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Genetic Diversity within Isolates of Mutans Streptococci Recognized by an rRNA Gene Probe

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A total of 79 clinical isolates of mutans streptococci and five laboratory strains representing serotypes c, d, e, f, and g were genotyped by a nonradioactive hybridization method with the *rrnB* rRNA operon of the *Escherichia coli* chromosome as a probe. The hybridization patterns of chromosomal DNA fragments obtained by digestion with restriction endonucleases *Hin*dIII, *Sma*I, and *Bam*HI revealed genotypic heterogeneity among the serotypes and among isolates of the same serotype recovered from unrelated subjects. Diversity also existed among isolates obtained from a single subject. For 5 of 13 subjects studied, two or three genotypes within serotypes were found, while eight subjects harbored the same number of genotypes as serotypes. The data show that the method utilizing the rRNA gene probe is of value in determining the molecular epidemiology of isolates of mutans streptococci.

Mutans streptococci have been divided into eight serotypes, a through h, based on the serological specificity of cell wall carbohydrate antigens (10). *Streptococcus mutans*, which includes serotypes c, e, and f, and *Streptococcus sobrinus*, which includes serotypes d and g, have been isolated from humans. Studies utilizing bacteriocin typing have revealed differences between isolates of mutans streptococci (12, 13). All isolates of the same serotype from one individual have a very similar bacteriocin pattern, but there is a wide variation in bacteriocin activity between individuals (1, 17). This finding suggests genetic differences in the isolates.

Restriction endonuclease analysis (REA) has been widely used to distinguish among different strains within many species of microorganisms. REA has shown the existence of restriction fragment length polymorphisms among isolates of mutans streptococci (6, 15). A major disadvantage of this method is the large number of chromosomal bands to be analyzed. The amount of DNA must be carefully adjusted for each identification, and several digests usually have to be run in different combinations before judgments of identity or nonidentity between the strains examined can be made. By hybridizing chromosomal digests with specific probes, it is possible to reduce the number of bands detected.

rRNA probes have proved useful in distinguishing between different strains of many bacterial species (4, 8, 9, 14, 16, 23). In this study, we hybridized mutans streptococcal chromosomal digests to plasmid pKK3535, which contains the *rrnB* rRNA operon of the *Escherichia coli* chromosome (5), to investigate the genotypic heterogeneity of mutans streptococcal isolates.

MATERIALS AND METHODS

Bacterial strains. A total of 79 isolates of mutans streptococci and five laboratory strains representing serotypes c (MT8148), d (B13), e (LM7), f (OMZ175), and g (6715) were included in the material. The clinical isolates were obtained from plaque or saliva samples from 13 subjects, of whom 11 were Caucasian and two were Asian. Three to 10 isolates per subject were sero- and genotyped in order to show the diversity of mutans streptococci in a single subject. Cultures of bacteria originally isolated from MSB agar were initiated for serotyping and chromosomal DNA isolation from frozen stocks.

E. coli DH5α carrying plasmid pKK3535 (kindly provided by P. A. Lawson, London Hospital Medical College, London) was grown in LB broth (20) supplemented with 50 μg of ampicillin per ml.

Serotyping. All isolates were serotyped by the immunodiffusion technique. Antigen extracts were prepared by autoclaving the bacterial cells (19). Antisera against serotypes c, d, e, f, and g were prepared in New Zealand White rabbits by using lyophilized whole-cell antigens. Antisera against serotypes d and g showed cross-reactions, and serotype-specific antisera were prepared by absorption with cross-reactive strains (11).

Isolation of DNA. The method used to isolate chromosomal DNA from the clinical isolates of mutans streptococci was slightly modified from the method of Palva (18). Briefly, bacteria from frozen stocks were grown in 5 ml of brain heart infusion broth (BHI) (Difco), incubated overnight in candle jars. Three milliliters of this culture was used to inoculate 200 ml of BHI and allowed to grow to the stationary phase at 37°C. The cells were washed with 1.5 mM trisodium citrate–15 mM NaCl and suspended in 2 ml of 20% (wt/vol) sucrose–50 mM Tris-HCl solution (pH 8.0). After incubation of the cells with 20 mg of lysozyme (Sigma) for 30 min at 37°C, 20 mg of proteinase K (Sigma) and 3 ml of 1% (wt/vol) Sarkosyl in 0.1 M EDTA (pH 8.0) were added, and incubation was continued at 37°C overnight. Proteins were denatured and removed by several extractions with phenol-chloroform-isoamyl alcohol (25:24:1, by volume) and once with chloroform-isoamyl alcohol (24:1, vol/vol). DNA was precipitated with ethanol and finally purified with the GeneClean II kit according to the manufacturer's instructions (Bio 101, Inc., La Jolla, Calif.).

Plasmid DNA was isolated from *E. coli* DH5α by standard techniques (20).

Restriction endonuclease digestion and agarose gel electro-

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phoresis. Chromosomal DNA (2 to 3 μg) was digested to completion with the restriction endonuclease *Sma*I, *Bam*HI, or *Hin*dIII (Boehringer Mannheim GmbH) according to the manufacturer's instructions. The isolates from five subjects were genotyped with all three enzymes. Isolates from eight subjects were genotyped only with *Hin*dIII and *Sma*I because *Bam*HI could not detect as much variation in the genotypes as the other two enzymes. Bacteriophage lambda DNA digested with *Eco*RI and *Hin*dIII (marker III; Boehringer Mannheim GmbH) was used as a molecular size marker in gel electrophoresis. The restriction fragments were separated by electrophoresis through 0.8% (wt/vol) agarose gels at 2 V/cm for 17 h in Tris-borate buffer. The gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination with the Polaroid MP4 system and 667 film.

Southern blotting, hybridization, and detection. Gels were sequentially soaked in 0.25 M HCl for 20 min, 1.0 M NaCl–0.5 M NaOH twice for 15 min each, and then 0.5 M Tris (pH 7.4)–3 M NaCl twice for 15 min each and blotted (21) on a Nytran-N 13 nylon membrane (Schleicher & Schuell) either by the capillary blotting technique (20) or by the VacuGene XL vacuum-blotting system (Pharmacia LKB Biotechnology, Uppsala, Sweden) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as a transfer solution. The blotted DNA was fixed by UV irradiation at 302 nm for 3 min. Fragments were hybridized to the recombinant plasmid pKK3535, which consists of the cloning vector pBR322 and the *rrnB* rRNA operon of the *E. coli* chromosome (5). Plasmid pBR322 alone did not hybridize to the chromosomal DNA of mutans streptococci, so we were able to use the whole pKK3535 as a probe. Plasmids pKK3535 and pBR322 were labeled by the random primed labeling method, in which digoxigenin-labeled dUTP is incorporated into newly synthesized DNA. Labeling, hybridization, and detection were performed with the nonradioactive digoxigenin DNA labeling and detection kit according to the manufacturer's instructions (Boehringer Mannheim GmbH). The substrate for alkaline phosphatase in color reactions was either 5-bromo-4-chloro-3-indolylphosphate (toluidinium salt) in combination with nitroblue tetrazolium chloride, included in the DNA labeling and detection kit, or naphthol-AS-GR-phosphate (0.02% in 100% dimethylformamide) (Sigma) in combination with fast brown RR salt (0.006% in 70% dimethylformamide). 5-Bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride were replaced by naphthol-AS-GR-phosphate and diazonium salt to speed up the color reaction and to diminish the formation of background (22).

The lambda III molecular size marker DNA was occasionally blotted on the nylon membrane to aid in evaluation of the mobility of the DNA fragments. Plasmid pKK3535 contains a short sequence of lambda DNA (5), and thus it hybridizes to one of the lambda III marker fragments.

RESULTS

Distribution of serotypes. Among the 79 isolates, there were 52 of serotype c, 15 of serotype g, 7 of serotype e, 4 of serotype f, and 1 of serotype d. Six subjects harbored two serotypes, and seven harbored only a single serotype (Table 1).

Hybridization patterns of serotype isolates. Chromosomal DNA from the mutans streptococcal strains (both clinical and laboratory strains) representing each serotype was tested for cleavage with restriction enzymes *Hin*dIII, *Sma*I, and *Bam*HI. DNA was digested separately with either the first two or all three enzymes, and the fragments were separated by electrophoresis and hybridized to *E. coli* plasmid pKK3535. *Sma*I and *Bam*HI produced from four to six major bands, and *Hin*dIII produced from 4 to 10 major bands. The band sizes varied from 1.8 to ca. 21 kbp for *Hin*dIII, from 4.8 to more than 20 kbp for *Sma*I, and from 3.4 to more than 20 kbp for *Bam*HI. In general, differences in the patterns of hybridized DNA fragments were most clearly seen when the chromosomal DNA was digested with *Hin*dIII. The genotypes of mutans streptococcal strains isolated from unrelated subjects were always different regardless of the serotype of the strains when genotyping was performed with *Hin*dIII. Figure 1A shows the genetic diversity of the laboratory strains representing serotypes c, d, e, f, and g, and Fig. 1B shows four examples of serotype c isolates from unrelated subjects.

TABLE 1. Distribution of serotypes and genotypes among 13 subjects

Subject	Age[a] (yr)	No. of strains isolated	Serotype(s)	No. of genotypes within each serotype
A	5, 10	7	c	1
B	5	6	c	1
C	5, 10	5	e	1
D	5, 10	5	c	1
E	10	4	f	1
F	12	6	2 e, 4 c	1, 1
G	6	6	4 c, 2 g	1, 1
H	5	3	c	2
I	61	10	5 c, 5 g	2, 1
J	67	10	5 c, 5 g	1, 2
K	67	4	c	3
L	5	6	3 g, 3 c	1, 1
M	6	7	1 d, 6 c	1, 3

[a] For subjects A, C, and D, strains of mutans streptococci were isolated on two occasions with an interval of 5 years.

Genotypic diversity among isolates obtained from a single subject. Five subjects harbored strains representing two or three genotypes within a serotype, while the rest of the subjects harbored the same number of genotypes as sero-

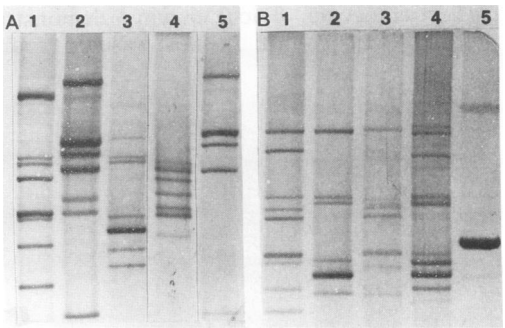

FIG. 1. Examples of hybridization patterns of isolates of various serotypes of mutans streptococci. The chromosomal DNA was digested with *Hin*dIII. (A) Laboratory strains representing serotypes c (MT8148, lane 1), d (B13, lane 2), e (LM7, lane 3), f (OMZ175, lane 4), and g (6715, lane 5). (B) Four serotype c strains (lanes 1 to 4) isolated from unrelated subjects. Lane 5, lambda DNA size marker.

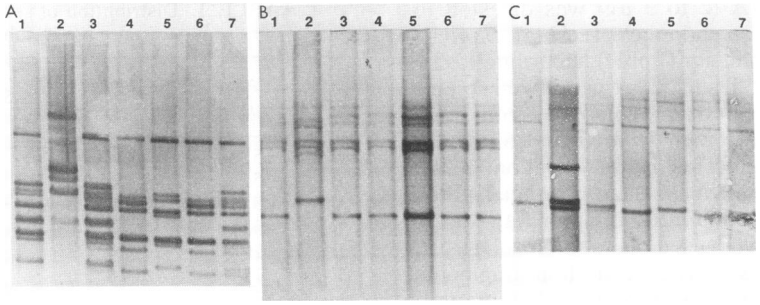

FIG. 2. Hybridization patterns of serotype c (lanes 1, 3, 4, 5, 6, and 7) and d (lanes 2) isolates of mutans streptococci obtained from subject M. DNA was digested with *Hin*dIII (A), *Bam*HI (B), or *Sma*I (C).

types. Diversity was found in serotypes c and g. An example of multiple genotypes in the same individual is shown in Fig. 2, which shows subject M, harboring serotype c and d isolates. Three different genotypes were found within serotype c. The differences between the isolates could be clearly seen when the chromosomal DNA was digested with *Hin*dIII (Fig. 2A). No differences in the band patterns could be detected with *Bam*HI (Fig. 2B), and with *Sma*I, the differences were only slight (Fig. 2C). Of the five subjects who harbored strains representing more than one genotype within a serotype, the isolates from four (subjects I, J, K, and M) had band patterns that closely resembled those of the others from the same individual. The difference in genotypes could be as small as a single band with changed mobility (Fig. 3). Mutans streptococcal isolates had been recovered from three subjects (A, C, and D) on two occasions, the follow-up period being 5 years. No change in the band patterns of these isolates could be detected.

DISCUSSION

In the present study, the restriction fragments of mutans streptococcal chromosomal DNA were hybridized to the digoxigenin-labeled plasmid pKK3535, which contains the *rrnB* rRNA operon of the *E. coli* chromosome. With this technique, the diversity of clinical isolates of mutans streptococci could be clearly visualized. The size and the number of bands made the judgment of identity or nonidentity easier with this method than with REA.

Three restriction enzymes, *Sma*I, *Bam*HI, and *Hin*dIII, were tested for their ability to differentiate between isolates of mutans streptococci. Each enzyme demonstrated a unique hybridization pattern. In most cases, the diversity among the strains was best visualized when *Hin*dIII was used for digestion. Compared with *Hin*dIII and *Sma*I, *Bam*HI had only a limited ability to discriminate among the isolates. The hybridization patterns were reproducible. No change could be detected in patterns after repeated DNA extractions and hybridizations.

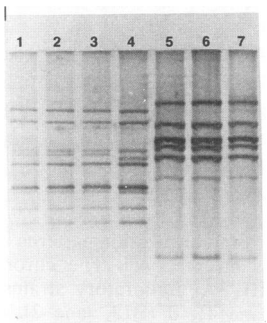

FIG. 3. Hybridization patterns of serotype c (lanes 1 to 4) and g (lanes 5 to 7) isolates of mutans streptococci obtained from subject I. DNA was digested with *Hin*dIII.

The information on genetic diversity within isolates of a particular serotype obtained from a single subject has been limited. Kulkarni et al. (15) investigated the diversity of REA patterns of *S. mutans* serotype c strains isolated from four unrelated subjects. They could find only one REA pattern per subject, but when they investigated strains isolated from other subjects (serotype designations were not given), as many as five REA patterns could be found in isolates from one individual. Our results showed that 5 of 13 subjects had strains of two or three different genotypes within the isolates representing serotypes c and g. Diversity was not revealed with all the enzymes used in the study. It is possible that even greater genetic diversity could have been found if more enzymes or more strains had been included in the material. The occurrence of multiple genotypes within a serotype may be due to infection with several different strains of mutans streptococci. Another alternative is a mutation in the chromosome of the original strain. The finding that, for isolates from four subjects, genotypes within a serotype had several bands of the same size supports the possibility of a mutation in these cases.

When an individual harbored mutans streptococci of two serotypes, the genotypes of the strains were always different. This implies that the finding of double serotypes was not caused by the serotypic switching of a single bacterial clone, a phenomenon observed, for example, in *Borrelia* species causing relapsing fever (2).

Bacteriocin typing has revealed that the bacteriocin activity of a mutans streptococcal strain from one individual is different from that of a strain from another individual. This might reflect genetic diversity among mutans streptococcal strains obtained from unrelated subjects, as shown in our study and previously by others using REA (6, 15). Some studies also suggest that many subjects harbor strains of more than one bacteriocin type (3, 7). This can partly be explained by the fact that an individual can harbor strains of two or more serotypes, since each serotype has its own bacteriocin activity (1, 17). However, strains of the same serotype but with dissimilar bacteriocin activity can also be found in the same individual (1). We analyzed two such serotype c isolates by the method presented here and found that the isolates had different hybridization profiles (data not shown).

In conclusion, the results showed that by using probe-specific DNA fingerprinting, genetic diversity between serotypes and among the isolates of mutans streptococci of the same serotype recovered from unrelated subjects could be clearly visualized. Furthermore, several genotypes of mutans streptococci of the same serotype could be isolated from a single subject. This method, utilizing the rRNA gene probe, can be applied to epidemiological studies in which the acquisition, transmission, and stability of infecting mutans streptococci are examined.

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