Agrococcus carbonis sp. nov., isolated from soil of a coal mine

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An actinobacterial strain, designated G4^T, isolated from a coal mine was subjected to polyphasic taxonomic characterization. Cells were Gram-stain-positive, yellow-pigmented, non-motile and non-spore-forming cocci. This organism possessed a type B peptidoglycan with diaminobutyric acid as diagnostic diamino acid. The major respiratory quinones were MK-9, MK-10 and MK-11. The major fatty acids were anteiso-C_{15:0} (41.6 %) and anteiso-C_{17:0} (32.8 %). The predominant cellular polar lipids were diphosphatidylglycerol and phosphatidylglycerol. Cell wall sugars comprised galactose, glucose, ribose and rhamnose. 16S rRNA gene sequence analysis of strain G4^T showed high similarity with *Agrococcus baldri* (98.9 %), *Agrococcus citreus* (97.8 %), *Agrococcus jenensis* (97.3 %) and *Agrococcus terreus* (97.0 %). Sequence similarity with the type strains of the other species of the genus *Agrococcus* was less than 97.0 %. The DNA–DNA relatedness of strain G4^T with the type strains of *Agrococcus baldri*, *Agrococcus citreus*, *Agrococcus jenensis* and *Agrococcus terreus* was less than 70 %. On the basis of the physiological, biochemical and chemotaxonomic characteristics, strain G4^T should be classified as the type strain of a novel species of the genus *Agrococcus*, for which the name *Agrococcus carbonis* sp. nov. is proposed. The type strain is G4^T (=MTCC 10213^T =DSM 22965^T).

The genus *Agrococcus* was created by Groth *et al.* (1996) to accommodate two novel coryneform bacterial strains which were markedly different from the closely related members of the genera *Agromyces*, *Clavibacter* and *Rathayibacter* in the family *Microbacteriaceae*. The members of the genus *Agrococcus* form a distinct lineage within the family *Microbacteriaceae* (Park *et al.*, 1993; Rainey *et al.*, 1994; Stackebrandt *et al.*, 1997). At the time of writing, the genus *Agrococcus* comprises eight species with validly published names, *Agrococcus jenensis* (Groth *et al.*, 1996), *Agrococcus citreus* (Wieser *et al.*, 1999), *Agrococcus baldri* (Zlamala *et al.*, 2002), *Agrococcus lahoulensis* (Mayilraj *et al.*, 2006), *Agrococcus casei* (Bora *et al.*, 2007), *Agrococcus jejuensis* (Lee, 2008), *Agrococcus versicolor* (Behrendt *et al.*, 2008) and *Agrococcus terreus* (Zhang *et al.*, 2010). All of these strains have been isolated from diverse environmental niches like forest soil, dried seaweed, plant phyllosphere,

smear-ripened cheese, soil of Lahaul-spiti valley (Indian Himalayas), air, medieval wall paintings, compost soil and sandstone surface. While screening bacterial isolates from a soil sample of a coal mine, collected from Bonjemehari colliery, West Bengal, India, a novel species of the genus *Agrococcus* was isolated, which was characterized using a polyphasic approach.

Strain G4^T was isolated from a soil sample collected from a coal mine by the standard method of dilution plating on tryptic soy agar medium (TSA; HiMedia). The pure isolate was routinely cultured on TSA plates at 30 °C and also preserved in 10 % glycerol solution at –70 °C. The reference type strains *Agrococcus baldri* MTCC 6457^T, *Agrococcus citreus* MTCC 6410^T, *Agrococcus jenensis* MTCC 6413^T and *Agrococcus terreus* MTCC 10319^T were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), an International Depository Authority, Chandigarh, India.

Colony and cell morphology was studied according to standard methods as described by Murray *et al.* (1994). Gram staining was done using a Gram staining kit

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain G4^T is GQ504748.

Two supplementary tables and a supplementary figure are available with the online version of this paper.

(HiMedia). Motility was determined by a hanging drop method by observing slides under a phase-contrast microscope (Zeiss). For anaerobiosis, TSA plates were streaked and placed in an anaerobic jar (MART), which was evacuated and flushed using an Anoxomat unit (MART) with an anaerobic gas mixture consisting of nitrogen (85%), carbon dioxide (10%) and hydrogen (5%). Plates were incubated at 30 °C for 5 days. Physiological tests (temperature, pH and NaCl tolerance) to determine optimum growth conditions were performed in tryptic soy broth (HiMedia). Catalase activity, citrate utilization (using Simmons' citrate agar), H₂S production and urea hydrolysis were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch and gelatin, indole production, methyl red and Voges–Proskauer tests, oxidase activity and endospore staining were assessed as described by Smibert & Krieg (1994). Nitrate reduction was tested by the method of Lányi (1987). Additional physiological and biochemical tests were performed using API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Susceptibility to various antibiotics was studied using antibiotic discs (HiMedia) on TSA plates. Utilization of various substrates as sole carbon and energy sources was tested using Biolog GP2 MicroPlates in accordance with the manufacturer's instructions, except that TSB medium was used instead of Biolog Universal Growth agar medium (Mayilraj *et al.*, 2006). Plates were incubated for 48 h and the results were read with a MicroPlate reader (Biolog Microstation ELx808BLG) using Microlog 4.2 computer software. Acid production from various carbohydrates was tested as described by Smith *et al.* (1952).

The differential physiological, biochemical and chemotaxonomic characteristics of strain G4^T and the four type strains of *Agrococcus baldri*, *Agrococcus citreus*, *Agrococcus jenensis* and *Agrococcus terreus* are summarized in Table 1. All these five strains are positive for β -galactosidase (*p*-nitrophenyl β -D-galactopyranosidase) and negative for fermentation of glucose, arginine dihydrolase, urease, hydrolysis of aesculin, assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid as determined using API 20NE strips. They are also positive for esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and α -glucosidase and negative for alkaline phosphatase, lipase (C14), valine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase as determined using API ZYM strips. The isolate is clearly distinguishable from the other species of the genus *Agrococcus* on the basis of utilization of various substrates on GP2 Biolog MicroPlates (Supplementary Table S1, available in IJSEM Online). Strain G4^T differs from closely related species of the genus *Agrococcus* in the utilization of 41 substrates.

For chemotaxonomic studies, bacterial biomass was lyophilized following cultivation in tryptic soy broth for 3 days at 30 °C. Respiratory isoprenoid quinones were extracted by using the method described by Minnikin *et al.* (1984). The purified menaquinones were separated by reversed phase HPLC (Shimadzu) (Kroppenstedt, 1982). Polar lipids were extracted following the method of Bligh & Dyer (1959). Polar lipid spots were detected using the following spray reagents: molybdatophosphoric acid (5%, w/v, in absolute ethanol; TLC plate developed at 120 °C overnight), molybdenum blue spray reagent (spots appear immediately), ninhydrin (0.2%, w/v, in acetone; TLC plate developed at 100 °C for 5 min) and anisaldehyde reagent (TLC plate heated at 100 °C for 10 min) for detection of total lipids, phospholipids, aminolipids and glycolipids, respectively. Whole-cell sugars were analysed by using the methods described by Stanek & Roberts (1974). The diagnostic cell wall diamino acid and peptidoglycan structure were determined by using a hydrolysate of purified cell walls, according to Schleifer & Kandler (1972). For cellular fatty acid analysis, strains were grown on TSA medium at 30 °C. Fatty acid methyl ester analysis was performed by using gas chromatography according to the instructions of the Sherlock Microbial Identification System (MIDI, Sasser, 1990) as described previously (Pandey *et al.*, 2002).

The major respiratory quinones of strain G4^T are MK-9 (31.2%), MK-10 (35.2%) and MK-11 (28.4%) with MK-12 (5.0%) as a minor component. Fatty acid profiles of strain G4^T and other type strains of the genus *Agrococcus* were characterized by major amounts of saturated and iso- or anteiso- branched fatty acids. Small amounts of unsaturated fatty acids were also detected in some strains. Anteiso-C_{15:0} (41.6%) and anteiso-C_{17:0} (32.8%) were the major fatty acid components in strain G4^T, consistent with those found in members of the genus *Agrococcus*. The comparative fatty acid profiles of strain G4^T and other related species of genus *Agrococcus* are summarized in Supplementary Table S2. Strain G4^T showed a B-type peptidoglycan containing the amino acids alanine, glycine, threonine, diaminobutyric acid, aspartic acid and glutamic acid in a molar ratio of 1.9:1.3:0.6:0.5:0.7:1.0. The diagnostic cell wall diamino acid was diaminobutyric acid. The peptidoglycan of strain G4^T shows similarity to those of *Agrococcus jenensis* and *Agrococcus lahaulensis* but differs from those of *Agrococcus citreus* and *Agrococcus baldri* in the presence of threonine. The composition of the cell wall is an important chemotaxonomic marker to substantiate the affiliation of the isolate to the genus *Agrococcus*. The cell-wall sugars were glucose, galactose, ribose and minor amounts of rhamnose. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, two unknown glycolipids and several unknown lipids (Supplementary Fig. S1).

For 16S rRNA gene sequencing, genomic DNA extraction and amplification were performed as described previously (Mayilraj *et al.*, 2005). The 16S rRNA gene was amplified

Table 1. Differential characteristics of strain G4^T and the type strains of its close phylogenetic neighbours

Strains: 1, G4^T; 2, *Agrococcus baldri* MTCC 6457^T; 3, *Agrococcus citreus* MTCC 6410^T; 4, *Agrococcus jenensis* MTCC 6413^T; 5, *Agrococcus terreus* MTCC 10319^T. All data from this study, except where indicated. All strains are positive for catalase activity but negative for oxidase, hydrolysis of casein, methyl red and Voges–Proskauer reactions, citrate utilization, indole production, urea hydrolysis, H₂S production and nitrate reduction. They are positive for acid production from cellobiose and sucrose and negative for acid production from dulcitol, galactose, inositol, inulin, lactose, rhamnose, salicin and sorbitol. All strains are susceptible to the following antibiotics (µg per disc): vancomycin (30), amoxicillin (10), tetracycline (30), fusidic acid (30), chloramphenicol (10), penicillin G (10), ciprofloxacin (5), nitrofurantoin (300), neomycin (30), polymyxin B (300), streptomycin (10), lincomycin (2), bacitracin (10), novobiocin (30), teicoplanin (30), imipenem (10) and ampicillin (10); and resistant to norfloxacin (10) and ceftazidime (30). +, Positive; –, negative; ND, not determined; R, resistant; S, sensitive; PG, phosphatidylglycerol; DPG, diphenylphosphatidylglycerol; GL, glycolipids; UL, unknown lipids.

Characteristics	1	2	3	4	5
Origin of isolation	Coal mine soil	Air	Wall paintings	Soil	Forest soil
Growth at 37 °C	+	–	–	–	+
Growth with 9 % NaCl	–	–	–	–	+
Hydrolysis of:					
Gelatin	–	–	–	–	+
Starch	–	–	–	+	+
Acid production from:					
Adonitol	+	–	+	–	–
Arabinose	+	–	+	–	–
Glucose	+	+	+	–	+
Fructose	+	–	+	+	+
Maltose	+	+	+	–	–
Mannitol	–	+	–	+	+
Mannose	+	–	–	–	+
Melibiose	+	+	+	–	–
Raffinose	+	+	+	–	–
Trehalose	+	+	+	–	–
Xylose	+	+	+	–	–
Antibiotic susceptibility (µg per disc)					
Gentamicin (10)	R	S	R	S	S
Oxacillin (1)	R	R	R	S	S
Erythromycin (15)	S	S	S	S	R
Meticillin (5)	R	S	S	R	S
Cefoxitin (30)	R	S	S	S	S
Kanamycin (30)	R	R	S	S	S
Cephalothin (30)	R	R	S	S	S
Trimethoprim (5)	R	S	R	R	S
Colistin (10)	R	S	S	S	S
Polar lipids	PG, DPG, 2 GL, 6 UL	PG, DPG, 2 GL, 1 UL ^a	PG, DPG, 2 GL, 4 UL ^b	PG, DPG, 2 GL, 1 UL ^c	PG, DPG, 2 GL ^d
Whole cell sugars	Galactose, glucose, ribose, rhamnose	Galactose, glucose, ribose ^a	Glucose, rhamnose, mannose, ribose, galactose, trehalose ^b	Glucose, mannose ^c	ND ^d
Major menaquinones	MK-9, MK-10, MK-11	MK-9, MK-10, MK-11, MK-12 ^a	MK-11, MK-12 ^b	MK-10, MK-11, MK-12, MK-13 ^c	MK-10, MK-11 ^d
DNA G + C content (mol%)	73.8	73.8–74.9 ^a	74 ^b	74 ^c	75.9 ^d

*Data from: a, Zlamala *et al.* (2002); b, Wieser *et al.* (1999); c, Groth *et al.* (1996); d, Zhang *et al.* (2010).

by using the universal primers 27F and 1492R. Sequencing of the amplified product was done by the dideoxy chain terminator method using the Big Dye terminator kit followed by capillary electrophoresis on an ABI 310 genetic analyser (Applied Biosystems). An almost complete 16S rRNA gene sequence (1470 bases) of strain G4^T was determined for phylogenetic analysis. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). 16S rRNA gene sequences of members of the genus *Agrococcus* as well as other members of the family *Microbacteriaceae* were retrieved from the EzTaxon server. Sequences were aligned and phylogenetic trees were reconstructed by using the neighbour-joining and maximum-parsimony algorithms using MEGA software version 4 (Tamura *et al.*, 2007). Bootstrap analysis was performed to assess the confidence limits of branching. Strain G4^T showed the highest degree of sequence similarity with *Agrococcus baldri* IAM 15147^T (98.9%), *Agrococcus citreus* IAM 15145^T (97.8%), *Agrococcus jenensis* DSM 9580^T (97.3%) and *Agrococcus terreus* DNG5^T (97.0%). Sequence similarities with other type strains of the genus *Agrococcus*

were less than 97.0%, being 96.6% with *Agrococcus jejuensis* SSW1-48^T, 96.5% with *Agrococcus lahaulensis* K22-21^T, 95.6% with *Agrococcus versicolor* DSM 19812^T and 94.8% with *Agrococcus casei* LMG 22410^T. A maximum-parsimony tree based on 16S rRNA gene sequences showed that the novel organism formed a coherent branch with *Agrococcus baldri* (Fig. 1). This relationship was further supported by a high bootstrap value (78%) in the maximum-parsimony tree. The topology of the phylogenetic tree including members of the family *Microbacteriaceae* changes depending on the strain set selected for analysis (Stackebrandt *et al.*, 2007). DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1988). DNA–DNA hybridization experiments revealed $57.5 \pm 1\%$ relatedness between strain G4^T and *Agrococcus baldri* MTCC 6457^T, $63.8 \pm 0.5\%$ between strain G4^T and *Agrococcus citreus* MTCC 6410^T, $38.7 \pm 1\%$ between strain G4^T and *Agrococcus jenensis* MTCC 6413^T and $49.9 \pm 1\%$ between strain G4^T and *Agrococcus terreus* MTCC 10319^T, which is below the 70% value recommended for the delineation of bacterial species (Wayne *et al.*, 1987). The G + C content of the genomic DNA of strain G4^T was determined spectrophotometrically (Lambda 35; Perkin Elmer) using the

Fig. 1. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationship between strain G4^T and other members of the genus *Agrococcus*. *Macroccoccus equiperis* H8h3^T (Y15712) was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at nodes. Filled circles (●) represent common branches retrieved in maximum-parsimony and neighbour-joining phylogenetic trees. Bar, 50 substitutions per nucleotide position.

thermal denaturation method (Mandel & Marmur, 1968). The DNA G + C content of strain G4^T was 73.8 mol%. On the basis of the data presented above, strain G4^T is clearly distinguishable from other members of the genus *Agrococcus*. Therefore, strain G4^T is considered to represent a novel species of the genus *Agrococcus*, for which we propose the name *Agrococcus carbonis* sp. nov.

Description of *Agrococcus carbonis* sp. nov.

Agrococcus carbonis (car.bo'nis. L. gen. n. *carbonis* of coal, of charcoal).

Cells are Gram-stain-positive, coccoid (1.0–1.5 µm in diameter) and arranged in pairs, tetrads and clusters. Strictly aerobic and non-motile. Colonies are circular, yellow and 0.4–3.0 mm in diameter on TSA medium. Grows at 15–37 °C (optimum 30 °C) and pH 5–11 (optimum 7.5); no growth at 42 °C. Catalase-positive and oxidase-negative. Grows in the presence of ≤7% NaCl. Negative for starch hydrolysis, gelatin hydrolysis, citrate utilization, indole production, nitrate reduction and the methyl red and Voges–Proskauer tests. Hydrogen sulphide is not produced. Positive for utilization of the following substrates: dextrin, Tween 40, L-arabinose, D-fructose, D-galactose, D-gluconic acid, α-D-glucose, maltose, D-mannose, α-methyl D-galactoside, palatinose, D-psicose, L-rhamnose, D-ribose, sedoheptulose, sucrose, D-tagatose, turanose, D-xylose, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-malic acid, succinic acid monomethyl ester, propionic acid, pyruvic acid, L-glutamic acid, L-serine, putrescine, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine and thymidine 5'-monophosphate. Negative for utilization of α-cyclodextrin, β-cyclodextrin, glycogen, inulin, mannan, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, amygdalin, D-arabitol, arbutin, cellobiose, L-fucose, D-galacturonic acid, gentiobiose, *myo*-inositol, lactose, lactulose, maltotriose, D-mannitol, melezitose, melibiose, β-methyl D-galactoside, 3-methyl D-glucose, α-methyl D-glucoside, β-methyl D-glucoside, α-methyl D-mannoside, raffinose, salicin, D-sorbitol, stachyose, trehalose, xylitol, acetic acid, α-hydroxybutyric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, pyruvic acid methyl esters, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, glycyl L-glutamic acid, L-pyrroglutamic acid, 2,3-butanediol, adenosine 5'-monophosphate, uridine 5'-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. Acid is produced aerobically from adonitol, arabinose, cellobiose, glucose, fructose, maltose, mannose, melibiose, raffinose, xylose, sucrose and trehalose but not from dulcitol, galactose, inositol, inulin, lactose, mannitol, rhamnose or salicin. Presence of B-type peptidoglycan containing the amino acids alanine, glycine, threonine, aspartic acid, glutamic acid and the diagnostic diaminobutyric acid. The predominant fatty acids are anteiso-C_{15:0} and

anteiso-C_{17:0}. The major menaquinones are MK-9, MK-10 and MK-11. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, two unknown glycolipids and six unknown lipids. Cell wall sugars include galactose, glucose, ribose and rhamnose. The DNA G + C content of the type strain is 73.8 mol%.

The type strain, G4^T (=MTCC 10213^T =DSM 22965^T), was isolated from a soil sample collected from a coal mine of West Bengal, India.

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