Staphylococcus devriesei sp. nov., isolated from teat apices and milk of dairy cows

Karlién Supré,¹ Sarne De Vlieghe,¹ Ilse Cleenwerck,² Katrien Engelbeen,² Stefanie Van Trappen,² Sofie Piepers,¹ Otlis C. Sampimon,³ Ruth N. Zadoks,⁴ Paul De Vos² and Freddy Haesebrouck⁵

Correspondence

Karlién Supré
Karlien.Supre@UGent.be

¹Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

²BCCM/LMG Bacteria Collection and Laboratory of Microbiology, Faculty of Sciences, Ghent University, K. L. Ledeganckstraat 35, 9000 Ghent, Belgium

³Animal Health Service, Deventer, The Netherlands

⁴Royal (Dick) School of Veterinary Studies, University of Edinburgh, and Moredun Research Institute, Penicuik, Scotland, UK

⁵Department of Pathology, Bacteriology, and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium

Ten non-motile, Gram-stain-positive, coagulase-negative staphylococci were isolated from bovine milk and teat apices. All isolates were catalase-positive, with anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0}, iso-C_{17:0} and C_{18:0} as predominant fatty acids and diphosphatidylglycerol and phosphatidylglycerol as major polar lipids. The results of sequence analysis of the 16S rRNA gene and four housekeeping genes (*rpoB*, *hsp60*, *tuf* and *dnaJ*) in combination with tRNA-intergenic spacer length analysis showed that the isolates form a separate branch within the genus *Staphylococcus*. Based on 16S rRNA gene sequencing, the phylogenetically most closely related species are *Staphylococcus haemolyticus*, *S. hominis* and *S. lugdunensis*, with >98.7 % sequence similarity. The DNA G + C content varies from 33.3 to 33.7 mol%, and DNA–DNA hybridization with the nearest neighbours, based on 16S rRNA gene sequences, confirmed that the isolates represent a novel *Staphylococcus* species. All isolates induced a small zone of complete haemolysis on Columbia agar with 5 % sheep blood and exhibited a homogeneous biochemical fingerprint that is discriminative from the phylogenetically most closely related species. Based on these results, it is proposed to classify the ten isolates as *Staphylococcus devriesei* sp. nov., with strain KS-SP 60^T (=LMG 25332^T =CCUG 58238^T) as the type strain.

Abbreviations: CNS, coagulase-negative staphylococci; tDNA-PCR, tRNA-intergenic spacer PCR.

The GenBank/EMBL/DDBJ accession numbers for the *rpoB*, *hsp60*, *tuf*, *dnaJ* and 16S rRNA gene sequences of isolates KS-SDV 16 and 19 and KS-SP 11, 16, 18, 20, 27, 60^T, 65 and 66 are respectively FJ389223–FJ389225, FJ389227–FJ389230 and FJ389232–FJ389234 (failed reaction for KS-SP 20) (*rpoB*), FJ389210, FJ389211 and FJ389215–FJ389220 (failed reaction for KS-SP 27 and 66) (*hsp60*), FJ389240–FJ389242, FJ389244–FJ389246 and FJ389248 (failed reaction for KS-SP 16, 65 and 66) (*tuf*), FJ907448–FJ907455 (failed reaction for KS-SDV 16 and KS-SP 66) (*dnaJ*) and FJ938168, FJ389197, FJ389198, FJ389200–FJ389203 and FJ389206–389208 (16S rRNA gene).

Details of strain isolation and amplification primers, phenotypic results for individual strains and dendrograms based on tDNA-PCR results and *dnaJ*, *rpoB*, *hsp60* and *tuf* sequences are available as supplementary material with the online version of this paper.

In well-managed dairy farms in many parts of the world, coagulase-negative staphylococci (CNS) have become the predominant pathogens in milk samples from cows (Piepers *et al.*, 2007; Schukken *et al.*, 2009). Recent publications report on subclinical/clinical mastitis due to CNS (Gillespie *et al.*, 2009), but beneficial effects have also been suggested (De Vlieghe *et al.*, 2003, 2004; Schukken *et al.*, 2009). tRNA-intergenic spacer PCR (tDNA-PCR) has been shown to be useful for rapid, inexpensive and accurate identification of CNS at the species level (Supré *et al.*, 2009). During a recent validation study applying the latter technique to 288 bovine field isolates from milk and teat apices, ten CNS isolates remained unidentified. The tDNA-PCR patterns of these isolates were highly similar to each other and closely related to those of *Staphylococcus capitis*, *S. simulans*, *S. haemolyticus* and *S. xylosus*, but they formed a well-defined, separate cluster (Supplementary

Fig. S1, available in IJSEM Online), indicating that the ten isolates could represent an undefined *Staphylococcus* species. The aim of this study was to characterize these isolates further and to determine whether they constitute a hitherto-unknown CNS species.

The ten isolates originated from six animals from five herds in Belgium and the Netherlands. One isolate originated from milk of a subclinical infection of the udder (KS-SP 11) and nine originated from teat apices (Supplementary Table S1). The isolates were preliminarily identified as CNS following the guidelines of the NMC (a global organization for mastitis control and milk quality; <http://www.nmconline.org/>), including determination of catalase and coagulase activity and detection of haemolysis (Hogan *et al.*, 1999).

After aerobic incubation at 35 °C for 24 and 48 h on Colombia agar with 5 % sheep blood, all ten isolates showed homogeneous growth. After 48 h of incubation, colonies were circular with a diameter of 3–4 mm, smooth and glistening, and they showed a small zone (1 mm) of complete haemolysis. The majority of the colonies were grey–yellow, but KS-SP 27, KS-SP 66 and KS-SDV 16 were yellow and KS-SP 20 and KS-SP 65 showed a yellow to orange pigmentation. Incubation at 42 °C resulted in larger colonies, whereas incubation at 25 °C and under anaerobic conditions resulted in smaller colonies. All isolates were catalase-positive. Gram-staining and phase-contrast microscopy showed typical staphylococcal, non-spore-forming, non-motile, Gram-stain-positive cells, occurring in pairs and small clusters.

The cellular fatty acid patterns of the ten isolates were determined as described previously (Mergaert *et al.*, 2001). Cells were harvested from cultures grown on trypticase soy agar (BBL 11768) at 28 °C for 24 h. All ten isolates possessed very similar fatty acid profiles, and the mean profile consisted of anteiso-C_{15:0} (48.2 %), anteiso-C_{17:0} (19.1 %), iso-C_{17:0} (6.1 %), C_{18:0} (5.9 %), iso-C_{15:0} (5.1 %), C_{20:0} (3.6 %), anteiso-C_{19:0} (3.3 %), iso-C_{16:0} (2.5 %), iso-C_{19:0} (1.9 %), iso-C_{14:0} (1.5 %), C_{16:0} (1.4 %) and iso-C_{18:0} (1.1 %), which corresponds to that of other CNS (Kotilainen *et al.*, 1991).

Polar lipids were extracted and separated by using two-dimensional TLC according to Tindall (1990a, b). The total lipid profile was visualized by spraying with molybdatophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates) and α -naphthol (specific for sugars). Biomass used for the extraction was grown on trypticase soy agar (BBL) at 28 °C for 24 h. The polar lipid profiles of the three representative isolates tested, KS-SP 11, KS-SP 60^T and KS-SDV 19, were very similar. They consisted of the major lipids diphosphatidylglycerol and phosphatidylglycerol. Moderate amounts of three unknown glycolipids, of which two corresponded to GL1 and GL2 as described by Nahaie *et al.* (1984), were detected. Also, minor to trace amounts of unknown aminolipids were observed. These profiles are in agreement

with other *Staphylococcus* species profiles (Nahaie *et al.*, 1984; Nováková *et al.*, 2010).

An almost-complete fragment of the 16S rRNA gene and partial fragments of the housekeeping genes coding for the β -subunit of the RNA polymerase (*rpoB*), the heat-shock protein 60 (*hsp60*), the elongation factor Tu (*tuf*) and the heat-shock protein DnaJ (*dnaJ*), were amplified and sequenced according to previously described procedures (for *rpoB*, *hsp60* and *tuf*, Supré *et al.*, 2009; for *dnaJ*, Shah *et al.*, 2007), except that other primers were used for 16S rRNA gene sequencing (Mergaert *et al.*, 2001; Coenye *et al.*, 1999) (Supplementary Table S2). Forward and reverse sequences were aligned with the Vector NTI Advance 10 software (Invitrogen Life Technologies) and compared to GenBank sequences via the nucleotide–nucleotide BLAST algorithm (<http://ncbi.nlm.nih.gov/>). For alignment of nucleotide sequences, the CLUSTAL W program (Thompson *et al.*, 1994) was used. Neighbour-joining and maximum-parsimony (Saitou & Nei, 1987) trees were obtained with the PHYLIP program (Felsenstein, 1989) and drawn with the software TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). DNADIST was utilized for distance analysis according to Kimura's two-parameter model (Kimura, 1980). Bootstrap values were determined based on 1000 replications.

The discriminatory power of the 16S rRNA gene is limited in the genus *Staphylococcus* (CLSI, 2007; Shah *et al.*, 2007), whereas the housekeeping genes *rpoB*, *hsp60*, *tuf* and *dnaJ* show more variation and are preferred over 16S rRNA gene sequencing for species identification (Zadoks & Watts, 2009). The cut-off values previously reported for species identification were 98.7 % for 16S rRNA (Jousson *et al.*, 2007), 94 % for *rpoB* (Mellmann *et al.*, 2006) and 97 % for *hsp60*, *tuf* and *dnaJ* (CLSI, 2007). For each of the genes, the sequences of the ten isolates were compared against each other. Within each gene, the sequence similarities were high, namely 99.6–100 % for the 16S rRNA gene sequence, 99.1–100 % for *dnaJ*, 99.7–100 % for *rpoB*, 98.6–100 % for *hsp60* and 99.6–100 % for *tuf*. The overall mean intraspecies divergence per housekeeping gene was 0.7 %, which is within the variability noted for *Staphylococcus* species (Shah *et al.*, 2007). The high similarities therefore indicate that the isolates probably belong to a single species. Neighbour-joining phylogenetic trees based on 16S rRNA (Fig. 1) and *dnaJ* (Supplementary Fig. S2) gene sequences were constructed. The phylogenetic position of the ten isolates was in the genus *Staphylococcus*, confirming the previous data. The topology of the maximum-parsimony tree was comparable (not shown). Sequencing of all five genes revealed seven different species to be the closest relatives, namely *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. pasteurii*, *S. warneri*, *S. caprae* and *S. piscifermentans* (not shown). Partial phylogenetic trees for the *rpoB*, *hsp60* and *tuf* genes, in which the unknown cluster and only these closest relatives were included, are shown in Supplementary Fig. S3. Because *S. piscifermentans* was distant from the unknown cluster in the 16S rRNA gene tree (Fig. 1), this species was not included in the

partial trees. Within each gene, sequences of the unknowns were compared to those of the three closest relatives based on the 16S rRNA gene (*S. haemolyticus*, *S. hominis* and *S. lugdunensis*) and similarities were calculated (Table 1). The similarities obtained were below the cut-off values used previously for species identification, which suggests that the isolates represent a novel *Staphylococcus* species.

In some phylogenetic trees, namely in those based on 16S rRNA, *dnaJ* and *hsp60* gene sequences, the isolates formed a cluster divided into subclusters, with KS-SDV 19 belonging to the smallest subcluster (Fig. 1 and Supplementary Figs S2 and S3b). Therefore, three of the unknown isolates (KS-SP 11, KS-SP 60^T and KS-SDV 19), taken from the subclusters and covering the total branch of the isolates in

Fig. 1. Phylogenetic tree, constructed using the neighbour-joining method, based on the 16S rRNA gene sequences of strains of *Staphylococcus devriesei* sp. nov. and reference sequences of *Staphylococcus* strains available online. *Macroccoccus caseolyticus* ATCC 13548^T was chosen as the outgroup. Bootstrap values, calculated from 1000 resamplings, are given at nodes if higher than 70 %. Bar, 1 % sequence divergence. *, Type strain of *Staphylococcus pulvereri*.

Table 1. Intraspecies and interspecies sequence similarity of the ten strains of *Staphylococcus devriesei* sp. nov.

Values are mean (range) percentage similarity. Sequences of the ten novel strains were compared with sequences from the type strains of the three phylogenetically most closely related species on the basis of the 16S rRNA gene sequence. Gene-specific cut-off percentages for each gene are given as cited in literature.

Strain(s)	16S rRNA	<i>dnaJ</i>	<i>rpoB</i>	<i>hsp60</i>	<i>tuf</i>
<i>S. devriesei</i> sp. nov. (<i>n</i> =10)	99.9 (99.6–100)	99.6 (99.1–100)	99.9 (99.7–100)	99.6 (98.6–100)	99.9 (99.6–100)
<i>S. haemolyticus</i> LMG 13349 ^T	99.2 (98.6–99.3)	83.4 (82.8–84.0)	92.4 (92.0–93.3)	90.0 (88.3–91.6)	95.5 (94.6–98.4)
<i>S. hominis</i> LMG 13348 ^T	98.9 (98.8–98.9)	82.6 (82.2–82.9)	90.7 (90.4–91.1)	89.5 (88.2–91.2)	94.5 (93.3–96.9)
<i>S. lugdunensis</i> LMG 13346 ^T	98.8 (98.8–98.9)	80.2 (79.8–80.9)	89.4 (89.0–90.4)	87.4 (86.0–89.3)	94.1 (93.0–97.6)
Gene-specific cut-off (%) [*]	98.7 ^a	97.0 ^b	94.0 ^c	97.0 ^b	97.0 ^b

^{*}Obtained from: *a*, Jousson *et al.* (2007); *b*, CLSI (2007); *c*, Mellmann *et al.* (2006).

all sequencing trees, were selected for DNA–DNA hybridizations. Hybridization was performed with the type strains of the three phylogenetically most closely related species based on 16S rRNA gene sequence analysis (*S. haemolyticus* LMG 13349^T, *S. hominis* LMG 13348^T and *S. lugdunensis* LMG 13346^T). DNA was extracted as described by Gevers *et al.* (2001). DNA–DNA hybridizations were carried out according to a modification of the microplate method (Ezaki *et al.*, 1989) as detailed by Goris *et al.* (1998) and Cleenwerck *et al.* (2002). The hybridization temperature was 34 °C. Reciprocal reactions (e.g. A × B and B × A) showed variations within the limits of the method, i.e. mean SD of 7 % (Goris *et al.*, 1998). DNA–DNA hybridizations revealed 97–99 % DNA binding between isolates KS-SP 11, KS-SP 60^T and KS-SDV 19. Although KS-SDV 19 belonged to a different subcluster based on 16S rRNA, *dnaJ* and *hsp60* gene sequences, it showed similarly high hybridization with KS-SP 11 and KS-SP 60^T and, therefore, hybridization of this strain with type strains of other species was not deemed necessary. There was a low level of DNA binding between isolates KS-SP 11 and KS-SP 60^T and the type strains *S. haemolyticus* LMG 13349^T (17 %), *S. hominis* LMG 13348^T (23 %) and *S. lugdunensis* LMG 13346^T (27 %). These data indicate that the ten isolates belong to a single novel species within the genus *Staphylococcus* (Wayne *et al.*, 1987).

The DNA base composition (mol%) of KS-SDV 16, KS-SP 11, KS-SP 16, KS-SP 20 and KS-SP 60^T was determined in triplicate by HPLC, according to Mesbah *et al.* (1989), using DNA extraction procedures described by Gevers *et al.* (2001). The DNA G + C content of KS-SDV 16, KS-SP 11, KS-SP 16 and KS-SP 20 was 33.7 mol% and that of KS-SP 60^T was 33.3 mol%. The range of DNA base composition is lower than 2 mol%, as generally accepted within a single species, and is consistent with that of members of the genus *Staphylococcus* (Kocur *et al.*, 1971).

Phenotypic characteristics of all ten isolates were obtained using API 50 CH with CHB/E medium (bioMérieux), API Staph ID 32 (bioMérieux) and Staph Zym (Rosco) test kits. Tube coagulase (coagulase plasma; Difco Laboratories) and clumping factor (Devriese, 1979) were also examined. The

DNase reaction was assessed using DNase agar (Oxoid). The phenotypic features of the ten isolates were compared with those of the three phylogenetically most closely related species based on 16S rRNA gene sequences (*S. haemolyticus*, *S. hominis* and *S. lugdunensis*). Differentiating biochemical characteristics are listed in Table 2 and individual phenotypes of each isolate are given in

Table 2. Phenotypic characteristics of *S. devriesei* sp. nov. compared with the phylogenetically most closely related *Staphylococcus* species

Species: 1, *S. devriesei* sp. nov. (for variable tests, reaction of strain KS-SP 60^T in brackets); 2, *S. haemolyticus*; 3, *S. hominis*; 4, *S. lugdunensis*. Closely related species were selected on the basis of the 16S rRNA gene sequence. Data for reference taxa were obtained from Freney *et al.* (1999). +, 90 % or more isolates positive; –, 90 % or more isolates negative; a, 11–50 % of isolates positive; b, 51–89 % of isolates positive; c, 11–89 % of isolates positive; ND, no data available. All tested strains were positive for acetyl methyl carbinol utilization and negative for starch utilization, alkaline phosphatase and β-galactosidase. Oxidation and fermentation of D-glucose was positive. Acid was produced from D-fructose, maltose, trehalose and L-sorbose. There was no acid production from amygdalin, D-adonitol, arabinose, arabitol, cellobiose, D-fucose, D-xylose, raffinose, D-sorbitol, D-tagatose, xylose, erythritol, gentiobiose, salicin or xylitol. Utilization of glycogen, inositol, inulin, potassium 2-ketogluconate, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside and methyl α-D-glucopyranoside was negative.

Characteristic	1	2	3	4
Arginine dihydrolase	+	+	c	–
β-Glucosidase	+	c	–	ND
Acid production from:				
D-Mannitol	b [+]	c	–	–
D-Mannose	a [–]	–	–	+
Melezitose	b [+]	–	c	ND
D-Ribose	+	c	–	–
Nitrate reduction	a [–]	c	c	+
Urease	b [+]	–	+	c

Supplementary Table S3. Tube coagulase, clumping and the DNase reaction were negative for all isolates. All isolates were considered sensitive to novobiocin.

In conclusion, the data from this study demonstrate that the ten isolates are members of a novel *Staphylococcus* species, for which the name *Staphylococcus devriesei* sp. nov. is proposed. In ongoing field studies in Belgium, additional isolates belonging to this species have been detected in milk and on teat apices of dairy heifers and cows.

Description of *Staphylococcus devriesei* sp. nov.

Staphylococcus devriesei (de.vrie'se.i. N.L. masc. gen. n. *devriesei* of Devriese, named in honour of the Belgian microbiologist Dr Luc A. Devriese for his contribution to the taxonomy of staphylococci).

This description is based on the characteristics of nine isolates originating from teat apices and one isolate originating from milk of dairy cows. Cells are Gram-stain-positive, non-spore-forming cocci that occur in pairs or small clusters. Colonies are 3–4 mm in diameter after 48 h of growth on Colombia blood agar with 5% sheep blood at 35 °C, have a zone of complete haemolysis of 1 mm and are grey–yellow, yellow or yellow–orange, smooth and glistening. Coagulase- and oxidase-negative and catalase-positive. Negative for starch utilization and alkaline phosphatase, β -glucuronidase and β -galactosidase activities. Acid is produced from D-galactose, lactose, D-ribose, sucrose, turanose, D-fructose, maltose, trehalose and L-sorbose. No acid production from melibiose, L-fucose, L-rhamnose, amygdalin, D-adonitol, arabinose, arabitol, cellobiose, D-fucose, D-lyxose, raffinose, D-sorbitol, D-tagatose, xylose, erythritol, gentiobiose, salicin or xylitol. Utilization of glycogen, dulcitol, inositol, inulin, potassium 2-ketogluconate, potassium 5-ketogluconate, pyrrolidonyl aminopeptidase, methyl α -D-mannopyranoside, methyl β -D-xylopyranoside and methyl α -D-glucopyranoside are negative. Acid is not produced from D-mannose for eight of the tested strains. Variable acid production is seen from D-mannitol (eight strains positive), melezitose (seven positive) and glycerol (nine positive). Aesculin ferric citrate reaction is weakly positive for nine of the strains, the remaining strain tested negative. Oxidation and fermentation of D-glucose is positive for all tested isolates. Utilization of acetyl methyl carbinol and arginine dihydrolase and β -glucosidase reactions are positive. Variable reactions are detected for the utilization of arbutin (four strains positive), potassium gluconate (seven positive) and N-acetylglucosamine (seven positive) and urease activity (six positive), as well as reduction of nitrate (four positive). The major fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0}, iso-C_{17:0} and C_{18:0}.

The type strain, KS-SP 60^T (=LMG 25332^T =CCUG 58238^T), was isolated from the teat apex of a Belgian dairy heifer; its characteristics are in agreement with those given in the species description. In addition, the type strain is

positive for acid production from D-mannitol, melezitose and glycerol, acid is not produced from D-mannose, the aesculin ferric citrate reaction is weakly positive, utilization of potassium gluconate, N-acetylglucosamine and arbutin is negative, urease activity is positive and nitrate is not reduced. The DNA G+C content of the type strain is 33.3 mol%. Details of the ten isolates studied are included in Supplementary Table S1.

Acknowledgements

This study was funded by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) (grant no. 61459) and by the Prime Minister's Services – Federal Office for Scientific, Technical and Cultural Affairs, Belgium. Arlette Vandekerckhove, Liesbeth Lebbe and An Coorevits are acknowledged for their excellent technical assistance and Venessa Eeckhaut for her assistance with bootstrap analysis.

References

- Cleenwerck, I., Vandemeulebroecke, K., Janssens, D. & Swings, J. (2002). Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. *Int J Syst Evol Microbiol* **52**, 1551–1558.
- CLSI (2007). *Interpretive criteria for microorganism identification by DNA target sequencing. Proposed Guideline*. CLSI document MM18-P (ISBN 1-56238-646-8). Wayne, PA: Clinical and Laboratory Standards Institute.
- Coenye, T., Enevold, F., Vancanneyt, M., Hoste, B., Govan, J. R. W., Kersters, K. & Vandamme, P. (1999). Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *Int J Syst Bacteriol* **49**, 405–413.
- De Vlieghe, S., Laevens, H., Devriese, L. A., Opsomer, G., Leroy, J. L., Barkema, H. W. & de Kruif, A. (2003). Prepartum teat apex colonization with *Staphylococcus chromogenes* in dairy heifers is associated with low somatic cell count in early lactation. *Vet Microbiol* **92**, 245–252.
- De Vlieghe, S., Opsomer, G., Vanrolleghem, A., Devriese, L. A., Sampimon, O. C., Sol, J., Barkema, H. W. & Haesebrouck, F. (2004). *In vitro* growth inhibition of major mastitis pathogens by *Staphylococcus chromogenes* originating from teat apices of dairy heifers. *Vet Microbiol* **101**, 215–221.
- Devriese, L. A. (1979). Identification of clumping factor negative staphylococci isolated from cows' udders. *Res Vet Sci* **27**, 313–320.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Freney, J., Kloos, W. E., Hajek, V., Webster, J. A., Bes, M., Brun, Y. & Vernozy-Rozand, C. (1999). Recommended minimal standards for description of new staphylococcal species. *Int J Syst Bacteriol* **49**, 489–502.
- Gevers, D., Huys, G. & Swings, J. (2001). Application of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31–36.

- Gillespie, B. E., Headrick, S. I., Boonyayatra, S. & Oliver, S. P. (2009). Prevalence and persistence of coagulase-negative *Staphylococcus* species in three dairy research herds. *Vet Microbiol* **134**, 65–72.
- Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol* **44**, 1148–1153.
- Hogan, J. S., Gonzles, R. N., Harmon, R. J., Nickerson, S. C., Oliver, S. P., Pankey, J. W. & Smith, K. L. (1999). *Laboratory Handbook on Bovine Mastitis*, revised edn. Madison, WI: National Mastitis Council.
- Jousson, O., Di Bello, D., Vanni, M., Cardini, G., Soldani, G., Pretti, C. & Intorre, L. (2007). Genotypic versus phenotypic identification of staphylococcal species of canine origin with special reference to *Staphylococcus schleiferi* subsp. *coagulans*. *Vet Microbiol* **123**, 238–244.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kocur, M., Bergan, T. & Mortensen, N. (1971). DNA base composition of Gram-positive cocci. *J Gen Microbiol* **69**, 167–183.
- Kotilainen, P., Huovinen, P. & Eerola, E. (1991). Application of gas-liquid chromatographic analysis of cellular fatty acids for species identification and typing of coagulase-negative staphylococci. *J Clin Microbiol* **29**, 315–322.
- Mellmann, A., Becker, K., van Eiff, C., Keckevoet, U., Schumann, P. & Harmsen, D. (2006). Sequencing and staphylococci identification. *Emerg Infect Dis* **12**, 333–336.
- Mergaert, J., Verhelst, A., Cnockaert, M. C., Tan, T.-L. & Swings, J. (2001). Characterization of facultative oligotrophic bacteria from polar seas by analysis of their fatty acids and 16S rDNA sequences. *Syst Appl Microbiol* **24**, 98–107.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Nahaie, M. R., Goodfellow, M., Minnikin, D. E. & Hajek, V. (1984). Polar lipid and isoprenoid quinine composition in the classification of *Staphylococcus*. *J Gen Microbiol* **130**, 2427–2437.
- Novkov, D., Pantek, R., Hublek, Z., Falsen, E., Busse, H.-J., Schumann, P. & Sedlek, I. (2010). *Staphylococcus microti* sp. nov., isolated from the common vole (*Microtus arvalis*). *Int J Syst Evol Microbiol* **60**, 566–573.
- Piepers, S. L., De Meulemeester, L., de Kruif, A., Opsomer, G., Barkema, H. W. & De Vlieghe, S. (2007). Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *J Dairy Res* **74**, 478–483.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schukken, Y. H., Gonzalez, R. N., Tikofsky, L. L., Schulte, H. F., Santisteban, G. C., Welcome, F. L., Bennett, G. J., Zurakowski, M. J. & Zadoks, R. N. (2009). CNS mastitis: nothing to worry about? *Vet Microbiol* **134**, 9–14.
- Shah, M. M., Iihara, H., Noda, M., Song, S. X., Nhung, P. H., Ohkusu, K., Kawamura, Y. & Ezaki, T. (2007). *dnaJ* gene sequence-based assay for species identification and phylogenetic grouping in the genus *Staphylococcus*. *Int J Syst Evol Microbiol* **57**, 25–30.
- Supr, K., De Vlieghe, S., Sampimon, O. C., Zadoks, R. N., Vaneechoutte, M., Baele, M., De Graef, E., Piepers, S. & Haesebrouck, F. (2009). Use of tRNA-intergenic spacer PCR combined with capillary electrophoresis to identify coagulase-negative *Staphylococcus* species originating from bovine milk and teat apices. *J Dairy Sci* **92**, 3204–3210.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tindall, B. J. (1990a). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.
- Tindall, B. J. (1990b). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Zadoks, R. N. & Watts, J. L. (2009). Species identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. *Vet Microbiol* **134**, 20–28.