***Globicatella sulfidifaciens* sp. nov., isolated from purulent infections in domestic animals**

¹ Laboratorium voor Microbiologie, Faculteit Wetenschappen, Universiteit Gent, Ledeganckstraat 35, B-9000 Ghent, Belgium

² Regional Veterinary Investigation Centre, Industrielaan 15, B8820 Torhout, Belgium

³ BCCMTM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Faculteit Wetenschappen, Universiteit Gent, Belgium

⁴ Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B9820 Merelbeke, Belgium

P. Vandamme,¹ J. Hommez,² C. Snauwaert,³ B. Hoste,³ I. Cleenwerck,³ K. Lefebvre,³ M. Vancanneyt,³ J. Swings,^{1,3} L. A. Devriese⁴ and F. Haesebrouck⁴

Author for correspondence: P. Vandamme. Tel: +32 9 264 51 13. Fax: +32 9 264 50 92.
e-mail: Peter.Vandamme@rug.ac.be

DNA–DNA hybridization experiments and comparative 16S rDNA sequence analysis revealed that six isolates from purulent joint and lung infections in calves, from a lung lesion in a sheep, and from a joint infection of a pig represented a novel species belonging to the genus *Globicatella*. Whole-cell protein electrophoresis and biochemical activity testing revealed that the isolates formed a homogeneous group differing from *Globicatella sanguinis*, the only species of this genus described to date. These animal isolates were classified as *Globicatella sulfidifaciens* sp. nov. with LMG 18844^T (= CCUG 44365^T), isolated from the lung of a calf, as the type strain. A detailed description of its phenotypic characteristics is presented. Hydrogen sulphide production was found to be a very useful diagnostic feature.

Keywords: *Globicatella sulfidifaciens* sp. nov., cattle, sheep, pig

INTRODUCTION

Globicatella has been described as a new genus comprising the single species *Globicatella sanguis* (Collins *et al.*, 1992). This specific epithet was later corrected to ‘*sanguinis*’ (Trüper & de’Clari, 1997). The description was based on the study of strains from human clinical sources including blood, urine and spinal fluid (Collins *et al.*, 1992). Phenotypically, the strains showed some similarity to *Streptococcus uberis* and the ‘*bovis*’ and ‘*viridans*’ streptococcal groups, as well as the aerococci. Later studies demonstrated that the genus is phylogenetically most closely related to the recently described *Emerococcus* genus, to *Ignavigranum*, *Facklamia* and *Abiotrophia*, and to the well-established genus *Aerococcus* (Collins *et al.*, 1999).

The present paper describes the characterization and classification of relatively slow-growing Gram-variable cocci from purulent infections in animals as members of the genus *Globicatella*. Despite having a certain resemblance to *G. sanguinis*, the animal isolates were distinct and appeared to constitute a new species

– *Globicatella sulfidifaciens* sp. nov. – a description of which is given below.

METHODS

Strains. The present study covers six field isolates which were obtained from severe (usually purulent) lesions in lungs of calves (three strains), from a lung sample from a lamb with acute bronchopneumonia, and from joint fluids of a pig and a calf with polyarthritis (one strain each) (Table 1). All were from necropsies carried out on animals from different farms in Belgium. The infections appeared to be sporadic and of a mixed nature. The bacteria were obtained in large numbers from lesions, but only one strain was found in pure culture in a joint-fluid sample from a calf. *Arcanobacterium pyogenes* was co-isolated in three out of the six cases. *G. sanguinis* reference strains and their sources are listed in Table 1.

SDS-PAGE of whole-cell proteins. All strains were grown for 24 h on brain–heart infusion (BHI) agar (Difco) and incubated at 36–37 °C in a microaerobic atmosphere containing approximately 5% O₂, 10% CO₂ and 85% N₂. Preparation of cellular protein extracts, PAGE, densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed as described by Pot *et al.* (1994) using the GelCompar version 4.2 software package (Applied Maths). The profiles were recorded and stored on a PC. The similarity between all pairs of traces was expressed by the Pearson product-moment correlation coefficient converted, for convenience, to a percentage value.

Abbreviations: AMC, 7-amino-4-methyl coumarin; MU, methyl-umbelliferyl.

The GenBank accession number for the 16S rRNA sequence of strain LMG 18844^T is AJ297627.

Table 1. *Globicatella* strains investigated

CCUG, Culture Collection, University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium.

Species and LMG no.	Received as:	Source
<i>G. sulfidifaciens</i>		
18837	GEM 618	Cattle lung
18839	T2137-2	Pig joint
18842	GEM 571	Sheep lung
18844 ^T	GEM 604 ^T	Cattle lung
18846	GEM 565	Cattle joint
18847	GEM 615	Cattle lung
<i>G. sanguinis</i>		
14367	CCUG 26778	Human urine
18987 ^T	CCUG 32999 ^T	Human blood
18988	CCUG 33000	Human blood
18996	CCUG 33367	Human blood

Preparation of high-molecular-weight DNA. High-molecular-weight DNA was prepared as described by Pitcher *et al.* (1989), modified as outlined below. Prior to lysis using guanidium thiocyanate, cells were incubated for 1 h at 37 °C in a buffer containing RNase A (200 mg l⁻¹; Sigma), mutanolysin (100 U ml⁻¹; Sigma) and lysozyme (250 mg ml⁻¹; Serva). This was followed by treatment with proteinase K (200 mg l⁻¹; Merck) for 15 min at 37 °C.

16S rRNA gene sequence analysis. Part of the rDNA operon, comprising the nearly complete 16S DNA, was amplified by using the PCR. The forward primer was 5'-CTGGCTCAGGAC/TGAACGCTG-3', corresponding to positions 19–38 of the *Escherichia coli* 16S rRNA numbering system. The reverse primer was 5'-AAGGAGGTGATCCAGCCGCA-3', complementary to positions 1541–1522 of the *E. coli* 16S rRNA numbering system. PCR-amplified 16S rDNAs were purified using the QIAquick PCR purification kit (Qiagen). Sequence analysis was performed using an Applied Biosystems 377 DNA sequencer and the protocols of the manufacturer (Perkin-Elmer/Applied Biosystems), using the BigDye Terminator Cycle Sequencing Ready Reaction kit (with AmpliTaq DNA Polymerase, Fs). The sequencing primers were those described by Coenye *et al.* (1999). Sequence assembly was performed by using the program AUTOASSEMBLER (Applied Biosystems). The consensus sequence of strain LMG 18844^T and the sequences of strains belonging to the same phylogenetic group (retrieved from the EMBL database) were aligned and a phylogenetic tree was constructed on the basis of the neighbour-joining method by using the BIONUMERICS software package (Applied Maths). The nearly complete sequences (1375 bases) of the 16S rRNA genes were used for the calculation of similarity levels and for tree construction; unknown bases were excluded from the calculations.

Determination of the DNA base composition. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC, using a Waters Symmetry Shield C8 column with the thermostat set at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% (v/v) acetonitrile. Non-methylated lambda-phage DNA (Sigma) was used as the calibration reference.

DNA–DNA hybridization. DNA–DNA hybridization experiments were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 50 °C in 50% (v/v) formamide. Each value given is the mean of at least two hybridization experiments.

Growth characteristics and biochemical activity. Cultural characteristics were studied as described previously (Devriese *et al.*, 1998). API 20 STREP, API 50CH (bioMérieux) and the BBLCRYSTAL Gram-positive ID System (Becton Dickinson) were used to test biochemical activity.

RESULTS AND DISCUSSION

SDS-PAGE of whole-cell protein

The whole-cell-protein profiles of the six *G. sanguinis*-like isolates were compared with each other and with those of *G. sanguinis* reference strains that were of human origin. The result of the numerical analysis is shown in Fig. 1. The six *G. sanguinis*-like isolates had virtually identical whole-cell protein profiles that were

Fig. 1. Dendrogram derived from the unweighted pair group average linkage of correlation coefficients between the whole-cell-protein patterns of all *Globicatella* strains examined.

Fig. 2. Neighbour-joining phylogenetic tree of *Globicatella sulfidifaciens* and related bacteria, based on a comparison of 1375 bases of the 16S rDNA sequences. Bar, 5% sequence dissimilarity.

clearly different from those of *G. sanguinis* reference strains. In addition, the latter reference isolates had very similar protein profiles.

16S rRNA gene sequence analysis

The 16S rDNA sequence of strain LMG 18844^T was examined to determine its phylogenetic position (GenBank no. AJ297627). The level of similarity to the 16S rRNA gene of *G. sanguinis* LMG 18987^T was 99.2%; the level of similarity to the 16S rRNA genes of other organisms was below 94%. Fig. 2 shows the result of neighbour-joining cluster analysis of the 16S rDNA sequences of strain LMG 18844^T and related bacteria.

DNA base composition analysis and DNA–DNA hybridization results

The close relationship between both taxa was confirmed by DNA base composition analysis and DNA–DNA hybridization experiments. DNA was prepared from strains LMG 18844^T and LMG 18842, and from the *G. sanguinis* type strain, LMG 18987^T. The DNA base ratio of strains LMG 18844^T and LMG 18842 was 35.8 mol%; that of LMG 18987^T was 35.7%. The DNA–DNA binding value between strains LMG 18844^T and LMG 18842 was 90%. The mean value for binding with *G. sanguinis* LMG 18987^T was calculated as 68%.

Morphology, growth characteristics and biochemical activity

The protein electrophoretic differences between these organisms were further supported by salient biochemical differences (Table 2). The cells were predominantly Gram-negative, although Gram-positive cells were always present in the preparations. Most cells tended to occur separately or in pairs, but chains were formed occasionally. Colonies on blood agar were pinpoint-sized after 1 d and became grey-white with a diameter of 0.5–1 mm after 2 d incubation. They appeared crumbly and dry when removed. Corrosion of the agar surface was seen underneath the colonies. Growth occurred at 25 °C but was better at 37 and 42 °C.

Supplementation of the incubation atmosphere with 5% CO₂ did not influence growth. The strains showed homogeneous growth after 1 d in BHI broth, but this sedimented after 2 d. Double-zone haemolysis (an inner, opaque, brownish zone and an outer, semi-transparent, greenish ring) was seen on sheep-blood agar.

The strains were unable to grow on bile–aesculin medium but they grew on Edwards agar, and hydrolysed aesculin on this medium. Growth was seen in 6.5% NaCl BHI broth after 2 d incubation. Most characteristically, the strains produced a zone of blackening along the stab inoculation in Kligler's iron agar, indicating hydrogen sulphide production from sulphur-bearing amino acids. These zones were narrow at the tops of the agar columns (in contact with air) and broadened towards the bottom. The slants and the remaining parts of the agar columns turned yellow because of glucose fermentation. Weak amylase production was evident on starch-containing plates. All strains were resistant to neomycins and to the macrolide antibiotics erythromycin, clindamycin and lincomycin. Other biochemical test results are given in Table 2.

Taxonomic position and identification of the *G. sanguinis*-like isolates

Together, these data indicate that the human and animal isolates examined are closely related and obviously represent the same genus. Given the clear biochemical differences between both taxa, the differences in whole-cell-protein patterns, and the mean DNA–DNA binding level of 68%, it is appropriate to accommodate the six animal isolates into a novel *Globicatella* species, for which we propose the name *G. sulfidifaciens*.

The production of hydrogen sulphide, as demonstrated by the deposition of black iron sulphide in Kligler and similar media, is a reaction widely used in the identification of Gram-negative bacteria. The test can be applied to the recognition of *Erysipelothrix*, but, otherwise, the ability to produce hydrogen in these media is apparently rare in Gram-positive bacteria. The four strains of *G. sanguinis* available for study

Table 2. Biochemical characteristics and differentiation between *Globicatella sulfidifaciens* and *Globicatella sanguinis*

Differential results are given in body of the table. *Globicatella sulfidifaciens* strains gave positive reactions in the following tests: α -galactosidase, 4-MU β -D-glucoside, 4-MU α -D-glucoside, L-pyrogutamic acid AMC, *p*-nitrophenyl β -D-glucoside, *p*-nitrophenyl β -D-cellobioside, L-tryptophan AMC (except LMG 18842), proline and leucine-*p*-nitroanilide, *p*-nitrophenyl α -D-maltoside, ONPG and *p*-nitrophenyl α -D-galactoside, acid production from arbutin, cellobiose, aesculin, D-glucose, glycogen, D-fructose, maltose, D-mannose, melibiose, D-raffinose, saccharose, starch, trehalose and D-xylose; the strains were negative in tests for Voges-Proskauer, hippurate, pyrrolidonyl arylamidase, alkaline phosphatase, arginine dihydrolase, urease, L-valine AMC, L-arginine AMC, 4-MU-phosphate, L-leucine AMC, L-isoleucine AMC, *p*-nitrophenyl-phosphate, acid from adonitol, D- and L-arabitol, amygdaline, D-arabinose, dulcitol, erythritol, galactose, β -gentiobiose, gluconate, methyl α -D-glucoside, *N*-acetyl D-glucosamine, glycerol, D- and L-fucose, inositol, lactose, D-lyxose, 2- and 5-ketogluconate, methyl α -D-mannoside, melezitose, rhamnose, sorbitol, L-sorbose, D-tagatose, turanose, xylitol, methyl β -xyloside and L-xylose. Variable reactions were seen with reagents for leucine arylamidase, β -galactosidase and L-arabinose.

Characteristic	<i>G. sulfidifaciens</i>	<i>G. sanguinis</i> *
H ₂ S production (Kligler's iron agar)	+	—
β -Galactosidase	—	+
β -Glucuronidase	+	—
Hippurate hydrolysis	—	+
L-Phenylalanine AMC hydrolysis	+	—
4-MU <i>N</i> -acetyl β -D-glucosaminide	—	+
4-MU- β -D-glucuronide hydrolysis	+	—
Acid from:		
Inulin	+	—
Mannitol	—	+
Maltotriose	—	+
Ribose	—	+
Methyl β -glucoside	—	+

* This table is based on the results of Collins *et al.* (1999) and our results obtained with the four collection strains used in this study for comparative purposes.

were negative in this test. This indicates that sulphide production is possibly useful in the search for *G. sulfidifaciens* isolates, and in their identification. However, as only a few strains of the two species were investigated, this characteristic should be used cautiously. Other characteristics differentiating the two *Globicatella* species are listed in Table 2. The differential scheme given by Collins *et al.* (1999) can be useful for differentiating the *Globicatella* strains from other recently described phylogenetically related genera. The reactions listed in this table, which may be of use with respect to *G. sanguinis*, are not, however, entirely applicable to *G. sulfidifaciens*. Notably, the positive hippurate, β -galactosidase, ribose and mannitol reactions cannot be used as genus-specific characteristics, since they are absent in *G. sulfidifaciens*. Acid production from melibiose appears to be a characteristic of the genus *Globicatella* not shared by the related genera as described to date.

G. sulfidifaciens was found most often in clinical samples from ruminants, despite the fact that this category of samples is not the most frequently examined in the laboratory in which the strains were

detected. As most of the strains were not isolated in pure culture, the pathological significance of these bacteria is a matter of conjecture. Certainly, their role in lung infections is secondary (much like the situation with *A. pyogenes*). The natural habitat of *G. sulfidifaciens* remains unknown.

Description of *Globicatella sulfidifaciens* sp. nov.

Globicatella sulfidifaciens (sul'fi.di.fa'ci.ens. L. n. *sulfidum* sulphide; L. v. *facere* produce; N.L. adj. *sulfidifaciens* sulphide-producing).

Gram-variable cocci showing the characteristics of Gram-positive cocci, occurring singly, in pairs and in short chains. Colonies are dry and corroding. Growth takes 2 d. α -Haemolytic. Facultatively anaerobic and catalase-negative. Grows in 6.5% NaCl but not on bile-aesculin agar. Glucose is fermented. Other characteristics are given in Table 2. G+C content of the DNA is 35–36 mol%. Isolated from lung and joint lesions in ruminants and a pig. Type strain is LMG 18844^T (= CCUG 44365^T), which was isolated from a bovine lung in Belgium; its G+C content is

35.8 mol%. Its biochemical characteristics are as described above for the species.

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