Micrococcus cohnii sp. nov., isolated from the air in a medical practice

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Three Gram-reaction-positive bacteria, isolated from the air in a medical practice (strains WS4601T, WS4602) or a pharmaceutical clean room (strain WS4599), were characterized using a polyphasic approach. Phylogenetic analyses based on 16S rRNA and recA gene sequences of the three novel strains showed that they formed a distinct lineage within the genus Micrococcus, sharing 16S rRNA gene sequence similarities of 96.1–98.0 % with other species of this genus. Chemotaxonomic features also supported the classification of the three novel strains within the genus Micrococcus. The major cellular fatty acids of strain WS4601T were anteiso-C₁₅ : ₀ and iso-C₁₅ : ₀, the cell-wall peptidoglycan was of type A3α (L-Lys–L-Ala), and the predominant respiratory quinones were MK-7(H₂) and MK-8(H₂). The polar lipid profile contained diphasphatidylglycerol and phosphatidylglycerol, but no phosphatidylinositol. The G+C content of the genomic DNA was 70.4 mol%. Numerous physiological properties were found that clearly distinguished strains WS4599, WS4601T and WS4602 from established members of the genus Micrococcus. Based on the phenotypic and phylogenetic data, strains WS4599, WS4601T and WS4602 are considered to represent three different strains of a novel species of the genus Micrococcus, for which the name Micrococcus cohnii sp. nov. is proposed. The type strain is WS4601T (DSM 23974T = LMG 26183T).

The genus Micrococcus was first described by Cohn (1872) and emended by Stackebrandt et al. (1995) and Wieser et al. (2002). At the time of writing, the genus Micrococcus encompassed eight species: Micrococcus luteus (Cohn, 1872; Wieser et al., 2002), Micrococcus lylae (Kloos et al., 1974; Wieser et al., 2002), Micrococcus antarcticus (Liu et al., 2000), Micrococcus flavus (Liu et al., 2007), Micrococcus endophyticus (Chen et al., 2009), Micrococcus yunnanensis (Zhao et al., 2009), Micrococcus terreus (Zhang et al., 2010), and Micrococcus lactis (Chittipurna et al., 2011). In this study, we report the taxonomic positions of three strains, WS4599, WS4601T and WS4602, belonging to a novel species of the genus Micrococcus as determined by following a polyphasic approach that included morphological, physiological, chemotaxonomic and phylogenetic analyses.

To investigate the microbial biodiversity of the air of specific environments, and to upgrade Fourier-transform infrared (FTIR) spectral libraries with the spectra of bacterial isolates from such habitats, air samples were collected from different locations of medical practices and pharmaceutical clean rooms in Germany (G. Rieser, S. von Brehmer, G. Schuffenhauer, S. Scherer & M. Wenning, unpublished). Cells were incubated on tryptone soy agar (TSA) at 30 °C for 24 h. FTIR samples were then prepared and spectra acquired as described by Oberreuter et al. (2002), using a Tensor 27 FTIR spectrometer (Bruker Optics) coupled to a HTS-XT high-throughput device (Bruker Optics). Prior to identification, spectra were pre-processed according to Oberreuter et al. (2002). Identification was performed using the OPUS 5.5 software package (Bruker), in combination with libraries of reference spectra. Isolates that displayed FTIR spectra that did not match any reference in the libraries were identified by gene sequence analysis before the corresponding spectra were integrated into the system. During the course of this study, three novel strains, designated strains WS4599, WS4601T and WS4602, were identified as being members of the genus Micrococcus. Strains WS4601T and WS4602 were isolated from the examination room and cloakroom of a single medical practice, respectively, whereas strain WS4599 was isolated from a pharmaceutical clean room.
Morphological features were observed after cultivation on TSA for 3 days at 30 °C. Cell shape and motility were observed under a phase-contrast light microscope (Laborlux S, Leitz). The Gram reaction was determined by the KOH method of Gregersen (1978). Oxidase activity was evaluated using Bactident oxidase strips (Merck) according to the instructions of the manufacturer. Catalase activity, the hydrolysis of starch, aesculin and casein and H2S production were analysed according to the methods of Smibert & Krieg (1994). Gelatin hydrolysis, indole production, nitrate reduction and arginine dihydrolase, β-galactosidase and urease activities were investigated by using the API 20NE system (bioMérieux) according to the instructions of the manufacturer. Substrate utilization tests were performed by using ISP9 (Shirling & Gottlieb, 1966) as the basal medium. Each substrate was added at a final concentration of 0.2% (w/v) according to Kämpfer et al. (1991). Aerobic production of acids from carbohydrates was determined by using API 50CH test kits (bioMérieux) with API 50CHB/E medium (bioMérieux) in combination with 96-well microtitre plates as described by Schmidt et al. (2012). Growth at different temperatures (6, 15, 20, 28, 30, 37, 45 and 50 °C) and NaCl concentrations (0, 1, 3, 5, 7, 10 and 15 %, w/v) was tested in tryptone soy broth (TSB) containing (l-1) 17 g tryptone, 3 g soya peptone, 2.5 g K2HPO4, 2.5 g glucose and 5 g NaCl, pH 7.2. The pH range for growth was investigated at pH 4.0–11.0 (at intervals of 1.0 pH unit) using the buffer system described by Xu et al. (2005) and TSB as the basal medium. The ability to grow under anaerobic conditions was determined on TSA in an anaerobic jar containing the anaerobic catalyst Anaerocult A (Merck). Results are given in the species description.

The phylogenetic positions of the three novel strains were determined by sequence analysis of the 16S rRNA and recA genes. The genomic DNA for these analyses came from cells that had been lysed with zirconia silica beads (0.1 mm; Roth), as described by Büchel et al. (2008). Almost-complete 16S rRNA gene sequences (1407 bp) were amplified by PCR, as described previously (Oberreuter et al., 2002), using Thermo-Start Taq DNA polymerase (Thermo Scientific) and a thermocycling program of 15 min at 95 °C, 35 cycles of 20 s at 95 °C, 40 s at 52 °C and 1 min 40 s at 72 °C, and then a final extension step of 6 min at 72 °C. All PCR products were purified using the GenElute PCR Clean-Up kit (Sigma-Aldrich) before sequencing was performed by GATC Biotech AG (Konstanz, Germany). To confirm the genotypic distinction of strains WS4599, WS4601T and WS4602 from recognised species of the genus Micrococcus, sequences of the protein-encoding recA gene were used as a basis for further phylogenetic analyses. recA gene fragments (844 bp) were amplified by using the degenerated primers GPRA-UF2 and GPRA-UR2, which were originally designed by van Waasbergen et al. (2000) to amplify recA gene fragments of species of the genus Arthrobacter. PCR products were obtained for the three novel strains and the type strains of Micrococcus lylae, M. flvus and M. yunnanensis, but not for the type strains of the other three species, M. luteus, M. endophyticus and M. terres indicating that the primer sequences did not match with recA gene sequences of all species of the genus Micrococcus. The PCR products were then sequenced by using the primer GPRA-UF2, and based on the obtained sequences and the recA gene sequence of M. luteus (GenBank accession no. CP001628), primers 606FW (5’-AGATCGCGGTGGTCCGCGC-3’) and 307Rev (5’-GTTCCACGCGAGGTGG-3’) were designed in order to elongate the recA sequences at the 5’ and 3’ ends. The elongated sequences were then used to design a new reverse primer, 870Rev (5’-GGTGAACC-ABGCRCGGAA-3’), in an attempt to establish a set of primers applicable to all recognised members of the genus Micrococcus. Subsequently, the missing recA genes of M. endophyticus and M. terres were amplified by using the primer pair GPRA-UF2 and 870Rev. Cycle conditions were the same as those described for the 16S rRNA gene amplification except that the annealing temperature was 68 °C. For sequencing, primer 870Rev was used. The obtained sequences were aligned using CLUSTAL_X, version 2.0 (Thompson et al., 1997) and distance matrices of the resulting multiple sequence alignments were calculated using TREECON (Van de Peer & De Wachter, 1997). Rooted phylogenetic trees were then constructed by the neighbour-joining method, within the TREECON program. The values for sequence similarities among the most closely related strains were determined using the EzTaxon server (Chun et al., 2007).

The 16S rRNA gene sequences of strains WS4599, WS4601T and WS4602 (1407–1411 nt bases) were identical across 1407 nucleotide positions. The three novel strains showed the highest 16S rRNA gene sequence similarities to M. yunnanensis YIM 65004T (98.0 %), M. antarcticus T2T (97.9 %), M. luteus DSM 20030T (97.9 %) and M. terres NBRC 104258T (97.9 %). Similarity values between the 16S rRNA gene sequences of strain WS4601T and recognised species of the genus Micrococcus are given in Table S1 (available in IJSEM Online). In the neighbour-joining phylogenetic tree based on the almost-complete 16S rRNA gene sequences, the three novel strains formed one distinct lineage within the genus Micrococcus (Fig. 1).

The results of the recA gene sequence analysis, based on the comparison of 815 nucleotide positions, are presented in Fig. 2. Although M. antarcticus T2T appeared closely related to each novel strain in terms of its 16S rRNA gene sequence similarity (97.9 %), it was not included in the phylogenetic analysis based on recA gene sequences as it was unavailable from either of the culture collections preserving this strain [i.e. the China General Microbiological Culture Collection (CGMCC) and the Japan Collection of Microorganisms (JCM)] because of uncertainties over the strain’s identity. Strains WS4601T and WS4599 showed identical recA gene sequences and each had 99.3 % recA gene sequence similarity to strain WS4602. Based on recA gene sequences, the three novel strains were most closely related to M. lylae DSM 20315T (88.7–88.8 % recA gene sequence similarity) and M. endophyticus DSM 17945T (87.0–87.1 %). Additional
information on the recA gene sequence similarities between strain WS4601T, strain WS4602 and closely related members of the genus Micrococcus is given in Table S2. From these data it can be concluded that strains WS4599, WS4601T and WS4602 represent a distinct lineage within the genus Micrococcus and can be considered a single novel species.

All analyses for the chemotaxonomic characterization of the novel strains were performed by the Identification Service of the DSMZ. Biomass for the analyses of respiratory quinones, polar lipids, genomic DNA G+C content and cell wall peptidoglycan was obtained by cultivation in shaken flasks (150 r.p.m.) of TSB at 30 °C for 2 days. For the analysis of cellular fatty acids, cells were cultivated on TSA for 24 h at 28 °C according to the instructions of the Microbial ID system. In order to ensure that the cells of the different strains subjected to fatty acid analysis were of similar physiological age, a particular sector of the quadrant streak was chosen for harvesting cell material. Cellular fatty acids were extracted and analysed as described by Verbiag et al. (2008), using an Agilent 6890N gas chromatograph and version 6.1 of the Sherlock MIS software with TSBA 40 4.10 database (MIDI, Inc.). The predominant cellular fatty acids of strain WS4601T were anteiso-C15 : 0 (78.2 %) and iso-C14 : 0(4.3 %) and iso-C16 : 0(4.3 %) were also detected in amounts > 1 % (Table S3). The results are in accordance with those obtained for other species of the genus Micrococcus which also have anteiso-C15 : 0 and iso-C15 : 0 as the predominant fatty acids. A detailed comparison of the fatty acid profiles of strain WS4601T and other species of the genus Micrococcus is given in Table S3.

Polar lipids and respiratory quinones were analysed by the Identification Service of the DSMZ and Dr Brian J. Tindall (DSMZ). Lipids were extracted from freeze-dried cells using a chloroform/methanol/0.3 % (w/v) aqueous NaCl mixture [modified from Bligh & Dyer (1959)] and were then analysed by two-dimensional TLC, as described by Tindall et al. (2007). The polar lipid profile of strain WS4601T included diphosphatidylglycerol, phosphatidylglycerol, two unidentified phospholipids and three unidentified glycolipids (Fig. S1). Phosphatidinositol was not detected in strain WS4601T but has been found in all of the polar lipid profiles that have been reported for established

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the relationships between strains WS4599, WS4601T, WS4602 and related taxa. Bootstrap values (>50 %) based on 500 replications are shown at branch points. Kytococcus sedentarius DSM 20547T was used as the outgroup. Bar, 5 % sequence divergence.
Table 1. Differential phenotypic characteristics of strain WS4601<sup>T</sup> and related species of the genus *Micrococcus*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<td>−</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>w&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>w&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>w&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>−</td>
<td>+</td>
<td>−&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>DNA G + C content (mol%)†</td>
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<td>70.0</td>
<td>70.0</td>
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<td>72.9</td>
<td>71.4</td>
<td>69.0</td>
<td>66.4</td>
<td>68.0</td>
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<td>Respiratory quinones‡</td>
<td>MK-7(H&lt;sub&gt;2&lt;/sub&gt;), MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-7(H&lt;sub&gt;2&lt;/sub&gt;), MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-7(H&lt;sub&gt;2&lt;/sub&gt;), MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-7, MK-7(H&lt;sub&gt;2&lt;/sub&gt;), MK-8, MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-7, MK-7(H&lt;sub&gt;2&lt;/sub&gt;), MK-8, MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-7(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
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</table>

*Different results were reported by: a, Wieser et al. (2002); b, Zhang et al. (2010); c, Zhao et al. (2009); d, Chen et al. (2009).
†The data shown for taxa 2–7 are those reported by Zhao et al. (2009), Wieser et al. (2002), Zhang et al. (2010), Chen et al. (2009), Wieser et al. (2002) and Liu et al. (2007), respectively.

Micrococcus cohnii sp. nov.
members of the genus *Micrococcus* (Table 1); the polar lipid profiles of *M. flavus* and *M. antarcticus* have yet to be determined. Quinones were extracted from freeze-dried cells with methanol/hexane (Tindall, 1990a, b), separated by TLC and analysed by HPLC (Altenburger et al., 1996). The predominant menaquinones of strain WS4601T were MK-8(H2) (65 %) and MK-7(H2) (35 %), which is in accordance with established members of the genus *Micrococcus* (Table 1).

For the evaluation of genomic DNA G+C content, cells of strain WS4601T were disrupted by French pressing and the DNA was purified by chromatography on hydroxyapatite, as described by Cashion et al. (1977), then hydrolysed with P1 nuclease. The resulting nucleotides were dephosphorylated using bovine alkaline phosphatase (Mesbah et al., 1989) and deoxyribonucleosides were then analysed by HPLC (Tamaoka & Komagata, 1984) and the DNA G+C content (mol%) was calculated from the ratio of deoxyguanosine and thymidine (Mesbah et al., 1989). The genomic DNA G+C content determined for strain WS4601T was 70.4 mol%, which was well within the range reported for established members of the genus *Micrococcus* (Table 1).

Analysis of the cell-wall peptidoglycan was performed according to Schleifer & Kandler (1972); Schleifer (1985) and MacKenzie (1987). After treatment with hydrofluoric acid (HF), the hydrolysate of the cell-wall peptidoglycan of strain WS4601T was found to contain alanine (Ala), aspartic acid (Asp), glutamic acid (Glu) and lysine (Lys) in a molar ratio of 2.4:0.1:0.4:1.0. The peptides L-Ala–D-Glu, L-Ala–L-Lys, L-Lys–D-Ala, L-Ala–L-Lys–D-Ala, D-Ala–L-Ala and L-Ala–D-Glu were detected in a partial hydrolysate. Without HF treatment, glycine and the peptide Asp–L-Ala were found, suggesting that these peptides, and also the small fraction of aspartic acid detected after HF treatment, may originate from a peptidoglycan-associated polymer. Based on these findings, it was concluded that the peptidoglycan of strain WS4601T was of type A3a (L-Lys–L-Ala). Although this cell wall type has not been detected in other species of the genus *Micrococcus*, the composition of amino acids in the cell wall peptidoglycan of strain WS4601T is characteristic of those reported for members of the genus *Micrococcus*. The peptidoglycan type, as defined by Schleifer & Kandler (1972), varies within the genus *Micrococcus*, with type A4x reported for *M. lylae* DSM 20315T (Wieser et al., 2002) and *M. luteus* DSM 20369T (Chittpurna et al., 2011) and type A2 reported for *M. luteus* DSM 20030T (Wieser et al., 2002). The A3x cell wall peptidoglycan type determined for strain WS4601T was consistent with the assignment of this strain to a novel species in the genus *Micrococcus*.

Based on the chemotaxonomic and phylogenetic data, it is clear that strains WS4599, WS4601T and WS4602 should be considered members of the genus *Micrococcus*. Although the three novel strains appear indistinguishable in terms of their phenotypic characteristics and 16S rRNA gene sequences, they are not identical since small differences in their recA gene sequences were found. All three novel strains can be distinguished from established members of the genus *Micrococcus* genetically and on the basis of the phenotypic traits given in Table 1. Therefore strains WS4599, WS4601T and WS4602 are considered to represent three strains of a single novel species in the genus *Micrococcus*, for which the name *Micrococcus cohnii* sp. nov. is proposed.

**Description of *Micrococcus cohnii* sp. nov.**

*Micrococcus cohnii* (coh’ni.i. N.L. masc. gen. n. cohnii of Cohn; named after the German botanist and bacteriologist Ferdinand Cohn, who described the genus *Micrococcus* in 1872).

Cells are Gram-reaction-positive, non-motile, non-spore-forming, aerobic cocci (1.0–1.3 μm in diameter). Colonies on TSA are smooth, circular and cream–white. Grows at 15–37 °C, at pH 7–9, and with 0–10 % (w/v) NaCl. Cells are positive for oxidase, catalase and urease activities and the hydrolysis of casein, starch and gelatin, but negative for arginine dihydrolase and β-galactosidase activities, nitrate reduction, indole and H2S production, and aesculin hydrolysis. Utilizes D-glucose, maltose, sucrose and trehalose, but not D-mannose, D-mannitol, melibiose, D-sorbitol, L-arabinose or L-rhamnose. Produces acid from D-glucose, maltose, sucrose, trehalose and turanose, but not from any other substrate provided in the API 50CH test strips. The cell wall peptidoglycan is of the A3a (L-Lys–L-Ala) type, and the predominant menaquinones are MK-7(H2) and MK-8 (H2). The major cellular fatty acids are anteiso-C15:0 and iso-C15:0 and the polar lipid profile contains diphosphatidylglycerol and phosphatidylglycerol as major components.

The type strain, WS4601T (=DSM 23974T =LMG 26183T), was isolated from an air sample collected in a medical practice in Germany. The genomic DNA G+C content of the type strain is 70.4 mol%. Two additional strains of the species, WS4602 and WS4599, were isolated from the air of a medical practice and a pharmaceutical clean room, respectively.

**References**


