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Whole-cell protein electrophoretic analysis of viridans streptococci: evidence for heterogeneity among *Streptococcus mitis* biovars

P. Vandamme,[1,2] U. Torck,[1] E. Falsen,[3] B. Pot,[4] H. Goossens[2] and K. Kersters[1]

Author for correspondence: P. Vandamme. Tel: +32 9 264 51 13. Fax: +32 9 264 50 92.
e-mail: Peter.Vandamme@rug.ac.be

[1,4] Laboratory of Microbiology[1], and BCCM™/LMG Culture Collection[4], Ledeganckstraat 35, University of Ghent, B-9000 Ghent, Belgium

[2] Laboratory of Medical Microbiology, University Hospital Antwerp UIA, Antwerp, Belgium

[3] CCUG Culture Collection, Department of Clinical Bacteriology, University of Göteborg, S-413 46 Göteborg, Sweden

One hundred reference strains representing all species belonging to the different phylogenetic lineages of the viridans streptococci were examined by means of one-dimensional whole-organism protein electrophoresis. For most of the species examined, multiple strains characterized by DNA–DNA hybridization were included and, wherever described, representatives of different biochemical variants were analysed. Most species were clearly differentiated. The data support the viewpoint that members of the *Streptococcus anginosus* group constitute a single species and indicate that *Streptococcus mitis* biovar 2 is a heterogeneous taxon comprising strains from several streptococcal species.

Keywords: viridans streptococci, *Streptococcus mitis*, whole-cell protein analysis

INTRODUCTION

The differentiation of species belonging to the viridans streptococci has always been tedious, and species-level identification of all of these species is beyond the reach of many laboratories. Amongst the streptococci, several major phylogenetic lineages can be discriminated, four of which comprise viridans species: the *Streptococcus anginosus* rRNA homology group with the species *S. anginosus*, *Streptococcus intermedius* and *Streptococcus constellatus*; the *Streptococcus mitis* rRNA homology group with *S. mitis*, *Streptococcus oralis*, *Streptococcus gordonii*, *Streptococcus sanguis* and *Streptococcus parasanguis*; the *Streptococcus mutans* rRNA homology group with *S. mutans*, *Streptococcus cricetus*, *Streptococcus downei*, *Streptococcus ferus*, *Streptococcus macacae*, *Streptococcus rattus* and *Streptococcus sobrinus*; and finally, the *Streptococcus thermophilus* rRNA homology group with *S. thermophilus*, *Streptococcus salivarius* and *Streptococcus vestibularis* (2, 9). The phylogenetic position of *Streptococcus crista* is yet to be determined, but this species shares rather high DNA–DNA hybridization values with *S. sanguis* and *S. gordonii* strains, indicating that it belongs to the *S. mitis* rRNA homology group as well (7). In addition, it has been demonstrated that *Streptococcus pneumoniae* also belongs to the *S. mitis* rRNA homology group (2). Most pneumococci are however easily identified, have a different pathology and are often not included in comparative studies of viridans streptococci. Pneumococcal reference strains were examined but they did not grow under the conditions used in the present study. The remaining major streptococcal phylogenetic lineages comprise the pyogenic streptococci and the species belonging to the *Streptococcus bovis* group (2, 9).

In previous studies, we demonstrated the ability of comparative whole-organism protein electrophoresis for the differentiation of pyogenic streptococci (15, 16). As in many other Gram-positive and Gram-negative bacteria, streptococcal strains with very similar whole-cell protein patterns shared high DNA–DNA hybridization values as was deduced from the inclusion of strains for which DNA–DNA hybridization data were available. In the present study, we evaluate the suitability of whole-cell protein electrophoresis for the differentiation of viridans streptococci by examining a collection of well-characterized reference strains, including a considerable number of strains for which DNA–DNA hybridization data are available.

Recently, the specific epithets of several streptococcal species were corrected to conform to Rule 12c of the

Table 1. Strains examined

Strain	Other strain no.*	Reference	Strain information
***Streptococcus anginosus* group**			
Streptococcus anginosus LMG 14502^{T}	CCUG 27298, NCTC 10713	Whiley & Beighton (20)	Throat
Streptococcus anginosus LMG 14696	CCUG 223, NCTC 8037	Whiley & Beighton (20)	Respiratory tract
Streptococcus anginosus LMG 17666	CCUG 28191, LHMC PC4890	Whiley & Beighton (20)	Dental plaque
Streptococcus anginosus LMG 17832	CCUG 28192, LHMC NMH 10	Whiley & Beighton (20)	Perforated ulcer
Streptococcus anginosus LMG 17833	CCUG 28195, CDC 2236-81	Whiley & Beighton (20)	Blood
Streptococcus constellatus LMG 14503	CCUG 4215, NCTC 5389	Whiley & Beighton (20)	Unknown
Streptococcus constellatus LMG 14504	CCUG 9569, NCTC 10708	Whiley & Beighton (20)	Dental abscess
Streptococcus constellatus LMG 14507^{T}	CCUG 24889, NCFB 2226	Whiley & Beighton (20)	Purulent pleurisy
Streptococcus constellatus LMG 17665	CCUG 28196, LHMC AM 699	Whiley & Beighton (20)	Dental plaque
Streptococcus constellatus LMG 17835	CCUG 28197, LHMC NMH 1	Whiley & Beighton (20)	Brain abscess
Streptococcus constellatus LMG 17836	CCUG 28198, LHMC UNS 55	Whiley & Beighton (20)	Appendix
Streptococcus constellatus LMG 17837	CCUG 28199, LHMC EF 52	Whiley & Beighton (20)	Dental plaque
Streptococcus constellatus LMG 17838	CCUG 28200, LHMC PC 67	Whiley & Beighton (20)	Dental plaque
Streptococcus constellatus LMG 17839	CCUG 28201, LHMC NMH 12	Whiley & Beighton (20)	Submandibular abscess
Streptococcus intermedius LMG 17840^{T}	CCUG 17827, NCFB 2227	Whiley & Beighton (20)	Unknown
Streptococcus intermedius LMG 14548	CCUG 28203, LHMC NHM 2	Whiley & Beighton (20)	Brain abscess
Streptococcus intermedius LMG 17841	CCUG 28204, LHMC GN72	Whiley & Beighton (20)	Dental plaque
Streptococcus intermedius LMG 14550	CCUG 28205, LHMC AC4817	Whiley & Beighton (20)	Dental plaque
***Streptococcus mitis* group**			
Streptococcus cristatus LMG 14512	LMG 17205, CCUG 30424, LHMC AK1	Handley *et al.* (7)	Throat swab
Streptococcus cristatus LMG 16320^{T}	CCUG 33481, CR311	Handley *et al.* (7)	Periodontal abscess
Streptococcus cristatus LMG 17206	LHMC CCSA	Handley *et al.* (7)	Dental plaque
Streptococcus cristatus LMG 17207	LHMC CR3	Handley *et al.* (7)	Dental plaque
Streptococcus cristatus LMG 17842			Human blood
Streptococcus gordonii LMG 14515	CCUG 4216, NCTC 10231, SK 5	Kilian *et al.* (10)	(Biovar 2)
Streptococcus gordonii LMG 14516	LMG 14648, CCUG 18374, NCTC 3165, SK 51	Kilian *et al.* (10)	(Biovar 3)
Streptococcus gordonii LMG 14517	CCUG 25607, SK 6	Kilian *et al.* (10)	(Biovar 1)
Streptococcus gordonii LMG 14518^{T}	CCUG 25608, SK 3	Kilian *et al.* (10)	Endocarditis (biovar 2)
Streptococcus gordonii LMG 14519	CCUG 27309, SK 86	Kilian *et al.* (10)	Dental plaque (biovar 2)
Streptococcus gordonii LMG 17843	CCUG 27509, Carlsson 30	Carlsson (3)	Human oral cavity
Streptococcus gordonii LMG 17844	CCUG 27510, Carlsson 31	Carlsson (3)	Human oral cavity
Streptococcus gordonii LMG 17845	CCUG 27511, Carlsson 32	Carlsson (3)	Human oral cavity
Streptococcus gordonii LMG 17846	CCUG 27515, Carlsson 36	Carlsson (3)	Human oral cavity
Streptococcus mitis LMG 14552	R-107,† CCUG 4212, NCTC 10712, SK 113	Kilian *et al.* (10)	Human sputum (biovar 1)
Streptococcus mitis LMG 14554	CCUG 27740, SK135	Kilian *et al.* (10)	Human oral cavity (biovar 1)
Streptococcus mitis LMG 14555	CCUG 27741, SK 138	Kilian *et al.* (10)	Human dental plaque (biovar 1)
Streptococcus mitis LMG 14557^{T}	CCUG 31611, NCTC 12261, SK 142	Kilian *et al.* (10)	Human oral cavity (biovar 1)
Streptococcus mitis LMG 14553	CCUG 25611, SK 34	Kilian *et al.* (10)	Human oral cavity (biovar 2)
Streptococcus mitis LMG 14556	CCUG 27743, SK 79	Kilian *et al.* (10)	Human oral cavity (biovar 2)
Streptococcus mitis LMG 17594	CCUG 27742, SK 71	Kilian *et al.* (10)	Human oral cavity (biovar 2)
Streptococcus mitis LMG 17595	CCUG 21026, SK 132	Kilian *et al.* (10)	Ulcerated sore throat (biovar 2)
Streptococcus mitis LMG 17596	CCUG 35789, SK 96	Kilian *et al.* (10)	Human oral cavity (biovar 2)
Streptococcus mitis LMG 17597	CCUG 35792, SK 148	Kilian *et al.* (10)	Human dental plaque (biovar 2)
Streptococcus oralis LMG 14532^{T}	CCUG 24891, SK 23	Kilian *et al.* (10)	Human oral cavity
Streptococcus oralis LMG 14533	LMG 14649, CCUG 24892, SK 2, ATCC 10557	Kilian *et al.* (10)	Endocarditis
Streptococcus oralis LMG 14534	CCUG 27680, Carlsson 50	Carlsson (3)	Human oral cavity
Streptococcus oralis LMG 14535	CCUG 27681, Carlsson 51	Carlsson (3)	Human oral cavity
Streptococcus oralis LMG 14536	CCUG 27682, Carlsson 39	Carlsson (3)	Human oral cavity
Streptococcus oralis LMG 17851	CCUG 27679, Carlsson 49	Carlsson (3)	Human oral cavity
Streptococcus parasanguinis LMG 14537^{T}	CCUG 30417, LHMC SS898	Whiley *et al.* (21)	Throat
Streptococcus parasanguinis LMG 14538	R-112,† CCUG 30418, LHMC SS897	Whiley *et al.* (21)	Throat
Streptococcus parasanguinis LMG 14539	CCUG 30419, LHMC 2059-87	Whiley *et al.* (21)	Blood
Streptococcus parasanguinis LMG 14540	CCUG 30420, LHMC 2156-81	Whiley *et al.* (21)	Blood
Streptococcus parasanguinis LMG 17852	CCUG 30423, LHMC MGH 413	Whiley *et al.* (21)	Urine
Streptococcus parasanguinis LMG 17853	CCUG 30422, LHMC MGH 143	Whiley *et al.* (21)	Urine
Streptococcus sanguinis LMG 14637	R-105,† CCUG 25606, SK 45	Kilian *et al.* (10)	Human dental plaque (biovar 4)
Streptococcus sanguinis LMG 14638	R-115,† CCUG 27744, SK 72	Kilian *et al.* (10)	Human dental plaque (biovar 1)
Streptococcus sanguinis LMG 14639	R-116,† CCUG 27745, SK 77	Kilian *et al.* (10)	Human dental plaque (biovar 1)
Streptococcus sanguinis LMG 14656	CCUG 25604, SK 164	Kilian *et al.* (10)	Human dental plaque (biovar 2)
Streptococcus sanguinis LMG 14657	CCUG 25605, SK 150	Kilian *et al.* (10)	Human dental plaque (biovar 3)
Streptococcus sanguinis LMG 14702^{T}	R-104,† CCUG 17826, ATCC 10556, SK1	Kilian *et al.* (10)	Endocarditis (biovar 1)
***Streptococcus mutans* group**			
Streptococcus cricetus LMG 14508^{T}	CCUG 27300, NCFB 2720, strain HS6	Coykendall (4)	Hamster caries lesion
Streptococcus cricetus LMG 14511	CCUG 28071, strain AHT	Coykendall (4)	Human caries lesion
Streptococcus downei LMG 14514^{T}	CCUG 24890, NCTC 11391	Whiley *et al.* (19)	Dental plaque, monkey
Streptococcus ferus LMG 16520^{T}	CCUG 34784, DSM 20646	Coykendall (5)	Oral cavity, rat
Streptococcus macacae LMG 15097^{T}	NCTC 11558, strain 25-1	Beighton *et al.* (1)	Dental plaque, monkey
Streptococcus mutans LMG 14558^{T}	CCUG 11877, NCTC 10449	Coykendall (4)	Human decayed tooth
Streptococcus mutans LMG 14560	CCUG 32092		Human blood, endocarditis
Streptococcus ratti LMG 14650^{T}	CCUG 27502, ATCC 19645	Coykendall (4)	Laboratory rat
Streptococcus ratti LMG 14651	CCUG 27641, strain BHT	Coykendall (4)	Human
Streptococcus sobrinus LMG 14641^{T}	CCUG 24717, DSM 20742	Coykendall (5)	Human dental plaque
Streptococcus sobrinus LMG 14640	CCUG 21019, NCTC 10921	Coykendall (5)	Human dental plaque

Table 1 *(cont.)*

Strain	Other strain no.*	Reference	Strain information
***Streptococcus thermophilus* group**			
Streptococcus salivarius LMG 11489^{T}	NCFB 1779, CCUG 17825	Whiley & Hardie (22)	Human blood
Streptococcus salivarius LMG 13103	NCFB 1777		Unknown
Streptococcus salivarius LMG 14652	CCUG 32152		Human blood
Streptococcus salivarius LMG 14653	CCUG 32452		Human blood
Streptococcus salivarius LMG 13108	NCFB 2701, strain A 385		Unknown
Streptococcus salivarius LMG 13107	NCFB 2700, strain M36	Whiley & Hardie (22)	Unknown
Streptococcus thermophilus LMG 7952	NCFB 574		Pasteurized milk
Streptococcus thermophilus LMG 7953	NCFB 575		Pasteurized milk
Streptococcus thermophilus LMG 13100	NCFB 1242		Unknown
Streptococcus thermophilus LMG 13101	NCFB 489		Unknown
Streptococcus thermophilus LMG 13102^{T}	NCFB 573, CCUG 21957	Schleifer *et al.* (10)	Pasteurized milk
Streptococcus vestibularis LMG 13516^{T}	CCUG 24893, MM1, NCTC 12166	Whiley & Hardie (22)	Human vestibular mucosa
Streptococcus vestibularis LMG 14645	CCUG 24684, LV71, Carlsson 85	Whiley & Hardie (22)	Human vestibular mucosa
Streptococcus vestibularis LMG 14646	CCUG 24685, PV91, Carlsson 86	Whiley & Hardie (22)	Human vestibular mucosa
Streptococcus vestibularis LMG 14647	CCUG 24686, LV81, Carlsson 87	Whiley & Hardie (22)	Human vestibular mucosa
Streptococcus vestibularis LMG 17854	R-410,† CCUG 24683, OV71, Carlsson 83	Whiley & Hardie (22)	Human vestibular mucosa
Streptococcus vestibularis LMG 17855	CCUG 24687, HV81, Carlsson 88	Whiley & Hardie (22)	Human vestibular mucosa
Streptococcus vestibularis LMG 17856	R-411,† CCUG 24688, OP81, Carlsson 89	Whiley & Hardie (22)	Human vestibular mucosa

* ATCC, American Type Culture Collection, Rockville, MD, USA; CCUG, Culture Collection University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden; CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LHMC, Department of Oral Microbiology, the Royal London Hospital Medical College, London, UK; LMG, BCCM/LMG Culture Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium; NCFB, The National Collection of Food Bacteria, Agricultural and Food Research Council, Institute of Food Research, Reading Laboratory, Reading, UK; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; NMH, M. W. D. Wren, North Middlesex Hospital, London, UK; UNS, P. Unsworth, Central Public Health Laboratory, Colindale, London, UK.

† Strains included in a research collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium.

International Code of Nomenclature of Bacteria (14). The names *Streptococcus cristatus*, *Streptococcus ratti*, *Streptococcus sanguinis* and *Streptococcus parasanguinis* were proposed for the former *S. crista*, *S. rattus*, *S. sanguis* and *S. parasanguis*, and will be used throughout the present manuscript.

METHODS

Bacterial strains. All strains examined are listed in Table 1. The majority of the strains examined are type and well-characterized reference strains included in various taxonomic studies of viridans streptococci. Appropriate references are listed in Table 1. Bacteriological purity was checked by plating and examining living and Gram-stained cells.

Whole-cell protein analysis. All strains were grown for 24 h on Brain–Heart Infusion agar (Difco) and incubated at 36–37 °C in a microaerobic atmosphere containing approximately 5% O_2, 10% CO_2 and 85% N_2. Preparation of cellular protein extracts and PAGE were performed as described previously (12). Briefly, discontinuous gels (1·5 mm thick) were run overnight at constant current (6 mA per gel) and temperature in a vertical slab apparatus. The separation gel was 12·6 cm long and contained 12% total acrylamide [the monomer solution contained 30% total acrylamide with 2·67% cross-linking in 0·375 M Tris/HCl (pH 8·8) and 0·1% SDS]; the stacking gel was 12 mm long and contained 5% total acrylamide [the monomer solution again contained 30% total acrylamide with 2·67% cross-linking in 0·125 M Tris/HCl (pH 6·8) and 0·1% SDS]. Protein bands are stained with Coomassie blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid. These conditions allow separation of proteins and peptides in the molecular mass range of 14–116 kDa.

The densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed using the GelCompar 4.0 software package (Applied Maths, Kortrijk, Belgium).

RESULTS

Reproducibility

The reproducibility of the method was verified by running the same extracts on multiple gels and by including duplicate cultures of 11 strains (duplicate cultures are subcultures of the same strain, received on different occasions or from different depositors) (see Table 1). The inter-run reproducibility was at least 94% (data not shown). Duplicate cultures always belong to the same cluster and group closely together (Fig. 1). The similarity level between these pairs of patterns was always higher than 91%.

Cluster analysis of the protein patterns of the strains examined

Fig. 1 is a dendrogram obtained after numerical comparison and cluster analysis of the protein patterns of all strains examined. In this analysis, all reference

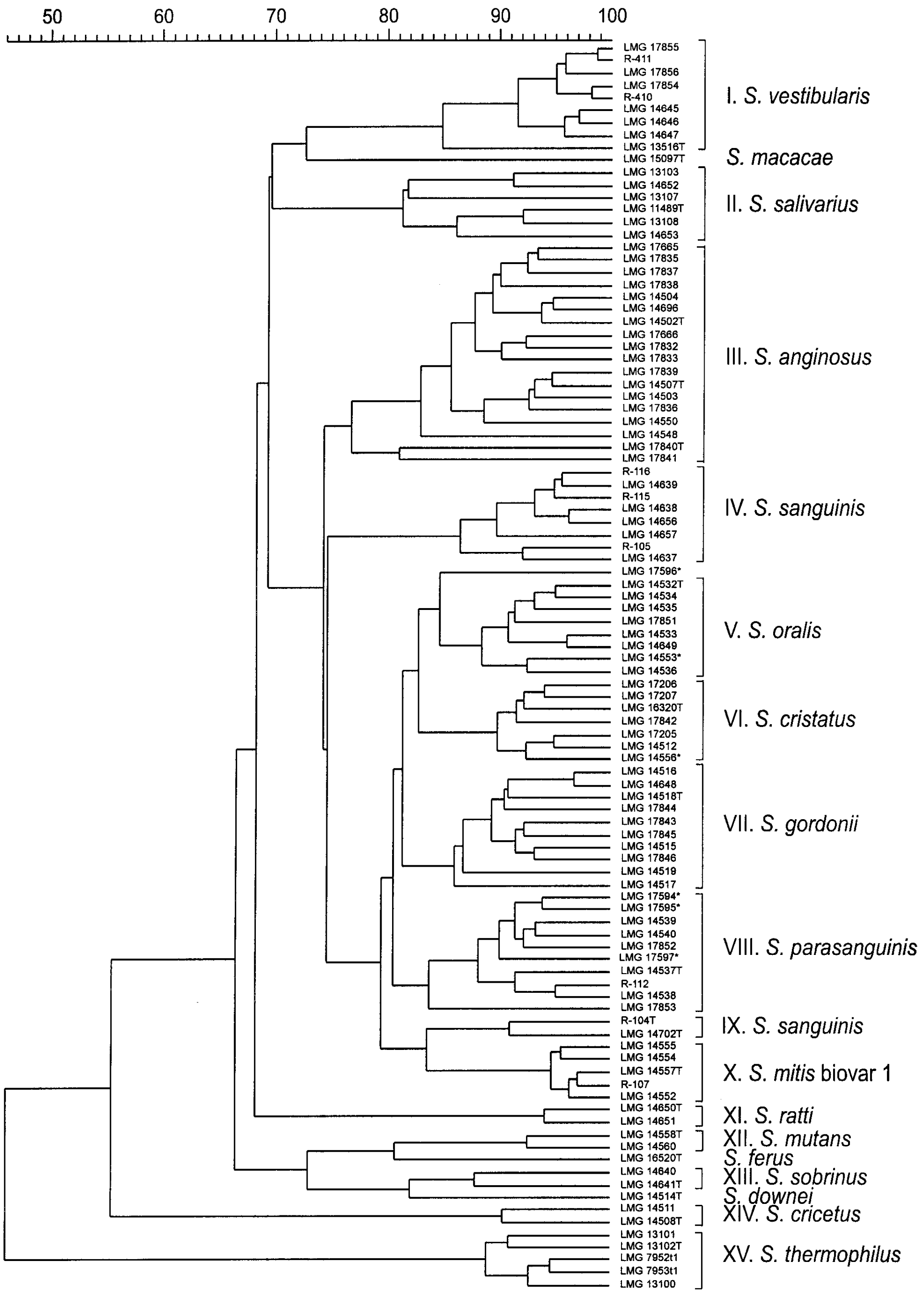

Fig. 1. Dendrogram derived from the unweighted pair group average linkage of correlation coefficients (expressed for convenience as a percentage) between whole-cell protein patterns of all of the strains examined. Strains marked by an asterisk were received as *S. mitis* biovar 2.

strains of the *S. anginosus* rRNA homology group form a single protein electrophoretic cluster (cluster III). Whole-cell protein patterns of representative strains are shown in Fig. 2.

The three species of the *S. thermophilus* rRNA homology group each constitute a clearly separated protein electrophoretic cluster (cluster I, *S. vestibularis*; cluster II, *S. salivarius*; cluster XV, *S. thermophilus*). Whole-cell protein patterns of representative strains are shown in Fig. 3.

Most of the species of the *S. mitis* rRNA homology group each constitute a distinct protein electrophoretic

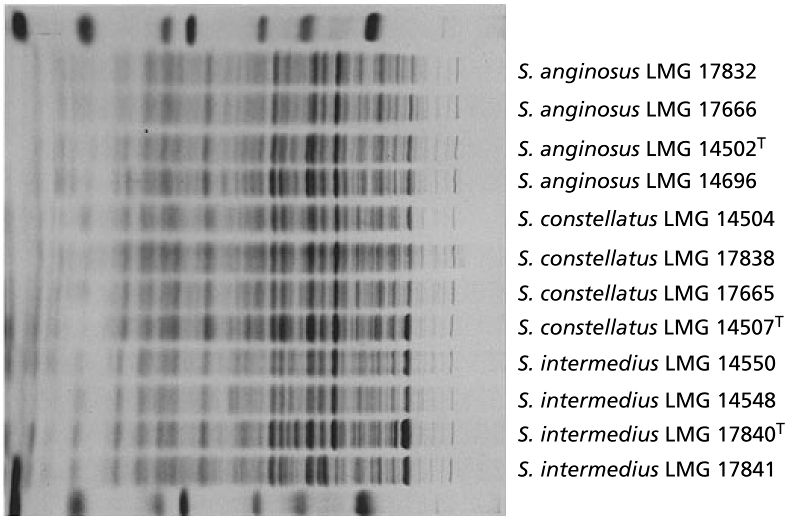

Fig. 2. Electrophoretic protein profiles of a selection of strains belonging to the *S. anginosus* group. The molecular mass markers used (indicated in the top and bottom lanes) are indicated from left to right: lysozyme, 14 kDa; trypsin inhibitor, 20·1 kDa; trypsinogen, 24 kDa; carbonic anhydrase, 29 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; egg albumin, 45 kDa; and bovine albumin, 66 kDa.

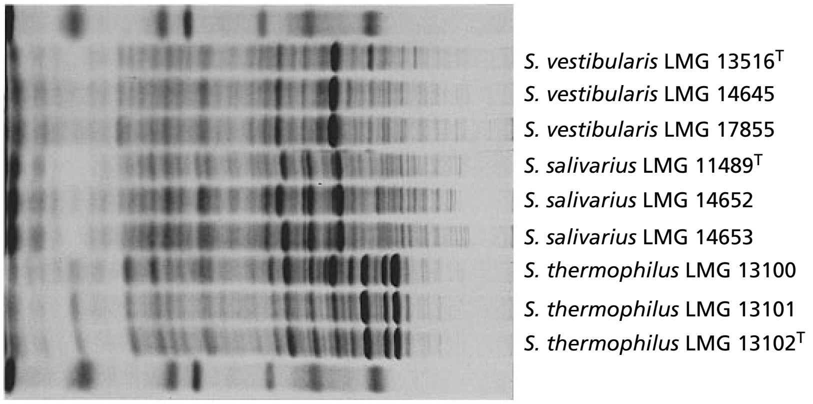

Fig. 3. Electrophoretic protein profiles of a selection of strains belonging to the *S. thermophilus* group. The molecular mass markers used (indicated in the top and bottom lanes) are as in Fig. 2.

cluster: *S. oralis* (cluster V), *S. cristatus* (cluster VI), *S. gordonii* (cluster VII) and *S. parasanguinis* (cluster VIII). Whole-cell protein patterns of representative strains are shown in Fig. 4. *S. sanguinis* strains are separated into two distinct clusters: cluster IV contains the majority of strains, whereas cluster IX comprises the two subcultures of the type strain. All *S. mitis* biovar 1 strains constitute a single protein electrophoretic cluster (cluster X). However, *S. mitis* biovar 2 strains are distributed over several clusters. One strain (LMG 14553) belongs to the *S. oralis* cluster; a second strain (LMG 17596) groups near the *S. oralis* cluster; one strain (LMG 14556) belongs to the *S. cristatus* cluster; and three strains (LMG 17594, 17595 and 17597) belong to the *S. parasanguinis* cluster.

Finally, species of the *S. mutans* rRNA homology group occupy distinct positions in the dendrogram. *S. mutans*, *S. cricetus*, *S. sobrinus* and *S. ratti* strains form separate protein electrophoretic clusters (clusters XII, XIV, XIII and XI, respectively), while the type strains of *S. macacae*, *S. downei* and *S. ferus* occupy distinct positions in the dendrogram (only a single strain of these species was examined). Whole-cell protein patterns of representative strains are shown in Fig. 5.

DISCUSSION

In previous studies on the relationships of pyogenic streptococci, results of whole-organism protein electrophoresis correlated with those of DNA–DNA hybridization experiments, i.e. strains with very similar whole-cell protein patterns shared high DNA–DNA hybridization values (15, 16). The aim of the present study was to evaluate whether whole-cell protein electrophoresis could be used as a method for species-level identification for the viridans streptococci. For this purpose, we collected 100 type and well-characterized reference strains of the various species. Most of these strains were included in previous taxonomic studies and, per species, several DNA–DNA hybridization data were available.

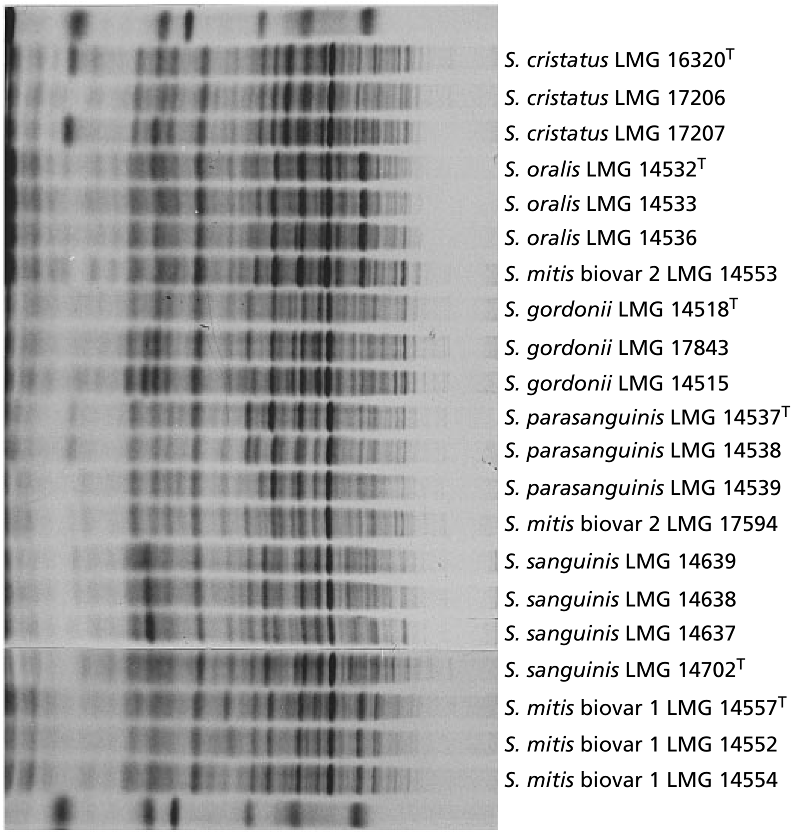

Fig. 4. Electrophoretic protein profiles of a selection of strains belonging to the *S. mitis* group. The molecular mass markers used (indicated in the top and bottom lanes) are indicated from left to right: trypsin inhibitor, 20·1 kDa; trypsinogen, 24 kDa; carbonic anhydrase, 29 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; egg albumin, 45 kDa; and bovine albumin, 66 kDa.

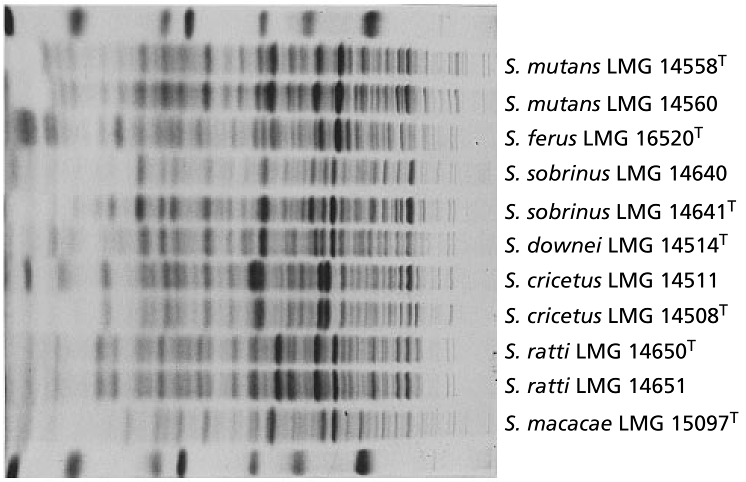

Fig. 5. Electrophoretic protein profiles of strains belonging to the *S. mutans* group. The molecular mass markers used (indicated in the top and bottom lanes) are as in Fig. 2.

The *S. mitis* rRNA homology group (excluding *S. pneumoniae*)

The taxonomic relationships between some members of the *S. mitis* rRNA homology group were studied in detail by Kilian *et al.* (10). These authors described or emended the description of *S. mitis*, *S. oralis*, *S. gordonii* and *S. sanguinis*, and delineated multiple biovars in several of these species. Their revised classification was based on an extensive phenotypic analysis and on a compilation of published DNA–DNA hybridization values amongst well-chosen strains.

S. sanguinis**.** Four biovars were described within *S. sanguinis*. Strains of two of these (biovars 1 and 2) were proven by DNA–DNA hybridization experiments to constitute a single species, whereas no DNA–DNA hybridization data were available for strains of biovars 3 and 4. Of all of the *S. sanguinis* strains examined, the type strain (LMG 14702) was the most aberrant (10).

In our analysis, six strains studied by Kilian *et al.* (10)

representing all four biovars were included. We found that five of these strains (and duplicate cultures of three of them) representing the four biovars formed a single protein electrophoretic cluster (cluster IV, Fig. 1). The type strain, however, differed from the others by the absence of a prominent protein band with an approximate molecular mass of 24 kDa (Fig. 4). As a consequence, both subcultures of the type strain cluster separately in the dendrogram (cluster IX) in the vicinity of *S. mitis* biovar 1 strains (cluster X). These protein electrophoretic data therefore confirm the aberrant nature of the *S. sanguinis* type strain and suggest that strains of biovars 3 and 4 share high DNA–DNA hybridization levels with strains of biovars 1 and 2.

***S. gordonii*.** Three biovars were described within *S. gordonii*. Strains representing all three biovars were included in DNA–DNA hybridization analyses which demonstrated that they formed a single species.

In our analysis, five strains studied by Kilian *et al.* (10) representing all three biovars were included. All five strains (and duplicate cultures of one) and the strains LMG 17843 through LMG 17846 formed a single homogeneous cluster VII. The latter four strains were received as *S. gordonii* from J. Carlsson and were included in a numerical taxonomic study of oral streptococci where they were classified as cluster I:B (3). Carlsson's cluster I:B is therefore not synonymous to *S. sanguinis* as discussed by Kilian *et al.* (10). In our analysis, the former type strain of *S. mitis* (LMG 14516) which was reclassified as *S. gordonii* (10, 18), behaved as a typical *S. gordonii* strain, in contrast with some of its other phenotypic characteristics (10).

***S. oralis*.** *S. oralis* does not comprise multiple biovars. We included two representative strains examined by Kilian *et al.* (10) and four strains from Carlsson's study representing Carlsson's cluster I:A (3). All six strains (and duplicate cultures of one) formed a single homogeneous cluster V which also comprised strain *S. mitis* LMG 14553 (see below).

***S. mitis*.** Two biovars were delineated within *S. mitis*. Strains representing *S. mitis* biovar 1 were included in DNA–DNA hybridization analyses and proved to constitute a single species (10). Thirteen additional isolates were classified as *S. mitis* biovar 2 (10). However, DNA–DNA hybridization data confirming that these strains belonged to *S. mitis* were not available and important phenotypic variability was found, suggesting that biovar 2 was heterogeneous (10).

In our analysis, four of the *S. mitis* biovar 1 strains were included. These four strains (and duplicate cultures of one) formed a single homogeneous cluster X. However, the six *S. mitis* biovar 2 strains which were examined did not cluster with *S. mitis* biovar 1 strains, nor did they constitute a single phenon. Three of these strains belonged to the *S. parasanguinis* cluster and one belonged to the *S. cristatus* cluster (Figs 1 and 4). Neither species had been described when Kilian's study was published (7, 21). Finally, one of the strains (LMG 14553) belonged to the *S. oralis* cluster (Figs 1 and 4), while another strain (LMG 17849) occupied a distinct position in the dendrogram with *S. oralis* as its closest neighbour. Obviously, *S. mitis* biovar 2 is a heterogeneous taxon and our results suggest that strains classified as such may belong to various other streptococcal species.

***S. parasanguinis*.** *S. parasanguinis* was described by Whiley *et al.* (21) for ten strains tentatively classified in the *S. anginosus* species group (23). All strains shared high DNA–DNA binding values with *S. sanguinis* as closest relative. In the present analysis, we included six of these strains (and duplicate cultures of one) which all formed a single homogeneous cluster (cluster VIII).

***S. cristatus*.** Similarly, *S. cristatus* was described by Handley *et al.* (7) for four strains superficially resembling *S. sanguinis* and previously referred to as 'the CR group' or the 'tufted fibril group'. Three of these strains were examined by DNA–DNA hybridization and showed high hybridization values. All four strains (including duplicate cultures of one) and a recent human blood isolate also formed a single homogeneous protein electrophoretic cluster (cluster VI).

The *S. anginosus* rRNA homology group

The taxonomic status of strains classified as *S. anginosus*, *S. constellatus* and *S. intermedius* has been the subject of much debate [reviewed by Whiley & Beighton (20)]. One view is that these three taxa are phenotypically virtually indistinguishable and that, in spite of some genotypic heterogeneity, they constitute a single species, *S. anginosus* (6). The other view is that there are sufficient differential phenotypic characteristics between these three taxa and that the DNA–DNA hybridization levels between the three groups are sufficiently low to warrant their classification as three distinct species (20). The reported DNA–DNA hybridization levels between the three named species are often near the borderline of species delimitation, while the phenotypic differentiation between at least two groups is not straightforward [*S. intermedius* is readily differentiated from the others using the scheme of Whiley & Beighton (20)]. Group-specific polypeptide patterns (peptides in the molecular mass range of 25·5–34 kDa, i.e. the low-molecular-mass region) as revealed by SDS-PAGE (23), 16S rRNA probe hybridization results (8) and Py-MS (11) support the distribution of these isolates in three, four or five subgroups, respectively.

In our protein electrophoretic analysis, we included five strains classified by Whiley & Beighton (20) as *S. anginosus*, nine as *S. constellatus* and four as *S. intermedius*. Whereas numerical analysis of whole-cell protein patterns enabled clear differentiation between all other streptococcal species, these 18 isolates formed a single, though heterogeneous, cluster (cluster III) (Fig. 1). Fig. 4 illustrates the whole-cell protein patterns of four reference strains of each of these taxa. The *S. intermedius* strains were clearly the most aberrant ones (the differences between *S. intermedius* strains and strains from the other two species are mainly situated in the region between the 20·1 and 24 kDa marker proteins) but there was no clear subdivison in three taxa, not even when the analysis was restricted to the low-molecular-mass region of the protein patterns (23) (data not shown). It is well-known that culture conditions and electrophoretic conditions may influence the protein patterns as revealed by SDS-PAGE. The discrepancy between the results from our study and the one of Whiley and Hardie (23) may be due to different conditions used. However, as discussed above, the conditions used in our laboratory generate protein patterns which correlate with DNA–DNA hybridization results as demonstrated for a wide variety of both Gram-negative and Gram-positive species (17), including the majority of streptococcal species (15, 16, this study). It is therefore not unlikely that the differences between polypeptide patterns as revealed in the study by Whiley & Beighton (20) correspond to infraspecific subdivisions of a single species.

We conclude that our analysis of whole-cell protein patterns supports the viewpoint that these three taxa constitute a single species which should be referred to as *S. anginosus* (6).

The *S. thermophilus* rRNA homology group

The *S. thermophilus*, *S. salivarius* and *S. vestibularis* reference strains constitute distinct protein electrophoretic clusters each (Figs 1 and 3), confirming that also within the *S. thermophilus* rRNA homology group, whole-cell protein electrophoresis allows a clear-cut species differentiation.

The *S. mutans* rRNA homology group

Few reference strains of species belonging to the *S. mutans* rRNA homology group were included. Whole-cell protein analysis was used by Beighton *et al.* (1) for the differentiation of mutans streptococci and was found appropriate. In our analysis we found considerable similarities between *S. downei* LMG 14514^T and *S. sobrinus* strains (Figs 1 and 5), while all other species were clearly identifiable.

Conclusion

In previous studies we demonstrated that members of the pyogenic species group (*Streptococcus agalactiae*, *Streptococcus canis*, *Streptococcus dysgalactiae*, *Streptococcus equi*, *Streptococcus iniae*, *Streptococcus parauberis*, *Streptococcus phocae*, *Streptococcus porcinus*, *Streptococcus pyogenes* and *Streptococcus uberis*) could all be differentiated by means of whole-cell protein electrophoresis (15, 16). In the present study, we included 100 strains representing all 19 species belonging to the different phylogenetic lineages of the so-called viridans or oral streptococci. For most of the species examined, multiple strains characterized by DNA–DNA hybridization were available and, wherever described, representatives of different biochemical variants were included. The differentiation of most of these species by means of SDS-PAGE of cellular proteins was straightforward. Problems were only encountered in the *S. anginosus* rRNA homology group and in *S. mitis*, and our data confirmed the aberrant nature of the *S. sanguinis* type strain. Our data support the viewpoint that members of the *S. anginosus* group constitute a single species and indicate that *S. mitis* biovar 2 is a heterogeneous taxon comprising strains from several streptococcal species.

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