Intraspecies Variations in Nutritionally Variant Streptococci: rRNA Gene Restriction Patterns of Streptococcus defectivus and Streptococcus adjacens

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The rRNA gene restriction patterns of two species of nutritionally variant streptococci, Streptococcus defectivus and Streptococcus adjacens, were determined, and the results were compared with the electrophoretic migration profiles of penicillin-binding proteins. Reference strains belonging to various streptococcal species were used as controls. Our results correlated with the results of DNA-DNA hybridization experiments and confirmed the delineation of these two species. Moreover, they demonstrated that intraspecies variations occur and suggested that there are two subspecies of S. defectivus.

The nutritionally variant streptococci (NVS) are fastidious bacteria which were first recognized by their growth as satellite colonies around other microorganisms (8). These organisms have also been shown to grow in complex media supplemented with l-cysteine or pyridoxal (6, 8), and balanced growth has also been obtained in a semisynthetic medium (3). Two species of NVS, Streptococcus defectivus and Streptococcus adjacens, which can be recognized by their phenotypic characteristics (4), have been delineated recently on the basis of DNA relatedness data (1).

The major interest in these bacteria results from their pathogenicity for humans. Indeed, they are responsible for 5% of human bacterial endocarditis cases, including most of the so-called blood culture-negative endocarditis cases (for reviews see references 2 and 17), which are often severe forms of the disease. In addition, NVS have been isolated from the throats and urogenital and intestinal floras of normal subjects (9, 15, 17).

Hence, it seems important to analyze the possible intraspecies variations of these pathogens in order to further investigate their sites of entrance and develop a prophylactic treatment for endocarditis. In this study we examined the rRNA gene restriction patterns of representative strains and demonstrated such variations in the two NVS species mentioned above.

MATERIALS AND METHODS

Bacterial strains. Five strains of S. defectivus and seven strains of S. adjacens, including type strains SC10 (= ATCC 49176) and GaD (= ATCC 49175), were studied. These strains were isolated from clinical specimens taken from 12 patients suffering either from bacterial endocarditis (blood cultures, 8 cases; cardiac vegetations, 1 case) or from bacteremia (1 case), urinary tract infection (1 case), or pharyngitis (1 case). All of the strains have been investigated previously by using DNA-DNA hybridization techniques (1), and their penicillin-binding protein patterns have been determined (4, 12). The following reference strains, which represented 20 other Streptococcus species, were also examined as controls: S. alactolyticus CIP 103.244T (T = type strain), S. agalactiae CCUG 4208T, S. bovis CIP 102.302T, S. canis CIP 103.223T, S. dysgalactiae CIP 102.914T, S. downei CIP 103.222T, S. equi CIP 102.910T, S. equinus CIP 102.504T, S. ferus AC 851 (= ATCC 33477T), S. gordoni CCUG 18374, S. iniae CIP 102.508T, S. milleri ATCC 12395, AC UC7895, and AC MGH611, S. mitis CDC SS-429, S. oralis CIP 102.922T, AC 9811, AC 0551, and API 063.07.78, S. pneumoniae CIP 102.911T, S. pyogenes CIP 56.41T, S. rattus AC FA1T (= ATCC 19645T), S. sanguis ATCC 10556T, S. suis CIP 103.217T, and S. uberis CCUG 17930T. These reference strains were received from following sources: the collection of Alan Coykendall (AC), University of Connecticut, Farmington, Conn.; API System (API), La Balme-les-Grottes, France; American Type Culture Collection (ATCC), Rockville, Md.; Culture Collection, University of Göteborg (CCUG), Göteborg, Sweden; Richard R. Facklam, Centers of Disease Control (CDC), Atlanta, Ga.; and Collection de l'Institut Pasteur (CIP), Paris, France.

DNA preparation. The strains were grown in Todd-Hewitt broth supplemented with 10 mg of pyridoxal hydrochloride (Sigma Chemical Co., St. Louis, Mo.) per liter (6). DNA from bacteria treated with mutanolysin and lysozyme (12) was extracted and purified by using the method of Brenner (5).

Gel electrophoresis of endonuclease-cleaved DNA. Purified DNA samples (5 μg) were cleaved with restriction endonucleases HindIII and PstI according to the instructions of the manufacturer (Appligene, Strasbourg, France). DNA was treated twice for 2 h at 37°C with various concentrations of HindIII (3 U/mg of DNA for strains SC10T, J14, CHO4, PE7, DA5, GaD7, J18, G40, and C28, 8 U/mg for strains LAM22 and C50, and 20 U/mg for strain L61). Alternatively, DNA was treated twice for 2 h at 37°C with PstI (6 U/mg for strains C50 and L61, 7.5 U/mg for strains GaD7 and G40, or 12 U/mg for strain LAM22). DNA fragments were separated by performing horizontal electrophoresis for 16 h at 50 V in a 0.8% (wt/vol) agarose gel (Sigma) with a Tris-acetate buffer (0.04 M Tris-acetate, 0.02 M EDTA; pH 8.1). The size markers used were either Raoul I (Appligene) or rRNA gene restriction fragments of Xenorhabdus sp. strain 278 obtained after cleavage with EcoRI; the latter markers were directly visualized with the same probe as streptococcal rRNA genes. The DNA fragments were blotted onto a Hybond-N nylon membrane (Amersham International, Amersham, En-
Radioactive labeling and hybridization. Ribosomal 16 + 23S RNA from Escherichia coli (Boehringer, Mannheim, Germany) was end labeled by using [γ-32P]ATP (Amersham) and T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.), following the exchange reaction procedure and the instructions provided by the manufacturer.

Hybridization with the 32P-labeled E. coli probe rRNA was performed as previously described (10), with the following modifications. Hybridizations were done at 60°C, and the membranes were washed five times at the same temperature for 15 min in 2× SSC-0.1% sodium dodecyl sulfate (1× SSC is 0.015 M trisodium citrate plus 0.15 M NaCl, pH 7.0) and then at 20°C for 30 min in 0.1× SSC-0.1% sodium dodecyl sulfate.

DNA from pBR322, which was used for revealing the DNA fragments of the Raoul I size marker by hybridization, was labeled by using [α-32P]dCTP and a nick translation kit (Amersham), following the instructions of the manufacturer.

Membranes were autoradiographed by using Hyperfilm MP film (Amersham) and an intensifying screen at −80°C for 7 days.

DNA fragment size determination. The algorithm of Schaffer and Sederoff (18) was used to derive an experimental function relating molecular size to electrophoretic migration distance and to interpolate the sizes of new fragments. DNA fragment size marker Raoul I and DNA fragments of Xenorhabdus sp. strain 278 were used to calibrate this function.

RESULTS

After HindIII cleavage of DNA, two closely related patterns were observed for the five strains of S. defectivus. Pattern 1 had four fragments (8.6, 4.6, 3.8, and 1.5 kbp) whereas pattern 2 differed by having a 6.1-kbp fragment instead of the 8.6-kbp fragment (Fig. 1 and 2). The seven strains of S. adjacens produced a clearly different pattern with three fragments (3.3, 3.1, and 1.2 kbp) (Fig. 1 and 2). None of these patterns was observed when we examined reference strains belonging to 20 other streptococcal species (Fig. 1).

More fragments were obtained with PstI endonuclease digestion than with HindIII digestion, and both species were found to be heterogeneous (Fig. 3 and 4). In S. defectivus, four of seven cleavage bands (15.9, 11.1, 8.3, and 3.2 kbp) were shared by strains CH04 and PE7; the sizes of the three other bands were clearly different (13.8, 12.8, and 5.2 kbp for strain PE7 and 16.1, 6.8, and 2.7 kbp for strain CH04) (Fig. 4). Similar intraspecies variations were observed in S. adjacens both in size and in the number of the fragments which ranged in size from 12.1 to 15.2 kbp. The band patterns of smaller fragments (size range, 1.2 to 11.4 kbp) were more homogeneous, since all strains shared six bands (11.4, 7.3, 5.1, 3.3, 1.4, and 1.2 kbp). We also observed the following additional fragments: an 8.1-kbp fragment in strains LAM22, C50, and G40, a 6.2-kbp fragment in strains GaD7 and L61, and a 5.9-kbp fragment in strains LAM22 and L61. Strains G40 and C50 exhibited similar patterns (Fig. 3 and 4).

In an attempt to further characterize this interstrain variation, we examined the previously described penicillin-binding protein patterns (4). We found that in S. defectivus,
strains SC10, J14, and CHO4 exhibited minor, but significant, variations in penicillin-binding protein patterns, compared with strains PE7 and DA5. The latter group had a 185-kDa band instead of a 188-kDa band. All strains produced four lower-molecular-mass bands, although there were minor interstrain variations (data not shown). We also observed minor variations in the penicillin-binding protein patterns of *S. adjacens* strains. Interestingly, strains G40 and C50 shared the same pattern.

**DISCUSSION**

In order to further characterize NVS strains for the study of their routes of entry into endocarditis patients, we investigated representative strains of *S. defectivus* and *S. adjacens* isolated from various human specimens. We confirmed the distinction between these two species and found both intraspecies and individual variations.

Since rRNAs are conserved ubiquitous molecules in bacteria, electrophoretic analysis of rRNA gene restriction fragments has become a useful tool for accurate identification of bacterial species (10, 11, 14, 16). One application of this technique is involved in phylogenetic analyses (10), and another application deals with molecular typing of bacterial pathogens during epidemiological studies (13, 19, 20).

Using HindIII cleavage, we observed clear-cut intraspecies variation within *S. defectivus*, leading us to subdivide this species into two genomic groups (Fig. 2). In contrast, *S. adjacens* was found to be homogeneous after cleavage with HindIII (Fig. 1 and 2). When PstI was used, the genomic subdivision of *S. defectivus* was confirmed, and the use of this enzyme also revealed interstrain variations within *S. adjacens* (Fig. 4) that allowed individual recognition of most strains.

An examination of our previous data on the penicillin-binding protein patterns of NVS showed that *S. defectivus*...
strains also exhibit slight variations in the apparent molecular mass of one band. These variations correlated with the rRNA genomic subdivision. Similarly, interstrain variations in *S. adjacens* patterns after cleavage with *PstI* are in agreement with the low level of individual heterogeneity of the two doublets observed by penicillin-binding protein analysis.

The NVS were first described as satellite streptococci by Frenkel and Hirsch (8). Initially, these bacteria were considered variants of other viridans streptococci (6, 7, 17); then they were recognized as specific entities (4, 21), and finally they were described as two different species (1). In this paper we show that both *S. defectivus* and *S. adjacens* exhibit interstrain variations and that *S. defectivus* includes two related genomic groups, which may represent two subspecies. These findings lead to the further use of genomic analysis both in etiopathogeny and in epidemiological investigations of the severe forms of human endocarditis caused by streptococci.

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REFERENCES


