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Clonal Diversity of *Streptococcus mitis* Biovar 1 Isolates from the Oral Cavity of Human Neonates

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The clonal diversity of 101 isolates of the pioneer bacterium *Streptococcus mitis* biovar 1 obtained from the oral cavities of 40 human neonates 1 to 3 days, 2 weeks, and 1 month postpartum was examined by using rRNA gene restriction patterns. There was a high degree of genetic diversity, with the 101 isolates comprising 93 unique *Pvu*II ribotypes. There were eight identical pairs of ribotype patterns, and seven of the eight pairs were obtained from individual neonates. Only one identical pair comprised isolates obtained from different neonates. In all but two cases, isolates with matching ribotypes were obtained at one visit. Two pairs of isolates with matching ribotype patterns were obtained from neonates on successive visits. The ribotype patterns of the isolates were examined by cluster analysis. The isolates forming each cluster were very similar, yet each cluster was well separated from its neighbors. When several isolates were obtained from individual neonates at a particular visit, in some instances they were contained in a single cluster, whereas in other cases each isolate was contained in a separate cluster. Isolates obtained from individual neonates on successive visits tended to be contained in different clusters. This high degree of diversity, which has been observed in other mucosal commensal bacteria, may serve as a mechanism for avoiding immune elimination of these bacteria.

The epidemiology, diversity, and population structure of a number of pathogenic bacteria have been studied by several techniques that include serological analysis (9), analysis of whole-cell (6) and outer membrane protein (9) profiles, multilocus enzyme electrophoresis (36, 37, 43, 44), restriction endonuclease cleavage of chromosomal DNA (6, 51), and the detection of specific restriction fragment length polymorphisms (RFLPs) at specific loci or within rRNA operons (2, 4, 6, 24, 48, 49). These techniques have been extremely valuable in identifying strains responsible for nosocomial infections and their transmission and maintenance in patients and in studies of bacterial pathogenesis.

Studies of the genetic diversity and population structures among selected bacterial pathogens from different genera have indicated that they can be clonal in nature, although they may show diversity in individual characteristics (34, 45, 53). However, in pathogens and other bacteria recombination and other genetic events, particularly in panmictic situations, can result in more extensive diversity, and the organisms lose any clonal structure and their populations become nonclonal in nature. Even within the same phenotypic species, such as *Neisseria meningitidis*, clonal and nonclonal population structures may be detected (47). Maynard Smith et al. (34) have applied a measure of association statistic to a number of multilocus enzyme electrophoresis data sets and have shown that bacterial population structures can range from those that are effectively panmictic to those that are clonal at all levels. However, even in clonal populations, there is evidence that horizontal gene transfer, which can also be related to virulence, may occur in nature (16, 52). Also, specific clones may be associated with infections, also suggesting a relationship between clonal type and virulence (33, 34, 52, 54). Less is known of the population structures of truly autochthonous bacteria associated with humans. However, although it seems most likely that these autochthonous bacteria show extensive genetic diversity, in some cases relatively few clonal types may be associated with an individual host.

As a result of the increasing use of immunosuppressive agents for tissue and organ transplantation and immunosuppression resulting from infection with the human immunodeficiency virus, infections with opportunistic pathogens from among the resident microbiota of humans have assumed a new relevance. Appreciation of the genetic variation among such bacteria is important in defining any specific clones which may be associated with infections among compromised patients and the identification of any associated virulence factors. A significant factor in the establishment and survival of the resident microbiota is the immune response by the host and the consequent possibility of the immune elimination of species from the human oral cavity.

A longitudinal study of the relationships between oral colonization of infants by commensal bacteria and the development of the secretory immune response identified *Streptococcus mitis* biovar 1 as the principal pioneer bacterium colonizing the oral cavity of human neonates (38). This report describes the genetic diversity of these bacteria determined by their rRNA gene restriction patterns (ribotypes) (48). The extent of genetic diversity and the persistence of specific ribotypes in an individual may relate to the generation and diversity of the immunoglobulin A (IgA) antibodies of the secretory immune response.

MATERIALS AND METHODS

Study population. Forty healthy, full-term infants were enrolled in the study without regard to race or sex and were included in the study after obtaining

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signed, informed consent from their mothers. Details of the study population and the inclusion and exclusion criteria have been described previously (18).

Sample collection and processing. The mucosal surfaces of the cheeks, buccal sulci, edentulous ridges, tongue, and hard palate of each neonate were swabbed with a swab from a Vacutainer Anaerobic Specimen Collector (Becton Dickinson Microbiology Systems, Cockeysville, Md.) 1 to 3 days, 2 weeks, and 4 weeks postpartum.

Recovery and identification of pioneer viridans group streptococci. Streptococci were recovered and identified as previously described in detail (38). Briefly, the isolates were examined for hemolysis, Gram stained, catalase tested, and subjected to the following biochemical tests: fermentation of mannitol, sorbitol, raffinose, melibiose, trehalose, amygdalin, inulin, lactose, arbutin, *N*-acetylglucosamine, and glucose; hydrolysis of arginine and esculin; and production of hydrogen peroxide, neuraminidase, α-L-fucosidase, β-D-fucosidase, β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, α-glucosidase, β-glucosidase, α-arabinosidase, and IgA1 protease. In addition, isolates were tested for their ability to bind salivary amylase (15, 25). One hundred seven isolates were identified as *S. mitis* biovar 1. Chromosomal DNA was obtained from 101 isolates.

Preparation of DNA. Genomic DNA was extracted and purified from the *S. mitis* biovar 1 isolates by using the Puregene DNA isolation kit (Gentra Systems Inc., Minneapolis, Minn.). DNA concentration and purity were determined spectrophotometrically by measuring the A_{260} and A_{280} (HP 8425A diode array spectrophotometer; Hewlett-Packard Company, Roseville, Calif.).

Restriction endonuclease digestion and electrophoresis of streptococcal DNA. In order to identify restriction endonucleases that would yield patterns with a high level of diversity for ribotyping, genomic DNAs from several reference strains of *S. mitis* biovar 1 were digested with each of a panel of restriction endonucleases. *Pvu*II and *Sal*I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were selected because the ribotypes generated with either enzyme were unique for each reference strain and yielded between 2 and 13 bands that were suitable for analysis. Genomic DNA (≈3 μg) obtained from the *S. mitis* biovar 1 isolates obtained from infants 1 to 3 days, 2 weeks, and 4 weeks postpartum was digested with 10 U of *Pvu*II according to the manufacturer's instructions. To ensure that isolates with matching *Pvu*II ribotypes were identical, the *Sal*I digests of these isolates were also ribotyped. Only isolates with matching *Pvu*II and *Sal*I ribotypes were considered to represent the same clone.

Digested DNA was separated by horizontal agarose electrophoresis with 25.0-cm, 0.8% agarose gels for 19 h at 20 V in 1× TAE buffer (89 mM Tris base, 89 mM boric acid, 2.4 mM EDTA [pH 8.3]) containing 0.05 μg of ethidium bromide per ml. Digoxigenin-labeled *Hin*dIII-digested bacteriophage λ DNA (Boehringer Mannheim Biochemicals) was run on each gel to serve as a DNA size standard.

Synthesis of digoxigenin-labeled *Escherichia coli* rRNA cDNA probes. By using 16S and 23S rRNA from *E. coli* as a template, digoxigenin-labeled rRNA cDNA probes were synthesized by using the Genius 1 nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemicals) according to the manufacturer's instructions.

Southern blot hybridization. DNA was transferred to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, Ill.) as described previously (42) and fixed by baking at 80°C for 1.5 h. The membranes were hybridized with the digoxigenin-labeled probe at 42°C overnight. The hybridized probe was detected by incubation with anti-digoxigenin Fabγ fragments conjugated with alkaline phosphatase and development with Nitro Blue Tetrazolium and X-phosphate.

Analysis of ribotype patterns. The ribotype pattern generated from each streptococcal isolate and the *Hin*dIII-digested λ DNA standards were scanned with a laser densitometer (Ultrascan XL; Pharmacia Biotech Inc., Piscataway, N.J.). The resulting densitometric profiles were imported into a commercial software package (GRAMS/386; Galactic Industries Corp., Salem, N.H.). Standard curves were constructed by plotting the migration distances of the *Hin*dIII-digested λ DNA fragments against the logarithm of their size (in kilobases). Ribotype band migration distances were then converted to kilobases by interpolation into these standard curves.

Cluster analysis. The ribotype patterns were clustered hierarchically by using the Pearson product-moment correlation coefficient by the average linkage method (46) and SAS software (SAS/PC 6.08; SAS Institute Inc., Cary, N.C.).

RESULTS

The *S. mitis* biovar 1 *Pvu*II ribotype patterns contained between 2 and 13 hybridization bands ranging in size from 2 to 21 kb. If isolates displayed identical *Pvu*II ribotypes, then *Sal*I ribotypes were also obtained. Isolates showing matching *Pvu*II and *Sal*I ribotype patterns were considered to represent the same clone. Table 1 provides the distribution of the *S. mitis* biovar 1 isolates among the 40 neonates. The isolate number and number of the cluster containing each isolate is provided for each neonate. Ninety-three of the 101 *Pvu*II ribotype patterns from the isolates of *S. mitis* biovar 1 were unique (Fig. 1). It was considered that each individual ribotype pattern represented a distinct clone of the wild-type *S. mitis* biovar 1 isolates. Figure 2 depicts a dendrogram showing the result of cluster analysis of the *Pvu*II ribotype patterns. Ninety-eight percent of the total variance of the ribotype patterns could be explained by assigning the patterns to 12 clusters. There were eight identical pairs of ribotype patterns, and for seven of the eight pairs of patterns both isolates were obtained from individual neonates. Only one identical pair comprised isolates obtained from different neonates. In all but two cases isolates with matching ribotypes were obtained at one visit. Two pairs of isolates with matching ribotype patterns were obtained from neonates on successive visits. The isolates forming each cluster were very similar, yet each cluster was well separated from its neighbors. When several isolates were obtained from individual neonates at a particular visit, in some instances they were contained in a single cluster, whereas in other cases each isolate was contained in a separate cluster. Isolates obtained from individual neonates on successive visits tended to be contained in different clusters (Table 1).

DISCUSSION

S. mitis biovar 1 represents a numerically important pioneer streptococcus in the human oral cavity and pharynx (38) and remains a predominant species in these habitats throughout life (23). Commensal bacteria appear to exist, for the most part, in a state of dynamic equilibrium with the host (50). Despite the secretory immune system and nonimmune salivary host factors, commensal bacteria colonize and persist in the mouth. The mechanisms by which commensal oral bacteria avoid immune elimination remain obscure; however, a number of mechanisms are possible (12). Many oral microorganisms possess proteases that are capable of degrading various antibody isotypes. For example, oral bacteria such as *Streptococcus sanguis*, *Streptococcus oralis*, and a significant number of strains of *S. mitis* biovar 1 produce an IgA1 protease capable of cleaving IgA1 antibodies (13, 40). Secreted antigens may bind antibody away from the bacterial cell surface (29), or host salivary macromolecules may mask bacterial cell-surface antigens from immune recognition. Chronic exposure of the host to resident bacteria may give rise to the induction of low-avidity antibody (14). In addition, resident bacteria may undergo antigenic variation or drift (5) or clonal replacement (23).

The genetic diversity of the resident microbiota has received little attention in comparison with the attention given to the genetic diversity of extrinsic pathogens. However, it has been reported that there is more single-locus and multilocus genotypic diversity in nonpathogenic species than in pathogenic species (44). Zambon et al. (55) examined 124 isolates of the oral bacterium *Actinobacillus actinomycetemcomitans* from the United States, Korea, and Norway by RFLP analysis. The isolates showed little diversity, with only three RFLP patterns. Similar limited diversity was observed by Saarela et al. (41). In contrast to the limited diversity observed with *A. actinomycetemcomitans* in those studies, considerable genetic diversity has been observed in other investigations of *A. actinomycetemcomitans* (10, 20, 21, 39) and other commensal oral bacteria. The genetic structure of natural populations of the anaerobic, asaccharolytic, gram-negative coccobacillus *Porphyromonas gingivalis* has been examined by RFLP analysis, multilocus enzyme electrophoresis, and random amplified polymorphic DNA fingerprinting (11, 30–32, 35). Those studies demonstrated extensive genetic diversity among *P. gingivalis* isolates, with the majority of subjects harboring a single clonal type of *P. gingivalis*. A study of the transmission of *Prevotella melaninogenica* from mother to child revealed 101 distinct ribotypes among 248 isolates, with both mothers and children harboring

TABLE 1. Distribution of *S. mitis* biovar 1 isolates among the neonates

Neonate	Isolate no. (cluster no.) for isolates obtained at the following times postpartum[a]:		
	1–3 days	2 wk	1 mo
1		87 (7)	127 (12)
2			190 (6)
3	64 (9)		
4			214 (4), 216 (7), 217 (10)
5	79 (10)		
6			247 (10), 249 (5), 264 (7)
7			282 (10), 283 (10)
8			339 (10), 340 (11)
9			373 (8)
10	**393** (10), **401** (10)	445 (5)	515 (7), 533 (12)
11		479 (8)	
12	483 (11)	552 (9), 554 (7)	629 (10)
13		592 (4), 602 (8), 604 (7)	647 (4)
14	506 (10), 514 (7)	622 (10)	
15	544 (7), **556** (9), 558 (10)	**616** (9)	696 (8)
16	651 (10)	**752** (10), **761** (10)	
17	Not enrolled	823 (6)	
18	Not enrolled	827 (9)	859 (9), 943 (6)
19		1015 (5)	1233 (2), 1278 (3)
20	868 (8)		**1347** (7), 1376 (7), 1379 (7)
21	857 (10)		1288 (4)
22	Not enrolled	938 (2), 962 (8)	
23		1067 (10), 1071 (1)	1183 (11)
24	940 (7), 958 (10), 960 (6), 964 (10)	1073 (11)	
25	**1196** (10)	1374 (7)	**1583** (6), **1609** (6)
26A	Not enrolled	1512 (10), 1522 (3)	
26B	Not enrolled	1622 (6)	
27		1429 (10)	1640 (6), 1657 (11)
28	Not enrolled	1421 (3), 1425 (6)	
29	1339 (6)	1495 (9), 1496 (7)	1596 (7), 1615 (10), 1616 (10), 1617 (10)
30	**1414** (4), **1417** (4)		1674 (10), 1682 (10)
31	Not enrolled		1637 (12)
32	Not enrolled		1752 (2)
33			2003 (4)
34	Not enrolled	1827 (7)	
35	Not enrolled	1954 (9)	
36	Not enrolled	2074 (10)	
37		2070 (9)	
38	Not enrolled	**2094** (10), 2122 (9)	**2403** (10)
39	Not enrolled	2405 (7), 2366 (7)	2505 (5), 2532 (10)

[a] Isolates with identical ribotypes are indicated in boldface type.

up to 7 ribotypes (26). Similarly, isolates of *Streptococcus mutans* obtained from more than 30 individuals demonstrated unique RFLP profiles (7). Mothers and their infants harbored two to three strains. Kulkarni et al. (28) examined 396 strains of mutans group streptococci from a small group of families. Restriction endonuclease patterns were unique between each subject. Identical patterns were seen only within subjects and families. Up to five restriction endonuclease types were seen in many subjects. There was evidence of intrafamily transmission, but also sources of infection outside of the family. Interestingly, a pair of identical twins each harbored unique strains. Hohwy and Kilian (23) analyzed the restriction enzyme patterns of 106 isolates of *S. mitis* biovar 1 isolated from three members of one family. Their study revealed considerable genetic diversity. Twenty-four genotypes were detected, with each individual carrying 6 to 13 types. There was limited sharing of genotypes between family members and between the pharynx and buccal mucosa of each individual. The results of our study of the genetic diversity of *S. mitis* biovar 1 in 40 human neonates are consistent with those of Hohwy and Kilian (23). The 101 *S. mitis* biovar 1 isolates comprised 93 genotypes. Because some infants harbored several *S. mitis* biovar 1 clones and only a single between-infant match was observed, it was concluded that colonization of the neonates by one or more dominant clonotypes did not occur. In concert with the findings of Kulkarni et al. (28) for *S. mutans*, we also found that a set of twins (neonates 26BA and 26BB) in our study harbored unique clonotypes of *S. mitis* biovar 1. It is possible that the extensive genetic diversity observed among *S. mitis* biovar 1 isolates reflects the turnover of clones as this pioneer species becomes established in the oral cavity of the neonate.

Bacteria that constitute the normal microbiota are considered to be constant components of the microbial community in a particular habitat (17). Caugant et al. (8) studied the *E. coli* population in the feces of a single human over a period of 11 months using multilocus electrophoresis. Five hundred fifty clones were assigned to 53 distinct electrophoretic types. Although two electrophoretic types were isolated many times over an extended period, one of which represented 46% of the clones, most electrophoretic types appeared on only 1 day or a few days and were considered transients. The investigators concluded that most of the genetic diversity in this species

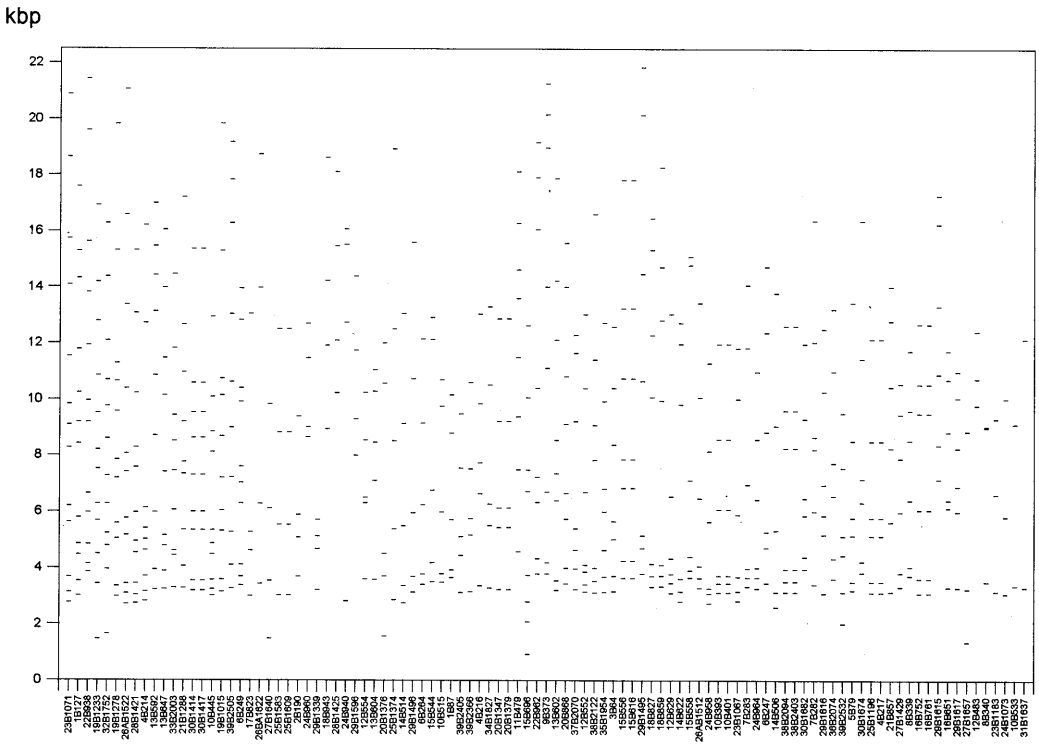

FIG. 1. Schematic of ribotype patterns of 101 isolates of *S. mitis* biovar 1 obtained from the oral cavities of 40 neonates 1 to 3 days, 2 weeks, and 1 month postpartum. Genomic DNA was digested with *Pvu*II and separated by electrophoresis. Digested DNA was transferred to nylon membranes and was probed with digoxigenin-labeled *E. coli* 16S and 23S rRNA cDNA. Hybridized bands were detected with antidigoxigenin Fabγ fragments conjugated with alkaline phosphatase and developed with Nitro Blue Tetrazolium and X-phosphate. The number before the letter B (baby) refers to the code number for each neonate. The number after the letter B refers to the isolate number. The set of twins are neonates 26A and 26B. The ribotype patterns of the isolates are arranged in the same order listed in the dendrogram (Fig. 2).

resulted from repeated infection from environmental sources. However, this conclusion assumes that the composition of the fecal flora reflects that of the resident microbiota of the large intestine, an assumption that may not be warranted (17). Furthermore, the assertion that the genetic diversity of *E. coli* isolates results from repeated infection from environmental sources is inconsistent with the stability of climax communities of indigenous bacteria and the difficulty in introducing bacteria into the normal microbiota of experimental animals or humans (19). In addition, accumulating data suggest that clones of some species of resident bacteria may be stable over time (1, 3, 7, 26, 27, 30).

It is likely that the different methods used to determine genetic diversity vary in their discriminatory potentials. RFLP analysis is probably the most sensitive discriminator, particularly if a difference of a single band is taken as evidence of diversity (23). Depending on the choice of endonuclease, ribotyping may be less discriminating, although more stable, than RFLP analysis (22). However, the endonucleases used in our study were selected on the basis of preliminary experiments for their ability to reveal a high level of diversity within *S. mitis* biovar 1 isolates. Nevertheless, our ribotype clusters could represent groups of organisms that may show greater diversity by other methods.

If ribotypes are more stable than RFLP types, our results could suggest that each cluster collects together strains which are less influenced by recombination and other events, which can result in differences in phenotypic characters and RFLP patterns. Unfortunately, we did not compare ribotyping with other molecular typing methods for these strains.

If we assume that the cluster analysis grouped ribotypes of *S. mitis* biovar 1 without undue influence of minor genomic changes, then the data support the concept that the strains are transient and the species niche in the habitat is maintained by consistent clonal replacement. Elimination of clones may be related to the development of the salivary immune response in the infant, in which a given ribotype may exhibit antigenic diversity that allows it to survive for a given period of time. Indeed, Hohwy and Kilian (23) reported remarkable diversity in carbohydrate and protein antigens between the 24 genotypes of *S. mitis* biovar 1 in their study. It may be that each ribotype cluster contains strains with similar antigenic structures. Therefore, it might be expected that a sequence of organisms from different clusters would be found colonizing an infant, and indeed, this was observed in the present study. Cluster analysis of the *S. mitis* biovar 1 isolates revealed clusters of closely related strains, with each cluster being well separated. The identification of *S. mitis* biovar 1 is based largely on negative biochemical tests (23, 38). Therefore, it is possible that the phenotypic characters for *S. mitis* biovar 1 do not identify a well-defined species, that *S. mitis*, as presently defined, represents a diverse group of organisms, and that this diversity may be identified through genetic and antigenic differences.

In conclusion, the data from this and other studies indicate that species of commensal bacteria are extremely diverse. Whether clones of resident bacteria are stable over time or exhibit clonal exchange and whether the diversity of pioneer species is influenced by the development of the climax community remain to be determined. The isolates of *S. mitis* biovar 1 collected during the course of our longitudinal study may help to answer these questions.

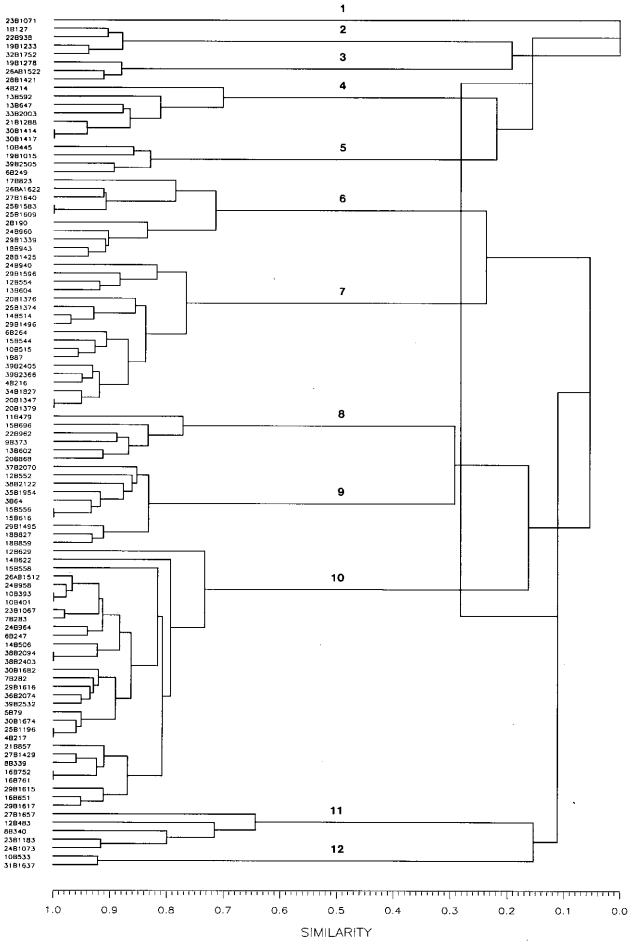

FIG. 2. Dendrogram showing the relatedness of *Pvu*II ribotype patterns of 101 isolates of *S. mitis* biovar 1 obtained from the oral cavities of 38 neonates 1 to 3 days, 2 weeks, and 1 month postpartum. The number before the letter B (baby) refers to the code number for each neonate. The number after the letter B refers to the isolate number. The set of twins are neonates 26A and 26B. Clusters are assigned the numbers 1 to 12.

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