Identification of clinically relevant viridans streptococci by analysis of transfer DNA intergenic spacer length polymorphism

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The utility of PCR analysis of transfer DNA intergenic spacer length polymorphism (tDNA-ILP) for the identification to the species level of clinically relevant viridans streptococci was evaluated with a collection of reference strains of 15 species of the salivarius, anginosus, mitis and mutans rRNA homology groups. PCR products generated by using fluorescent, outwardly directed, consensus tDNA primers were analysed by electrophoresis on denaturing polyacrylamide gels and by laser fluorescence scanning. Eleven species showed specific and distinct tDNA patterns: *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus sanguinis*, *Streptococcus parasanguinis*, *Streptococcus anginosus*, *Streptococcus mutans*, *Streptococcus criceti* and *Streptococcus rattii*. Indistinguishable patterns were obtained among two groups of species: *Streptococcus vestibularis* and *Streptococcus salivarius* on the one hand and *Streptococcus constellatus* and *Streptococcus intermedius* on the other. *S. mitis* strains produced heterogeneous patterns that could be separated into three groups: a group containing *S. mitis* biovar 1 and two *S. mitis* biovar 2 groups, one of which clustered with *S. parasanguinis* strains while the other showed patterns unrelated to other species. These results agree in part with protein electrophoretic analysis showing that *S. mitis* biovar 2 strains belong to several streptococcal taxa. In conclusion, PCR analysis of tDNA-ILP holds promise for rapid identification of viridans streptococci that are difficult to identify by phenotypic tests.

Keywords: viridans streptococci, transfer DNA intergenic spacer length polymorphism analysis, genotypic identification

INTRODUCTION

The viridans streptococci currently encompass 20 species, which are commensal inhabitants of the oropharyngeal cavity and the gastrointestinal and genital tracts of mammals (Hardie & Whaley, 1994). On the basis of 16S rRNA sequence homology, these bacteria are categorized in four groups: the salivarius rRNA homology group, including *Streptococcus thermophilus*, *Streptococcus vestibularis* and *Streptococcus salivarius*; the mitis group, including *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus pneumoniae*,

Streptococcus sanguinis and *Streptococcus parasanguinis*; the anginosus group, including *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*; and the mutans group, including *Streptococcus mutans*, *Streptococcus criceti*, *Streptococcus downei*, *Streptococcus ferus*, *Streptococcus macacae*, *Streptococcus rattii* and *Streptococcus sobrinus* (Hardie & Whaley, 1994; Bentley *et al.*, 1991; Kawamura *et al.*, 1995). In this study, we focused on the 15 species that are described as facultative pathogens in humans, causing a variety of infections including abscesses, bacteraemia, endocarditis and meningitis. Consequently, *S. thermophilus*, *S. downei*, *S. ferus* and *S. macacae*, which to our knowledge were not isolated from humans, and *S. sobrinus*, which is rarely reported in human infections, were not included.

Abbreviation: tDNA-ILP, transfer DNA intergenic spacer length polymorphism.

Table 1. Bacterial strains and tDNA-ILP patterns

Culture collections are abbreviated as follows: ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden; CDC, Centers for Disease Prevention and Control, Atlanta, GA, USA; LHMC, Department of Oral Microbiology, Royal London Hospital Medical College, London, UK; LMG, BCCM/LMG Culture Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium; NCFB, National Collection of Food Bacteria, Institute of Food Research, Reading Laboratory, Reading, UK; NMH, M. W. D. Wen, North Middlesex Hospital, London, UK. The tDNA-ILP types are as shown in Fig. 4.

Species	Strain	Other strain numbers	Reference	Strain isolation	tDNA-ILP type		
					Visual comparison	Pearson coefficient comparison	
Salivarius group							
<i>S. salivarius</i>	LMG 11489 ^T	NCFB 1779 ^T , CCUG 17825 ^T	Whiley & Hardie (1988)	Human blood	9	I	
	LMG 13103	NCFB 1777		Unknown	9	I	
	LMG 13107	NCFB 2700, strain M36	Whiley & Hardie (1988)	Unknown	9	I	
	LMG 13108	NCFB 2701, strain A385		Unknown	9	I	
<i>S. vestibularis</i>	LMG 13516 ^T	CCUG 24893 ^T , MM1 ^T , NCTC 12166 ^T	Whiley & Hardie (1988)	Human vestibular mucosa	9	I	
	LMG 14647	CCUG 24686, LV81, Carlsson87	Whiley & Hardie (1988)	Human vestibular mucosa	9	I	
	LMG 14645	CCUG 24684, LV71, Carlsson85	Whiley & Hardie (1988)	Human vestibular mucosa	9	I	
	LMG 14646	CCUG 24685, PV91, Carlsson86	Whiley & Hardie (1988)	Human vestibular mucosa	9	I	
Anginosus group							
<i>S. anginosus</i>	LMG 14502 ^T	CCUG 27298 ^T , NCTC 10713 ^T	Whiley & Hardie (1988)	Throat	2	B	
	LMG 14696	CCUG 223, NCTC 8037	Whiley & Hardie (1988)	Respiratory tract	2	B	
	LMG 17666	CCUG 28191, LHMC PC4890	Whiley & Hardie (1988)	Dental plaque	2	B	
	LMG 17832	CCUG 28192, LHMC NMH10	Whiley & Hardie (1988)	Perforated ulcer	2	B	
<i>S. constellatus</i>	LMG 17833	CCUG 28195, CDC 2236-81	Whiley & Hardie (1988)	Human blood	2	B	
	LMG 14503	CCUG 4215, NCTC 5389	Whiley & Hardie (1988)	Unknown	1	A	
	LMG 14504	CCUG 9569, NCTC 10708	Whiley & Hardie (1988)	Dental abscess	1	A	
	LMG 14507 ^T	CCUG 24889 ^T , NCFB 2226 ^T	Whiley & Hardie (1988)	Purulent pleurisy	1	A	
<i>S. intermedius</i>	LMG 17665	CCUG 28196, LHMC AM699	Whiley & Hardie (1988)	Dental plaque	1	A	
	LMG 17835	CCUG 28197, LHMC NMH1	Whiley & Hardie (1988)	Brain abscess	1	A	
	LMG 14548	CCUG 28203, LHMC NMH2	Whiley & Hardie (1988)	Brain abscess	1	A	
	LMG 14549	CCUG 28204, LHMC GN72	Whiley & Hardie (1988)	Unknown	1	A	
<i>Mitis group</i>	LMG 14550	CCUG 28205, LHMC AC4817	Whiley & Hardie (1988)	Dental plaque	1	A	
	LMG 17840 ^T	CCUG 17827 ^T , NCFB 2227 ^T	Whiley & Hardie (1988)	Unknown	1	A	
	<i>S. cristatus</i>	LMG 14512	CCUG 30424, LHMC AK1	Handley <i>et al.</i> (1991)	Throat swab	8	H
		LMG 16320 ^T	CCUG 33481 ^T , CR311 ^T	Handley <i>et al.</i> (1991)	Periodontal abscess	8	H
LMG 17206		LHMC CCSA	Handley <i>et al.</i> (1991)	Dental plaque	8	H	
LMG 17207		LHMC CR3	Handley <i>et al.</i> (1991)	Dental plaque	8	H	
<i>S. gordonii</i>	LMG 17842			Human blood	8	H	
	LMG 14515	CCUG 4216, NCTC 10231, SK5	Kilian <i>et al.</i> (1989)	(biovar 2)	4	D	
	LMG 14516	CCUG 18734, NCTC 3165, SK51	Kilian <i>et al.</i> (1989)	(biovar 3)	4	D	
	LMG 14517	CCUG 25607, SK6	Kilian <i>et al.</i> (1989)	(biovar 1)	4	D	
<i>S. oralis</i>	LMG 14518 ^T	CCUG 25608 ^T , SK3 ^T	Kilian <i>et al.</i> (1989)	Endocarditis (biovar 2)	4	D	
	LMG 17844	CCUG 25510, Carlsson31	Carlsson (1968)	Human oral cavity	4	D	
	LMG 14532 ^T	CCUG 24891 ^T , SK23 ^T	Kilian <i>et al.</i> (1989)	Human oral cavity	11	K	
	LMG 14533	CCUG 24892, SK2, ATCC 10557	Kilian <i>et al.</i> (1989)	Endocarditis	11	K	
<i>S. sanguinis</i>	LMG 14534	CCUG 27680, Carlsson50	Carlsson (1968)	Human oral cavity	11	K	
	LMG 14535	CCUG 27681, Carlsson51	Carlsson (1968)	Human oral cavity	11	K	
	LMG 14536	CCUG 27682, Carlsson39	Carlsson (1968)	Human oral cavity	11	K	
	LMG 14637	CCUG 25606, SK45	Kilian <i>et al.</i> (1989)	Human dental plaque (biovar 4)	7	G	
<i>S. mitis</i>	LMG 14638	CCUG 27744, SK72	Kilian <i>et al.</i> (1989)	Human dental plaque (biovar 1)	7	G	
	LMG 14639	CCUG 27745, SK77	Kilian <i>et al.</i> (1989)	Human dental plaque (biovar 1)	7	G	
	LMG 14656	CCUG 25604, SK164	Kilian <i>et al.</i> (1989)	Human dental plaque (biovar 2)	7	G	
	LMG 14657	CCUG 25605, SK150	Kilian <i>et al.</i> (1989)	Human dental plaque (biovar 3)	7	G	
<i>S. parasanguinis</i>	LMG 14702 ^T	CCUG 17286 ^T , ATCC 10556 ^T , SK1 ^T	Kilian <i>et al.</i> (1989)	Endocarditis (biovar 1)	7	G	
	LMG 14554	CCUG 27740, SK135	Kilian <i>et al.</i> (1989)	Human oral cavity (biovar 1)	10	J	
	LMG 14555	CCUG 27741, SK138	Kilian <i>et al.</i> (1989)	Human dental plaque (biovar 1)	10	J	
	LMG 14557 ^T	CCUG 31611 ^T , NCTC 12261 ^T , SK142 ^T	Kilian <i>et al.</i> (1989)	Human oral cavity (biovar 1)	10	J	
<i>S. pneumoniae</i>	LMG 14553	CCUG 25611, SK34	Kilian <i>et al.</i> (1989)	Human oral cavity (biovar 2)	12	L	
	LMG 14556	CCUG 27743, SK79	Kilian <i>et al.</i> (1989)	Human oral cavity (biovar 2)	12	L	
	LMG 17594	CCUG 27742, SK71	Kilian <i>et al.</i> (1989)	Human oral cavity (biovar 2)	14	M	
	LMG 17595	CCUG 21026, SK132	Kilian <i>et al.</i> (1989)	Ulcerated sore throat (biovar 2)	13	M	
<i>S. pneumoniae</i>	LMG 17596	CCUG 35789, SK96	Kilian <i>et al.</i> (1989)	Human oral cavity (biovar 2)	12	L	
	LMG 14537 ^T	CCUG 30417 ^T , LHMC SS898 ^T	Whiley & Hardie (1988)	Throat	13	M	
	LMG 14538	CCUG 30418, LHMC SS897	Whiley & Hardie (1988)	Throat	13	M	
	LMG 14540	CCUG 30420, LHMC 3156-81	Whiley & Hardie (1988)	Blood	13	M	
<i>S. pneumoniae</i>	LMG 17852	CCUG 30423, LHMC MGH 413	Whiley & Hardie (1988)	Urine	13	M	
	LMG 14542	CCUG 2839A		Human blood	6	F	
	ATCC 6301				6	F	
	LMG 14544	CCUG 10708		Acute otitis	5	E	
	ATCC 49619		Jorgensen <i>et al.</i> (1992)	Sputum	5	E	
	ATCC 49620			Sputum	5	E	

Table 1 (cont.)

Species	Strain	Other strain numbers	Reference	Strain isolation	tDNA-ILP type	
					Visual comparison	Pearson coefficient comparison
Mutans group						
<i>S. mutans</i>	LMG 14558 ^T	CCUG 11877 ^T , NCTC 10449 ^T	Coykendall (1977)	Human decayed tooth	3	C
	LMG 14560	CCUG 32092		Human blood, endocarditis	3	C
<i>S. criceti</i>	LMG 14508 ^T	CCUG 27300 ^T , NCFB 2720 ^T , strain HS6 ^T	Coykendall (1977)	Hamster caries lesion	15	N
	LMG 14511	CCUG 28071, strain AHT		Human caries lesion	15	N
<i>S. rattii</i>	LMG 14650 ^T	CCUG 27502 ^T , ATCC 19645 ^T	Coykendall (1977)	Laboratory rat	16	O
	LMG 14651	CCUG 27641, strain BHT		Human	16	O

Analysis of transfer DNA intergenic spacer length polymorphism (tDNA-ILP), based on PCR amplification of spacers between tRNA genes, was proposed by McClelland *et al.* (1992) to distinguish streptococcal strains of groups A, B and G and *S. mutans* strains at the species level. This technique has been applied successfully to the identification of *Staphylococcus* (Welsh & McClelland, 1991, 1992; Maes *et al.*, 1997), *Acinetobacter* (Ehrenstein *et al.*, 1996) and *Listeria* species (Vanechoutte *et al.*, 1998). In this study, we evaluated the discriminatory power of tDNA-ILP analysis, using laser scanning of fluorescently labelled amplicons, for identification of reference and type strains of clinically significant species of viridans streptococci. In addition, the clustering of tDNA-ILP patterns was compared with phylogenetic trees obtained from sequence data from 16S rRNA (Kawamura *et al.*, 1995), superoxide dismutase (Poyart *et al.*, 1998) and D-alanine:D-alanine ligase (Garnier *et al.*, 1997) genes to determine its phylogenetic significance.

METHODS

Bacterial strains. Type and reference strains ($n = 66$) from the BCCM/LMG and ATCC culture collections are described in Table 1.

Preparation of DNA. Bacteria were cultured on blood-agar plates for 24 h at 37 °C in an atmosphere supplemented with 10% CO₂. Genomic DNA was extracted from cells in a procedure that used sequentially lysozyme, guanidium thiocyanate, ammonium acetate, chloroform/isoamyl alcohol, 2-propanol and ethanol, as described previously (Pitcher *et al.*, 1989).

tDNA-ILP PCR assay. PCR was carried out with the outwardly directed tDNA consensus primers T5A (5'-AGTCCGGT-GCTCTAACCAACTGAG) and T3B (5'-AGGTCGCG-GGTTCTGAATCC) described by Welsh & McClelland (1991). Primer T5A was 5'-end-labelled with carbocyanine dye Cy5 (Pharmacia Biotech). Fifty microlitres of PCR solution contained 1.25 U *Taq* polymerase (Cetus Corp), 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 8.3), 1.5 mM MgCl₂, 0.5 µM of each primer, 0.2 mM of the four dNTPs and 5 µl DNA extract. The reaction mixture was overlaid with a drop of mineral oil. Amplification conditions were as described previously (Welsh & McClelland, 1991).

Analysis of tDNA-ILP. The amplification products were separated by electrophoresis through 6% (w/v)

acrylamide/bisacrylamide/7 M urea denaturing gels (ReadyMix Gel, ALF grade; Pharmacia) in 1 × TBE buffer. A fluorescein-labelled molecular marker (Cy5 Sizer 50–500; Pharmacia), including ten fragments ranging from 50 to 500 bp, was used as an external sizing ladder and placed in every sixth lane. Samples including 1.25 µl of the PCR products, 5 µl gel-loading solution (Pharmacia) and 0.4 µl each of 50 bp and 1000 bp internal reference standards were denatured at 100 °C for 3 min and loaded onto the gel. Electrophoretic separation was done by using the ALFexpress DNA sequencer (Pharmacia) at 1500 V, 60 mA and 30 W for 4 h at 40 °C. Gels were normalized in two steps: by alignment firstly with external standards and then with internal standards. Fluorescence densitograms were produced by using the FRAGMENT MANAGER software (Pharmacia) and compared visually. Digitized densitograms were exported to and analysed with the GELCOMPAR software (Applied Maths). Similarity matrices of Pearson product-moment correlation coefficients between pairs of PCR patterns were used for construction of a similarity dendrogram by using the unweighted pair group method with averages (UPGMA) algorithm.

Reproducibility evaluation. Duplicate bacterial lysates of each type strain were co-amplified in the same PCR experiment and in separate PCR experiments. These repeat samples were analysed in the same gel for between-run and between-extract pattern reproducibility. Between-gel reproducibility was assessed by analysing one amplicon (*S. cristatus* LMG 16320^T) in three different gels. Fluorescence densitograms were compared visually and the reproducibility of normalized tDNA-ILP patterns was evaluated by using Pearson product-moment correlation coefficients.

RESULTS

Reproducibility

Between-extract, between-run and between-gel comparisons of tDNA-ILP patterns showed complete reproducibility by visual analysis of fluorescence densitograms. The number and size of DNA fragments (densitogram peak positions) were highly reproducible. Small variations occurred only in amplified product concentration (peak heights). Normalized tDNA-ILP patterns showed a mean 95% similarity coefficient (range 91–99%) for between-extract and between-run comparisons of the 15 species. Between-gel comparison of tDNA-ILP patterns showed a mean 94.2% similarity coefficient (range 92.9–96.3%). On

Fig. 1. Fluorescence densitogram of tDNA-ILP patterns of 15 type strains of clinically relevant viridans streptococci. The molecular mass marker was a 50–500 bp Cy5-labelled external standard.

the basis of these data, the threshold of pattern identity was defined as 91 % similarity.

Visual comparison of tDNA-ILP patterns

Type and reference strains ($n = 66$) produced 16 distinct patterns of eight to twelve DNA fragments, which varied in size from 60 to 470 bp (Table 1 and Figs 1–3). Easily distinguishable, species-specific patterns were produced by strains of *S. cristatus*, *S. gordonii*, *S. oralis*, *S. mitis* biovar 1, *S. pneumoniae*, *S. sanguinis*, *S. parasanguinis*, *S. anginosus*, *S. mutans*, *S. criceti* and *S. ratti*. Indistinguishable patterns were obtained among two groups of species: *S. vestibularis* and *S. salivarius* on the one hand and *S. constellatus* and *S. intermedius* on the other (Fig. 1 and Table 1).

Reference strains of a given species produced patterns indistinguishable from the corresponding type strain, with the exception of *S. mitis* and *S. pneumoniae*. *S.*

Fig. 2. Fluorescence densitogram of tDNA-ILP patterns of *S. parasanguinis* type strain and *S. mitis* strains. tDNA-ILP types are indicated in parentheses by numbers as defined by visual comparison and upper-case letters as defined by computer analysis. The arrow points to the additional peak of 153 bp in *S. mitis* strain LMG 17594. Molecular mass marker as in Fig. 1.

mitis biovar 1 strains produced patterns identical to the type strain, whereas *S. mitis* biovar 2 strains produced patterns separated into two groups. The first group included two strains: *S. mitis* LMG 17595, which produced a tDNA-ILP pattern indistinguishable from the *S. parasanguinis* type strain, and LMG 17594, which produced a pattern that differed from the *S. parasanguinis* type strain pattern by the presence of an additional peak of 153 bp (Fig. 2, arrow). The second group included three strains: *S. mitis* LMG 14556, LMG 17596 and LMG 14553, which produced common patterns, distinct from all others (Fig. 2). *S. pneumoniae* strains produced two patterns: strains LMG 14542 and ATCC 6301 produced identical

Fig. 3. Fluorescence densitogram of tDNA-ILP patterns of *S. pneumoniae* strains. tDNA-ILP types are indicated in parentheses as in Fig. 2. Horizontal arrows indicate an additional 156 bp peak in three strains. Molecular mass marker as in Fig. 1.

patterns, distinct from the pattern shared by strains LMG 14544, ATCC 49619 and ATCC 49620, which produced an additional peak of 156 bp (Fig. 3, horizontal arrows).

Computer-assisted analysis of tDNA-ILP patterns

At the 91% similarity cut-off level, 15 clusters were obtained by comparison of the Pearson similarity coefficients of normalized tDNA-ILP patterns, which displayed between 30 and 99% similarity (Fig. 4). Reference and type strains within the same species clustered together at a mean of 95.4% (range 92–99%) pattern similarity, except for *S. pneumoniae* and *S. mitis* strains. *S. salivarius* and *S. vestibularis* strains grouped in a single cluster, with 91% pattern similarity, as did *S. constellatus* and *S. intermedius* strains, with 92% similarity. These two latter species displayed 90% pattern similarity with *S. anginosus* strains, which were then grouped separately on the basis of this borderline value below the pattern identity level.

Fig. 4. Dendrogram of tDNA-ILP pattern relatedness of type and reference strains of viridans streptococci constructed by the UPGMA method. The threshold of pattern identity is indicated by a vertical dotted line. The minor discrepancy noted between visual and computer-assisted pattern clustering is indicated in bold type.

Only one minor discrepancy was noted between the visual and computer-assisted pattern clustering. *S. mitis* LMG 17594 was more than 91% similar to the *S. parasanguinis* cluster, whereas a minor additional DNA fragment (approximate size 153 bp) was observed by visual inspection (Fig. 2, arrow).

DISCUSSION

Identification of viridans streptococci to the species level is clinically relevant for several reasons. Firstly, certain species (e.g. *S. anginosus*, *S. constellatus* and *S. intermedius*) have been associated with different sites of infections (Hardie & Whiley, 1994; Whiley *et al.*, 1990). Secondly, antimicrobial susceptibility profiles vary among species (Hardie & Whiley, 1994; Doern *et al.*, 1996). For instance, strains of *S. mitis* are more frequently reported as penicillin resistant than other

species of viridans streptococci. In the clinical laboratory, the currently available methods of identification of streptococci are based on phenotypic characters, including colonial morphology, growth characteristics and biochemical reactions, such as those assayed in the API Rapid ID32 Strep system (Coykendall, 1989; Hilman *et al.*, 1989; Kilian *et al.*, 1989; Tardif *et al.*, 1989; Whiley *et al.*, 1990; Beighton *et al.*, 1991; Freney *et al.*, 1992). However, intrinsic strain variability and overlapping characters between species frequently leads to equivocal results or inaccurate identification of certain species (Hilman *et al.*, 1989; Kilian *et al.*, 1989; Tardif *et al.*, 1989). Strains of the anginosus and mitis groups are the most problematic in this regard (Coykendall, 1989; Kilian *et al.*, 1989; Beighton *et al.*, 1991; Freney *et al.*, 1992).

Molecular methods have been developed for the identification of viridans streptococci to the species level (Rudney & Larson, 1993; Ezaki *et al.*, 1988; Jayarao *et al.*, 1992; McClelland *et al.*, 1992; Whiley *et al.*, 1995; Garnier *et al.*, 1997; Poyart *et al.*, 1998; Vandamme *et al.*, 1998). Among recently described genotypic techniques, size analysis of internal portions of the D-alanine:D-alanine ligase gene by PCR allowed the identification of *S. mitis*, *S. gordonii*, *S. oralis*, *S. sanguinis*, *S. mutans* and *S. salivarius*, and the distinction at the group level of strains of *S. anginosus*, *S. constellatus* and *S. intermedius* (Garnier *et al.*, 1997). Sequence analysis of the superoxide dismutase gene enabled identification of strains of all species of viridans streptococci except for *S. cristatus*, which was not studied (Poyart *et al.*, 1998). A molecular phenotypic method, electrophoresis of whole-organism proteins, differentiated most species among viridans streptococci except for strains of the anginosus group, which could not be distinguished (Vandamme *et al.*, 1998). In addition, *S. mitis* biovar 2 strains were shown by this method to consist of several taxa.

In this study, we examined the usefulness of tDNA-ILP analysis, which has been proposed as a genotypic method for bacterial identification (Welsh & McClelland, 1991, 1992; McClelland *et al.*, 1992). These authors reported that this method allowed the distinction between species of staphylococci and streptococci, based on a limited sample of species and strains. In our study, we extended their findings by applying the method to the identification of 66 well-characterized viridans streptococcal strains belonging to 15 species, known to be clinically relevant and difficult to identify by conventional methods (Coykendall, 1989; Hardie & Whiley, 1994; Hilman *et al.*, 1989; Kilian *et al.*, 1989; Tardif *et al.*, 1989; Whiley *et al.*, 1990; Beighton *et al.*, 1991; Freney *et al.*, 1992; Kawamura *et al.*, 1995). Since tDNA spacers vary in size between species of a given genus by only a few base pairs (Vold, 1985; McClelland *et al.*, 1992), we amplified these spacers using fluorescently labelled primers and analysed DNA fragments by PAGE on a DNA sequencer, as reported previously (Ehrenstein *et al.*, 1996; Maes *et al.*, 1997).

The salivarius rRNA homology group

Surprisingly, tDNA-ILP analysis could not distinguish between *S. salivarius* and *S. vestibularis*, two species of the salivarius rRNA homology group associated with human infection (Coykendall, 1989; Hardie & Whiley, 1994; Whiley & Hardie, 1988). This is in contrast with results reported with various methods, including DNA-DNA hybridization, biochemical tests (Whiley & Hardie, 1988; Beighton *et al.*, 1991; Freney *et al.*, 1992), superoxide dismutase gene sequence determination (Poyart *et al.*, 1998) and whole-cell protein electrophoretic analysis (Vandamme *et al.*, 1998), which demonstrated unambiguously that these taxa represent two different species.

The anginosus rRNA homology group

There is an ongoing controversy among taxonomists who argue for and against the inclusion of organisms of this rRNA homology group into a single species, as described by Coykendall *et al.* (1987). The currently accepted subdivision into three species, *S. anginosus*, *S. constellatus* and *S. intermedius*, is supported by distinguishing phenotypic characteristics (Whiley & Beighton, 1991), by clinical correlates such as distinct organ distribution of pyogenic infections (Whiley *et al.*, 1990) and by sequence divergence of the superoxide dismutase gene (Poyart *et al.*, 1998). On the other hand, whole-cell protein analysis (Vandamme *et al.*, 1998) and sequence analysis of the D-alanine:D-alanine ligase gene (Garnier *et al.*, 1997) showed this taxon to be homogeneous. In the present study, tDNA-ILP differentiated *S. anginosus* strains, by a minor variation only, from *S. constellatus* and *S. intermedius*, which showed a common pattern. Strains of all three species, however, clustered closely together. In contrast to our observation, minor variation in 16S-23S rRNA intergenic spacers distinguished *S. constellatus* from the two other species of this group (Whiley *et al.*, 1995). This discrepancy illustrates the divergence between tRNA and rRNA gene organization, as described in *Bacillus* species (Vold, 1985).

The mitis rRNA homology group

Five of seven species in this group displayed species-specific tDNA-ILP patterns, irrespective of their biovar status (Table 1). Heterogeneous patterns were observed among *S. pneumoniae* strains, suggesting variation of tDNA gene organization. This is in contrast with superoxide dismutase gene sequence data, which showed that *S. pneumoniae* strains displayed the highest level of intraspecies sequence identity of all the species studied by Poyart *et al.* (1998). However, all *S. pneumoniae* strains could be identified correctly by tDNA-ILP analysis, as they clustered separately from all other taxa.

S. mitis strains showed an even more marked heterogeneity of tDNA-ILP patterns and were separated into three distantly related groups. Biovar 1 strains

clustered in a separate group, close to the *S. oralis* cluster. Biovar 2 strains were subdivided into two groups: strains LMG 17594 and LMG 17595 clustered with *S. parasanguinis*, while strains LMG 14556, LMG 17596 and LMG 14553 produced a distinct pattern. In contrast, by whole-cell protein analysis, LMG 14556 was identified as a strain of *S. cristatus* and LMG 17594 as *S. oralis*, while LMG 17596 occupied a distinct position in the dendrogram (Vandamme *et al.*, 1998). The nature of the discrepancies between tDNA-ILP analysis and whole-cell protein analysis is unknown. *S. mitis* biovar 2 strains were previously divided into two sub-groups on the basis of phenotypic characteristics by Kilian *et al.* (1989). These groups were the same as those observed for tDNA-ILP patterns with the exception of strain LMG 17594, which produced a tDNA-ILP pattern identical to that of *S. parasanguinis*. Interestingly, the need for further characterization of *S. mitis* biovar 2 strains was proposed by Kilian *et al.* (1989). We agree with this proposal and propose that whole-genome DNA-DNA hybridization would be needed in order to establish their correct identity.

The mutans rRNA homology group

The three species examined produced different patterns and could easily be distinguished from the other species, in agreement with the resolution provided by other molecular methods (Poyart *et al.*, 1998; Vandamme *et al.*, 1998).

Comparison with phylogenetic data

The topology of the phenogram of tDNA-ILP patterns above species level did not exhibit congruence with phylogenetic trees derived from sequences of 16S rRNA genes (Bentley *et al.*, 1991; Kawamura *et al.*, 1995) and the superoxide dismutase gene (Poyart *et al.*, 1998). This is not unexpected, and is presumably related to the small size of the genomic region examined for polymorphism by this assay. However, in another study of tDNA-ILP analysis of staphylococci, Maes *et al.* (1997) observed a congruence between the phenogram topology based on this assay and that of ribotype analysis.

Conclusions

PCR analysis of tDNA-ILP of reference strains of viridans streptococci known to be human pathogens revealed significant species homogeneity of tDNA gene organization and a high degree of interspecies heterogeneity, allowing the identification of 11 species at the species level and of four species at the rRNA homology group level. Observation of heterogeneous patterns in two species emphasizes the need to evaluate more than one reference strain of each species. The assay is rapid to perform and computer-assisted pattern analysis would allow easy classification of unknown strains. We conclude that this method should

be evaluated on a large set of clinical isolates. The assay offers potential usefulness in the clinical laboratory, especially as a back-up method for the identification of problem strains that can not be identified by routine phenotypic tests, e.g. *S. mitis* and *S. oralis* strains.

ACKNOWLEDGEMENTS

This work was supported in part by a grant to Y. D. from the Fondation Erasme. P. V. is indebted to the Fund for Scientific Research-Flanders (Belgium) for a position as a post-doctoral research fellow. We thank N. Maes, A. Deplano, F. Brancart, E. Godfroid and C. Liesnard for technical advice and critical comments.

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