NOTE

***Citricoccus muralis* gen. nov., sp. nov., a novel actinobacterium isolated from a medieval wall painting**Petra Altenburger,^{1,2} Peter Kämpfer,³ Peter Schumann,⁴ Robert Steiner,² Werner Lubitz² and Hans-Jürgen Busse^{1,2}¹ Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria² Institut für Mikrobiologie und Genetik, Universität Wien, A-1030 Wien, Austria³ Institut für Angewandte Mikrobiologie, Justus Liebig-Universität Giessen, D-35392 Giessen, Germany⁴ DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, D-38124 Braunschweig, Germany

Author for correspondence: Hans-Jürgen Busse. Tel: +43 1 25077 2128. Fax: +43 1 25077 2190. e-mail: hans-juergen.Busse@vu-wien.ac.at

A Gram-positive, aerobic, spherical actinobacterium, designated strain 4-0^T, was isolated from a medieval wall painting and characterized to determine its taxonomic position. The peptidoglycan of strain 4-0^T was of type A4 α , with lysine as the diagnostic cell wall diamino acid and an interpeptide bridge of Lys–Gly–Glu. Its quinone system contained predominantly MK-9(H₂) (64 %) and its polar lipid profile consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, four unknown glycolipids, two unknown phospholipids and an unknown lipid. The fatty acid profile of strain 4-0^T was represented by significant amounts of ai-C15:0 and moderate amounts of ai-C17:0, i-C16:0 and i-C15:0 fatty acids. Spermidine was predominant in the polyamine pattern. The G+C content of the genomic DNA was 68 mol %. Comparative 16S rDNA sequence studies revealed the highest similarity values (95.4–96.1 %) between strain 4-0^T and species of the genus *Micrococcus* and certain species of the genus *Arthrobacter*, including *Arthrobacter pascens* DSM 20545^T, *Arthrobacter ramosus* DSM 20546^T, *Arthrobacter protophormiae* DSM 20168^T, *Arthrobacter nicotianae* DSM 20123^T and *Arthrobacter globiformis* DSM 20124^T. Phylogenetic analyses demonstrated that strain 4-0^T branches deeply on the *Micrococcus* lineage. Because it is almost equidistant phylogenetically from the genera *Micrococcus* and *Arthrobacter* and possesses significant differences in chemotaxonomic characteristics from members of these genera, it is suggested that strain 4-0^T be classified as a novel species in a new genus; the name *Citricoccus muralis* gen. nov., sp. nov. is proposed. The type strain is strain 4-0^T (= DSM 11442^T = CCM 4981^T).

Keywords: *Citricoccus muralis* gen. nov., sp. nov., 16S rDNA sequence, chemotaxonomy, phenotypic characteristics

Recently, several bacterial strains were isolated from a damaged medieval wall painting in the church of St Georgen, Styria (Austria). They have been characterized by a polyphasic approach, including physiological, biochemical and chemotaxonomic methods, as well as analyses of partial 16S rRNA gene sequences (Altenburger *et al.*, 1996). Originally, these strains

were identified as members of the genus *Bacillus*, the class *Actinobacteria* and the α -*Proteobacteria*. Recently, the name *Georgenia muralis* (Altenburger *et al.*, 2002) has been proposed for two of these isolates. Isolate 4-0^T (originally designated G4; Altenburger *et al.*, 1996), which was initially identified as a member of the genus *Kocuria* based on its morphology, quinone system and fatty acid profile, was re-examined to determine its taxonomic position due to its distinctive characteristics.

PYES medium (Altenburger *et al.*, 2002) was used for general laboratory cultivation and for morphological examinations. Cell morphology and Gram behaviour

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Abbreviations: pNA, p-nitroanilide; pNP, p-nitrophenyl.

The EMBL accession number for the 16S rDNA sequence of strain 4-0^T is AJ344143.

were studied as described previously (Moaledj, 1986; Altenburger *et al.*, 2002). Cells of strain 4-0^T were Gram-positive cocci (approx. 1 µm in diameter) and occurred singly, in short chains or in packets. Endospores and motile cells were not observed. Colonies reached a maximum size (3 mm in diameter) after 2 weeks incubation at room temperature on PYES agar. They were yellow, circular, convex and opaque.

Anaerobic growth, growth at different temperatures, tolerance to NaCl and pH tolerance were examined as described by Altenburger *et al.* (2002). Growth was not observed under anaerobic conditions. Aerobically, cells grew moderately well at 4 and 28 °C, but not at 37 °C.

Strain 4-0^T grew well in the presence of 0–7% (w/v) NaCl and moderately in 10% (w/v) NaCl. No growth was observed in the presence of 15% (w/v) NaCl. The pH range for growth was pH 6–10. Good growth was observed at room temperature on media with rich nutrient status such as nutrient agar (Altenburger *et al.*, 1996) and tryptone soy agar (Oxoid), as well as on media with low nutrient status such as PYES agar, CasMM agar (Altenburger *et al.*, 1996) and R2A agar (Oxoid).

Carbon source utilization tests, sugar fermentation and qualitative enzyme tests were carried out according to Kämpfer *et al.* (1991). Hydrolysis of starch, Tween 80, Tween 20 and casein were determined on PYES agar respectively supplemented with 1% potato starch, 0.5% Tween 80, 0.5% Tween 20 or 5% casein. Tests for oxidase, catalase, nitrate reduction, H₂S production, indole production and urease activity were performed as described by Smibert & Krieg (1994). The isolate was catalase-positive and oxidase-negative. Detailed physiological and biochemical properties are given in the species description.

16S rDNA sequence amplification and analyses were performed according to Wieser *et al.* (1999). The DNA sequence was aligned with sequences currently available in the EMBL database using the program PILEUP (Genetics Computer Group, version 8). Pairwise evolutionary distances were calculated by the method of Jukes & Cantor (1969). The neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood methods contained in the PHYLIP package (Felsenstein, 1993) and the method for parsimony (Swofford, 1999) were used in the construction of phylogenetic dendrograms. Bootstrap analysis (1000 replications) was done according to Felsenstein (1993).

An almost complete 16S rDNA sequence of 1455 nt was determined for strain 4-0^T. Phylogenetic analysis based on a dataset containing 1421 unambiguous nucleotides between positions 39 and 1460 (*Escherichia coli* numbering; Brosius *et al.*, 1978) showed that the novel isolate clustered within the radiation of the family *Micrococcaceae* (Stackebrandt *et al.*, 1997). Highest 16S rDNA sequence similarity values were detected with *Micrococcus antarcticus* AS 1.2372^T, *Arthrobacter pascens* DSM 20545^T and *Arthrobacter*

Fig. 1. Phylogenetic tree (constructed by the neighbour-joining method) based on 16S rRNA sequence comparisons indicating the phylogenetic positions of strain 4-0^T within the family *Micrococcaceae*. *Agrococcus jenensis* DSM 9580^T (X92492) was included as an outgroup. Bar, 1 nt substitution per 100 nt.

ramosus DSM 20546^T (all three at 96.1%), *Arthrobacter protophormiae* DSM 20168^T (96.0%), *Micrococcus lylae* DSM 20315^T and *Arthrobacter nicotianae* DSM 20123^T (both at 95.8%), *Arthrobacter globiformis* DSM 20124^T (95.6%) and *Micrococcus luteus* ATCC 381 (95.4%). All three treeing algorithms, neighbour-joining (Fig. 1), maximum-likelihood and parsimony (results not shown), placed strain 4-0^T branching deeply on the *Micrococcus* lineage. Confidence in this branching point was given by a bootstrap value of 726 (Fig. 1).

Affiliation of strain 4-0^T to the family *Micrococcaceae* was also supported by the presence of almost all family-specific signature nucleotides in its 16S rDNA sequence (Stackebrandt *et al.*, 1997). The only deviation found was the replacement of a U by a C at position 859 (*E. coli* numbering).

Chemotaxonomic characteristics, including the peptidoglycan type, quinone type and polar lipids, were determined as described previously (Tindall, 1990; Ventosa *et al.*, 1993; Groth *et al.*, 1997). The peptidoglycan of strain 4-0^T contained Ala, Gly, Glu and Lys in a molar ratio of 1.3:0.8:2.0:1.0 and the interpeptide bridge consisted of Lys–Gly–Glu (peptidoglycan type A4α, murein type A11.40/56; DSMZ, 2001). The quinone system of strain 4-0^T consisted of MK-9(H₂) (64%), MK-7(H₂) (24%) and MK-8(H₂) (14%). Species of *Arthrobacter* group I, such as *A. ramosus* and *A. pascens*, which are close

Fig. 2. Two-dimensional TLC of a polar lipid extract from strain 4-0^T stained with molybdotophosphoric acid. DPG, Diphosphatidylglycerol; PG, diphosphatidylglycerol; PI, phosphatidylinositol; PL, unknown phospholipid; GL, unknown glycolipid; L, unknown polar lipid (i.e. gave negative reactions to all specific spray reagents).

relatives of strain 4-0^T, also have a quinone system containing predominantly MK-9(H₂), but they are characterized by the peptidoglycan type L-Lys-L-Ala₁₋₄, variation A3 α and an interpeptide bridge that contains only monocarboxylic amino acids (Koch *et al.*, 1995). In contrast, species of *Arthrobacter* group II, such as *Arthrobacter nicotinovorans* DSM 420^T, which are phylogenetically separated from group I, are characterized by a combination of peptidoglycan type A4 α with a dicarboxylic acid in the interpeptide bridge (murein type 11.31; DSMZ, 2001) and a quinone system with completely unsaturated menaquinones (Koch *et al.*, 1995; Stackebrandt & Schumann, 2000; Stackebrandt *et al.*, 1983). Within the genus *Micrococcus*, the peptidoglycan variation A4 α (murein type A11.31; DSMZ, 2001) is found in *M. luteus* biovar III (Wieser *et al.*, 2002) and in *M. lylae* (Stackebrandt *et al.*, 1995), whereas *M. luteus* biovars I and II and *M. antarcticus* possess peptidoglycan type A2 (L-Lys peptide subunit) (Wieser *et al.*, 2002). The quinone systems of *Micrococcus* species clearly differ from that of strain 4-0^T in containing predominantly either MK-8 and MK-8(H₂) or MK-8(H₂) only (Wieser *et al.*, 2002; Stackebrandt *et al.*, 1995; Liu *et al.*, 2000).

The polar lipid pattern of strain 4-0^T consisted of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol (Fig. 2). In addition, four unknown glycolipids (GL2 showing the same chromatographic behaviour as one of the components of the yellow pigment), two unknown phospholipids and one unknown lipid were detected. This pattern is more

complex than the polar lipid profiles reported for *Micrococcus* and *Arthrobacter* species (Wieser *et al.*, 2002; Stackebrandt *et al.*, 1995; Jones & Collins, 1986). After development of the polar lipid extract in the first dimension by TLC, the yellow pigment separated into four spots, indicating that it is made up of four components. Thus, the combined chemotaxonomic characteristics, peptidoglycan type, quinone system and polar lipid profile do not fit those described for either *Micrococcus* or *Arthrobacter*.

Analysis of the fatty acids (Kämpfer *et al.*, 1997) of strain 4-0^T revealed a profile containing ai-C15:0 (55.6%), ai-C17:0 (27.0%), i-C16:0 (8.0), i-C15:0 (6.4%), ai-C13:0 (1.2%), i-C17:0 (1.1%) and C16:0 (0.8%). Similar fatty acid patterns are present in *A. ramosus* ATCC 13727^T, *A. pascens* ATCC 13346^T and other *Arthrobacter* and *Micrococcus* species (Funke *et al.*, 1996; Stackebrandt *et al.*, 1995; Wieser *et al.*, 2002; Liu *et al.*, 2000). However, members of the genus *Micrococcus* are reported to contain large amounts of ai-C15:0 and smaller amounts of i-C15:0 fatty acids. Significant levels of the ai-C15:1 fatty acid present in *M. antarcticus* AS 1.2372^T were not found in strain 4-0^T. This isolate is furthermore discriminated from the three *Micrococcus* species by their lack of significant amounts of ai-C17:0.

It has been shown in two independent studies that *M. luteus* DSM 20030^T and *M. lylae* DSM 20315^T are characterized by polyamine patterns that contain predominantly spermidine along with significant amounts of spermine (Hamana, 1994; Gvozdiak *et al.*, 1998). To explore the taxonomic significance of polyamine patterns within the family *Micrococcaceae*, several *Arthrobacter* species from our strain collection, as well as strain 4-0^T, were analysed for their polyamine content. Polyamine analysis was carried out as described by Altenburger *et al.* (1997). The majority of strains investigated, including strain 4-0^T, contained polyamine patterns with spermidine as the predominant compound, along with varying amounts of 1,3-diaminopropane, putrescine, cadaverine and spermine (Table 1). *Arthrobacter atrocyaneus* DSM 20127^T, *Arthrobacter uratoxydans* DSM 20647^T and *Arthrobacter crystallopoietes* DSM 20117^T could be clearly distinguished from all the other strains by their significantly lower polyamine content, which was close to the detection limit of our system. However, our polyamine analyses, as well as the data of Hamana (1994) and Gvozdiak *et al.* (1998), indicate that the majority of members of the family *Micrococcaceae* are characterized by a polyamine pattern with spermidine as the predominant compound, but differentiation between groups within the family does not appear to be possible based on this character. The polyamine pattern of strain 4-0^T (Table 1) was in agreement with the polyamine patterns within the family, but it could not be distinguished from either *Micrococcus* or *Arthrobacter*. DNA isolation and analysis of the G+C content were performed according to Groth *et al.*

Table 1. Polyamine patterns of strain 4-0^T and several species of the genus *Arthrobacter*

Strains are indicated as: 1, strain 4-0^T; 2, *Arthrobacter ramosus* DSM 20546^T; 3, *Arthrobacter pascens* DSM 20545^T; 4, *Arthrobacter histidinovorans* DSM 20115^T; 5, *Arthrobacter ilicis* DSM 20138^T; 6, *Arthrobacter globiformis* DSM 20124^T; 7, *Arthrobacter oxydans* DSM 20119^T; 8, *Arthrobacter aureus* DSM 20116^T; 9, *Arthrobacter ureafaciens* DSM 20126^T; 10, *Arthrobacter polychromogenes* DSM 20136^T; 11, *Arthrobacter nicotianae* DSM 20123^T; 12, *Arthrobacter atrocyaneus* DSM 20127^T; 13, *Arthrobacter uratoxydans* DSM 20647^T; 14, *Arthrobacter crystallopoietes* DSM 20117^T. Amounts are given as µmol (g dry weight)⁻¹; tr, Trace.

Polyamine	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1,3-Diaminopropane	0.01	0.07	—	tr	0.05	tr	0.08	0.08	tr	0.08	tr	tr	0.02	0.03
Putrescine	0.04	0.03	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.02	tr
Cadaverine	0.05	0.24	0.10	0.42	tr	tr	0.17	0.11	0.05	tr	tr	0.03	tr	0.04
Spermidine	2.30	5.64	5.56	2.00	1.28	1.11	1.01	0.99	2.41	2.58	0.74	0.08	0.02	0.15
sym-Homospermine	—	tr	—	—	—	tr	—	tr	tr	tr	tr	—	—	—
Spermine	0.05	0.05	0.06	0.04	tr	0.06	0.03	0.04	0.04	0.06	0.48	0.03	0.14	0.06
Tyrosine	0.02	—	—	—	tr	—	tr	tr	—	tr	tr	tr	tr	tr

Table 2. Differential characteristics of *Citricoccus* gen. nov. and related taxa

Data for reference taxa were taken from Stackebrandt *et al.* (1995) (*Micrococcus*, *Arthrobacter*), Wieser *et al.* (2002) and Liu *et al.* (2000) (*Micrococcus*) and Funke *et al.* (1996), Jones & Collins (1986) and Collins & Kroppenstedt (1983) (*Arthrobacter*).

Characteristic	<i>Citricoccus</i>	<i>Micrococcus</i>	<i>Arthrobacter</i> group I	<i>Arthrobacter</i> group II
Cell morphology	Coccoid	Coccoid	Rod-coccus cycle	Rod-coccus cycle or coccoid
Major menaquinone(s)	MK-9(H ₂)	MK-8 and MK-8(H ₂) or MK-8(H ₂) only	MK-9(H ₂)	MK-8, MK-9
Major fatty acids	ai-C15:0, ai-C17:0, i-C16:0, i-C15:0	ai-C15:0, i-C15:0	ai-C15:0, ai-C17:0, i-C15:0, i-C16:0	ai-C15:0, ai-C17:0, i-C15:0, i-C16:0
Polar lipids*	DPG, PG, PI, PL, GL	DPG, PG, PI, GL, PL	DPG, PG, PI, DMDG	DPG, PG, DMDG
Peptidoglycan type	A4α	A2 or A4α	A3α	A4α
G + C content (mol %)	68	66–76	61–66	60–65

* DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; DMDG, dimannosyldiacylglycerol; PL, unknown phospholipid; GL, unknown glycolipid.

(1996). The G + C content of strain 4-0^T was determined to be 68 mol %.

Based on phylogenetic studies, strain 4-0^T clusters within the radiation of *Arthrobacter* and *Micrococcus* species (Fig. 1) and it shares with them family-specific 16S rDNA signature nucleotides. Its taxonomic position separate from both genera is indicated by comparable levels of 16S rDNA sequence similarity to certain members of the two genera and it can be distinguished from members of the two genera by its chemotaxonomic characteristics (menaquinone system and peptidoglycan type) (Table 2). Hence, it cannot be considered as a member of either of these genera. Also, the preliminary identification of strain 4-0^T as a member of the genus *Kocuria*, based on biochemical characteristics, the presence of lysine in the cell wall and similarities in the polar lipid patterns and fatty acid profiles (Altenburger *et al.*, 1996), is not supported

by the results presented here. Our results suggest that strain 4-0^T is a novel species belonging to a new genus, for which the name *Citricoccus muralis* gen. nov., sp. nov. is proposed.

Description of *Citricoccus* gen. nov.

Citricoccus (Cit.ri.coc'cus. L. n. *citrus* lemon, citron tree or citrus, an African tree; M.L. n. *coccus* a sphere; N.L. n. *Citricoccus* lemon-yellow-pigmented coccus).

Cells are non-motile, Gram-positive, non-spore-forming cocci, occurring singly, in short chains or in clusters, about 1 µm in diameter. Aerobic. Oxidase-negative and catalase-positive. The peptidoglycan type is Ala/Gly/Glu/Lys with Lys–Gly–Glu in the interpeptide chain (variation A4α). The quinone system contains MK-9(H₂) as the predominant menaquinone and moderate amounts of MK-7(H₂) and MK-8(H₂). Polar lipids are diphosphatidylglycerol, phosphatidyl-

glycerol, phosphatidylinositol, four unknown glycolipids, two unknown phospholipids and an unknown lipid. The major fatty acids are ai-C15:0, ai-C17:0, i-C16:0 and i-C15:0. Spermidine is predominant in the polyamine pattern. Phylogenetically, this genus is a member of the family *Micrococcaceae*. The type species is *Citricoccus muralis*.

Description of *Citricoccus muralis* sp. nov.

Citricoccus muralis (mu.ra'lis. L. adj. *muralis*, -le pertaining or belonging to walls).

Morphological, chemotaxonomic and general characteristics are as described for the genus. Colonies reach a maximum size of 3 mm after 2 weeks incubation. They are yellow-pigmented, circular, convex and opaque. No acid is produced from glucose, sucrose, salicin, inositol, rhamnose, trehalose, cellobiose or D-mannose. Gluconate, D-glucose, D-maltose, D-mannose, D-sucrose, D-trehalose, acetate, propionate, *cis*-aconitate, fumarate, glutarate, DL-3-hydroxybutyrate, DL-lactate, L-malate, oxoglutarate, pyruvate, L-aspartate, L-leucine, L-phenylalanine, L-proline, L-serine, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate are assimilated. *N*-Acetyl D-galactosamine, *N*-acetyl D-glucosamine, L-arabinose, *p*-arbutin, D-cellobiose, D-fructose, D-galactose, α -D-melibiose, L-rhamnose, D-ribose, salicin, D-xylose, adonitol, *i*-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, itaconate, mesaconate, suberate, L-alanine, β -alanine, L-histidine, L-ornithine and L-tryptophan are not assimilated. Aesculin, *p*-nitrophenyl (*p*NP) α -D-glucopyranoside, 2-deoxythymidine-5'-*p*NP phosphate, L-alanine *p*-nitroanilide (L-alanine *p*NA) and L-proline *p*NA are hydrolysed. *p*NP β -D-Galactopyranoside, *p*NP β -D-glucuronide, *p*NP β -D-glucopyranoside, *p*NP β -D-xylopyranoside, bis-*p*NP phosphate, *p*NP phenylphosphonate, *p*NP phosphorylcholine and L-glutamate- γ -3-carboxy *p*NA are not hydrolysed. Tweens 20 and 80 are decomposed. Casein and starch are not decomposed. Nitrate is not reduced to nitrite. H₂S and indole production are negative. Urease-positive. Good growth occurs at room temperature and at 28 °C. Moderate growth occurs at 4 °C and no growth is seen at 37 °C. Good growth occurs at NaCl concentrations up to 7% (w/v), weak growth at 10% (w/v) and no growth at 15% (w/v). Growth is observed at initial pH values between 6 and 10. The DNA G + C content is 68 mol% (by HPLC). Isolated from a medieval wall painting in the church of St Georgen in Styria, Austria. The type strain is strain 4-0^T (= DSM 11442^T = CCM 4981^T).

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