***Streptococcus infantarius* sp. nov., *Streptococcus infantarius* subsp. *infantarius* subsp. nov. and *Streptococcus infantarius* subsp. *coli* subsp. nov., isolated from humans and food**

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Eighteen strains isolated from human specimens or from food products were characterized as atypical variants of mannitol-negative *Streptococcus bovis*. They were tested for extended biochemical criteria, ribotyping and DNA–DNA hybridization in order to define their taxonomic status. These strains were demonstrated to constitute a DNA relatedness group that includes strains of DNA group 4 of Farrow *et al.* (1984). Comparative analysis of 16S rRNA sequences demonstrated that these strains represent a new species which belongs to the *Streptococcus bovis*/*Streptococcus equinus* complex and which has been provisionally named *S. infantarius* by Bouvet *et al.* (1997). Biotyping and ribotyping allowed differentiation of these strains from the aesculin-positive strains of *S. bovis* belonging to the previously described biotypes I, II.1 and II.2. The results of the ribotyping and hybridization assays demonstrated the presence of two different DNA subgroups within the 18 strains. On the basis of these data, the names *S. infantarius* subsp. *infantarius* (aesculin-negative for five strains out of seven, including the type strain HDP 90056^T = NCDO 599^T) and *S. infantarius* subsp. *coli* (aesculin-positive, reference strain HDP 90248 = NCDO 2620) are proposed as the names for these two subspecies within the *S. infantarius* species.

Keywords: *Streptococcus bovis*, *Streptococcus infantarius*, ribotyping, DNA–DNA hybridization, 16S rRNA gene

INTRODUCTION

Since the delineation of the genus *Enterococcus* from the non-enterococcal strains of the *Streptococcus bovis*/*Streptococcus equinus* complex (Schleifer & Kilpper-Bälz, 1984), the classification of the group D streptococci has progressively changed according to the descriptions of additional species (Bouvet *et al.*, 1997; Farrow *et al.*, 1984; Osawa *et al.*, 1995; Tsakalidou *et al.*, 1998). Isolates are commonly identified by their ability to grow in the presence of bile, by the inhibition of growth at pH 9.6 or in a hypertonic broth and by the presence of the Lancefield

group D antigen, associated with the production of β -glucosidase leading to hydrolysis of aesculin (Edberg *et al.*, 1985; Facklam, 1972; Ruoff *et al.*, 1984, 1989). The initial delineation of *S. bovis* and *S. equinus* strains was based on the ability or inability to produce α -galactosidase and to hydrolyse lactose or raffinose (Bridge & Sneath, 1983; Facklam, 1972; Garvie & Bramley, 1979; Hardie, 1986; Jones *et al.*, 1972). These differences were also correlated with different host specificity: *S. equinus* strains were isolated from horses, whereas *S. bovis* is known as a major causative agent of bovine mastitis and was also isolated from human specimens including the faeces of 10% of healthy humans (Facklam, 1972; Garvie & Bramley, 1979; Hardie, 1986; Klein *et al.*, 1977; Nelms *et al.*, 1995; Ruoff *et al.*, 1989).

The GenBank accession number for the sequence of the 16S rRNA gene determined in this study is AF177729.

Phenotypic characterization leads to a classification of *S. bovis* strains based on their biotype. Biotype I designates mannitol-positive strains, whereas biotype II designates mannitol-negative strains. The strains of biotype II.1 ferment starch, melibiose and glycogen, but not trehalose; they do not produce β -glucuronidase or β -galactosidase (Coykendall & Gustafson, 1985; Garvie & Bramley, 1979; Garvie & Farrow, 1981). The strains of biotype II.2 ferment trehalose, but not starch or glycogen; they produce β -glucuronidase and β -galactosidase (Coykendall & Gustafson, 1985; Knight & Shlaes, 1985). This terminology is commonly used for the identification of clinical strains (Ruoff *et al.*, 1989).

Genotypic characterization has led to a reappraisal of this classification and six genomic groups have been described by Farrow *et al.* (1984). The type strains of *S. bovis* and of *S. equinus* both belong to genomic group 1 (Farrow *et al.*, 1984; Kilpper-Bälz *et al.*, 1982; Nelms *et al.*, 1995), which mainly includes mannitol-negative *S. bovis* biotype II.1 strains of bovine origin. Genomic group 2 has been identified as a new species, called *Streptococcus gallolyticus* (Osawa *et al.*, 1995). This species includes the strains formerly identified as *S. bovis* biotype I. Most of the mannitol-positive group D streptococci isolated from blood, which are responsible for human endocarditis associated with colonic cancer, might be reclassified in this species (Klein *et al.*, 1977; Nelms *et al.*, 1995; Osawa *et al.*, 1995; Ruoff *et al.*, 1989). Rare atypical mannitol-positive or -negative strains of *S. bovis*/*S. equinus* were identified by Farrow *et al.* (1984) as strains of genomic group 3. Genomic group 4 is heterogeneous (Farrow *et al.*, 1984). It includes both strains sharing an unusual character (the absence of hydrolysis of aesculin) and aesculin-positive strains of human origin associated with a biotype II.1-related biochemical pattern. The level of chromosomal DNA hybridization confirmed that the aesculin-negative strains correspond to an individual species, designated as *Streptococcus infantarius* (Bouvet *et al.*, 1997). Preliminary investigations of the strains that hydrolysed aesculin have suggested their close relationships with this new species. Genomic groups 5 and 6 of Farrow *et al.* (1984) include mannitol-positive strains; they have been identified as two new species, named *Enterococcus saccharolyticus* and *Streptococcus alactolyticus*, respectively (Farrow *et al.*, 1984; Rodriguez & Collins, 1990). Recently, a new species named *Streptococcus macedonicus* has been described from mannitol-negative and aesculin-negative strains (Tsakalidou *et al.*, 1998).

In the present study, 18 strains of *S. infantarius* (previously identified as *S. bovis* biotype II.1) were analysed and compared with strains of *S. bovis* biotype II.2 and with representative strains of the DNA homology groups of the *S. bovis*/*S. equinus* complex. We demonstrate the existence of two distinct subspecies within the novel species *S. infantarius*, for which we propose the names *S. infantarius* subsp.

infantarius (β -glucosidase- and aesculin-variable) and *S. infantarius* subsp. *coli* (β -glucosidase- and aesculin-positive).

METHODS

Strains. Eighteen *S. bovis* biotype II.1 strains were compared with representative strains of the different DNA homology groups defined by Farrow *et al.* (1984) and with *S. gallolyticus*, *S. alactolyticus*, *E. saccharolyticus* and the recently described *S. macedonicus*. The 40 strains examined are listed in Table 1, together with their former identifications according to the phenotypic and genotypic studies. These strains were obtained from the bioMérieux collection of bacteria (bioMérieux, La Balme-les-Grottes, France), the Australian Collection of Microorganisms (ACM, University of Queensland, St Lucia, Australia), the Collection de l'Institut Pasteur (CIP, Institut Pasteur, Paris, France), the Laboratorium voor Microbiologie of Gent Culture Collection (LMG, University of Ghent, Belgium), the National Collection of Food Bacteria (NCDO/NCFB, Aberdeen, UK) and from our collection of French National Reference Centre for Streptococci (HDP, Hôtel-Dieu, Paris, France). Cultures were stored at -80°C in brain-heart infusion broth supplemented with 15% (w/v) glycerol. Subculturing was performed on blood Columbia agar (bioMérieux) or in buffered dextrose broth (Sanofi-Diagnostics Pasteur).

Phenotypic characteristics. The strains were characterized for their morphology, their growth and their biochemical properties. The production of acetoin, enzymic reactions and fermentation of carbohydrates were determined using API 20 STREP and the Rapid ID32 STREP system (Facklam *et al.*, 1984; Freny *et al.*, 1992) according to the manufacturer's instructions (bioMérieux). All strains were cultured on agar plates supplemented with 40% bile/aesculin, 5% sucrose or 0.04% tellurite sodium. Growth was tested in broth containing 6.5% (w/v) NaCl (Sanofi-Diagnostics Pasteur) and gas production was assayed in MRS broth (Sanofi-Diagnostics Pasteur). Isolates were agglutinated for the Lancefield group D antigen with a latex bead agglutination reagent after enzymic lysis at 37°C for 30 min (Pastorex; Sanofi-Diagnostics Pasteur).

Preparation of DNA. Bacteria were grown in 1.5 l buffered dextrose broth for 5–7 h to the mid-exponential phase at 37°C . Glycine (Sigma) was added to a final concentration of 30 g l^{-1} and incubation was prolonged for an additional hour. Bacteria were then harvested by centrifugation, washed in TE buffer (10 mM Tris, 1 mM Na_2EDTA , pH 8.0) and suspended in lysis buffer (10 mM Tris, 1 mM Na_2EDTA , 1 M sucrose, pH 8.0). Mutanolysine (5 U ml^{-1} ; Sigma) and lysozyme (10 mg ml^{-1} ; Boehringer Mannheim) were added and the mixture was incubated overnight at 37°C (Bentley & Leigh, 1995; Bentley *et al.*, 1991; Grimont & Grimont, 1995). Bacterial membrane disruption was then achieved with proteinase K (0.4 mg ml^{-1}) and SDS (1%, w/v). Sometimes, overnight incubation of the mixture at 37°C was necessary for extended lysis. Genomic DNA was extracted and purified using the phenol/chloroform method of Brenner *et al.* (1982).

Ribotyping. DNA (2–5 μg) was cleaved by restriction endonuclease *Hind*III (40 U ml^{-1} ; Boehringer Mannheim) and *Pst*I (50 U ml^{-1} ; Gibco-BRL) at 37°C for 4 h, according to the manufacturers' instructions. Restriction fragments were separated by horizontal gel electrophoresis in 0.8%

Table 1. Strains of the *Streptococcus bovis*/*Streptococcus equinus* complex used in the study

Strain designation	Former identification and designation(s)*	Source
<i>S. infantarius</i> subsp. <i>infantarius</i>		
HDP 90056 ^T	<i>S. infantarius</i> , <i>S. bovis</i> , group 4, NCDO 599	Infant's faeces
HDP 90104	<i>S. infantarius</i>	Dairy product
HDP 90247	<i>S. infantarius</i> , <i>S. bovis</i> , group 4, NCDO 2602	Frozen peas
HDP 91257		Endocarditis
HDP 97027	<i>S. bovis</i> biotype II.1, API 92.03.336	From human
HDP 97029	<i>S. bovis</i> biotype II.1, API 88.12.012	Human blood
HDP 98032		Human blood
<i>S. infantarius</i> subsp. <i>coli</i>		
HDP 90062		Medical case
HDP 90246	<i>S. bovis</i> biotype II.1, group 4, NCDO 964	Unknown source
HDP 90248	<i>S. bovis</i> biotype II.1, group 4, NCDO 2620	Infant's faeces
HDP 90249	<i>S. bovis</i> biotype II.1, group 4, NCDO 2632	Medical case
HDP 90256		Endocarditis
HDP 91356		Endocarditis
HDP 97028	<i>S. bovis</i> biotype II.1, API 82.10.072	Human urinary tract infection
HDP 97317	<i>S. bovis</i> biotype II.1, NCDO 1600	From human
HDP 97318	<i>S. bovis</i> biotype II.1, NCDO 1610	Infant's faeces
HDP 97319	<i>S. bovis</i> biotype II.1, NCDO 1616	From human
HDP 97322	<i>S. bovis</i> biotype II.1, NCDO 2759	Human blood
<i>S. equinus</i>		
HDP 89506 ^T	<i>S. equinus</i> , group 1, NCDO1037 ^T , CIP 102504 ^T	Horse faeces
<i>S. bovis</i> biotype II.1		
HDP 89505 ^T	<i>S. bovis</i> , group 1, NCDO 597 ^T , CIP 102302 ^T	Cow faeces
HDP 90083	<i>S. bovis</i> biotype II.1, API 86.06.003	Unknown source
<i>S. bovis</i> biotype II.2		
HDP 90084	<i>S. bovis</i> , API 79.04.159	Human vaginal discharge
HDP 90382		Human urinary tract infection
HDP 91130		Endocarditis
HDP 91303		Endocarditis
HDP 91317		Endocarditis
HDP 91321		Endocarditis
HDP 91333		Endocarditis
HDP 91338		Endocarditis
HDP 97320	<i>S. bovis</i> , NCDO 2756	From human
HDP 97321	<i>S. bovis</i> , NCDO 2758	Human blood
HDP 97323	<i>S. bovis</i> , NCDO 2760	Human blood
HDP 97324	<i>S. bovis</i> , NCDO 2761	Human blood
Genomic group 3		
HDP 90058	<i>S. bovis</i> , group 3, NCDO 2127, CIP 103245	Mastitis
<i>S. gallolyticus</i> (formerly <i>S. bovis</i> , biotype I)		
HDP 98035 ^T	<i>S. gallolyticus</i> , ACM 36.11 ^T , CIP 105428 ^T	Koala faeces
HDP 90055	<i>S. bovis</i> , group 2, NCDO 2019	Bovine mastitis
HDP 90299	<i>S. bovis</i> biotype I, API 84.03.028	Unknown source
<i>S. alactolyticus</i>		
HDP 90057 ^T	<i>S. alactolyticus</i> , group 6, NCDO 1091 ^T	Pig intestine
<i>S. macedonicus</i>		
HDP 98362 ^T	<i>S. macedonicus</i> , LMG 18488 ^T , CIP 105683 ^T	Greek cheese
<i>E. saccharolyticus</i>		
HDP 90059 ^T	<i>E. saccharolyticus</i> , group 5, NCDO 2594 ^T	Straw bedding

* DNA homology groups are designated according to Farrow *et al.* (1984).

Table 2. Biochemical characterization of species belonging to the *S. bovis*/*S. equinus* complex (no. of positive strains for each reaction)

Strains: 1, *S. infantarius* subsp. *infantarius*; 2, *S. infantarius* subsp. *coli*; 3, *S. bovis* biotype II.1; 4, *S. equinus* (type strain); 5, *S. bovis* biotype II.2; 6, *S. gallolyticus*; 7, genomic group 3; 8, *S. alactolyticus* (type strain); 9, *S. macedonicus* (type strain); 10, *Enterococcus saccharolyticus* (type strain). Enzymic activities and carbohydrate assimilation were determined using the API 20 STREP and Rapid ID32 STREP products. Readings were taken at 4 h and after overnight incubation. All strains produced acid from maltose and sucrose; acid was not produced from L-arabinose or ribose. All strains were leucine arylamidase-positive, and arginine dihydrolase- and pyrrolidonyl-arylamidase-negative.

Characteristics	1	2	3	4	5	6	7	8	9	10
No. of strains tested	7	11	2	1	12	3	1	1	1	1
Hydrolysis of:										
Aesculin	2	11	2	1	12	3	1	1	0	1
Hippurate	0	0	0	0	0	0	1	0	0	0
Urea	0	0	0	0	0	0	0	1	0	0
Production of:										
Acetoin	7	11	2	1	12	3	0	1	1	0
APPA*	7	11	2	1	12	3	1	1	1	0
α -Galactosidase	7	10	2	0	8	3	0	1	0	1
β -Galactosidase	0	0	0	0	12	0	1	0	1	0
β -Glucosidase	2	11	2	1	12	3	1	1	0	1
β -Glucuronidase	0	0	0	0	12	0	0	0	0	0
N-Acetyl β -glucosaminidase	0	0	0	0	1	0	0	0	0	1
GTA†	0	0	0	0	0	0	1	0	0	1
β -Mannosidase	0	0	0	0	12	2	0	0	0	0
Production of acid from:										
D-Arabitol	0	0	0	0	0	0	0	0	0	1
Cyclodextrin	0	0	0	0	0	0	1	0	0	1
Glycogen	6	0	2	0	0	3	1	0	0	0
Inulin	0	0	2	0	0	3	1	0	0	1
Lactose	6	11	2	0	12	3	1	0	1	1
D-Mannitol	0	0	0	0	0	3	1	1	0	1
Melezitose	0	0	0	0	6	0	0	0	0	1
Melibiose	5	0	2	0	2	0	0	0	0	1
Methyl β -D-glucopyranoside	2	11	2	0	12	3	1	1	0	0
Pullulan	6	0	0	0	0	3	1	0	0	0
D-Raffinose	5	5	2	0	8	2	1	0	0	1
Sorbitol	0	0	0	0	0	0	0	0	0	1
D-Tagatose	0	0	0	0	3	0	1	0	0	0
Trehalose	0	0	0	1	12	3	1	0	0	1
Starch	7	4	2	0	0	3	1	0	1	1

* APPA, Alanyl-phenylalanyl-proline arylamidase.

† GTA, Glycyl-tryptophan arylamidase.

(w/v) agarose gel (Appligene) in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.025 M Na₂EDTA, pH 8) for 16 h at 1.5 V cm⁻¹. DNA fragments were blotted on to a positively charged nylon membrane (Hybond-N; Amersham) using a VacuGene vacuum-transfer system (Pharmacia Biotech). Hybridization with five specific digoxigenin-labelled oligonucleotides (28 pmol each) was conducted as previously described (Régnauld *et al.*, 1997) and according to the instructions provided with the DIG Easy Hyb kit (Boehringer Mannheim). Bound digoxigenin was detected using an alkaline-phosphatase-conjugated anti-digoxigenin antibody and a mixture of nitroblue tetrazolium and 5-bromo-4-

chloro-3-indolylphosphate as the enzyme substrate (all chemicals were from Sigma) (Machado *et al.*, 1998; Régnauld *et al.*, 1997). Membranes were scanned and the sizes of DNA fragments calculated using the Taxotron package (Institut Pasteur) as described elsewhere (Machado *et al.*, 1998). DNA extracted from *Citrobacter koseri* CIP 105177 and cleaved with *Mlu*I (2 U ml⁻¹) was used as the external standard (Grimont & Grimont, 1986; Machado *et al.*, 1998).

Quantitative DNA-DNA hybridization. DNA from *S. bovis* HDP 89505^T and *S. infantarius* HDP 90104 and HDP 90246 was labelled *in vitro* with [³H]adenine triphosphate, [³H]-thymidine triphosphate, [³H]guanosine triphosphate and

Fig. 1. Normalized graph of the rRNA gene restriction patterns obtained after digestion with *Hind*III and *Pst*I. (Fragment size values were determined using *Citrobacter koseri* CIP 105177 digested with *M*luI as the molecular mass standard.) A dendrogram was derived from the mean linkage analysis of the restriction patterns. Letters indicate clusters, as discussed in the text.

[³H]cytosine triphosphate using a Megaprim DNA-labelling reaction kit (all from Amersham). DNA-DNA hybridizations were performed in stringent conditions at 60 °C for 16 h according to the S1 nuclease/trichloroacetic acid precipitation method of Crosa *et al.* (1973), with minor modifications (Grimont *et al.*, 1980). The temperature (T_m) at which 50 % of the reassociated DNA became hydrolysable by the S1 nuclease was determined (Grimont *et al.*, 1980) and the differences (ΔT_m) between the melting temperatures of homoduplexes and heteroduplexes were calculated in order to estimate DNA divergence between strains with high levels of DNA homology.

16S rRNA sequence determination and analysis. PCR of 16S rRNA encoding DNA and sequencing of amplified fragments were performed as described previously (Janvier & Grimont, 1995). Alignment with the available streptococcal sequence from GenBank and phylogenetic analysis of the 16S rRNA data were performed with the MEGALIGN program from the DNASTAR package.

RESULTS

Phenotypic characterization

All streptococcal strains shared common characteristics of morphology and growth. They were Gram-positive, non-motile, non-sporulating and catalase-negative cocci occurring in pairs or short chains. Their growth in buffer dextrose and in brain-heart infusion broths was homogeneous. They grew in MRS broth without gas production and they did not grow in 6.5 % (w/v) NaCl broth. *E. saccharolyticus* strain HDP 90059^T yielded similar characteristics, but grew in 6.5 % (w/v) NaCl broth and on bile/aesculin agar. All of the strains except the reference strain of genomic group 3 and the type strain of *E. saccharolyticus* were positive for the Voges-Proskauer test. The strains formerly called *S. bovis* produced leucine aminopeptidase, alanyl-phenylalanyl-proline arylamidase

Table 3. Quantitative hybridization with labelled DNA from strains

ND, Not determined.

Ribotyping cluster and strain no.	HDP 90104		HDP 90246		HDP 89505 ^T %
	%	ΔT_m	%	ΔT_m	
Cluster B, <i>S. infantarius</i> subsp. <i>infantarius</i>					
HDP 90104	100	0	67	3	47
HDP 90056 ^T	79	2	60	3	39
HDP 90247	77	0	66	4	40
HDP 91257	77	1	65	3	40
HDP 97027	80	2	68	5	40
HDP 97029	82	1	62	4	43
HDP 98032	70	0	60	6	38
Cluster C, <i>S. infantarius</i> subsp. <i>coli</i>					
HDP 90246	64	1	100	0	42
HDP 90062	64	3	88	2	48
HDP 90248	56	4	90	ND	46
HDP 90249	69	2	94	1	49
HDP 91356	57	ND	90	ND	43
HDP 97028	55	2	83	0	41
HDP 97317	69	1	93	ND	50
HDP 97318	63	ND	100	ND	47
Cluster D, <i>S. bovis</i> and <i>S. equinus</i>					
HDP 89505 ^T	33	ND	46	ND	100
HDP 89506 ^T	42	ND	45	ND	71
HDP 90083	42	ND	46	ND	75
Cluster A, <i>S. bovis</i> biotype II.2					
HDP 90084	29	ND	37	ND	30
HDP 91303	29	ND	37	ND	30
HDP 91317	28	ND	33	ND	50
HDP 91323	28	ND	39	ND	31
Cluster E, <i>S. gallolyticus</i>					
HDP 98035 ^T	32	ND	38	ND	33
HDP 90055	33	ND	33	ND	41
HDP 90299	42	ND	26	ND	43
Other strains tested					
HDP 98362 ^T , <i>S. macedonicus</i>	30	ND	34	ND	53
HDP 90057 ^T , <i>S. alactolyticus</i>	15	ND	23	ND	21
HDP 90058, genomic group 3	2	ND	4	ND	5
HDP 90059 ^T , <i>E. saccharolyticus</i>	2	ND	3	ND	5

and α -galactosidase. They did not produce arginine dihydrolase or pyrrolidonyl arylamidase. The biochemical characteristics of *S. infantarius* strains were similar to those of the other strains of *S. bovis* of biotype II.1 including the type strain of *S. bovis*. Eight strains out of 18 reacted with the group D antiserum. They differ by the lack of fermentation of inulin and by the variation of some characteristics, such as the hydrolysis of aesculin, the production of β -glucosidase and the fermentation of melibiose, pullulan, raffinose and starch (Table 2). The absence of the production of β -galactosidase and β -mannosidase and the lack of acidification of trehalose allowed the delineation of *S. infantarius* and *S. bovis* biotype II.2. Other biochemical

tests that differentiate this species from representative strains of *S. equinus*, *S. gallolyticus*, DNA homology group 3, *S. alactolyticus*, *S. macedonicus* and *E. saccharolyticus* are shown in Table 2.

The two subgroups of *S. infantarius* differed in terms of the presence or absence of some characteristics, such as the hydrolysis of aesculin, the presence of β -glucosidase and the acidification of starch, glycogen, melibiose, methyl β -D-glucopyranoside and pullulan (Table 2). The strains of the first subgroup, except strains HDP 97027 and HDP 91257, fail to hydrolyse aesculin and to produce β -glucosidase. Most strains produce acid from glycogen, pullulan and melibiose.

Conversely, all of the strains of the second subgroup were aesculin- and β -glucosidase-positive. None of them produced acid from glycogen, pullulan or melibiose.

Ribotyping

The patterns of rRNA gene restriction analysis after digestion with *Hind*III and *Pst*I are shown in Fig. 1. Mean linkage statistical analysis of the size of DNA fragments delineated five clusters of strains which were designated A to E. Statistical analysis of restriction patterns using two other clustering methods (single-linkage and unweighted pair group method with arithmetic averages) displayed similar results. Cluster A contained all of the strains identified as *S. bovis* biotype II.2. Cluster B contained the seven strains of the first subgroup of *S. infantarius*, i.e. three strains that were previously designated *S. infantarius* and four additional isolates. A second subgroup of *S. infantarius*, designated as cluster C, comprised 11 strains, which had been identified as *S. bovis* biotype II.1. Cluster D contained the type strain of *S. equinus* and two strains identified as *S. bovis* biotype II.1, including the type strain of *S. bovis*. Cluster E corresponded to *S. gallolyticus*. The four additional isolates tested (which are the type strains of *S. alactolyticus*, *S. macedonicus* and *E. saccharolyticus*) and the reference strain of DNA homology group 3 are distant from the former clusters.

DNA–DNA hybridization

The results obtained in hybridization experiments with radiolabelled DNA from HDP 90104 (*S. infantarius*, cluster B), HDP 90246 (*S. infantarius*, cluster C) and HDP 89505^T (*S. bovis*, cluster D) are shown in Table 3. High levels of homology were found between DNA of strains belonging to the same cluster, B, C or D (i.e. 70–100% and $\Delta T_m < 3$ °C). The close relationship between ribogroups B and C was confirmed by the results of DNA–DNA hybridization assays with radio-labelled DNA from *S. infantarius* strains HDP 90104 and HDP 90246. Moreover, low hybridization levels (26–50%) were observed between strains of clusters B and C and strains belonging to clusters A, D and E. The reference strains of the other species of the *S. bovis*/*S. equinus* complex including the type strains of *S. alactolyticus*, *S. macedonicus* and *E. saccharolyticus* and DNA homology group 3 yielded low homology with both HDP 90104 and HDP 90246 (2–34%).

16S rRNA gene sequencing

Comparative analysis of the determined sequence for *S. infantarius* HDP 90104 confirmed the inclusion of this species in the genus *Streptococcus* (sequence similarity between 89 and 99% with the other type strains of described species in the genus). The closest relations were *S. bovis* (98.2%), *S. macedonicus*

Fig. 2. Unrooted phylogenetic tree of *Streptococcus infantarius* strain HDP 90104 and other major streptococcal species (using sequences available from GenBank) based on comparative analysis of the 16S rRNA gene sequences. The dendrogram was generated by using the neighbour-joining algorithm. Bar, evolutionary distance value (K_{nuc}) between sequences.

(97.9%) and *S. gallolyticus* (95.8%), as shown in Fig. 2.

DISCUSSION

The comparison of phenotypic and genotypic characteristics of group D streptococcal strains, mostly of human origin, confirms the heterogeneity of the *S. bovis*/*S. equinus* complex and the description of a new species called *S. infantarius*. In addition, analysis of 18 strains of *S. infantarius* allows the delineation of two different subspecies.

Using ribotyping, we have demonstrated that, among the mannitol-negative strains, the strains formerly designated as *S. bovis* biotype II.1 can be separated into two different clusters (B and C) associated with different biochemical properties. Their ribotypes were distinct from those of cluster D (which includes the type strains of *S. bovis* and *S. equinus*), cluster A (*S. bovis* biotype II.2) and other group D streptococcal species. The determination of rRNA gene restriction patterns has been shown to be a general method for

Table 4. Characteristics useful for the differentiation of *S. infantarius* from *S. bovis* and major species belonging to the *S. bovis*/*S. equinus* complex [description based on results obtained with strains in this study and data from Farrow *et al.* (1984), Knight & Shlaes (1985), Nelms *et al.* (1995), Osawa *et al.* (1995) and Tsakalidou *et al.* (1998)].

Strains: 1, *S. infantarius* subsp. *infantarius*; 2, *S. infantarius* subsp. *coli*; 3, *S. bovis* biotype II.1; 4, *S. equinus*; 5, *S. bovis* biotype II.2; 6, *S. gallolyticus*; 7, *S. macedonicus*. +, More than 85% of the strains positive; –, less than 15% of the strains positive; v, 15–85% of the strains positive; X, not designated.

Characteristics	1	2	3	4	5	6	7
Hydrolysis of aesculin	v	+	+	+	+	+	–
Production of:							
α -Galactosidase	+	+	+	–	v	+	–
β -Galactosidase	–	–	–	v	+	–	–
β -Glucosidase	v	+	+	+	+	+	–
β -Glucuronidase	–	–	–	–	+	–	–
β -Mannosidase	–	–	–	–	+	v	–
Production of acid from:							
Glycogen	+	–	+	–	–	+	–
Inulin	–	–	+	v	–	+	–
Lactose	+	+	+	–	+	+	+
D-Mannitol	–	–	–	–	–	+	–
Melibiose	+	–	+	–	–	–	–
Methyl β -D-glucopyranoside	v	+	+	v	+	+	v
Pullulan	+	–	–	–	–	+	–
Trehalose	–	–	–	+	+	+	–
Starch	+	v	+	–	–	+	+
Cluster (ribotyping)	B	C	D	D	A	E	X

taxonomic analysis of bacteria (Grimont & Grimont, 1986). This method is a powerful tool for species identification and also for distinguishing between subspecies or biotypes within species (Bouvet *et al.*, 1991; Doit *et al.*, 1994; Rudney & Larson, 1994). The combination of patterns obtained with two different restriction enzymes may generate more diversity between isolates and strengthen the results of the cluster analysis (Grimont & Grimont, 1986; Machado *et al.*, 1998). Cluster B includes *S. infantarius* HDP 90104 and six other strains which were mostly β -glucosidase-negative, whereas cluster C comprises closely related β -glucosidase- and aesculin-positive *S. bovis* biotype II.1 strains. The genetic relatedness, evaluated by the level of hybridization and the difference in melting temperature, was superior between strains belonging to the same cluster (i.e. B or C) than between strains from the two clusters. Since the difference did not meet the requirement of the Ad Hoc Committee and did not allow the distinction of two different species (Wayne *et al.*, 1987), we propose the delineation of two subspecies. Ribotyping analysis demonstrated also that these two clusters are distinct from cluster D, which includes the type strains of *S. bovis* and *S. equinus*. The similarity of the restriction patterns of these two

type strains and their quantitative DNA–DNA hybridization are consistent with the levels previously reported (Farrow *et al.*, 1984; Kilpper-Bälz *et al.*, 1982; Nelms *et al.*, 1995). Moreover, our results confirm the lack of homology between strains of cluster A (*S. bovis*, biotype II.2), cluster E (*S. gallolyticus*, formerly *S. bovis* biotype I) and the type strain of *S. bovis* (Coykendall & Gustafson, 1985; Farrow *et al.*, 1984; Garvie & Bramley, 1979). In addition, the strains identified as *S. bovis* biotype II.2 belong to a distinct cluster (A). This delineation is in accordance with previous observations on a limited number of strains (Coykendall & Gustafson, 1985; Garvie & Bramley, 1979) and is under investigation in our laboratory. *S. gallolyticus*, *S. alactolyticus* and *E. saccharolyticus* appeared distantly related to *S. infantarius* and *S. bovis* or *S. equinus* according to ribotyping and DNA hybridization assays. *S. macedonicus* (Tsakalidou *et al.*, 1998) has been described recently as an aesculin-negative species belonging to the *S. bovis*/*S. equinus* complex. However, a reduced 16S rRNA similarity (97.9%) and a low level of DNA–DNA hybridization (30–34%) warranted the separation of this species from *S. infantarius*.

Results of ribotyping, DNA–DNA hybridization assays and 16S rRNA sequence analysis confirm the inclusion of *S. infantarius* within the *S. bovis*/*S. equinus* complex and its distinction from previously described species. The comparative analysis of the strains confirms the presence of two distinct subspecies for which the names *S. infantarius* subsp. *infantarius* (β -glucosidase- and aesculin-variable) and *S. infantarius* subsp. *coli* (aesculin- and β -glucosidase-positive) are proposed.

Description of *Streptococcus infantarius* sp. nov.

Streptococcus infantarius (in.fan.ta'ri.us. L. adj. *infantarius* relating to infants, the source of the type strain).

The cells are Gram-positive cocci that occur in pairs or short chains and are non-motile, non-sporulating and catalase-negative. Colonies on blood agar are circular, 1 mm in diameter after a 24 h incubation at 37 °C, unpigmented and α -haemolytic. Growth is enhanced in a 5% CO₂ atmosphere. Strains show homogeneous growth in buffer dextrose and in brain-heart infusion broths. Growth also occurs in MRS broth, without gas production. No growth in 6.5% (w/v) NaCl broth. No exopolysaccharide production on 5% sucrose medium. The Lancefield group D antigen is present in 40% of the strains. Strains are positive for Voges–Proskauer, leucine aminopeptidase and alanyl-phenylalanyl-proline arylamidase tests. Arginine dihydrolase, alkaline phosphatase and pyrrolidonyl-arylamidase tests are negative. Urea and hippurate are not hydrolysed. Aesculin hydrolysis is variable. Nearly all strains are α -galactosidase-positive. They are *N*-acetyl- β -glucosaminidase-, β -galactosidase-, β -glucuronidase-, glycyl-tryptophan arylamidase- and β -

mannosidase-negative. All strains produce acid from lactose, maltose and sucrose. They do not produce acid from arabinose, arabitol, cyclodextrine, inulin, D-mannitol, melezitose, ribose, sorbitol, D-tagatose or trehalose. Variable results occur with glycogen, melibiose, methyl β -D-glucopyranoside, pullulan, D-raffinose and starch. Characteristics useful in their differentiation from related organisms and also in the delineation between the two subspecies are listed in Table 4.

Most of the *S. infantarius* subsp. *infantarius* strains do not hydrolyse aesculin (five out of seven) and are β -glucosidase- and methyl β -D-glucopyranoside-negative. They produce acid from glycogen, melibiose, pullulan and starch, with some exceptions. The type strain of *S. infantarius* subsp. *infantarius* HDP 90056^T (= NCDO 599^T) is aesculin- and β -glucosidase-negative. It produces acid from glycogen, pullulan, D-raffinose and starch, but not from melibiose or methyl β -D-glucopyranoside. The type strain was isolated from the faeces of an infant human. Other isolates have been obtained from clinical specimens (three from blood, including one from a case of endocarditis) or food products (a dairy product and frozen peas). *S. infantarius* subsp. *coli* is β -glucosidase- and aesculin-positive. Glycogen, starch, melibiose and pullulan are not fermented. Ten strains of this subspecies were isolated from human specimens including faeces from two infants, urine (one isolate) and blood (three isolates, including one from a case of endocarditis). The reference strain for this subspecies is HDP 90246 (= NCDO 964).

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