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Identification of streptococci from Greek Kasseri cheese and description of *Streptococcus macedonicus* sp. nov.

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Taxonomic studies were performed on some *Streptococcus*-like organisms isolated from naturally fermented Greek Kasseri cheese. By SDS-PAGE analysis of whole-cell proteins the group was found to be quite different from *Streptococcus thermophilus*. Comparative 16S and 23S rRNA sequence analyses showed that the isolates represent a new species within the genus *Streptococcus*, where they are most closely related to the *Streptococcus bovis* cluster. On the basis of these phylogenetic results and some phenotypic differences, a new species, *Streptococcus macedonicus*, is proposed. The type strain is ACA-DC 206.

Keywords: Kasseri cheese, *Streptococcus macedonicus* sp. nov., phenotypic analysis, protein electrophoresis, rRNA sequencing

INTRODUCTION

Streptococci *sensu lato* are widely distributed as human and animal pathogens, in the mouth and intestines of man and animals, in raw milk and dairy products, and on plant material. The genus was described in 1884 by Rosenbach (25). Sherman (29) divided the streptococci into four groups, the enterococci, the 'lactic', the 'viridans' and the 'pyogenic' streptococci. The identification and classification of streptococci relied too long and too heavily on the serological groupings introduced by Lancefield (16). Other taxonomic markers were neglected until new physiological and biochemical data were used by Jones (13) to extend and modify the original proposal of Sherman. Jones (13) maintained the 'pyogenic' and the 'lactic' streptococci, replaced the names 'viridans' and 'enterococci' by 'oral' and 'faecal', respectively, and added the groupings 'pneumococci', 'anaerobic' and 'other' streptococci. Bridge & Sneath (3) performed a numerical taxonomy study and divided the 'pyogenic' and 'oral' groups into two or three further groups.

Based on 16S rRNA cataloguing (17), DNA–rRNA hybridizations (11, 15, 26, 27) and serological studies using superoxidase dismutase antisera (27), the streptococci were subdivided into three genera: (i) *Streptococcus sensu stricto* comprising the majority of the known species, (ii) *Enterococcus* for the enterococcal group and (iii) *Lactococcus* for the lactic streptococci (28). 16S rRNA sequence analysis performed on lactococci, enterococci and other Gram-positive reference strains (4) are in good agreement with the proposed subdivision. The most recent phylogenetic study (14) based on 16S rRNA sequence comparison, confirms the presence of different groupings within the genus *Streptococcus*: the anginosus, bovis, mitis, mutans, pyogenic and salivarius rRNA groups were delineated.

In the course of a survey of the lactic acid bacterial flora of naturally fermented Greek Kasseri cheese, we isolated a group of 26 strains phenotypically assigned to *Streptococcus*. However, using SDS-PAGE analysis of whole-cell proteins the group was found to be quite different from *Streptococcus thermophilus* (32), which is frequently found in Greek cheese. In this paper we describe the phenotypic characteristics of these isolates, the results of the comparative SDS-PAGE analysis of whole-cell proteins and a phylogenetic analysis of their rRNA. A new species, *Streptococcus macedonicus*, is proposed for the isolates from Greek Kasseri cheese. *S. macedonicus* was shown to belong to the bovis rRNA group, whilst *S. thermophilus*, which shares with *S. macedonicus* the same habitat, belongs to the salivarius group (14).

The EMBL accession numbers for the 16S and 23S rRNA sequences reported in this paper are Z94012 and Z94013, respectively.

METHODS

Bacterial strains. A total of 26 lactic acid bacteria were isolated from traditional Greek Kasseri cheese prepared from ewes' milk (Table 1). All strains are deposited in the ACA-DC Collection (Laboratory of Dairy Research, Agricultural University of Athens, Greece). For the isolation, samples were collected aseptically and kept under cooling until use (maximum time of storage 24 h). Ten gram hard cheese samples were diluted in 2% (w/v) KH_2PO_4 (pH 4·40). After stirring, 10^{-5}, 10^{-6} and 10^{-7} dilutions were prepared in Ringer's solution [0·9% (w/v) NaCl] and plated on M17 agar (Oxoid). M17 agar plates were incubated aerobically at 37 °C for 48 h. Colonies were collected according to their shape and colour and then examined for Gram staining and catalase production. For further purification, the Gram-positive and catalase-negative strains, which were also able to grow in skimmed milk [10% (w/v)], were inoculated three times on M17 agar. Pure cultures were stored at −80 °C in 10% skimmed milk containing 5% (v/v) glycerol.

Table 1. *S. macedonicus* strains used in the SDS-PAGE analysis

All strains were isolated from Greek Kasseri cheese except strain LMG 15061, which was isolated from sour mash. t2, different colony type of the same strain.

ACA-DC no.*	LAB/LMG no.†
185	LAB 591
186	LAB 592
243	LAB 593
244	LAB 596
245	LAB 597
246	LAB 598
247	LAB 600
248	LAB 601
249	LAB 603
250	LAB 604
251	LAB 605
198	LAB 606
199	LAB 607
200	LAB 608
255	LAB 609
256	LAB 610
203	LAB 612
204	LAB 614
205	LAB 616
206^T	LAB 617^T
207	LAB 618
208	LAB 620t2
209	LAB 621
210	LAB 624
211	LAB 625
266	LAB 627
	LMG 15061

* ACA-DC, Culture Collection of the Laboratory of Dairy Research, Agricultural University of Athens, Greece.

†, LAB and LMG, BCCM/LMG Culture Collection of the Laboratory of Microbiology Ghent, University of Ghent, Belgium.

Phenotypic characterization. All strains were characterized by morphology and simple physiological tests (12). Ability to grow at different temperatures was determined in M17 broth (Oxoid) at 10 °C for 10 d and at 45 °C for 48 h. Growth in the presence of 2, 4 and 6·5% NaCl was observed in Nutrient Broth (Oxoid). The ability to grow at pH 9·6 was tested in broth containing 10 g tryptone, 5 g yeast extract, 10 g glucose, 2·5 g NaCl, 0·5 g Tween 80, 2·5 g K_2HPO_4 and 5·3 g Na_2CO_3 in 1 litre H_2O. Finally, all strains were tested for growth in 10% skimmed milk containing 0·1% (w/v) methylene blue and in litmus milk at 37 °C for 7 d.

Growth characteristics and biochemical activities of the following 10 strains were phenotypically further studied as described previously (7, 22): ACA-DC 185, ACA-DC 186, ACA-DC 204, ACA-DC 207, ACA-DC 206^T, ACA-DC 208, ACA-DC 211, ACA-DC 248, ACA-DC 256 and LMG 15061. Experiments on strain ACA-DC 256 were repeated on two independently received subcultures (designated ACA-DC 256A and ACA-DC 256B).

PAGE of whole-cell proteins. Strains were grown on MRS agar in Roux flasks at 37 °C for 24 h. Roux flasks were inoculated from a 24-h-grown 10 ml MRS broth culture. Cell-free extracts were prepared as described by Pot *et al.* (23).

Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains by the Pearson product–moment correlation coefficient (r) and UPGMA cluster analysis were performed by techniques described by Pot *et al.* (23) using the software package GelCompar (35). The reproducibility of the SDS-PAGE method was estimated by running single extracts on multiple gels and by running independently received subcultures of the same strain on different gels. The reproducibility of multiple runs of a single extract was at least 95% (data not shown); r values are shown as percentages for convenience. Duplicate extracts always clustered above 92% (data not shown).

Identification of the new isolates was performed by comparison of their protein patterns with a database containing the normalized protein fingerprints of reference strains from all known species of lactic acid bacteria (Table 2). Pattern storage and comparison was performed on a MS-DOS compatible PC using GelCompar (35).

rRNA gene sequence determination and analysis. *In vitro* amplification of 16S and 23S rRNA-encoding DNA and direct sequencing of amplified fragments were performed as previously described (18, 30). The new rRNA sequences were added to alignments of homologous primary structures available from public databases (6, 19, 34). Phylogenetic analyses of the 16S rRNA data were performed by applying distance matrix, maximum parsimony and maximum likehood methods on different data sets. Distance matrix and maximum parsimony analyses were performed using data sets comprising all available 16S rRNA primary structures from representatives of the phylum of the Gram-positive bacteria with a low DNA G+C content, as well as reference sequences from selected members of the remaining major phylogenetic groups of bacteria. Maximum likehood methods were used to analyse subsets of about 120 sequences comprising the homologous primary structures from streptococci and selected representatives of related phylogenetic groups of the phylum such as lactococci and enterococci.

Table 2. Reference strains used in the SDS-PAGE analysis

Name as received	LMG no.*	Strain no. as received†	Name according to SDS-PAGE grouping	Strain information*
S. alactolyticus	14588	Devriese F143	*S. alactolyticus*	Pig, intestine
S. alactolyticus	14589	Devriese F156	*S. alactolyticus*	Pig, intestine
S. alactolyticus	14591	Devriese T118		Pig
S. alactolyticus	14808^T	NCFB 1091^T	*S. alactolyticus*	
S. bovis	14617	Devriese 94/551	*S. alactolyticus*	Chicken
S. bovis/equinus	14872t1	92/2214	*S. alactolyticus*	Dog
S. equinus	14897^T	CCUG 27302^T		
S. equinus	15050	NCFB 2079		Homology group 2
S. equinus	15051	NCFB 2080		Homology group 2
S. equinus	15059	NCFB 2445		Homology group 1; horse faeces
S. equinus	15067	NCFB 964		Homology group 4
S. equinus	15115	NCFB 2600		Homology group 1
S. equinus	15119	NCFB 2620		Homology group 4; babies' faeces
S. equinus	15121	NCFB 2632	*S.* species 2	Homology group 4; medical case
S. salivarius	11489^T	NCFB 1779	*S. salivarius*	
S. salivarius	13104	NCFB 2685	*S. salivarius*	
S. salivarius	13105	NCFB 2686	*S. salivarius*	
S. salivarius	13106	NCFB 2699	*S. salivarius*	
S. salivarius	13108	NCFB 2701	*S. salivarius*	
S. salivarius	13109	NCFB 2698	*S. salivarius*	
S. salivarius	14653	CCUG 32452	*S. salivarius*	
S. thermophilus	6896^T	NCIB 8510^T	*S. thermophilus*	Pasteurized milk
S. thermophilus	7952t1	NCFB 574	*S. thermophilus*	Pasteurized milk
S. thermophilus	7952t2	NCFB 574	*S. thermophilus*	Pasteurized milk
S. thermophilus	7953t1	NCFB 575	*S. thermophilus*	Pasteurized milk
S. thermophilus	7953t2	NCFB 575	*S. thermophilus*	Pasteurized milk
S. thermophilus	7953t3	NCFB 575	*S. thermophilus*	Pasteurized milk
S. thermophilus	13048	DSM 20479	*S. thermophilus*	LMG 13101
S. thermophilus	13100	NCFB 1242	*S. thermophilus*	
S. thermophilus	13101	NCFB 489	*S. thermophilus*	LMG 13048
S. thermophilus	13102^T	NCFB 573^T	*S. thermophilus*	Pasteurized milk
S. thermophilus	LAB 510	ACA-DC 144	*S. thermophilus*	Feta cheese
S. thermophilus	LAB 511	ACA-DC 80	*S. thermophilus*	Feta cheese
S. thermophilus	LAB 594	ACA-DC 145	*S. thermophilus*	Kasseri cheese
S. thermophilus	LAB 595t1	ACA-DC 164	*S. thermophilus*	Kasseri cheese
S. vestibularis	13516^T	CCUG 24893^T	*S. vestibularis*	
S. vestibularis	14645	CCUG 24684	*S. vestibularis*	
S. vestibularis	14646	CCUG 24685	*S. vestibularis*	
S. vestibularis	14647	CCUG 24686	*S. vestibularis*	

* LMG and LAB, BCCM/LMG Culture Collection of the Laboratory of Microbiology Ghent, University of Ghent, Belgium; t1, t2 and t3, different colony types of the same strain.

† ACA-DC, Culture Collection of the Laboratory of Dairy Research, Agricultural University of Athens, Greece.

The data sets varied with respect to the reference sequences and alignment positions included. The variabilities of the individual alignment positions were determined and used as criteria to successively remove highly variable positions from the data set. This was done to recognize and minimize treeing artefacts resulting from alignment errors, database inconsistencies and 'false' identities (multiple base changes) in highly variable regions. The calculations and data analyses were performed using the ARB program package (31).

The design of specific rRNA-targeted oligonucleotide probes was perfomed applying the respective tools of the ARB program package (31). The specificity of the probe was evaluated by Southern hybridization to *in-vitro*-amplified rRNA gene fragments of reference organisms. The oligonucleotides were labelled with dioxigenin at their 3′ ends using the DIG Oligonucleotide 3′-End Labeling kit (Boehringer Mannheim), according to the instructions of the manufacturer. Porablot NY Plus membranes (0·45 μm;

Machery & Nagel) were used for Southern hybridization of the *in-vitro*-amplified rRNA. Prehybridization was done in 5× SSC (standard saline citrate; 150 mM NaCl, 15 mM sodium citrate, pH 7·0) containing 0·1 % *N*-lauroylsarcosine, 0·02 % SDS and 1·5 % blocking reagent (DIG Nucleic Acid Detection kit; Boehringer Mannheim) for 1 h at 46 °C. Hybridization was carried out in the same buffer containing in addition 15 pmol probe DNA for 4 h at 46 °C (16S rRNA probe) and 54 °C (23S rRNA probe). Subsequently, the membranes were incubated twice in washing buffer (2× SSC, pH 8·0; 0·01 % SDS) for 5 min at 52 °C (16S rRNA probe) and 60 °C (23S rRNA probe). Visualization of the hybrids was achieved by using the DIG Nucleic Acid Detection kit (Boehringer Mannheim). Labelling of the probes with fluorescent dyes and *in situ* cell hybridization of *p*-formaldehyde- (16S rRNA probe) and formamide- (23S rRNA probe) fixed cells was done as described previously (2).

RESULTS AND DISCUSSION

In Greece, even nowadays, cheese is mainly produced from ewes' and goats' milk without the use of industrial starter cultures. The specific characteristics such as taste, aroma and texture of these traditional fermented products may be attributed to the presence of 'wild' starter cultures. To improve on understanding the relationship between the organoleptic quality of cheese and the microbial flora responsible, large numbers of isolates from a wide variety of fermented substrates need to be sampled and submitted to a rapid and reliable identification and typing method. In the present work, taxonomic studies were performed on some unidentified *Streptococcus*-like organisms isolated from naturally fermented Greek Kasseri cheese.

Kasseri cheese is a Greek 'pasta filata' variety, prepared from ewes' milk or a ewes' and goats' milk mixture. It is mainly produced in the regions of Macedonia, Thessalia and Epirus, and it is one of the most popular cheeses in Greece. Its annual production is second among the semi-hard/hard cheeses. In spite of its economical significance, limited information is available concerning its physico-chemical and microbiological characteristics (20).

Phenotypic characterization

All 26 Kasseri cheese isolates were found to be phenotypically homogeneous. They were Gram-positive, catalase-negative cocci and occurred in pairs or in small groups. Most strains showed homogeneous growth in Brain Heart Infusion broth after 1 d incubation. Growth at 42 °C was similar to that at 37 °C and definitely better than at 30 °C. After 1 d very slight growth was seen at 25 °C. No growth occurred in 6·5 % NaCl broth after 3 d at 37 °C. Tests on strain ACA-DC 256 were repeated using two independently received subcultures (256A and 256B), which showed almost identical reactions in all cases, confirming the reproducibility of the phenotypic analysis.

The new isolates shared with *S. thermophilus* both habitat and resistance to high temperatures (45 °C). However, their colonies were larger and growth in

Table 3. Differentiation of *S. macedonicus* from *S. thermophilus*, *S. alactolyticus* and *S. bovis–S. equinus*-like strains on the basis of biochemical activity

−, Negative; +, positive; d, variable; d+, over 75 % positive; NT, not tested.

Characteristic	*S. macedonicus*	*S. thermophilus**	*S. alactolyticus*†	*S. bovis–S. equinus*‡
Acid from:				
Amygdalin	−	−	d	d+
β-Gentibiose	−	NT	d	d+
Cellobiose	+	−	NT	NT
Glycogen	−	NT	−	d+
Inulin	−	NT	−	d+
Maltose	+	−	NT	NT
Mannitol	−	NT	d+	d
N-Acetylglucosamine	+	−	NT	NT
Aesculin hydrolysis	−	NT	d	+
Bile resistance	−	NT	NT	+
Urease	−	NT	+	NT
Production of:				
Acetoin	+	d+	NT	NT
β-Galactosidase	−	+	NT	NT

* Based on data in reference (12).

† Based on descriptions in references (10) and (8).

‡ Based on the description of DNA homology groups 1, 2 and 4 in reference (10).

broth was diffuse. Furthermore, they could be differentiated easily from *S. thermophilus* by their growth characteristics and biochemical activities, such as acid production from *N*-acetylglucosamine, cellobiose and maltose, as well as the negative reaction for β-galactosidase (Table 3). The new isolates could also quite easily be differentiated from the phylogenetically most closely related organisms *Streptococcus bovis*, *Streptococcus equinus* and *Streptococcus alactolyticus* by their failure to produce blackening reactions in media containing aesculin. Other characteristics useful in the differentiation from related organisms are shown in Table 3. The new streptococcal isolates could be distinguished from the enterococci by their lack of resistance to bile, their negative aesculin and β-glucosidase reactions and their inability to produce acid from β-gentiobiose. All enterococcal species known to date, are positive in these tests (7).

PAGE of whole-cell proteins

All 26 protein patterns obtained by SDS-PAGE were compared to a library of protein fingerprints representing most species of lactic acid bacteria. The reproducibility of the SDS-PAGE technique was sufficient to allow reliable conclusions from the clusters obtained. The dendrogram obtained after numerical comparison, using the Pearson product–moment correlation coefficient followed by a UPGMA cluster analysis of the protein patterns of all strains and some relevant reference strains, is shown in Fig. 1. The group was found to be quite different from the already described *Streptococcus* species. In the analysis, all strains formed a single protein electrophoretic cluster (cluster I, r values $\geqslant$ 86·2%). Twenty-six strains from Kasseri cheese formed one separate cluster at a similarity level of 90·5% (correlation coefficient $r \times 100$). One strain (LMG 15061), isolated from sour mash and not from cheese, was found to take a slightly separate position in cluster I (correlation of 86·2%). The occurrence of at least one strain not originating from cheese suggests that other sources of isolation may be found. The five species of the genus *Streptococcus* included as references each clearly constitute separate protein electrophoretic clusters (cluster II, *S. thermophilus*; cluster III, *Streptococcus salivarius*; cluster IV, *Streptococcus vestibularis*; cluster V, *S. alactolyticus*; cluster VI, *S. bovis–S. equinus*).

Previous taxonomic studies performed on streptococci have shown that results of whole-cell protein electrophoresis correlated well with those of DNA–DNA hybridizations at the species level (9, 33). Thus, the protein electrophoretic differences encountered, as illustrated in Fig. 1, not only showed the presence of a new group of closely related strains, but also encouraged us to perform a genotypic study to reveal the exact phylogenetic position of this group of strains. Since the results of SDS-PAGE analysis cannot be interpreted above the species level, a comparative analysis of 16S rRNA sequences was performed.

rRNA gene sequence determination and analysis

The 16S and 23S rRNA sequences of the type strain ACA-DC 206^T were determined by direct sequencing of *in-vitro*-amplified rRNA gene fragments. Comparative 16S rRNA sequence analysis confirmed the conclusions from the phenotypic analyses by clearly assigning strain ACA-DC 206^T to the genus *Streptococcus*. Overall, 16S rRNA sequence similarities of ACA-DC 206^T and all other streptococci for which data are available were 93·8% or higher (Table 4). The closest relatives according to 16S rRNA data were *S. bovis* (98·3%), *S. equinus* (98·4%) and *S. alactolyticus* (97·5%). The corresponding values for all other complete or partial sequences available for streptococci were 96% or lower. The corresponding 23S rRNA similarity values were slightly higher (Table 4). The phylogenetic tree in Fig. 2 shows the relationships of ACA-DC 206^T and those type strains of streptococcal species for which at least 90% complete 16S rRNA primary structures (in comparison with the homologous *Escherichia coli* molecule) were available in public databases.

Specific hybridization probes targeted against 16S (ACA-DC 206/81; 5′ CTTCCAACTCTAGCAAGC 3′) and 23S (ACA-DC 206/274; 5′ GACTTCCCA-CAGCGCAGT 3′) rRNA were designed for strain ACA-DC 206^T. All available small and large subunit rRNA sequences were included for probe design applying the corresponding tool of the ARB program package (31). Experimental evaluation of the designed specificity was done by Southern as well as *in situ* cell hybridization, including target molecules or cells from the closest relatives of ACA-DC 206^T, respectively (Table 5). The target region of the probes corresponds to positions 81–99 and 274–291 within the 16S and 23S rRNA from *E. coli*, respectively.

Based on the currently available 16S rRNA sequence data, strain ACA-DC 206^T phylogenetically represented a member of the streptococci clearly separated from the other species. The 23S rRNA sequence data support these findings. However, the limited data set of available reference sequences did not allow a detailed phylogenetic analysis. The two specific probes which were designed for 16S and 23S rRNA targets, respectively, in combination with conventional nucleic acid or *in situ* cell hybridization should be valuable tools for assisting rapid identification of the new species. Given that different molecules are targeted, the combined application of both probes is recommended for reliable identification.

The comparative analysis thus confirmed the presence of a new species for which the name *Streptococcus macedonicus* is proposed. The relationship of *S. macedonicus* to the *S. bovis* rRNA group was surprising, since a closer relationship to *S. thermophilus* was expected on phenotypic grounds. *S. bovis* belongs to the viridans streptococci (29) and was described originally by Orla-Jensen (21), for strains mainly isolated from bovine faeces. Initially, a considerable

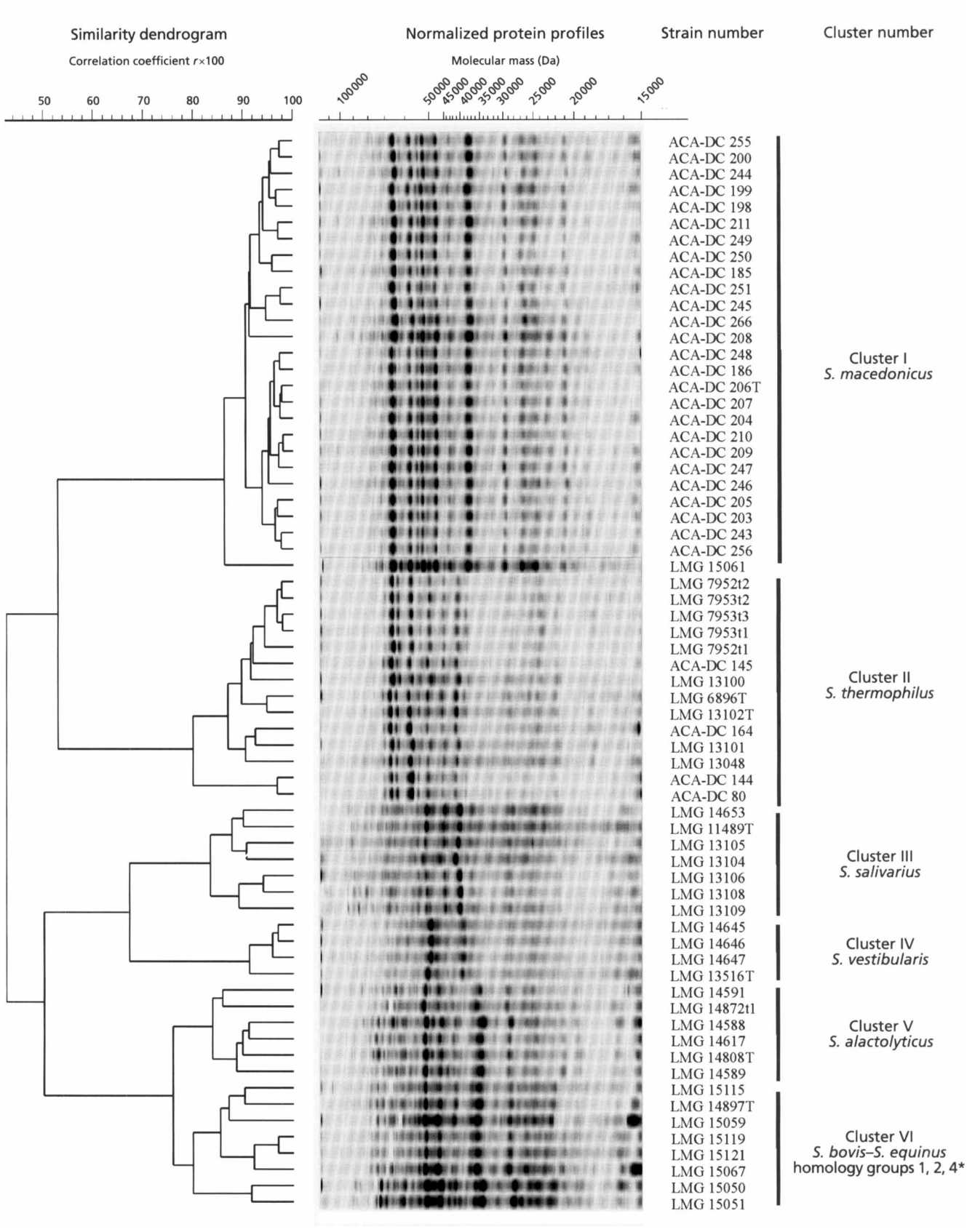

Fig. 1. Grouping of SDS-PAGE protein patterns. On the left side of the figure the mean correlation coefficients expressed as percentages ($r \times 100$) are represented as a dendrogram that was constructed by the unweighted pair group method with averages (UPGMA) for a number of *Streptococcus* species. Points 10 to 325 of the 400-point traces were used to calculate similarities between the individual pairs of traces. In the centre of the figure is a computer-processed printout

Table 4. Overall similarities (%) of 16S (lower left) and 23S (upper right) rRNA primary structures from strain ACA-DC 206^T, its closest relatives (*S. bovis*, *S. equinus* and *S. alactolyticus*) and selected streptococci and lactococci

Only sequence positions which have been unambiguously determined in both sequences were included for the calculations of binary similarity values.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*S. macedonicus* ACA-DC 206^T						97·0						96·2	96·0						89·0
2	*S. bovis*	98·3																		
3	*S. equinus*	98·4	99·5																	
4	*S. alactolyticus*	97·5	97·0	97·3																
5	*S. salivarius*	95·8	96·2	96·7	95·9															
6	*S. thermophilus*	95·8	95·7	96·2	95·4	99·0							96·2	96·0						89·7
7	*S. pneumoniae*	94·7	94·4	94·9	95·1	95·7	94·7													
8	*S. mitis*	94·8	94·3	95·0	95·3	95·3	94·5	99·0												
9	*S. gordonii*	94·7	94·5	95·4	95·6	95·1	94·5	96·5	96·9											
10	*S. parasanguinis*	95·4	95·1	96·0	95·9	95·8	95·2	96·5	96·7	95·3										
11	*S. sanguinis*	96·0	95·3	96·1	96·4	96·0	95·3	97·4	97·3	96·5	97·3									
12	*S. uberis*	95·1	95·1	95·5	95·1	95·2	94·7	93·8	93·5	93·7	94·9	94·9		97·5						89·4
13	*S. parauberis*	95·1	95·2	95·4	95·0	94·9	94·3	94·4	94·3	93·3	95·7	95·8	97·5							89·0
14	*S. milleri*	94·6	94·3	95·2	96·1	93·7	93·1	94·3	94·3	94·5	94·6	95·1	93·2	93·4						
15	*S. anginosous*	95·9	95·4	96·5	95·9	94·9	94·5	94·3	93·8	94·9	94·6	94·7	93·7	93·4	98·5					
16	*S. ratti*	95·2	94·8	96·2	96·2	93·9	93·5	93·2	93·1	92·8	93·8	94·5	93·6	93·1	94·2	94·2				
17	*S. mutans*	93·8	94·1	95·3	93·7	94·0	93·5	93·2	92·7	93·2	93·4	94·3	92·6	92·7	92·4	93·1	95·6			
18	*S. equi*	94·9	94·7	95·5	94·8	94·3	93·8	93·5	93·0	93·2	93·6	94·5	95·4	95·0	93·8	94·5	94·1	93·4		
19	*L. lactis*	90·3	90·3	92·2	91·3	89·3	89·1	89·0	89·9	89·3	90·0	89·7	89·0	90·1	88·5	89·3	89·0	88·9	89·3	
20	*L. raffinolactis*	92·1	91·9	92·9	92·4	91·3	90·9	90·5	90·6	90·6	90·7	91·1	91·6	91·6	90·6	91·3	90·3	90·5	91·9	91·7

phenotypic diversity was described (1, 5, 21, 29). Later, DNA–DNA hybridizations confirmed the presence of at least six DNA homology groups (10) in a large collection of *S. bovis* and *S. equinus* strains of a variety of origins. In our SDS-PAGE analysis we included representative strains from the DNA homology groups 1, 2 and 4, as well as *S. alactolyticus* (DNA homology group 6), which is also a member of the *S. bovis* rRNA group. A more detailed taxonomic analysis of this rRNA group has recently been performed (24).

Description of *Streptococcus macedonicus* sp. nov.

Streptococcus macedonicus (ma.ce.'do.ni.cus. M.L. adj. *macedonicos* from Macedonia, Northern Greece, from where the bacterium was first isolated).

All strains are Gram-positive cocci and occur in pairs or in small groups. Most strains show homogeneous growth in Brain Heart Infusion broth after 1 d incubation. Growth at 42 °C is similar to that at 37 °C and definitely better than at 30 °C. After 1 d, very slight growth is seen at 25 °C. No growth occurs in 6·5% NaCl broth after 3 d at 37 °C. A slight growth enhancement is noted when plates are incubated in 5% CO_2. Colonies on Columbia blood agar of all strains except ACA-DC 256 and LMG 15061 are α-haemolytic. The strains are not pigmented and non-motile. All strains grow on Edward's medium but they do not lyse aesculin. They grow as very small colonies on kanamycin aesculin azide agar, but produce no blackening on this medium. Growth also occurs on Slanetz & Bartley medium. The small colonies appearing on this medium are slightly pink. The strains are unable to grow on bile aesculin agar. All strains are positive in the Voges–Proskauer and leucine arylamidase tests. All except one (strain ACA-DC 207) are alanyl-phenylalanylproline-arylamidase-positive and all except strains ACA-DC 211 and LMG 15061 are α-galactosidase-positive. All strains reduce litmus milk and produce strong clots after 1 d incubation. All strains produce acid from D-glucose, galactose, D-fructose, D-mannose, *N*-acetylglucosamine, cellobiose, maltose, lactose, saccharose and D-raffinose, and are negative in tests for arginine dihydrolase, β-glucosidase, β-glucuronidase, alkaline phosphatase, β-galactosidase, pyrrolidonyl arylamidase, glycyltryptophan arylamidase, hippurate, *N*-acetyl-β-glucosaminidase, β-mannosidase and urease. All strains are negative in tests with adonitol, amidon, amygdalin, D- and L-arabitol, D- and L-arabinose, β-gentiobiose, D- and L-ketogluconate, dulcitol, erythritol, D- and L-fucose, methyl α-D-glucoside, gluconate, glycogen, inositol, inulin, D-lyxose, methyl α-D-mannoside, mannitol, melezitose, raffinose, rhamnose, ribose, sorbitol, L-sorbose, trehalose, D-furanose, D- and L-xylose, xylitol and β-methylxyloside. Only strain LMG 15061 produces strong clearing reactions on starch, whilst most other strains show only a weak or delayed amylase reaction. Variable reactions occur with arbutin (four

of positions 0 (top of gel, left side of the pattern) to 360 (front of gel, right side of the pattern) of the digitized and normalized protein patterns of all strains compared. On the right are strain designations (ACA-DC, Culture Collection of the Laboratory of Dairy Research, Agricultural University of Athens, Greece; LMG, BCCM/LMG Culture Collection of the Laboratory of Microbiology Ghent, University of Ghent, Belgium) and delineation of clusters. The suffix T indicates type strain, and t1, t2 and t3 indicate different colony types. *, See reference (10) and Table 2.

Table 5. Reference organisms used for specificity evaluation of probes ACA-DC 206/81 and ACA-DC 206/274

In-vitro-amplified rDNA or bacterial cells were used as targets for Southern or *in situ* hybridization, respectively.

Organism	Strain	Hybridization with ACA-DC 206/81		Hybridization with ACA-DC 206/274	
		Southern	*In situ*	Southern	*In situ*
S. macedonicus	ACA-DC 206^T	+	+	+	+
S. bovis	25481*	–	–	–	–
S. equinus	NCTC 9812	–	–	–	–
S. equinus	53364*	–	–	–	–
S. equinus	DSM 20062	–	–	–	–
S. salivarius	DSM 20560	–	–	–	–
S. salivarius	45807*	–	–	–	–
L. lactis	89211*	–	–	–	–

* Bundesanstalt fuer Milchforschung, Kiel, Germany.

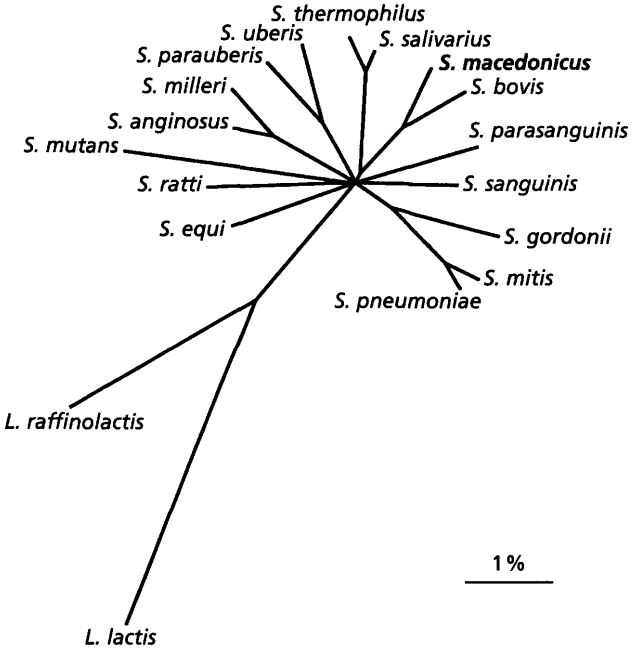

Fig. 2. Phylogenetic tree showing the relationships of *S. macedonicus* strain ACA-DC 206^T with selected streptococci. The tree is based on a maximum likehood analysis. The tree topology was evaluated and corrected according to the results of maximum parsimony and distance matrix treeing methods. Multifurcations connect branchings for which relative order could not be unambiguously determined or a common order was not supported applying different treeing methods. The bar indicates 1% estimated sequence divergence. Only alignment positions which share identical residues in at least 50% of all streptococcal 16S rRNA sequences were included for tree reconstruction. Only type strains of which at least 90% complete 16S rRNA sequences are available are shown.

strains positive). Six react with salicin and eight with melibiose. One strain is weakly positive with aesculin and three with methyl β-D-glucopyranoside. Only LMG 15061 produces acid from starch, pullulan and glycogen. All strains are sensitive to vancomycin, clindamycin, erythromycin and oxacillin. The G+C content is 38 mol%. Type strain is ACA-DC 206, isolated from Greek Kasseri cheese.

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