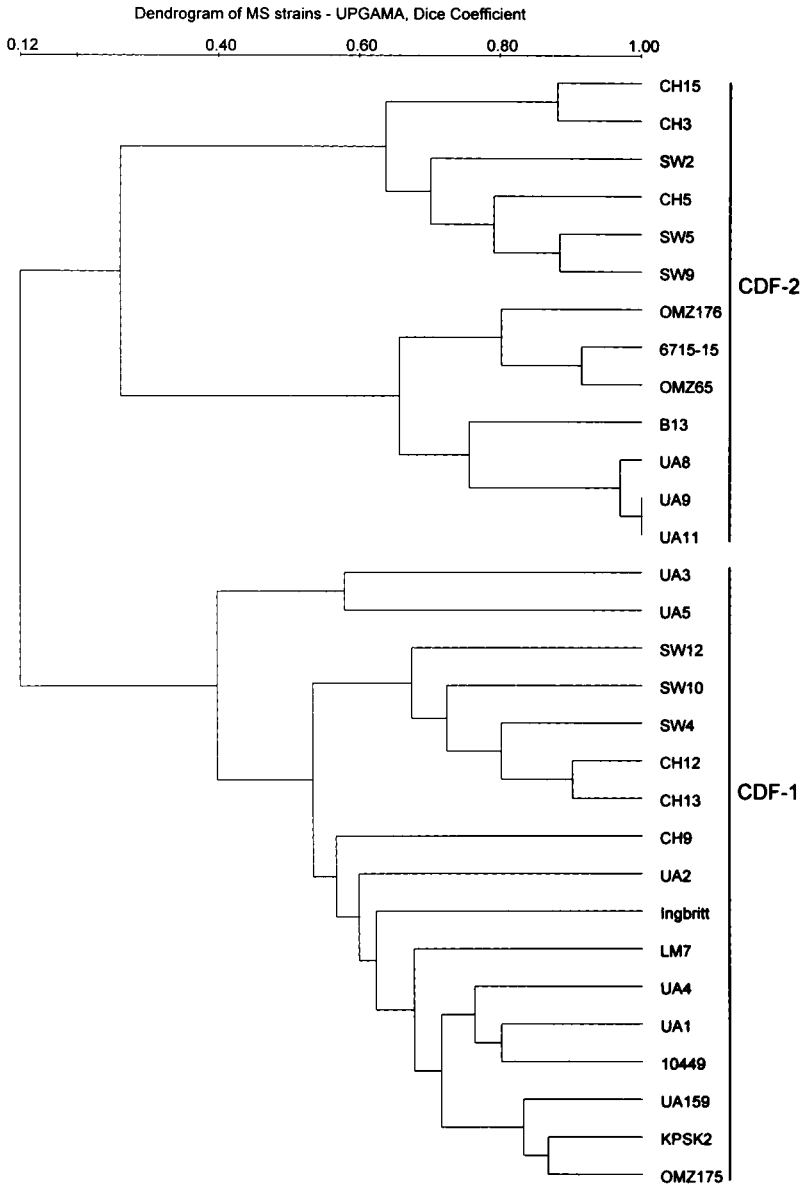

Fig. 4. Dendrogram based on the AP-PCR profiles. The Dice coefficient was generated from UPGMA clustering analysis based upon the comparison of the similarity matrices of all 30 strains.

individuals display distinctive DNA fingerprint profiles. The uniqueness of the DNA profiles aids in the study of MS acquisition and transmission. These studies lead us to the recognitions that DNA fingerprints of MS strains generally fell into two distinct patterns. CDF-1 consisted of a greater proportion of larger DNA fragments and more frequently occurred among MS serotypes c/e/f strains. On the other hand, CDF-2 consisted of smaller DNA fragments and mostly occurred among MS serotypes d/g strains. On the basis of the statistical probability of G+C-rich sequences, we attributed these observations to differences in G+C content between *S. mutans* and *S. sobrinus*. Because *Hae*III recognizes and cleaves only the particular base sequence of GG↓CC, the outcome of DNA restriction fragments is dependent on the G+C mol% of a particular bacterial genome. Theoretically, if these four bases occur with equal frequency, the probability of the site occurring is $P_{rob}=(1/4)^4=1/256$. Assuming the size of the chromosomal DNA molecule of MS equals 2.4×10^6 and the GGCC sequence is random distributed. One would expect to find this site, on the average, once in every stretch of 256 bp on the molecule. Because *S. mutans* and *S. sobrinus* differ in G+C content (38% *vs* 46%), *Hae*III cuts are less frequently for *S. mutans* than for *S. sobrinus*. The average fragment size for *S. mutans* can be calculated as $(0.38)^4\times(2.4\times10^6$ bp)/4=191.8 bp. The average fragment size for *S. sobrinus* can be calculated as $(0.46)^4\times(2.4\times10^6$ bp)/4=89.3 bp. Therefore, one can expect that molecule with high G+C of 42% to be cut by *Hae*III about twice more frequently than that of G+C of 38%. Our CDF patterns not only reflected the differences in G+C content between species but also correctly predicted that enzymes other than *Hae*III, such as *Hin*dIII (A↓AGCTT) or *Eco*RI (G↓AATTC), failed to generate a readable fingerprint profile (data not shown).

To further validate the difference in MS genotypic characterization, we randomly selected 10 prototypes and 20 clinical MS isolates for G+C content analysis. Coykendall and other investigators reported that mol% G+C was 36–38% for MS serotype c/e/f strains, and 44–46% for d/g strains (7, 8, 30). In our study, the mean T_m value for serotypes c/e/f strains (reference strains+clinical isolates, $n=18$) was 69.6±1.2, with a mol% G+C mean of 37.6±3.0. The mean T_m value for serotypes d/g strains (reference strains+clinical isolates, $n=9$) was 73.1±0.3, with G+C mol% mean of 46.1±0.8. For three clinical MS strains, the chromosomal DNA fingerprint belonging to the CDF-2 group could not be classified within either serotype group. Their T_m values (mean=72.8) and mol% G+C (mean=46.2) were similar to those of serotypes d/g strains as identified by the chromosomal DNA fingerprint. The results from the present study suggested that chromosomal DNA fingerprint method is comparable to G+C base analysis in differentiating serotypes c/e/f strains (*S. mutans*) from serotypes d/g strains (*S. sobrinus*) of mutans streptococci.

Certain limitations exist in determining the presence and relative quantification of *S. mutans* and *S. sobrinus* in dental plaque. Different selective medium such as MSB are known to selectively suppress *S. sobrinus* relatively to *S. mutans*. Phenotypic identification methods such as sugar fermentation tests may not be sufficient because the

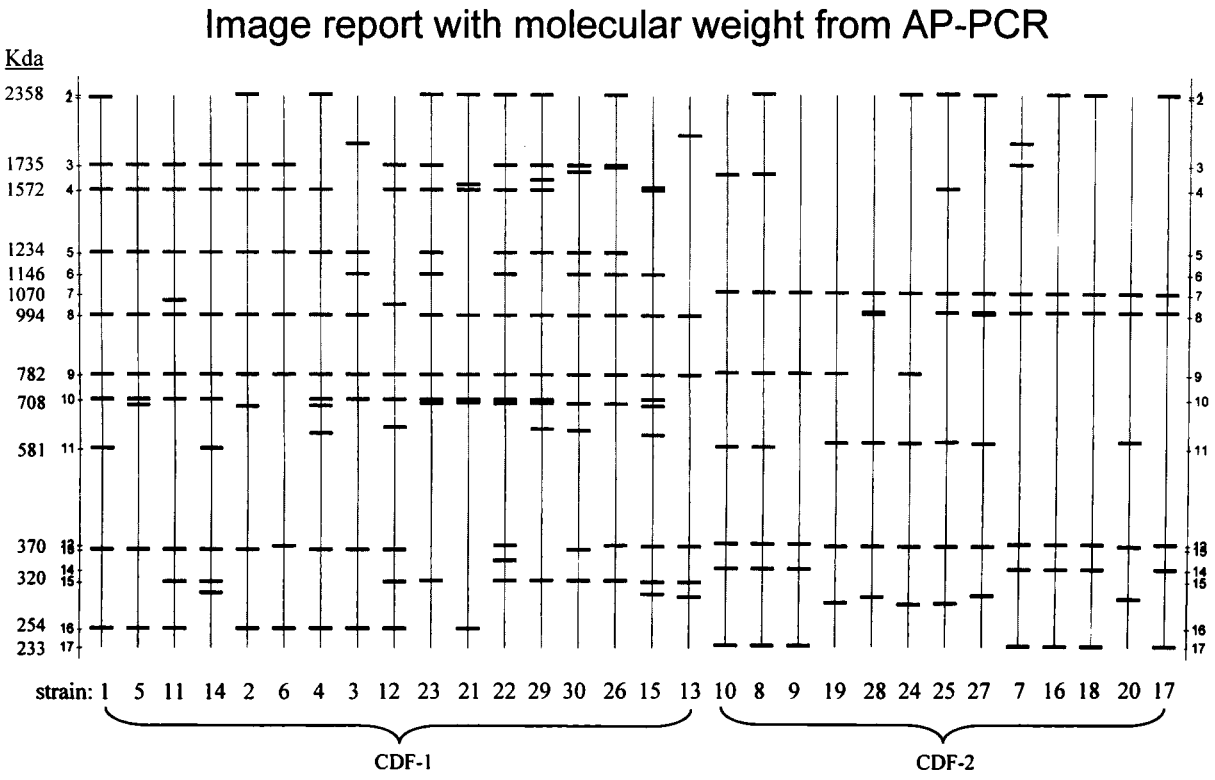

Fig. 5. Image report with molecular weight from AP-PCR generated by the Diversity Database (Bio-Rad Laboratories). All of the CDF-1 strains displayed a fragment sized 782 bp. All of the CDF-2 strains presented a fragment of 1070 bp.

systems are based on *S. mutans* ability to produce acid from raffinose and melibiose, while *S. sobrinus* does not. In our study, we used the Minitek system and showed that 11% of the *S. sobrinus* strains examined may have been incorrectly identified. A similar result was obtained by other studies (11). Compared with the gold standard of the chromosomal DNA fingerprint, the biotype presented 38.5% specificity and 88.2% sensitivity in differentiating the two types of MS. Hence, *S. mutans* strains could not always be distinguished from *S. sobrinus* strains by biochemical tests alone.

In addition, the serotyping assay also proved that all CDF-1 strains belong to MS serotypes c/e/f group and that all CDF-2 reference strains belong to MS serotypes d/g group. Notice that 10% of the clinical MS strains in the study could not be classified into a serotype group. It indicates that serotyping may not be the best method to distinguish the two species. In recent years, the use of DNA-DNA hybridization, monoclonal antibodies or polyconal antisera assays showed promising results in differentiating *S. mutans* and *S. sobrinus* (1, 2, 9). Nevertheless, they still depend on conventional bacterial culture techniques. A more reliable technique for identification of MS is determination of the DNA base composition. It has been used as a benchmark method for bacteria speciation for three decades since it was first described in 1962 (24). Its limitation is that it is labor- and time-intensive.

More recently, PCR and AP-PCR have been widely applied in genotypic characterization of many different bacterial species. We reported that AP-PCR had the power to discern both homogeneity and heterogeneity of MS (19) among unrelated individuals. Results from the present study demonstrated intensively that the agreement between AP-PCR and chromosomal DNA fingerprint in delineating *S. mutans* from *S. sobrinus* was 100%, suggesting that AP-PCR could be an alternative method for identification of MS strains.

One problem with comparing DNA fingerprint profiles is the lack of an objective analytical method. In the current literature, UPGMA cluster is the most commonly employed method. Unlike the study of genomic structure or sequence evolution, DNA fingerprint profile analyses are based not on the accurate oligonucleotide sequence information but on a fingerprint pattern. To compare the fingerprint patterns of bacterial strains among different individuals, the UPGMA clustering analysis based upon similarity matrices is an appropriate tree-construction algorithm. In this study, we chose the Bio-Rad Diversity Database to analyze our data and to test our hypotheses. Clearly, the program separated all 30 MS strains tested into two groups. In addition, two interesting observations were found that related to AP-PCR. First, more bands and more diversity were displayed within the CDF-1 strains than in the CDF-2 strains. It is not clear how the G+C-rich primer (OPA02, G+C=70%) could amplify more fragments for strains with less G+C content than for strains with more G+C content. Second, all of the CDF-1 strains, presumably *S. mutans*, exclusively displayed a fragment sized 782 bp. All of the CDF-2 strains, presumably *S. sobrinus*, uniquely presented a fragment of 1070 bp. It would be

worthwhile to investigate what the molecular significance could be for these two fragments. Information generated from such a study could help further development of species-specific probes for identification of *S. mutans* and *S. sobrinus*.

In summary, the most common cariogenic bacteria related to dental caries in humans are *S. mutans* and *S. sobrinus.* Studies have shown that young children with both *S. mutans* and *S. sobrinus* in their saliva had significantly more dental caries than those with either *S. mutans* or *S. sobrinus* alone (14, 17). In an animal model, it has been suggested that *S. sobrinus* could be more acidogenic than the other species of MS (10). The prevalence and the level of *S. mutans* and *S. sobrinus* have been used as biological markers for caries prediction. However, organisms that are genetically heterogeneous might present phenotypes that are homogeneous, creating more confusion and difficulty for bacterial identification. This study demonstrated that discrimination between the two species was possible by means of the chromosomal DNA fingerprint and AP-PCR method. Relying on the less variable genotype for identification of organisms would inherently lead to greater test stability and would yield more accurate epidemiological information.

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References

1. Babaahmady KG, Challacombe SJ, Marsh PD, Newman HN. Ecological study of *Streptococcus mutans*, *Streptococcus sobrinus and Lactobacillus* spp. at sub-sites from approximal dental plaque from children. Caries Res 1998: **32**: 51–58.
2. Bentley RW, Leigh JA. Development of PCR-based hybridization protocol for identification of streptococcal species. J Clin Microbiol 1995: **33**: 1296–1301.
3. Bratthall D. Mutans streptococci – dental, oral and global aspects. J Indian Soc Pedod Prev Dent 1991: **9**: 4–12.
4. Bratthall D. Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. Odontol Revy 1970: **21**: 143–152.
5. Bratthall D. Immunofluorescent identification of *Streptococcus mutans*. Odontol Revy 1972: **23**: 181–196.
6. Caufield PW, Walker T. Genetic diversity within *Streptococcus mutans* evident by chromosomal DNA restriction fragment length polymorphisms. J Clin Microbiol 1989: **27**: 274–278.
7. Coykendall AL. Four types of *Streptococcus mutans* based on their genetic, antigenic and biochemical characteristics. J Gen Microbiol 1974: **83**: 327–338.
8. Coykendall AL. Base composition of deoxyribonucleic acid isolated from cariogenic streptococci. Arch Oral Biol 1970: **15**: 365–368.
9. de Soet JJ, van Dalen PJ, Russell RR, de Graaff J. Identification of mutans streptococci with monoclonal antibodies. Antonie van Leeuwenhoek 1990: **58**: 219–225.
10. de Soet JJ, Toors FA, de Graaff J. Acidogenesis by oral streptococci at different pH values. Caries Res 1989: **23**: 14–17.
11. de Soet JJ, van Dalen PJ, Appelmelk BJ, de Graaff J. Identification of *Streptococcus sobrinus* with monoclonal antibodies. J Clin Microbiol 1987: **25**: 2285–2288.
12. Emanuelsson IR, Li Y, Bratthall D. Genotyping shows different strains of mutans streptococci between father and child and within parental pairs in Swedish families. Oral Microbiol Immunol 1998: **13**: 271–277.
13. Gibbons RJ, Cohen L, Hay DI. Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors. Infect Immun 1986: **52**: 555–561.
14. Hirose H, Hirose K, Isogai E, Miura H, Ueda I. Close association between *Streptococcus sobrinus* in the saliva of young children and smooth-surface caries increment. Caries Res 1993: **27**: 292–297.
15. Holloway Y, Schaareman M, Dankert J. Identification of viridans streptococci on the Minitek Miniaturised Differentiation System. J Clin Pathol 1979: **32**: 1168–1173.
16. Kiehn TE, Brennan K, Ellner PD. Evaluation of the Minitek system for identification of *Enterobacteriaceae*. Appl Microbiol 1974: **28**: 668–671.
17. Kohler B, Bjarnason S. Mutans streptococci, lactobacilli and caries prevalence in 11- and 12-year-old Icelandic children. Community Dent Oral Epidemiol 1987: **15**: 332–335.
18. Li Y, Caufield PW. The fidelity of initial acquisition of mutans streptococci by infants from their mothers. J Dent Res 1995: **74**: 681–685.
19. Li Y, Caufield PW. Arbitrarily primed polymerase chain reaction fingerprinting for the genotypic identification of mutans streptococci from humans. Oral Microbiol Immunol 1998: **13**: 17–22.
20. Li Y, Caufield PW, Thornqvist L, Emanuelsson IR, Bratthall D. Differentiation of *S. mutans* and *S. sobrinus* via DNA fingerprint profiles. J Dent Res 1995: **74**: 505.
21. Li Y, Wang W, Caufield PW. The fidelity of mutans streptococci transmission and caries status correlate to breast-feeding experience among Chinese families. Caries Res 2000: **34**: 123–132.
22. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986: **50**: 353–380.
23. Madison KM, Bowen WH, Pearson SK, Falany JL. Enhancing the virulence of *Streptococcus sobrinus* in rats. J Dent Res 1991: **70**: 38–43.
24. Marmur J, Doty P. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 1962: **5**: 109–118.
25. Perch B, Kjems E, Ravn T. Biochemical and serological properties of *Streptococcus mutans* from various human and animal sources. APMIS 1974: **82**: 357–370.
26. Schleifer KH, Kilpper-Balz R, Kraus J, Gehring F. Relatedness and classification of *Streptococcus mutans* and "mutans-like" streptococci. J Dent Res 1984: **63**: 1047–1050.
27. Shklair IL, Keene HJ. A biochemical scheme for the separation of the five varieties of *Streptococcus mutans*. Arch Oral Biol 1974: **19**: 1079–1081.
28. SPSS. SPSS 10.0 for Windows. SPSS Inc., Chicago, IL, 1999.
29. Swofford DL, Olsen GJ, Waddell PJ, Hillis DV. Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, ed. Molecular systematics. 2nd edn. Sunderland, MA: Sinauer Associates, 1996: 407–514.
30. Whiley RA, Beighton D. Current classification of the oral streptococci. Oral Microbiol Immunol 1998: **13**: 195–216.