***Streptococcus pluranimalium* sp. nov., from cattle and other animals**

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Strains from subclinical mastitis, from the genital tract and tonsils of cattle, from tonsils of a goat and a cat and from the crop and the respiratory tract of canaries were found to constitute a new streptococcal species, for which the name *Streptococcus pluranimalium* sp. nov. is proposed. Sequencing of 16S rRNA showed that *Streptococcus thoraltensis* and *Streptococcus hyovaginalis* were its closest known phylogenetic relatives. The new species showed some phenotypic resemblance to the poorly described species *Streptococcus acidominimus*, but whole-cell protein analysis and 16S rRNA sequencing revealed that the new species was only distantly related to the type strain of *S. acidominimus*. Identification of these bacteria, which showed heterogeneous biochemical reaction patterns, was most reliably made by whole-cell protein analysis. Nevertheless, a number of biochemical reactions can be used to differentiate *S. pluranimalium* from other animal streptococci. Strain LMG 14177^T, isolated from mastitic milk of a dairy cow, was designated as the type strain of *S. pluranimalium* sp. nov.

Keywords: *Streptococcus pluranimalium* sp. nov., mastitis, genital tract flora, domestic animals

INTRODUCTION

During an investigation of unidentified aesculin-hydrolysing mastitis streptococci, a number of aesculin-hydrolysing isolates were found to share many characteristics with certain strains from the genital tract and tonsils of cows, as well as with strains from very diverse animal hosts. Streptococci from bovine mastitis reacting positively in aesculin-containing isolation media are traditionally grouped with *Streptococcus uberis* in routine diagnostic bacteriology. Upon closer inspection, however, these bacteria are frequently found to be very different from this major streptococcal pathogen, especially when they originate from subclinical mastitis (Bentley *et al.*, 1993; McDonald & McDonald, 1976). In this paper, we report the results of a polyphasic study of a group of unknown aesculin-hydrolysing mastitis strains and of similar strains from other body sites as well as from

other animal hosts. On the basis of the results of this study, a new species *Streptococcus pluranimalium* sp. nov. is described.

Many of the phenotypic characteristics of the strains investigated were found to correspond to those of the poorly described species *Streptococcus acidominimus*, which has been reported to occur in the bovine vagina and udder (Ayers & Mudge, 1922; Hardie, 1986). Particular attention was given, therefore, to the differentiation of the new species from *S. acidominimus*, as well as from other phenotypically similar or phylogenetically related streptococcal species from animals.

METHODS

Strains. Twenty-three strains (Table 1) were investigated and compared with reference strains of known species. The strains had been isolated from the genital tract (cervix, vaginal mucus) and from subclinical mastitis in cows, from lungs and from the crop of canaries and from the tonsils of cattle, a cat and a goat. One strain (LMG 14257) was isolated from the cervix of a cow in Germany. LMG 14256, another bovine vaginal strain, originated from France. All others were from animals of different farms or owners in Belgium.

Abbreviations: AMC, 7-amido-4-methyl-coumarin; MU, methylumbelliferyl.

The GenBank accession number for the 16S rRNA sequence of *Streptococcus pluranimalium* sp. nov. strain LMG 14177^T is Y18026.

Table 1. Strains of *Streptococcus pluranimalium* investigated in this study

Abbreviations used are LMG (BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium), CCUG (Culture Collection of Göteborg, Department of Clinical Microbiology, Göteborg, Sweden), L.A.D. (L. A. Devriese) and J.H. (J. Hommez).

Strain number		Depositor	Isolation
LMG	As received		
13589	Ton444	L.A.D.	Goat, tonsil
13592	Ton200	L.A.D.	Cat, tonsil
13612	Ton427	L.A.D.	Calf, tonsil
13613	Ton263	L.A.D.	Calf, tonsil
13614	Ton213	L.A.D.	Calf, tonsil
13615	STR820	J.H.	Cow, mastitis
13623	Ton512	L.A.D.	Calf, tonsil
13632	Ton403	L.A.D.	Calf, tonsil
13649	K24	L.A.D.	Canary, crop
14099	D153	L.A.D.	Cow, vagina
14177 ^T	T70 ^T	L.A.D.	Cow, mastitis
14256	CCUG 11675	CCUG	Cow, vagina
14257	CCUG 28589	CCUG	Cow, cervix
14385	ESC101	J.H.	Cow, mastitis
14482	D54	J.H.	Cow, vagina
14483	D53	J.H.	Cow, vagina
14827	D244	J.H.	Cow, vagina
14829	D38	J.H.	Cow, vagina
14831	94/241	L.A.D.	Canary, lung
15722	POK3	L.A.D.	Canary, pox lesion
16621	T1368	J.H.	Cow, mastitis
16622	T1387	J.H.	Cow, mastitis
16653	M95/2121	L.A.D.	Canary, lung

SDS-PAGE of whole-cell protein. Preparation of cellular protein extracts, PAGE, densitometric analysis, normalization of the protein profiles and numerical analysis were performed as described previously (Pot *et al.*, 1994; Vandamme *et al.*, 1998) by using the GELCOMP 4.0 software package (Applied Maths). The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient converted, for convenience, to a percentage similarity value.

Determination of the DNA G+C content. The mean DNA G+C content of strain LMG 14177^T was determined as described by Mesbah *et al.* (1989). DNA was enzymically degraded into nucleosides and the nucleoside mixture obtained was then separated by HPLC using a Waters SymmetryShield C8 column thermostatted at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene (corresponding to positions 30–1521 of the *Escherichia coli* 16S rRNA gene) was amplified by PCR and sequenced directly by using a Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The

sequences of the closest relatives of the unknown strains were retrieved from the GenBank or Ribosomal Database Project (RDP) databases and aligned with the newly determined sequences by using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and a phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of grouping was assessed by bootstrap analysis (200 replicates) (Felsenstein, 1989). In addition, parsimony analysis was performed (Felsenstein, 1989).

Growth characteristics and biochemical activity. These were studied as described previously (Devriese *et al.*, 1997). Additionally, biochemical activity was tested in the BBL CRYSTAL Gram-positive kit (Becton Dickinson).

RESULTS

SDS-PAGE of whole-cell protein

A numerical analysis of the whole-cell protein patterns of all of the *S. pluranimalium* and *S. acidominimus* strains, together with reference strains of *Streptococcus thoraltensis* and *Streptococcus hyovaginalis* obtained from a previous study (Devriese *et al.*, 1997), is shown in Fig. 1. Three clusters of strains are clearly distinguished. The *S. acidominimus* type strain, NCFB 2025^T, occupies a distinct position. One cluster consists of all *S. pluranimalium* strains, grouping above a

Fig. 1. Dendrogram derived from the unweighted pair group mean linkage of correlation coefficients (expressed as percentages) between whole-cell protein patterns of *S. pluranimalium* strains, the *S. acidominimus* type strain and strains belonging to the phylogenetically related species *S. thoraltensis* and *S. hyovaginalis*.

Fig. 2. Unrooted tree constructed by the neighbour-joining method based on 16S rRNA sequences showing the phylogenetic position of *Streptococcus pluranimalium* within the genus *Streptococcus*. *, Sequence from IFR database. Bar represents 1% sequence divergence. Only bootstrap values of 90% or more are shown, except for the value of 65% for the species *S. pluranimalium* and *S. hyovaginalis*.

correlation level of 85%. The remaining clusters are formed by the *S. thoralensis* and *S. hyovaginalis* strains, grouping above correlation levels of 91 and 87%, respectively.

Determination of the DNA G + C content

The mean DNA G + C content of strain LMG 14177^T was 38.5 mol %.

16S rRNA gene sequence analysis

The 16S rRNA genes of two strains (LMG 14257 and LMG 14177^T) were amplified by PCR and sequenced directly. Almost complete sequences (>1400 bases) were determined for each strain and pairwise analysis revealed 99.9% sequence identity (corresponding to one nucleotide difference). Sequence searches of the GenBank and RDP databases showed that the unknown organisms were most closely related to *S. hyovaginalis* (3% sequence divergence) and *S. thoralensis* (3.5% divergence). A tree, constructed by using the neighbour-joining method, depicting the position of the bacterium (as exemplified by LMG 14177^T) within the genus *Streptococcus* is shown in Fig. 2. The treeing analysis confirmed *S. hyovaginalis* as the closest relative of LMG 14177^T, followed by *S. thoralensis*. All other streptococcal species were distantly related. Parsimony analysis confirmed the results of the neighbour-joining analysis.

Table 2. Variable characteristics of *Streptococcus pluranimalium* strains

Characteristic	No. positive/ no. tested
Growth in 6.5% NaCl broth (2 d)	11/23
Aesculin hydrolysis on Edward's agar	21/23
Brown discoloration on Edward's agar	16/23
Aesculin hydrolysis in API 20 STREP	12/23
Activity of:	
Pyrrolidonyl arylamidase	7/23
α -Galactosidase	10/23
β -Galactosidase	3/23
β -Glucuronidase	9/23
β -Glucosidase	15/23
Alkaline phosphatase	20/23
Arginine dihydrolase	4/23
N-Acetyl β -glucosaminidase	6/23
Hydrolysis of:	
4-MU β -glucoside	7/13
L-Valine AMC	11/13
4-MU α -D-glucoside	12/13
L-Pyrogutamic acid AMC	3/13
L-Arginine AMC	12/13
4-MU N-acetyl- β -D-glucosaminide	7/13
4-MU β -D-glucuronide	8/13
L-Isoleucine AMC	11/13
p-Nitrophenyl β -D-glucoside	8/13
p-Nitrophenyl β -D-cellobioside	8/13
Proline and leucine p-nitroanilide	11/13
Production of acid from:	
N-Acetylglucosamine	17/23
Amygdalin	10/23
Arbutin	11/23
Cellobiose	13/23
Galactose	15/23
β -Gentiobiose	12/23
Lactose	15/23
D-Mannose	17/23
Mannitol	12/23
Maltose	18/23
Melezitose	10/23
Melibiose	6/23
Methyl β -glucopyranoside	13/23
Raffinose	11/23
Ribose	11/23
Saccharose	18/23
Salicin	13/23
Sorbitol	9/23
D-Tagatose	4/23

AMC, 7-amido-4-methyl-coumarin; MU, methylumbelliferyl.

Growth characteristics and resistance

All strains were Gram-positive cocci and formed chains, pairs or small groups. The chain lengths were very variable. The cells were relatively small, definitely smaller than those of the well-known β -haemolytic

Table 3. Characteristics that differ between bovine strains of *Streptococcus pluranimalium* from the genital tract and from other sources

No. positive/no. tested is shown for each characteristic.

Characteristic	Genital tract strains	Other strains
Growth in 6.5% NaCl broth	0/7	8/10
Browning on Edward's medium	2/7	9/10
Activity of:		
β -Glucuronidase	6/7	1/10
β -Glucosidase	1/7	8/10
Production of acid from:		
Amygdalin	0/7	10/10
Arbutin	0/7	10/10
Cellobiose	0/7	10/10
β -Gentiobiose	0/7	10/10
Mannitol	1/7	9/10
Melezitose	0/7	8/10
Raffinose	0/7	10/10
Ribose	0/7	8/10
Sorbitol	0/7	8/10

pyogenic cocci. Most strains produced precipitates with clear supernatants in Brain Heart Infusion broth after 1 d incubation. Growth at 42 °C was similar to that at 37 °C. The strains did not grow or grew only faintly after 1 d at 25 °C. Slightly enhanced growth was noted with certain strains when plates were incubated in 5% CO₂ at 37 °C. On Columbia sheep blood agar, colonies were not pigmented and were

regular, transparent and small, less than 1 mm in diameter. All strains were α -haemolytic. Colonies on blood agar were usually surrounded by very pronounced greening zones with sharply demarcated edges. The strains were not motile.

Growth in 6.5% NaCl broth was variable (Table 2). All strains grew on Edward's medium and all but two lysed aesculin on this medium. All but five bovine genital tract strains and two strains from other sources produced colonies with strong browning or blackening effects on the medium surrounding the bacterial growth. They grew to very small colonies on kanamycin aesculin azide agar but did not produce blackening in this medium. Growth on Slanetz and Bartley medium was poor or absent.

The *S. acidominimus* type strain NCFB 2025^T showed similar growth and resistance characteristics. It was moderately capnophilic and did not grow on blood agar at 42 °C.

Biochemical activity

All strains reacted positively in the following tests: hippurate hydrolysis, activity of leucine arylamidase and alanine-phenylalanine-proline arylamidase, hydrolysis of 4-methylumbelliferyl (MU)-phosphate, L-phenylalanine 7-amido-4-methyl-coumarin (AMC) and L-tryptophan AMC, acid production from glucose, fructose and trehalose. Carbohydrate reactions were very weak and slow to develop in certain strains. All were negative in the amylase plate test, in Voges-Proskauer tests, in tests for catalase and β -mannosidase activity, hydrolysis of *p*-nitrophenyl α -D-maltoside, urease activity and acid production from

Table 4. Characteristics differentiating *Streptococcus pluranimalium* from phylogenetically related and phenotypically similar species

v, Variable reaction: v+, most strains (>75%) positive; v-, most strains (>75%) negative. D, Reaction depends on strain origin (see Table 3).

Characteristic	<i>S. pluranimalium</i>	<i>S. thoraltensis</i>	<i>S. hyovaginalis</i>	<i>S. suis</i>	<i>S. acidominimus</i>
Strong greening on blood agar	+	—	—	—	+
Voges-Proskauer reaction	—	+	+	—	—
Hippurate hydrolysis	+	v	+	v—	—
Activity of:					
β -Glucuronidase	D	+	—	v+	+
Alkaline phosphatase	v+	v	+	v—	—
Production of acid from:					
L-Arabinose	—	+	—	—	—
Glycogen	—	—	—	+	—
Inulin	—	+	—	v+	Delayed +
Mannitol	D	+	—	v—	Delayed +
Ribose	D	+	v	—	—
Trehalose	+	+	+	+	—
Sorbitol	D	+	+	—	—
Starch	—	+	—	+	—

adonitol, starch, D- and L-arabitol, D- and L-arabinose, D-cyclodextrin, 2- and 5-ketogluconate, dulcitol, erythritol, D- and L-fucose, methyl α -D-glucoside, gluconate, glycogen, inositol, inulin, D-lyxose, methyl α -D-mannoside, pullulan, rhamnose, L-sorbose, D-turanose, D- and L-xylose, xylitol and methyl β -xyloside. Variable reactions are listed in Table 2. Among the bovine strains, a number of characteristics differed according to body location. The strains from vagina and cervix were biochemically less reactive than strains from subclinical mastitis and from tonsils. Only the β -glucuronidase reaction showed a clearly opposite tendency. These differing reactions are tabulated here (Table 3) because they facilitate biochemical identification.

Characteristics of the *S. acidominimus* type strain NCFB 2025^T differing from the strains included in the present study are listed in Table 4. In addition, it was noted that this strain did not react in tests for acid production from salicin and cellobiose. This is in contradiction to results described in the literature (Kilpper-Bälz *et al.*, 1985).

DISCUSSION

In contrast to most streptococcal species, which are associated with only one or a few phylogenetically related animal hosts (Devriese, 1991), the strains investigated were found in very different animal host species. Moreover, the body locations of the species were unusually diverse: genital tract, udder and tonsils of mammals; crop, skin and respiratory tract of birds. SDS-PAGE analysis of whole-cell protein revealed that the strains were phenotypically related at the species level. The results described above, obtained by very different types of tests, all demonstrated that the strains included in this study belong to a new species of the genus *Streptococcus*. 16S rRNA gene sequence analysis demonstrated that strains LMG 14257 and LMG 14177^T were closely related to each other genealogically and constituted a new subline within this genus. The bacteria described here displayed a specific association (Fig. 2) with the recently described species *S. hyovaginalis* and *S. thoralensis* from sows. However, sequence divergence values of approximately 3% and 3.5%, respectively, indicate that the isolates represent a hitherto unknown species. Although a precise correlation between 16S rRNA sequences and DNA–DNA pairing is not possible, the 3% or greater levels of 16S rRNA sequence divergence between the unknown species and *S. hyovaginalis* and *S. thoralensis* are supportive of a new species (Stackebrandt & Goebel, 1994). The phylogenetic distinctiveness of the unknown bacterium is consistent with its separateness as revealed by whole-cell protein analysis and it is proposed that organisms of this species be designated *Streptococcus pluranimalium* sp. nov.

A special feature of the strains described here is the relatively large number of variable biochemical char-

acteristics (Table 2). This metabolic variability possibly relates to the very diverse habitats of this species. This infra-specific aspect falls outside the scope of the present paper but clearly merits further study. The divergent reactions of the bovine genital (vagina and cervix) strains, as shown in Table 3, are merely an indication of the possible existence within *S. pluranimalium* of ecovars showing typical host-specific or even site-specific characteristics, as is well known in other streptococci (Devriese, 1991). Although the species was associated with subclinical mastitis in dairy cows and was isolated from lesions in canaries, its pathogenic significance remains unclear. Moreover, the preponderance of bovine strains in the collection examined in this study is probably a reflection of the animal material at our disposal.

The presence of *S. pluranimalium* on blood-agar isolation plates can be suspected when colonies are surrounded by broad, sharply demarcated zones of strong greening haemolysis. On aesculin-containing blood agars such as Edward's medium, colonies are usually intensely brown or black, as is the surrounding medium. Characteristics useful in the differentiation of *S. pluranimalium* from phylogenetically related or phenotypically similar species are shown in Table 4. The usefulness of some of these characteristics may depend on the origin of the strains (Tables 3 and 4). *S. pluranimalium* may also be confused with strains of the *Streptococcus bovis* species group and even with the greening enterococci of the *Streptococcus avium* species group (Devriese *et al.*, 1993) and with the lactococci. The negative Voges–Proskauer reaction of *S. pluranimalium* differentiates the new species unequivocally from these groups.

Certain strains (LMG 14256, = CCUG 11675; LMG 14257, = CCUG 28589) were originally identified as *S. acidominimus*. It has long been recognized that the taxonomy of *S. acidominimus* is unsatisfactory and that the species as defined (Ayers & Mudge, 1922; Hardie, 1986) embraces many misidentified strains. In view of these difficulties, the type strain *S. acidominimus*, NCFB 2025^T, which has been shown by 16S rRNA sequence analysis to represent a separate species (Bentley *et al.*, 1993), was included in our study. This strain differs markedly from *S. pluranimalium* (Figs 1 and 2, Table 4). Its biochemical characteristics, as determined here, were in agreement with the original species description. It is pertinent to note that they differed from those listed by Kilpper-Bälz *et al.* (1985) in not less than five of eleven tests.

Description of *Streptococcus pluranimalium* sp. nov.

Streptococcus pluranimalium (plur.an.im.al'i.um. L. adj. *pluris* many; L. n. gen. pl. *animalium* from animals; M.L. gen. n. *pluranimalium* from many animals).

Cells are small, Gram-positive cocci arranged in chains or groups. Non-motile. Grows at 37 and 42 °C. Most strains produce precipitates and clear supernatants in

broth. Colony size less than 1 mm. Produces greening haemolysis. May grow in 6.5 % NaCl broth. Reacts positively in tests for hydrolysis of hippurate, 4-MU-phosphate, L-phenylalanine AMC and L-tryptophan AMC, leucine arylamidase, alanine-phenylalanine-proline arylamidase, 4-MU-phosphate hydrolysis, acid production from glucose, fructose and trehalose. Other carbohydrate reactions are very variable and, in some strains, weak and slow to develop. Negative in the Voges-Proskauer test. The DNA G + C content of the type strain is 38.5 mol%. The type strain is LMG 14177^T; this strain was isolated from subclinical mastitis in a cow.

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