Microbacterium agarici sp. nov., *Microbacterium humi* sp. nov. and *Microbacterium pseudoresistens* sp. nov., isolated from the base of the mushroom *Agaricus blazei*

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Three Gram-positive, rod-shaped bacteria (strains CC-SBCK-209^T, CC-12309^T and CC-5209^T) were isolated from the stalk of the edible mushroom *Agaricus blazei* grown in the laboratory. 16S rRNA gene sequence analysis indicated that all three isolates clearly belonged to the genus *Microbacterium*. Strains CC-SBCK-209^T and CC-12309^T were most related closely to the type strain of *Microbacterium halotolerans* (95.9 and 96.1 % 16S rRNA gene sequence similarity, respectively). These two novel strains shared 97.9 % 16S rRNA gene sequence similarity. Levels of similarity to the type strains of all other recognized *Microbacterium* species were lower than 95.5 %. The third strain (CC-5209^T) showed the highest 16S rRNA gene sequence similarity to the type strain of *Microbacterium resistens* (97.6 %); levels of similarity to the type strains of all other recognized *Microbacterium* species were lower than 96 %. The quinone systems of strains CC-SBCK-209^T, CC-12309^T and CC-5209^T consisted of MK-11/MK-12, MK-11/MK-10 and MK-13 as major compounds, respectively. All three strains contained ornithine in their peptidoglycan. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. The polyamine pattern consisted of spermidine and spermine as predominant components. Fatty acid profiles (anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} as major components) supported the affiliation of all three strains to the genus *Microbacterium*. The results of physiological and biochemical tests and DNA–DNA hybridization experiments allowed the clear phenotypic and genotypic differentiation of strains CC-SBCK-209^T and CC-12309^T from *M. halotolerans* and other closely related *Microbacterium* species. Strain CC-5209^T could be differentiated clearly from *M. resistens* both genotypically and phenotypically. Based on these data, the novel strains are considered to represent three novel species of the genus *Microbacterium*. The names proposed for these organisms are *Microbacterium agarici* sp. nov. [type strain CC-SBCK-209^T (=DSM 21798^T=CCM 7686^T)], *Microbacterium humi* sp. nov. [type strain CC-12309^T (=DSM 21799^T=CCM 7687^T)] and *Microbacterium pseudoresistens* sp. nov. [type strain CC-5209^T (=DSM 22185^T=CCM 7688^T)].

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains CC-SBCK-209^T, CC-12309^T and CC-5209^T are FJ807673, FJ865215 and FJ865214, respectively.

A table giving the fatty acid profiles of strains CC-12309^T, CC-SBCK-209^T and CC-5209^T and the type strains of closely related *Microbacterium* species is available as supplementary material with the online version of this paper.

The genus *Microbacterium* was described by Orla-Jensen (1919) and comprises a diverse collection of Gram-positive, non-spore-forming rods that were isolated from various environmental habitats, including plants, soil, water, steep liquor and milk products, and also from humans. Collins *et al.* (1983) emended the description of the genus, and Takeuchi & Hatano (1998) emended the description again with the unification of the genera *Microbacterium* and *Aureobacterium*.

Here we describe the phenotypic and genotypic properties of three novel *Microbacterium* strains isolated from an edible mushroom.

Strains CC-SBCK-209^T, CC-12309^T and CC-5209^T were isolated from the stalk of the mushroom *Agaricus blazei* grown in the laboratory, on nutrient agar (NA; Hi-Media) after incubation at 30 °C for 48 h, and were maintained on NA. The strains were preserved at -80 °C in nutrient broth (NB; Hi-Media) with 20 % (v/v) glycerol or by lyophilization.

Morphological properties, Gram-staining and cell morphology were observed by using phase-contrast microscopy as described by Kämpfer & Kroppenstedt (2004).

The 16S rRNA gene sequences of all three strains were determined and analysed as described by Young *et al.* (2005). Multiple sequence alignment and analysis of the data were performed by using the software package MEGA version 4 (Tamura *et al.*, 2007). Genetic distance calculations (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method (Fig. 1) and maximum-parsimony method (results not shown) were performed by using bootstrap values based on 1000 replications. The 16S rRNA gene sequences of strains CC-SBCK-209^T, CC-12309^T and CC-5209^T were continuous stretches of 1480, 1430 and 1489 bp, respectively. Strains CC-SBCK-209^T and CC-12309^T shared 97.9 % 16S rRNA gene sequence similarity; these strains were related most closely to *Microbacterium halotolerans* DSM 15855^T (95.9 and 96.1 % similarity, respectively). Levels of 16S rRNA gene sequence similarity between strains CC-SBCK-209^T and CC-12309^T and the type strains of all other recognized *Microbacterium* species were below 95.5 %.

Strain CC-5209^T was related most closely to the type strain of *Microbacterium resistens* (97.6 % 16S rRNA gene sequence similarity). Levels of less than 96 % similarity were found to the type strains of all other recognized *Microbacterium* species.

The peptidoglycan of strains CC-SBCK-209^T and CC-12309^T grown in trypticase soy yeast extract medium (medium 92; <http://www.dsmz.de>) and of strain CC-5209^T grown in brain heart infusion broth (Becton Dickinson) supplemented with haemin (1 mg l⁻¹) at 28 °C was extracted after disruption of the cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). Amino acids were analysed by

using GC (GC 14A; Shimadzu) and GC-MS (320-MS Quadrupole GC/MS; Varian) of *N*-heptafluorobutyl amino acid isobutyl esters (MacKenzie, 1987; Groth *et al.*, 1996). The hydrolysates (4 M HCl, 100 °C, 16 h) of the purified peptidoglycan of strains CC-SBCK-209^T, CC-12309^T and CC-5209^T contained the amino acids ornithine, glycine, alanine and glutamic acid, but did not contain homoserine. Strain CC-5209^T differed from the other two novel strains because a relatively high amount of glutamic acid in the peptidoglycan was replaced by 3-hydroxyglutamic acid (Hyg) and the ratio of glycine to ornithine was lower. Peptides in the cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using the solvent systems described by Schleifer (1985). The peptides Gly→D-Glu and D-Ala→D-Orn were detected in the partial hydrolysates (4 M HCl, 100 °C, 0.75 h) of the peptidoglycan of all three strains. The peptides Gly→D-Orn and Gly→D-Orn←D-Ala were detected only in strains CC-SBCK-209^T and CC-12309^T. These data suggested that the peptidoglycan of strains CC-SBCK-209^T and CC-12309^T is of type B2α with an interpeptide bridge D-Glu→Gly→D-Orn (Schleifer & Kandler, 1972; B12 according to http://www.dsmz.de/microorganisms/main.php?content_id=35). The data supported the view that the peptidoglycan of strain CC-5209^T is of type B2α with an interpeptide bridge D-Glu→D-Orn (Schleifer & Kandler, 1972; B9 according to http://www.dsmz.de/microorganisms/main.php?content_id=35), which was found also in its phylogenetic neighbour, *M. resistens* (Funke *et al.*, 1998). For polyamine, quinone and polar lipid analyses, cells were grown at 30 °C for 48 h in PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2). Polyamines were extracted and analysed as described by Altenburger *et al.* (1997). Analyses of quinones and polar lipids were carried out according to Tindall (1990a, b) and Altenburger *et al.* (1996). The HPLC system used for analysis of quinones and polyamines was equipped as described by Stolz *et al.* (2007).

The polyamine pattern of strain CC-SBCK-209^T contained 4.2 µmol spermidine (g dry weight)⁻¹ and 3.6 µmol spermine (g dry weight)⁻¹, the polyamine pattern of strain CC-12309^T contained 0.9 µmol spermidine (g dry weight)⁻¹ and 1.3 µmol spermine (g dry weight)⁻¹, and the polyamine pattern of strain CC-5209^T contained 0.3 µmol spermidine (g dry weight)⁻¹ and 0.1 µmol spermine (g dry weight)⁻¹. The three novel strains also contained trace amounts of putrescine or putrescine and cadaverine [<0.1 µmol (g dry weight)⁻¹]. The relatively high polyamine contents in strains CC-SBCK-209^T and CC-12309^T can be used to differentiate the two strains from recognized *Microbacterium* species. This polyamine pattern is rare among other examined microbacteria (Altenburger *et al.*, 1997). Only *Microbacterium aurum* was reported to contain these major polyamines in concentrations corresponding to those of strain CC-12309^T. The quinone system of strain CC-12309^T consisted of MK-11 (52 %), MK-10 (37 %) and MK-12 (11 %), similar to that reported for *M. halotolerans* (Li *et al.*, 2005).

Fig. 1. Neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences showing the positions of strains CC-SBCK-209^T, CC-12309^T and CC-5209^T among the type strains of selected *Microbacterium* species available from GenBank/EMBL/DBJ (accession numbers are given in parentheses). Bootstrap values based on 1000 replications are listed as percentages at branch points. Bar, 0.01 substitutions per nucleotide position.

The quinone system of strain CC-SBCK-209^T consisted of MK-11 (59%), MK-12 (36%) and MK-10 (5%), clearly distinguishing it from strain CC-12309^T and from *M. halotolerans*. The quinone system of strain CC-5209^T comprised MK-13 (92%) as the major compound, with minor amounts of MK-12 (4%) and MK-14 (4%). It can be assumed that examination of additional strains of any of these species would show very similar quinone systems, as it has been shown that the relative amounts of the major quinones for four strains of *Microbacterium paraoxydans*

are quite stable (Buczolits *et al.*, 2008). Strains CC-SBCK-209^T, CC-12309^T and CC-5209^T exhibited polar lipid profiles consisting of the major components diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid that showed identical behaviour during TLC analyses of the extracts of the three strains. These major polar lipids have been detected in numerous *Microbacterium* species (Zlamala *et al.*, 2002; Rivas *et al.*, 2004; Kim *et al.*, 2005; Shivaji *et al.*, 2007; Buczolits *et al.*, 2008; Park *et al.*, 2006) and they have also been listed in the emended description

of the genus (Takeuchi & Hatano, 1998). In addition, in the extract from strain CC-SBCK-209^T, minor amounts of a second unknown glycolipid and two unknown phospholipids were detected (Fig. 2). Although no additional lipids were detected in strain CC-12309^T, trace amounts of three lipids not detectable with any of the specific reagents used (molybdenum blue, ninhydrin, α -naphthol) were detected in the extract of strain CC-5209^T. However, these polar lipid profiles are in accordance with the placement of the three strains in the genus *Microbacterium*.

Fatty acid analysis was performed according to Kämpfer & Kroppenstedt (1996). The fatty acid profiles of all three strains were very similar to those of closely related *Microbacterium* species (see Supplementary Table S1, available in IJSEM Online). A fatty acid profile typical of the genus, with anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} as major components, was found.

Results of comparative physiological characterization under identical test conditions (Kämpfer *et al.*, 1991) are given for strains CC-SBCK-209^T and CC-12309^T in comparison with the type strains of *M. halotolerans*, *Microbacterium gubbeenense* and *Microbacterium barkeri* in Table 1 and in the species descriptions below. DNA–DNA hybridization experiments were performed with strains CC-SBCK-209^T and CC-12309^T and *M. halotolerans* DSM 15855^T according to the method of Ziemke *et al.* (1998). Levels of DNA–DNA relatedness between strains CC-SBCK-209^T and CC-12309^T were 45 and 49 % (reciprocal analyses). The level of DNA–DNA relatedness between strain CC-SBCK-209^T and *M. halotolerans* DSM 15855^T was 26 % (14.1 % reciprocal analysis) and between strain CC-12309^T and *M. halotolerans* DSM 15855^T was 21.5 % (21.1 % reciprocal analysis).

Table 1 also provides a comparison of the phenotypic characteristics of strain CC-5209^T and the type strain of *M. resistens*. The level of DNA–DNA relatedness between strain CC-5209^T and *M. resistens* CCUG 38312^T was 42 % (34.1 % reciprocal analysis).

Peptidoglycan types, quinone systems, fatty acid and polar lipid profiles as well as 16S rRNA gene sequence analyses showed unambiguously that strains CC-SBCK-209^T, CC-12309^T and CC-5209^T are affiliated with the genus *Microbacterium*. On the basis of observed phenotypic differences, the results of DNA–DNA hybridization experiments and differences in 16S rRNA gene sequences we propose that these strains represent novel species of the genus *Microbacterium*, for which the names *Microbacterium agarici* sp. nov., *Microbacterium humi* sp. nov. and *Microbacterium pseudoresistens* sp. nov. are proposed.

Description of *Microbacterium agarici* sp. nov.

Microbacterium agarici (a.ga.ri'ci. N.L. gen. n. *agarici* of *Agaricus*, the generic name of the mushroom *Agaricus blazei* from where the type strain was isolated).

Cells are short rods, about 2–3 μ m in length and 1–1.2 μ m in width. Cells are Gram-positive, oxidase-positive and catalase-positive, showing aerobic respiratory metabolism. Good growth occurs after 3 days incubation on R2A agar (Oxoid) and NA at 25–30 °C; colonies on NA are yellow. The peptidoglycan is of type B2 α with an interpeptide bridge D-Glu→Gly→D-Orn. The quinone system is composed of the major components MK-11 and MK-12 and minor amounts of MK-10. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid, plus minor amounts of two unknown

Fig. 2. Polar lipid profile of strain CC-SBCK-209^T after two-dimensional TLC and detection with molybdatophosphoric acid. Abbreviations: DPG, diphosphatidylglycerol; GL1, GL2, unknown glycolipids; PG, phosphatidylglycerol; PL1, PL2, unknown phospholipids.

Table 1. Differential physiological characteristics between strains CC-12309^T, CC-SBCK-209^T, CC-5209^T and the type strains of closely related *Microbacterium* species

Strains: 1, CC-12309^T (*M. humi* sp. nov.); 2, CC-SBCK-209^T (*M. agarici* sp. nov.); 3, *M. halotolerans* DSM 15855^T; 4, *M. gubbeenense* DSM 15944^T; 5, *M. barkeri* JCM 1343^T; 6, CC-5209^T (*M. pseudoresistens* sp. nov.); 7, *M. resistens* CCUG 38312^T. All data are from the present study. Acid production from D-arabitol, dulcitol, erythritol, maltose, melibiose, methyl D-glucoside, raffinose, rhamnose, salicin, sorbitol and trehalose was negative for all strains. All strains hydrolysed aesculin, pNP- α -D-glucopyranoside, pNP- β -D-glucopyranoside and L-alanine-pNA (oNP, *o*-nitrophenyl; pNP, *p*-nitrophenyl; pNA, *p*-nitroanilide). All strains utilized cellobiose, D-fructose, D-glucose, DL-lactate, D-mannose, maltose, D-mannitol, L-proline, sucrose and trehalose as sole carbon source. None of the strains was able to utilize *N*-acetyl-D-galactosamine, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, glutarate, DL-3-hydroxybutyrate, itaconate, L-leucine, mesaconate, phenylacetate, L-phenylalanine, suberate or L-tryptophan. +, Positive; –, negative, (+), weakly positive.

Characteristic	1	2	3	4	5	6	7
Acid production from:							
Adonitol	–	(+)	–	–	–	–	–
L-Arabinose	–	–	–	–	+	–	–
Cellobiose	–	–	–	–	+	–	–
Glucose	(+)	–	–	–	+	–	–
Lactose	(+)	–	–	–	–	–	–
D-Mannitol	+	+	+	+	+	–	–
D-Mannose	–	–	–	–	+	–	–
Sucrose	+	–	–	–	–	–	–
D-Xylose	–	–	–	–	+	–	–
Hydrolysis of:							
Aesculin	+	(+)	+	+	+	+	+
oNP- β -D-galactopyranoside	+	+	+	+	+	–	+
pNP- β -D-glucuronide	+	–	–	–	–	–	–
pNP- β -D-xylopyranoside	+	+	+	+	+	–	+
Bis-pNP-phosphate	+	–	+	+	+	–	+
pNP-phenyl-phosphonate	+	–	+	+	+	+	+
pNP-phosphoryl-choline	–	–	–	–	–	–	+
2-Deoxythymidine-5'-pNP-phosphate	–	–	–	–	+	–	+
L-Glutamate- γ -3-carboxy-pNA	–	–	–	–	–	–	+
L-Proline-pNA	–	+	+	+	+	+	+
Assimilation of:							
<i>N</i> -Acetyl-D-glucosamine	+	–	+	+	+	+	+
L-Arabinose	+	+	+	+	+	–	+
<i>p</i> -Arbutin	–	–	+	+	+	–	+
D-Galactose	+	+	+	+	+	–	+
Gluconate	–	–	+	+	+	–	+
α -Melibiose	+	–	–	–	+	–	–
L-Rhamnose	+	+	+	+	+	–	+
D-Ribose	+	+	+	+	+	+	+
Salicin	–	+	+	+	+	+	+
D-Xylose	+	+	+	–	+	–	(+)
Adonitol	–	+	–	–	–	–	(+)
i-Inositol	–	–	–	–	+	–	–
Maltitol	+	+	–	–	+	–	+
D-Sorbitol	+	+	+	–	–	–	(+)
Putrescine	–	+	–	–	+	–	–
Acetate	–	–	–	(+)	+	–	+
Propionate	–	–	–	(+)	+	–	+
<i>cis</i> -Aconitate	–	–	+	+	+	–	–
Citrate	–	–	+	+	+	–	+
Fumarate	+	–	–	–	+	(+)	–
L-Malate	+	+	–	–	+	–	–
Oxoglutarate	+	–	–	–	+	–	–
Pyruvate	+	–	+	+	+	–	+
L-Alanine	–	–	+	+	+	–	+

Table 1. cont.

Characteristic	1	2	3	4	5	6	7
β -Alanine	–	–	–	+	–	–	–
L-Aspartate	+	+	+	+	+	–	–
L-Histidine	–	–	–	+	+	–	–
L-Ornithine	–	–	–	+	–	–	–
L-Serine	–	–	–	+	+	–	–
3-Hydroxybenzoate	–	–	+	–	+	–	–
4-Hydroxybenzoate	–	–	–	–	+	–	–

phospholipids and another glycolipid. The predominant polyamines are spermidine and spermine. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. Differential physiological characteristics are given in Table 1. Many sugar and other compounds are utilized, including salicin, D-adonitol and putrescine, but not *N*-acetyl-D-glucosamine, α -melibiose or fumarate.

The type strain, CC-SBCK-209^T (=DSM 21798^T=CCM 7686^T), was isolated from the stalk of the edible mushroom *Agaricus blazei* grown in the laboratory.

Description of *Microbacterium humi* sp. nov.

Microbacterium humi (hu'mi. L. gen. n. *humi* of earth, soil, of soil, the source of the type strain).

Cells are short rods, about 2–3 μ m in length and 1–1.2 μ m in width. Cells are Gram-positive, oxidase-positive and catalase-positive, showing aerobic respiratory metabolism. Good growth occurs after 3 days incubation on R2A agar and NA at 25–30 °C; colonies on NA are yellow. The peptidoglycan is of type B2 α with an interpeptide bridge D-Glu→Gly→D-Orn. The quinone system is composed of the major components MK-11 and MK-10 and minor amounts of MK-12. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. The predominant polyamines are spermidine and spermine. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. Differential physiological characteristics are given in Table 1. Many sugars and other compounds are utilized, including *N*-acetyl-D-glucosamine, α -melibiose and fumarate, but not salicin, D-adonitol or putrescine.

The type strain, CC-12309^T (=DSM 21799^T=CCM 7687^T), was isolated from the stalk of the edible mushroom *Agaricus blazei* grown in the laboratory.

Description of *Microbacterium pseudoresistens* sp. nov.

Microbacterium pseudoresistens (pseu.do.re.sis'tens. Gr. adj. *pseudes* false; L. part adj. *resistens* resisting and also a bacterial specific epithet; N.L. part. adj. *pseudoresistens* the false *Microbacterium resistens*).

Cells are short rods, about 2–3 μ m in length and 1–1.2 μ m in width. Cells are Gram-positive, oxidase-positive and

catalase-positive, showing aerobic respiratory metabolism. Good growth occurs after 3 days incubation on R2A agar and NA at 25–30 °C; colonies on NA are yellow. The peptidoglycan is of type B2 α with an interpeptide bridge D-Glu→D-Orn. The quinone system is composed of the predominant menaquinone MK-13 with minor amounts of MK-12 and MK-14. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid and trace amounts of three unknown lipids. The predominant polyamines are spermidine and spermine. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. Differential physiological characteristics are given in Table 1. Many sugar and other compounds utilized by *M. resistens* CCUG 38312^T are not utilized, including D-galactose, L-rhamnose and acetate.

The type strain, CC-5209^T (=DSM 22185^T=CCM 7688^T), was isolated from the stalk of the edible mushroom *Agaricus blazei* grown in the laboratory.

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