rDNA sequence analyses of *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from pigs

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The nucleotide sequences of 16S and 23S rRNA genes (rDNA) were determined for 11 isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* from slaughtered pigs with endocarditis, arthritis or lymphadenitis and strain ATCC 35666, designated as a strain of subspecies *equisimilis*. The sequences of each of the genes were compared phylogenetically with the corresponding sequences of *S. dysgalactiae* subsp. *dysgalactiae* ATCC 43078^T and ATCC 27957, which were also determined in this study. Based on the 16S rDNA analysis, the isolates of *S. dysgalactiae* subsp. *equisimilis* were divided into two distinct groups, designated groups 1 and 2. *S. dysgalactiae* subsp. *equisimilis* ATCC 35666 was closely related to the group 2 strains. The *S. dysgalactiae* subsp. *dysgalactiae* strains seemed to be associated with the group 1 strains, which was not consistent with the conventional subspecific classification of *S. dysgalactiae*. In contrast, the 23S rDNA analysis distinguished *S. dysgalactiae* subsp. *dysgalactiae* strains from subsp. *equisimilis* strains. This inconsistency between phylogenies based on 16S and 23S rDNA indicates that 23S rDNA is a more rigid marker for determining the phylogenetic relationships and taxonomic position of these organisms than is 16S rDNA.

INTRODUCTION

The species *Streptococcus dysgalactiae* is known to include a variety of strains that are biochemically and genetically very close but differ in serological grouping, type of haemolysis, host association and pathogenicity (Jones, 1980; Farrow & Collins, 1984; Kilpper-Bälz & Schleifer, 1984; Hommez *et al.*, 1991; Efstratiou *et al.*, 1994; Bert *et al.*, 1997). Vandamme *et al.* (1996) showed that these bacteria could be divided into two subspecies (*S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis*) based on their whole-cell protein profiles and biochemical properties. Recently, Vieira *et al.* (1998) proposed a new classification for the organisms based on multilocus enzyme electrophoresis typing and genomic DNA relatedness. These analyses defined α -haemolytic or non-haemolytic strains of Lancefield group C as *S. dysgalactiae* subsp. *dysgalactiae* and β -haemolytic strains of group C, G or L antigen as *S. dysgalactiae* subsp. *equisimilis*. This is the traditional and

most widely used method of classification to date. Furthermore, genealogical divergences between *S. dysgalactiae* isolates have also been detected by means of multilocus enzyme electrophoresis (Bert *et al.*, 1995), random amplified polymorphic DNA (RAPD) (Bert *et al.*, 1996) and 16S–23S rRNA gene (rDNA) intergenic spacer analyses (Chanter *et al.*, 1997).

The phylogenetic position of the species *S. dysgalactiae* within the genus *Streptococcus* has been estimated by sequencing analyses of 16S rRNA (Bentley *et al.*, 1991; Kawamura *et al.*, 1995a, b). However, subspecific information for sequences of *S. dysgalactiae* strains is limited and, therefore, the details of the phylogenetic relationship among strains of the organism remain unclear.

It is evident that the resolving power of 16S rDNA sequences is limited when closely related organisms are examined (Rogall *et al.*, 1990; Amann *et al.*, 1992; Fox *et al.*, 1992), although analysis based on such sequences is a valuable phylogenetic marker, as is DNA reassociation (Stackebrandt & Goebel, 1994). On the other hand, 23S rRNA is about twice as long as 16S rRNA and contains more variable regions. Therefore, 23S rRNA is considered to be a more

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The GenBank/EMBL/DDBJ accession numbers for the 16S and 23S rDNA sequences determined in this paper are given in Fig. 2.

informative phylogenetic marker (Ludwig & Schleifer, 1999).

S. dysgalactiae subsp. *equisimilis* is classified into four subgroups of host-associated ecovars based on serogrouping and biotyping: human groups C and G, animal group C and group L (Devriese, 1991). Bacteria of the latter two groups are frequently isolated from pigs, in which the organisms cause septicaemia, arthritis or valvular endocarditis (Hommeze *et al.*, 1991). In this study, we used porcine *S. dysgalactiae* subsp. *equisimilis* isolates associated with arthritis, lymphadenitis or valvular endocarditis to examine subspecific relationships among *S. dysgalactiae* strains through a comparison of the nucleotide sequences of their 16S and 23S rDNA.

METHODS

Bacteria. Streptococcal isolates and strains used for 16S and 23S rDNA analyses are shown in Table 1. Eleven porcine isolates of *S. dysgalactiae* subsp. *equisimilis*, whose origins have been described previously (Katsumi *et al.*, 1997), were examined along with strain ATCC 35666 of *S. dysgalactiae* subsp. *equisimilis* and two strains of *S. dysgalactiae* subsp. *dysgalactiae*. In addition, *Streptococcus agalactiae* ATCC 13813^T was used as an outgroup.

Phenotypic characterization. The Streptex agglutination procedure (Murex Diagnostics) was used for detection of the Lancefield group A–D, F and G antigens and the group L antigen was examined by precipitation in capillary tubes with hyperimmune serum (Lancefield, 1938). Streptokinase activity was determined on fibrin-plasma plates as described previously (Vandamme *et al.*, 1996).

DNA–DNA reassociation. DNA preparation and microplate hybridization were performed using the method of Ezaki *et al.* (1989) at an optimal renaturation temperature of 30 °C in 50 % formamide.

Sequence determination of rDNA. Genomic DNA was extracted from bacterial colonies with a DNA extraction kit (SMITEST; Sumitomo Kinzoku Kogyo) according to the manufacturer's instructions. rDNA sequences were determined directly from DNA fragments produced by PCR amplification (Hultman *et al.*, 1989, 1991). Primers and conditions for 16S rDNA PCR were described previously (Takahashi *et al.*, 1997). The 23S rDNA sequence was analysed in the same manner. Furthermore, sequence determination was carried out for DNAs cloned into a pZero-2 vector plasmid (Invitrogen) for precise analysis. Oligonucleotide sequences of PCR primers used for the 23S rDNA are listed in Table 2. These and most of the intergenic sequencing primers were designed on the basis of data from a streptococcal 23S rDNA multiple alignment and sequences from previous investigations (Lane *et al.*, 1991; Ash *et al.*, 1992; Ludwig *et al.*, 1992; Van Camp *et al.*, 1993; Takahashi *et al.*, 1997).

Denatured PCR fragments or recombinant plasmids were submitted to a sequencing reaction using an Auto-Read sequencing kit and an ALF Express II DNA sequencer (Amersham Pharmacia Biotech).

Sequence alignment, calculation of similarity values and phylogenetic tree construction. Nucleotide sequences of the 16S and 23S rDNA determined in this study were assembled and aligned manually using Gene Jockey II version 2.1 (Biosoft). Evolutionary distance values were estimated by Kimura's two-parameter method (Kimura, 1980) using the BioResearch SINCA program package (Fujitsu). The neighbour-joining method of Saitou & Nei (1987) was employed to construct a phylogenetic tree using the BioResearch SINCA program. The topology of the tree was evaluated by a bootstrapping method (Felsenstein, 1985).

Table 1. Streptococci used for sequence analysis of 16S and 23S rDNA

S. dysgalactiae subsp. *equisimilis* strains are identified as arthritis-associated (prefix A), lymphadenitis-associated (L) or valvular endocarditis-associated (V) strains. NT, Not typable.

Strain	Type of haemolysis on sheep blood	Lancefield group	Streptokinase activity on human plasminogen
<i>S. agalactiae</i> ATCC 13813 ^T	β	B	Not tested
<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>			
ATCC 43078 ^T	α	C	NT
ATCC 27957	α	C	NT
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>			
ATCC 35666	β	C	+
A1	β	C	–
A7	β	L	–
A20	β	L	–
A24	β	C	–
L1	β	C	–
L2	β	C	–
L8	β	C	–
L21	β	L	–
L23	β	C	–
V21	β	C	–
V26	β	C	–

Table 2. Primers used for amplification of 23S rDNA

Primer positions are based on the *E. coli* numbering system (Brosius *et al.*, 1981).

PCR product	Primer	Sequence (5'–3')	Position
Fragment 1	Forward	GTACACACCGCCCGT	1392–1406 (16S)
	Reverse	GGGTTYCCCCATTCGG	115–130
Fragment 2	Forward	AAGGGCGCACGGTGGATGCC	13–32
	Reverse	TTCTCCCGAAGTTACGG	1685–1701
Fragment 3	Forward	AAACCGACACAGGTAG	1608–1623
	Reverse	CTTTTATCCGTTGAGCGA	2419–2436
Fragment 4	Forward	AGTTTGACTGGGGCGGT	2241–2257
	Reverse	TTAACTTCCGTGTTCCGGTATGGG	36–58 (5S)

RESULTS AND DISCUSSION

The results of Lancefield grouping and streptokinase activity on human plasminogen of the 11 porcine isolates of *S. dysgalactiae* subsp. *equisimilis* are summarized in Table 1. Each of the isolates reacted with antibodies against the C or L antigen, which are considered to be the group antigens possessed by *S. dysgalactiae* subsp. *equisimilis* of animal origin. On the other hand, an atypical reaction was observed for streptokinase activity. Both Vieira *et al.* (1998) and Vandamme *et al.* (1996) have reported that a positive reaction for streptokinase activity on human plasminogen is a key characteristic for distinguishing subspecies *equisimilis* from subspecies *dysgalactiae*. However, the porcine isolates in this study did not exhibit streptokinase activity. These results were consistent with the results reported by Vandamme *et al.* (1996), in which most animal isolates of *S. dysgalactiae* lacked streptokinase activity, but were

inconsistent with the classification proposed by Vieira *et al.* (1998). Streptokinase activity on human plasminogen may not be useful for identifying porcine isolates at the subspecies level as defined by Vieira *et al.* (1998).

Vieira *et al.* (1998) have reported that the levels of DNA relatedness between the two subspecies are 79% or less. However, the porcine isolates in this study showed high levels of DNA relatedness (85–88%) against both *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* strains (Table 3). In this study, we tentatively designated the isolates as *S. dysgalactiae* subsp. *equisimilis* because of their β -haemolysis, which is a possible key factor for distinguishing one subspecies from the other based on the results reported by Vieira *et al.* (1998).

Alignment data from nucleotide residues 85 to 1500 (based on the *Escherichia coli* numbering system; Brosius *et al.*,

Table 3. DNA relatedness of porcine *S. dysgalactiae* strains against strains of the two subspecies of *S. dysgalactiae*

Source of unlabelled DNA	Relative binding (%) with labelled DNA from:			
	1	2	6	12
1. <i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i> ATCC 43078 ^T	100	84.1	87.0	86.4
2. <i>S. dysgalactiae</i> subsp. <i>equisimilis</i> ATCC 35666	80.2	100	86.6	86.6
3. A1	87.5	84.8	84.1	92.3
4. A7	86.5	84.9	85.5	82.2
5. A20	85.6	86.1	85.4	87.0
6. A24	85.2	87.5	100	81.9
7. L1	84.9	84.7	87.8	81.9
8. L2	86.7	85.0	87.7	86.3
9. L8	85.0	87.0	81.7	86.3
10. L21	86.0	85.7	88.0	83.0
11. L23	88.1	86.5	84.3	91.9
12. V21	84.7	88.0	88.5	100
13. V26	85.3	85.2	87.9	83.4
14. <i>S. agalactiae</i> ATCC 13813 ^T	19.0	22.7	17.6	17.4

Fig. 1. Sequence alignment of residues 181–194 of the 16S rDNA of strains of *S. dysgalactiae* subsp. *dysgalactiae* (ATCC 43078^T and ATCC 27957) and *S. dysgalactiae* subsp. *equisimilis* (A1 to V21). Dots indicates bases identical to the residue of strain ATCC 43078^T. Numbers 1 and 2 on the right correspond to subgroups designated in this study. Boxed sequences may form a helix structure with a minimum of free energy. Numbering of positions follows the convention for *E. coli* (Brosius *et al.*, 1981).

1981) of the 16S rDNA were used for comparative analyses of the streptococcal strains. Of the 1429 bases of the 16S rDNA, nucleotide changes within the species *S. dysgalactiae* were found at 24 residues. Thirteen of the 24 residues were located in variable region V3 (residues 179–220) designated by Raué *et al.* (1990) and the others were scattered in and around the other variable regions.

Fig. 1 shows part of the sequence alignment from residues 181 to 194 of the 16S rDNA. This substantial domain provided a criterion for subdividing strains of *S. dysgalactiae* subsp. *equisimilis* into two subgroups. One consisted of strains V26, L21, A1, A24 and L2, which all have sequences similar to that of *S. dysgalactiae* subsp. *dysgalactiae*. The other group consisted of the rest of the organisms, which were similar to each other but distinct from the other strains. We hypothetically designated the former and the latter as subgroups 1 and 2 (Fig. 1). The predicted secondary structures of rRNA estimated from these sequences were hairpin loop motifs. Although the primary sequences were significantly different at residues 181–194, the hairpin loop motifs were very similar. All the motifs consisted of helices, 10 or 11 bp in length, and a 4-base loop. Furthermore, the numbers of canonical and non-canonical base pairs were nearly identical in each.

In the complete 23S rDNA sequences (2904 bp), 21 positions varied between strains of the species *S. dysgalactiae*. However, no significant differences were apparent except at residues 294 and 652. In strains of *S. dysgalactiae* subsp. *dysgalactiae*, the bases at these positions were A and T, respectively, while, in most strains of *S. dysgalactiae* subsp. *equisimilis*, the bases were G and A.

It is apparent from the results of the treeing analyses based on 16S and 23S rDNA (Fig. 2) that the species *S. dysgalactiae*

was distinct from the type strain of *S. agalactiae* with large evolutionary distance values (divergence 2.43–3.45 and 3.27–3.47 %, respectively). However, the branching order and the length of sublines within the *S. dysgalactiae* clusters were significantly different. *S. dysgalactiae* subsp. *equisimilis* strains were subdivided into two distinct clades in the 16S rDNA tree. The subgroups were recovered in 99 or 74 % of the bootstrapped trees. Furthermore, one of the subgroups (subgroup 1) formed a distinct clade with the strains of *S. dysgalactiae* subsp. *dysgalactiae* (bootstrap value of the group, 99 %). This conflicts with the degree of difference between the two subspecies of *S. dysgalactiae* demonstrated by DNA–DNA reassociation analysis (Vieira *et al.*, 1998). Furthermore, the results based on 16S rDNA were inconsistent with those based on PFGE (Bert *et al.*, 1997) and PAGE profiling of bacterial whole-cell proteins (Vandamme *et al.*, 1996). These studies have shown no distinct groups within porcine isolates of *S. dysgalactiae*, although subtle differences, as in the biochemical properties of the isolates used in this study, were detected in individual strains.

In contrast, the subgroups produced by 16S rDNA treeing analysis were not distinguishable in the 23S rDNA tree. All the sublines of *S. dysgalactiae* strains branched at positions that were much closer to each other in the 23S rDNA tree (divergence, 0.39 % or less) than in the 16S rDNA tree (divergence, 1.51 % or less), although each of the clusters of *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* was monophyletic (bootstrap values 92 and 69 %, respectively). Subspecies-specific nucleotide differences were found at not more than three residues, though these could distinguish the two strains of *S. dysgalactiae* subsp. *dysgalactiae* from the other strains with a high bootstrap value in the treeing analysis. These findings were not inconsistent with the classification shown by Vieira *et al.* (1998).

It has been demonstrated that phylogenetic trees constructed from 16S and 23S rDNA sequence data are generally congruent with each other (Höpfel *et al.*, 1989; Ludwig & Schleifer, 1994; Sallen *et al.*, 1996; Ward *et al.*, 2000). However, in this study, the phylogenetic relationship derived from 16S rDNA analysis was not supported by the result of the 23S rDNA analysis. This indicated that phylogenetic trees derived from 16S and 23S rDNAs are not always in agreement at the intraspecific level and poses the question of which phylogenetic marker reflects the true relatedness. A DNA–DNA reassociation study (Vieira *et al.*, 1998) clearly subdivided *S. dysgalactiae* strains into two subspecies, one of which (β -haemolytic) reacts with Lancefield group C, G or L antigen and is positive for streptokinase activity on human plasminogen. The coincidence of these properties, except for streptokinase activity, in the porcine isolates used in this study suggests that the 23S rDNA analysis has an advantage over the 16S rDNA in the case of these taxa.

The strains used in our study carried either Lancefield C or L antigen. However, these serogroups were unrelated

Fig. 2. Unrooted neighbour-joining trees based on 16S and 23S rDNA sequences of 12 strains of *Streptococcus dysgalactiae* subsp. *dysgalactiae* and two strains of *S. dysgalactiae* subsp. *equisimilis*. Numbers at nodes are estimated confidence levels expressed as percentages and determined by bootstrap analysis with 1000 replications. Bar, evolutionary distance (K_{nuc}) between sequences, determined by measuring the lengths of horizontal lines connecting two organisms.

to the phylogenetic relationships deduced from 16S and 23S rDNA sequences. Homme *et al.* (1991) reported that Lancefield group C and L *S. dysgalactiae* from pigs have almost identical cultural and biochemical traits. Furthermore, it is nearly impossible to distinguish these *S. dysgalactiae* serogroups on the basis of genealogical analyses such as DNA–DNA hybridization (Farrow & Collins, 1984), RAPD (Bert *et al.*, 1996) and PFGE (Bert *et al.*, 1997). These data indicate that distinctions based on Lancefield C or L serogrouping have no practical value for the subgrouping of *S. dysgalactiae* subsp. *equisimilis*.

The present phylogenetic analysis based on 16S rDNA sequences indicates that two groups exist within *S. dysgalactiae* subsp. *equisimilis* of porcine origin and that this divergence does not seem to be related to the phylogenetic relationships of 23S rDNA. Even so, the unique sequence found in the group-2 porcine isolates will be a useful marker for epidemiological studies.

Due to limitations in the number and source of strains examined, genealogical relationships between porcine strains of *S. dysgalactiae* subsp. *equisimilis* and other groups of strains, such as ecovars or group G strains, are still unclear.

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