Macrococcus brunensis sp. nov., *Macrococcus hajekii* sp. nov. and *Macrococcus lamae* sp. nov., from the skin of llamas

Sylva Mannerová,¹ Roman Pantůček,² Jiří Doškař,² Pavel Švec,¹ Cindy Snauwaert,³ Marc Vancanneyt,³ Jean Swings³ and Ivo Sedláček¹

Correspondence

Ivo Sedláček

ivo@sci.muni.cz

¹Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Tvrdeho 14, 602 00 Brno, Czech Republic

²Department of Genetics and Molecular Biology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

³BCCM/LMG Bacteria Collection, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

Eight strains of Gram-positive, catalase- and oxidase-positive cocci were isolated from the skin of llamas (*Lama glama* L.) and characterized using a polyphasic approach. These strains were assigned to the genus *Macrococcus* on the basis of their phenotypic properties (resistance to bacitracin and sensitivity to furazolidone) and DNA base content (40–42 mol% G + C). Phylogenetic analysis based on 16S rDNA confirmed that the strains are members of the genus *Macrococcus*. They differed from all hitherto described macrococcal species in their production of phosphatase and reduction of nitrate (most strains) and the inability to produce acid from glycerol or to grow in 7.5 % NaCl. Ribotyping (*EcoRI*), macrorestriction analysis (*XbaI*) and fatty acid methyl ester analysis divided the strains from llamas into three stable clusters. Moreover, ribotyping differentiated the strains analysed not only from previously described macrococcal species but also from oxidase-positive staphylococci. DNA–DNA hybridization confirmed that the three clusters represent separate genomic groups (similarity values < 54 %). All the results showed that the strains represent three novel species, for which the names *Macrococcus hajekii* sp. nov. (type strain CCM 4809^T = LMG 21711^T), *Macrococcus brunensis* sp. nov. (type strain CCM 4811^T = LMG 21712^T) and *Macrococcus lamae* sp. nov. (type strain CCM 4815^T = LMG 21713^T) are proposed.

Gram-positive, catalase-positive cocci represent a large group of genera inhabiting different environments (water, soil, plants) as well as animals and humans. The skin and body surfaces of animals provide a large area for microbial colonization. Bacteria from these sources appear to be saprophytes, commensals or parasites, but they can also play an important role in various infections (Kloos *et al.*, 1992, 1998; Murray, 1992; Stackebrandt *et al.*, 1995). Staphylococcal and macrococcal strains are common on the skin of animals and may be differentiated from other Gram-positive, catalase- and oxidase-positive cocci by the G + C content of their DNA, their susceptibility to furazolidone (100 µg) (Baker, 1984; Kloos *et al.*, 1998) and resistance to bacitracin (0.04 U) (Falk & Guering, 1983; Kloos *et al.*,

1998). The genus *Macrococcus* was described by Kloos *et al.* (1998) with the reclassification of *Staphylococcus caseolyticus* Schleifer *et al.* 1982 as *Macrococcus caseolyticus* and the description of three novel species, *Macrococcus carouselicus*, *Macrococcus equiperficus* and *Macrococcus bovicus*. All these species originate from animal sources (ponies, horses, cows and a food-processing factory) (Kloos *et al.*, 1998). Macrococci are similar to oxidase-positive, novobiocin-resistant staphylococci (*Staphylococcus sciuri* complex) but differ in their somewhat higher G + C content, larger cells and the inability to produce acid from D-cellobiose (Kloos *et al.*, 1976, 1997, 1998; Webster *et al.*, 1994). Identification of macrococci at the species level on the basis of phenotypic tests alone is unreliable, and molecular and chemotaxonomic methods must be applied.

In the present study, eight atypical Gram-positive, catalase- and oxidase-positive cocci were isolated from the surface microflora of llamas. Phenotypic tests and selected molecular biological methods were used for their exact

Published online ahead of print on 16 May 2003 as DOI 10.1099/ijs.0.02683-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *M. hajekii* CCM 4809^T, *M. brunensis* CCM 4811^T and *M. lamae* CCM 4815^T are respectively AY119685–AY119687.

taxonomic characterization. All the results obtained indicated that these strains represent three hitherto undescribed macrococcal species, for which we propose the names *Macrococcus brunensis* sp. nov., *Macrococcus hajekii* sp. nov. and *Macrococcus lamae* sp. nov.

Bacterial strains

M. caseolyticus CCM 3540^T, *M. bovicus* CCM 4655^T and CCM 4656, *M. carouselicus* CCM 4650^T, CCM 4651 and CCM 4652, *M. equiperficus* CCM 4653^T and CCM 4654, *Staphylococcus vitulinus* CCM 4511^T, *Staphylococcus lentus* CCM 3472^T, *S. sciuri* subsp. *sciuri* CCM 3473^T, *S. sciuri* subsp. *rodentium* CCM 4657^T and CCM 4658 and *S. sciuri* subsp. *carnaticus* CCM 4835^T were used as reference strains. The strains isolated from llamas have been deposited in the Czech Collection of Microorganisms as *M. brunensis* CCM 4808, CCM 4810, CCM 4811^T (=LMG 21712^T) and CCM 4813, *M. lamae* CCM 4812, CCM 4814 and CCM 4815^T (=LMG 21713^T) and *M. hajekii* CCM 4809^T (=LMG 21711^T).

Strain isolation and cultivation

All the strains were isolated from the skin of llamas in Brno Zoo. Samples were collected using sterile swabs from non-hairy parts of the llama's bodies (nostrils, groin and abdomen, around the jaws and under the tail) and cultivated at 36 °C (body temperature of *Lama glama* L.) on nutrient agar (Oxoid) in the presence or absence of blood for 24–48 h.

Biotyping

Gram-stained cell morphology and cell arrangement were determined on P agar (Kloos *et al.*, 1992) after 24 h; colony morphology and pigment production were determined on P agar after 48–72 h. Cell size was assessed after 24 h on nutrient agar (Oxoid). Haemolysis was tested on tryptic soy agar (Difco) with 5 % sheep blood. Growth was observed on nutrient agar (Oxoid) at different salt concentrations (4, 7.5, 10 and 12 % NaCl) at 36 °C and at different temperatures (4, 15, 36 and 42 °C). Anaerobic growth was observed in thioglycolate semi-solid medium (Oxoid). Hydrolysis of Tween 80, gelatin (Páčová & Kocur, 1984), starch (Barrow & Feltham, 1993) and casein (Kurup & Babcock, 1979) and production of DNase (Oxoid), lecithinase (Owens, 1974) and catalase (Hanker & Rabin, 1975) was tested. Acid production from glycerol, D-salicin, D-sorbitol, D-galactose and melezitose (Anonymous, 1965) was studied by conventional methods. Oxidase (OXI test, Pliva-Lachema), pyrrolidonyl arylamidase (Pliva-Lachema) and coagulase (Itest-Plus) activity and the presence of clumping factor (Pastorex Staph Plus) were determined by commercial tests. Further biochemical properties were tested using API STAPH, ID 32 Staph and API ZYM kits (bioMérieux) according to the manufacturer's instructions. Antibiotic susceptibility was determined by disc-diffusion (Woods & Washington, 1995), involving incubation of cultures on

Mueller–Hinton agar (Oxoid) plates at 36 °C for 24 h with a standard set of antibiotic discs for staphylococci (Sanofi Diagnostic Pasteur, Pliva-Lachema, Itest-Plus). The following antibiotics discs were used: furazolidone (100 µg), bacitracin Taxo A (0.04 U), clindamycin (5 IU), novobiocin (1.6 µg), novobiocin (5 µg), oxacillin (1 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg), cephalothin (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), amoxicillin/clavulanic acid (20/10 µg), vancomycin (30 µg) and piperacillin/tazobactam (100/10 µg).

All strains were Gram-positive, catalase- and oxidase-positive cocci. Cells were arranged singly, in pairs and tetrads or in irregular clusters. Colonies grown at 36 °C were 2–5 mm in diameter after 24 h. Strains of *M. brunensis* sp. nov. and *M. hajekii* sp. nov. were non-pigmented; strains of *M. lamae* sp. nov. produced an orange pigment. None of the strains grew in the anaerobic portion of a thioglycolate semi-solid medium. The strains were non-haemolytic on sheep blood (5 %) agar. Phenotypic properties to distinguish the newly isolated taxa and to differentiate them from previously described macrococci and phenotypically closely related staphylococci are summarized in Table 1. All three novel species of macrococci from llamas are clearly separated from other previously described macrococci by their phosphatase production, absence of acid production from glycerol and inability to grow in 7.5 % NaCl. Strains of the three novel species can be distinguished from each other by a combination of the following tests: acid production from sucrose, esterase-lipase production, growth in 4 % NaCl and susceptibility to 5 µg novobiocin (Table 1). All the strains isolated are susceptible to furazolidone and resistant to bacitracin. They were also susceptible to clindamycin, oxacillin, trimethoprim/sulfamethoxazole, tetracycline, cephalothin, chloramphenicol, amoxicillin/clavulanic acid, gentamicin, vancomycin and piperacillin/tazobactam.

Phylogenetic analysis based on 16S rDNA sequences

The 16S rRNA gene was amplified using primers U24 (5'-AGAGTTTGATCAGGGCTCAGGATG-3') and L20 (5'-AAGGAGGTGATCCAGCCGCA-3'). PCR was performed according to Švec *et al.* (2001a). Both strands of the amplified DNA were sequenced by dideoxynucleotide chain termination sequencing with primers U24 and L20 followed by primer walking with forward primers P1 (5'-TTCGGATCGTAAACTCTGTTG-3') and P2 (5'-GATGGTACCAAGGGCAACGAAAC-3') and reverse primers P3 (5'-TCTCAGGTCGGCTACGCATCGTC-3') and P4 (5'-AATGATGGCAACTAAGGATAAGG-3'). Primer synthesis and DNA sequencing were performed according to Štěpán *et al.* (2001).

Calculations and data analyses were performed with PHYLIP version 3.6 (Felsenstein, 2001). For estimation of phylogenetic relationships among the species under study, a program was used that implements the maximum-likelihood

Table 1. Characteristics useful for differentiation of *M. brunensis* sp. nov., *M. hajekii* sp. nov. and *M. lamae* sp. nov., related macrococci and oxidase-positive staphylococci

Species: 1, *M. brunensis* sp. nov.; 2, *M. lamae* sp. nov.; 3, *M. hajekii* sp. nov.; 4, *M. caseolyticus*; 5, *M. carouselicus*; 6, *M. equipercicus*; 7, *M. bovicus* (data in columns 4–7 from Kloos *et al.*, 1998); 8, *S. lentus* (data from Kloos *et al.*, 1976; Webster *et al.*, 1994); 9, *S. vitulinus* (Webster *et al.*, 1994; Kloos & Bannerman, 1999); 10, *S. sciuri* subsp. *rodentium*; 11, *S. sciuri* subsp. *sciuri*; 12, *S. sciuri* subsp. *carnaticus* (data in columns 10–12 from Webster *et al.*, 1994; Kloos *et al.*, 1997). +, Positive; –, negative; d, variable reaction; ND, no data. The phenotypic profile for *M. hajekii* sp. nov. was determined on the basis of results for a single strain.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
G + C content (mol%)	42	41	40	38–39	41	45	42–44	34	34	35	35–36	36
Resistance to novobiocin (5 µg)	+	–	+	+	+	+	+	+	+	+	+	+
Pigment production	–	+	–	–	d	+	d	d	d	d	d	d
Growth in:												
4 % NaCl	+	–	–	+	+	+	+	+	+	+	+	+
7.5 % NaCl	–	–	–	+	+	+	+	+	+	+	+	+
Acid from:												
Glycerol	–	–	–	d	d	+	+	+	+	+	+	+
Sucrose	–	+	+	d	d	d	d	+	+	+	+	+
D-Cellobiose	–	–	–	–	–	–	–	+	d	d	+	d
Hydrolysis of:												
Aesculin	–	–	–	d	+	d	d	+	d	+	+	+
DNA	–	–	–	d	+	–	d	+	ND	ND	ND	d
Phosphatase production	+	+	+	–	–	–	–	+	–	d	d	d
Esterase-lipase production	–	+	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
Nitrate reduction	+	–	+	+	–	–	d	+	d	+	+	+

method for DNA sequences implementing the Hidden Markov Model method of inferring different rates of evolution of different sites (Felsenstein & Churchill, 1996).

The 16S rDNA sequences of type strains from each proposed macrococcal species were determined and compared with 16S rDNA sequences of other species of macrococci, oxidase-positive species of staphylococci and selected oxidase-negative staphylococci (Fig. 1). The phylogenetic positions on the tree confirm that the type strains of *M. brunensis* sp. nov., *M. hajekii* sp. nov. and *M. lamae* sp. nov. are members of the genus *Macrococcus*. Levels of 16S rDNA sequence similarity among the newly described species are in the range found in known macrococcal species, 98–99 %, except for *M. caseolyticus*, which shows lower similarity of only 97 %. Most differences in the 16S rDNA sequence (predominate transitions) were found in the region 61–228. The sequences of *M. brunensis* CCM 4811^T and *M. bovicus* CCM 4655^T are characterized by two 1 nt deletions at positions 72 and 84, where A and/or T are present in the other macrococcal species. In *M. lamae* CCM 4815^T and *M. brunensis* CCM 4811^T, there is a 1 nt insertion of G at position 217. The sequence of *M. hajekii* CCM 4809^T differs from the other species in a 1 nt insertion at position 568 and is closely related to the sequences of *M. equipercicus* CCM 4653^T and *M. carouselicus* CCM 4650^T.

Ribotyping

Isolation of genomic DNA and ribotyping were performed according to Švec *et al.* (2001b), modified only by using

brain heart infusion broth (Oxoid) for cultivation. Hybridization profile analysis and cluster analysis were done using GelCompar II software (Applied Maths). The dendrogram was calculated by UPGMA clustering using the Jaccard coefficient.

The dendrogram (Fig. 2) shows ribotyping (*EcoRI*) results of representative strains selected from all species analysed. Ribotyping clearly separated the macrococci and staphylococci (similarity was only 17 %). Furthermore, the hybridization profiles of all the macrococcal species are clearly distinguished. All three *M. lamae* strains have identical hybridization profiles. The *M. brunensis* strains formed one cluster, with 75 % similarity. The profile of *M. hajekii* confirmed its separate position.

Fatty acid methyl ester (FAME) analysis

Cultures were grown for 24 h at 28 °C on plates containing 30 g trypticase soy broth (BBL) supplemented with 15 g Bacto agar (Difco) l^{–1} distilled water. Cells were saponified, methylated to FAMES and extracted following the Sherlock Microbial Identification System version 3.0 (MIDI, 1999). FAMES were separated on an Agilent 6890A series gas chromatograph with 7683 autoinjector and autosampler tray module (Agilent Technologies). Separation of FAMES was achieved with a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5 % phenylmethyl silicone (film thickness 0.33 µm; HP Ultra2). H₂ served as carrier gas. Peak integration and identification were performed using

Fig. 1. Phylogenetic tree constructed on the basis of 16S rDNA sequences shows the relationship between *M. brunensis* sp. nov., *M. hajekii* sp. nov. and *M. lamae* sp. nov., previously described macrococci, oxidase-positive staphylococci and selected oxidase-negative staphylococci. *S.*, *Staphylococcus*. GenBank accession numbers are given in parentheses.

the Hewlett Packard Chemstation software and Sherlock software.

The results of this analysis are summarized in Table 2. The novel macrococcal species differ from one another and from previously described species in the composition and percentages of FAMES. Among the novel taxa, *M. brunensis* and *M. hajekii* strains contain predominantly saturated branched-chain (anteiso-15:0 and iso-15:0) and

unsaturated straight-chain (18:1 ω 9c, 16:1 ω 11c and iso-17:1 ω 10c) fatty acids. *M. lamae* strains contain predominantly saturated branched-chain (iso-15:0) and saturated straight-chain (16:0, 14:0 and 20:0) fatty acids.

Macrorestriction pattern analysis

Preparation of chromosomal DNAs, digestion with *Xba*I, *Not*I and *Sma*I (Roche Diagnostics) and macrorestriction

Fig. 2. Dendrogram of ribotyping (*Eco*RI) profiles of *M. brunensis* sp. nov., *M. hajekii* sp. nov. and *M. lamae* sp. nov. and other macrococcal species and oxidase-positive staphylococci.

Table 2. Fatty acid contents of whole-cell hydrolysates of *Macrococcus* strains

Strains: 1–4, *M. brunensis* sp. nov. strains CCM 4808 (1), CCM 4810 (2), CCM 4811^T (3) and CCM 4813 (4); 5, *M. hajekii* sp. nov. CCM 4809^T; 6–8, *M. lamae* sp. nov. strains CCM 4812 (6), CCM 4814 (7) and CCM 4815^T (8); 9, *M. bovicus* CCM 4655^T; 10, *M. caseolyticus* CCM 3540^T; 11, *M. carouselicus* CCM 4650^T; 12, *M. equipercicus* CCM 4653^T. Values are mean percentages of total FAMES. Only fatty acids accounting for more than 1.0% (mean amount) are indicated. –, Not detected; tr, trace amount (<1.0%). TBSA, Tuberculostearic acid (10-methyl-18:0).

Strain	1	2	3	4	5	6	7	8	9	10	11	12
iso-13:0	4.1	4.6	6.0	4.4	7.5	6.3	5.0	7.6	5.5	–	6.6	tr
anteiso-13:0	tr	tr	1.1	tr	tr	tr	tr	1.3	tr	–	tr	–
14:0	tr	2.8	1.8	2.2	2.2	15.6	19.6	14.1	1.4	16.5	10.2	–
iso-14:0	1.8	1.7	1.8	1.3	2.0	1.9	1.6	1.7	1.3	7.9	2.0	1.1
iso-15:0	14.5	11.9	12.6	13.4	16.0	22.1	17.2	26.1	10.4	–	4.9	24.7
anteiso-15:0	30.6	23.2	21.1	24.7	19.8	7.2	5.7	9.2	18.8	–	1.9	31.8
ECL 15:669	–	–	tr	tr	–	4.8	3.6	5.7	tr	–	–	4.4
16:0	tr	2.3	2.6	2.6	1.3	13.6	16.8	11.1	6.0	6.6	13.6	tr
Summed feature 4*	4.5	2.1	1.9	2.2	1.4	–	–	–	1.6	–	–	12.5
16:1 ω 11c	7.8	12.6	6.5	9.1	16.4	5.4	6.1	6.0	4.6	29.5	18.0	1.3
16:1 ω 7c alcohol	tr	–	tr	–	–	–	–	–	–	tr	–	2.6
16:0 N alcohol	1.0	1.3	tr	1.1	1.0	–	–	–	1.2	–	6.9	–
iso-17:0	tr	tr	1.0	tr	–	1.0	tr	tr	1.6	–	tr	1.1
anteiso-17:0	1.1	tr	1.1	1.0	–	–	–	–	1.4	–	–	1.3
iso-17:1 ω 10c	15.4	8.3	8.3	7.7	8.1	1.0	1.0	1.6	8.5	–	1.1	12.0
18:0	tr	2.2	3.0	2.6	tr	4.7	4.9	3.5	10.0	1.4	6.5	–
18:1 ω 9c	10.6	20.0	22.4	19.4	18.8	1.9	1.8	1.6	16.0	32.6	17.1	tr
18:3 ω 6c	tr	tr	1.1	1.4	3.3	–	–	–	5.5	–	8.2	–
TBSA	1.6	1.3	1.8	1.1	tr	–	–	–	2.0	–	–	3.1
20:0	tr	1.5	2.6	1.8	–	12.8	13.0	9.7	2.4	–	1.1	tr
20:1 ω 9c	1.6	1.1	1.4	1.2	tr	–	tr	–	–	2.0	–	tr

*Summed feature 4 consists of one or more of the following fatty acids, which could not be separated by the Microbial Identification System: 17:1 iso I, 17:1 anteiso B.

pattern analysis were performed as described by Pantůček *et al.* (1999) with the following modifications. The CHEF-Mapper (Bio-Rad) and multistate program conditions were: field inversion gel electrophoresis with increasing pulse times of 0.05 to 1.0 s for 7 h with forward and reverse voltage 8.5 and 5.5 V cm⁻¹, respectively, followed by standard PFGE with reorientation angle 120°, pulse times 4.0–20.0 s and voltage 5.5 V cm⁻¹ for 17 h. Concatemers of lambda phage DNA (Sigma) and molecular mass markers II and X (Roche Diagnostics) were used as size standards.

NotI cleaved the chromosomes of the newly proposed macrococcal species into small numbers of fragments (2–8), so the results were unsuitable for similarity determination. *SmaI* cleaved them into a larger number of fragments, showing substantial size differences in comparison with other macrococcal species. Restriction endonuclease *XbaI* was the most satisfactory, since it cleaved all the macrococcal chromosomes into 30–50 fragments that exhibited comparable size ranges; comparison of profiles demonstrated that *M. brunensis*, *M. lamae* and *M. hajekii* strains differed from the type strains of previously described macrococcal species (Fig. 3). All four strains of *M. brunensis* (CCM 4808, CCM

4810, CCM 4811^T and CCM 4813) had very similar macrorestriction patterns (98–99% similarity), differing in only one or two fragments. Likewise, the three *M. lamae*

Fig. 3. Dendrogram based on cluster analysis of macrorestriction fragments of *M. brunensis* sp. nov., *M. hajekii* sp. nov., *M. lamae* sp. nov. and type strains of previously described *Macrococcus* species.

strains formed one cluster with similarities of 97–100 %. *M. hajekii* CCM 4809^T showed unique macrorestriction patterns, different from those of *M. brunensis* and *M. lamae* strains.

DNA base compositions

DNA was isolated as described for DNA–DNA hybridization and degraded enzymically 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 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 calibration reference.

The G + C contents of the newly isolated strains ranged from 40 to 42 mol%, values which are within the range of those described for *Macrococcus* species (Kloos *et al.*, 1998) (Table 3). These values are higher than those of staphylococcal species (30–39 mol%; Kloos *et al.*, 1992) and significantly lower than those of other Gram-positive, oxidase- and catalase-positive cocci belonging to genera such as *Kocuria*, *Dermaecoccus*, *Kytococcus*, *Micrococcus* and *Deinococcus*, in which the G + C content is 62–76 mol% (Kloos *et al.*, 1992, 1998; Murray, 1992; Stackebrandt *et al.*, 1995).

DNA–DNA hybridization

The strains were cultivated on nutrient agar (Oxoid) at 28 °C for 24 h with the exception of *M. carouselicus* CCM 4650^T, which was cultivated in trypticase soy broth (BBL) at 28 °C for 7 h. DNA was extracted from 0.75–1.25 g wet weight of cells using the protocol described by Pitcher *et al.* (1989) with the following modifications. For strains *M. caseolyticus* CCM 3540^T, *M. equipercicus* CCM 4653^T, *M. hajekii* CCM 4809^T, *M. brunensis* CCM 4811^T and CCM 4813 and *M. lamae* CCM 4815^T, the washed cell pellet was resuspended and lysed in a buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) containing RNase (200 µg ml⁻¹; Sigma), mutanolysin (100 U ml⁻¹; Sigma) and lysozyme (25 mg ml⁻¹; SERVA) for 1 h at 37 °C. For strains

M. bovicus CCM 4655^T and *M. carouselicus* CCM 4650^T, lysostaphin (120 U ml⁻¹; Sigma) was used instead of mutanolysin. Before addition of GES reagent, proteinase K (200 µg ml⁻¹; Merck) was added to the mixture for 15 min. DNA–DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 35 °C in hybridization mixture (2 × SSC, 5 × Denhardt’s solution, 2.5 % dextran sulfate, 50 % formamide, 100 µg denatured salmon sperm DNA ml⁻¹ and 1250 ng biotinylated probe DNA ml⁻¹).

The DNA–DNA hybridization results are shown in Table 3. Representative strains from each phenotypic and ribotyping group were chosen to perform DNA–DNA hybridization experiments. The DNA–DNA relatedness values between previously described and newly proposed macrococcal species were lower than 54 %. These data confirmed that the strains from llamas represent three novel species.

Description of *Macrococcus brunensis* sp. nov.

Macrococcus brunensis (bru.nen’sis. L. adj. *brunensis* from *Bruna*, the Roman name of the city of Brno, Czech Republic, where the type strain was isolated).

Colonies reach 2–4 mm in diameter on P agar after 24 h. Cell diameter is 0.89–1.21 µm. Colonies are circular, smooth and glossy, without pigment. Growth is detected under anaerobic conditions at 15–36 °C and in 4 % NaCl but not at 4 or 42 °C. All strains hydrolyse casein and gelatin, but not Tween 80, starch, lecithin, aesculin or tyrosine. Alkaline and acid phosphatases are produced, nitrates are reduced and acid is produced from maltose, D-mannitol, D-fructose, D-trehalose and D-glucose. Acetoin, clumping factor and coagulase are not produced and activities of urease, haemolysis, arginine dihydrolase, arginine arylamidase, ornithine decarboxylase, β-galactosidase, β-glucuronidase, pyrrolidonyl arylamidase, esterase (C4), lipase (C14),

Table 3. G + C contents and DNA–DNA hybridization values of *Macrococcus* strains

Strain	DNA G + C content (mol%)	DNA–DNA hybridization with DNA from: (%)							
		1	2	3	4	5	6	7	8
1. <i>M. bovicus</i> CCM 4655 ^T	45	100							
2. <i>M. carouselicus</i> CCM 4650 ^T	41*	40	100						
3. <i>M. caseolyticus</i> CCM 3540 ^T	37	13	17	100					
4. <i>M. equipercicus</i> CCM 4653 ^T	44	25	33	16	100				
5. <i>M. hajekii</i> CCM 4809 ^T	40	31	43	18	24	100			
6. <i>M. brunensis</i> CCM 4811 ^T	42	54	49	19	26	34	100		
7. <i>M. brunensis</i> CCM 4813	42	53	45	20	28	35	100	100	
8. <i>M. lamae</i> CCM 4815 ^T	41	29	30	16	37	23	30	28	100

*Data from Kloos *et al.* (1998).

naphthol-AS-BI-phosphohydrolase, valine arylamidase, cystine arylamidase, α -galactosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase and esterase-lipase (C8) are negative. Acid is not produced from sucrose, melezitose, xylose, arabinose, turanose, xylitol, D-sorbitol, D-cellobiose, D-salicin, raffinose, D-galactose, lactose, ribose, D-melibiose, D-mannose, *N*-acetylglucosamine or methyl α -D-glucoside. All strains are resistant to novobiocin. Predominant fatty acids are iso-13:0, iso-15:0, anteiso-15:0, 16:1 ω 11*c*, iso-17:1 ω 10*c* and 18:1 ω 9*c* (Table 2). The G+C content of the DNA is 41–42 mol%.

The type strain, CCM 4811^T (= LMG 21712^T), was isolated from llama skin. Its characteristics are in full agreement with the species description. The G+C content of the DNA is 42 mol%.

Description of *Macrococcus hajekii* sp. nov.

Macrococcus hajekii (ha.je'ki.i. N.L. gen. n. *hajekii* of Hájek, named after Václav Hájek, a Czech microbial taxonomist).

Colonies reach 2–3 mm in diameter on P agar after 24 h. Mean cell diameter is 0.89 μ m. Colonies are circular, smooth and glossy, without pigment. Does not grow at 4 or 42 °C or in 4 % NaCl, but grows at 15–36 °C. Hydrolyses casein and gelatin, but not Tween 80, starch, lecithin, aesculin or tyrosine. Alkaline phosphatase is produced, nitrates are reduced and acid is produced from sucrose, maltose, D-mannitol, D-fructose, D-trehalose and D-glucose. Acetoin, clumping factor and coagulase are not produced and activities of urease, haemolysis, arginine dihydrolase, arginine arylamidase, ornithine decarboxylase, β -galactosidase, β -glucuronidase, pyrrolidonyl arylamidase, esterase (C4), lipase (C14), naphthol-AS-BI-phosphohydrolase, valine arylamidase, cystine arylamidase, α -galactosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, esterase-lipase (C8) and acid phosphatase are negative. Acid is not produced from melezitose, xylose, arabinose, turanose, D-sorbitol, D-cellobiose, xylitol, D-salicin, raffinose, D-galactose, lactose, ribose, D-melibiose, D-mannose, *N*-acetylglucosamine or methyl α -D-glucoside. Resistant to novobiocin. Dominant fatty acids are iso-13:0, iso-15:0, anteiso-15:0, 16:1 ω 11*c*, iso-17:1 ω 10*c*, 18:1 ω 9*c* and 18:3 ω 6*c* (Table 2). The G+C content of the DNA is 40 mol%.

The type strain, CCM 4809^T (= LMG 21711^T), was isolated from llama skin.

Description of *Macrococcus lamae* sp. nov.

Macrococcus lamae (la'mae. N.L. fem. gen. n. *lamae* of *Lama*, the zoological genus name of the llama).

Colonies reach 2–5 mm in diameter on P agar after 24 h. Cell diameter is 0.74–0.92 μ m. Colonies are circular, smooth and glossy and have an orange pigment. All the strains tested grow at 15–36 °C. Growth is not detected under anaerobic conditions, in 4 % NaCl or at 4 or 42 °C.

Strains hydrolyse casein and gelatin, but not Tween 80, starch, lecithin, aesculin or tyrosine. Alkaline phosphatase and esterase-lipase (C8) are produced. Acid is produced from sucrose, maltose, D-mannitol, D-fructose, D-trehalose and D-glucose. Acetoin, clumping factor and coagulase are not produced and activities of urease, haemolysis, arginine dihydrolase, arginine arylamidase, ornithine decarboxylase, β -galactosidase, β -glucuronidase, pyrrolidonyl arylamidase, esterase (C4), lipase (C14), naphthol-AS-BI-phosphohydrolase, valine arylamidase, cystine arylamidase, α -galactosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase are not observed. Nitrate is not reduced. Acid is not produced from melezitose, xylose, arabinose, turanose, D-sorbitol, D-cellobiose, D-salicin, raffinose, xylitol, D-galactose, lactose, ribose, D-melibiose, D-mannose, *N*-acetylglucosamine or methyl α -D-glucoside. Strain CCM 4812 does not produce acid phosphatase. Susceptible to novobiocin (5 μ g) and intermediate susceptibility to novobiocin (1.6 μ g). Predominant fatty acids are iso-13:0, 14:0, iso-15:0, anteiso-15:0, ECL 15:669, 16:0, 16:1 ω 11*c*, 18:0 and 20:0 (Table 2). The G+C content of the DNA is 41–42 mol%.

The type strain, CCM 4815^T (= LMG 21713^T), was isolated from llama skin. Its characteristics are in full agreement with the species description. The G+C content of the DNA is 41 mol%.

Acknowledgements

We would like to thank to Anthony Long for English correction of the manuscript. Special thanks to H. G. Trüper for checking the nomenclature. This study was partially supported by the projects of the Grant Agency of the Czech Republic (project numbers 301/02/1505 and 204/02/D099) and by long-term research program no. MSM143100008 of the Ministry of Education of the Czech Republic.

References

- Anonymous (1965).** Subcommittee on taxonomy of staphylococci and micrococci. Recommendations. *Int Bull Bacteriol Nomencl Taxon* **15**, 109–110.
- Baker, J. S. (1984).** Comparison of various methods for differentiation of staphylococci and micrococci. *J Clin Microbiol* **19**, 875–879.
- Barrow, G. I. & Feltham, R. K. A. (1993).** *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 2nd edn. Cambridge: Cambridge University Press.
- 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.
- Falk, D. & Guering, S. J. (1983).** Differentiation of *Staphylococcus* and *Micrococcus* spp. with the Taxo A bacitracin disk. *J Clin Microbiol* **18**, 719–721.
- Felsenstein, J. (2001).** PHYLIP: phylogeny inference package. Department of Genetics, University of Washington, Seattle, USA. <http://evolution.genetics.washington.edu/phylip.html>.
- Felsenstein, J. & Churchill, G. A. (1996).** A Hidden Markov Model approach to variation among sites in rate of evolution. *Mol Biol Evol* **13**, 93–104.

- Hanker, J. S. & Rabin, A. N. (1975). Color reaction streak test for catalase-positive microorganisms. *J Clin Microbiol* **2**, 463–464.
- Kloos, W. E. & Bannerman, T. L. (1999). *Staphylococcus* and *Micrococcus*. In *Manual of Clinical Microbiology*, 7th edn, pp. 264–282. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Tenover. Washington, DC: American Society for Microbiology.
- Kloos, W. E., Schleifer, K. H. & Smith, R. F. (1976). Characterization of *Staphylococcus sciuri* sp. nov. and its subspecies. *Int J Syst Bacteriol* **26**, 22–37.
- Kloos, W. E., Schleifer, K. H. & Götz, F. (1992). The genus *Staphylococcus*. In *The Prokaryotes: a Handbook on the Biology of Bacteria. Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. 2, pp. 1369–1403. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Kloos, W. E., Ballard, D. N., Webster, J. A. & 12 other authors (1997). Ribotype delineation and description of *Staphylococcus sciuri* subspecies and their potential as reservoirs of methicillin resistance and staphylolytic enzyme genes. *Int J Syst Bacteriol* **47**, 313–323.
- Kloos, W. E., Ballard, D. N., George, C. G., Webster, J. A., Hubner, R. J., Ludwig, W., Schleifer, K. H., Fiedler, F. & Schubert, K. (1998). Delimiting the genus *Staphylococcus* through description of *Macroccoccus caseolyticus* gen. nov., comb. nov. and *Macroccoccus equipericus* sp. nov., *Macroccoccus bovicus* sp. nov. and *Macroccoccus carouselicus* sp. nov. *Int J Syst Bacteriol* **48**, 859–877.
- Kurup, V. P. & Babcock, J. B. (1979). Use of casein, tyrosine, and hypoxanthine in the identification of nonfermentative Gram-negative bacilli. *Med Microbiol Immunol* **167**, 71–75.
- 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.
- MIDI (1999). *Sherlock Microbial Identification System Operating Manual*, version 3. Newark, DE: MIDI Inc.
- Murray, R. G. E. (1992). The genus *Deinococcus*. In *The Prokaryotes: a Handbook on the Biology of Bacteria. Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. 2, pp. 1369–1403. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Owens, J. J. (1974). The egg yolk reaction produced by several species of bacteria. *J Appl Bacteriol* **37**, 137–148.
- Páčová, Z. & Kocur, M. (1984). New medium for detection of esterase and gelatinase activity. *Zentbl Bakteriol Mikrobiol Hyg A* **258**, 69–73.
- Pantůček, R., Sedláček, I., Doškař, J. & Rosypal, S. (1999). Complex genomic and phenotypic characterization of the related species *Staphylococcus carnosus* and *Staphylococcus piscifermentans*. *Int J Syst Bacteriol* **49**, 941–951.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Schleifer, K. H., Kilpper-Bälz, R., Fischer, U., Faller, A. & Endl, J. (1982). Identification of “*Micrococcus candidus*” ATCC 14852 as a strain of *Staphylococcus epidermidis* and of “*Micrococcus caseolyticus*” ATCC 13548 and *Micrococcus varians* ATCC 29750 as members of a new species, *Staphylococcus caseolyticus*. *Int J Syst Bacteriol* **32**, 15–20.
- Stackebrandt, E., Koch, C., Gvozdiak, O. & Schumann, P. (1995). Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *Int J Syst Bacteriol* **45**, 682–692.
- Štěpán, J., Pantůček, R., Růžicková, V., Rosypal, S., Hájek, V. & Doškař, J. (2001). Identification of *Staphylococcus aureus* based on PCR amplification of species specific genomic 826 bp sequence derived from a common 44-kb *Sma*I restriction fragment. *Mol Cell Probes* **15**, 249–257.
- Švec, P., Devriese, L. A., Sedláček, I., Baele, M., Vancanneyt, M., Haesebrouck, F., Swings, J. & Doškař, J. (2001a). *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., isolated from water. *Int J Syst Evol Microbiol* **51**, 1567–1574.
- Švec, P., Sedláček, I., Pantůček, R., Devriese, L. A. & Doškař, J. V. (2001b). Evaluation of ribotyping for characterization and identification of *Enterococcus haemoperoxidus* and *Enterococcus moraviensis* strains. *FEMS Microbiol Lett* **203**, 23–27.
- Webster, J. A., Bannerman, T. L., Hubner, R. J., Ballard, D. N., Cole, E. M., Bruce, J. L., Fiedler, F., Schubert, K. & Kloos, W. E. (1994). Identification of the *Staphylococcus sciuri* species group with *Eco*RI fragments containing rRNA sequences and description of *Staphylococcus vitulus* sp. nov. *Int J Syst Bacteriol* **44**, 454–460.
- Woods, G. L. & Washington, J. A. (1995). Antibacterial susceptibility tests: dilution and disk-diffusion methods. In *Manual of Clinical Microbiology*, 6th edn, pp. 1327–1341. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Tenover. Washington, DC: American Society for Microbiology.