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Reappraisal of the taxonomy of the *Streptococcus bovis*/*Streptococcus equinus* complex and related species: description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *macedonicus* subsp. nov. and *S. gallolyticus* subsp. *pasteurianus* subsp. nov.

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'*Streptococcus bovis*/*Streptococcus equinus*' is a large bacterial complex including different species frequently isolated from infections of humans (*Streptococcus gallolyticus*, *Streptococcus infantarius*) or animals (*S. bovis*, *S. equinus*, *Streptococcus alactolyticus*). The separation of *S. bovis* into three different biotypes has been partially correlated with genetic differentiation. In addition, recent advances in bacterial phylogeny have led to the inclusion of *Streptococcus macedonicus* and *Streptococcus waius* in this complex. The aim of this study was to improve physiological differentiation between species related to the complex and to clarify their respective phylogenetic positions. In this study, physiological, genetic and phylogenetic analyses of a set of 88 streptococcal strains were performed. The diversity of strains of *S. bovis* biotype II was analysed, and it was confirmed that they belong to different species, either *S. equinus* or *S. infantarius*. It was demonstrated that *S. gallolyticus*, *S. bovis* biotype II.2, *S. macedonicus* and *S. waius* form a single DNA cluster separated into three different subspecies. They are delineated by different biochemical traits, limited DNA–DNA relatedness and noticeable divergence in 16S rDNA sequences. According to the current definition of species, the names *S. gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *pasteurianus* subsp. nov. and *S. gallolyticus* subsp. *macedonicus* subsp. nov. are proposed for these three subspecies.

INTRODUCTION

Twenty-five years ago, Klein *et al.* (1977) reported a strong association between *Streptococcus bovis* bacteraemia and colonic tumours in humans. This association led to great interest in the identification of group D streptococci, which are common inhabitants of the intestinal flora of humans and animals (Facklam, 1972; Farrow *et al.*, 1984; Osawa *et al.*, 1995). Since the initial antigen-based description of the group D streptococci by Lancefield (1933), *Streptococcus equinus* and *S. bovis* have been separated from *Streptococcus suis*, which may possess this antigen (Bentley *et al.*, 1991; Chatellier *et al.*, 1998; Farrow *et al.*, 1984; Jones *et al.*, 1972; Kawamura *et al.*, 1995, Kilpper-Bälz *et al.*, 1982).

A genetic classification of the group D streptococci was proposed by Farrow *et al.* (1984), who delineated six different DNA groups in the '*S. equinus*/*S. bovis*' complex. DNA group 1 included *S. equinus* and *S. bovis*. DNA groups 2, 3 and 4 corresponded to three different unnamed genospecies. DNA group 5 was named *Streptococcus saccharolyticus* and group 6 was named *Streptococcus alactolyticus*. Phylogenetic analysis of 16S rDNA sequences reinforced the separation of *S. equinus* and *S. bovis* from other streptococcal species (Bentley *et al.*, 1991) and assigned *S. saccharolyticus* to the genus *Enterococcus* (Rodriguez & Collins, 1990). *S. equinus*, *S. bovis* and *S. alactolyticus* are commonly identified according to the biochemical schemes proposed by Facklam (1972), Farrow *et al.* (1984) and Coykendall & Gustafson (1985). Human

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S. bovis strains are delineated into two biotypes according to their ability (biotype I) or inability (biotype II) to ferment mannitol. Furthermore, Osawa (1990) demonstrated that the *S. bovis* biotype I strains produce tannase. Indeed, hydrolysis of tannins and decarboxylation of gallic acid are characteristic of strains of DNA group 2 of Farrow *et al.* (1984), which Osawa *et al.* (1995) called *Streptococcus gallolyticus*. Simultaneously, Brooker *et al.* (1994) proposed that tannin-tolerant strains isolated from goats should be named *Streptococcus caprinus*. Phenotypic, genotypic and phylogenetic results have since indicated that *S. gallolyticus* and *S. caprinus* belong to the same species (Sly *et al.*, 1997).

Another medically important species, *Streptococcus infantarius* (Bouvet *et al.*, 1997; Schlegel *et al.*, 2000), two other novel species isolated from dairy products, *Streptococcus macedonicus* (Tsakalidou *et al.*, 1998) and *Streptococcus waius* (Flint *et al.*, 1999), and *S. suis* (Kilpper-Bälz & Schleifer, 1987) have not been included in these conventional schemes. Our laboratory has conducted genotypic studies on strains of *S. bovis* biotype II.1 (mannitol- and β-glucuronidase-negative and α-galactosidase-positive). Our results have shown that *S. bovis* biotype II.1 includes strains belonging to DNA group 1 of Farrow *et al.* (1984), whereas the strains of *S. infantarius* subsp. *infantarius*, which have a closely related phenotype, belong to DNA group 4 (Bouvet *et al.*, 1997; Schlegel *et al.*, 2000). Strains of *S. bovis*/*S. equinus* DNA group 1 have been isolated from humans, animals and food products, whereas strains of *S. infantarius* have mostly been obtained from human infections. *S. macedonicus* (Tsakalidou *et al.*, 1998) and *S. waius* (Flint *et al.*, 1999) have been included in the '*S. bovis*/*S. equinus*' complex on the basis of their 16S rDNA sequences. However, hybridization experiments have been carried out between the DNAs of the type strains of these two species and the DNA of only a limited number of reference strains (Manachini *et al.*, 2002). These considerations indicate that the relationships between these two species and their position in the '*S. bovis*/*S. equinus*' complex remain questionable.

In parallel with our investigations, Poyart *et al.* (2002) undertook sequence analysis and phylogenetic studies of the $sodA_{int}$ gene in group D streptococci. They propose the recognition of two additional species, which they called *Streptococcus lutetiensis* and *Streptococcus pasteurianus*, for *S. bovis* biotype II.1 and biotype II.2 strains, respectively. However, *S. lutetiensis* exhibits both phenotypic and genetic similarity to *S. infantarius* subsp. *coli* (Schlegel *et al.*, 2000), and preliminary results show a close relationship between *S. pasteurianus* and *S. gallolyticus*.

In the present study, we examined isolates of *S. bovis* biotype II.2 (mannitol-negative, β-glucuronidase- and β-mannosidase-positive) from various human infections. We have reappraised the classification of the '*S. bovis*/*S. equinus*' complex proposed by Farrow *et al.* (1984). Using DNA–DNA hybridizations and 16S rDNA sequences, we demonstrate that *S. macedonicus* and *S. waius* are subjective synonyms and that *S. bovis* biotype II.2 strains, *S. gallolyticus* and *S. macedonicus* are subspecies of a single species. The name *S. gallolyticus* has nomenclatural priority according to Rule 24b(2) of the *International Code of Nomenclature of Bacteria* (Lapage *et al.*, 1992). We propose to designate these three subspecies *S. gallolyticus* subsp. *pasteurianus* subsp. nov., *S. gallolyticus* subsp. *gallolyticus* subsp. nov. and *S. gallolyticus* subsp. *macedonicus* subsp. nov., respectively.

METHODS

Bacterial strains. A total of 88 strains were obtained from various collections (Table 1). Pure cultures were stored at −80 °C in brain heart infusion broth supplemented with 15 % (w/v) glycerol. All subcultures were carried out on Columbia blood agar (bioMérieux) or in buffered glucose broth (Bio-Rad).

Physiological tests. Growth characteristics were investigated in 5 % sheep blood/Columbia agar, buffered glucose broth, brain heart infusion broth and MRS broth (Bio-Rad). Conventional methods were used to test for the production of catalase, growth in 6·5 % NaCl, tolerance of 40 % bile and tolerance of 0·016 % (w/v) potassium tellurite (Facklam & Washington, 1991). Production of enzymes and fermentation of carbohydrates were determined using both the API 20 Strep and Rapid ID32 STREP strips, according to the instructions of the manufacturer (bioMérieux). Production of tannase was assayed by the colorimetric test of Osawa *et al.* (1995). Investigation of this enzyme was preferred to the assessment of gallate decarboxylase, since the former test is easier to carry out and because both enzymes are present in most strains of *S. gallolyticus* (Osawa *et al.*, 1995). Moreover, the hydrolysis of tannin is known to be the first step of this decarboxylation pathway (Chamka *et al.*, 2002). Isolates were investigated for the presence of the Lancefield group D antigen by using a latex-bead agglutination reagent after enzymic lysis at 37 °C for 30 min (Pastorex; Bio-Rad).

Preparation of DNA. Bacteria were grown in 1·5 l buffered glucose broth for 5–7 h to late exponential phase at 37 °C. One hour before harvesting of the bacteria, glycine (30 g l^{-1}; Sigma) was added to the culture broth to facilitate cell lysis. Bacteria were then harvested by centrifugation, washed in TE buffer (10 mM Tris/HCl, 1 mM disodium EDTA, pH 8) and suspended in lysis buffer (10 mM Tris/HCl, 1 mM disodium EDTA, 1 M sucrose, pH 8) (Bouvet *et al.*, 1991). Mutanolysin (5 U ml^{-1}; Sigma Aldrich) and lysozyme (10 mg ml^{-1}; Boehringer Mannheim) were added and the mixture was incubated overnight at 37 °C (Grimont & Grimont, 1995). Bacterial membrane disruption was achieved by adding proteinase K (0·4 mg ml^{-1}) and SDS (1 %, w/v). For several strains, lysis was completed with an additional 24 h incubation of the mixture at 37 °C. DNA was extracted and purified using the phenol/chloroform method (Grimont & Grimont, 1995).

Quantitative DNA–DNA hybridization. DNA from type or reference strains *S. bovis* HDP 89505^T, *S. equinus* HDP 89506^T, *S. gallolyticus* HDP 98035^T, *S. bovis* biotype II.2 HDP 90084, *S. infantarius* subsp. *infantarius* HDP 90056^T, *S. infantarius* subsp. *coli* HDP 90246^T, *S. alactolyticus* HDP 90057^T, *S. macedonicus* HDP 98362^T and *S. waius* HDP 99442^T was labelled *in vitro* with [^{3}H]ATP, [^{3}H]TTP, [^{3}H]GTP and [^{3}H]CTP using the Megaprime DNA labelling reaction kit (all from Amersham). Hybridizations of these labelled DNAs with DNA of representative strains of the '*S. bovis*/*S. equinus*' complex were carried out in a liquid medium under stringent conditions consisting of 60 °C for 16 h, according to a modification of the S1 nuclease/trichloracetic acid precipitation method (Crosa *et al.*, 1973; Grimont *et al.*, 1980). The temperature at which 50 % of the reassociated DNAs were hydrolysed by S1 nuclease (T_m) was determined.

Table 1. Streptococcal strains used in the study

Culture collections: ACA-DC, Culture Collection of the Laboratory of Dairy Research, Agricultural University of Athens, Greece; ACM, Australian Collection of Microorganisms, University of Queensland, Australia; Api, API-bioMérieux Collection, La Balme-les-Grottes, France; ATCC, American Type Culture Collection, Manassas, VA, USA; BioVac, Technopole d'Angers, Beaucouzé, France; CIP, Collection de l'Institut Pasteur, Paris, France; CDC, Centers for Disease Control, Atlanta, GA, USA; CNRZ, Centre National de Référence de Zootechnie, France; INRA, Institut National de la Recherche Agronomique, Centre de Tours, France; LMG, Culture Collection of the Laboratory of Microbiology Ghent, University of Ghent, Ghent, Belgium; NCDO, National Collection of Dairy Organisms, Reading, UK; NCTC, National Collection of Type Cultures, London, UK; NZRCC, New Zealand Reference Culture Collection, Palmerston North, New Zealand; UPL, Université Louis Pasteur de Strasbourg, France.

Strain designation	Original designation(s)	Source
Type and reference strains		
S. bovis HDP 89505^T	NCDO 597^T, ATCC 33317^T	Bovine faeces
S. equinus HDP 89506^T	NCDO 1037^T, ATCC 9812^T	Equine faeces
S. gallolyticus HDP 98035^T	ACM 3611^T	Koala faeces
S. caprinus HDP 99204^T	ACM 3969^T	Goat faeces
S. bovis biotype II.2 HDP 90084	Api 79.04.159	Human isolate
DNA homology group 3 strain HDP 90058	NCDO 2127	Bovine mastitis
S. infantarius subsp. *infantarius* HDP 90056^T	NCDO 599^T	Human baby faeces
S. infantarius subsp. *coli* HDP 90246^T	NCDO 964^T	Unknown source
S. alactolyticus HDP 90057^T	NCDO 1091^T	Porcine intestine
S. intestinalis HDP 90052^T	ATCC 43492^T, LMG 14906^T	Porcine intestine
S. macedonicus HDP 98362^T	ACA-DC 206^T, LMG 18488^T	Greek Kasseri cheese
S. suis HDP 90048^T	NCTC 10234^T	Porcine intestine
S. waius HDP 99422^T	NZRCC 20100^T	Dairy product
E. saccharolyticus HDP 90059^T	NCDO 2594^T	Straw bedding
Environmental and clinical isolates		
S. bovis		
HDP 90083	Api 86.06.003	Unknown source
HDP 94133	Our collection*	Human endocarditis
HDP 98354	CDC 3437.70	Human isolate
HDP 99171	CNRZ102	Bovine mastitis
HDP 99172	CNRZ99	Bovine mastitis
HDP 99298	INRA D2 38.21	Bovine mastitis
HDP 99307	INRA D5 551.01	Bovine mastitis
S. equinus		
HDP 89538	CIP TH1	Unknown source
HDP 99282	CIP 82.5, NCDO 2491	Equine faeces
HDP 99308	NCTC 10386	Equine faeces
HDP 99309	NCTC 10389	Equine faeces
HDP 99310	Api Benelux 75/289	Unknown source
HDP 99311	Api 81.02.022	Unknown source
HDP 99312	Api 83.12.025	Equine vagina
HDP 99315	Api 99.03.045	Unknown source
S. gallolyticus		
HDP 90055	NCDO 2019	Bovine mastitis
HDP 90299	Api 84.03.028	Unknown source
HDP 91381	Our collection	Human endocarditis
HDP 91291	Our collection	Human endocarditis
HDP 91201	Our collection	Human endocarditis
HDP 91230	Our collection	Human endocarditis
HDP 91242	Our collection	Human endocarditis
HDP 91255	Our collection	Human endocarditis
HDP 98354	CDC 3437.70	Human isolate
HDP 98386	NCDO 2080, ATCC 9809	Unknown source
HDP 98430	ULP T15125/98	Unknown source
S. infantarius subsp. *infantarius*		
HDP 90104	Our collection	Dairy product

Table 1. cont.

Strain designation	Original designation(s)	Source
HDP 90247	NCDO 2602	Frozen peas
HDP 91257	Our collection	Human endocarditis
HDP 97027	Api 92.03.336	Unknown source
HDP 97029	Api 88.12.012	Human blood
HDP 98032	Our collection	Human blood
HDP 98426	NCTC 8133	Human infection
HDP 99070	ULP 11B172	Human endocarditis
HDP 99071	ULP 12B42	Human endocarditis
S. infantarius subsp. *coli*		
HDP 90062	Our collection	Human infection
HDP 90248	NCDO 2620	Human baby faeces
HDP 90249	NCDO 2632	Human infection
HDP 90256	Our collection	Human endocarditis
HDP 91356	Our collection	Human endocarditis
HDP 97028	Api 82.10.072	Human urine
HDP 97317	NCDO 1600	Human isolate
HDP 97318	NCDO 1610	Human infant faeces
HDP 97319	NCDO 1616	Human isolate
HDP 97322	NCDO 2759	Human blood
HDP 98364	Api 78.06.120, NCTC 11436	Human blood
HDP 98367	Api 81.07.022	Human suppuration
HDP 98370	Api 87.06.001	Human blood
S. bovis biotype II.2		
HDP 90382	Our collection	Urinary infection
HDP 91130	Our collection	Human endocarditis
HDP 91303	Our collection	Human endocarditis
HDP 91317	Our collection	Human endocarditis
HDP 91321	Our collection	Human endocarditis
HDP 91333	Our collection	Human endocarditis
HDP 91338	Our collection	Human endocarditis
HDP 97320	NCDO 2756	Human isolate
HDP 97321	NCDO 2758	Human blood
HDP 97323	NCDO 2760	Human blood
HDP 97324	NCDO 2761	Human blood
HDP 98355	CDC 2460.71	Human blood
HDP 98356	CDC 1723.81, ATCC 43144	Human blood
HDP 98357	CDC 2266.81	Human blood
HDP 98358	CDC 663.82	Human blood
HDP 98365	Api 79.04.158	Urinary tract infection
HDP 98368	Api 82.02.073	Human suppuration
HDP 98369	Api 83.07.026	Human blood
HDP 98371	Api 88.12.010	Human blood
HDP 98372	Api 94.05.016	Urinary tract infection
DNA group 3		
HDP 99300	INRA D2 44.19	Bovine mastitis
S. alactolyticus		
HDP 99293	BioVac 57E	Porcine infection
HDP 00089	BioVac 9809141	Porcine infection
S. macedonicus		
HDP 99050	ACA-DC 0207	Greek Kasseri cheese
HDP 99051	ACA-DC 0210	Greek Kasseri cheese
HDP 99072	ACA-DC 0205	Greek Kasseri cheese

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The difference between the melting temperatures of homoduplexes and heteroduplexes (ΔT_m) allowed the estimation of DNA divergence between strains with high levels of DNA relatedness (Grimont *et al.*, 1980).

16S rDNA sequence determination and phylogenetic analysis. PCR amplification of 16S rRNA-encoding DNA and sequencing of amplified fragments were carried out as described previously (Janvier & Grimont, 1995) for *S. equinus* HDP 89506^T, *S. bovis* biotype II.2 HDP 90084, *S. infantarius* subsp. *infantarius* HDP 90056^T, *S. infantarius* subsp. *coli* HDP 90246^T and the reference strain of DNA group 3, HDP 90058. Alignment with a selection of the available sequences of 16S rDNA from GenBank and phylogenetic analysis of the 16S rDNA data were performed with the MEGALIGN program from the DNAstar package. Sequences were aligned by using the CLUSTAL multiple-sequence method. A distance matrix was then computed using a Kimura model for nucleotide substitution. Phylogenetic trees were generated from the distance matrices by using the neighbour-joining method.

RESULTS

Phenotypic characterization

All strains were Gram-positive cocci, non-motile, non-sporulating and occurred in pairs or in short chains. The catalase test was negative. The strains showed homogeneous growth in buffered glucose and brain heart infusion broths and did not produce gas in MRS broth. They were α- or non-haemolytic on sheep-blood agar in an aerobic atmosphere. They were tellurite-negative. None of the strains, except the type strain of *Enterococcus saccharolyticus*, grew on bile/aesculin agar or in 6·5 % NaCl broth. All the strains produced leucine aminopeptidase and alanyl-phenylalanyl-proline arylamidase. Table 2 shows the reactions obtained with the 84 strains representative of all species of group D streptococci *sensu stricto*, the two strains of DNA group 3 of Farrow *et al.* (1984) and the type strains of *S. suis* and *E. saccharolyticus*. Except for *S. suis* and *E. saccharolyticus*, they did not produce pyrrolidonyl arylamidase. Most strains produced acetoin according to the Voges–Proskauer test, and did not produce arginine dihydrolase. However, the strains of DNA group 3 and *E. saccharolyticus* were Voges–Proskauer-negative and the strain of *S. suis* was Voges–Proskauer-negative and arginine dihydrolase-positive. A limited number of biochemical tests allowed further assignment to different species within the '*S. bovis*/*S. equinus*' complex (Table 2). These included acidification of mannitol and hydrolysis of gallate (production of tannase) by *S. gallolyticus*, production of β-glucuronidase by *S. bovis* biotype II.2 and *S. suis*, production of β-mannosidase by *S. bovis* biotype II.2, absence of production of β-glucuronidase and production of β-galactosidase (β-GAR test) by *S. macedonicus* and *S. waius* and the absence of β-glucosidase by *S. macedonicus*, *S. waius* and by most of the strains of *S. infantarius* subsp. *infantarius*. The association of the production of β-glucuronidase, β-galactosidase (β-GAL test) and β-mannosidase with the acidification of trehalose clearly distinguished the *S. bovis* biotype II.2 strains from other species (Table 2).

DNA–DNA hybridization

Tables 3 and 4 summarize the results obtained in hybridization experiments with radiolabelled DNA of type and reference strains. The existence of four DNA homology groups within the '*S. bovis*/*S. equinus*' complex was demonstrated. We designated these DNA clusters using Roman numerals, instead of the Arabic numerals used for the DNA–DNA homology groups of Farrow *et al.* (1984). DNA cluster I is formed by the type strains of *S. equinus* and *S. bovis* (Table 3). *S. gallolyticus* HDP 98035^T, *S. macedonicus* HDP 98362^T, *S. waius* HDP 99422^T and a strain of *S. bovis* biotype II.2, HDP 90084, appeared to belong to the same DNA cluster, cluster II (Table 4). According to the DNA–DNA relatedness analysis, this cluster contains three different subgroups. Within each subgroup, the level of relatedness between strains was over 77 % ($\Delta T_m \leqslant 2\,^\circ C$), whereas the relatedness between the different subgroups ranged from 48 to 93 % ($\Delta T_m \leqslant 6\,^\circ C$). The first subgroup in this DNA cluster, cluster II, included the reference strain HDP 90055 of DNA homology group 2 of Farrow *et al.* (1984), the type strains of *S. gallolyticus* and *S. caprinus* and strain HDP 90299, identified biochemically as *S. gallolyticus* (81–100 % DNA relatedness). The second subgroup included the type strains of *S. macedonicus* and *S. waius* (77–100 % DNA relatedness, $\Delta T_m \leqslant 2\,^\circ C$). The last subgroup was formed by strains of *S. bovis* II.2, which were biochemically and genetically homogeneous (100 % DNA relatedness among the strains tested). A high level of DNA reassociation was also found for *S. infantarius* subsp. *infantarius* and *S. infantarius* subsp. *coli*, which constitute DNA cluster III (64–67 % between strains of the two subspecies). The strain of *S. alactolyticus* appeared to belong to another relatedness group (DNA cluster IV) within the '*S. bovis*/*S. equinus*' complex.

16S rDNA sequencing and phylogenetic analysis

We determined the 16S rDNA sequences of *S. infantarius* subsp *infantarius* HDP 90056^T, *S. infantarius* subsp. *coli* HDP 90246^T, *S. bovis* biotype II.2 HDP 90084, *S. equinus* HDP 89506^T and the reference strain for DNA group 3 of Farrow *et al.* (1984), HDP 90058. 16S rDNA sequences of other representative streptococcal strains were obtained from GenBank. We selected long (>1350 bp) and high-quality (<1 % undetermined positions) sequences of type or reference strains. The length of the alignment was further limited to 1350 sites to reduce the weight of gaps and mismatches at the beginning or end of the sequences. The corresponding phylogenetic tree (Fig. 1) may be divided into seven major clusters: the '*S. bovis*/*S. equinus*' complex, the 'thermophilic' streptococci, the '*milleri*' group, the pyogenic streptococci, the *S. suis* species, the *Streptococcus mitis* group and the '*mutans*' group. This 16S rDNA-based analysis led to classification of the DNA group 3 reference strain within the cluster of *S. suis*, as demonstrated by the high level of similarity of the sequence of strain HDP 90058 to *S. suis* type 22 (98·9 %). *S. bovis*, *S. equinus*, *S. gallolyticus*, *S. infantarius* subsp. *infantarius*, *S. infantarius* subsp. *coli*,

Table 2. Biochemical patterns of strains belonging to the '*S. bovis*/*S. equinus*' complex and related species

Phenotype: 1, *S. equinus* (9 strains examined); 2, *S. bovis* ($n=8$); 3, *S. gallolyticus* ($n=13$); 4, *S. macedonicus* ($n=4$); 5, *S. waius* ($n=1$); 6, *S. bovis* biotype II.2 ($n=21$); 7, *S. infantarius* subsp. *infantarius* ($n=10$); 8, *S. infantarius* subsp. *coli* ($n=14$); 9, *S. alactolyticus* ($n=4$); 10, *S. suis* ($n=1$); 11, DNA group 3 ($n=2$); 12, *E. saccharolyticus* ($n=1$). Results are expressed as numbers (percentages) of positive strains for each reaction.

Test	1	2	3	4	5	6	7	8	9	10	11	12
Hydrolysis of:												
Arginine (arginine dihydrolase)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Aesculin	9 (100)	8 (100)	13 (100)	0 (0)	0 (0)	21 (100)	5 (50)	14 (100)	4 (100)	1 (100)	2 (100)	1 (100)
Gallate (tannase activity)	0 (0)	0 (0)	13 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Production of:												
Acetoin (Voges–Proskauer)	9 (100)	8 (100)	13 (100)	4 (100)	1 (100)	21 (100)	10 (100)	14 (100)	4 (100)	0 (0)	0 (0)	0 (0)
β-Glucosidase	9 (100)	8 (100)	13 (100)	0 (0)	0 (0)	21 (100)	2 (20)	14 (100)	4 (100)	0 (0)	2 (100)	1 (100)
β-Glucuronidase	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
α-Galactosidase	0 (0)	8 (100)	8 (62)	1 (25)	1 (100)	15 (71)	10 (100)	13 (93)	4 (100)	1 (100)	1 (50)	1 (100)
β-Galactosidase (β-GAR test)	0 (0)	0 (0)	1 (8)	4 (100)	1 (100)	1 (5)	0 (0)	0 (0)	0 (0)	1 (100)	2 (100)	0 (0)
β-Galactosidase (β-GAL test)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	20 (95)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
β-Mannosidase	0 (0)	2 (25)	3 (23)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pyrrolidonyl arylamidase	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Acidification of:												
Glycogen	0 (0)	8 (100)	13 (100)	0 (0)	0 (0)	0 (0)	9 (90)	1 (7)	0 (0)	1 (100)	2 (100)	0 (0)
Inulin	0 (0)	8 (100)	12 (92)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	1 (100)	2 (100)	1 (100)
Lactose	0 (0)	8 (100)	13 (100)	4 (100)	1 (100)	21 (100)	10 (100)	14 (100)	0 (0)	1 (100)	2 (100)	1 (100)
Mannitol	0 (0)	0 (0)	13 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	1 (100)
Melibiose	0 (0)	8 (100)	0 (0)	0 (0)	0 (0)	2 (10)	8 (80)	1 (7)	0 (0)	0 (0)	0 (0)	1 (100)
Melezitose	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9 (43)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Methyl β-D-glucopyranoside	5 (56)	8 (100)	12 (92)	1 (25)	0 (0)	21 (100)	2 (20)	13 (93)	2 (50)	0 (0)	2 (100)	1 (100)
Raffinose	0 (0)	6 (75)	2 (15)	0 (0)	0 (0)	12 (57)	8 (80)	3 (21)	1 (25)	1 (100)	2 (100)	1 (100)
Starch	0 (0)	8 (100)	13 (100)	4 (100)	0 (0)	3 (14)	10 (100)	7 (50)	0 (0)	1 (100)	2 (100)	1 (100)
Trehalose	3 (33)	3 (38)	13 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	0 (0)	1 (100)	2 (100)	1 (100)
DNA homology group*	1	1	2	–	–	–	4	4	6	–	3	5
DNA cluster	I	I	II	II	II	II	III	III	IV	–	–	–

*Groups of Farrow *et al.* (1984).

Table 3. Levels of hybridization of total DNA from strains of the '*S. bovis*/*S. equinus*' complex

Hybridization levels within the same DNA cluster are boxed. ND, Not determined.

Source of unlabelled DNA	Source of labelled DNA					
	1	2	3	13	15	16
DNA cluster I						
1. *S. bovis* HDP 89505^T (=NCDO 597^T)	100	98	38	33	46	22
2. *S. equinus* HDP 89506^T (=NCDO 1037^T)	91	100	35	42	45	24
DNA cluster II						
S. gallolyticus subsp. *gallolyticus*						
3. *S. gallolyticus* HDP 98035^T (=ACM 3611^T)	33	48	100	32	38	19
4. *S. caprinus* HDP 99204^T (=ACM 3969^T)	ND	42	94	31	33	21
5. *S. gallolyticus* HDP 90055 (=NCDO 2019)	41	50	94	33	33	29
6. *S. gallolyticus* HDP 90299 (=Api 84.03.028)	43	36	81	42	26	21
S. gallolyticus subsp. *macedonicus*						
7. *S. macedonicus* HDP 98362^T (=ACA-DC 206^T)	53	47	54	43	57	18
8. *S. macedonicus* HDP 99050 (=ACA-DC 207)	ND	ND	54	44	ND	17
9. *S. waius* HDP 99422^T (=NZRCC 20100^T)	ND	ND	77	33	29	15
S. gallolyticus subsp. *pasteurianus*						
10. *S. bovis* biotype II.2 HDP 90084 (=Api 79.04.159)	30	55	65	29	37	ND
11. *S. bovis* biotype II.2 HDP 97323 (=NCDO 2760)	30	ND	ND	29	37	ND
12. *S. bovis* biotype II.2 HDP 97324 (=NCDO 2761)	50	ND	57	28	33	ND
DNA cluster III						
13. *S. infantarius* subsp. *infantarius* HDP 90056^T (=NCDO 599^T)	39	39	33	100	ND	40
14. *S. infantarius* subsp. *infantarius* HDP 90104	47	54	36	89	67	39
15. *S. infantarius* subsp. *coli* HDP 90246^T (=NCDO 964^T)	42	52	33	64	100	30
DNA cluster IV						
16. *S. alactolyticus* HDP 90057^T (=NCDO 1091^T)	21	ND	19	20	ND	100

S. alactolyticus, *S. macedonicus* and *S. waius* were grouped together within the '*S. bovis*/*S. equinus*' complex (Fig. 1).

Additional partial sequences of 16S rDNA of about 350 bp from the GenBank database have been analysed to construct a larger phylogenetic tree (Table 5; Fig. 2). Three major divisions corresponding to four DNA clusters were observed. The first division included *S. infantarius* (DNA cluster III) and '*S. bovis*/*S. equinus*' (DNA cluster I). The second division corresponded to *S. gallolyticus*, *S. macedonicus*, *S. waius* and *S. bovis* biotype II.2 (DNA cluster II). The third division included *S. alactolyticus* and *Streptococcus intestinalis* (DNA cluster IV). The 99·5 % similarity between *S. waius* and *S. macedonicus* favours the notion of a single species. In DNA cluster II, the divergence between the 16S rDNA sequences from *S. bovis* biotype II.2 HDP 90084, *S. gallolyticus* HDP 98035^T, *S. macedonicus* HDP 98362^T and *S. waius* HDP 99422^T ranged from 2·6 to 7·1 %.

DISCUSSION

S. bovis and *S. equinus* are important intestinal bacteria frequently isolated from human and animal specimens as well as from food and vegetables. These two species were first delineated according to antigenic (group D), cultural (tolerance of bile and of 6·5 % NaCl) and biochemical (hydrolysis of aesculin, acidification of sucrose and lactose) characteristics (Bridge & Sneath, 1983; Facklam & Washington, 1991; Farrow *et al.*, 1984; Hardie, 1986; Seeley & Dain, 1960). However, phenotypic heterogeneity was recognized among isolates in terms of carbohydrate fermentation, starch hydrolysis and production of glucan from sucrose. These variations led to the description of 'variant strains' of *S. bovis* by different authors (Coykendall & Gustafson, 1985; Facklam, 1972; Knight & Shlaes, 1985; Nelms *et al.*, 1995). The species *S. alactolyticus*, *S. suis*, *S. gallolyticus*, *S. macedonicus*, *S. waius* and *S. infantarius* were further described. In this study, we have collected a large number of strains to improve the classification of the '*S. bovis*/*S. equinus*' complex and related species. Most of these strains have already been subjected to genetic analyses such as DNA–DNA hybridization or 16S rDNA sequencing. Some additional fresh clinical isolates were included when necessary to establish the phenotypic description of species.

Our results are consistent with the data of Farrow *et al.* (1984) on group D streptococci. The type strain and isolates of *S. bovis* (identified as *S. bovis* biotype II.1) differed from *S. equinus* by a small number of positive biochemical reactions, such as the production of α-galactosidase and the acidification of starch, lactose and raffinose. DNA–DNA hybridization experiments using total DNA from *S. bovis* and *S. equinus* type strains displayed between 91 and 100 % relatedness (Table 3; DNA cluster I). Our results

Table 4. Levels of DNA hybridization and ΔT_m values of total DNA of strains of the '*S. bovis*/*S. equinus*' complex

Values are percentages of DNA–DNA hybridization with ΔT_m (°C) in parentheses. Hybridization levels that correspond to newly described subspecies within DNA cluster II are boxed. ND, Not determined.

Source of unlabelled DNA	Source of labelled DNA			
	3	7	9	10
DNA cluster I				
1. *S. bovis* HDP 89505^T (=NCDO 597^T)	38 (ND)	44 (ND)	31 (ND)	41 (ND)
2. *S. equinus* HDP 89506^T (=NCDO 1037^T)	35 (ND)	53 (ND)	29 (ND)	30 (ND)
DNA cluster II				
S. gallolyticus subsp. *gallolyticus*				
3. *S. gallolyticus* HDP 98035^T (=ACM 3611^T)	100 (0)	87 (1)	61 (2)	63 (4)
4. *S. caprinus* HDP 99204^T (=ACM 3969^T)	94 (2)	92 (0)	66 (ND)	54 (6)
5. *S. gallolyticus* HDP 90055 (=NCDO 2019)	94 (ND)	80 (ND)	70 (ND)	51 (ND)
6. *S. gallolyticus* HDP 90299 (=Api 84.03.028)	81 (ND)	93 (ND)	67 (ND)	50 (ND)
S. gallolyticus subsp. *macedonicus*				
7. *S. macedonicus* HDP 98362^T (=ACA-DC 206^T)	54 (2)	100 (0)	96 (2)	57 (3)
8. *S. macedonicus* HDP 99050 (=ACA-DC 207)	54 (ND)	80 (ND)	77 (ND)	54 (6)
9. *S. waius* HDP 99422^T (=NZRCC 20100^T)	77 (5)	97 (2)	100 (0)	72 (5)
S. gallolyticus subsp. *pasteurianus*				
10. *S. bovis* biotype II.2 HDP 90084 (=Api 79.04.159)	65 (2)	48 (4)	68 (4)	100 (0)
11. *S. bovis* biotype II.2 HDP 97323 (=NCDO 2760)	ND (ND)	57 (ND)	80 (ND)	100 (ND)
12. *S. bovis* biotype II.2 HDP 97324 (=NCDO 2761)	57 (3)	58 (ND)	80 (3)	100 (ND)
DNA cluster III				
13. *S. infantarius* subsp. *infantarius* HDP 90056^T (=NCDO 599^T)	33 (ND)	38 (ND)	27 (ND)	31 (ND)
14. *S. infantarius* subsp. *infantarius* HDP 90104	36 (ND)	37 (ND)	41 (ND)	29 (ND)
15. *S. infantarius* subsp. *coli* HDP 90246^T (=NCDO 964^T)	33 (ND)	25 (ND)	45 (ND)	32 (ND)
DNA cluster IV				
16. *S. alactolyticus* HDP 90057^T (=NCDO 1091^T)	19 (ND)	18 (ND)	17 (ND)	23 (ND)

are in accordance with previous studies (Garvie & Farrow, 1981; Kilpper-Bälz *et al.*, 1982; Farrow *et al.*, 1984, Knight & Shlaes, 1985; Nelms *et al.*, 1995) that have demonstrated that *S. bovis* and *S. equinus* form a single genospecies (DNA group 1 of Farrow *et al.*, 1984). In addition, we have determined the 16S rDNA sequence of *S. equinus* HDP 89506^T and compared it with those of the other type strains of the '*S. bovis*/*S. equinus*' complex. The 16S rDNA sequence of *S. bovis* HDP 89505^T differs from that of *S. equinus* HDP 89506^T by only 15 sites along a 1455-base fragment (99·0 % similarity). These high degrees of similarity both in total DNA–DNA hybridization and in 16S rDNA sequence confirm that *S. bovis* and *S. equinus* belong to a single species, according to the overall criteria of Stackebrandt & Goebel (1994), Drancourt *et al.* (2000) and Stackebrandt *et al.* (2002). Therefore, *S. equinus* and *S. bovis* can be considered definitively as a single species. The name *S. equinus* has nomenclatural priority, though *S. bovis* is widely used to designate these species in the medical literature.

DNA group 2 of Farrow *et al.* (1984) included mannitol-hydrolysing strains isolated from bovine mastitis specimens and from human blood cultures. The existence of tannase and gallate decarboxylase activities has been demonstrated in these strains, and it was proposed that they be designated as *S. gallolyticus* (Osawa, 1990; Osawa *et al.*, 1995). The initial report by these authors indicated the existence of atypical strains of *S. bovis* that lacked tannase activity and that yielded significant relatedness with *S. gallolyticus* (60–66 % DNA–DNA hybridization with the type strain ACM 3611^T). From this study, DNA cluster II includes *S. gallolyticus*, *S. bovis* biotype II.2, *S. macedonicus* and *S. waius* in three separate subgroups. The DNA relatedness ranged from 48 to 93 % ($\Delta T_m < 6$ °C) between different subgroups. The levels of hybridization of total DNA between *S. gallolyticus* and *S. bovis* biotype II.2 are similar to the results of Coykendall & Gustafson (1985), Garvie & Bramley (1979) and Osawa *et al.* (1995). Only a few biochemical characteristics (acidification of arbutin and melibiose) distinguish *S. macedonicus* from *S. waius*. Both the DNA–DNA relatedness of the type strains, HDP 98362^T and HDP 99422^T, and the phylogeny of the 16S rDNA sequences indicate a close relationship (Fig. 2; Table 5). Our results are in agreement with the proposition of Manachini *et al.* (2002) to group the two species together. The name *S. macedonicus*, which was the first to be validly published, must be maintained, according to the Bacteriological Code (Lapage *et al.*, 1992).

We show that the 16S rDNA sequence of strain CIP 105070^T, proposed by Poyart *et al.* (2002) as the type strain of the novel species *S. pasteurianus*, clusters with that of *S. bovis*

Fig. 1. Phylogenetic tree of streptococci, based on comparative analysis of the complete 16S rDNA sequences. Bar, 5% nucleotide sequence difference.

Table 5. Analysis of 16S rDNA sequences from strains belonging to the '*S. bovis*/*S. equinus*' complex

Strains are listed under their former classification. Divergence levels within the same DNA cluster are boxed.

Strain	Accession no.	Length (bp)	Divergence (%) from sequence:				
			1	2	3	4	5
DNA cluster I							
1. *S. bovis* ATCC 3317^T	AF002482	1457	–	0·5	8·1	1·0	8·2
2. *S. equinus* HDP 89506^T	AF429765	1463	0·5	–	7·2	1·0	7·6
S. equinus ATCC 9812^T	AF104116	356	1·3	0·0	7·2	1·6	8·3
S. equinus NCTC 9814	AB002514	1258	0·8	0·3	7·0	1·2	6·3
DNA cluster II							
S. gallolyticus subsp. *gallolyticus*							
3. *S. gallolyticus* ACM 3611^T	X94337	1501	8·1	7·2	–	8·2	11·2
S. bovis VAMC 3076	AF313408	534	4·6	3·7	2·8	4·5	8·2
S. caprinus ACM 3969^T	Y10868	1279	4·7	4·0	2·7	4·9	7·8
S. caprinus ACM 3968	Y10869	1371	4·8	4·1	2·6	5·0	7·6
S. gallolyticus ATCC 43143	AF104114	1541	5·9	4·7	2·7	5·7	8·5
S. gallolyticus subsp. *macedonicus*							
S. macedonicus ACA-DC 206^T	Z94012	1542	5·5	6·5	4·4	5·7	7·1
S. waius NZRCC 20100^T	AF088900	1484	4·8	7·1	4·1	5·3	7·8
S. macedonicus 3/1	U96621	343	5·4	6·8	4·6	5·9	7·7
S. gallolyticus subsp. *pasteurianus*							
S. pasteurianus CIP 105070^T	AF297216	1466	3·2	2·8	3·6	3·6	9·1
S. bovis biotype II.2 HDP 90084	AF429764	1430	3·2	2·8	3·7	3·6	8·3
S. bovis VAMC 3395	AF313406	535	4·0	3·1	3·3	3·9	8·2
S. bovis 00 UCUL 4487	AF313407	535	4·0	3·1	3·3	3·9	8·2
S. bovis ATCC 43144	AF104115	356	5·1	3·9	3·3	4·9	8·3
DNA cluster III							
S. infantarius subsp. *coli*							
S. infantarius subsp. *coli* HDP 90246^T	AF429763	1473	0·5	0·5	7·5	0·5	7·9
S. lutetiensis NEM 782^T	AF297215	1461	0·5	0·3	7·9	0·3	8·9
S. bovis 21-09-6c	AF104111	353	0·7	0·3	7·4	1·0	8·4
S. bovis JB1	AF104109	1541	0·6	0·3	7·2	0·9	8·2
S. bovis C14b1	AF396921	1539	0·5	0·3	7·2	0·8	7·9
S. bovis B315	AF396920	1540	0·5	0·5	7·2	0·8	7·9
S. bovis 1315	AF135453	1462	0·3	0·3	7·5	0·8	7·9
S. bovis 26	AF104110	352	0·7	0·3	7·4	1·0	8·4
S. bovis 45s1	AF104112	353	0·7	0·3	7·4	1·0	8·4
S. bovis H2-1	AF202223	1540	0·6	0·3	7·2	0·9	8·2
S. bovis NCFB 2476	AF396922	1539	0·5	0·3	7·2	0·8	7·9
S. bovis K27FF4	AF104113	352	1·0	0·6	7·7	1·0	8·7
S. bovis ATCC 27960	AB002481	1500	0·6	0·6	7·5	0·6	8·8
S. bovis VAMC 2818	AF353718	535	0·8	0·5	7·5	0·5	8·5
S. infantarius subsp. *infantarius*							
4. *S. infantarius* subsp. *infantarius* HDP 90056^T	AF429762	1386	1·0	1·0	8·2	–	9·0
S. infantarius subsp. *infantarius* HDP 90104	AF177729	1493	1·1	1·1	8·2	0·0	8·6
DNA cluster IV							
5. *S. alactolyticus* ATCC 43077^T	AF201899	1437	8·2	7·6	11·2	9·0	–
S. intestinalis ATCC 43492^T	AB002519	1491	10·9	10·3	14·7	11·0	2·5

biotype II.2 (strain HDP 90084; Fig. 2). In addition, the biochemical patterns of *S. pasteurianus* strains, including HDP 90084, and the DNA–DNA hybridization values reported by Poyart *et al.* (2002) indicate that *S. pasteurianus* is similar to our subgroup of *S. bovis* biotype II.2 strains. However, the levels of DNA–DNA hybridization reported by Poyart *et al.* (2002) and determined in the present study, as well as the values of ΔT_m (Table 4), do not allow these strains to be proposed as distinct species (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). Therefore, *S. gallolyticus*, *S. macedonicus*, *S. waius*, *S. pasteurianus* and *S. bovis* biotype II.2 can be merged into a single species. In addition,

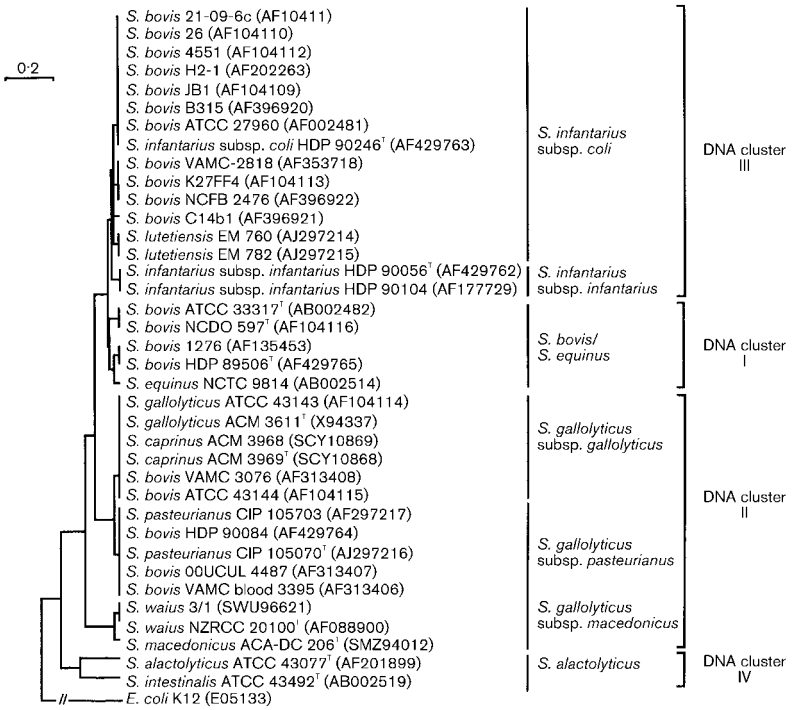

Fig. 2. Phylogenetic tree of the '*S. bovis*/*S. equinus*' complex, based on comparative analysis of partial 16S rDNA sequences. All available sequences were included, regardless of their length. Long sequences were truncated to 350–400 bp before the alignment was computed. Bar, 20 % nucleotide sequence difference.

phylogenetic analysis of 16S rDNA does not separate the subgroups into different species at the cut-off level of 97 % similarity. However, the topology of the phylogenetic trees that we have constructed (Figs 1 and 2) confirms the existence of separate subspecies. Taking together the variability of biochemical patterns between subgroups, the differences in reassociation levels between total DNAs of strains and the divergence of the 16S rDNA sequences, we propose the division of DNA cluster II into three subspecies. This cluster contains the reference strain of the unnamed DNA group 2 of Farrow *et al.* (1984) and the former type strains of *S. gallolyticus*, *S. macedonicus*, *S. waius* and *S. pasteurianus*. Taxonomic rules (Lapage *et al.*, 1992) imply use of the name of *S. gallolyticus*, as it was validated in 1996. We propose *S. gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *pasteurianus* subsp. nov. and *S. gallolyticus* subsp. *macedonicus* subsp. nov. as the names for the three different taxa, which were respectively formerly designated as *S. gallolyticus*, *S. bovis* biotype II.2 (or *S. pasteurianus* according to Poyart *et al.*, 2002) and *S. macedonicus* (or *S. waius*).

DNA cluster III includes the strains of *S. infantarius* subsp. *infantarius* and *S. infantarius* subsp. *coli* that we described previously (Schlegel *et al.*, 2000). This species demonstrates limited DNA–DNA hybridization with and an elevated percentage of divergence from the subspecies of *S. gallolyticus*. The analysis of 16S rDNA sequences yielded the phylogenetic relationships of *S. infantarius* with *S. bovis* or *S. equinus* (DNA cluster I). As shown in Fig. 2, the subspecies previously described as *S. infantarius* subsp. *coli* includes strains proposed as *S. lutetiensis* by Poyart *et al.* (2002). We have previously hybridized one of these strains (NCDO 1600 = NEM 1764 = HDP 97317) with other strains of *S. infantarius* (Schlegel *et al.*, 2000); under stringent conditions, we found DNA–DNA relatedness values of 93 % with strain HDP 90246^T of *S. infantarius* subsp. *coli* and 69 % ($\Delta T_m = 1\ ^\circ C$) with *S. infantarius* subsp. *infantarius* HDP 90104. These results are not in agreement with the conclusions of Poyart *et al.* (2002) based on phylogenetic analysis of $sodA_{int}$ genes and we therefore propose to maintain the two subspecies of *S. infantarius*. In our original description, no type strain was designated for *S. infantarius* subsp. *coli* (Schlegel *et al.*, 2000); we have therefore included descriptions of the two subspecies of *S. infantarius* below.

The 16S rDNA phylogenetic analysis also confirmed the inclusion of *S. alactolyticus* (DNA group 6 of Farrow *et al.*, 1984) and *Streptococcus intestinalis* (Robinson *et al.*, 1988) in the '*S. bovis*/*S. equinus*' complex as a single species, *S. alactolyticus*. Vandamme *et al.* (1999) had noticed that isolates identified as *S. alactolyticus* or *S. intestinalis* were indistinguishable using SDS-PAGE of whole-cell proteins. Our comparison of the 16S rDNA sequences of the two type strains displayed six nucleotide differences along a 1350 bp stretch (i.e. 0·4 % divergence), which is consistent with the existence of a single species. *S. alactolyticus* is presently the sole species belonging to DNA cluster IV.

No strain belonging to DNA group 3 has been identified since the study of Farrow *et al.* (1984). This is primarily due to the large variability of biochemical characteristics of these strains (Farrow *et al.*, 1984) or to a misidentification of these isolates. The analysis of 16S rDNA sequences leads to a change in the phylogenetic position of DNA group

3 from the '*S. bovis*/*S. equinus*' complex to the cluster of *S. suis*. Some controversial data may be found in the literature about the taxonomic position of *S. suis*. Because some strains of *S. suis* may react with antisera of serogroups R, S, T and D (Farrow *et al.*, 1984; Kilpper-Bälz & Schleifer, 1987), this species was initially associated with group D streptococci in the '*S. bovis*/*S. equinus*' complex. In the study of Farrow *et al.* (1984), *S. suis* exhibited relatively high DNA–DNA relatedness values with seven strains of DNA group 3 isolated from raw milk or from porcine mastitis specimens, although its apparent relationship with *S. bovis* decreased dramatically under stringent conditions (approx. 40 %). The species *S. suis* was constructed by successive addition of strains isolated from diseased pigs. These strains were selected because they shared identical biochemical characteristics (i.e. they are Voges–Proskauer-negative, arginine dihydrolase-positive, hydrolyse aesculin, produce β-glucuronidase and acidify trehalose and starch). They were distinguished according to a serologically specific scheme (Chatellier *et al.*, 1998; Gottschalk *et al.*, 1989; Kilpper-Bälz & Schleifer, 1987). The phylogenetic studies of Kawamura *et al.* (1995) and Chatellier *et al.* (1998) have shown that *S. suis* is distant from all other streptococcal species. Our 16S rDNA sequence analyses allow the inclusion of the sequence corresponding to the reference strain for DNA group 3, HDP 90058, within the cluster of *S. suis* (Fig. 1). The biochemical pattern of these strains appeared distinct from that of *S. suis* HDP 90052^T (Table 2). This is in agreement with the phenotypic variability previously recognized among the serotypes of *S. suis* (Tarradas *et al.*, 1994), which suggests that *S. bovis* DNA group 3 is similar to *S. suis* serotype 22, described in 1989 (Gottschalk *et al.*, 1989).

The taxonomic position of *E. saccharolyticus*, which previously belonged to streptococcal DNA group 5 of Farrow *et al.* (1984), has been clarified previously by Rodriguez & Collins (1990), who demonstrated these strains as being classified in the genus *Enterococcus*. The results obtained by reverse transcription base sequencing of cellular RNA were later confirmed by direct sequencing of the 16S rDNA (Kawamura *et al.*, 1995).

Our study provides an update of the classification and identification of streptococci belonging to the '*S. bovis*/*S. equinus*' complex *sensu stricto*. It includes seven species or subspecies: *S. equinus*, *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus*, *S. gallolyticus* subsp. *macedonicus*, *S. infantarius* subsp. *infantarius*, *S. infantarius* subsp. *coli* and *S. alactolyticus*. These can be identified according to differential biochemical reactions (Table 6). The successive changes in the classification of the '*S. bovis*/*S. equinus*' complex illustrate the usefulness of a polyphasic approach to bacterial identification when phenotypic, genetic or phylogenetic methods alone are insufficient for the recognition of different species and for establishing a classification.

Table 6. Differential biochemical characteristics used for identification of species and subspecies within the '*S. bovis*/*S. equinus*' complex

Taxa: 1, *S. equinus*; 2, *S. gallolyticus* subsp. *gallolyticus* subsp. nov.; 3, *S. gallolyticus* subsp. *pasteurianus* subsp. nov.; 4, *S. gallolyticus* subsp. *macedonicus* subsp. nov.; 5, *S. infantarius* subsp. *infantarius*; 6, *S. infantarius* subsp. *coli*; 7, *S. alactolyticus*. The major differential characteristics are boxed; +, ⩾80 % of strains positive; −, ⩽20 % of strains positive; V, 21–79 % of strains positive.

Characteristic	1*	2	3	4	5	6	7
Hydrolysis of:							
Aesculin	+	+	+	−	V	+	+
Gallate (tannase activity)†	−	+	−	−	−	−	−
Production of:							
β-Glucosidase	+	+	+	−	V	+	+
β-Glucuronidase	−	−	+	−	−	−	−
α-Galactosidase	−/+	+	V	V	+	+	+
β-Galactosidase (β-GAR test)	−	−	−	+	−	−	−
β-Galactosidase (β-GAL test)	−	−	+	V	−	−	−
β-Mannosidase	−	V	+	−	−	−	−
Acidification of:							
Starch	−/+	+	−	+	+	V	−
Glycogen	−/+	+	−	−	+	−	−
Inulin	−/+	+	−	−	−	−	−
Lactose	−/+	+	+	+	+	+	−
Mannitol	−	+	−	−	−	−	−
Methyl β-D-glucopyranoside	+	+	+	−	−	+	V
Raffinose	−/+	+	V	−	+	−	−
Trehalose	V	+	+	−	−	−	−

*Results are shown for *S. equinus*/*S. bovis* when different reactions were observed for isolates formerly assigned to the two species.
†Hydrolysis of methyl gallate into gallic acid (tannase activity) was determined according to Osawa *et al.* (1995).

Description of *Streptococcus infantarius* subsp. *infantarius* subsp. nov.

The description is identical to that effectively published by Schlegel *et al.* (2000). The type strain is strain HDP 90056^T (=ATCC BAA-102^T=CCUG 43820^T=CIP 103233^T=NCDO 599^T).

Description of *Streptococcus infantarius* subsp. *coli* subsp. nov.

The description is identical to that effectively published by Schlegel *et al.* (2000). The type strain is strain HDP 90246^T (=CCUG 43822^T=NCDO 964^T), formerly designated as a reference strain.

Emended description of *Streptococcus gallolyticus* Osawa *et al.* 1996

Colonies of about 1 mm in size, when grown on blood agar for 24 h at 37 °C, are circular, unpigmented, α-haemolytic

or non-haemolytic. They are catalase-negative and facultatively anaerobic, but their growth is enhanced by 5 % CO_2. Strains show homogeneous growth in buffered glucose broth and brain heart infusion broth. They grow in MRS broth without gas production. Growth in 6·5 % NaCl broth is variable. Cells are coccoid, Gram-positive and mostly grouped in pairs or in short chains. They are non-motile and non-sporulating. They do not produce exopolysaccharide in 5 % sucrose medium. All strains produce leucine aminopeptidase, alanyl-phenylalanyl-proline arylamidase and acetoin (Voges–Proskauer test is positive). Most of them produce α-galactosidase. They do not produce pyrrolidonyl arylamidase or alkaline phosphatase. Arginine, urea and hippurate are not hydrolysed. The production of β-galactosidase, β-glucuronidase, β-mannosidase and β-glucosidase is variable, as is the hydrolysis of aesculin. Strains produce acid from lactose, maltose and sucrose, but not from arabitol, tagatose, ribose, sorbitol or cyclodextrin. Variable results are observed with glycogen, inulin, mannitol, melibiose, melezitose, methyl β-D-glucopyranoside, pullulan, raffinose, trehalose and starch. Some strains may produce tannase or gallate decarboxylase. The presence of the Lancefield group D antigen is variable. The type strain is ACM 3611^T (=CCUG 35224^T=CIP 105428^T =JCM 10005^T=LMG 16802^T=HDP 98035^T).

Description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov.

Streptococcus gallolyticus subsp. *gallolyticus* (gal.lo.ly′ti.cus. N.L. n. *gallatum* gallate; N.L. adj. *lyticus* able to loosen; N.L. adj. *gallolyticus* gallate-digesting).

This subspecies includes the strains identified as *S. bovis* biotype I or *S. gallolyticus* according to the results of Osawa *et al.* (1995). They hydrolyse methyl gallate (tannase activity) and they decarboxylate gallic acid to pyrogallol. Most strains ferment mannitol, trehalose and inulin. They produce acid from starch and glycogen. Most strains have been isolated from the faeces of marsupials, such as koalas, kangaroos, brushtails and possums, as well as from various mammals, such as cows, horses, pigs, dogs and guinea pigs; some strains have been isolated from the sheep rumen and some were shown to be responsible for bovine mastitis (Osawa *et al.*, 1995; Sly *et al.*, 1997). Most of the human strains were isolated from blood or faeces; they were often responsible for endocarditis associated with a colonic cancer. The type strain is ACM 3611^T (=CCUG 35224^T=CIP 105428^T=JCM 10005^T=LMG 16802^T=HDP 98035^T).

Description of *Streptococcus gallolyticus* subsp. *macedonicus* subsp. nov.

Streptococcus gallolyticus subsp. *macedonicus* (ma.ce.do′ni.cus. L. adj. *macedonicus* of Macedonia, northern Greece, where the bacterium was first isolated).

This subspecies includes strains formerly identified as *S. macedonicus* Tsakalidou *et al.* 1998 or *S. waius* Flint *et al.* 1999. The strains are positive for β-galactosidase (β-GAR test), negative for β-glucosidase and they do not hydrolyse aesculin. They do not produce acid from glycogen or inulin. They do not produce tannase. They do not produce acid from melibiose. Production of acid from methyl β-D-glucopyranoside and starch is variable. Dairy-associated *S. macedonicus* strains were isolated from the naturally fermented Greek Kasseri cheese, from Italian cheese and from sour mash. Strains previously named *S. waius* were isolated from biofilms on stainless-steel samples exposed to pasteurized skimmed milk and from dairy products (Manachini *et al.*, 2002). The type strain is ACA-DC 206^T (=LAB 617^T=ATCC BAA-249^T=CCUG 39970^T=CIP 105683^T=JCM 11119^T =LMG 18488^T=HDP 98362^T).

Description of *Streptococcus gallolyticus* subsp. *pasteurianus* subsp. nov.

Streptococcus gallolyticus subsp. *pasteurianus* (pas.teur′i.a.nus. N.L. masc. adj. *pasteurianus* of Pasteur, referring to the Pasteur Institute, where the type strain was characterized).

This novel subspecies includes strains formerly identified as *S. bovis* biotype II.2 or *S. pasteurianus* Poyart *et al.* 2002. They produce β-glucosidase, β-glucuronidase, β-mannosidase and β-galactosidase (β-GAL test). They produce acid from lactose, trehalose and methyl β-D-glucopyranoside. Production of acid from melibiose, melezitose, raffinose and starch is variable. Production of acid from glycogen, inulin and mannitol is absent. Strains do not produce tannase, but some strains may yield a gallate decarboxylase activity (Osawa *et al.*, 1995). Strains have been isolated from various human infections, mostly bacteraemia and endocarditis. Some strains were isolated from urinary tract infections or from suppurative infections. The type strain is NEM 1202^T (=CIP 107122^T).

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