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Identification of Major Streptococcal Species by *rrn*-Amplified Ribosomal DNA Restriction Analysis

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Amplified ribosomal DNA restriction analysis (*rrn*-ARDRA) is based on PCR amplification and restriction of a fragment of rRNA genes including 16S and 23S genes and the intergenic spacer. *rrn*-ARDRA was evaluated for the identification of species within the genus *Streptococcus*. A total of 148 type and reference strains of pyogenic, oral, and group D streptococci were examined in order to construct a database for identification of streptococci. The amplified product was a single band approximately 4,500 bp long. This amplicon was digested separately with three (*Hha*I, *Mbo*II, and *Sau*3A) restriction endonucleases. Respectively, 27, 26, and 28 major patterns were observed after *Hha*I, *Mbo*II, and *Sau*3A restrictions. Streptococcal strains belonging to different species had different patterns or different combination of patterns. An identification system based upon a combination of the three restriction patterns in a single database was then proposed. *rrn*-ARDRA was successfully applied to 11 clinical isolates whose identification to the species level was difficult to obtain by phenotypic analysis. Using a database of well-characterized strains, *rrn*-ARDRA is a powerful method for the identification of streptococcal isolates.

Streptococci are major pathogens for animals and human beings. Phenotypic studies and DNA-DNA hybridization assays have demonstrated that the genus *Streptococcus* may be divided in three major groups of species: pyogenic, oral, and group D streptococci (5, 6, 17, 20, 21, 27).

Identification of streptococcal species is currently based on observation of the cultural and morphological characteristics, determination of the biochemical pattern (production of enzymes and production of acid from various carbohydrate sources) (4, 5, 6, 9, 11, 17, 20), and observation of the antigenic structure according to the classification of Rebecca Lancefield (22).

Molecular tools as ribotyping (3, 8, 25, 26), oligonucleotide probing (2), PCR-based protocols (13, 23), and DNA sequence analysis (1, 19, 24) are now proposed to provide an accurate identification of streptococcal isolates. Among these, restriction fragment length polymorphism analysis was proposed as a general method for bacterial identification and typing (15). Amplified ribosomal DNA (rDNA) restriction analysis (ARDRA) was recently reported to be a rapid and efficient method of identification of bacterial isolates to the species level in mollicutes (7) and in some genera such as *Acinetobacter* or *Mycobacterium* (30, 31). The initial protocol was substantially improved by amplification of a larger fragment of the *rrn* operon (16S rRNA gene, 16S-23S intergenic spacer, and 23S rRNA gene) followed by the digestion with one to three endonucleases selected according to the bacterial genus studied (12, 18). The latest modification of this method uses a unique combination of three restriction endonucleases (*Hha*I, *Mbo*II, and *Sau*3A) irrespective of the bacterial species studied. This modified protocol was successfully used for identification of the genus and the species of strains belonging to the family *Enterobacteriaceae* (G. Giammanco, P. Nogueira, O. Bouallegue, F. Grimont, and P. A. D. Grimont, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. R20, p. 482, 1998).

In the present study, we investigate the interest of the modified *rrn*-ARDRA protocol for identification of streptococci. One hundred and forty-eight strains were amplified and analyzed with *Hha*I, *Mbo*II, and *Sau*3A endonucleases. A database suitable for identification of streptococcal species was constructed and used to identify some clinical isolates.

MATERIALS AND METHODS

Bacterial strains. A total of one hundred and forty-eight reference strains were included in the study (Table 1). These strains were obtained from the Australian Collection of Microorganisms (ACM), St. Lucia, Australia; the bioMérieux collection of microorganisms (API), La-Balme-les-Grottes, France; the American Type Culture Collection (ATCC), Manassas, Va.; the Culture Collection of the University of Göteborg (CCUG), Göteborg, Sweden; the Centers for Disease Control and Prevention (CDC), Atlanta, Ga.; the Collection de l'Institut Pasteur (CIP), Paris, France; le Centre National de Référence des Pneumocoques (CNRP), Créteil, France; the National Collection of Dairy Organisms (NCDO), Sheffield, United Kingdom; the National Collection of Type Cultures (NCTC), London, United Kingdom; and other depositors. Strains were cultured onto blood Columbia agar plates or brain heart infusion (BHI) broth medium (bioMérieux, La Balme-les-Grottes, France) and conserved at −80°C in BHI broth supplemented with glycerol (15%, vol/vol). For pneumococci, BHI broth was supplemented with 5% bovine ascite-serum (Bio-Rad, Marnes-la-Coquette, France), and cultures were incubated in a 5% CO_2 atmosphere.

Preparation of DNA. Bacteria (10 ml of a mid-log-phase culture) were harvested by centrifugation and washed twice in TE buffer (10 mM Tris, 1.0 mM Na_2EDTA [pH 8.0]). Pellets were suspended in 500 μl of TES buffer (10 mM Tris, 1.0 mM Na_2EDTA, 1 M sucrose [pH 8.0]) for lysis. Mutanolysin (5 U/ml; Sigma, St. Louis, Mo.) and lysozyme (10 mg/ml; Boehringer, Mannheim, Germany) were added, and the mixture was incubated overnight at 37°C. Bacterial membrane disruption was then done with proteinase K (0.4 mg/ml) and sodium dodecyl sulfate (1%, vol/vol). High-molecular-weight bacterial genomic DNA

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TABLE 1. Identification, source, and restriction patterns of streptococcal strains studied for construction of database

Species	Strain no.	Designation in initial collection or other source	Restriction pattern		
			*Hha*I	*Mbo*II	*Sau*3A
S. agalactiae	HDP 89543^T	CCUG 4208^T	H-D1b	M-B1a	S-F1b
	HDP 96146	CIP 104971	H-D1a	M-B1b	S-F1a
	HDP 98299	CIP 105451	H-D1a	M-B1a	S-F1a
	CNRP 96/87	Serotype Ia	H-D1a	M-B1a	S-F1a
	CNRP 96/126	Serotype Ia	H-D1b	M-B1a	S-F1a
	CNRP 96/133	Serotype V	H-D1c	M-B1a	S-F1a
	CNRP 96/138	Serotype III	H-D1b	M-B1a	S-F1a
	CNRP 96/408	Serotype V	H-D1c	M-B1a	S-F1b
	CNRP 96/409	Serotype Ia	H-D1b	M-B1a	S-F1a
	CNRP 96/433	Serotype V	H-D1c	M-B1a	S-F1b
S. alactolyticus	HDP 90057^T	CIP 103244^T	H-B4	M-H1	S-T1
S. anginosus	HDP 89578^T	CIP 102921^T	H-E3	M-B5e	S-B1a
	HDP 89551	R. Whiley, strain UC 7895	H-F4a	M-B5b	S-B1a
	HDP 92259	R. Whiley, strain VR 348	H-F4a	M-B5e	S-B1a
	HDP 93171	R. Whiley, strain KR 455	H-F3	M-B5a	S-B1b
	HDP 93172	R. Whiley, strain KR 687	H-F3	M-B5a	S-B1b
	HDP 93173	CDC 1007-77	H-C1b	M-B5a	S-B1c
	HDP 93174	CDC 2236-81	H-F4a	M-B5c	S-B1a
	HDP 93179	NCTC 8037	H-C1a	M-B5c	S-B1c
	HDP 93180	ATCC 9895	H-F4b	M-B5e	S-B1a
S. bovis	HDP 89505^T	CIP 102302^T	H-B3b	M-E2	S-G1c
S. canis	HDP 90051^T	CIP 103223^T	H-F1	M-A3a	S-R1b
	HDP 99030	CIP 105029	H-F1	M-A3a	S-R1b
S. caprinus	HDP 99204^T	CIP 104887^T	H-B3a	M-B7b	S-G2
S. constellatus	HDP 89579^T	NCDO 2226^T	H-E2b	M-A4b	S-L1a
	HDP 93154	NCTC 5389	H-E2a	M-A4a	S-L1b
	HDP 93155	NCTC 11063	H-E2a	M-A5a	S-L1b
	HDP 93156	NCTC 10714	H-E2a	M-A4c	S-L1b
S. criceti	HDP 89549^T	A. Coykendall, strain H56	H-G5	M-F2	S-U7
S. difficilis	HDP 98301^T	CIP 103768^T	H-D1b	M-B1	S-F1a
	HDP 99031	CIP 103853	H-D1b	M-B1	S-F1a
S. downei	HDP 90050^T	CIP 103222^T	H-G1	M-F1	S-U6
S. dysgalactiae subsp. *dysgalactiae*	HDP 89550^T	CIP 102914^T	H-F2	M-A3a	S-R1a
	HDP 98005	CIP 55.123	H-F2	M-A3a	S-R2
	HDP 98110	CIP 55.119	H-F2	M-A3a	S-R2
S. dysgalactiae subsp. *equisimilis*	HDP 98055	CIP 105120, former type strain of *S. equisimilis*	H-F2	M-A3a	S-R2
	HDP 97340	ATCC 35666	H-F2	M-A3a	S-R1b
	HDP 98003	CIP 104.339	H-F2	M-A3a	S-R2
	HDP 98004	CIP 55.120	H-F2	M-A3a	S-R2
	HDP 99032	CIP 103684	H-F2	M-A3a	S-R1b
	HDP 99033	CIP 105034	H-F2	M-A3a	S-R1b
S. equi subsp. *equi*	HDP 89501^T	CIP 102910^T	H-G7b	M-B6a	S-N1
	HDP 99034	CIP 105022	H-G7b	M-B6a	S-N1
	HDP 99035	CIP 105023	H-G7b	M-B6a	S-N1
S. equi subsp. *zooepidemicus*	HDP 90053	CIP 103228, former type strain of *S. zooepidemicus*	H-G7a	M-B6c	S-E1a
	HDP 98092	CIP 52.209	H-G7d	M-B6a	S-E1b
	HDP 98093	CIP A7	H-G7d	M-B6b	S-E1b
	HDP 98297	CIP 105025	H-G7c	M-B6b	S-E1b
	HDP 99036	CIP 105024	H-G7c	M-B6a	S-E1b
S. equinus	HDP 89506^T	CIP 102504^T	H-B3b	M-E2	S-G1b
	HDP 99309	API 78.04.058, NCTC 10389	H-B3b	M-E2	S-G1a
	HDP 99313	API 88.07.126	H-B3b	M-E2	S-G1b
S. ferus	HDP 89545^T	A. Coykendall, strain 8S1	H-G6	M-G5	S-O1

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TABLE 1—*Continued*

Species	Strain no.	Designation in initial collection or other source	Restriction pattern		
			*Hha*I	*Mbo*II	*Sau*3A
S. gallolyticus	HDP 98035^T	ACM 36.11^T	H-B3c	M-B7b	S-G1a
	HDP 90055	NCDO 2019	H-B3b	M-B7a	S-G1a
	HDP 90299	API 84.03.028	H-B3b	M-B7a	S-G3
	HDP 98354	CDC 3437-70	H-B3b	M-B7a	S-G1a
S. gordonii	HDP 89552^T	API 91.01.022, NCTC 7865^T	H-B2a	M-G1a	S-H1a
	HDP 84022	API 77.09.010, NCTC 10231	H-B2b	M-G1a	S-H1a
	HDP 89542	CCUG 18374	H-B2a	M-G1b	S-H1a
	HDP 99101	CIP 103221	H-B2a	M-G1a	S-H1a
	HDP 99285	CIP 55.121	H-B2a	M-G1a	S-H1b
	HDP 99287	CIP 102337 (received as *Streptococcus* sp.)	H-B2a	M-G1a	S-H1c
S. infantarius subsp. *infantarius*	HDP 90056^T	NCDO 599^T, CIP 103233^T	H-B1	M-E1a	S-G1b
	HDP 90104	Our collection, dairy product	H-B1	M-E1c	S-G1b
S. infantarius subsp. *coli*	HDP 90246	NCDO 964	H-B1	M-E1b	S-G1b
	HDP 90247	NCDO 2602	H-B1	M-E1d	S-G1b
	HDP 90248	NCDO 2620	H-B1	M-E1d	S-G1b
S. intermedius	HDP 93161^T	NCDO 2227^T	H-E1b	M-B3	S-C2
	HDP 99286	CIP 105038 (received as *S. gordonii*)	H-E1a	M-B3	S-C2
	HDP 93162	CDC 415-87	H-E1a	M-B3	S-C2
	HDP 93167	A. Coykendall, strain AC 6425	E2c/E1a	M-A5b	S-C2
S. intestinalis	HDP 90052^T	CIP 103224^T	H-B4	M-H1	S-T1
S. macacae	HDP 89541^T	CIP 102912^T	H-D4	M-F3	S-U8
S. mitis	HDP 99284^T	CIP 103335^T, neo-type strain	H-A5c	M-B2c	S-A1d
	HDP 84001	API 78.04.067, NCTC 3168	H-A1b	M-B2a	S-A2b
	HDP 84002	CIP 103216 (received as *S. sanguis* biotype II)	H-A2a	M-B2b	S-A3
	HDP 84009	NCTC 10712 (received as *S. sanguis* biotype II)	H-A2b	M-G6	S-A3
	HDP 84010	API 78.11.144, ATCC 10232	H-A3a	M-F7	S-A2a
	HDP 89503	R. Facklam, strain SS-429	H-A1b	M-B2b	S-A1d
	HDP 89609	API 78.07.063 (received as *S. sanguis* biotype II)	H-A2a	M-E3c	S-A3
S. mutans	HDP 89502^T	NCTC 10449^T	H-G4	M-G3	S-I1a
	HDP 97299	ATCC 35668	H-G4	M-G3	S-I1b
S. oralis	HDP 89546^T	CIP 102922^T	H-A5a	M-E3a	S-A1a
	HDP 89527	NY SBE-844	H-A2a	M-B2b	S-A1b
	HDP 89535	A. Coykendall, strain AC 9811	H-A2a	M-E3e	S-A1a
	HDP 89537	A. Coykendall, strain OS51	H-A5b	M-E3b	S-A1c
S. parasanguinis	HDP 99102^T	CIP 104372^T	H-C1c	M-G1c	S-U5
S. parauberis	HDP 98049^T	CIP 103956^T	H-D5a	M-D2	S-M1
	HDP 90054	NCDO 2018	H-D5a	M-D2	S-M1
	HDP 98385	NCDO 2056	H-D5a	M-D2	S-M1
	HDP 98401	NCDO 642	H-D5a	M-D2	S-M1
	HDP 98402	NCDO 648	H-D5a?	M-D2	S-M1
	HDP 98403	NCDO 649	H-D5a?	M-D2	S-M1
	HDP 98404	NCDO 650	H-D5a?	M-D2	S-M1
	HDP 98405	NCDO 689	H-D5a?	M-D2	S-M1
	HDP 99184	CIP 105803	H-D5a	M-D2	S-M1
S. pneumoniae	HDP 89540^T	CIP 102911^T	H-A3a	M-G2a	S-A1g
	CNRP 70131	P. Geslin, serotype 23F	H-A4b	M-G2c	S-U4
	CNRP 70133	P. Geslin, serotype 23F	H-A4a	M-G2c	S-U3
	CNRP 70203	P. Geslin, serotype 23F	H-A3a	M-C1c	S-V2
	CNRP 70247	P. Geslin, serotype 19F	H-A3a	M-C1b	S-A1f
	CNRP 70265	P. Geslin, serotype 19F	H-A4a	M-G2b	S-A2a
	CNRP 70305	P. Geslin, serotype 19F	H-A4a	M-G2b	S-A2b
	CNRP 70350	P. Geslin, serotype 9V	H-A3a	M-C1a	S-A1e
	HDP 98040	ATCC 6301, serotype 1	H-A2b	M-G2a	S-A1c
	Strain R6	Nontypeable strain	H-A4c	M-G2a	S-A2c
S. porcinus	HDP 90049^T	CIP 103218^T	H-H2	M-A1	S-K1a
	HDP 98090	CIP 104385, serogroup U	H-H2	M-A1	S-K1b

Continued on following page

TABLE 1—*Continued*

Species	Strain no.	Designation in initial collection or other source[a]	Restriction pattern		
			*Hha*I	*Mbo*II	*Sau*3A
	HDP 98112	CIP 104338, serogroup P	H-H2	M-A1	S-K2
	HDP 98089	CIP 104384, serogroup E	H-H2	M-A1	S-K1b
	HDP 98111	CIP 104386, serogroup V	H-H2	M-A1	S-K1b
	HDP 98149	CIP 104389	H-H2	M-A1	S-K1a
	HDP 98150	CIP 104388	H-H2	M-A1	S-K1a
	HDP 98151	CIP 104387	H-H2	M-A1	S-K1a
S. pyogenes	HDP 89539^T	P. Kriz, CIP 56.41^T, biotype 1, serotype M1	H-H1b	M-A2a	S-Q1a
	HDP 93104	P. Kriz, PHLS SF130/13, biotype 1, serotype M1	H-H1b	M-A2a	S-Q2
	HDP 93106	P. Kriz, PZH GH1/Richard 55P serotype M3	H-H1b	M-A2a	S-Q2
	HDP 93109	P. Kriz, RU T11/137/1, serotype M11	H-H1b	M-A2c	S-Q1b
	HDP 93110	P. Kriz, 63595-H1, serotype M12	H-H1d	M-A2a	S-Q2
	HDP 93112	P. Kriz, NCTC 11/64, serotype M48	H-H1d	M-A2b	S-Q2
	HDP 98001	Our collection, biotype 3	H-H1b	M-A2b	S-Q2
	HDP 98052	Our collection, biotype 3	H-H1c	M-A2a	S-Q3
	HDP 98139	Our collection, biotype 3	H-H1a	M-A2a	S-Q3
	HDP 98173	Our collection, biotype 3	H-H1b	M-A2a	S-Q3
	HDP 98353	Our collection, biotype 5	H-H1a	M-A2a	S-Q3
	HDP 98359	Our collection, biotype 3	H-H1a	M-A2a	S-Q3
	HDP 98384	Our collection, biotype 3	H-H1a	M-A2a	S-Q3
S. ratti	HDP 89575^T	ATCC 19645^T	H-G3a	M-G4a	S-U1
	HDP 99416	CIP 74.15	H-G3b	M-G4b	S-U2
S. salivarius	HDP 89532^T	CIP 102503^T	H-C2	M-E3f	S-J1
S. sanguinis	HDP 84025^T	API 77.09.011, NCTC 7863^T	H-F5	M-E4	S-C1a
	HDP 93177	CDC 2405-81	H-C1a	M-D3	S-C1b
	HDP 99283	CIP 103.231	H-A1a	M-B2b	S-V1
S. sobrinus	HDP 89577^T	A. Coykendall, strain SL1	H-D3	M-F4	S-O1
	HDP 99417	CIP 74.16	H-G2	M-A5b	S-U9
S. suis	HDP 90048^T	CIP 103217^T	H-D2	M-D1a	S-P1
S. thermophilus	HDP89530^T	CIP 102303^T	H-C2	M-F5	S-J1
S. uberis	HDP 89504^T	CCUG 17930^T	H-E3	M-B4	S-D1a
	HDP 98091	CIP 101889	H-E3	M-B4	S-D1b
	HDP 98406	NCDO 640	H-E3	M-B4	S-D1a
	HDP 98407	NCDO 643	H-E3	M-B4	S-D1b
	HDP 98408	NCDO 658	H-E3	M-B4	S-D1a
	HDP 98409	NCDO 1513	H-E3	M-B4	S-D1b
	HDP 98410	NCDO 2022	H-E3	M-B4	S-D1b
	HDP 99182	CIP 105801	H-E3	M-B4	S-D1a
	HDP 99183	CIP 105802	H-E3	M-B4	S-D1a
S. vestibularis	HDP 90069^T	CIP 103363^T	H-C2	M-E3d	S-J1

was purified from lysates by two sequential phenol-chloroform extraction and ethanol precipitation steps. DNA was dissolved in a buffer containing 10 mM Tris, 1 mM EDTA, and RNase (20 mg/ml). Its concentration and its integrity were determined visually after agarose (Bioprobe) gel electrophoresis (0.8%, wt/vol; 100 V; 1 h). Gels were stained by immersion in a 50-μg/ml ethidium bromide solution. DNA samples were diluted to a concentration of 1 μg/μl and microdialyzed (pore size, 0.22 μm; Millipore) before amplification.

Amplification. Oligonucleotide primers Ad (5′-AGAGTTTGATCMTGGCT CAG) and O24/3 (5′-CGACATCGAGGTGCCAAA) were derived from conserved regions present at the 5′ end of the 16S rRNA gene and the 3′ end of the 23S rRNA gene, respectively (Giammanco et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol.). Purified DNA (1 μg) was added to a 74-μl aliquot of PCR mixture containing 0.4 pmol (each) of primers Ad and O24/3 (Genset, Paris, France), 250 μmol (each) of deoxynucleoside triphosphate (Pharmacia Biotech, Orsay, France), and 0.05 U of *Taq* polymerase (Gibco BRL, Cergy Pontoise, France) in 1× reaction buffer. Magnesium chloride (1.1 mM; Gibco BRL), 0.05% (vol/vol) wetting agent (Gibco BRL), and 0.08% (wt/vol) bovine serum albumin (Sigma) were added to the mixture. Amplification was done in a Perkin-Elmer 2400 system as follows: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 45 s and annealing at 60°C for 60 s, and extension at 72°C for 3 min. Finally, a 7-min extension period at 72°C was carried out. Positive (DNA from *S. agalactiae* CNRP 96/409) and negative controls were included in each run of amplification. The presence of a specific PCR product was controlled by agarose gel electrophoresis (0.8%, wt/vol; 100 V; 1 h). The molecular weight of the amplification product was calculated using a 1-kb marker from Sigma.

Restriction endonuclease digestion and analysis. Restriction was carried out for a 4-h period at 37°C in a 20-μl mixture containing 2 U of restriction enzymes *Hha*I, *Mbo*II, and *Sau*3AI (Amersham), 2 μl of the supplied specific incubation buffer, and 10 to 16 μl of PCR product. The volume of PCR product used in the restriction mixture was adjusted on the basis of the fluorescence intensity of the observed fragment in the control gel. Restriction was stopped by addition of 5 μl of 1× TE buffer (glycerol [50%, vol/vol], bromophenol blue 0.07%, wt/vol]). Before electrophoresis, restriction endonuclease digestions were heat inactivated as recommended by Giammenco et al. (14). Restriction fragment patterns were analyzed by gel electrophoresis at 120 V for 5 to 6 h in a 1.5% (wt/vol) Metaphor agarose (FMC BioProduct, Rockport, Maine) and 1.5% (wt/vol) standard agarose (Bioprobe) gel in 0.5× TAE buffer. DNA ladder 50-2000 (Bio-Rad) was loaded in four lanes of each gel for an accurate determination of the molecular

weights of the restriction fragments. After migration, gels were stained by immersion in a 50-μg/ml BET solution for 1 h. Gels were then photographed and video acquired using Visiomic software (Genomic, Grenoble, France). The produced TIFF-formatted file was then reopened with the RestrictoScan program from the Taxotron package (Institut Pasteur, Paris, France). In each lane, restriction fragments were detected and their positions were consecutively noted. Calculation of the molecular size of each fragment was done using the Schaffer and Sederoff algorithm implemented in RestrictoTyper program. The sizes of the fragments were then entered in a database. Clustering of restriction patterns based on the single-linkage, average-linkage, and unweighted pair-group method using arithmetic averages (UPGMA) algorithms were done using the Adanson and Dendrograph programs from the Taxotron package.

In silico analysis. In order to test the reliability of our method, virtual restriction analyses were performed on the nucleotide sequence of the *rrn* operon from *S. pyogenes* ATCC 700294 (serotype M1). The sequences were retrieved from the *S. pyogenes* sequence data published by the University of Oklahoma Health Sciences Center (28). In silico analysis was performed using the Geneman program from the Lasergene software (DNAStar, Madison, Wis.). Results were compared to those provided by the experimental restriction patterns of the amplified *rrn* operon of *S. pyogenes* HDP 89539^T (serotype M1), HDP 93106 (serotype M3), and HDP 93112 (serotype M48).

Identification of atypical streptococcal isolates. Eleven strains recently isolated from clinical specimens were studied. These isolates were identified as streptococci with conventional methods based upon morphological, cultural, and biochemical characteristics but exhibited either unexpected serological markers by the agglutination assay (Bio-Rad) or atypical biochemical traits when the rapid ID32STREP galleries (bioMérieux) were used. The three restriction patterns were first compared to their respective data base but, in an effort to allow a definitive identification, the three data bases were combined using the average-linkage algorithm. Restriction patterns of unknown isolates were then simultaneously compared to the complete data base, and a bacterial identification was then proposed. When analysis of the restriction patterns allowed identification of species represented in the library by a small number of strains, we confirmed the *rrn*-ARDRA identification by 16S rDNA sequence analysis as previously described (26).

RESULTS

Amplification and restriction of *rrn* operon. We have evaluated a total of 148 strains representative of 38 streptococcal species or subspecies mostly isolated from humans or animals. The PCR-based protocol allowed production of a single amplification product with an estimated size between 4,400 and 4,500 bp. This size was consistent with the size predicted after analysis of the published sequence of *S. pyogenes* ATCC 700294 (28). Electrophoretic analysis of the amplified product digested with *Hha*I, *Mbo*II, and *Sau*3A separately disclosed 7 to 14 bands ranging from 30 up to 1,500 bp (Fig. 1). BET staining and densitometric detection of smaller bands (i.e., bands under 100 bp) were not sufficiently reliable between runs, and these bands were not recorded for further analysis.

In silico restriction analysis. Since the published sequence of the entire chromosome of *S. pyogenes* ATCC 700294 includes six amplified fragments of the *rrn* operon, we considered different sequences corresponding to positions 268.758 to 273.233 (4,476 bp), 515.696 to 520.172 (4,477 bp), 1.587.525 to 1.583.047 (4,479 bp), 1.772.590 to 1.768.155 (4,476 bp), 1.828.809 to 1.824.334 (4,475 bp), and 1.834.809 to 1.830.335 (4,475 bp). Each fragment was virtually digested with the three endonucleases. The six restriction patterns were then combined together and compared with the experimental patterns obtained with *S. pyogenes* HDP 89539^T, HDP 93106, and HDP 93112. The real patterns displayed all the predicted bands and also few extra bands after *Hha*I and *Sau*3A digestion. However, these additional bands are very faint and may correspond to strain-specific alterations of the DNA sequence of only one or a few copies of the operon.

Restriction length polymorphism analysis. Visual observation of restriction patterns demonstrated a high interspecies variability and a low intraspecies variability (Fig. 1 and 2). In some species, the patterns successively obtained with the three restriction endonucleases were very homogeneous even though a large set of strains was studied (*S. agalactiae*, *S. equi*, *S. gordonii*, *S. parauberis*, *S. uberis*, and *S. pyogenes* strains). Thus, the construction of a similarity matrix and the statistical clustering of patterns improved the detection of small alterations in patterns obtained for strains of the same species. A computerized database was then built with restriction patterns using the average-linkage algorithm. The fragment size tolerance (experimental error) was set to 5%, which is superior to the usual interrun variations. Results of clustering analysis were identical when carried out by using UPGMA, single-linkage, or average-linkage analysis (data not shown). Initially the clustering analysis was done with each endonuclease separately, and a designation was assigned to each pattern, corresponding to a capital letter for major subdivisions of the dendrogram (distance between patterns > 0.25), an Arabic number for the different cluster of patterns inside these subdivisions (distance between 0.25 and 0.05), and a lowercase letter for minor variations of pattern (distance between patterns < 0.05) (Table 1).

*Hha*I analysis alone allowed the delineation of most of the species, except those within the "*mitis*" group, including *S. mitis*, *S. oralis*, and *S. pneumoniae* (cluster designated as H-A with large strain-specific variations). Patterns obtained for *S. bovis* and *S. gallolyticus* isolates are also combined together (H-B3). Subspecies of *S. dysgalactiae* and *S. equi* were not delineated after digestion with *Hha*I endonuclease (patterns H-F2). Contrarily, restriction patterns of strains belonging to *S. anginosus* were assigned to different clusters (H-C1, H-E3, H-F3, and H-F4).

In the *Mbo*II database, all *S. anginosus* strains were grouped together (M-B5 patterns). Restriction patterns obtained with *S. dysgalactiae* subsp. *equisimilis*, *S. dysgalactiae* subsp. *dysgalactiae*, and *S. canis* were also merged (M-A3). At the same time, subspecies of *S. equi* were not distinguishable (patterns M-B6).

Patterns obtained with *Sau*3A displayed a large variability among *S. pyogenes* or *S. porcinus* strains, which were divided in two or three clusters (S-Q1, -Q2, and -Q3; S-K1 and -K2). Within *S. dysgalactiae*, two clusters of patterns were observed (S-R1 and -R2) irrespective of the subspecies delineation according to reference identification. In contrast to *Hha*I and *Mbo*II, the patterns obtained with *Sau*3A for the two subspecies *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* were separated in two different clusters (S-N1 and S-E1, respectively). Restriction patterns obtained for oral streptococci belonging to the *mitis* group (*S. mitis*, *S. oralis*, and *S. pneumoniae*) were not grouped in the same branch of the dendrogram as was observed with *Hha*I or *Mbo*II endonuclease.

The simultaneous observation of restriction patterns obtained with the three endonucleases was then proposed to improve the differentiation of strains corresponding to the different species and subspecies (Fig. 1 and 2). Minor alterations observed in one of the restriction patterns (band shift or addition or suppression of a single band) have a limited effect

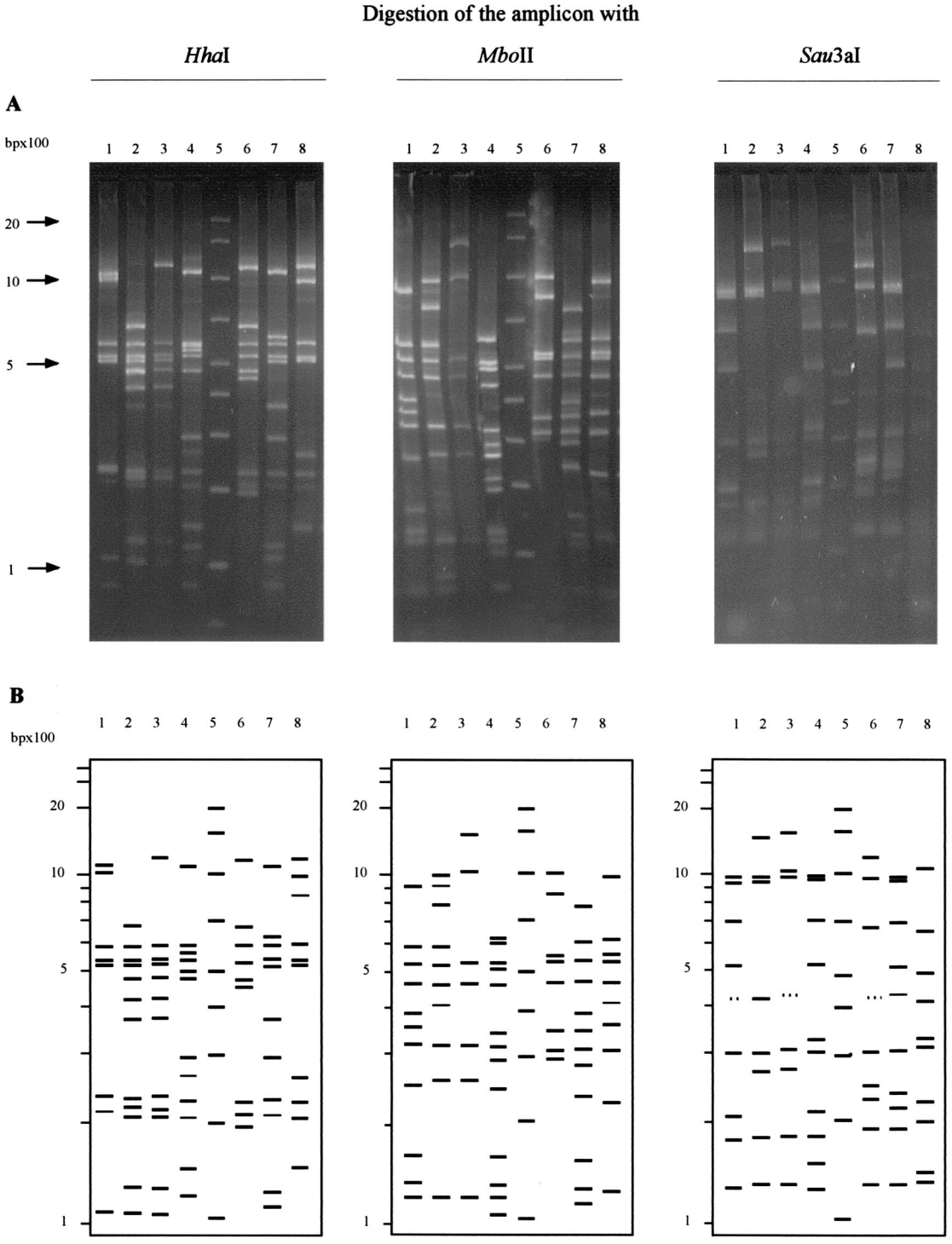

FIG. 1. Restriction analysis of amplifed operon with *Hha*I, *Mbo*II, and *Sau*3A restriction endonucleases. 1, *S. pyogenes* HDP 89539^T; 2, *S. infantarius* HDP 90056^T; 3, *S. equinus* HDP 89506^T; 4, *S. porcinus* HDP 90049^T; 5, molecular size marker; 6, *S. pneumoniae* HDP 89540^T; 7, *S. pyogenes* HDP 93110; 8, *S. constellatus* HDP 89579^T. (A) Visualization of the bands obtained after migration and BET staining. (B) Schematic representation of agarose gel. Only bands ranging from 100 to 2,000 bp are used for identification and are represented here.

upon clustering when the two patterns obtained with the other restriction endonucleases are similar. At the same time, strains belonging to different subspecies and distinguished only by patterns obtained with one endonuclease were separated in the cumulative database. All the strains belonging to the 36 species studied, including type strains, reference strains, and clinical isolates, were included in separated clusters. The phylogenic tree obtained for cumulative analysis of the three sets of data

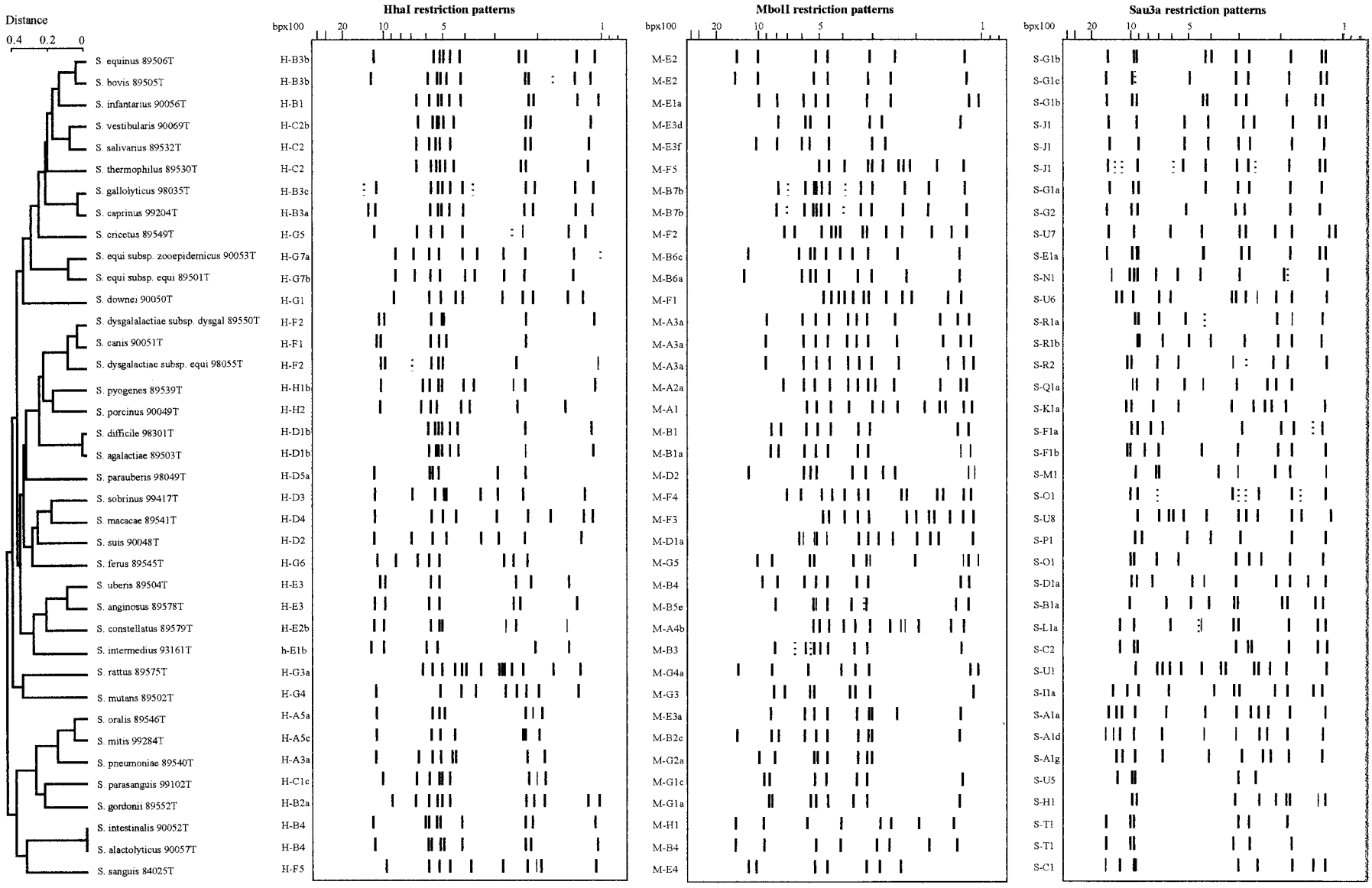

FIG. 2. Diagrammatic representation showing restriction patterns database for *rrn*-ARDRA obtained with type strains of major streptococcal species. Each restriction pattern was named separately in each restriction endonuclease database. The designation was assign to each digestion pattern corresponding to a capital letter for major subdivisions of the dendrogram (similarity index over 0.75), an arabic number for the different cluster of patterns inside of these subdivisions (similarity index between 0.75 and 0.95) and a lowercase letter for minor variations of pattern (similarity of patterns comprised between 0.95 and 0.99). Dendrogram was calculated using the UPGMA algorithm, for a database limited to the restriction pattern of the type strains of the species.

TABLE 2. Identification of atypical or phenotypically unidentified streptococcal strains by *rrn*-ARDRA

Strain no.	Conventional identification	Atypical characteristic(s)[a]	Restriction pattern			*rrn*-ARDRA identification
			*Hha*I	*Mbo*II	*Sau*3a	
HDP 99045	*S. porcinus*	Lancefield group B	H-H2	M-A1	S-K1a	*S. porcinus*
HDP 99520	*S. dysgalactiae*	Lancefield group A	H-F2	M-A3a	S-R2	*S. dysgalactiae*
HDP 99296	*S. suis*	β-GLU (−), MβDG (−), mannitol (+)	H-D2	M-D1a	S-P1	*S. suis*
HDP 91257	*S. bovis*	Trehalose (−), raffinose (+), pullulan (+)	H-B1	M-E1d	S-G1b	*S. infantarius*
HDP 99201	*S. sanguinis*	β-GAR (−), trehalose (+), tagatose (−), dextran (+)	H-C1	M-G1c	S-U5	*S. macacae*
HDP 99294	*S. suis*	ADH (−), β-GUR (−), β-MAN (+), raffinose (+)	H-B3b	M-E2	S-G1c	*S. equinus*
HDP 99374	*S. suis*	β-GAL (+), glycogen (−)	H-E1a	M-B3	S-C2	*S. intermedius*
HDP 99401	*S. parasanguinis*	ADH (−), dextran (−)	H-A4a	M-E3e	S-A1a	*S. oralis*
HDP 99419	*S. suis*	Lactose (−), β-GAL (+), β-NAG+, tagatose (+)	H-G2	M-A5a	S-P1	*S. sobrinus*
HDP 2000/341	*S. alactolyticus*	α-GAL (−), mannitol (−), pullulan (+)	H-C2	M-E3f	S-J1	*S. salivarius*
HDP 2000/051	Unidentified coccus from suppuration	ADH (−), β-GLU (−), VP (−), Lancefield group G	H-F4a	M-B5e	S-B1b	*S. anginosus*

[a] Atypical characteristics include atypical results for abilities to grow on the indicated substrates or to produce, the indicated compounds. Abbreviations: β-NAG, β-*N*-acetyl glycosylase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; β-GUR, β-glucuronidase; β-GLU, β-glucosidase; β-MAN, β-mannosidase; β-NAG, *N*-acetyl glycosidase; VP, production of acetoin; ADH, arginine dihydrolase; MβDG, methyl-β-D-glucopyranoside.

after UPGMA analysis of type strains is presented in Fig. 2. A distance index of <0.05 was found for four pairs of related streptococcal species: *S. equinus* and *S. bovis*, *S. gallolyticus* and *S. caprinus*, *S. agalactiae* and *S. difficilis*, and *S. alactolyticus* and *S. intestinalis*, respectively. These results suggest that each of these couples corresponds to a single species. *S. dysgalactiae* subsp. *dysgalactiae*, *S. dysgalactiae* subsp. *equisimilis*, and *S. canis* appeared close to each other in cumulative analysis. In contrast, restriction patterns obtained for *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* were clearly separated.

Identification of clinical isolates. A total of 11 streptococcal strains identified with atypical characteristics were subjected to *rrn*-ARDRA analysis. Biochemical identification was confirmed in three cases and changed to another species in seven cases (Table 2). In four cases, the *rrn*-ARDRA identification was confirmed by the 16S rDNA analysis, including one strain which was initially unidentified according to its biochemical patterns and assigned to *S. anginosus*.

DISCUSSION

Among molecular tools of bacterial identification, the determination of rRNA gene restriction patterns (ribotyping) has been extensively used (15). However, this methodology requires several steps, including transfer onto membranes and hybridization steps. Therefore, some alternative techniques have been proposed in an effort to obtain faster results. PCR amplification of DNA followed by restriction analysis of amplification products has attracted a growing interest. ARDRA (i.e., amplification and polymorphism of restriction analysis of 16S rRNA genes) has been proposed for the identification of species within some genera, such as *Acinetobacter*, *Mycobacterium*, and *Mycoplasma* (7, 30, 31). However, analysis restricted to the 16S rRNA gene appeared to be of limited value in some other genera (18). It was then suggested that inclusion of the 23S rRNA gene and 16-23S spacer in the analysis may improved the differentiation between species in some genera (18; Giammanco et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol.) as it allows the study of a larger amount of genetic information. Indeed, due to evolutionary constraints, there is a minimal variation into the 16S or the 23S rRNA sequences, whereas the 16S-23S intergenic sequence demonstrated an enhanced variability. The similarity of the sequences of 16S rRNA gene between different streptococcal species was determined to be between 91 and 99% (1). In contrast, the 16S-23S rRNA internal spacer is subjected to a minimal selective pressure compared to rRNA genes. Insertion, deletion, and single-base changes are frequent in this DNA fragment, and sequence identity between the intergenic spacers of different streptococcal species is limited to between 50 and 80% (10). In our study, a single band corresponding to the amplification of the *rrn* operon of streptococci was observed on the electropherogram. This indicates that the potential difference of lengths between the *rrn* operons of distinct species or between the six different *rrn* operons of the streptococcal strains (28) remains within the resolution of the agarose gel we used.

To determine the advantage and the reliability of the improved *rrn*-ARDRA protocol of identification, we have investigated 148 type and reference strains representative of the 39 major species and subspecies isolated from humans or animals. All these strains have been phenotypically characterized by their initial depositor, and most of them have also been identified by DNA-DNA hybridizations. A large interspecific variability of restriction profiles was detected among the species studied. When available, three to seven reference strains of each species were included in the study. The results of these investigations suggest that restriction patterns were conserved within species. So, restriction patterns obtained with strains of *S. pyogenes* were homogeneous and similar to the profiles predicted by DNA sequence analysis. In fact, controversial data were published about the presence or the absence of variable restriction sites in different copies of the 16S rRNA gene within the bacterial chromosome. Some studies reported the absence of intraspecies variations of 16S rRNA genes (18, 30), but a large variability of restriction patterns of 16S RNA was observed within *Abiotrophia adiacens* in the study of a large collection of *Abiotrophia* strains (23). Gurtler et al. (16) have also reported highly variable restriction patterns in a recent analysis of the clostridial species. Alterations of one or a few copies of the *rrn* operon may have resulted in faint bands on the electropherogram, but these additional bands, like the bands due to minor or incomplete digestion, did not appear

essential for the differentiation between the restriction patterns of different species (31). Some other minor variations of restriction patterns appeared unpredictable according to the species studied or to the restriction endonuclease used. These variations were assumed to be strain specific.

The construction of a combined database using the three restriction patterns obtained separately for each strain was proposed to overtake the strain variability and achieve a better delineation of species (Giammanco et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol.). Using the triple combination of *Hha*I, *Mbo*II, and *Sau*3A restriction endonucleases, strains belonging to the same species are found to exclusively cluster in the same restriction patterns. When 3 to 10 strains were studied, none of the strains were assigned to an inadequate cluster with the noticeable exceptions of *S. dysgalactiae* and *S. canis*, as well as *S. pneumoniae*, *S. mitis*, and *S. oralis*. We observed that enlargement of the available database of patterns usually induces a better clustering of the restriction patterns around the type strain of the species. The dendrogram that we obtained using average-linkage or UPGMA clustering methods confirms the major subdivisions of the classification of streptococci and the results based on rRNA (1) or superoxide dismutase (19, 24) sequence analysis

The classification of *S. anginosus*, *S. constellatus*, and *S. intermedius*, which has now been clarified (33), is clearly delineated using *rrn*-ARDRA. However, the similarity of their *Hha*I and *Sau*3A patterns confirms the phylogenic proximity of these three species (33). In the case of *S. uberis* and *S. parauberis*, where biochemical or serological differentiation is not possible (2, 34), *rrn*-ARDRA clearly distinguishes between the two species. We also noticed the inclusion of strains belonging to the species *S. bovis*, *S. caprinus*, *S. difficilis*, or *S. intestinalis* into the respective clusters of *S. equinus*, *S. gallolyticus*, *S. agalactiae*, and *S. alactolyticus*. Our data appear consistent with the results of DNA-DNA reassociation experiments which have recently demonstrated the synonymy of these species.

The strains of *S. porcinus*, *S. pyogenes*, or *S. pneumoniae* cluster together within their respective species, including whatever Lancefield's serogroups or serotypes they belong, allowing a rapid species identification. Since similar conserved patterns were observed for different strains within the same species, *rrn*-ARDRA may have a reduced epidemiological (typing) interest. *rrn*-ARDRA could not differentiate between the two subspecies of *S. dysgalactiae* and *S. canis*. These species are part of a large and heterogeneous group of C, G, or L streptococci associated with human and animal infections. *Hha*I, *Mbo*II, and *Sau*3A patterns are quite similar for these three species as well, although DNA homology between species is between 45 and 75% (32) and 16S rRNA similitude value is limited to 96 to 97% (1). However, the classification of this group remained controversial as no test was clearly capable of differentiating these species (9, 29, 32).

Similarly, identification of strains belonging to *S. pneumoniae*, *S. oralis*, and *S. mitis* to the species level is also difficult with *rrn*-ARDRA. These three species constitute a single cluster of restriction patterns and the distinct differences seen between the three type strains are not observed with other species. Similar results were found using analysis of superoxide dismutase (19, 24) or the 16S rRNA gene sequence (1). *S. pneumoniae*, *S. oralis*, and *S. mitis* were demonstrated to form a bacterial complex in which both biochemical or genotypical differentiation may be difficult (K. Poulsen and M. Kilian, ASM Conf. Streptococcal Genet., abstr. 2D-05, 1998). In contrast, species belonging to the streptococcal group of *S. sanguinis*, *S. parasanguinis*, and *S. gordonii* displayed restriction patterns clearly separated from the *S. pneumoniae*, *S. oralis*, and *S. mitis* complex.

The data presented here show that PCR amplification followed by restriction analysis of the amplified products is a reliable and easy-to-use method of identification of streptococci. It has been demonstrated on a limited scale to be capable of identifying clinical isolates that were phenotypically mis- or not identified and appears to be a promising method for identification of pathogenic streptococci.

As observed for identification of *Enterobacteriaceae* or *Acinetobacter*, the performances of *rrn*-ARDRA were found excellent for identification of gram-positive, catalase negative cocci belonging to the genus *Streptococcus*. In order to improve these performances, determination of restriction patterns of large set of bacteria have to be conducted and databases of patterns have to be extended. Using our modified *rrn*-ARDRA protocol, a single database which combine the three restriction patterns obtained for each strain, may allow a rapid identification irrespective of the Gram staining or the morphological characteristics of the isolate. We propose to use this method in reference centers for identification of phenotypically unidentified isolates after construction and evaluation of specific databases of interest.

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