Partial *recN* gene sequencing: a new tool for identification and phylogeny within the genus *Streptococcus*

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Partial sequences of the *recN* gene (1249 bp), which encodes a recombination and repair protein, were analysed to determine the phylogenetic relationship and identification of streptococci. The partial sequences presented interspecies nucleotide similarity of 56.4–98.2 % and intersubspecies similarity of 89.8–98 %. The mean DNA sequence similarity of *recN* gene sequences (66.6 %) was found to be lower than those of the 16S rRNA gene (94.1 %), *rpoB* (84.6 %), *sodA* (74.8 %), *groEL* (78.1 %) and *gyrB* (73.2 %). Phylogenetically derived trees revealed six statistically supported groups: *Streptococcus salivarius*, *S. equinus*, *S. hyovaginalis*/ *S. pluranimalium*/ *S. thoraltensis*, *S. pyogenes*, *S. mutans* and *S. suis*. The 'mitis' group was not supported by a significant bootstrap value, but three statistically supported subgroups were noted: *Streptococcus sanguinis*/ *S. cristatus*/ *S. sinensis*, *S. anginosus*/ *S. intermedius*/ *S. constellatus* (the 'anginosus' subgroup) and *S. mitis*/ *S. infantis*/ *S. peroris*/ *S. oralis*/ *S. oligofermentans*/ *S. pneumoniae*/ *S. pseudopneumoniae*. The partial *recN* gene sequence comparison highlighted a high percentage of divergence between *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis*. This observation is confirmed by other gene sequence comparisons (*groEL*, *gyrB*, *rpoB* and *sodA*). A high percentage of similarity was found between *S. intermedius* and *S. constellatus* after sequence comparison of the *recN* gene. To study the genetic diversity among the 'anginosus' subgroup, *recN*, *groEL*, *sodA*, *gyrB* and *rpoB* sequences were determined for 36 clinical isolates. The results that were obtained confirmed the high genetic diversity within this group of streptococci.

Representatives of the genus *Streptococcus* are Gram-positive, catalase-negative, non-motile cocci. They are commensals of the tegument or mucous membranes in both humans and animals. The normal presence of streptococci on the mucous membranes and the skin explains how they can frequently contaminate biological samples. Although their pathogenicity is variable, they can be responsible for meningitis, pneumonia, endocarditis and fasciitis. At the time of writing, 65 species and 12 subspecies with validly published names are recognized as members of the genus (Euzéby, 2010). Novel species are continually being isolated from human, animal and

environmental samples and have been characterized by 16S rRNA gene sequence comparisons in association with phenotypic criteria (Glazunova *et al.*, 2006; Milinovich *et al.*, 2008; Shewmaker *et al.*, 2007; Takada & Hirasawa, 2008; Vela *et al.*, 2009). The precise identification of these bacteria at the species level is quite laborious, particularly in the 'viridans' group. Streptococci were first identified based on the haemolytic reaction, group carbohydrate antigens (Lancefield serotyping) and phenotypic tests (Facklam, 2002). Commercial systems based on comparisons of phenotypic characteristics do not permit unambiguous identification at the species level, however, because of the limited number of biochemical traits that can be analysed, the variability of traits within a species, the poor reproducibility of some tests and the fact that not all species are included in the database. Therefore, it is impossible to obtain a real idea of the host–pathogen relationships of streptococci. According to Hoshino *et al.* (2005), the rate of correct identification of non-haemolytic streptococcal strains by commercial identification kits was below 50 % and varied significantly with regard to the studied species.

Abbreviation: ILD, incongruence length difference.

The GenBank/EMBL/DDBJ accession numbers for the partial *recN* gene sequences determined in this study are EU917226–EU917315, as detailed in Supplementary Table S1, and those of the other sequences determined in this study are FJ712038–FJ712187 (Supplementary Table S4).

Strain and primer details, including details of accession numbers, and details of inter- and intraspecies divergence and phylogenetic trees for the 'anginosus' subgroup are available as supplementary material with the online version of this paper.

Various molecular DNA-based methods have been proposed for the identification of *Streptococcus* species. Genes encoding D-alanine:D-alanine ligase (*ddl*) (Garnier *et al.*, 1997), autolysin (*lytA*) (Kawamura *et al.*, 1999), dextranase (*dex*) (Igarashi *et al.*, 2001), the 10 kDa and 60 kDa heat-shock proteins (*groESL*) (Hung *et al.*, 2005; Teng *et al.*, 2002), the RNA subunit of endoribonuclease P (*rnpB*) (Tapp *et al.*, 2003), elongation factor Tu (*tuf*) (Picard *et al.*, 2004), quinolone resistance-determining regions of DNA gyrase (*gyrA*) and topoisomerase subunit C (*parC*) (Kawamura *et al.*, 2005) and the 16S–23S rRNA intergenic spacer region (Chen *et al.*, 2004; Nielsen *et al.*, 2009) have been suggested. A good candidate for identification at the species level should present enough variability between different species but little variability within a species.

Zeigler (2003) studied 32 protein-encoding genes and determined a small set of protein-encoding genes in which the percentage of similarity between isolates could assign novel strains reliably to bacterial species and replace DNA–DNA hybridization. This set of genes included *recN* (recombination/repair protein), *thdF* (GTP-binding, thiophene oxidation) and *rpoA* (RNA polymerase, alpha subunit). For members of the family *Pasteurellaceae*, sequence comparisons of these three genes were proposed to replace DNA–DNA hybridization, to estimate the genome G+C content of a species and to infer phylogenetic analysis in association with other markers (Kuhnert & Korczak, 2006). Sequence comparison of the *recN* gene was applied to phylogenetic analysis and the assignment of isolates to species within the genus *Geobacillus* and presented a resolving power that was nearly an order of magnitude greater than that of the 16S rRNA gene (Zeigler, 2005). In this study, we explored the use of partial *recN* gene sequence comparisons in order to investigate taxonomy and phylogeny in the genus *Streptococcus*. In addition, our results were compared with those obtained previously for four other genes (*rpoB*, *sodA*, *groEL* and *gyrB*; Glazunova *et al.*, 2009).

The 89 streptococcal strains studied (representing 54 species and nine subspecies) were obtained from the Collection de l'Institut Pasteur (CIP, Paris, France) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and are listed in Supplementary Table S1 (available in IJSEM Online). *Enterococcus faecalis* CIP 103015^T was chosen as an outgroup for phylogenetic tree reconstructions. Clinical isolates obtained by our laboratory are characterized in Table 1.

Bacterial DNA was extracted using the MagNA Pure LC DNA isolation kit III (Roche) with the MagNA Pure LC instrument, as described by the manufacturer. Primers for amplification of the *recN* gene fragment (recNd/recNr) were selected from conserved regions after alignment of sequences from complete streptococcal genomes [*Streptococcus pneumoniae* R6 (GenBank accession no. AE007317), *S. pneumoniae* TIGR4 (AE005672), *S. agalactiae* 2603V/R (AE009948), *S. pyogenes* SF370 (AE004092) and *S. mutans* UA159

(AE014133)] and phylogenetically related bacterial genomes [*Enterococcus faecalis* V583 (AE016830), *Lactococcus lactis* Il1403 (AE005176), *Listeria innocua* Clip11262 (AL592022), *Listeria monocytogenes* EGD (AL591824) and *Staphylococcus aureus* N315 (BA000018)] which were available when the study was started. The PCR annealing temperature was 48 °C. Other primers were designed for the sequencing reactions. Primers are described in Supplementary Table S2. Fragments of the *rpoB*, *sodA*, *gyrB* and *groEL* genes were amplified by PCR as described previously (Glazunova *et al.*, 2009).

PCR products were purified using MultiScreen PCR plates (Millipore) and sequenced using a DNA sequencing kit (BigDye Terminator version 1.1 cycle sequencing kit; PE Biosystems) according to the manufacturers' instructions. Sequence products were purified and electrophoresis was performed with a 3100 Genetic Analyzer (Applied Biosystems).

Gene sequences were aligned using the multiple alignment program CLUSTAL_X version 1.8 (Thompson *et al.*, 1997). Phylogenetic relationships were determined using MEGA version 4 (Tamura *et al.*, 2007). Distance matrices were determined following the assumptions described by Kimura (1980) and were used to reconstruct dendrograms using the neighbour-joining method (Saitou & Nei, 1987). The maximum-parsimony algorithm was also used for phylogenetic analysis (Farris, 1970). Phylogenetic trees were also calculated by the maximum-likelihood method using the program DNAML in the PHYLIP software package (Felsenstein, 1989). The percentage of similarity between nucleotide sequences was calculated using BioEdit software. Bootstrap values were obtained for a consensus tree based on 100 randomly generated trees. DNA sequences of clinical isolates were compared using the BLAST2 program (Altschul *et al.*, 1990) (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>) with our local database.

The TaxonGap software tool (Slabbinck *et al.*, 2008) was applied to represent the resolution of the gene within and between taxonomic units. TaxonGap represents the *s*-heterogeneity and *s*-separability values as light-grey and dark-grey horizontal bars, respectively, for individual biomarkers. The same scale is used to plot both *s*-heterogeneity and *s*-separability in order to support optimal comparability of the values across the biomarkers. The name of the closest neighbour is identified to the right of the dark-grey bar. Distances used for the calculation of *s*-heterogeneity and *s*-separability values were determined using pairwise nucleotide sequence alignments with MEGA version 4.

To investigate incongruence between the *recN* data and the dataset for each gene sequence, the incongruence length difference (ILD) test of Farris *et al.* (1994) was performed using the PAUP 4.0 beta version (Swofford, 1998). The maximum-parsimony trees exploited for the ILD test were obtained from parsimony-informative sites determined using MEGA version 4.

Table 1. Identification of clinical isolates and reference strains of *S. anginosus*, *S. constellatus* and *S. intermedius* using phylogenetic analysis and BLAST comparison

Analysis resulted in identification as: SA, *S. anginosus*; SC, *S. constellatus*; SI, *S. intermedius*. Strains that could not be identified by phylogenetic analysis were identified by BLAST comparison, with identifications given in parentheses. NK, Not known.

Strain	Isolate no.	Biological sample	Identification based on:				
			<i>recN</i>	<i>groEL</i>	<i>sodA</i>	<i>rpoB</i>	<i>gyrB</i>
5400732	S1	Urine	SA	SA	SA	SA	SA
5400767	S2	NK	SI	SI	SI	SI	SI
5401630	S3	Subdural empyema	(SI)	SI	SI	SI	SI
5402532	S4	Right arm abscess	SI	SI	SI	SI	SI
5402857	S5	Bone sample	(SI/SC)	SC	SC	SC	SC
6400296	S6	Blood	SA	SA	SA	SA	SA
6400536	S7	Abdominal abscess	SA	SA	SA	SA	SA
6400598	S8	Blood	SI	SI	SI	SI	SI
6400954	S9	Left foot excision	SA	SA	SA	SA	SA
6400987	S10	Blood	SA	SA	SA	SA	SA
6400988	S11	Prosthesis	SA	SA	SA	(SA/SC)	SA
6401233	S12	Gastrectomy	SC	SC	SA	SC	SC
6401499	S13	Blood	SA	SA	SA	(SA/SC)	SA
6401637	S14	Abdominal biomembrane	SA	SA	SA	(SA/SC)	SA
6401638	S15	Blood	SA	SC	SA	SC	SA
6401858	S16	Brain abscess	SC	SC	(SC)	SC	SC
6402381	S17	Heel wound	SA	SA	SA	(SA/SC)	SA
6402538	S18	Cerebral abscess	SI	SC	SC	SC	SC
6402571	S19	Left leg abscess	SC	SC	SC	SC	SC
6402846	S20	Blood	SA	SA	SA	SA	SA
7401233	S21	Pleural sample	SC	SC	SC	SC	SI
7401381	S22	Hand abscess	SA	SA	SA	SA	SA
7401697	S23	Pleural drainage tube discharge	SI	SI	SI	SI	SI
7402390	S24	Urine	SA	SA	SA	SA	SA
7402568	S25	Blood	SA	SA	SA	SA	SA
7402928	S26	Subcutaneous sample	SA	SC	SA	SC	SA
7403049	S27	Blood	SA	SC	SC	SC	SC
8400103	S28	Blood	SC	SC	SA	SC	SC
8401570	S29	Subcutaneous sample	SA	SA	SA	SA	SA
8401387	S30	Blood	SI	SI	SI	SI	SI
CIP 105053	–	Pleural liquid	SI	SI	SI	SI	SI
CIP 105039	–	Cerebral abscess	SI	SI	SI	SI	SI
CIP 105046	–	Dental plaque	SI	SC	SC	SC	(SC)
CIP 105032	–	NK	SC	SC	SC	SC	SC
CIP 105055	–	Spittle	(SI)	SC	SC	SC	SC
CIP 105057	–	Skin	SA	SA	SA	(SA/SC)	SA

Phylogeny of streptococci derived from partial *recN* sequences

The sequenced fragment represented 75.3 % of the full-length *recN* gene sequence of *S. mutans* UA159 (NP_721018). A 1249 bp fragment was amplified from the majority of the 89 streptococcal strains studied using the *recN* primers designed in this study, with the exception of the fragment from *Streptococcus gallinaceus* CIP 107087^T, which was 1246 bp. A fragment of only 1212 bp was amplified from *Streptococcus parauberis* CIP 103956^T as a result of unsuccessful sequencing of the 3' end of the gene. Accession numbers for all submitted sequences are given in Supplementary Table S1.

Phylogenetic analysis was inferred from partial *recN* sequences using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. Identical organization was obtained for the three analyses with slight variations. The highest bootstrap values were obtained for the tree inferred from the neighbour-joining method and therefore we have concentrated on this analysis in the discussion. The neighbour-joining phylogenetic tree for type strains of species and subspecies is presented in Fig. 1.

Compared with the phylogenetic trees inferred from previously described sequence comparisons of various genes, the tree inferred from sequence comparison of the

Fig. 1. Phylogenetic tree of members of the genus *Streptococcus* inferred from comparison of partial *recN* gene sequences using the neighbour-joining method. Bar, 0.05 changes per nucleotide position. The sequence of *Enterococcus faecalis* CIP 103015^T was used as the outgroup. Bootstrap values ≥ 50 (from 100 replicates) are indicated.

recN gene resulted in the most stable nodes. The numbers of nodes presenting bootstrap values higher than 50, 80 and 95 % are detailed in Table 2. In the *recN* tree, 29 (49.2 %) nodes presented bootstrap values ≥ 95 %, which was higher than the value found for *groEL* (24, 40.7 %), which we had considered the best tool for inference of phylogenetic relationships of streptococci in a previous study (Glazunova *et al.*, 2009).

Although the representatives of the 'mitis' group formed the major cluster, it was not supported by a significant bootstrap value. Three subgroups were observed and were supported by high bootstrap values. The first included *Streptococcus cristatus*, *S. sanguinis* and *S. sinensis*, the second included *Streptococcus anginosus*, *S. intermedius* and *S. constellatus* and the third included *Streptococcus infantis*, *S. peroris*, *S. oralis*, *S. oligofermentans*, *S. pneumoniae*, *S. pseudopneumoniae* and *S. mitis*. In addition, *Streptococcus parasanguinis* and *S. entericus* grouped together, and *Streptococcus gordonii* and *S. massiliensis* were found as singletons.

Six other clusters were noted: (i) *Streptococcus thermophilus*, *S. vestibularis* and *S. salivarius*; (ii) the representatives of the 'equinus' group, which included *Streptococcus alactolyticus*, *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus*, *S. gallolyticus* subsp. *macedonicus*, *S. infantarius* subsp. *infantarius*, *S. infantarius* subsp. *coli* and *S. equinus*; (iii) *Streptococcus hyovaginalis*, *S. pluranimalium* and *S. thoralensis*; (iv) *Streptococcus pyogenes*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. dysgalactiae* subsp. *equisimilis*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus* and *S. castoreus*; (v) the representatives of the 'mutans' group, which included *Streptococcus mutans*, *S. macacae*, *S. devriesei* and *S. rattii*; and (vi) *Streptococcus suis*, *S. acidominimus*, *S. gallinaceus*, *S. minor* and *S. ovis*. All of the other species clustered with non-significant bootstrap values at the nodes.

In the tree constructed by the maximum-parsimony method, the same clusters were noted. By this method, *S. entericus* was

not included in the 'mitis' group but rather was closely related to the 'mutans' group, even though the bootstrap value at the node was not significant (20 %). *S. equi* and *S. castoreus* were closely related to the 'pyogenes' group, but the bootstrap values at the nodes were low (56 and 54 %, respectively). In the tree constructed by the maximum-likelihood method, the major groups were congruent with those obtained using the neighbour-joining method and were supported by statistically significant bootstrap values that ranged from 85 to 100 %. Slight modifications were noted in the 'mitis' group. *S. massiliensis* was closely related to the 'anginosus' subgroup, but the bootstrap value at the node was low (60 %). *S. gordonii* grouped with the 'cristatus' subgroup, also with a low bootstrap value (59 %).

Compared with the tree obtained from sequence comparison of the *groEL* gene fragment (Glazunova *et al.*, 2009), some differences were noted. *S. acidominimus* and *S. suis* were not included in the 'mitis' group and clustered with *S. gallinaceus*, *S. minor* and *S. ovis*. *Streptococcus ferus* was closely related to the 'mutans' group with a bootstrap value of 89 %. *S. equi* clustered with *S. pyogenes*, *S. dysgalactiae* and *S. castoreus*. In the 'mitis' group, *S. gordonii* did not cluster with *S. sanguinis*, *S. cristatus* and *S. sinensis*. The other groups or subgroups that were described for phylogenetic analysis inferred from *recN* sequence comparisons were identical to those inferred from *groEL* sequence comparisons (Glazunova *et al.*, 2009).

To improve some of these inconsistencies, we considered concatenating the sequences that were obtained from different gene fragments. Most systematists consider data partitions to be combinable if they are not strongly incongruent with one another and use tests, such as the ILD test, to evaluate incongruence (Planet, 2006). Our results from the ILD test demonstrated no congruence between the *recN* data and the data for the previously studied genes (not shown). Therefore, we did not concatenate sequences that were obtained from different genes.

Table 2. Nodes that presented bootstrap values greater than the indicated threshold values

Bootstrap values were calculated from 100 trees. Corresponding percentages of all nodes in the trees are indicated in parentheses. Phylogenetic trees were inferred from different genetic targets using the neighbour-joining method. Accession numbers of the sequences used are listed in Supplementary Table S1 and by Glazunova *et al.* (2009).

Gene	Bootstrap values		
	≥ 50	≥ 80	≥ 95
<i>recN</i>	42 (71.2)	35 (59.3)	29 (49.2)
<i>groEL</i>	38 (64.4)	32 (54.2)	24 (40.7)
<i>gyrB</i>	40 (67.8)	25 (42.4)	17 (28.8)
<i>rpoB</i>	33 (55.9)	22 (37.3)	15 (25.4)
<i>sodA</i>	34 (57.6)	21 (35.6)	17 (28.8)

Sequence similarity for streptococcal species

The TaxonGap output for the *Streptococcus* species that were studied by *recN* gene sequence comparisons is shown in Fig. 2. The *s*-separability is a measure of the divergence between the different species, which is subsequently referred to as interspecies divergence. The *s*-heterogeneity is a measure of the heterogeneity between different isolates of the same species, which is subsequently referred to as intraspecies divergence. The left panel shows a neighbour-joining tree inferred from sequence comparison of partial *recN* genes of type strains.

The similarity of the *recN* sequences between members of different species ranged from 56.4 % between *Streptococcus halichoeri* and *S. entericus* to 98.2 % between *S. constellatus* and *S. intermedius*. Compared to previously studied genes, *recN* gene sequence comparisons presented lower mean similarity values between different species (Table 3).

Fig. 2. TaxonGap output for *Streptococcus* species studied by partial *recN* gene sequence comparison. The left panel shows a neighbour-joining tree inferred from partial *recN* gene sequence comparison. For each of the species in the phylogenetic tree, the right panel depicts the interspecies and intraspecies variability. Separability values between different species are represented as dark-grey bars with the names of the closest relatives. Heterogeneity values among isolates of the same species are represented as pale-grey bars.

Sequence comparison of the partial *recN* gene was also applied to streptococci at the subspecies level. In the genus *Streptococcus*, most of the subspecies were highly related (95.6–98%). The most closely related subspecies were *S. gallolyticus* subsp. *gallolyticus* and *S. gallolyticus* subsp. *macedonicus* (98%) and the least closely related subspecies were *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* (89.8%). Higher mean divergence values between subspecies were obtained with the *sodA* gene than

with the *recN* gene (Tables 3 and 4). Fewer than 1% of species and subspecies pairs exhibited levels of sequence similarity $\geq 90\%$. The majority of species and subspecies pairs (85.6%) were situated in the range of 60–70% similarity. Several isolates from some species or subspecies were studied: *S. mitis* (3), *S. oralis* (2), *S. constellatus* (3), *S. intermedius* (3), *S. anginosus* (2), *S. gordonii* (2), *S. infantarius* subsp. *coli* (2), *S. infantarius* subsp. *infantarius* (3), *S. gallolyticus* subsp. *macedonicus* (2), *S. gallolyticus*

Table 3. Nucleotide sequence similarities between species and divergence between subspecies of the genus *Streptococcus*

Values are means with minimum–maximum ranges in parentheses. Accession numbers of the sequences used are listed in Supplementary Table S1 and by Glazunova *et al.* (2009).

Gene	Interspecies similarity (%)	Intersubspecies divergence (%)
16S rRNA	94.1 (88.8–99.7)	1.11 (0.03–2.1)
<i>rpoB</i>	84.6 (76.1–97.6)	1.31 (0.57–2.1)
<i>sodA</i>	74.8 (63.6–100)	5.92 (1.2–12.9)
<i>groEL</i>	78.1 (72.6–96.6)	2.96 (1.2–6.1)
<i>gyrB</i>	73.2 (64.6–97.5)	3.29 (1.6–6.5)
<i>recN</i>	66.6 (56.4–98.2)	4.32 (1.9–10.2)

subsp. *gallolyticus* (4), *S. gallolyticus* subsp. *pasteurianus* (3), *S. equinus* (3) and *S. agalactiae* (8). Although only relatively few isolates were studied, the intraspecies variability seemed low for most species (<4%), with the exception of *S. mitis* (6.7%). These observations will need to be confirmed by studying more isolates.

In the phylogenetic analysis inferred from the *recN* gene sequence comparison, the two subspecies of *S. dysgalactiae* did not cluster together. The species *S. dysgalactiae* was proposed by Garvie *et al.* (1983), and its description was amended by Farrow & Collins (1984). The latter authors showed, based on DNA–DNA hybridization, that the species '*Streptococcus equisimilis*' should be included in *S. dysgalactiae*. Two subpopulations of this species were subsequently characterized by Vandamme *et al.* (1996), from which two subspecies, *S. dysgalactiae* subsp. *dysgalactiae* (animal origin) and *S. dysgalactiae* subsp. *equisimilis* (human origin), were proposed. A multilocus enzyme electrophoresis study confirmed the existence of the two subgroups in *S. dysgalactiae*, but the isolates included in each subspecies differed (Vieira *et al.*, 1998). In our study, the divergence between the two subspecies of *S. dysgalactiae* was high (10.2%). This particular divergence was the highest for each of the gene sequence comparisons: *groEL* (6.1%), *rpoB* (2.1%) and *sodA* (12.9%). The values found for other genes are reported in Table 4. DNA–DNA hybridization is

considered the standard reference technique in bacterial identification. Values $\geq 70\%$ (commonly held as the threshold value) were found for strains of the two subspecies of *S. dysgalactiae* by Farrow & Collins (1984) (92–99%) and Vieira *et al.* (1998) (75–77%), although the reported values are very different. Therefore, *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* are representatives of the same species, although genetic analysis of more isolates could improve their clustering into subspecies.

The 'anginosus' subgroup

Another interesting result of this study was the high similarity between *S. constellatus* and *S. intermedius*. *S. anginosus*, *S. constellatus* and *S. intermedius* were described in 1906, 1924 and 1925, respectively. Previous studies have shown considerable genetic similarity within the group, even though it is biochemically heterogeneous (Farrow & Collins, 1984; Coykendall *et al.*, 1987), and proposed that the three species should be included in a single species. Based on DNA–DNA hybridization, Whiley & Beighton (1991) again proposed the recognition of three distinct species. However, a lack of reciprocity was observed in the levels of DNA relatedness between the type strains of *S. intermedius* and *S. constellatus* (36 and 65%). Taken together, these observations demonstrated the complexity in defining species within the 'anginosus' subgroup. To understand better the phylogenetic evolution of this group, we determined the sequences of the *recN*, *groEL*, *gyrB*, *sodA* and *rpoB* genes for 30 clinical strains isolated in our laboratory as well as isolates of *S. anginosus* (1), *S. intermedius* (2) and *S. constellatus* (3) obtained from the CIP collection (Supplementary Table S3). Two approaches were used to identify the isolates at the species level: phylogenetic analysis (Supplementary Fig. S1) and BLAST comparison when identification by phylogenetic analysis was impossible. In the phylogenetic analysis, sequences that grouped into one cluster with a significant bootstrap value at the corresponding node ($>85\%$) were considered to belong to the same species. Sequences that were isolated or which grouped on separated branches with non-significant bootstrap values at the corresponding nodes were subjected to analysis by the BLAST program. Strain identifications are detailed in Table 1. Two strains identified as *S. anginosus* in the CIP, strains CIP 103053 and CIP

Table 4. Nucleotide sequence divergence between subspecies

Subspecies comparison	Mean divergence (%)					
	<i>recN</i>	<i>groEL</i>	<i>gyrB</i>	<i>rpoB</i>	<i>sodA</i>	16S rRNA
<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i> vs subsp. <i>equisimilis</i>	10.2	6.1	3.3	2.1	12.9	1.3
<i>S. equi</i> subsp. <i>equi</i> vs subsp. <i>zooepidemicus</i>	3.7	1.6	1.6	2.0	1.2	2.1
<i>S. infantarius</i> subsp. <i>infantarius</i> vs subsp. <i>coli</i>	2.1	2.93	6.5	1.42	10.27	0.03
<i>S. gallolyticus</i> subsp. <i>gallolyticus</i> vs subsp. <i>macedonicus</i>	2.0	1.08	2.17	0.67	2.1	1.45
<i>S. gallolyticus</i> subsp. <i>gallolyticus</i> vs subsp. <i>pasteurianus</i>	3.5	1.52	2.7	0.57	3.97	0.95
<i>S. gallolyticus</i> subsp. <i>macedonicus</i> vs subsp. <i>pasteurianus</i>	4.4	2.33	3.5	1.1	5.1	0.8

103055, grouped with the species *S. intermedius* and *S. constellatus*, respectively. Identification at the species level (*S. intermedius*) was impossible using *recN* gene sequence comparison for isolate S5. Isolates of *S. constellatus* and *S. intermedius* clustered in the phylogenetic tree inferred from sequence comparisons of the *recN*, *groEL*, *sodA* and *gyrB* genes (Supplementary Fig. S1). Using *rpoB* gene sequence comparison, the type strain of *S. intermedius*, CIP 103248^T, clustered with the ‘anginosus’ species isolates, and another cluster included the other isolates that represented *S. intermedius*. However, this cluster was not related to the cluster that included the *S. constellatus* isolates. The identification of *S. anginosus* and *S. constellatus* isolates was more difficult using *rpoB* gene sequence comparison, as it was impossible to identify five isolates (S11, S13, S14, S17 and CIP 105057) at the species level. Identification of two isolates (S15 and S26) was impossible at the species level; they were identified as either *S. anginosus* or *S. constellatus*, depending on which gene sequence was analysed. To try to explain the difficulty in differentiating isolates of *S. anginosus* and *S. constellatus*, inter- and intraspecies percentages of divergence were calculated for each species and for each gene (Supplementary Table S4). The interspecies divergence was lower for *S. intermedius*/*S. constellatus* and the intraspecies divergence was higher for *S. anginosus*. The most likely explanation is that genetic recombination has occurred between some isolates of *S. anginosus* and *S. constellatus*.

Conclusions

Partial sequence comparison of the *recN* gene differentiates members of the genus *Streptococcus* clearly and allows phylogenetic relationships between the different species to be inferred with a high level of statistical support. In comparison with previously studied genes (16S rRNA, *groEL*, *gyrB*, *rpoB* and *sodA*), *recN* represents the best tool to identify streptococci and to study evolution in this group of bacteria. Six clusters are well defined: the ‘pyogenes’ group, the ‘equinus’ group, the ‘salivarius’ group, the ‘mutans’ group, the ‘hyovaginalis’ group and the ‘suis’ group. In general, however, the streptococci constitute a heterogeneous group, as the phylogenetic position of many representatives differs with the studied gene. DNA–DNA hybridization has been the ‘gold-standard’ method for identification at the species level, but partial gene sequencing analyses highlight some peculiarities, such as the high divergence between *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* or the high similarity between *S. intermedius* and *S. constellatus*. In the past, contradictory results have been published concerning these species and subspecies and, in future, the use of new approaches could result in re-evaluation of their taxonomic status.

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