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Restriction Fragment Length Polymorphism Analysis of 16S Ribosomal DNA of *Streptococcus* and *Enterococcus* Species of Bovine Origin

B. M. JAYARAO,[1] J. J. E. DORÉ, JR.,[2] AND S. P. OLIVER[1]*

Department of Animal Science[1] and Department of Pathobiology,[2] College of Veterinary Medicine, Institute of Agriculture, University of Tennessee, Knoxville, Tennessee 37901-1071

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Twelve bacterial species including *Streptococcus uberis*, *S. parauberis*, *S. agalactiae*, *S. dysgalactiae*, *S. bovis*, *S. mitis*, *S. salivarius*, *S. saccharolyticus*, *Enterococcus faecium*, *E. faecalis*, *E. avium*, and *Aerococcus viridans* were examined for their 16S ribosomal DNA fingerprint patterns. Oligonucleotide primers complementary to 16S rRNA genes were used to amplify by the polymerase chain reaction 16S ribosomal gene fragments from genomic DNAs. The molecular sizes of the amplified 16S ribosomal DNA (rDNA) fragments from the 12 species examined ranged from 1,400 to 1,500 bp. Restriction fragment length polymorphism analysis of 16S rDNA was performed with 11 different restriction endonucleases. All 12 species examined could be differentiated on the basis of characteristic 16S rDNA fingerprint patterns by using the restriction endonucleases *Hha*I, *Rsa*I, and *Msp*I. A scheme for the differentiation of the 12 species is presented. Eleven isolates representing 11 species were obtained from cows with intramammary infections and were examined by 16S rDNA fingerprinting. All 11 species isolated from cows were differentiated by using *Hha*I, *Rsa*I, and *Msp*I restriction endonucleases. The results of this study demonstrate the potential application of 16S rDNA fingerprinting for the identification and differentiation of bacterial species.

Bovine mastitis is a widespread disease that affects dairy cows (5, 17, 19, 20, 26). The predominant species involved in bovine mastitis are *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Streptococcus agalactiae*. Other species encountered less frequently include *Streptococcus equinus*, *Streptococcus mitis*, *Streptococcus salivarius*, *Streptococcus saccharolyticus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus avium*, and *Aerococcus viridans* (27–29).

In most laboratories, identification of bacterial species is based on analysis of phenotypic characteristics that are determined by using biochemical tests, serotyping, and enzymatic profiles. Diagnostic laboratories involved in the isolation and identification of pathogens that cause mastitis have frequently reported the inability to identify 1 to 10% of isolates belonging to the genera *Streptococcus* and *Enterococcus* because of atypical biochemical and enzyme profile test results (15, 17, 21, 28, 29). Similar observations have been reported with *Streptococcus* and *Enterococcus* species isolated from human sources (1, 2, 8, 9, 18).

DNA-DNA hybridization and sequence analysis of 16S rRNA have resulted in restructuring of the family *Streptococcaceae*. The enterococci now belong to the genus *Enterococcus* (7, 25). Sequence comparisons of 16S rRNA of *S. uberis* type I and II strains resulted in reclassification of *S. uberis* type II as a separate species, *S. parauberis* (30). *S. equisimilis* and group L and human group G streptococci are now within a single species, *S. dysgalactiae* (10). The group D nonenterococcal organisms, *S. equinus* and *S. bovis* now reside within a single species, *S. equinus* (11). On the basis of 16S rRNA sequence analysis, Rodrigues and Collins (23) suggested that *S. saccharolyticus* should be transferred to the genus *Enterococcus*.

* Corresponding author.

Recent developments in nucleic acid technology such as the polymerase chain reaction (PCR) (24) and analysis of 16S rRNA (31) have resulted in new methods that can be used for genotypic analysis of bacteria. Comparative analysis of 16S rRNAs has shown that highly conserved sequences are interspersed with regions of variable sequences. Analysis of variable portions permits the determination of phylogenetic and evolutionary relationships (31). Conserved or variable regions of 16S rRNA sequences have been used as targets for primer-directed DNA amplification by PCR for the identification of microorganisms (4, 12–14).

In this study, PCR-amplified 16S ribosomal DNAs (rDNAs) of 12 bacterial species were examined for their restriction fragment length polymorphisms (RFLPs). RFLP was used to evaluate the potential application of 16S rDNA fingerprinting for species identification of members of the genera *Streptococcus* and *Enterococcus* associated with bovine mastitis.

MATERIALS AND METHODS

Bacteria. Twelve American Type Culture Collection (ATCC; Rockville, Md.) strains, including *S. uberis* ATCC 27958, *S. parauberis* ATCC 13386, *S. agalactiae* ATCC 27956, *S. dysgalactiae* ATCC 27957, *S. mitis* ATCC 9811, *S. salivarius* ATCC 13419, *S. equinus* ATCC 27960, *S. saccharolyticus* ATCC 43076, *E. faecium* ATCC 35667, *E. faecalis* ATCC 27959, *E. avium* ATCC 14025, and *A. viridans* ATCC 11563, were used in this study. In addition, 11 species, including *S. uberis*, *S. agalactiae*, *S. dysgalactiae*, *S. mitis*, *S. salivarius*, *S. equinus*, *S. saccharolyticus*, *E. faecium*, *E. faecalis*, *E. avium*, and *A. viridans*, isolated from bovine mammary secretions were also examined. All ATCC strains and isolates from cows (Table 1) were identified to the species level by the Gram-Positive Identification System (Vitek Systems Inc., Hazelwood, Mo.), API Rapid Strep

TABLE 1. Identification profiles of *Streptococcus* and *Enterococcus* species isolated from bovine mammary secretions used for 16S rDNA fingerprinting

Organism[a]	Serogroup[b]	% Correct identification or organism identified by Vitek[c]	API result[d]	Atypical profile[e]
S. uberis	Untypeable	99	EX	Raf^+ β-gur^-
S. agalactiae	B	42	AC	Arg^- Rib^-
S. dysgalactiae	G	Unidentified	VG	Arg^- Rib^-
S. mitis	C	Unidentified	LD	Hip^+
S. salivarius	D	91	AC	
S. equinus	D	*E. faecium*	LD	Arg^- Rib^-
S. saccharolyticus	D	Unidentified	LD	Esc^+
E. faecium	D	*E. durans*	LD	Raf^- Ara^-
E. faecalis	D	70	VG	
E. avium	D	86	VG	Hip^+
A. viridans	Untypeable	72	AC	Ara^+

[a] Identified by conventional biochemical tests.
[b] Lancefield's serotype; untypeable, negative for groups A, B, C, D, F, and G.
[c] Vitek Gram-Positive Identification System.
[d] API Rapid Strep Identification system. EX, excellent; VG, very good; AC, acceptable; LD, low discrimination.
[e] Atypical profile, test reactions that were identified by the Vitek GPI or API Rapid Strep system that varied from conventional biochemical test results. Esc, esculin; Arg, arginine; Rib, ribose; Raf, raffinose; Hip, hippurate; Ara, arabinose; β-Gur, β-glucuronidase; +, positive; −, negative.

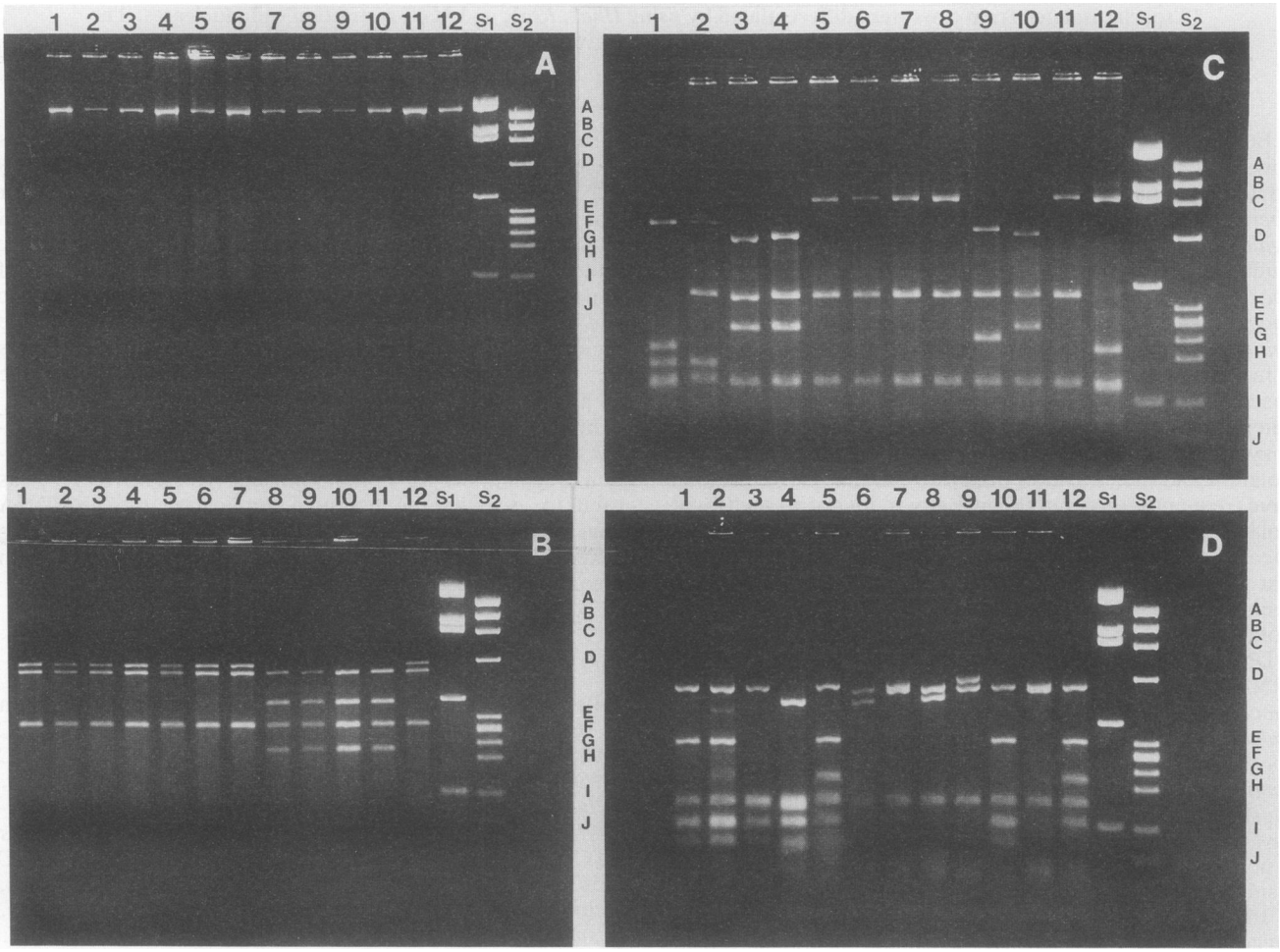

FIG. 1. The 16S rDNA fingerprint patterns of 12 species belonging to the family *Streptococcaceae*. Lanes 1 through 12, *S. uberis*, *S. parauberis*, *S. agalactiae*, *S. dysgalactiae*, *S. mitis*, *S. salivarius*, *S. equinus*, *S. saccharolyticus*, *E. faecium*, *E. faecalis*, *E. avium*, and *A. viridans*, respectively; lane S_1, *Bst*NI-digested pBR322 DNA; lane S_2, *Hae*III-digested ϕX174 DNA; A to J, restriction fragment size markers of 1,353, 1,078, 872, 603, 310, 281 and 271, 234, 194, 118, and 72 bp, respectively. (A) Undigested 16S rDNA; (B) 16S rDNA digested with *Hha*I; (C) 16S rDNA digested with *Rsa*I; (D) 16S rDNA digested with *Msp*I.

System (Analytab Inc., Plainview, N.Y.), and conventional biochemical tests and were serotyped as described previously (15). In this study, *S. bovis* ATCC 27960 was designated *S. equinus* (11), and *S. uberis* type II was designated *S. parauberis* (30).

Isolation of DNA. The chromosomal DNAs of the 12 organisms were isolated by the method described by Jayarao et al. (14).

DNA primers and PCR amplification. The oligonucleotide primers used in this study were synthesized by the Analytical Services Laboratory, University of Tennessee, Knoxville. Sequences corresponded to those within the 16S rRNA gene of *S. uberis* (30). The 5′ primer 5′-CCAAGCTTGCT CAGGACGAACGCT-3′ corresponded to nucleotides 20 to 35, with a *Hin*dIII restriction site (underlined) incorporated at the 5′ end. The 3′ primer 5′-CGGGATCCCGCCCGG GAACGTATTCAC-3′ was similar to the broad-range primer p13B described by Chen et al. (6) and corresponded to nucleotides 1374 through 1392 of 16S rRNA (30), with a *Bam*HI restriction site (underlined) incorporated at the 5′ end. Both primers had a theoretical melting temperature of 58°C. PCR amplification was performed as described previously (14). Briefly, PCR was done in a total volume of 100 μl containing 20 ng of the bacterial DNA preparation, 0.5 μM each primer, 100 μM each deoxynucleoside triphosphate (Boehringer Mannheim, Indianapolis, Ind.), 10 μl of 10× *Taq* buffer, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.). Each reaction mixture was overlaid with 50 μl of mineral oil, and the reaction was run for 35 cycles in a GTC Thermocycler (Precision Scientific Inc., Chicago, Ill.). One cycle consisted of 90 s at 93°C, 90 s at 56°C, and 90 s at 75°C. On completion, each sample was extracted with chloroform, precipitated with 2 volumes of ethanol, and resuspended in 50 μl of distilled water.

Enzymatic digestion of amplified DNA. DNA (5 μl) was digested for 2 h at 37°C in 20-μl volumes with *Ava*I, *Ava*II, *Dde*I, *Hha*I, *Mbo*II, *Msp*I, *Mva*I, *Pst*I, *Rsa*I, *Sca*I, and *Scr*FI (New England Biolabs, Beverly, Mass.). Determination of suitable restriction endonucleases for RFLP analysis was done by restriction analysis of *S. uberis*, *S. parauberis* (30), and *S. saccharolyticus* (23) 16S rRNA sequences with IBI/Pustell sequence analysis programs (22). Digestion of amplified 16S rDNA was repeated to establish reproducibility.

Agarose gel electrophoresis and RFLP analysis. Restriction endonuclease-digested 16S rDNA amplification products were electrophoresed in 2% agarose by using Tris-borate-EDTA buffer (0.09 M Tris base, 0.09 M sodium borate, 2.5 mM EDTA [pH 8.3]) in gels (20 by 20 cm) at 100 V for 4 h. The gels were stained with ethidium bromide (1.0 μg/ml), and DNA was visualized by UV transillumination (Fotodyne Inc., New Berlin, Wis.) and photographed with type 55 Polaroid film. *Bst*NI-digested pBR322 DNA and *Hae*III-digested φX174 DNA (both from New England Biolabs) were used as molecular weight markers. The negative of the Polaroid film was scanned with a computer-integrated laser densitometer (Ultroscan XL; LKB Produkter AB, Bromma, Sweden). Scans were evaluated with the Gelscan XL version 2.0 software package (Pharmacia LKB Biotechnology, Uppsala, Sweden) to determine the numbers and sizes of DNA fragments.

RESULTS

Amplification of 16S rDNA by PCR. The sizes of the amplified 16S rDNA products ranged from 1,400 to 1,500 bp (Fig. 1) relative to the DNA size markers, as determined by scanning laser densitometric evaluation.

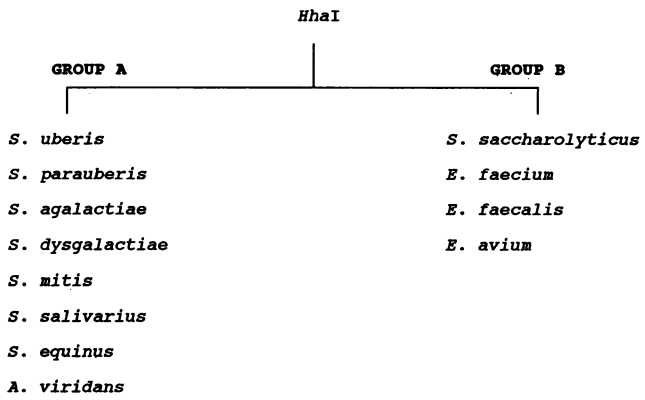

FIG. 2. Differentiation of eight *Streptococcus*, three *Enterococcus*, and one *Aerococcus* species into two groups, designated A and B, based on 16S rDNA fingerprinting with restriction endonuclease *Hha*I.

RFLP analysis. The amplified 16S rDNAs were digested with 11 different restriction endonucleases. With the combination of three restriction endonucleases (*Hha*I, *Rsa*I, and *Msp*I), the 12 organisms could be differentiated on the basis of unique RFLPs. Digestion of 16S rDNA fragments with *Hha*I allowed separation of organisms into two distinct groups, designated A and B (Fig. 2 and 3), with each group having a characteristic 16S rDNA RFLP pattern. In group A, *S. uberis*, *S. parauberis*, *S. agalactiae*, *S. dysgalactiae*, *S. mitis*, *S. salivarius*, *S. equinus*, and *A. viridans* had similar

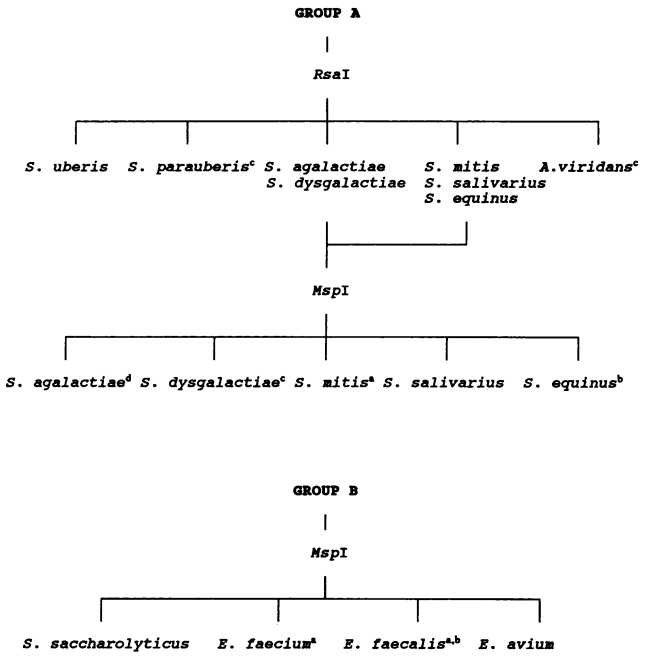

FIG. 3. Differentiation scheme for organisms in groups A and B. Organisms in group A were differentiated with restriction endonucleases *Rsa*I and *Msp*I. Organisms in group B were differentiated with the restriction endonuclease *Msp*I. Confirmation of organisms identified in group A or B was done with *Scr*FI (a), *Dde*I (b), *Ava*II (c), and *Mbo*II (d).

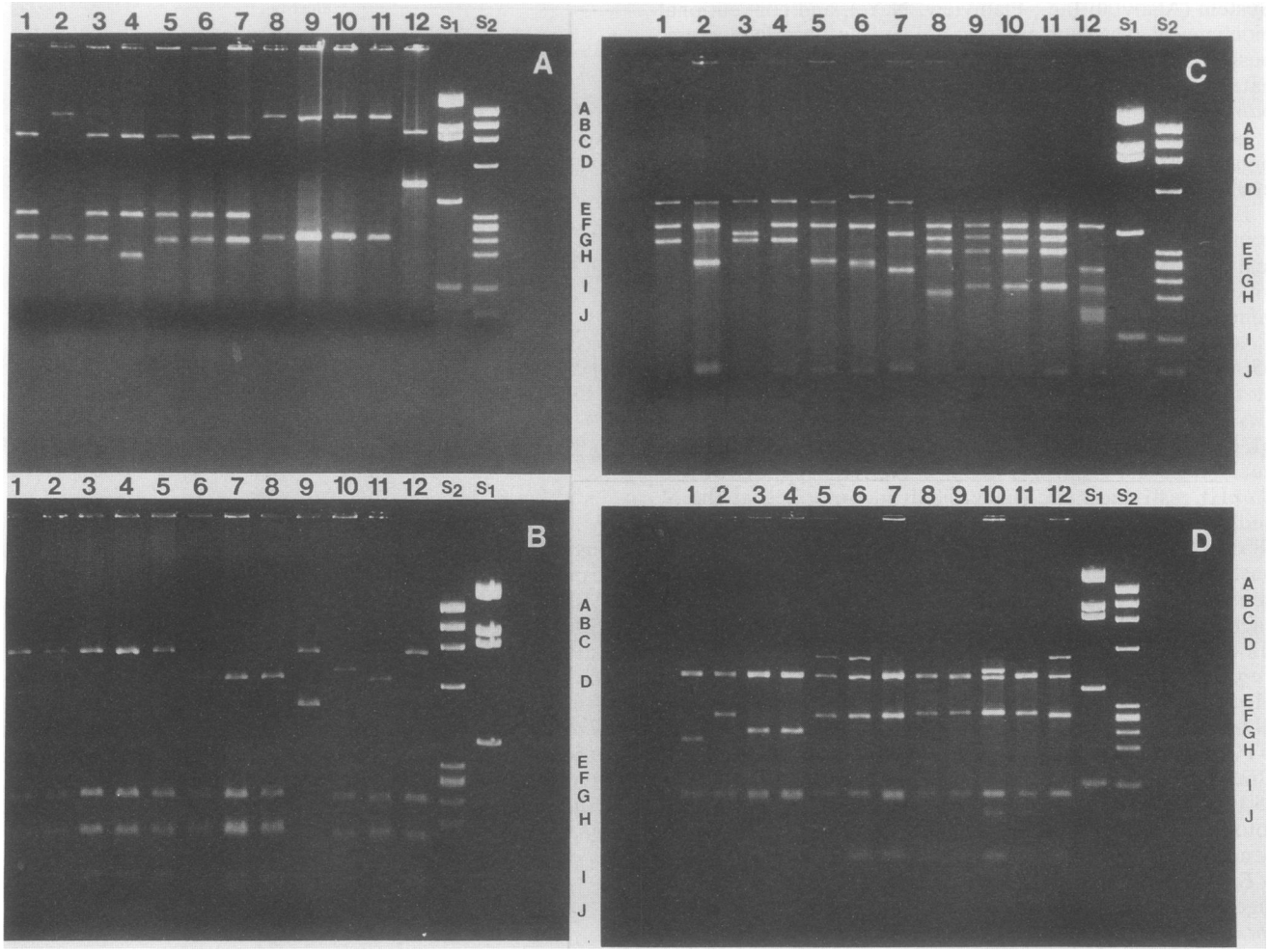

FIG. 4. 16S rDNA fingerprint patterns of 12 species belonging to the family *Streptococcaceae*. Lanes 1 through 12, *S. uberis*, *S. parauberis*, *S. agalactiae*, *S. dysgalactiae*, *S. mitis*, *S. salivarius*, *S. bovis*, *S. saccharolyticus*, *E. faecium*, *E. faecalis*, *E. avium*, and *A. viridans*, respectively; lanes S_1, *Bst*NI-digested pBR322 DNA; lane S_2, *Hae*III-digested ϕX174 DNA; A to J, restriction fragment size markers of 1,353, 1,078, 872, 603, 310, 281 and 271, 234, 194, 118, and 72 bp, respectively. (A) 16S rDNA digested with *Ava*II; (B) 16S rDNA digested with *Scr*FI; (C) 16S rDNA digested with *Mbo*II; (D) 16S rDNA digested with *Dde*I.

16S rDNA RFLP patterns which consisted of three fragments of 280, 550, and 600 bp (Fig. 1 and 2). In group B, four fragments of 220, 280, 380, and 550 bp were observed in *S. saccharolyticus*, *E. faecium*, *E. faecalis*, and *E. avium* (Fig. 1 and 2).

Identification of organisms in group A. The 16S rDNAs of *S. uberis*, *S. parauberis*, *S. agalactiae*, *S. dysgalactiae*, *S. mitis*, *S. salivarius*, *S. equinus*, and *A. viridans* were digested with *Rsa*I. A 200-bp restriction fragment was observed only in *S. uberis* and *S. parauberis*. A 270-bp fragment was present in only *S. agalactiae* and *S. dysgalactiae*. A 930-bp fragment was observed only in *S. mitis*, *S. salivarius*, and *S. equinus*. *A. viridans* could be differentiated from the other organisms by the absence of the 200-, 270-, and 370-bp fragments which were present in other organisms of this group. *S. uberis* could be differentiated from *S. parauberis* by the absence of a 370-bp and presence of a 240-bp fragment in *S. uberis*. Thus, 16S rDNA digestion with *Rsa*I allowed identification of *S. uberis*, *S. parauberis*, and *A. viridans*. Digestion of the 16S rDNA of *S. parauberis* with restriction endonucleases *Sca*I and *Ava*II and of the 16S rDNA of *A. viridans* with *Ava*II produced characteristic restriction fragments, confirming the identities of the two species (Fig. 2 to 4).

Digestion of 16S rDNAs with *Msp*I allowed differentiation of *S. agalactiae*, *S. dysgalactiae*, *S. mitis*, *S. salivarius*, and *S. equinus* (Fig. 1 to 4). The presence of a 570-bp fragment in *S. agalactiae* and a 490-bp fragment in *S. dysgalactiae* differentiated these two species (Fig. 1). Further differentiation of *S. agalactiae* and *S. dysgalactiae* was confirmed by digestion of 16S rDNA with *Mbo*II and *Ava*II, respectively (Fig. 4). Digestion of 16S rDNA with *Msp*I allowed differentiation of *S. mitis* from *S. salivarius* and *S. equinus* (Fig. 1 to 4). The restriction fragments of 100, 130, and 330 bp found in *S. mitis* were not observed in either *S. salivarius* or *S. equinus*. A restriction fragment of 490 bp was observed only in *S. salivarius*, while in *S. equinus* none of the differentiating fragments present in *S. mitis* and *S. salivarius* were observed (Fig. 1). Confirmation of *S. mitis* was achieved by 16S rDNA digestion with *Scr*FI, while *S. equinus* was confirmed by 16S rDNA digestion with *Dde*I (Fig. 4).

Identification of organisms in group B. Digestion of 16S rDNAs with *Msp*I produced characteristic restriction fragments that permitted differentiation of *S. saccharolyticus*, *E.*

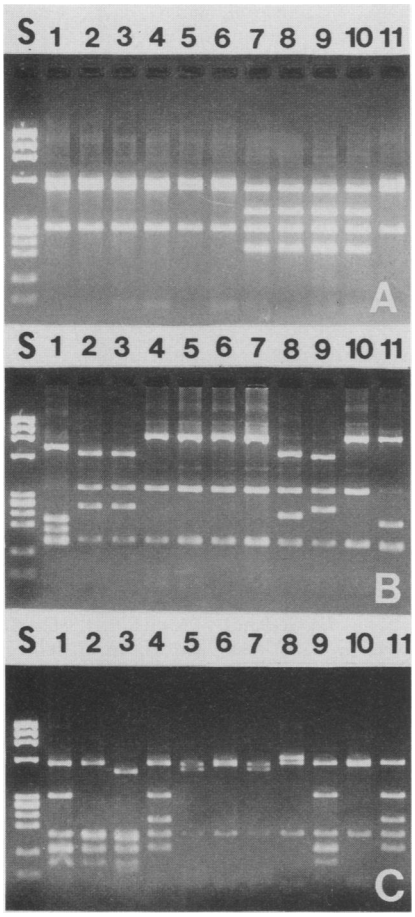

FIG. 5. The 16S rDNA fingerprint patterns of 11 species belonging to the family *Streptococcaceae* isolated from bovine mammary secretions. Lanes 1 through 11, *S. uberis*, *S. agalactiae*, *S. dysgalactiae*, *S. mitis*, *S. salivarius*, *S. bovis*, *S. saccharolyticus*, *E. faecium*, *E. faecalis*, *E. avium*, and *A. viridans*, respectively; lane S, *Hae*III-digested φX174 DNA restriction fragment size markers (in base pairs). (A) 16S rDNA digested with *Hha*I; (B) 16S rDNA digested with *Rsa*I; (C) 16S rDNA digested with *Msp*I.

faecium, *E. faecalis*, and *E. avium* (Fig. 1 to 4). Unique restriction fragments of 490, 590, and 330 bp were observed in *S. saccharolyticus*, *E. faecium*, and *E. faecalis*, respectively (Fig. 1); these unique fragments allowed differentiation and identification of these three species from *E. avium*. Confirmation of *E. faecium* was achieved with *Scr*FI, while *E. faecalis* was confirmed with *Scr*FI and *Dde*I (Fig. 4).

Identification of isolates of bovine origin. The 11 isolates representing 11 species were examined by 16S rDNA fingerprinting on the basis of a scheme developed for ATCC type strains. The isolates selected for this study had either atypical biochemical profiles or a low identification level (Table 1). The organisms were identified to the species level by using the scheme proposed for the identification and differentiation of streptococcal and enterococcal organisms (Fig. 5).

DISCUSSION

Sequences of 16S rRNAs have been used to determine phylogenetic relationships (31). Amplification of 16S rRNA genes by PCR by using taxon-specific oligonucleotide probes complementary to the 16S rRNA sequence has resulted in detection systems for the identification and differentiation of bacterial species (4, 12–14).

In this study, we developed a scheme for fingerprinting 16S rDNAs for the identification and differentiation of streptococcal and enterococcal species of bovine origin. In our earlier study (14), we used 16S rDNA fingerprinting to differentiate *S. uberis* type I and type II strains. *S. uberis* type II was recently reclassified as *S. parauberis* (30). The oligonucleotide primers used for amplification of the 16S rDNAs of *S. uberis* and *S. parauberis* were used successfully in this study to amplify 16S rDNAs from other streptococcal and enterococcal species. The RFLPs of 16S rDNAs were determined by using a variety of restriction endonucleases. Characteristic fingerprint patterns generated with *Hha*I, *Rsa*I, and *Msp*I resulted in the development of a scheme for species identification of the 12 organisms examined.

A preliminary evaluation of the proposed scheme was done by using organisms of bovine origin. The isolates that were selected had atypical biochemical and/or low identification profiles when they were determined by using commercially available diagnostic kits. Using the proposed scheme, organisms were identified correctly to the species level, and the identifications were confirmed by conventional biochemical tests.

At the time of this study, the 16S rRNA sequences of *S. uberis*, *S. parauberis* (30), and *S. saccharolyticus* (23) had been reported. Recently, Bentley et al. (3) reported the 16S rRNA sequences of five additional streptococcal species of bovine origin. Comparison of the *S. saccharolyticus* fingerprint patterns obtained with *Hha*I, *Ava*I, *Pst*I, *Ava*II, and *Mbo*II indicated that the patterns were more similar to those of the enterococcal species than to those of the streptococcal species. This agrees with the observation of Rodrigues and Collins (23), who performed phylogenetic analysis of *S. saccharolyticus* based on 16S rRNA sequencing and showed that *S. saccharolyticus* is phylogenetically a member of the genus *Enterococcus*.

Identification of streptococcal and enterococcal species of bovine origin is now possible by using 16S rDNA fingerprinting as an alternative to species identification based on phenotypic characteristics. With more 16S rRNA sequences being reported, use of taxon-specific probes (4) and colonies isolated from blood agar plates as templates for 16S rDNA amplification, as shown by Joshi et al. (16), could be used in future 16S rDNA fingerprinting studies. Integration of these steps could result in more rapid identification of streptococcal and enterococcal species of bovine origin. This identification system could also serve as a model for the development of identification systems for other bacterial species.

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