Staphylococcus agnetis sp. nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis

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Thirteen Gram-positive-staining coagulase-variable staphylococci were isolated from subclinical and mild clinical mastitic bovine milk (n = 12) and a teat apex (n = 1). The results of sequence analysis of the 16S rRNA gene and two housekeeping genes, rpoB and tuf, and DNA fingerprinting with amplified fragment length polymorphism (AFLP) analysis showed that the isolates formed a separate branch within the genus Staphylococcus. The phylogenetically most closely related species were Staphylococcus hyicus and Staphylococcus chromogenes. DNA–DNA hybridization with S. hyicus DSM 20459T and S. chromogenes DSM 20674T confirmed that the isolates belonged to a separate species. The predominant fatty acids were i-C15 : 0, ai-C15 : 0, i-C17 : 0 and C20 : 0 and the peptidoglycan type was A3α L-Lys–Gly5. Based on the results of genotypic and phenotypic analyses, it is proposed that the thirteen isolates represent a novel species, for which the name Staphylococcus agnetis sp. nov. is proposed. Strain 6-4T (DSM 23656T = CCUG 59809T) is the type strain.

Coagulase-negative staphylococci (CNS) are the bacteria most frequently isolated in milk samples from bovine intramammary infections (IMI) in well-managed dairy herds in many countries (for examples see Koivula et al., 2007; Piepers et al., 2007; Schukken et al., 2009). In routine diagnostic methods for IMI, CNS are not identified at the species level but are considered as a uniform group, although differences in clinical characteristics between species may exist (Taponen, 2008). Few species belonging to this group are in fact coagulase-variable. The identification of CNS species using the phenotypic reactions of these bacteria has been shown to be inaccurate (Sampimon et al., 2009) and new methods based on the bacterial genotype have been developed (Goh et al., 1997; Heikens et al., 2005; Suprê et al., 2009; Piessens et al., 2010). During a study on the clinical characteristics of CNS mastitis (Taponen et al., 2006), amplified fragment length polymorphism (AFLP) was compared with the phenotypic API Staph ID 32 identification scheme (bioMérieux). In this study, 82 % of isolates assigned to the species Staphylococcus hyicus by the API test (>90 % probability), did not cluster with the type strain of S. hyicus (CCM 2368T) or any other type strain of the genus Staphylococcus using AFLP. In later studies, isolates with the same unknown genotypic fingerprint were encountered (Taponen et al., 2007, 2008). The aim of this study was to further examine 13 of these isolates and to determine whether they constitute an undescribed CNS species.

In total, 13 CNS isolates, 12 from bovine subclinical or mild clinical IMI and one from a teat apex colonization, originating from 13 cows from eight dairy herds in...
Southern Finland, were available. The isolates were preliminarily identified as CNS following the guidelines of the National Mastitis Council, based on colony morphology on blood agar, Gram-staining and catalase and coagulase activity (Hogan et al., 1999). The strains were facultatively anaerobic. After 24 h aerobic incubation on bovine blood agar at 37 °C, the smooth, circular and slightly convex colonies reached 2–3 mm in diameter. They were opaque, light grey and non-haemolytic. Gram-staining showed Gram-positive, non-spore-forming cocci, which occurred singly, in pairs and in small clusters. Cells were non-motile when observed with phase-contrast microscopy. The isolates were catalase-positive and oxidase-negative. Coagulase activity was tested using tube coagulase (BBL Coagulase Plasma Rabbit; Becton Dickinson and Company) and was not visible after 4 h. However, three isolates (isolates 43-1, 55-1 and 64-2) became positive after 24 h. Growth characteristics were determined on P broth (Freney et al., 1999) with different NaCl concentrations, temperatures and pH values. With 2% or 10% NaCl (w/v), intensive growth occurred in 24 h, in 48 h with 15% NaCl and in 72 h with 18% NaCl. With 19% NaCl, growth was still weak after 72 h and no growth was seen with 20% NaCl, even after three weeks. Intensive growth occurred in 24 h at 15 °C and 45 °C, and less intensive growth took place at 10 °C and 47 °C. No growth was seen at 5 °C. Growth was intensive in 24 h at pH 6 and pH 10, and less intensive at pH 5. Less intensive growth was seen at pH 12 after 48 h. Anaerobic growth was studied using Brewer thioglycollate medium (Sigma-Aldrich) and was found to be positive. The clumping factor test (Devriese, 1979) was negative. The DNase reaction was tested on DNase agar plates (Tammer-Tutkan Maljat Oy) and was positive.

Phenotypic tests were applied to all of the 13 isolates. Susceptibility to novobiocin, lysostaphin and lysozyme was tested on P agar (novobiocin N-1628; lysostaphin L-7386; lysozyme L-6876, Sigma-Aldrich). Susceptibility to polymyxins and deferoxamine was tested in STAPH Zym gallery (Rosco). All isolates were susceptible to novobiocin and lysostaphin, and resistant to lysozyme, polymyxins and deferoxamine. Acid production from carbohydrates was tested in API 50 CH galleries (bioMérieux) under paraffin cover and other biochemical reactions were tested with API Staph ID 32 (bioMérieux) and STAPH Zym (Rosco) galleries according to the manufacturers’ instructions. The characteristics of the new isolates are summarized in the species description below.

Biochemical features were compared with those of the type strains of the phylogenetically most closely related species, S. hyicus ATCC 11249T and Staphylococcus chromogenes ATCC 43764T. The novel isolates were phenotypically very close to S. hyicus and S. chromogenes. Within the novel isolates, all but seven reactions gave identical results. The novel isolates could be distinguished from S. hyicus and S. chromogenes on the basis of these seven same reactions (listed in Table 1).

Phenotypically the novel species is a typical representative of the genus Staphylococcus. It could be differentiated from the coagulase-positive species of the genus, such as Staphylococcus aureus, Staphylococcus intermedius and Staphylococcus pseudointermedius, in the coagulase test reaction, which was negative after 4 h, and by the lack of haemolysis. The novel strains could be differentiated from most coagulase-negative species by means of the DNase test. The DNase reaction of the novel strains was strong, whereas coagulase-negative staphylococi usually give negative reaction in the DNase test (Devriese & van de Kerckhove, 1979). However, it was not possible to differentiate the novel strains from S. hyicus on the basis of these phenotypic characteristics.

All 13 isolates were further characterized using 16S rRNA, rpoB and tuf gene sequence comparisons, AFLP analysis and phenotypic properties following the recommendations for the description of new staphylococcal species (Freney et al., 1999). The nearly complete (1409 bp) sequences of the 16S rRNA gene and partial sequences of the housekeeping genes encoding the β-subunit of the RNA

### Table 1. Phenotypic characteristics of the 13 S. agnetis sp. nov. strains compared with the most closely related species of the genus Staphylococcus

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polymerase, rpoB (507 bp), and the elongation factor tu, tuf (180 bp), were determined for all the 13 isolates using previously described methods (Kuhnert et al., 2002; Drancourt & Raoult, 2002 and Heikens et al., 2005, respectively). The primers used for sequencing are shown in Supplementary Table S1 (available in IJSEM Online). The sequences of these isolates and of representative type strains of the members of the genus *Staphylococcus* (retrieved from GenBank, http://www.ncbi.nlm.nih.gov, using the nucleotide–nucleotide BLAST algorithm) were aligned and phylogenetic trees were constructed from the global alignment by the neighbour-joining algorithm (see Supplementary Figs S1, S2 and S3) and the maximum-parsimony algorithm (data not shown) using the BioNumerics 4.61 software package (Applied Maths). The tree topology was similar with both methods.

In the BLAST analyses, the 16S rRNA gene sequences of the isolates showed the highest similarities with *S. hyicus* ATCC 11249T (99.7 % for isolates 66-1, 69-4, 100-4, 67B, 02-644, 02-650 and 02-675; 99.6 % for isolates 6-4T², 43-1, 55-1, 59-1, 64-2 and 71-4) and *S. chromogenes* ATCC 43764T (99.1 % for all). Considering the previously described cut-off value for species identification based on 16S rRNA gene sequences (98.7 %) (Jousson et al., 2007), a species designation was not possible. As shown in the 16S rRNA, rpoB and tuf gene trees (Supplementary Figs S1, S2 and S3), the phylogenetic position of the 13 isolates was within the genus *Staphylococcus*. The strains clustered closely together (indicating that they belonged to a single species), within the *S. hyicus/S. intermedius* group. However, the cluster was clearly distinct from the closest phylogenetic neighbours *S. hyicus* ATCC 11249T and *S. chromogenes* ATCC 43764T. The 16S rRNA and rpoB gene sequence trees revealed the formation of two similar subclusters within the unknown group. Three strains that covered both subclusters, strains 6-4T, 55-1 and 02-650, were chosen for further examination. In the tuf gene sequence tree, the subdivision within the unknown cluster was less clear.

AFLP analysis was performed using the restriction enzyme pair HindIII-MseI as previously described by Ketontinenen et al. (2003). In the cluster analysis of the AFLP patterns of the 13 new isolates and 48 type strains of the genus *Staphylococcus*, the 13 isolates formed a clear cluster with high similarity (see Supplementary Figure S4). None of the type strains of members of the genus *Staphylococcus* clustered with these 13 isolates. The similarity of the cluster of the new isolates with the AFLP profiles of the type strains of members of the genus *Staphylococcus* (DSMZ 20459T and *S. chromogenes* DSM 20674T) was 100, with 96.5 %. Reassociation values, given as the mean of duplicate tests for the DNA–DNA hybridization pairs, between strains 6-4T, 55-1 and 02-650 varied from 89.8 to 96.5 %. Reassociation values between the novel strains and *S. hyicus* DSM 20459T or *S. chromogenes* DSM 20674T were only 44.7 % and 21.6 %, respectively. Considering the recommendations of a threshold value of 70 % DNA–DNA hybridization for the definition of bacterial species (Wayne et al., 1987), these results confirmed that isolates 02-650, 55-1 and 6-4T all belonged to a single species that was separate from *S. hyicus* and *S. chromogenes*.

The DNA base composition (mol%) of strain 6-4T was analysed by the DSMZ. The DNA was isolated and purified as described by Cashion et al. (1977). After hydrolysis, the DNA was analysed by HPLC (Shimadzu) at 45 °C as described by Mesbah et al. (1989), using the solvent 0.3 M (NH₄)₂HPO₄ with acetonitrile (Tamaoka & Komagata, 1984). Non-methylated λ-DNA (Sigma), with a DNA G+C content of 49.858 mol % (Mesbah et al., 1989) and three DNA samples from strains with published complete genome sequences, *Bacillus subtilis* DSM 402 (43.518 mol %), *Xanthomonas campestris* pv. *campestris* DSM 3586T (65.69 mol %) and *Streptomyces violaceoruber* DSM 40783 (72.119 mol %), were used as the calibration references. The DNA G+C content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah et al. (1989). The G+C content of the strain 6-4T was 37.2 mol %. This result was within the DNA G+C content range of 33–40 mol % (Götz et al., 2006) expected for species of the genus *Staphylococcus*.

The peptidoglycan of the cell walls of strains 6-4T, 55-1 and 02-650 was isolated and the structure was studied at the DMSZ using TLC (MacKenzie, 1987). The 1D- and 2D-TLC of the total hydrolysated peptidoglycan (4 M HCl, 16 h at 100 °C) revealed the presence of the amino acids lysine, alanine, glutamic acid and glycine (the latter in high amounts) in all three strains. After derivation according to MacKenzie (1987), the molar amino acid ratio was determined by GC. The ratios were as follows for strain 6-4T²: 2.4 Ala : 5.2 Gly : 1.0 Glu : 1.0 Lys; for strain 55-1: 1.7 Ala : 3.8 Gly : 1.0 Glu : 1.0 Lys; and for strain 02-650: 2.2 Ala : 5.0 Gly : 1.0 Glu : 0.9 Lys. The 2D-TLC of the partial hydrolysate of the peptidoglycan (4 M HCl, 0.75 h at 100 °C) revealed the presence of the following peptides in all three strains: L-Ala–D-Glu, D-Ala–Gly, L-Lys–D-Ala, oligo-Gly, L-Lys–Gly, Gly–L-Lys–D-Ala. On the basis of these results, the peptidoglycan type of the three novel strains was A3α L-Lys–Gly5 (A11.2 according to http://www.dsmz.de/microorganisms/main.php?content_id=35), which corresponded to that of other staphylococci (Schleifer & Kandler, 1972).

The cellular fatty acid patterns of strains 6-4T, 55-1 and 02-650 were determined at the DSMZ as described previously (Miller, 1982; Kuykendall et al., 1988). The fatty acid
profiles of the three isolates were very similar and consisted of (values are means): i-C_{15:0} (50.6 %), ai-C_{15:0} (18.7 %), i-C_{17:0} (8.9 %), C_{20:0} (6.1 %), C_{18:1} (3.8 %), C_{16:0} (3.3 %), ai-C_{17:0} (2.6 %), i-C_{19:0} (2.1 %), C_{14:0} (1.3 %), i-C_{16:0} (0.9 %), i-C_{13:0} (0.7 %), i-C_{14:0} (0.6 %), ai-C_{19:0} (0.4 %), C_{19:0} (0.1 %), i-C_{18:0} (0.05 %).

On the basis of the phenotypic and chemotaxonomic results presented in this study, it is suggested that the thirteen isolates represent a novel species for which the name *Staphylococcus agnetis* sp. nov. is proposed. Strain 6-4T (= DSM 23656^T = CCUG 59809^T) is designated as the type strain.

**Description of Staphylococcus agnetis** **sp. nov.**

*Staphylococcus agnetis* [ag.n.e’tis. N.L. gen. n. *agnetis* of Agnes, named in honour of Europe’s first female veterinary surgeon, the Finn, Agnes Sjöberg (1888–1964), who struggled her way into the profession, despite resistance from her male colleagues].

Cells are Gram-positive-staining, non-motile, non-spore-forming, facultatively anaerobic cocci, which occur singly, in pairs and in small clusters. After 24 h growth at 37 °C, colonies reach 2–3 mm in diameter, are circular, slightly convex, smooth, opaque, light grey and non-haemolytic on bovine blood agar. Catalase-positive and oxidase-negative. Susceptible to novobiocin and lysozyme, resistant to lysozyme, polymyxins and deoxyrribonuclease. Coagulase-negative after 4 h, but 20–25 % of the isolates are coagulase-positive after 24 h. Negative in tests for clumping factor. Hydrolyses DNA at 37 °C generating a degradation halo. Produces acid aerobically from D-glucose, D-fructose, D-mannose, lactose, sucrose and D-ribose. Variable reactions for acid production from D-galactose (69.2 % positive), trehalose (53.8 % positive), melibiose (7.7 % positive), gentiobiose (7.7 % positive) and amygdalin (7.7 % positive). Does not produce acid aerobically from maltose, D-mannitol, raffinose, cellobiose, and amygdalin (7.7 % positive). Does not produce acid from D-galactose and is negative in tests for amygdalin, melibiose, trehalose and gentiobiose. This species description is based on the characteristics of 12 isolates originating from milk samples of dairy cows with subclinical or mild clinical IMI and one isolate from a teat apex colonization.

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**References**


