Corynebacterium mastitidis sp. nov., Isolated from Milk of Sheep with Subclinical Mastitis

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Fourteen strains of a hitherto unknown catalase-positive, aerobic, gram-positive coryneformlike organism were isolated from the milk of sheep with subclinical mastitis from different regions of Spain. The strains phenotypically closely resembled one another and biochemically were similar to Corynebacterium urealyticum and Corynebacterium afermentans subspp. lipophilum. The results of chemotaxonomic investigations were consistent with membership in the genus Corynebacterium, and comparative 16S rRNA gene sequencing studies showed that the unknown bacterium from sheep was indeed a member of the genus Corynebacterium. Within the genus Corynebacterium the new bacterium formed a distinct subline that exhibited >4% sequence divergence with other species. Based on both phenotypic and phylogenetic findings, a new species, Corynebacterium mastitidis, is proposed for the organisms from mastitic sheep. The type strain of *C. mastitidis* is CECT 4843 (=S-8).

**MATERIALS AND METHODS**

**Bacterial strains and cultivation.** Fourteen strains were isolated from milk samples from 14 sheep suffering from subclinical mastitis. All of the strains were grown aerobically at 37°C on Columbia blood agar (Biomerieux s.a., Lyon, France). Milk samples (10 ml) were taken aseptically from mammary glands without clinical abnormalities after the teat ends were disinfected and the first steams of milk were discarded. The milk samples were kept at 4°C during transportation to the laboratory for microbiological analysis. All of the isolates were recovered in pure culture from apparently normal milk samples with bacterial counts ranging from 1 × 10³ to 4 × 10⁴ CFU/ml and positive for California mastitis tests. Mammary glands with the characteristics observed (no clinical abnormalities, apparently normal milk secretion, bacteriologically positive, and positive for California mastitis tests) are considered to have subclinical mastitis (25). Isolates were recovered from four different dairy flocks located in Alava and Guipuzcoa (north of Spain) and Madrid and Guadalajara (central region of Spain). The breeds of the sheep were Latxa (Alava and Guipuzcoa), Manchega (Madrid), and Assaf (Guadalajara).

**Biochemical tests.** The API Coryne system (Biomerieux) was used according to the manufacturer’s instructions except that the time of incubation at 37°C was 48 h. Enzymatic activities were determined with the API ZYM system (Biomerieux) after 24 h of incubation at 30°C. Utilization of carbohydrates (under aerobic and anaerobic conditions), motility, and CAMP test reactions (with *Staphylococcus aureus*) were determined as described by von Graevenitz and Funke (26). Lipophilims were determined by growing the isolates in brain heart infusion agar supplemented with 1% Tween 80 and comparing the results with the results obtained with brain heart infusion agar lacking lipids. Antimicrobial susceptibilities were determined by the diffusion method by using Mueller-Hinton agar supplemented with 1% Tween 80 (1).

**Chemotaxonomic tests.** Cell wall murine was prepared by mechanically disrupting cells, and complete acid hydrolysates were analyzed as described by Schleifer and Kandler (22). Fatty acid methyl esters were prepared and analyzed as described by Kämpfer and Kroppenstedt (13). The presence of mycolic acids was determined by the thin-layer chromatography method of Mintikin et al. (16). The presence of mycolic acids was also investigated by performing a gas-liquid chromatography analysis of trimethylsilylated derivatives (14).

**Phylogenetic analysis.** For phylogenetic studies, a large fragment of the 16S rRNA gene was amplified from DNA by PCR using universal primers PA (positions 8 to 28; *Escherichia coli* numbering) and PH (positions 1542 to 1522). The amplified product was sequenced directly by using primers for conserved regions of the rRNA. Sequencing was performed with a PRISM DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom), and reaction products were electrophoresed by using a model 373A automated DNA sequencer according to the protocols of the manufacturer (Applied Biosystems). To establish the relatives of the unknown strains, preliminary searches in the EMBL Data Library were performed with the program FASTA.

*Mastitis is one of the most serious health problems of sheep used for milk production and can result in substantial economic losses. Although coagulase-negative staphylococci are the most important organisms associated with subclinical mastitis in sheep (4, 7), corynebacteria are also associated with a significant proportion of cases (25). Corynebacterium pseudotuberculosis and Corynebacterium bovis are the species most commonly identified from goats and cows (3, 12, 15). However, the identities of *Corynebacterium* species that produce subclinical mastitis in sheep are poorly understood (4). Within the coryneform group of bacteria, the *Corynebacterium* presently comprises the largest number of species, and many new *Corynebacterium* species associated with disease in humans have been described in the past decade (see reference 8 for a review). Despite the importance of this genus in clinical and veterinary medicine, the methods used for routine identification of *Corynebacterium* species are generally unreliable, although some improvements (e.g., availability of miniaturized kits and improved databases) have been made (8, 24). Our knowledge of the phylogenetic relationships within the genus *Corynebacterium* has been revolutionized in recent years by 16S rRNA gene sequence analysis (17). Furthermore, the high degree of specificity of 16S rRNA and the cumulative nature of sequence data have provided diagnosticians with immensely powerful tools for the recognition of new species (9, 10, 17, 19, 20). Indeed, the use of this phylogenetic approach (17) in combination with chemical and biochemical characterization (8) is resulting in a much improved taxonomy for this important group of bacteria.

In a surveillance analysis of bacteria associated with subclinical mastitis of sheep from different regions of Spain, we isolated, from milk samples from several animals, 14 gram-positive coryneformlike rod-shaped organisms similar to, but different in some respects from, *Corynebacterium afermentans* subspp. lipophilum and *Corynebacterium urealyticum*. In this report, we present the phenotypic characteristics of these isolates and the results of a comparative 16S rRNA gene sequence analysis. Based on our phenotypic and molecular genetic findings, we formally propose a new species, *Corynebacterium mastitidis*, for the new bacterium from sheep.
TABLE 1. Levels 16S rRNA sequence similarity between C. mastitidis and closely related species

<table>
<thead>
<tr>
<th>Species</th>
<th>% 16S rRNA sequence similarity to C. mastitidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corynebacterium accolens (X80500)</td>
<td>95.5</td>
</tr>
<tr>
<td>Corynebacterium afermentans (X80255)</td>
<td>94.5</td>
</tr>
<tr>
<td>Corynebacterium ammoniagenes (X84440)</td>
<td>93.2</td>
</tr>
<tr>
<td>Corynebacterium amycolatum (X84244)</td>
<td>93.7</td>
</tr>
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<td>Corynebacterium auris (X82493)</td>
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</tr>
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<td>Corynebacterium bovis (X84444)</td>
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<td>Corynebacterium callidulae (X84251)</td>
<td>92.5</td>
</tr>
<tr>
<td>Corynebacterium coryneforme (X96497)</td>
<td>94.6</td>
</tr>
<tr>
<td>Corynebacterium cytopathicum (X84252)</td>
<td>93.9</td>
</tr>
<tr>
<td>Corynebacterium diphtheriae (X84248)</td>
<td>94.2</td>
</tr>
<tr>
<td>Corynebacterium “fastidiosum” (X84245)</td>
<td>94.8</td>
</tr>
<tr>
<td>Corynebacterium flavescens (X84441)</td>
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<td>Corynebacterium “genitalium” (X84253)</td>
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<tr>
<td>Corynebacterium glucuronolyticum (X86688)</td>
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<td>Corynebacterium glutamicum (X84257)</td>
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</tr>
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<td>Corynebacterium jeikeium (X84250)</td>
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<td>Corynebacterium maegleyi (X80499)</td>
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<td>Corynebacterium matruchoti (X84443)</td>
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<td>Corynebacterium minutissimum (X84678)</td>
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<td>Corynebacterium mycetoides (X84241)</td>
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<td>Corynebacterium pslorum (X84246)</td>
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</tr>
<tr>
<td>Corynebacterium pseudopseudotubercolosis (X84528)</td>
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</tr>
<tr>
<td>Corynebacterium pseudogenitalium (X81872)</td>
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</tr>
<tr>
<td>Corynebacterium pseudotubercolosis (X84255)</td>
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</tr>
<tr>
<td>Corynebacterium renale (X84249)</td>
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</tr>
<tr>
<td>Corynebacterium “segmentosum” (X84437)</td>
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<td>Corynebacterium striatum (X84442)</td>
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<td>Corynebacterium tuberculosisrense (X84247)</td>
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<td>Corynebacterium ulcerans (X84256)</td>
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<td>Corynebacterium ulei (X84439)</td>
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<td>Corynebacterium variabilis (X53185)</td>
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<tr>
<td>Corynebacterium vulgaris (X84680)</td>
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</tr>
<tr>
<td>Corynebacterium yersini (X84446)</td>
<td>93.6</td>
</tr>
</tbody>
</table>

* The numbers in parentheses are EMBL 16S rRNA nucleotide sequence accession numbers.

Sequences of the close relatives were retrieved from the EMBL Data Library and aligned with the newly determined sequences by using the program PILEUP (5). The RNA alignment was corrected manually, and approximately 100 bases at the 5’ end of the molecule were omitted from further analyses because of alignment uncertainties due to highly variable region VI. Levels of sequence similarity were calculated and corrected for substitution rates by using Kimura’s parameters. A phylogenetic tree was constructed by the neighbor-joining method (21), and the stability of relationships was assessed by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE of the PHYLIP package (6).

Nucleotide sequence accession number. The nucleotide sequence of the 16S rRNA of strain S-81 has been deposited in the EMBL Data Library under accession no. X08906.

RESULTS AND DISCUSSION

All of the isolates exhibited a typical coryneform morphology. The cells were short, gram positive, nonmotile, and rod shaped and occurred singly or in palisades or V-shapes. The isolates grew slowly on sheep blood agar at 37°C, forming small colonies that were nonhemolytic. All of the strains were aerobic, catalase positive, and oxidase negative. The isolates did not hydrolyze esculin or gelatin and did not reduce nitrate. Urease activity was variable (7 of the 14 strains did not hydrolyze urea). The isolates did not produce acid from glucose, ribose, xylose, mannitol, lactose, maltose, sucrose, and glycogen and were pyrrolidonylarylamidase, β-glucuronidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, and N-acetyl-β-glucosaminidase negative. The isolates displayed pyrazinamide and alkaline and acid phosphatase activities. All of the isolates displayed esterase (E), esterase lipase (L), lipase (P), leucine arylamidase, valine arylamidase, and cystine arylamidase activities but were negative for trypsin and chymotrypsin. The strains were all lipophilic. Two different biochemical patterns were obtained with the API Coryne strips: eight strains (S-1, S-6, S-29, S-74, S-82, S-85, S-86, and S-90) gave profile 2100004, corresponding to doubtful discrimination between the Corynebacterium ANF group and Brevibacterium spp., and six strains (S-87, S-36, S-63, S-67 S-83 and S-87) gave profile 2101004, corresponding to good identification as members of Corynebacterium group D2. The two numerical profiles differ only in the variable reactions observed for urease. This marker is usually considered constant in most Corynebacterium species (8); however, five and four strains that had numerical profiles 2100004 and 2101004, respectively, were totally or partially sequenced, and the results indicated that the expression of urease activity is really variable in the new species. All of the isolates were susceptible to penicillin G, ampicillin, amoxicillin, clavulanic acid, gentamicin, cefalotine, and nalidixic acid. It is pertinent that C. urealyticum, which biochemically resembles the mastitic isolates, is resistant to these antimicrobial agents (11). The sheep isolates were also readily distinguished from C. afermentans subsp. lipophilum by their inability to grow in the presence of 6.5% NaCl. The strains of C. afermentans subsp. lipophilum examined in this study grew in the presence of 6.5% NaCl, which is consistent with previous reports (18).

A cell wall murein analysis of a representative (strain S-81) of the unknown bacterium from sheep revealed that meso-
diaminopimelic acid is the dibasic amino acid, which is consistent with identification as a member of the genus \textit{Brevibacterium} or the genus \textit{Corynebacterium}. The cellular fatty acids of two isolates (S-87 and S-87) also resembled those of corynebacteria, with hexadecanoic acid (C16:o) (26 to 28%), octadecanoic acid (C18:o) (26 to 28%), and octadecenoic acid (C18:1, w9c) (26 to 28%) predominating. Tuberculostearic acid was not detected. The absence of the latter acid distinguishes the new isolate from \textit{C. urealyticum}, which produces small amounts of tuberculostearic acid. In contrast, \textit{brevibacteria} contain high levels of anteiso methyl-branched acids in addition to straight-chain saturated acids. Similarly, the thin-layer chromatography analysis of whole-cell methanolysates and the gas-liquid chromatography analysis of trimethylsilylated derivatives demonstrated the presence of short-chain mycolic acids (C \textsubscript{34}) in the two strains, which is consistent with the fatty acid content of true corynebacteria (2).

To establish the phylogenetic affinities of the isolates from sheep, their partial 16S rRNA gene sequences were examined. The sequence of a large fragment (>1,400 nucleotides) from four strains (S-87, S-29, S-36, and S-85) and short sequences (>700 nucleotides) from five strains (S-74, S-83, S-86, S-87, and S-90) were determined. Comparative analysis revealed 100% sequence similarity among the strains, thereby demonstrating their genealogical homogeneity. To determine the generic position of the unidentified bacterium, the 16S rRNA sequence of strain S-87 was compared with the 16S rRNA sequences of other gram-positive bacteria with high G+C contents. The unidentified bacterium displayed high sequence similarities (generally >90%) with species of the genus \textit{Corynebacterium} (Table 1). Significantly lower levels of relatedness were observed with other actinomycete and coryneform taxa (data not shown). A tree depicting the phylogenetic relationships of the unidentified bacterium within the genus \textit{Corynebacterium} is shown in Fig. 1. The new bacterium formed a distinct subline loosely associated with, but distinct from, \textit{Corynebacterium renale}. Levels of 16S rRNA sequence divergence of approximately 5% with \textit{C. renale} and 6 to 10% with other \textit{Corynebacterium} species (Table 1) clearly demonstrate that the unidentified bacterium represents a new species of the genus.

Based on the phenotypic and phylogenetic findings, it is evident that the unidentified coryneform from milk is a hitherto unrecognized species of the genus \textit{Corynebacterium}. The name \textit{Corynebacterium mastitidis} sp. nov. is formally proposed for this organism. \textit{C. mastitidis} can be readily distinguished from its closest phylogenetic relative (\textit{C. renale}), from \textit{C. bovis} and \textit{C. pseudotuberculosis}, which are also associated with mastitis in sheep, and from other lipophilic corynebacteria by the characteristics shown in Table 2. It is pertinent to note that most strains of \textit{C. mastitidis} were isolated in pure culture from the udders of sheep with subclinical mastitis and from flocks located in well-separated geographical areas (north and central regions) of Spain. These facts point out the ecological and clinical relevance of the species in subclinical mastitis of sheep when mechanical milking practices are used.

### Table 2. Characteristics that differentiate \textit{C. mastitidis} from \textit{C. renale}, \textit{C. pseudotuberculosis}, \textit{C. bovis}, and other lipophilic \textit{Corynebacterium} species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>\textit{C. mastitidis}</th>
<th>\textit{C. renale}</th>
<th>\textit{C. pseudotuberculosis}</th>
<th>\textit{C. bovis}</th>
<th>\textit{C. jeikeium}</th>
<th>\textit{C. urealyticum}</th>
<th>\textit{C. afermentans} subsp. lipophilum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation of(^a):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Sucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Production of(^b):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrrolidonylarnilamidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>β-Galactosidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>Pyrazinamidase</td>
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<td>Hydrolysis of(^c):</td>
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</tr>
<tr>
<td>2-Naphthylminristate</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1-Leuetyl-2-naphthylamide</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>2-Naphthylphosphate (phosphatase)</td>
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<td>+</td>
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<tr>
<td>Naphthol-AS-BI-phosphate</td>
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<td>V</td>
<td>V</td>
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<td>V</td>
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<tr>
<td>CAMP</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
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<td>Lipophilic</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Presence of tuberculosteric acid</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Data for \textit{Corynebacterium} species other than \textit{C. mastitidis} were obtained from the API Coryne system database.

\(^b\) Negative according to Funke et al. (8).

\(^c\) Variable according to Funke et al. (8).

\(^d\) Negative according to Funke et al. (8).

\(^e\) Reverse CAMP (see reference 8).
catalase positive and oxidase negative. On blood agar, small (diameter, less than 1 mm), rough, whitish, low convex colonies are formed after 3 days of incubation at 37°C; these colonies are nonhemolytic. Esculin and gelatin are not hydrolyzed. Nitrate is not reduced. Hydrolysis of urea is variable. Acid is not produced from glucose, ribose, xylose, mannitol, lactose, maltose, sucrose, and glycogen. Pyrazinamidase, alkaline phosphatase, acid phosphatase, esterase (C₄), esterase lipase (C₈), lipase (C₁₄), leucine arylamidase, valine arylamidase, and cysteine arylamidase are produced. Pyrrolidonylarylamidase, β-glucuronidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, N-acetyl-β-glucosaminidase, trypsin, and chymotrypsin are not produced. The cell wall contains meso-diaminopimelic acid. Short-chain mycolic acids are present. The major long-chain fatty acids are C₁₆:0, C₁₈:0, and C₁₈:1ω₇c. Tuberculostearic acid is not produced. The strains are susceptible to penicillin G, ampicillin, amoxicillin-clavulanic acid, gentamicin, cephalothin, and nalidixic acid. The strains do not grow in the presence of 6.5% NaCl. Type strain S-8 has been deposited in the Spanish Type Culture Collection as strain CECT 4843. The type strain has the characteristics of the species described above except that it is urease and naphthol-AS-BI-phosphate positive. The type strain was isolated from the udder of a sheep with subclinical mastitis.

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REFERENCES