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Phylogenetic Analysis of *Streptococcus dysgalactiae* Isolates from Pigs Based on the 16S rDNA Sequence

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Introduction

The species of *Streptococcus dysgalactiae* currently includes a variety of strains that are biochemically and genetically very close but differ in serological grouping, type of hemolysis, host association and pathogenicity[3,9,10,13,16,20]. Recently, two subspecies were proposed for the species of *S. dysgalactiae* by Vandamme *et al*[29] based on whole-cell protein analysis and physiological properties. According to the new classification, the habitats of the two subspecies *dysgalactiae* and *equisimilis* are animals and humans, respectively. The robustness of the heterogeneity within the species of *S.dysgalactiae* was supported by the results of chromosomal DNA analysis using pulsed-field gel electrophoresis (PFGE)[3], which showed a distinction between *S.dysgalactiae* isolates of human and animal origin. Such genealogical divergences have also been detected by means of multilocus enzyme electrophoresis[5], random amplified polymorphic DNA (RAPD)[4], and 16S-23S rDNA intergenic spacer analyses[7].

The phylogenetic position of the species of *S. dysgalactiae* in the genus *Streptococcus* was defined by comprehensive sequencing analysis of 16S ribosome RNA (rRNA)[2,8,19]. However, the sequence information of *S.dysgalactiae* is limited, and therefore the details of phylogenetic relationships among subspecific types of the organisms remain unclear.

Devriese[8] classified *S.dysgalactiae* into 5 groups of the strains as host-associated ecovars of the species based on serogrouping and biotyping. Three ecovars associated with animal hosts should be regarded as the types within *S. dysgalactiae* subsp. *dysgalactiae*[29]: the bovine group C ecovar, porcine group C ecovar and the group L ecovar. The main host of the latter two groups is the pig, in which the bacteria cause septicemia, arthritis and valvular endocarditis[26], and are frequently isolated from lesions[13,16,24]. However, there have been few studies on genealogical typing of porcine strains of *S.dysgalactiae*.

Riising[24] found different biotypes among the streptococcal isolates from swines, and we have also encountered biochemical differences among the isolates of *S.dysgalactiae* from slaughtered pigs[17]. Using the porcine *S.dysgalactiae* isolates associated with arthritis, lymphadenitis or valvular endocarditis, we detected subtle differences between *S.dysgalactiae* strains by comparing the nucleotide sequences of their 16S rDNA.

Materials and Methods

Bacteria

Streptococcal strains used for the 16S rDNA analysis are shown in Table 1. Five sequences of *S.acidominimus*, *S.anginosus*, *S.iniae*, *S.mutans*, and *S.salivarius*, which have been reported by Bentley *et al*[2], were retrieved from the GenBank database. Two bovine strains of *S.dysgalactiae* (ATCC 43078T and ATCC 27957) and 10 strains of the other species of the genus *Streptococcus* were used for determination of the 16S rDNA sequence. Eight streptococcal type strains were

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Table 1 Streptococci used for sequence analysis of 16S rDNA

Streptococcal strain		Accession no. of 16S rRNA/rDNA sequence
S.dysgalactiae	ATCC 43078^{T}[a]	AB002485
	ATCC 27957	AB002484
	A1[b]	AB002486
	A5	AB002490
	A6	AB002491
	A7	AB002492
	A20	AB002487
	A24	AB002488
	A25	AB002489
	L1[c]	AB002494
	L2	AB002498
	L4	AB002505
	L5	AB002506
	L7	AB002507
	L8	AB002508
	L9	AB002509
	L13	AB002493
	L21	AB002495
	L23	AB002496
	L27	AB002497
	L31	AB002499
	L32	AB002500
	L33	AB002501
	L34	AB002502
	L35	AB002503
	L36	AB002504
	V21[d]	AB002510
	V24	AB002511
	V26	AB002512
	V36	AB002513
S.acidominimus	NCDO 2025^{T}	X58301[e]
S.agalactiae	ATCC 13813^{T}	AB002479
S.anginosus	NCTC 10713^{T}	X58309[e]
S.bovis	ATCC 33317^{T}	AB002482
S.canis	ATCC 43496^{T}	AB002483
S.equi	ATCC 33398^{T}	AB002515
S.gordonii	MAFF 911479	AB002520
S.hyointestinalis	ATCC 49169^{T}	AB002518
S.iniae	NCDO 2772^{T}	X58316[e]
S.mutans	NCTC 10449^{T}	X58303[e]
S.porcinus	ATCC 43138^{T}	AB002523
S.pyogenes	MAFF 910217^{T}	AB002521
S.salivarius	NCDO 1779^{T}	X58320[e]
S.suis	ATCC 43765^{T}	AB002525
S.uberis	ATCC 19436^{T}	AB002526

[a]T, type strain.
[b]A, strain associated with arthritis.
[c]L, strain associated with lymphadenitis.
[d]V, strain associated with valvular endocarditis.
[e]Nucleotide sequence was retrieved from GenBank.

selected for the sequence analysis as members of the pyogenic group classified by Kawamura *et al*[18]: *S.agalactiae*, *S.canis*, *S.equi*, *S.hyointestinalis*, *S.iniae*, *S.porcinus*, *S.pyogenes*, and *S.uberis*. The type strain of a species in each of the other groups, *S.anginosus*, *S.bovis*, *S.mitis*, *S.mutans*, *S. salivarius*, *S.acidominimus* and *S.suis*, was also selected as a representative of each group except for a strain of *S.gordonii* instead of *S.mitis*.

Twenty-eight porcine isolates of *S.dysgalactiae*

were also used for sequence analysis and for biochemical examinations. These bacteria isolated from lesions of arthritis, lymphadenitis and valvular endocarditis were designated as strains A, L and V (followed by numerals), respectively, as shown in Table 1. The isolation of the bacteria has been described previously[17].

The bacteria were grown aerobically on a brain-heart-infusion agar (BBL, Becton Dickinson and Company, Cockeysville, MD) plate for 24 hours at 37℃, and the pure culture was used for the examinations.

Lancefield serogrouping and biochemical tests were performed for the 28 porcine isolates and the type strain of *S.dysgalactiae*. The Streptex agglutination procedure (Murex Diagnostics Ltd., Dartford, U.K.) was used for the detection of the Lancefield group antigens of A, B, C, D, F and G, and the group L antigen was examined by the precipitation in capillary tubes with hyperimmune serum[22]. Biochemical characteristics were examined using an API 20 STREP (BioMérieux, Marcy-l'Étoile, France).

Sequence determination of 16S rDNA

The purified culture was used for the extraction of bacterial genomic DNA with a DNA extraction kit (SMITEST; Sumitomo Kinzoku Kogyo Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. To amplify the 16S rDNA, polymerase chain reaction (PCR) was performed with a pair of generic primers for Gram-positive bacteria[28] and with Pfu DNA polymerase (Stratagene, La Jolla, CA). During the PCR amplification, Biotin was introduced into one of the strands of the PCR product, using one of the primers biotinylated in the 5' end. The PCR products of the DNA were then converted into a single-stranded template by immobilization onto ferrous beads (Dynabeads; Dynal A.S., Oslo, Norway) with coupled streptavidin on the surface and denaturation with NaOH, as described by Hultman *et al*[14,15]. The immobilized template was used for Sanger dideoxy DNA sequencing, as modified by Zimmermann *et al*[32], with a panel of oligonucleotide primers designed for Gram-positive bacteria[28]. The 5' termini of the sequence primers were labeled with fluorescein isothiocyanate, and the procedures for polyacrylamide gel electrophoresis and for the determination of DNA sequences were performed according to the manufacturer's instructions for the A.L.F. DNA Sequencer II (Pharmacia Biotech, Upsala, Sweden).

Sequence alignment, calculation of similarity values, and phylogenetic tree construction

The nucleotide sequences of the 16S rDNA determined in this study were aligned manually along with the sequences retrieved from the DNA database (Table 1). Evolutionary distance values were estimated by Kimura's two-parameter method[21] with the BioResearch SINCA program package (Fujitsu, Japan). Percent similarity between individual sequences (A and B) was calculated with the Lasergene Megalign program package (DNASTAR Inc., Madison, Wis.) as follows: similarity (A, B) = 100X sum of the matches/[length − gap residues(A) − gap residues(B)]. The neighbor-joining method of Saitou and Nei[25] was employed to construct a phylogenetic tree with the BioResearch SINCA program. The topology of the tree was evaluated by a bootstrapping method[11].

Secondary structure estimates of 16S rRNA

We constructed an estimated secondary structure of the V3 region of the 16S rRNA by replacement with each nucleotide corresponding to one marked on a model map of the molecule made by Weisburg *et al*[30], the construction of which was based on 20 representative Gram-positive 16S rRNA sequences.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession numbers listed in Table 1.

Results

Nucleotide sequence of 16S rDNAs of *Streptococcus dysgalactiae*

In this study, nucleotide sequences were determined for the 16S rDNAs of 30 strains of *S. dysgalactiae*, including 2 bovine strains and 28 porcine isolates, and for 10 strains of the other species of the genus *Streptococcus* (Table 1).

They were analyzed along with the sequences appearing in nucleotide databases[2]. Alignment was performed from 25 to 1,492 (the numbering system is based on that of *Escherichia coli*[6]). Unknown regions of some sequences that were determined to be shorter were eliminated for comparative analysis. In the sequences of 1,475 base pairs from *S.dysgalactiae* strains, nucleotide differences were found at 23 residues. Thirteen of the divergences were detected within the variable region V3, whose residues are 179 to 220[23], while the other ten residues were scattered in other variable and conserved regions (data not shown).

Some of the 16S rRNA V3 sequences in *S. dysgalactiae* strains are shown in Fig. 1. In this figure, the secondary structures of the region are also shown for two types of *S.dysgalactiae* sequences, designated as groups I and II, although the former sequences have 2 ambiguous residues. Group I consists of 2 bovine strains (ATCC 43078T and ATCC 27957) and 16 porcine isolates (A1, A5, A6, A24, A25, L2, L4, L5, L7, L9, L13, L21, L32, L34, V26 and V36), while group II consists of 12 porcine isolates (A7, A20, L1, L8, L23, L27, L31, L33, L35, L36, V21 and V24). The difference between the sequence stretches of the two groups are reflected in the predicted hairpin-loop structures. The length of the stem region in group I is shorter (10 base pairs) than that in group II (11 base pairs and a bulge structure).

Similarity values of the 16S rDNA in *Streptococcus dysgalactiae* strains

Table 2 shows percent similarities and evolutionary distance values for combinations of the 16S rDNA sequences in *Streptococcus dysgalactiae* strains and the type strain of *S.agalactiae*. The combinations of strains within the species of *S. dysgalactiae* exhibited a level of sequence similarity of 98.4% or more and 1.4 or fewer substitutions per 100 nucleotides. In contrary, *S. dysgalactiae* strains and *S.agalactiae* were distinct,

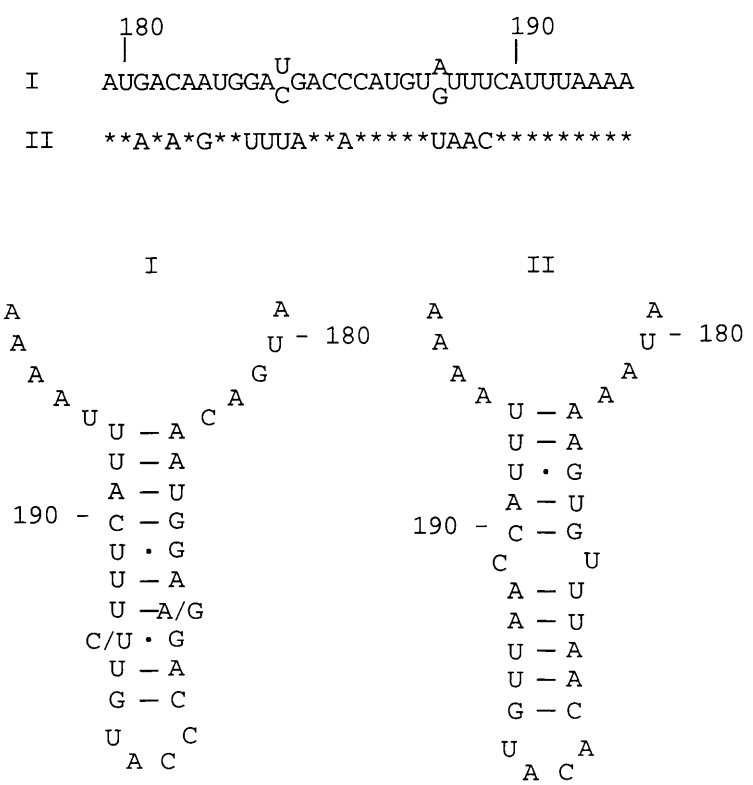

Fig. 1 Primary and predicted secondary structures of the V3 regions for two types of *Streptococcus dysgalactiae* 16S rRNA. The numbering positions of strain groups I and II followed the *Escherichia coli* convention. Base pairs in the secondary structural models are indicated by connecting lines for G-C or A-U, and by dots for G·U.

Table 2 Percent similarity and evolutionary distance for the 16S rDNA sequences of *Streptococcus dysgalactiae* and *S.agalactiae* strains[a]

	ATCC43496[T]	ATCC27957	V26	A5	A1	A24	A7	A20	L1	V21	ATCC13813[T]
ATCC 43496[Tb]		99.8	99.6	99.5	99.4	99.2	98.6	98.6	98.5	98.4	96.6
ATCC 27957	0.2		99.6	99.6	99.5	99.3	98.7	98.6	98.6	98.5	96.6
V26 (1)[c]	0.4	0.4		99.9	99.9	99.6	98.9	99.0	98.9	98.9	97.0
A5 (3)	0.5	0.4	0.1		99.9	99.7	98.9	99.0	98.9	98.9	97.0
A1 (8)	0.6	0.5	0.1	0.1		99.8	99.0	99.1	99.0	98.9	97.1
A24	0.8	0.7	0.4	0.3	0.2		99.8	98.9	98.8	98.7	96.9
A7 (2)	1.2	1.1	0.9	0.9	0.9	1.1		99.9	99.9	99.8	97.7
A20 (5)	1.3	1.2	0.9	0.9	0.8	1.0	0.1		99.9	99.9	97.8
L1 (1)	1.4	1.3	0.9	0.9	0.9	1.1	0.1	0.1		99.8	97.7
V21	1.4	1.4	1.0	1.0	0.9	1.1	0.2	0.1	0.2		97.6
ATCC 13813[Td]	3.1	3.0	2.6	2.6	2.6	2.8	2.2	2.1	2.2	2.3	

[a] Boxes enclose values within a strain group. Solid-lined boxes are for strain groups I and II having a specific sequence in the V3 region (Fig.1). Dotted-lined boxes are for subgroups of bovine and porcine strains within strain group I. Percent similarity is shown in the upper right of the chart. Evolutionary distance, expressed as percent diversity, is shown in the lower left half.

[b] T, type strain

[c] The number in parenthesis is the number of streptococcal strains that have identical 16S rDNA sequences. Identical strains are as follows: with V26, V36; with A5, A6, A25, and L21; with A1, L2, L4, L5, L7, L9, L13, L32, and L34; with A7, L23 and L31; with A20, V24, L27, L33, L35, and L36; with L1, L8.

[d] *S.agalactiae* type strain.

their similarity values were ranged from 96.6 to 97.8%. The two types of sequences shown in Fig. 1 also appeared as groups with higher similarities (and fewer substitutions) in Table 2. The percent similarities between strains of group I represented by ATCC 43078T, ATCC 27957, V26, A5, A1 and A24 were 99.2% or more within the group but showed similarities of 99.1% or less with other sequences. Such a sequence group was clearer in the strains represented by A7, A20, L1 and V21, whose percent similarities were 99.8% or more. The two bovine strains of *S. dysgalactiae*, regarded as members of strain group I, were more closely related with each other (similarity value of 99.8%) than with porcine isolates of the group.

Phylogenies of streptococcal 16S rDNAs

To construct a phylogenetic tree, evolutionary distance values were calculated for the region between nucleotide residues 107 to 1,430. The topology of the phylogenetic tree (Fig. 2) exhibited a substantial intrageneric heterogeneity in the genus *Streptococcus*. Phylogenetic positions for the strains of *S.dysgalactiae* were specified in the phylogenetic tree. They were located within a cluster of pyogenic streptococci and formed a monophyletic clade with the type strain of *S. agalactiae* (bootstrap value for the clade, 84%). The strains of *S.dysgalactiae* exhibited a specific association that was recovered in 99% of the bootstrapped trees. Two significant (99% bootstrap value for each) clusters were formed by the strain groups divided by the V3 sequence stretches described above (Fig. 1). The close relationships of the porcine isolates in each strain group detected in the similarity table (Table 2) were also confirmed by treeing analyses. The type strain of *S.dysgalactiae* and another bovine strain, which were nearly identical to each other, appeared to form a relatively short subline within group I strains. The bootstrap value for this subgroup was 100%.

Lancefield serogrouping and API 20 STREP profiles of *Streptococcus dysgalactiae*

The results of Lancefield grouping of 28 porcine isolates of *S.dysgalactiae* are summarized for each strain group in Table 3. Twenty-seven isolates reacted with antibodies against C or L antigen, which was considered as the possible group antigen possessed by *S.dysgalactiae* of ani-

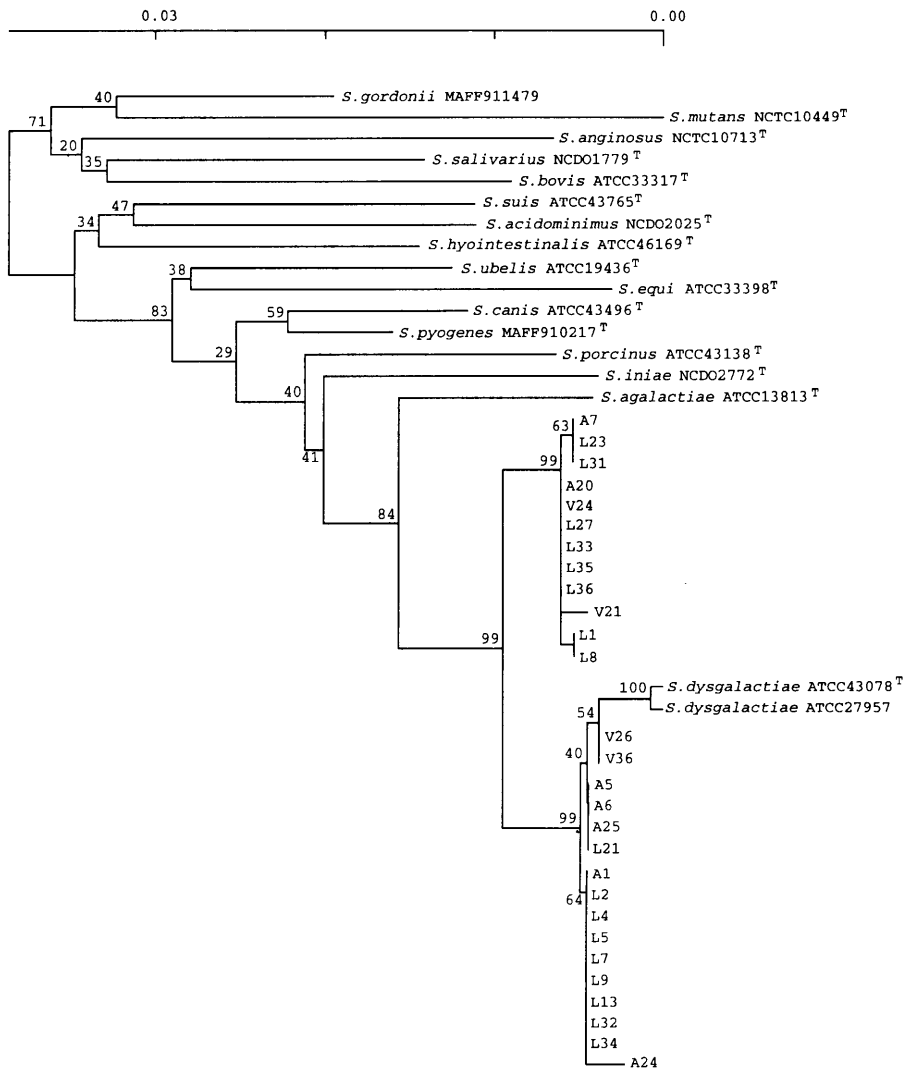

Fig. 2 Unrooted neighbor-joining tree based on 16S rDNA sequences in the 28 porcine isolates of *Streptococcus dysgalactiae* and 16 species of the genus *Streptococcus*. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, determined by bootstrap analysis[11] with 1,000 replications. The scale bar indicates the evolutionary distance value (*K*nuc) between sequences, determined by measuring the lengths of the horizontal lines connecting two organisms.

mal origin. Fourteen of 16 strains in strain group I and 7 of 12 strains in strain group II were group C, which was also detected in the type strain of the species. Group L antigens were detected in 2 and 4 strains in the strain groups I and II, respectively.

Table 3 Number of strains reacting with Lancefield antigens between *S.dysgalactiae* isolates of strain groups I and II

Strain group	No. of strains	Lancefield group		
		C	L	Untypeable
I	16	14	2	0
II	12	7	4	1

The 28 porcine isolates and the type strain of *S. dysgalactiae* were examined for their biochemical properties using the API 20 STREP system. These strains were either all positive (+) or negative (−) for β-D-glucuronidase (+), alkaline phosphatase (+), L-leucine aminopeptidase (+), arginine dehydrolase (+), α-D-galactosidase (−), β-D-galactosidase (−), ribose (−), trehalose (−), starch (−), and mannitol fermentation (−). The results of ten other tests using the API 20 STREP

system are summarized for each strain group in Table 4. Atypical reaction was observed positively in one to 3 strains of strain group I for each of the following 6 items: Voges-Proskauer (VP) reaction, hippurate hydrolysis, L-pyrrolidone aminopeptidase, arabinose, inuline, and raffinose fermentation. In strain group II, two strains each reacted atypically for VP and L-pyrrolidone aminopeptidase, respectively.

Discussion

Our treeing analyses based on the 16S rDNA sequences divided porcine isolates of *Streptococcus dysgalactiae* subsp. *dysgalactiae (S.dysgalactiae)* into two distinct groups. Specific associations were shown by the bootstrap analyses within the strain groups. Furthermore, the results of multiple sequence alignment of *S.dysgalactiae* exhibited substantial domains where the group specific stretches were detected. Nucleotide changes between two strain groups of *S.dysgalactiae* were found concentrically in the domain V3 and probably reflect secondary structures of the rRNA molecule. This indicates that the 16S rDNA is a suitable marker for the classification of *S. dysgalactiae* to the subspecific level.

In proposals of subspecific division in the species of *S.dysgalactiae*, Vandamme *et al* described the distinct taxonomic status between the strains of animal and human origin based on whole-cell protein analysis[29]. Similar divergence was also shown by Bert *et al*[3] in PFGE analysis using genomic DNA. In their study, *S. dysgalactiae* isolates from cows, pigs and foxes were further subdivided into 3 groups: one was composed only of bovine strains, but another included isolates from pigs and cows, and a third subgroup consisted of isolates from foxes[3]. Distinction between two strain groups of porcine isolates detected in the present study suggests that four or more groups of genealogically different strains may exist within the taxon of *S. dysgalactiae* subsp. *dysgalactiae*.

Table 4 Comparison of biochiemical characteristics between *Streptococcus dysgalactiae* isolates of strain groups I and II

Biochemical characteristics[a]	ATCC 43078[T]	Strain group	
		I	II
Voges-Proskauer reaction	−	3/16[b]	2/12
Hippurate hydrolysis	−	2/16	0/12
Esculin hydrolysis	−	6/16	2/12
L-Pyrrolidone aminopeptidase	−	3/16	2/12
Acid porduction from			
arabinose	−	1/16	0/12
sorbitol	+	8/16	3/12
lactose	+	11/16	6/12
inuline	−	1/16	0/12
raffinose	−	1/16	0/12
glycogen	−	8/16	10/12

[a]Items of the API 20 STREP system.
[b]Number of positives/number tested.
T, type strain.

The two bovine strains of *S.dysgalactiae* used in our study appeared to have specific relatedness (100% bootstrap value). Although these strains were much less distant from one porcine group than from another, and the number of strains examined was too small to draw conclusions from, this is similar to the distinction of a bovine ecovar of *S.dysgalactiae* classified by Devriese[8]. On the other hand, other ecovars of porcine origin were different from our strain groups in their Lancefield group antigens. The porcine ecovars show distinction in carrying either C or L antigens, while two genealogically separated groups contain both group C and L streptococci. Hommez *et al*[13] reported that group C and L *S. dysgalactiae* from pigs have almost identical cultural and biochemical traits. Furthermore, it is nearly impossible to distinguish these serogroups of *S.dysgalactiae* by means of genealogical analysis such as DNA-DNA hybridization[10], RAPD[4] and PFGE[3]. Therefore, the distinctions based on the serogrouping may have no practical value for subgrouping of *S.dysgalactiae* subsp. *dysgalactiae*. Further examination of the relationships between the 16S rDNA sequence and serogroups is necessary.

Six porcine isolates of strain group I were unable to be identified biophysiologically as *S. dysgalactiae* due to their atypical reactions in VP, hippurate hydrolysis, arabinose, inulin and/or raffinose fermentation. This was also the case in two isolates of group II, reacting positively in VP. Although other atypically reacted items of L-pyrrolidone aminopeptidase did not influence identification by the API 20 STREP system, these

characteristics may be strain-dependent in *S. dysgalactiae*. These results and the results of successful identification of *S.dysgalactiae* by the 16S rDNA sequence indicate that genealogical identification methods confer a considerable advantage on reliability.

Results from subspecific analysis in *S. dysgalactiae* based on the whole genome using RAPD[4] and PFGE[3] suggest that theese analyses can detect subtle differences among a variety of *S.dysgalactiae* strains, and these techniques also have the advantage of being easy to perform. Sequence determination of more than 1,000 nucleotides has become rapid and inexpensive[12,27]. Particularly, the integration of 16S rDNA sequence data has enabled taxonomic assignment of any bacterial organism by the standard technique. Although analysis based on the 16S rDNA sequence is a valuable phylogenetic marker[27], the resolution power of the sequence is limited when closely related organisms are being examined[1,12]. However, as described in Results, the difference in primary and secondary structures found in the V3 region discriminated the organisms into two types. Considering the conserved coordination of the secondary structures of 16S rRNA in other Gram-positive bacteria[30,31], the divergence within a single species is notable. Chanter *et al*[7] showed that the 16S-23S rRNA intergenic spacer regions of *S.dysgalactiae* were highly variable. This result and the results in the present study suggest that nucleotide sequencing for a short DNA fragment is a valuable approach for identification and typing *S.dysgalactiae* strains. Detecting a specific sequence stretch in order to identify this organisms is important because of the possibility of identification of the organisms directly from clinical specimens without isolation procedures.

The present phylogenetic analysis based on 16S rDNA sequences indicates that two groups exist within *S.dysgalactiae* subsp. *dysgalactiae* of porcine origin. These groups are able to be differentiated by their group-specific sequence stretches in the V3 region. In addition, two bovine strains, including the type strain, were also distinct. Due to limitations in the number and the source of the strains examined, however, it is necessary to complete the 16S rDNA sequence database for all of the typical strains of the undetermined taxa of *S.dysgalactiae* subsp. *dysgalactiae* and subsp. *equisimilis*. This will clarify the probable correspondence between 16S rDNA typing and other valuable classifications such as ecovars[8] and PFGE typing[3].

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要　約

多様な病態を示す豚から分離されたβ溶血性*Streptococcus dysgalactiae*の遺伝的相違を検出するために，16Sリボソーム RNA 遺伝子配列を決定し，近縁菌と比較系統解析を行った。*S.dysgalactiae* 株はいずれも近縁な関係にあることが示された。しかし*S.dysgalactiae* 分離株は99%の確率で二つの系統に分かれることが判明した。標準株である牛由来株に近縁なグループとその他の菌株のグループの間の塩基変化は21カ所で認められ，その多くは塩基変化が高頻度に認められる事で知られている V3 領域に集中していた。これらの系統と血清型や生化学性状との間の対応関係はみとめられなかったが，このような塩基変化の配列領域は本菌の分子疫学を研究する上で有用なマーカーとなり得ることが示唆された。

Summary

The nucleotide sequences of 16S ribosomal DNA (rDNA) were determined for 28 isolates of β-hemolytic *Streptococcus dysgalactiae* from slaughtered pigs with endocarditis, arthritis or lymphadenitis. The sequences were compared phylogenetically with the gene sequences of two bovine strains of *S.dysgalactiae* and the other species of the genus *Streptococcus*. In the neighbor-joining tree, *S.dysgalactiae* strains were more closely related to each other than to the other *Streptococcus* species (similarity values, 98.4% or more; bootstrap value, 99%). The strains of *S.dysgalactiae* were divided into two monophyletic groups. Two bovine strains including the type strain of the species and 16 porcine isolates formed a distinct clade recovered in 99% of the bootstrapped tree. The other strains of 12 porcine isolates also exhibited a specific association (level of sequence similarity, 99.8% or more; bootstrap value, 99%). These cluster groups of *S. dysgalactiae* strains, designated as groups I and II, respectively, were differentiated by 21 nucleotide changes, and most of them were concentrated in the V3 region of the 16S rRNA molecule. Although Lancefield groups or biochemical characteristics examined were not associated with the two genealogical groups, the group-specific nucleotide stretches can be a reliable tool for molecular epidemiological study of the organisms.