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Use of Restriction Fragment Polymorphism Analysis of rRNA Genes To Assign Species to Unknown Clinical Isolates of Oral Viridans Streptococci

JOEL D. RUDNEY* AND CHRISTOPHER J. LARSON

Department of Oral Science, School of Dentistry, University of Minnesota, 17-252 Moos Tower, 515 Delaware St. SE, Minneapolis, Minnesota 55455

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This study evaluated restriction fragment length polymorphisms of rRNA genes (ribotyping) for genotypic identification of 53 oral isolates classified as "*Streptococcus sanguis*" by colony morphology. Isolates were from 8-h buccal plaque on lower first permanent molars of 20 subjects. DNA was digested with *Aat*II and hybridized with digoxygenin-labeled cDNA of *Escherichia coli* 16S and 23S rRNA. Strains were ribotyped again with *Alw*NI or *Pvu*II on the basis of the presence or absence of a 2,290-bp *Aat*II band. Band patterns were compared with reference ribotypes for *Streptococcus gordonii*, *Streptococcus sanguis*, *Streptococcus crista*, *Streptococcus oralis*, *Streptococcus mitis*, and *Streptococcus parasanguis* strains. Forty-eight isolates could be assigned to a species (22 *S. sanguis*, 14 *S. oralis*, 12 *S. gordonii*). Multiple species were seen in 14 subjects; multiple strains of the same species occurred in 11 subjects. Our findings suggest that ribotyping can be used for genotypic identification of *S. sanguis*, *S. oralis*, and *S. gordonii* isolates.

Strains formerly placed within the "*Streptococcus sanguis*" group may represent six distinct genetic groups. Those groups have been assigned the species names *Streptococcus gordonii*, *S. sanguis*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus parasanguis*, and *Streptococcus crista* (14, 18, 34). The newly defined species appear to occupy distinct oral sites, which may change as dental plaque matures (11, 20, 23). Their prevalence may also differ between caries-resistant and caries-susceptible individuals (24). Accurate identification of strains recovered from the mouth is thus a current issue in clinical studies of streptococcal acquisition, transmission, and ecology.

The species mentioned above are not distinguishable on the basis of colony morphology (7, 18). Current identification protocols therefore rely on phenotypic tests. One product now in use is the API Rapid Strep system (Analytab Products, Plainview, N.Y.). The species names used by the API system predate the reclassification of the "*S. sanguis*" group (10, 29). However, some studies have used that system in taxonomic studies (7, 30). *S. gordonii*, *S. sanguis*, *S. oralis*, and *S. mitis* were formally defined by Kilian et al. (18), who described a comprehensive set of phenotypic tests to identify those species. Others have developed phenotypic approaches which include *S. parasanguis* and *S. crista* (2, 14, 34). A potential problem with phenotypic tests is that not all strains in a species may be positive for a common trait (2, 18). Another potential problem is suggested by observations of phenotypic shifts in laboratory strains of streptococci. The same strain may give different results on different occasions without corresponding changes in genotype (15, 33). Phenotypic classifications based on reference strains thus might not identify all clinical isolates.

We have investigated ribotyping as an approach for genotypic identification of streptococcal species (27). Ribotyping compares patterns produced when probes for rRNA genes are hybridized to restriction digests of chromosomal DNA (32). This approach has been used to discriminate related species from a number of genera (8, 12, 13, 17, 22, 25, 31). In a previous study of laboratory strains classified into "*S. sanguis*" group species by DNA hybridization, we found that *S. oralis*, *S. mitis*, and *S. parasanguis* could be distinguished from *S. gordonii*, *S. sanguis*, and *S. crista* by the presence or absence of a 2,290-bp band in ribotypes generated with restriction endonuclease *Aat*II (27). Common bands in *Aat*II and *Pvu*II ribotypes could be used to separate *S. gordonii* from *S. sanguis* and *S. crista*, while common bands in *Alw*NI ribotypes could be used to separate *S. oralis* from *S. mitis* and *S. parasanguis* (the last two species were not distinguished by any of seven enzymes used) (27). Those findings suggested that clinical isolates might be identified by matching *Aat*II, *Pvu*II, and *Alw*NI ribotypes to reference strains of known species. This study evaluated that identification strategy by ribotyping stored clinical isolates previously classified as "*S. sanguis*" on the basis of colony morphology. The following major research questions were asked. (i) Could isolates be assigned to new "*S. sanguis*" group species? (ii) What was the distribution of species among subjects? (iii) Were multiple species or multiple strains of the same species present within subjects? (iv) Did species assignments by ribotyping agree with those made by colony morphology, the API Rapid Strep system, and other phenotypic tests?

MATERIALS AND METHODS

Origin of clinical isolates. Clinical isolates used in this study were taken in 1989 during a study of saliva and plaque from college dormitory residents (26). Samples of 8-h supragingival plaque from both mandibular first molars were pooled in reduced transport buffer. "*S. sanguis*" strains were identified by colony morphology on mitis salivarius agar. Morphological identifications were supplemented with results from API Rapid Strep tests (26). A representative example of each morph identified as "*S. sanguis*" was picked and subcultured twice, as described below. Broth

* Corresponding author. Fax: (612) 626-2651. Electronic mail address: jrudney@maroon.tc.umn.edu.

cultures were then lyophilized in skim milk. Fifty-three isolates were used in this study. They had been taken from 20 subjects found to be at opposite extremes for resting whole saliva flow rate and concentrations of antimicrobial and total protein. Fifteen of the subjects were represented by two to four isolates.

Culture methods. Lyophiles reconstituted in sterile distilled water were streaked onto hemin- and menadione-supplemented sheep blood agar and mitis salivarius agar (Di-Med, St. Paul, Minn.). Plates were incubated anaerobically in Gas-Pak jars (BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 37°C. Representative colonies were transferred to a second set of plates and incubated as before. Colonies selected from those plates were incubated anaerobically in 100 ml of sterile Todd-Hewitt broth (Difco, Detroit, Mich.) overnight at 37°C.

Phenotypic tests. The API Rapid Strep system was used to classify 45 clinical isolates. Procedures were performed as described by Rudney et al. (28), who also provide data on the reproducibility of API system results. Two phenotypic tests which have been employed in the identification of "*S. sanguis*" group species were also used. Most strains of *S. gordonii*, *S. mitis*, *S. crista*, and *S. parasanguis* bind amylase, while *S. sanguis* and *S. oralis* do not (9, 19). Forty-eight clinical isolates were tested for amylase binding according to the method of Douglas et al. (9). Cells were incubated with clarified human whole saliva. Supernatants were then assayed for amylase activity in starch-agarose plates. Most strains of *S. oralis* and some strains of *S. mitis* produce sialidase, while other species typically do not (2, 3). Beighton and Whiley's (3) method was used to test 32 clinical isolates for sialidase activity. Cells were incubated with 100 μg of 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Sigma, St. Louis, Mo.) per ml in 96-well plates; activity was detected as blue fluorescence under UV light.

Ribotyping. Clinical isolates were ribotyped with Rudney and Larson's (27) protocols for laboratory strains. Briefly, washed cells were embedded in low-melting-point agarose (Sigma) beads by emulsification with paraffin oil. Embedded cells were lysed with 2 mg of lysozyme per ml–1,000 U of achromopeptidase per ml–42.5 U of mutanolysin per ml (all from Sigma), followed by 2% sodium dodecyl sulfate and then 200 μg of proteinase K per ml (GIBCO BRL, Gaithersburg, Md.), all at 40°C. Washed packed beads were equilibrated overnight in restriction buffers and treated with one of three restriction endonucleases, *Aat*II, *Alw*NI (New England Biolabs, Beverly, Mass.), or *Pvu*II (GIBCO BRL). *Aat*II gave optimal results when DNA was embedded in beads. Digestion was accelerated by heating samples in a microwave oven at full power for three 10-s intervals. Digests were run in horizontal 0.7% agarose gels (15 by 15 cm, 200 ml) at 30 V in TBE buffer (0.089 M Tris-borate, 20 mM EDTA [pH 8.3]) for 17 h at 25°C with size standards of digoxygenin-labeled DNA molecular weight marker II (Boehringer-Mannheim, Indianapolis, Ind.) loaded into outer and middle wells. A model 750 vacuum blotter (Bio-Rad, Richmond, Calif.) was used to transfer depurinated and denatured restriction fragments from gels to Hybond N^+ nylon membrane (Amersham, Arlington Heights, Ill.). Membranes were then hybridized with a digoxygenin-labeled cDNA probe prepared by reverse transcription of *Escherichia coli* 16S and 23S rRNA (25 μl [Boehringer Mannheim]), as described by Baloga and Harlander (1). Bound probe was detected with the Genius 3 kit from Boehringer Mannheim, as per the manufacturer's instructions. Blots were digitized at a resolution of 1,024 by 1,024 pixels with a Visage 110 image analysis system (Bio-Image, Ann Arbor, Mich.). Visage program WHOLE BAND was then used to determine band sizes (27, 28).

TABLE 1. Strains in panel of reference ribotypes[a]

Strain
S. gordonii
ATCC 10558 (type strain)
ATCC 12396
ATCC 33399
Blackburn
SPED3
S7
M5
S. sanguis
ATCC 10556 (type strain)
HPC1
804
S. crista CR3[b]
S. parasanguis
ATCC 15909
ATCC 15911
ATCC 15912 (type strain)
MGH 145
MGH 413
UC 4989
FW 213
S. mitis
ATCC 903
NCTC 10712 (type strain)
NCTC 12261
S. oralis
ATCC 10557
ATCC 35037 (type strain)
CR 834
9811

[a] Sources and citations for strains listed here are given by Rudney and Larson (27).

[b] The type strain for *S. crista* is CR311 (14). We previously found CR3 to be genotypically indistinguishable from CR311 by DNA fingerprinting and ribotyping (27, 28).

Strategy for species assignment. Isolates were first digested with *Aat*II. Visage program WB COMPARE was then used to match *Aat*II ribotypes for clinical isolates to those for *S. gordonii*, *S. sanguis*, *S. oralis*, *S. mitis*, *S. crista*, and *S. parasanguis* reference strains (27). The reference panel is listed in Table 1. Isolates were tentatively assigned to a species if they shared *Aat*II bands with reference strains of that species. Undigested beads from isolates showing a 2,290-bp *Aat*II band were then treated with *Alw*NI, and ribotypes were compared with reference patterns for *S. oralis*, *S. mitis*, and *S. parasanguis*. Undigested beads from isolates lacking a 2,290-bp *Aat*II band were digested with *Pvu*II, and ribotypes were compared with reference patterns for *S. gordonii*, *S. sanguis*, and *S. crista*. Final species assignments combined *Aat*II results with those for *Alw*NI or *Pvu*II.

RESULTS

Matching to reference patterns. Figure 1 gives examples of reference *Aat*II ribotypes for each species. Figure 2 gives examples of clinical isolates with matches to those patterns.

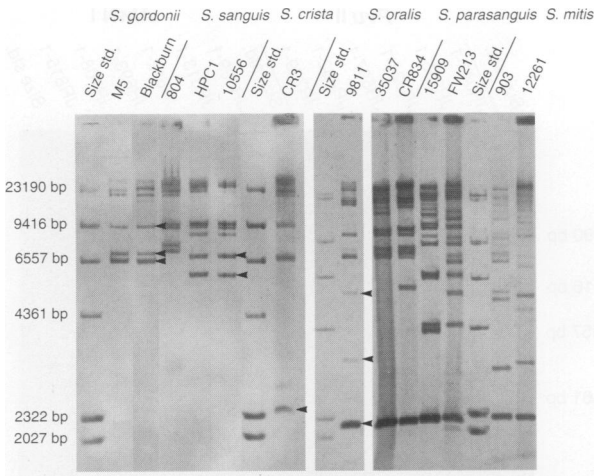

FIG. 1. *Aat*II ribotypes for examples of strains from the reference panel, compiled from blots presented in Rudney and Larson (27 [with permission of the publisher]) by using Adobe Photoshop 2.0.1 and Quark Express 3.11 software with a Macintosh IIcx computer and a Sharp 450 scanner. Species and strain names are above the lanes (species are separated by angled lines). Size std., DNA Molecular Weight Marker II (Boehringer Mannheim). Band sizes are given to the left. Arrowheads denote bands used for assigning isolates to species.

Twelve isolates showed an *Aat*II band at approximately 9,400 bp, followed by a doublet at approximately 7,100 and 6,600 bp (in three cases, the 9,400-bp band was present as the upper band of a doublet). Strains JR505-4, JR669-4, JR815-4, and JR981-4 in Fig. 2 provide examples of that pattern, which occurred in *Aat*II ribotypes for all *S. gordonii* reference strains (Fig. 1). All 12 isolates showing those bands were tentatively assigned to *S. gordonii*.

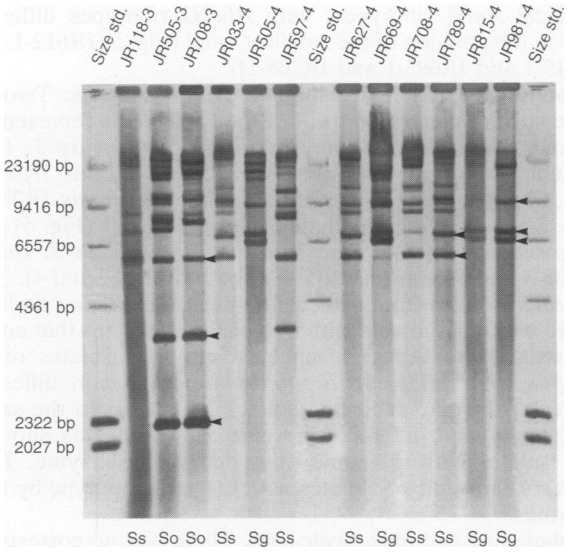

FIG. 2. Blot of representative *Aat*II ribotypes for clinical isolates. Labeling was added by using Adobe Photoshop 2.0.1 and Quark Express 3.11 software with a Macintosh IIcx computer and a Sharp 450 scanner. Isolate names are above the lanes. The number following the prefix JR is the subject number; different isolates from the same subject are distinguished by a numerical suffix (e.g., isolates JR708-3 and JR708-4 were obtained from the same person). Arrowheads denote examples of bands used for assigning isolates to species. Assigned species are indicated at the base of the lanes as Sg for *S. gordonii*, Ss for *S. sanguis*, and So for *S. oralis*. Size std., DNA Molecular Weight Marker II (Boehringer Mannheim).

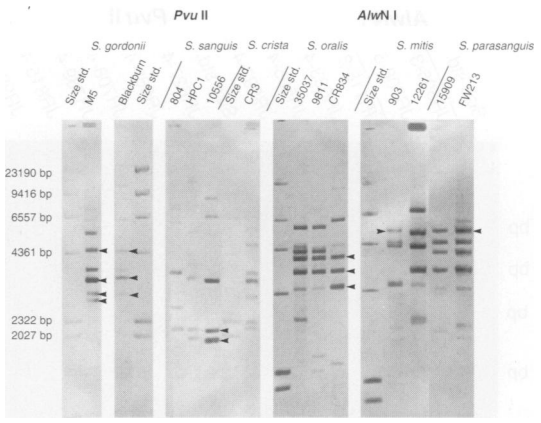

FIG. 3. *Pvu*II and *Alw*NI ribotypes for examples of strains from the reference panel, compiled from blots presented in Rudney and Larson (27 [with permission of the publisher]) by using Adobe Photoshop 2.0.1 and Quark Express 3.11 software with a Macintosh IIcx computer and a Sharp 450 scanner. Annotation is as in Fig. 1.

Six isolates shared five *Aat*II bands with *S. sanguis* reference strains HPC1 and 10556 (Fig. 1). That pattern is demonstrated by strains JR033-4 and JR789-4 in Fig. 2. Twelve other isolates showed *Aat*II bands in common with reference strains HPC1 and 10556, including a band at approximately 5,900 bp, which was the smallest band in 11 of those strains. Examples include JR118-3, JR597-4, JR627-4, and JR708-4 in Fig. 2. All 18 isolates were tentatively assigned to *S. sanguis*. Nine isolates without a 2,290-bp *Aat*II band did not match patterns for any *S. gordonii*, *S. sanguis*, or *S. crista* reference strain; that group included four strains not digested by *Aat*II.

Fourteen isolates showed a 2,290-bp *Aat*II band. All of them shared bands at 5,800 and 3,550 bp with *S. oralis* reference strain 9811 (Fig. 1 and strains JR505-3 and JR708-3 in Fig. 2). Those isolates were tentatively assigned to *S. oralis*. No isolates matched *Aat*II patterns for *S. mitis* or *S. parasanguis* reference strains.

Figure 3 presents *Pvu*II and *Alw*NI ribotypes for reference strains; Fig. 4 and 5 show clinical isolate examples for those enzymes. Seven of the 12 strains tentatively assigned to *S. gordonii* by *Aat*II showed *Pvu*II ribotypes similar to that of *S. gordonii* reference strain M5 (Fig. 3). Those strains shared bands at 3,475, 3,020, and 2,850 bp (strains JR815-4 and JR981-4 are examples in Fig. 4). *Pvu*II ribotypes for the other five tentative *S. gordonii* isolates were more similar to that of *S. gordonii* reference strain Blackburn. The 2,850-bp band was absent in those strains (JR505-4 and JR669-4 are examples in Fig. 4, and JR324-1 and JR739-1 are examples in Fig. 5).

Seventeen isolates shared *Pvu*II ribotype bands at approximately 2,100 and 1,730 bp with *S. sanguis* reference strains HPC1 and 10556 (Fig. 3); these included strains which did not share five *Aat*II bands with those reference strains and the four strains not digested by *Aat*II. The examples shown are JR579-4, JR627-4, and JR708-4 in Fig. 4 and JR047-1, JR597-1, and JR662-1 in Fig. 5. Three other isolates showed only one of those two *Pvu*II bands (JR033-4 in Fig. 4 and JR255-1 in Fig. 5). Two other isolates with poorly defined

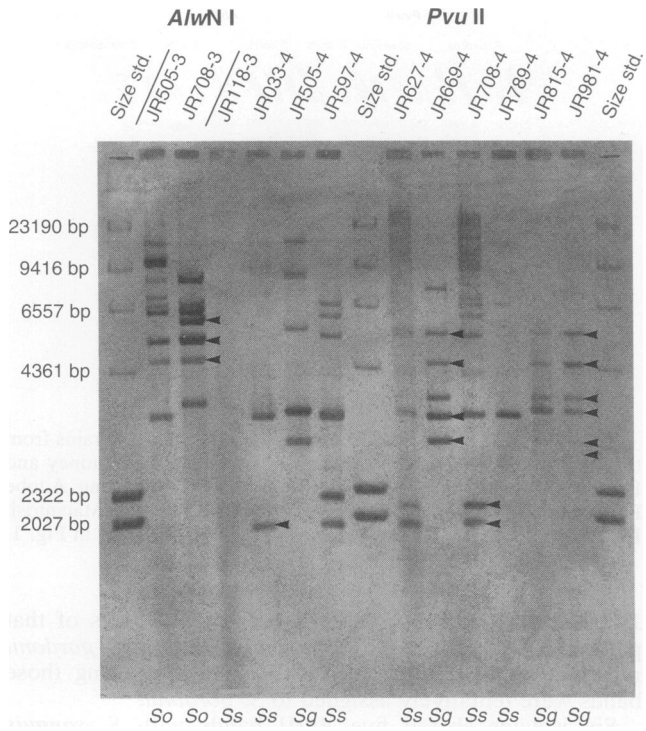

FIG. 4. Blot of representative *Pvu*II and *Alw*NI ribotypes for clinical isolates. Labeling was added by using Adobe Photoshop 2.0.1 and Quark Express 3.11 software with a Macintosh IIcx computer and a Sharp 450 scanner. Annotation is as in Fig. 2.

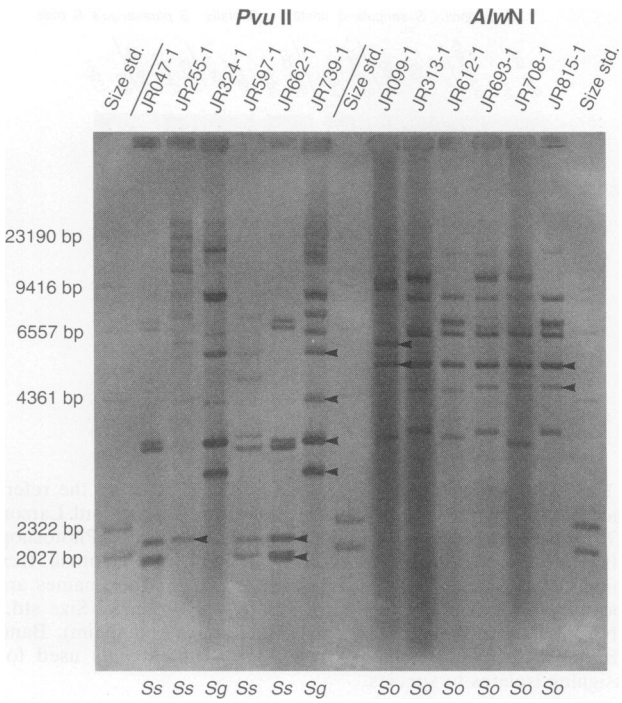

FIG. 5. Blot of representative *Pvu*II and *Alw*NI ribotypes for clinical isolates. Labeling was added by using Adobe Photoshop 2.0.1 and Quark Express 3.11 software with a Macintosh IIcx computer and a Sharp 450 scanner. Annotation is as in Fig. 2.

*Pvu*II patterns were assigned to *S. sanguis* on the basis of their *Aat*II ribotypes (JR118-3 and JR789-4 [Fig. 2 and 4]). A total of 22 isolates were assigned to *S. sanguis* by *Aat*II or *Pvu*II. Five isolates with unassignable *Aat*II ribotypes were also unassignable by *Pvu*II.

The 14 isolates found to have a 2,290-bp *Aat*II band shared at least two of three *Alw*NI bands at approximately 6,025, 5,300, and 4,600 bp with *S. oralis* reference strains (Fig. 3). Examples include JR505-3 and JR708-3 in Fig. 4 and JR099-1, JR313-1, JR612-1, JR693-1, JR708-1, and JR815-1 in Fig. 5. None of those isolates displayed a 7,600-bp band seen in all *S. mitis* and *S. parasanguis* reference strains (Fig. 3). Their assignment to *S. oralis* was thus supported by *Alw*NI results.

In summary, 48 (91%) of 53 clinical isolates could be matched to a reference pattern. Isolates matched with *S. sanguis* were the most common (42%), followed by strains assignable to *S. oralis* (26%) and *S. gordonii* (23%). No isolates could be assigned to *S. crista*, *S. mitis*, or *S. parasanguis*, and five strains (9%) could not be matched.

Species and strain distributions among subjects. Species prevalences among the 20 subjects appeared to reflect prevalences in the isolate collection. Strains assigned to *S. sanguis* were recovered from 14 subjects (70%). Putative *S. oralis* strains were recovered from 11 subjects (55%), and *S. gordonii* strains were recovered from 9 subjects (45%). The five unassignable isolates were distributed among five subjects (25%). In three cases, strains that appeared identical by two enzymes were recovered from different subjects. Examples include putative *S. gordonii* strains JR815-4 and JR981-4 (Fig. 2 and 4), *S. sanguis* JR033-4 and JR789-4 (Fig. 2 and 4 [that pattern was seen for strains from two other subjects]), and *S. sanguis* JR047-1 and JR662-1 (Fig. 5). In two cases, putative *S. oralis* strains from different subjects showed identical *Aat*II ribotypes, with *Alw*NI ribotypes differing only in the position of the smallest band (Fig. 5 [JR612-1 and JR815-1 and JR693-1 and JR708-1]).

Species and strain distributions within subjects. Two or three species were seen in 14 of the 15 subjects represented by multiple isolates. Many combinations occurred. One example is provided by putative *S. oralis* strains JR708-1 (Fig. 5) and JR708-3 (Fig. 2 and 4) with *S. sanguis* JR708-4 (Fig. 2 and 4). Others include *S. oralis* JR815-1 (Fig. 5) and *S. gordonii* JR815-4 (Fig. 2 and 4), as well as *S. oralis* JR505-3 and *S. gordonii* JR505-4 (both in Fig. 2 and 4). The *S. oralis* isolates from subject 708 illustrate another finding. There were 11 subjects with two or three isolates that could be assigned to the same species (multiple isolates of *S. sanguis*, *S. oralis*, and *S. gordonii* occurred in different subjects). In nine subjects, isolates assignable to the same species showed different ribotypes. Two subjects showed two isolates with the same ribotype by one enzyme. Two subjects showed two isolates with the same ribotype by two enzymes.

Ribotyping and phenotypic tests. There was no correspondence between colony morphology on mitis salivarius agar and ribotype patterns. Hard, embedded colonies were seen for some strains assigned to *S. gordonii*, *S. sanguis*, and *S. oralis*, while other strains assigned to *S. gordonii* and *S. oralis* showed soft, flat colonies. Hard and soft morphs were sometimes observed on the same plate. In those cases, the ribotypes for both morphs were identical.

API Rapid Strep identifications for isolates are cross-tabulated with ribotype species assignments in Table 2. Phenotypic diversity was greatest among strains ribotyped

TABLE 2. API Rapid Strep results for 41 clinical isolates

API Rapid Strep assignment[a]	No. of strains assigned by ribotyping to:			
	S. sanguis	*S. oralis*	*S. gordonii*	Unknown
S. sanguis I/1	4		7	
S. sanguis I/2	5			1
S. sanguis II		7		2
S. mitis		3	2	1
S. milleri III	1			
S. morbillorum	4	2	1	
S. pneumoniae 2	3			
S. uberis	1			
G. haemolysans		1		

[a] API Rapid Strep species designations predate current reclassifications of oral streptococcal species (10, 14, 18, 29, 34).

to *S. sanguis*, which were identified by the API system as *S. sanguis* I/2, *S. sanguis* I/1, *Streptococcus morbillorum*, *Streptococcus pneumoniae* 2, *Streptococcus milleri* III, and *Streptococcus uberis*. Isolates assigned to *S. oralis* were identified as *S. sanguis* II, *S. mitis, S. morbillorum*, and *Gemella haemolysans*. Strains assigned to *S. gordonii* showed the least amount of phenotypic diversity. Isolates were identified as *S. sanguis* I/1, *S. mitis*, and *S. morbillorum*. Four of the five isolates not assignable by ribotyping were tested with the API system; they were identified as *S. sanguis* II, *S. mitis*, and *S. sanguis* I/2.

Nine of 48 isolates tested for amylase binding had been assigned to *S. gordonii*. Eight of those isolates bound amylase, while one did not. All other isolates were negative for amylase binding. Ten of 32 isolates tested for sialidase activity had been assigned to *S. oralis*. Eight of those strains showed activity, as did one of two unassignable isolates tested. Sialidase activity was seen for one of eight isolates assigned to *S. gordonii*. A second isolate from the same subject with an identical ribotype pattern by two enzymes was negative for sialidase. Twelve putative *S. sanguis* isolates were negative for sialidase.

DISCUSSION

This study suggests that Rudney and Larson's (27) ribotyping strategy can be used to assign clinical isolates of oral viridans streptococci to *S. gordonii*, *S. sanguis*, and *S. oralis*. The strategy matches isolates to reference strains previously grouped by DNA hybridization. The number of strains that have been classified by hybridization is small, so the reference panel may not include a full range of ribotypes for each species. However, 90% of these isolates shared bands with the reference panel. This suggests that reference patterns may be representative of those that are seen in clinical samples.

Rudney and Larson (27) proposed that the presence or absence of a 2,290-bp *Aat*II band be used to separate *S. oralis*, *S. mitis*, and *S. parasanguis* from *S. gordonii*, *S. sanguis*, and *S. crista*, with *Alw*NI or *Pvu*II then being used to discriminate species within each group. In this study, *Aat*II ribotypes were often sufficient to assign isolates to *S. gordonii* and *S. sanguis*. However, *Pvu*II ribotypes were needed to assign a subgroup of strains not digested by *Aat*II to *S. sanguis*. Five isolates showed *Aat*II and *Pvu*II ribotypes different from those of all of the reference strains (two were similar to each other). DNA hybridization studies may be required to identify those isolates. *S. oralis* 9811 showed 5,800- and 3,350-bp *Aat*II bands that were common to all isolates with a 2,290-bp *Aat*II band. *Alw*NI ribotypes were not needed to assign those isolates to *S. oralis* but did confirm that they were not assignable to *S. mitis* or *S. parasanguis*.

The species distribution of these isolates is partly consistent with those from previous studies. Kilian and coworkers found that *S. sanguis* and *S. oralis* were more common than *S. gordonii* in supragingival plaque, but they also found large numbers of *S. mitis* biovar 1 (11, 20, 23, 24). The absence of *S. mitis*, *S. parasanguis*, or *S. crista* in this collection may indicate that those species were uncommon in supragingival plaque from these subjects. However, that finding might also be related to the way colonies were selected for storage.

Kilian's group took large random samples of isolates from small numbers of subjects, with the objective of identifying all streptococcal species by phenotype (11, 20, 23, 24). Rudney et al.'s (26) goal was to quantify "*S. sanguis*" in a large number of subjects differing in saliva composition. Quantification was primarily on the basis of colony morphology, and isolates were taken only for morphs that were counted. Colonies of *S. mitis*, *S. parasanguis*, or *S. crista* thus might have been present without being recognized.

Variability in the expression of phenotypic traits is an issue in either explanation. Heterogeneity of colony morphology was observed for isolates assignable to *S. sanguis*, *S. oralis*, and *S. gordonii*, and one morph was represented in all three species. Colonies of our *S. mitis* and *S. parasanguis* reference strains also show morphological variability. We likewise noted multiple morphs for colonies with identical ribotypes. Similar observations have been made for *S. gordonii* Challis (33). Those findings indicate that it is very difficult to distinguish these species of oral streptococci by colony morphology, although it may be possible to generate crude counts that include multiple species (e.g., *S. sanguis*, *S. oralis*, and *S. gordonii*).

Phenotypic variation may also affect biochemical tests. Strains assignable to the same species by ribotyping were placed in different species by the API Rapid Strep system. The range of API system identifications was greater for *S. sanguis* and *S. oralis*, which may indicate that phenotypic variation is greater in those species. Rudney et al. (28) reported inconsistent API system identification of *S. oralis* reference strains classified by DNA hybridization. They also noted variable API system results for cultures of a clinical isolate showing identical DNA fingerprint patterns. That isolate was later assigned to *S. sanguis* by ribotyping (27). Other studies of oral streptococci have noted that strains placed in the same species by DNA hybridization may be assigned to multiple species by the API system (9, 30). The API system was devised before current reclassifications of oral streptococci (10). It is thus possible that reference strains placed together in the API system coding scheme may not have been members of the same species. In a comparative study of API and other commercial systems, Hinnebusch et al. (16) reported that supplemental phenotypic tests were often needed to identify oral viridans streptococci. This may indicate that some of the traits used in those systems are not expressed consistently in oral isolates.

Other traits may show less variability. Amylase binding and sialidase activity were largely restricted to *S. gordonii* and *S. oralis*, respectively. That result agrees well with results from other investigators (2, 3, 9, 19). Well-chosen phenotypic traits thus may identify oral streptococcal species, as proposed by Kilian et al. (18), Douglas et al. (9), Whiley et al. (34), and Beighton et al. (2). Phenotypic tests

are easier to apply than ribotyping, since cells can be evaluated directly after culture. Ribotyping requires the additional steps of DNA isolation in agarose beads, gel electrophoresis, and Southern blotting. Visual comparison of blots is probably adequate for species identification, but image analysis may be needed when many strains are to be compared with each other. The additional labor required for ribotyping is best justified when it is important to identify strains that do not fit standard phenotypic profiles or strains that vary in their responses to phenotypic tests. Ribotyping may also be a method of choice in studies of epidemiology and transmission, since it allows detection of different strains of the same species.

Many subjects in this study harbored multiple strains of the same species. In most cases, the strains were distinct genotypically by ribotyping and phenotypically by colony morphology or biochemical tests. Phenotypic diversity could account for the presence of different clones. Each strain might play a distinct role in the ecology of supragingival plaque. Clones might also coexist within the same ecological niche as analogs to individual members of animal species. Rates of change in ribotype patterns are not known, and different strains might arise from a common ancestor within the lifetime of a host. However, the presence of *S. gordonii*, *S. sanguis*, and *S. oralis* strains with identical ribotypes in unrelated subjects suggests that some lineages are widely distributed. DNA fingerprinting studies of *Streptococcus mutans* also have shown the presence of multiple strains within subjects (6). Carriage of multiple strains was more common in adults, although plasmid profiles differed between subjects of European and African heritage (4, 5). Independently isolated *S. mutans* strains likewise have hybridized identically to a probe derived from a mobile genetic element (21). Those findings and present findings suggest that complex factors govern the ecology and transmission of oral viridans streptococci. Further studies are needed to address these issues. Ribotyping can play an important role in that research, since it may allow genotypic identification of "*S. sanguis*" group streptococci by strain and by species.

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