Macrococcus canis sp. nov., a skin bacterium associated with infections in dogs

Stefanie Gobeli Brawand, Kerstin Cotting, Elena Gómez-Sanz,† Alexandra Collaud, Andreas Thomann, Isabelle Brodard, Sabrina Rodriguez-Campes, Christian Strauss and Vincent Perreten*

Abstract

Gram-stain-positive cocci were isolated from miscellaneous sites of the skin of healthy dogs as well as from infection sites in dogs. The closest relative by sequencing of the 16S rRNA gene was Macrococcus caseolyticus with 99.7 % sequence identity, but compared with M. caseolyticus, the novel strains shared only 90.8 to 93.5 % DNA sequence identity with cpn60, dnaJ, rpoB and sodA partial genes, respectively. The novel strains also exhibited differential phenotypic characteristics from M. caseolyticus, and the majority displayed a visible haemolysis on sheep blood agar, while M. caseolyticus did not have any haemolytic activity. They generated different matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) MS spectral profiles compared with the other species of the genus Macrococcus. Furthermore, strain KM 45013T shared only 53.7 % DNA–DNA relatedness with the type strain of M. caseolyticus, confirming that they do not belong to the same species. The DNA G+C content of strain KM 45013T was 36.9 mol%. The most abundant fatty acids were \( C_{16:0} \); \( C_{18:2\omega 6c} \) (6, 9, 12) and \( C_{16:1\alpha 7c} \) alcohol. MK-6 was the menaquinone type of KM 45013T. Cell-wall structure analysis revealed that the peptidoglycan type was \( A3\alpha \), \( \text{L-Lys-Gly}_{2} \), \( \text{L-Ser} \). Based on genotypic and chemotaxonomic characteristics, we propose to classify these strains within a novel species of the genus Macrococcus for which the name Macrococcus canis sp. nov. is proposed. The type strain is KM 45013T (=DSM 101690'=CCOS 969'=CCUG 68920').

The genus Macrococcus contains seven species at the time of writing (Macrococcus caseolyticus, M. carouselicus, M. equipericus, M. bovicus, M. brunensis, M. hajekii and M. lamae) and consists of Gram-stain-positive bacteria which have been found on the skin of different mammals as well as in milk and meat [1–3]. The recent isolation of a haemolytic strain (strain KM 45013T) from a Bernese mountain dog with mucopurulent rhinitis, at that time assigned to the species M. caseolyticus, caught our attention since this species is not known to have any haemolytic activity [4]. Furthermore, M. caseolyticus has not been associated with an infection except for one reported case of abscesses in lambs [3, 5]. Additionally, the 16S rRNA gene sequence of strain KM 45013T, obtained from Ion Torrent next-generation sequencing [4], showed only 99.7 % identity with that of the M. caseolyticus type strain CCUG 15606T, and matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) MS (Bruker Microflex LT) failed to give a clear identification as M. caseolyticus. In the meantime, three other strains belonging to the genus Macrococcus with haemolytic activities were recovered from skin infection sites of three individual dogs. Following this discovery, we analysed samples taken from the skin and oral mucosa of 122 healthy dogs for the presence of this novel species of the genus Macrococcus and found it in 17 different animals (Table S1, available with the online Supplementary Material).

Samples were taken using swabs, which were streaked onto trypticase soy agar containing 5 % sheep blood (TSA-SB; Becton Dickinson). Plates were incubated aerobically at 37 °C for 24 h. To facilitate identification of this novel species, the mass spectra of strain KM 45013T, M. carouselicus CCUG 38360T, M. equipericus CCUG 38363T, M. bovicus CCUG 38365T, M. brunensis CCUG 47200T, M. hajekii CCUG 47201T and M. lamae CCUG 47199T were introduced as references into the MALDI-TOF MS database following the manufacturer’s instructions, and all the colonies growing on TSA-SB were submitted for MALDI-TOF MS identification (Bruker Microflex LT; Biotyper version 3.1). A MALDI-TOF spectra similarity dendrogram illustrated that the different species of the genus Macrococcus clustered into different branches and showed score values below 1.2 as compared with strain KM
The dendrogram was created using Pearson’s correlation with UPGMA clustering using Bionumerics 7.6 (Applied Maths) (Fig. S1). Isolates exhibiting spectra similar to the one of strain KM 45013\(^T\) with score values above 2.0 (Fig. S1) were subsequently analysed by 16S rRNA gene sequencing according to the method of Kuhnert et al. [6], revealing 100 % sequence identity between all strains. BLAST analysis showed that the strains had the highest sequence identity (99.7 %) with the 16S rRNA gene of \textit{M. caseolyticus} ATCC 13548\(^T\) (=CCUG 15606\(^T\)) (GenBank acc. no. Y15711). All species of the genus \textit{Macrococcus} shared similar 16S rRNA gene sequences, with sequence divergence being less than 3 %. Similarity matrix and comparative phylogenetic analysis of the 16S rRNA gene sequence of KM 45013\(^T\) with those of other species of the genus \textit{Macrococcus} and with \textit{Staphylococcus aureus} as a representative of the most closely related genus was performed using Bionumerics 7.5 (Applied Maths). The tree was generated by the neighbour-joining method [multiple alignment, open gap penalty (OG): 100 %, unit gap penalty (UG): 0 %] using the Jukes–Cantor correction. Bootstrap values were determined from 1000 replications (Fig. 1).

Based on genetic diversity as determined by enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) [7] (Fig. S2), the type strain and nine additional non-clonally related strains were further used for comparative phenotypic and genotypic analyses to distinguish the novel species of the genus \textit{Macrococcus} from the \textit{M. caseolyticus} type strain CCUG 15606\(^T\). Growth was assessed in brain heart infusion broth under aerobic conditions at 18, 37 and 43 °C and in 9 and 12 % NaCl solution at 37 °C. Anaerobic growth was tested on TSA-SB agar at 37 °C. For the additional chemotaxonomic analyses, cells belonging to members of the genus \textit{Macrococcus} were routinely grown in brain heart infusion broth at 37 °C under aerobic conditions unless stated otherwise. Gram staining was observed by light microscopy, and motility of cells was evaluated in 0.85 % sodium chloride solution by dark-field microscopy. The oxidase and catalase activities of the isolates were analysed using Oxidase and Catalase Reagent Drops (Becton Dickinson). DNase activity was tested on BD BBL DNase test agar (Becton Dickinson). Thermonuclease activity was determined by spotting 20 ml filtered overnight culture supernatant onto DNase test agar (BBL, Becton Dickinson) subsequently incubated for 2 h at 60 °C and flooded with 1 M HCl. Additional phenotypic characteristics were assessed using the GP card from the Vitek2 System (bioMérieux) and were compared with those of \textit{M. caseolyticus} CCUG 15606\(^T\). The diameter of the cells of the novel strains was determined by scanning electron microscopy (secondary electron imaging) with a DSM 982 Gemini microscope (Zeiss).

**Fig. 1.** Phylogenetic tree (a) and percentage similarity matrix (b) obtained from alignment of 16S rRNA gene sequences of \textit{Macrococcus canis} sp. nov. with those of other previously described species of the genus \textit{Macrococcus} and with \textit{Staphylococcus aureus} as a representative of the next most closely related genus. GenBank accession numbers are indicated in parentheses. The tree and similarity matrix were generated by neighbour-joining analysis [multiple alignment, open gap penalty (OG): 100 %, unit gap penalty (UG): 0 %; discard unknown bases; use active zones only] and the Jukes–Cantor correction using Bionumerics 7.6 (Applied Maths). Bootstrap values are shown at each node as percentages of 1000 replicates.
and by transmission electron microscopy after in toto preparation and negative staining with a CM12 microscope (FEI). The diameter of the cells was determined on the basis of corresponding micrographs at a primary magnification of ×10 000 (Division of Veterinary Anatomy, University of Berne, Switzerland).

The isolates were able to grow aerobically and anaerobically and at 18, 37 and 43 °C, and in 9 % and 12 % NaCl solution at 37 °C. They were Gram-stain-positive, non-motile cocci with a measured diameter of 0.8 µm. Catalase and oxidase tests were positive. Weak DNase production was observed from D-mannitol and the inability to produce acid from lactose. The presence of a complete haemolysis is also a specific characteristic of the majority of the novel strains (Table 1).

DNA sequences were determined for specific regions of the cpn60, dnaJ, rpoB and sodA genes, which are markers that have been shown to allow good discrimination between species of the genus Staphylococcus [8]. New PCR protocols were developed for the amplification of these markers in Macrococcus canis sp. nov. and M. caseolyticus (Table S2). DNA sequence alignment of these markers showed that all the M. canis sp. nov. strains were closely related with cpn60, dnaJ, rpoB and sodA fragments sharing more than 97.7, 91.2, 98.5 and 98.7 % DNA sequence identity, respectively. On the other hand, cpn60, dnaJ, rpoB and sodA fragments of the M. canis sp. nov. and M. caseolyticus type strains only shared 91.0, 83.3, 93.5 and 90.8 % DNA sequence identity, respectively, indicating that they were genetically distant. Cluster analysis of each marker was made for the M. canis sp. nov. and M. caseolyticus strains. The trees were generated by a multiple alignment (correction: Jukes and Cantor) and UPGMA clustering using Bionumerics 7.6 (Applied Maths) (Fig. S3).

The G+C content of the DNA, the cellular fatty acid composition and the peptidoglycan structure of KM 45013T were analysed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Analyses of polar lipids and respiratory quinones were performed by the Identification Service and Dr Brian Tindall, DSMZ.

The DNA G+C content of strain KM 45013T was determined by HPLC according to the method of Mesbah et al. [9]. The DNA G+C content measured was 36.9 mol%, which is slightly lower than the DNA G+C content reported for the other species of the genus Macrococcus (38–45 mol% [2, 3]).

Fatty acid analysis was performed using gas chromatography (www.midi-inc.com). Fatty acid methyl esters were obtained from 40 mg cells scraped from trypticase soy broth agar plates incubated at 28 °C for 24 h by saponification, methylation and extraction using minor modifications of the method of Miller [10] and Kuykendall et al. [11]. The fatty acid methyl esters mixtures were separated using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID) which consisted of an Agilent model 6890N gas chromatograph fitted with a 5 % phenylmethyl silicone
The polar lipids found in KM 45013\textsuperscript{T} were diphosphatidylglycerol, phosphatidylglycerol, one unknown aminolipid, two unknown glycolipids and two unknown phospholipids. Respiratory lipoquinones were extracted from 100 mg of freeze-dried cell material using the two-stage method described by Tindall [14, 15]. Respiratory quinones were extracted using methanol/hexane [14, 15], followed by phase separation into hexane. Respiratory lipoquinones were separated into their different classes by TLC on silica gel (Macherey-Nagel) using hexane/tert-butyl methyl ether (9:1, v/v) as the solvent. UV-absorbing bands corresponding to the different quinone classes were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical HPLC system (Thermo Separation Products) fitted with a reverse-phase column (Macherey-Nagel, 2 \times 125 mm, 3 \mu m, RP18) using methanol/heptane 9:1 (v/v) as the eluent. Respiratory lipoquinones were detected at 269 nm. The quinone type of strain KM 45013\textsuperscript{T} was MK-6.

The peptidoglycan structure was determined according to the protocol of Schumann [16]. The total hydrolysate (100 °C, 4 M HCl, 16 h) of the peptidoglycans of KM 45013\textsuperscript{T} contained the amino acids, alanine, glycine, serine, glutamic acid and lysine. The amino acids in the peptidoglycan hydrolysate were quantified by GC/MS after derivatization to yield N-heptafluorobutryric amino acid isobutyl esters. The identities of all amino acids were confirmed by agreement between their gas-chromatographic retention times and those of authentic standards and by the characteristic mass spectrometric fragment ions of their derivatives. The molar ratio of the amino acids in the peptidoglycan hydrolysate was as follows: 2.9 Ala: 1.7 Gly: 1.1 Ser: 1.0 Glu: 0.9 Lys. After hydrolysis under milder conditions (100 °C, 4 M HCl, 0.75 h), the hydrolysate also contained the peptides L-Ala–D-Glu, L-Lys–D-Ala, L-Lys–Gly, D-Ala–L-Lys–Gly. Based on these data, it was concluded that KM 45013\textsuperscript{T} belongs to the peptidoglycan type A3\alpha L-Lys–Gly\textsubscript{2}–L-Ser.

DNA–DNA hybridization was performed by the DSMZ. Cells were disrupted by using a French pressure cell (Thermo Spectronic), and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. [17].

DNA–DNA hybridization was performed as described by De Ley et al. [18] with the modifications described by Huss et al. [19] using a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 \times 6 multicell changer and a temperature controller with an in situ temperature probe (Varian). Hybridization was performed at a temperature of 69 °C. The DNA–DNA hybridization results revealed that KM 45013\textsuperscript{T} does not belong to the species M. caseolyticus, the closest relative according to the 16S rRNA gene sequence with 53.7 % relatedness, according to the recommended threshold value of 70 % DNA–DNA similarity for the definition of bacterial species by the ad hoc committee [20].

### Table 2. Fatty acid composition of strain KM 45013\textsuperscript{T} and M. caseolyticus CCUG 15606\textsuperscript{T}

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>KM 45013\textsuperscript{T}</th>
<th>M. caseolyticus</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C\textsubscript{13}-0</td>
<td>0.9</td>
<td>–</td>
</tr>
<tr>
<td>iso-C\textsubscript{14}-0</td>
<td>1.2</td>
<td>35.5</td>
</tr>
<tr>
<td>C\textsubscript{14}-0</td>
<td>29.5</td>
<td>10.7</td>
</tr>
<tr>
<td>anteiso-C\textsubscript{15}-0</td>
<td>–</td>
<td>2.9</td>
</tr>
<tr>
<td>C\textsubscript{16}-n alcohol</td>
<td>20.7</td>
<td>–</td>
</tr>
<tr>
<td>iso-C\textsubscript{16}-0</td>
<td>–</td>
<td>1.2</td>
</tr>
<tr>
<td>C\textsubscript{16}:1ω9c</td>
<td>–</td>
<td>19.5</td>
</tr>
<tr>
<td>C\textsubscript{16}:1ω11c</td>
<td>18.4</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{18}-0</td>
<td>5.0</td>
<td>1.9</td>
</tr>
<tr>
<td>C\textsubscript{17}:1ω9c</td>
<td>–</td>
<td>1.2</td>
</tr>
<tr>
<td>C\textsubscript{17}-10 methyl</td>
<td>–</td>
<td>3.1</td>
</tr>
<tr>
<td>C\textsubscript{18}:ω6c\textsubscript{(6,9,12)}</td>
<td>22.3</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{18}:ω6c\textsubscript{(anteiso-C\textsubscript{16}-0)}</td>
<td>–</td>
<td>2.4</td>
</tr>
<tr>
<td>C\textsubscript{18}:ω9c</td>
<td>–</td>
<td>11.1</td>
</tr>
<tr>
<td>C\textsubscript{19}-0</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td>C\textsubscript{20}-0</td>
<td>0.7</td>
<td>–</td>
</tr>
</tbody>
</table>

Fatty acids amounting to less than 1 % of the total fatty acids of all strains were not included. All data are from this study.
Based on the different genotypic and phenotypic characteristics observed during this study, the strains belonging to the genus *Macrococcus* isolated from dogs represent a novel species which differs from the species *M. caseolyticus*, and the novel species was named *Macrococcus canis* sp. nov. with strain KM 45013T as the type strain.

**DESCRIPTION OF MACROCOCCUS CANIS SP. NOV.**


Cells are Gram-stain-positive, non-motile, facultatively anaerobic cocci about 0.8 µm in diameter. Colonies reach 2 mm in diameter, are circular, smooth, flat, glossy, opaque and greyish white after 24 h of growth at 37 °C on TSA-5B agar. On the same agar, some strains exhibit complete haemolysis. Haemolysis is visible in all strains following a CAMP reaction with strain KM 45013.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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