Taxonomic dissection of the *Streptococcus bovis* group by analysis of manganese-dependent superoxide dismutase gene (*sodA*) sequences: reclassification of '*Streptococcus infantarius* subsp. *coli*' as *Streptococcus lutetiensis* sp. nov. and of *Streptococcus bovis* biotype II.2 as *Streptococcus pasteurianus* sp. nov.

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The taxonomic dissection of the *Streptococcus bovis*–*Streptococcus equinus* group was carried out upon obtaining sequences for the manganese-dependent superoxide dismutase gene (*sodA*) of the type strains of *S. bovis*, *Streptococcus caprinus*, *S. equinus*, *Streptococcus gallolyticus*, *Streptococcus infantarius*, *Streptococcus macedonicus* and *Streptococcus waius*. The *sodA* sequences of 29 streptococcal strains of animal and human origin that were related to *S. bovis* were also sequenced. A phylogenetic analysis of the *sodA* sequences revealed that the *S. bovis*–*S. equinus* group comprises five different clusters that correspond to five distinct species. The type strains of *S. bovis* and *S. equinus* were associated in the same cluster, corresponding to the species *S. equinus*. The type strains of *S. caprinus*, *S. gallolyticus*, *S. macedonicus* and *S. waius* were associated in the same cluster, which defined a single species containing *S. gallolyticus* and its junior synonym *S. caprinus*, and *S. macedonicus* and its junior synonym *S. waius*. The two subspecies thought to constitute the species *S. infantarius*, namely *S. infantarius* subsp. *infantarius* and '*S. infantarius* subsp. *coli*', were located in two distinct clusters. One of these clusters defined the species *S. infantarius* and included the type strain of *S. infantarius* subsp. *infantarius*. The other cluster defined '*S. infantarius* subsp. *coli*', leading to the proposal of its reclassification as the novel species *Streptococcus lutetiensis* (NEM 782^T = CIP 106849^T). The remaining cluster comprised all of the strains previously identified as belonging to *S. bovis* biotype II.2, leading to the proposal to reassign these strains to the novel species *Streptococcus pasteurianus* (NEM 1202^T = CIP 107122^T). The results of the phylogenetic analysis were confirmed by DNA–DNA hybridization experiments, thus demonstrating that sequence databases of defined DNA targets, such as *sodA*, may constitute a valuable alternative approach for modern bacterial systematics.

Keywords: *Streptococcus bovis*, *Streptococcus lutetiensis* sp. nov., *Streptococcus pasteurianus* sp. nov., superoxide dismutase gene (*sodA*), 16S rRNA gene

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Abbreviation: *sodA*_{int}, internal fragment of *sodA*.

The GenBank accession numbers for the *sodA*_{int} sequences reported in this study can be found in Table 1. The GenBank accession numbers for the 16S rDNA sequences of *S. lutetiensis* NEM 782^T and *S. pasteurianus* NEM 1202^T are AJ297189 and AJ297195, respectively.

INTRODUCTION

Streptococcus bovis is a normal inhabitant of the ruminant and human gut. In humans, it has been reported to be the causative agent of meningitis, septicaemia and endocarditis, and numerous reports have suggested a potential relationship between increased faecal carrier levels of *S. bovis* and human gastrointestinal disease (Duval *et al.*, 2001; Grant *et al.*, 2000; Manfredi *et al.*, 1999; Zarkin *et al.*, 1990). Therefore, the correct identification of *S. bovis* isolates is important in clinical microbiology laboratories.

The taxonomic status of *S. bovis* strains has been evolving in the last few decades and has progressively changed according to the description of new species originally identified as *S. bovis*. In the 1990s, four new species were described, *Streptococcus gallolyticus* (Osawa *et al.*, 1995), *Streptococcus macedonicus* (Tsakalidou *et al.*, 1998), *Streptococcus waius* (Flint *et al.*, 1999) and *Streptococcus infantarius* (Bouvet *et al.*, 1997). In clinical laboratories, the accurate identification of these streptococci is based on phenotypic characteristics that permit the classification of *S. bovis* strains into two biotypes (Facklam *et al.*, 1984; Knight & Schlaes, 1985; Ruoff *et al.*, 1984, 1989). However, these phenotypic characterizations are impaired due to the variable expression of certain traits and because of the frequent ambiguity in the interpretation of such data. Consequently, nucleic-acid-based technologies, such as DNA–DNA hybridization or the amplification of selected targets, have been developed to complement and improve the identification of streptococci at the species level (Garnier *et al.*, 1997; Kawamura *et al.*, 1995, 1999; Poyart *et al.*, 1998). Farrow *et al.* (1984) demonstrated that on the basis of DNA–DNA hybridization data *S. bovis* strains could be classified into six genomic groups that exhibited between 40 and 60% DNA similarity with each other. These authors also demonstrated that biotype I strains were genotypically homogeneous and distinct from biotype II strains, which include the type strains of *S. bovis* and *Streptococcus equinus*. More recently, based on 16S rDNA sequence analysis, Clarridge *et al.* (2001) have suggested that *S. bovis* biotype II.2 strains constitute a separate genus/species that is distinct from *S. bovis*, *S. gallolyticus* and *S. infantarius*.

The interpretation of 16S rDNA sequence data may be complicated by the fact that divergent 16S rDNA sequences may exist within a single organism (Ueda *et al.*, 1999) or, alternatively, by the fact that closely related species may have nearly identical 16S rDNA sequences (Fox *et al.*, 1992). The latter has been shown for members of the genus *Streptococcus*, namely *Streptococcus pneumoniae*, *Streptococcus mitis* and *Streptococcus oralis* (Kawamura *et al.*, 1995). The 16S rDNA sequences of the type strains of the *S. bovis* group (*S. bovis*, *Streptococcus caprinus*, *S. equinus*, *S. gallolyticus*, *S. infantarius* and *S. macedonicus*) exhibit a percentage of identity ranging from 97.1 (e.g. *S. equinus* and *S. gallolyticus*, and *S. gallolyticus* and *S.*

infantarius) to 99.8% (*S. bovis* and *S. infantarius*). To differentiate such strains, it is possible to use alternative single-copy target sequences that exhibit greater sequence divergence than that of 16S rDNA. The *sodA* gene of the Gram-positive cocci, which encodes the manganese-dependent superoxide dismutase (Mn-SOD), fulfils these criteria. We have previously described a PCR assay, based on the utilization of degenerate primers, which enabled the amplification of an internal fragment representing approximately 83% of the *sodA* gene encoding Mn-SOD in various Gram-positive bacteria, including streptococci and enterococci (Poyart *et al.*, 1995). We have also reported that sequencing the *sodA* PCR product, with the same degenerate primers, constitutes a valuable approach to the genotypic identification of species belonging to the genera *Streptococcus* and *Enterococcus* (Poyart *et al.*, 1995, 1998, 2000). This target gene has also been used for the identification of other bacteria at the species level, including coagulase-negative staphylococci (Poyart *et al.*, 2001) and mycobacteria (Zolg & Philippi-Schulz, 1994). In this work, we carried out a taxonomic analysis of the *S. bovis* group, by using the same approach as described previously and demonstrated the usefulness of a *sodA*-based database for the species identification of strains belonging to the *S. bovis*–*S. equinus* complex. Furthermore, phylogenetic studies of *sodA* gene sequences and DNA–DNA hybridization experiments support the recognition of two distinct novel species within the genus *Streptococcus*, for which the names *Streptococcus lutetiensis* (formerly '*S. infantarius* subsp. *coli*') and *Streptococcus pasteurianus* (formerly *S. bovis* biotype II.2) are proposed.

METHODS

Bacterial strains and culture conditions. The main characteristics of the strains used in this study, including the type strains, are listed in Table 1. The isolates of *S. bovis* were of various origins and were collected over a period of at least 10 years. All strains were grown at 37 °C on Columbia horse blood agar (bioMérieux) or in brain-heart infusion (BHI) broth under anaerobic conditions. Cultures were stored at –80 °C in BHI broth (Difco) supplemented with 10% (w/v) glycerol until required.

Phenotypic characteristics. The strains were characterized for their morphological, growth and biochemical properties. The production of acetoin, enzymic reactions and fermentation of carbohydrates were determined using the API 20Strep and Rapid ID 32Strep systems, according to the manufacturer's recommendations (bioMérieux). All strains were tested for growth on agar plates supplemented with 40% bile/aesculin, 5% sucrose or 0.04% sodium tellurite. Growth was tested in broth containing 6.5% (w/v) NaCl and gas production was assayed in MRS broth (Bio-Rad). The presence of the Lancefield's group D antigen was determined with the Streptex test, according to the manufacturer's recommendations (bioMérieux).

PFGE. High-molecular-mass DNA from the growth obtained from a single plate for each strain was extracted in agarose plugs by conventional methods, digested with *Sma*I and

Table 1. Characteristics of the streptococcal strains used in this study

Species*	Source	Rapid ID 32Strep biotype	<i>sodA</i> _{int} cluster†	<i>sodA</i> _{int} -based identification	GenBank no. for <i>sodA</i> _{int} sequence
Type strains					
<i>S. alactolyticus</i> CIP 103244 ^T	Pig intestine	ND	–	<i>S. alactolyticus</i>	Z95894
<i>S. bovis</i> CIP 102302 ^T	Cow faeces	ND	C	<i>S. bovis</i>	Z95896
<i>S. caprinus</i> CIP 104887 ^T	Goat rumen	ND	E	<i>S. caprinus</i>	AJ297182
<i>S. equinus</i> CIP 102504 ^T	Horse faeces	ND	C	<i>S. equinus</i>	Z95903
<i>S. gallolyticus</i> CIP 105428 ^T	Koala faeces	ND	E	<i>S. gallolyticus</i>	AJ297183
<i>S. infantarius</i> subsp. <i>infantarius</i> CIP 103233 ^T	Infant faeces	ND	A	<i>S. infantarius</i> subsp. <i>infantarius</i>	AJ297184
<i>S. macedonicus</i> CIP 105683 ^T	Kasseri cheese	ND	E	<i>S. macedonicus</i>	AJ297186
<i>S. salivarius</i> CIP 102503 ^T	Human blood	ND	–	<i>S. salivarius</i>	Z95916
<i>S. waius</i> CIP 106079 ^T	Milk	ND	E	<i>S. waius</i>	AJ297187
Clinical isolates					
<i>S. bovis</i> NEM 760	Infant faeces	22053003110	B	<i>S. lutetiensis</i>	AJ297188
<i>S. bovis</i> NEM 782 ^T , CIP 106849 ^{T‡}	Human isolate	22053003110	B	<i>S. lutetiensis</i>	AJ297189
<i>S. bovis</i> NEM 1101, CIP 103567	Human isolate	22273063150	E	<i>S. gallolyticus</i>	AJ297190
<i>S. bovis</i> NEM 1195, CIP 105064	Human isolate	22273063150	E	<i>S. gallolyticus</i>	AJ297191
<i>S. bovis</i> NEM 1196, CIP 105065	Human blood	22273063150	E	<i>S. gallolyticus</i>	AJ297192
<i>S. bovis</i> NEM 1197, CIP 105066	Human blood	22273063150	E	<i>S. gallolyticus</i>	AJ297193
<i>S. bovis</i> NEM 1201, CIP 105069	Human blood	22273063150	E	<i>S. gallolyticus</i>	AJ297194
<i>S. bovis</i> NEM 1202 ^T , CIP 107122 ^{T§}	Human cerebrospinal fluid	63077003150	D	<i>S. pasteurianus</i>	AJ297195
<i>S. bovis</i> NEM 1203, CIP 105071	Human blood	22273063150	E	<i>S. gallolyticus</i>	AJ297196
<i>S. bovis</i> NEM 1204, CIP 105072	Human blood	22273063150	E	<i>S. gallolyticus</i>	AJ297197
<i>S. bovis</i> NEM 1205, CIP 105073	Human blood	63077203150	D	<i>S. pasteurianus</i>	AJ297198
<i>S. bovis</i> NEM 1206, CIP 105074	Human blood	63077201150	D	<i>S. pasteurianus</i>	AJ297199
<i>S. bovis</i> NEM 1227, CIP 105068	Human blood	63077003150	D	<i>S. pasteurianus</i>	AJ297200
<i>S. bovis</i> NEM 1350, CIP 105284	Foal faeces	22273063150	E	<i>S. gallolyticus</i>	AJ297201
<i>S. bovis</i> NEM 1351, CIP 105285	Foal faeces	22273063150	E	<i>S. gallolyticus</i>	AJ297202
<i>S. bovis</i> NEM 1352, CIP 105286	Foal faeces	22273063150	E	<i>S. gallolyticus</i>	AJ297203
<i>S. bovis</i> NEM 1353, CIP 105287	Dog uterus	22273063150	E	<i>S. gallolyticus</i>	AJ297204
<i>S. bovis</i> NEM 1603	Human cerebrospinal fluid	22053003110	B	<i>S. lutetiensis</i>	AJ297205
<i>S. bovis</i> NEM 1771	Human urine	63077001150	D	<i>S. pasteurianus</i>	AJ297206
<i>S. bovis</i> NEM 1773	Human blood	22273063150	E	<i>S. gallolyticus</i>	AJ297207
<i>S. bovis</i> NEM 1772	Human blood	22273063150	E	<i>S. gallolyticus</i>	AJ297208
<i>S. bovis</i> NEM 1774, CIP 105070	Human cerebrospinal fluid	63077003150	D	<i>S. pasteurianus</i>	AJ297209
<i>S. bovis</i> NEM 1775, CIP 103560	Human isolate	22273063150	E	<i>S. gallolyticus</i>	AJ297210
<i>S. equinus</i> NEM 1761, CIP 103232	Milk, bovine mastitis	22273063150	E	<i>S. gallolyticus</i>	AJ297211
<i>S. equinus</i> NEM 1764, CIP 56.23	Human isolate	02013001100	B	<i>S. lutetiensis</i>	AJ297212
<i>S. equinus</i> NEM 1760, CIP 82.5	Horse faeces	20003001110	C	<i>S. equinus</i>	AJ297213
<i>'S. infantarius</i> subsp. <i>coli</i> ' NEM 1867, NCDO 964	Unknown source	22053001110	B	<i>S. lutetiensis</i>	AJ306978
<i>S. infantarius</i> subsp. <i>infantarius</i> NEM 1868, CIP 106106	Infant faeces	2205306610	A	<i>S. infantarius</i>	AJ306979
<i>S. infantarius</i> subsp. <i>infantarius</i> NEM 1869, CIP 106107	Human blood	0205306610	A	<i>S. infantarius</i>	AJ306980

ND, Not determined.

* CIP, Collection de l'Institut Pasteur; NEM, Necker-Enfants Malades; NCDO, National Collection of Dairy Organisms.

† The *sodA*_{int} cluster refers to the divisions shown in Fig. 1.

‡ Type strain of *S. lutetiensis*.

§ Type strain of *S. pasteurianus*.

*Bss*HII and separated through a 1 % agarose gel by using a clamped-homogeneous-field electrophoresis apparatus (CHEF MAPPER DRII, Bio-Rad), as described previously (Poyart *et al.*, 1997).

DNA–DNA hybridization. Genomic DNA was extracted as described previously (Poyart *et al.*, 1997). DNA samples were diluted in twofold serial dilutions to provide concentrations of between 0.0625 and 1 µg DNA (100 µl)^{–1}. Six replicate 100 µl samples of each dilution were loaded onto Nylon blotting membranes (Hybond-N⁺; Amersham) using a dot-blotting apparatus. Probes were prepared for six *Streptococcus* strains (*S. bovis* CIP 102302^T, *S. gallolyticus* CIP 105428^T, *S. macedonicus* CIP 105683^T, *S. infantarius* subsp. *infantarius* CIP 103233^T, *S. lutetiensis* NEM 782^T and *S. pasteurianus* NEM 1202^T) by labelling them with ³²P using the Megaprime DNA Labelling System (Amersham). Hybridizations were performed as follows. Prehybridization and hybridization were carried out for 2 h and 18 h, respectively, at 65 °C in 10 ml of Rapid-hyb buffer (Amersham), followed

by two washes in 2 × SSC (1.5 M NaCl, 0.15 M trisodium citrate) containing 0.1 % SDS at 65 °C for 15 min and by two washes in 1 × SSC containing 0.1 % SDS at 65 °C for 15 min. Images were revealed with a STORM phosphorimager (Molecular Dynamics) and interpreted with the software XdotsReader (COSE), which quantified the intensity of the signal associated with each dot.

PCR amplification and sequencing. The rapid extraction of bacterial genomic DNA collected from 2 ml of an overnight culture was performed with the InstaGene Matrix (Bio-Rad). The *sodA* degenerate primers *d1* (5'-CCITAYICITAYGAYGCIYTIGARCC-3') and *d2* (5'-ARRTARTAGC-RTGYTCCCAIACRTC-3') were used to amplify an internal fragment of *sodA* (*sodA*_{int}), representing approximately 82 % of the complete *sodA* gene. These primers match at positions 25–51 (*d1*) and 487–510 (*d2*) of the 609 bp long *sodA* gene of the *S. bovis* type strain, which was taken as a reference. PCRs were performed on a Gene Amp System 2400 thermal cycler (Perkin Elmer) in a final volume of 50 µl

containing 250 ng of DNA as template, 0.5 µM of each primer, 200 µM of each dNTP and 1 U of AmpliTaq Gold DNA polymerase (Perkin Elmer) in a 1 × amplification buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂]. The PCR mixtures were denatured (3 min at 95 °C) and then subjected to 30 cycles of amplification (60 s of annealing at 37 °C, 60 s of elongation at 72 °C and 30 s of denaturation at 95 °C). PCR products were purified on a S-400 Sephadex column (Pharmacia) and directly sequenced on both strands with the degenerate primers *d1* and *d2* by using the ABI-PRISM BigDye Terminator Sequencing Kit and a Genetic ABI-PRISM 310 Sequencer Analyser (Perkin Elmer), as described previously (Poyart *et al.*, 2000). Determination of the 16S rDNA sequences was done as follows. The PCR fragments obtained by using the pairs of primers R1 and R2 (R1, R2, R7 and R8), R3 and R4 (R3, R4, R9 and R10) and R5 and R6 (R5, R6, R11 and R12) were sequenced on both DNA strands by using the primers indicated in parentheses, as described previously (Poyart *et al.*, 2000). The resulting sequences were assembled to generate a single contig of approximately 1450 bp that corresponded to the 16S rDNA sequence. The sequences of the primers used were: R1, 5'-TAACACATGCAAGTCGAACG-3'; R2, 5'-CCTGCGCTCGCTTTACGCCC-3'; R3, 5'-GTGCCAG-CAGCCGCGGTAAT-3'; R4, 5'-ACACGAGCTGACG-ACAGCCA-3'; R5, 5'-GGGGGCCCCGACAAGCGG-3'; R6, 5'-AGGAGGTGATCCAACCGCA-3'; R7, 5'-GGCC-ACGATGCATAGCCG-3'; R8, 5'-GACTGCTGCCTCC-CGTAG-3'; R9, 5'-CTGAGGCTCGAAAGCGTGGG-3'; R10, 5'-CCCACGCTTTCGAGCCTCAG-3'; R11, 5'-GAGGAAGGTGGGGATGACGT-3'; R12, 5'-CGTCATCC-CCACCTTCCTCC-3'.

Sequence analysis. The nucleotide sequences were analysed with Perkin Elmer software (Sequence Analysis, Sequence Navigator and AutoAssembler). Multiple alignments of the *sodA* and 16S rRNA gene sequences were carried out with the CLUSTAL X program (Jeanmougin *et al.*, 1998). The construction of the unrooted phylogenetic trees was performed with both the neighbour-joining (Saitou & Nei, 1987) and the maximum-parsimony (Fitch, 1971) methods, using the PHYLIP package (version 3.57c; Felsenstein, 1995). The reliability of the tree nodes was evaluated by calculating the percentage of 1000 bootstrap re-samplings that supported each topological element. Phylogenetic trees were also generated based on the translated partial SodA protein sequences.

RESULTS AND DISCUSSION

By using the primers *d1* and *d2*, we amplified and sequenced *sodA*_{int} from the type strains of *S. bovis*, *S. caprinus*, *S. equinus*, *S. gallolyticus*, *S. infantarius*, *S. macedonicus* and *S. waius* (Table 1). In this study, we also included 29 streptococcal strains of animal and human origin that were originally identified as *S. bovis*-*S. equinus* by conventional microbiological tests. The strains used in this study were analysed by PFGE after digestion with *Sma*I and *Bss*HI. The analysis of the restriction profile patterns obtained with these two enzymes for all the strains studied were different from each other by at least five fragments, demonstrating their unrelatedness (data not shown).

A single DNA fragment, corresponding to the expected 480 bp amplification product *sodA*_{int}, was observed

in all cases following agarose-gel electrophoresis and ethidium-bromide staining (data not shown). Analysis of the sequences of these amplicons revealed that they were actually fragments of *sodA*, since the corresponding deduced polypeptides revealed that they all possessed three histidyl residues and one aspartyl residue, supposedly serving as metal ligands at positions characteristic of manganese- or iron-dependent superoxide dismutases (Parker & Blake, 1988a, b). A multiple alignment of the streptococcal *sodA*_{int} sequences was carried out by using the CLUSTAL X program. The sequences of the degenerate primers *d1* and *d2* and alignment gaps were not taken into consideration for calculations. Phylogenetic analyses of the *sodA*_{int} sequences (430 bp) were performed using both the neighbour-joining and maximum-parsimony methods, as contained within the PHYLIP software package (version 3.57c; Felsenstein, 1995). The consensus trees derived from the two methods were virtually identical, hence only the consensus tree constructed by the neighbour-joining method is shown here (Fig. 1). This phylogenetic tree revealed that the *S. bovis* group can be divided into five major clusters (A, B, C, D and E; Fig. 1), which were supported by significant bootstrap values. The *sodA*_{int} sequence identity within each cluster was greater than 97.7% (Fig. 1), whereas it varied from 80 to 96% if one considered a pair of sequences from two strains belonging to different clusters (data not shown). The consensus tree based on SodA protein sequences had a similar topology but the reliability of the tree nodes, determined by a bootstrap analysis, was weaker than that in the DNA consensus trees (data not shown). These findings reflect the fact that DNA sequences are generally more divergent than those of the corresponding protein sequences, due to the degeneracy of the genetic code.

Cluster A (Fig. 1) comprises three strains formerly identified as *S. infantarius* subsp. *infantarius*, including the type strain of the species *S. infantarius* (Bouvet *et al.*, 1997; Schlegel *et al.*, 2000). The *sodA*_{int} sequences of these three strains displayed less than 1% sequence divergence. The species *S. infantarius*, which belongs to biotype II.1, had been previously identified on the basis of DNA-DNA hybridization and ribotyping and was shown to contain two subgroups, leading to the description of two subspecies, *S. infantarius* subsp. *infantarius* and '*S. infantarius* subsp. *coli*' (Schlegel *et al.*, 2000).

Cluster B (Fig. 1) includes the strain previously defined as '*S. infantarius* subsp. *coli*' (Schlegel *et al.*, 2000). Comparison of the *sodA*_{int} sequence of '*S. infantarius* subsp. *coli*' NEM 1867 with those of the *S. infantarius* subsp. *infantarius* strains (Cluster A) revealed 10.3% sequence divergence between the two clusters. These results are in agreement with those obtained from DNA-DNA hybridization experiments which demonstrated that the two subspecies displayed less than 70% homology (Table 2); ≥ 70% homology is indicative of two strains belonging to the same species

Fig. 1. Phylogenetic tree showing the relationships among the *sodA_{int}* sequences from various streptococcal strains. The tree was constructed using the neighbour-joining method, and the *sodA_{int}* sequences of the type strains of *S. salivarius* and *S. alactolyticus* were used as an outgroup to root the tree. Relevant bootstrap values, expressed as a percentage of 1000 replications, are indicated at the appropriate nodes. The scale bar (neighbour-joining distance) represents the percentage sequence divergence. The accession numbers for the *sodA_{int}* sequences used in this study can be found in Table 1.

Table 2. DNA–DNA hybridization values among species belonging to the *S. bovis*–*S. equinus* complex

Species: 1, *S. infantarius* subsp. *infantarius* CIP 103233^T; 2, *S. lutetiensis* NEM 782^T; 3, *S. bovis* CIP 102302^T; 4, *S. pasteurianus* NEM 1202^T; 5, *S. gallolyticus* CIP 105428^T; 6, *S. macedonicus* CIP 105683^T. The *sodA_{int}* cluster refers to the divisions shown in Fig. 1.

Species	<i>sodA_{int}</i> cluster	Homology when following DNAs were used as probes (%)					
		1	2	3	4	5	6
<i>S. infantarius</i> CIP 103233 ^T	A	100	61.67	49.27	46.41	34.92	44.08
<i>S. lutetiensis</i> NEM 782 ^T	B	62.78	100	50.07	53.24	40.57	42.9
<i>S. bovis</i> CIP 102302 ^T	C	46.6	49.97	100	40.21	38.33	32.78
<i>S. equinus</i> CIP 102504 ^T	C	51.14	48.77	91.03	49.16	39.61	40.65
<i>S. pasteurianus</i> NEM 1202 ^T	D	43.34	37.83	37.76	100	60.38	56.63
<i>S. gallolyticus</i> CIP 105428 ^T	E	35.79	38.58	42.97	60.67	100	69.09
<i>S. caprinus</i> CIP 104887 ^T	E	34.59	38.1	43.83	57.12	97.5	70.31
<i>S. macedonicus</i> CIP 105683 ^T	E	43.09	39.95	42.53	56.85	66.3	100
<i>S. waius</i> CIP 106079 ^T	E	54.26	41.75	40.05	55.12	68.45	93.33

(Wayne *et al.*, 1987). Taken together, these data demonstrate that *S. infantarius* subsp. *infantarius* and ‘*S. infantarius* subsp. *coli*’ do not belong to the same species. Cluster B also contains four human clinical strains identified as belonging to *S. bovis* biotype II.1 and one strain (NEM 1764) initially identified by

phenotypic tests as *S. equinus*. All of the strains belonging to this cluster possessed a similar Rapid ID 32Strep biotype and exhibited less than 0.2% divergence between their *sodA_{int}* sequences (Table 1 and data not shown). Thus, we propose the description of the strains belonging to this cluster as members of the

novel species *Streptococcus lutetiensis*, which will incorporate '*S. infantarius* subsp. *coli*'.

Cluster C (Fig. 1) is composed of three strains, including the type strains of *S. bovis* and *S. equinus*. The *sodA*_{int} sequences of these strains displayed less than 1% divergence. DNA–DNA hybridization experiments confirmed that strains belonging to this cluster were highly homologous to each other (> 90%) but that they were poorly related to strains belonging to the other clusters (≤ 53%; Table 2). These data confirm previous results (Farrow *et al.*, 1984) which indicated that these bacteria represent a single species. According to Rule 24b(2) of the *Bacteriological Code* (1990 revision) (Lapage *et al.*, 1992), we propose to designate all strains belonging to Cluster C as *S. equinus*. Among the 29 clinical isolates characterized in this study, only one strain (NEM 1760) was assigned to this cluster. This strain, isolated from horse faeces, was identified as *S. equinus* on the basis of its Rapid ID 32Strep biotype. These results confirm previous assertions that isolates of the species *S. equinus*, including its heterotypic synonym *S. bovis*, are rarely encountered among human clinical isolates (Devriese *et al.*, 1998; Farrow *et al.*, 1984; Nelms *et al.*, 1995; Whitehead & Cotta, 2000).

Cluster D (Fig. 1) encompasses six human clinical isolates identified by phenotypic methods as belonging to *S. bovis* biotype II.2 and it does not include any of the type strains used in this study. The *sodA*_{int} sequences of these strains were highly similar (≤ 1.1% divergence). In addition, their Rapid ID 32Strep biotypes were nearly identical and differed from those of strains belonging to the other clusters (Table 1). DNA–DNA hybridization experiments performed with NEM 1202^T used as a probe yielded low hybridization levels (38–61%) with the other type strains studied (Table 2). These results are in agreement with those obtained previously from DNA–DNA hybridization assays, ribotyping and analyses of 16S rRNA gene sequences (Clarridge *et al.*, 2001; Farrow *et al.*, 1984; Schlegel *et al.*, 2000) and support the description of a novel species within the *S. bovis*–*S. equinus* complex. The name *S. pasteurianus* is proposed for this novel species. A remarkable phenotypic characteristic of the members of this new species is that they all produced activity for all of the enzymes involved in sugar metabolism (i.e. β -glucosidase, α -galactosidase, β -glucuronidase, β -galactosidase and β -mannosidase) tested in the API 20Strep and Rapid ID 32Strep systems (Tables 1 and 3). It is also worth noting that among the six unrelated clinical isolates which define this cluster two were isolated from cerebrospinal fluid and were responsible for meningitis. This observation is consistent with a recent report in which all streptococcal strains belonging to Lancefield's group D responsible for central nervous system infections were identified as *S. bovis* biotype II.2 and clustered in the same group (Clarridge *et al.*, 2001). Further investigations are required to determine whether the strains

belonging to this species possess specific virulence genes responsible for this neuropathogenicity.

Cluster E (Fig. 1) contains the type strains of *S. gallolyticus*, *S. caprinus*, *S. macedonicus* and *S. waius* (Brooker *et al.*, 1994; Flint *et al.*, 1999; Osawa *et al.*, 1995; Tsakalidou *et al.*, 1998), and 15 strains displaying the same Rapid ID 32Strep biotype and assigned as *S. bovis* biotype I. The *sodA*_{int} sequences of these strains are highly similar and show less than 2.3% divergence. The fact that the *sodA*_{int} sequences of the *S. gallolyticus* and *S. caprinus* type strains are almost identical (98.9%) confirms that these species are subjective synonyms, as reported by Sly *et al.* (1997). It is worth noting that the strains of human ($n = 10$) and animal ($n = 5$) origin could not be differentiated by this genotypic method (Table 1 and Fig. 1). Conversely, the complete sequence identity observed between the *sodA*_{int} sequences of the type strains of *S. macedonicus* and *S. waius* suggests that they should be associated in a single species (Fig. 1). The validity of these sequencing data are confirmed by the high hybridization levels observed between *S. macedonicus* and *S. waius* (93.3%; Table 2). Accordingly, besides the fact that they were both isolated from dairy products, these two streptococcal strains share many common phenotypic characters and their 16S rRNA genes possess less than 0.2% sequence divergence, as described by Flint *et al.* (1999), Tsakalidou *et al.* (1998) and this work. Therefore, according to Rule 24b(2) of the *Bacteriological Code* (1990 revision) (Lapage *et al.*, 1992), *S. macedonicus* should have nomenclatural priority. It is therefore possible that Cluster E contains two distinct species, *S. gallolyticus* and *S. macedonicus*. Alternatively, it may contain a single species, *S. gallolyticus*, and in this case *S. macedonicus* may constitute an aesculin-negative variant of this species. Our DNA–DNA hybridization data, which revealed that the genomes of *S. macedonicus*/*S. waius* and *S. gallolyticus*/*S. caprinus* display > 70% homology, favours this latter hypothesis (Table 2).

The sequences of the 16S rRNA genes of *S. bovis*, *S. caprinus*, *S. equinus*, *S. gallolyticus*, *S. infantarius* subsp. *infantarius* and *S. macedonicus* have been published previously. We therefore determined the 16S rDNA sequences of three strains (NEM 760, NEM 782^T and NEM 1603) and two strains (NEM 1202^T and NEM 1205) belonging to Clusters B and D, respectively. Sequence analysis revealed that within both of these clusters the 16S rDNA sequences were identical (data not shown). The sequences of the 16S rDNA of the strains from Cluster B were almost identical to those of the type strains of *S. bovis* (99.9%) and *S. infantarius* (99.9%), whereas the sequences of the strains belonging to Cluster D were highly related to that of the *S. caprinus* type strain (99.8%). However, from the phylogenetic tree shown in Fig. 1 it can be seen clearly that the *sodA*_{int} sequences of strains belonging to Clusters B and D are not closely related to those of *S. bovis*, *S. infantarius*, *S. caprinus* or to any of

the other type species studied here. These results confirm our proposal that the *sodA* gene is a more discriminative target sequence than the 16S rRNA gene for differentiating closely related species belonging to the genera *Streptococcus* and *Enterococcus* (Poyart *et al.*, 1998, 2000).

In conclusion, we have determined the *sodA*_{int} sequences of 36 strains belonging to the *S. bovis*–*S. equinus* group, including those for the type strains of *S. bovis*, *S. equinus*, *S. gallolyticus*, *S. caprinus*, *S. infantarius*, *S. macedonicus* and *S. waius*. The results obtained from our analyses demonstrate that this group comprises five different clusters that might correspond to five distinct species. Three of these species correspond to the previously characterized species *S. equinus*, *S. gallolyticus* and *S. infantarius*. Of the two remaining species, one comprised all of the strains identified by phenotypic methods as belonging to *S. bovis* biotype II.2. We propose the reassignment of these strains to a novel species, *S. pasteurianus*. The other species corresponds to strains previously identified as '*S. infantarius* subsp. *coli*', for which the name *S. lutetiensis* is proposed. This work demonstrates the usefulness of a *sodA*-based database for the species identification of related streptococcal isolates, and suggests that sequence databases of defined DNA targets, such as *sodA*, constitute a valuable alternative approach for modern bacterial systematics. It also confirms that the species *S. equinus sensu stricto* is almost never isolated from human specimens and that human clinical isolates are mostly composed of *S. gallolyticus*, *S. infantarius*, *S. pasteurianus* and *S. lutetiensis*.

Description of *Streptococcus lutetiensis* sp. nov.

Streptococcus lutetiensis (lu.te'ti.en.sis. L. masc. n. *lutetia* of Paris, where the species was characterized).

One of the strains of this species has been characterized previously as '*S. infantarius* subsp. *coli*' (Schlegel *et al.*, 2000). Cells are Gram-positive cocci that occur in pairs or short chains. They are non-motile, non-sporulating, catalase-negative and facultatively anaerobic. Most strains show homogeneous growth in BHI and glucose broths after 18 h incubation at 37 °C. Growth also occurs in MRS broth without gas production. No growth occurs in 6.5% NaCl broth. Colonies on blood agar or nutrient agar are circular, smooth, entire and non-pigmented. α -Haemolytic on blood agar. Characteristics useful in the differentiation of *S. lutetiensis* from its related streptococci are listed in Table 3. The type strain of *Streptococcus lutetiensis* (NEM 782^T = CIP 106849^T) is a human isolate of unknown origin. Three strains of this species were isolated from human specimens (stool, cerebrospinal fluid and unknown origin). Some other characteristics for *S. lutetiensis* can be found in Schlegel *et al.* (2000).

Table 3. Tests useful in differentiating *S. lutetiensis* and *S. pasteurianus* from major species belonging to the *S. bovis*–*S. equinus* complex

Species: 1, *S. lutetiensis*; 2, *S. pasteurianus*; 3, *S. infantarius*; 4, *S. gallolyticus*; 5, *S. macedonicus*; 6, *S. bovis*; 7, *S. equinus*. The data presented here are based on the results obtained by testing our strains and on data from Clarridge *et al.* (2001), Devriese *et al.* (1998), Farrow *et al.* (1984), Nelms *et al.* (1995), Osawa *et al.* (1995), Schlegel *et al.* (2000) and Tsakalidou *et al.* (1998). The *sodA*_{int} cluster refers to the divisions shown in Fig. 1. +, More than 85% of the strains tested positive; –, more than 85% of the strains tested negative; v, variable test reaction. α -GAL, α -Galactosidase; β -GAL, β -galactosidase; β -GLU, β -glucosidase; β -GLC, β -glucuronidase; β -MAN, β -mannosidase. None of the species hydrolysed hippurate or urea. None of the species produced arginine dihydrolase, alkaline phosphatase, pyrrolidonyl arylamidase, *N*-acetyl β -glucosamidase or glycyl tryptophan arylamidase. All of the species tested produced acetoin, alanyl phenylalanyl proline arylamidase and leucine aminopeptidase. Acid was not produced from L-arabinose, D-arabitol, cyclodextrin, ribose or sorbitol by any of the species tested. Acid was produced from maltose, sucrose and methyl β -D-glucopyranoside by all of the species.

Characteristics	1	2	3	4	5	6	7
<i>sodA</i>_{int} cluster	B	D	A	E	E	C	C
Hydrolysis of:							
Aesculin	+	+	v	+	–	+	+
Production of:							
α -GAL	+	+	+	+	–	+	–
β -GAL	–	+	–	–	–	–	–
β -GLU	+	+	v	–	–	+	v
β -GLC	–	+	–	–	–	–	–
β -MAN	–	+	–	–	–	–	–
Acid from:							
Glycogen	–	–	+	+	–	–	–
Lactose	+	+	+	+	+	+	–
D-Mannitol	–	–	–	+	–	–	–
Meleizitose	–	–	–	+	–	–	–
Melibiose	–	+	+	–	–	–	–
Pullulan	–	–	+	+	–	–	–
Raffinose	+	+	v	+	+	–	–
Tagatose	–	v	–	–	–	–	–
Trehalose	–	+	–	+	–	–	+
Starch	+	–	+	+	+	+	–

Description of *Streptococcus pasteurianus* sp. nov.

Streptococcus pasteurianus (pas.teu'ri.an.us. N.L. n. *pasteurianus* of the Pasteur Institute, where the species was characterized).

Strains of this species have been characterized previously as belonging to *S. bovis* biotype II.2 (Farrow *et al.*, 1984; Clarridge *et al.*, 2001). Cells are Gram-positive cocci that occur in pairs or short chains. They

are non-motile, non-sporulating, catalase-negative and facultatively anaerobic. Most strains show homogeneous growth in BHI and glucose broths after 18 h incubation at 37 °C. Growth also occurs in MRS broth without gas production. No growth occurs in 6.5% NaCl broth. Colonies on blood agar or nutrient agar are circular, smooth, entire and non-pigmented. α -Haemolytic on blood agar. Characteristics useful in the differentiation of streptococci related to *S. pasteurianus* are listed in Table 3. The type strain of *Streptococcus pasteurianus* (NEM 1202^T = CIP 107122^T) is a human isolate responsible for meningitis and was isolated from the cerebrospinal fluid of an infant. The five other strains of this species were isolated from human specimen cultures (cerebrospinal fluid, $n = 1$; blood, $n = 3$; urine, $n = 1$). Some other characteristics for *S. pasteurianus* can be found in Clarridge *et al.* (2001) and Schlegel *et al.* (2000).

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During the preparation of this paper, the reclassification of *S. waius* as *S. macedonicus* was proposed by Manachini *et al.* (2002).

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