Agrococcus lahaulensis sp. nov., isolated from a cold desert of the Indian Himalayas

S. Mayilraj,¹ K. Suresh,¹ P. Schumann,² R. M. Kroppenstedt² and H. S. Saini³

Correspondence

S. Mayilraj

mayil@imtech.res.in

¹Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Sector 39-A, Chandigarh, 160 036, India

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

³Department of Microbiology, Guru Nanak Dev University (GNDU), Amritsar, 143 005, India

The taxonomic position of a lemon-yellow-pigmented actinobacterium, strain K22-21^T, isolated from a soil sample from Lahaul-Spiti Valley in the Indian Himalayas, was determined using a polyphasic approach. The strain had phenotypic and chemical properties that were consistent with its classification in the genus *Agrococcus*. Alignment of the 16S rRNA gene sequence of strain K22-21^T with sequences from *Agrococcus jenensis* DSM 9580^T, *Agrococcus baldri* DSM 14215^T and *Agrococcus citreus* DSM 12453^T revealed similarities of 98.5, 96.8 and 96.6%, respectively. However, the level of DNA–DNA relatedness between strain K22-21^T and *A. jenensis* was 55.1%. The novel strain could be distinguished from type strains of the three species of the genus *Agrococcus* using DNA–DNA relatedness and phenotypic data. Based on these differences, strain K22-21^T (=MTCC 7154^T = DSM 17612^T) should be classified as the type strain of a novel species of *Agrococcus*, for which the name *Agrococcus lahaulensis* sp. nov. is proposed.

The genus *Agrococcus* forms a separate lineage within the family *Microbacteriaceae* (Park *et al.*, 1993; Rainey *et al.*, 1994; Stackebrandt *et al.*, 1997; Suzuki *et al.*, 1997). At the time of writing, the genus *Agrococcus* comprises three species, *Agrococcus jenensis* (Groth *et al.*, 1996), *Agrococcus citreus* (Wieser *et al.*, 1999) and *Agrococcus baldri* (Zlamala *et al.*, 2002).

Strain K22-21^T was isolated from a soil sample collected from the Lahaul-Spiti Valley in the Indian Himalayas by the dilution-plating technique on tryptic soy agar medium (TSA; HiMedia) and maintained as glycerol stocks at –70°C. The reference strains *Agrococcus jenensis* MTCC 6413^T, *Agrococcus citreus* MTCC 6410^T and *Agrococcus baldri* MTCC 6457^T were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

Colony and cell morphology were studied according to standard methods (Murray *et al.*, 1994). Gram reaction was determined using a Gram staining kit (HiMedia) according to the manufacturer's instructions. Growth at different

temperatures, pH and NaCl concentrations was examined by growing the strain on basal TSA medium. Catalase production, oxidase activity (with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride) and urea hydrolysis were determined as described by Cowan & Steel (1965). The following characters were analysed as described by Smibert & Krieg (1994): decomposition of casein, gelatin, Tween 20 and starch; indole and hydrogen sulfide production; methyl red and Voges–Proskauer tests; acid-fast and endospore staining; and motility. Nitrate reduction was assayed as described by Lanyi (1987). Utilization of various carbon sources was tested using Biolog GP2 MicroPlates; plates were used according to the manufacturer's instructions with the modification that TSA medium was used instead of Biolog Universal Growth agar medium. The inoculated plates were incubated for 24 h and results were read with a MicroPlate Reader using Microlog 4.2 software to perform automated reading. Acid production from various sugars was tested as described by Smith *et al.* (1952). Standard procedures were used to determine the cell-wall sugars (Staneck & Roberts, 1974). Preparation of cell walls and determination of peptidoglycan structure were done as described by Schleifer & Kandler (1972), with the modification that TLC on cellulose sheets (Merck) was used instead of paper chromatography. Polar lipids and menaquinones were extracted and analysed according to Minnikin *et al.* (1984) and Kroppenstedt (1982). The absence of mycolic acids was demonstrated by TLC (Minnikin & Goodfellow,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K22-21^T is DQ156908.

The fatty acid compositions of strain K22-21^T and the type strains of the three known *Agrococcus* species are available as supplementary material in IJSEM Online.

Table 1. Physiological and biochemical properties of strain K22-21^T and type strains of *Agrococcus* species

Strains: 1, K22-21^T; 2, *A. baldri* MTCC 6457^T; 3, *A. citreus* MTCC 6410^T; 4, *A. jenensis* MTCC 6413^T. All data are from the present study. All four strains do not produce endospores, are catalase-positive and oxidase-negative and grow in the presence of 2 and 4 % NaCl. In Biolog GP2 tests, all are positive for utilization of L-arabinose, D-fructose, D-mannitol, D-ribose and D-xylose and all are negative for utilization of α -cyclodextrin, β -cyclodextrin, glycogen, inulin, mannan, Tweens 40 and 80, *N*-acetyl-D-glucosamine, *N*-acetyl- β -D-mannosamine, amygdalin, D-arabitol, arbutin, D-cellobiose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, *myo*-inositol, α -D-lactose, lactulose, maltose, D-melezitose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, 3-methyl glucose, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, palatinose, D-raffinose, salicin, sedoheptulosan, stachyose, sucrose, D-trehalose, xylitol, acetic acid, α - and β -hydroxybutyric acids, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, α -ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D- and L-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, D- and L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-serine, L-pyroglyutamic acid, putrescine, 2,3-butanediol, uridine, AMP, TMP, UMP, α -D-glucose 1-phosphate, D-glucose 6-phosphate and DL- α -glycerol phosphate as sole carbon sources. Acid is produced from D-fructose and D-trehalose^{a*} but not from D-galactose, D-glucose, salicin, D-xylose^b or *myo*-inositol. Negative for growth at pH 5.2, utilization of citrate, methyl red and Voges-Proskauer tests, nitrate reduction, indole and urease production. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4
Decomposition of:				
Aesculin	–	–	+	+
Tyrosine	–	–	+	–
Casein	+	–	–	– ^{c*}
Gelatin	+	–	–	–
Growth in the presence of 6.5 % NaCl	+	+	+	+
H ₂ S production	–	–	– ^b	– ^c
Acid production (aerobically) from:				
L-Arabinose	w	–	+	– ^c
D-Cellobiose	–	–	+	+
D-Maltose	–	+	–	+
D-Mannitol	–	+	+	+
Glycerol	–	+	– ^b	– ^c
L-Rhamnose	+	– ^a	– ^b	– ^c
D-Raffinose	–	–	–	–
Sucrose	–	+	+	–
Utilization of substrate as sole carbon source (using Biolog GP2 MicroPlates)				
Dextrin	+	–	–	–
D-Gluconic acid	–	+	+	+
α -D-Glucose	–	+	–	+
Maltotriose	+	–	–	–
D-Mannose	–	+	+	+
D-Psicose	–	+	+	+
L-Rhamnose	+	–	–	–
D-Sorbitol	–	+	–	–
D-Tagatose	–	+	–	+
Turanose	–	–	+	–
γ -Hydroxybutyric acid	+	–	–	–
L-Alaninamide	–	–	–	+
Glycerol	+	+	–	–
Adenosine	+	+	–	–
2'-Deoxyadenosine	+	–	–	+
Inosine	+	–	–	–

Table 1. cont.

Characteristic	1	2	3	4
Thymidine	+	+	–	–
D-Fructose 6-phosphate	–	–	+	–

*Result not in line with data reported in the following studies: a, Zlamala *et al.* (2002); b, Wieser *et al.* (1999); c, Groth *et al.* (1996).

1976). The glycolic acid content of the bacterial cell wall was determined as described by Uchida & Aida (1984). For cellular fatty acid analysis, the strain was grown on TSA medium at 30 °C for 36 h and fatty acid methyl ester analysis was performed using the Sherlock Microbial Identification System (MIDI) as described previously (Pandey *et al.*, 2002). Freeze-dried cells for other chemotaxonomic analyses were prepared following growth of the strains in tryptic soy broth (HiMedia) for 4 days at 30 °C. Genomic DNA extraction and amplification, 16S rRNA gene sequencing and phylogenetic analysis were performed as described previously (Mayilraj *et al.*, 2005). DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987). The G+C content of the genomic DNA was determined as described by Saha *et al.* (2005).

The organism had phenotypic properties, including chemical properties, that were consistent with its classification in the genus *Agrococcus*. The detailed differential physiological and biochemical properties are listed in Table 1 and further characteristics are given in the species description. The fatty acid compositions of strain K22-21^T and the type strains of the three *Agrococcus* species are detailed in Supplementary Table S1 in IJSEM Online. To elucidate the phylogenetic position of strain K22-21^T within the genus *Agrococcus*, an almost complete sequence of the 16S rRNA gene was determined (1454 bases). The 16S rRNA gene sequence of strain K22-21^T generated in this work was aligned with those of the other *Agrococcus* species retrieved from GenBank. A phylogenetic tree was constructed using the 16S rRNA gene sequences of the three *Agrococcus* type strains and strain K22-21^T; *Agromyces ramosus* DSM 43045^T was used as an outgroup. Strain K22-21^T formed a separate lineage within the genus *Agrococcus* (Fig. 1). 16S rRNA gene sequence similarities between strain K22-21^T and *Agrococcus jenensis* DSM 9580^T, *Agrococcus baldri* DSM 14215^T and *Agrococcus citreus* DSM 12453^T were 98.5, 96.8 and 96.6 %, respectively. However, the DNA–DNA relatedness value between strain K22-21^T and *Agrococcus jenensis* was 55.1 %, which is well below the 70 % cut-off point recommended for the delineation of bacterial species (Wayne *et al.*, 1987). DNA–DNA relatedness values between strain K22-21^T and the two other *Agrococcus* type strains were not determined, as it has been shown that organisms with more than 3 % 16S rRNA gene sequence dissimilarity belong to different genomic species (Stackebrandt & Goebel, 1994). On the basis of the polyphasic data presented above, strain K22-21^T should

be placed in the genus *Agrococcus* as a representative of a novel species; the name *Agrococcus lahaulensis* sp. nov. is proposed.

Description of *Agrococcus lahaulensis* sp. nov.

Agrococcus lahaulensis (la.haul.en'sis. N.L. masc. adj. *lahaulensis* pertaining to Lahaul Valley, located in the Indian Himalayas, where the type strain was isolated).

Gram-positive, non-acid-fast, aerobic bacterium. Forms lemon-coloured, circular, glistening, opaque colonies with an entire margin on TSA medium. Colony sizes are 0.7–3.0 mm. Cells are irregularly spherical, occurring in pairs and clusters. Cells vary in size (0.6–1.0 µm wide by 1.0–1.5 µm long). Does not form endospores. Non-motile. No mycelial growth phase is observed. Catalase-positive and oxidase-negative. Tolerates up to 7.0 % NaCl and grows at temperatures between 25 and 37 °C, with optimum growth at 30 °C. Growth occurs at between pH 6 and 10; optimum growth is at pH 8.0. Positive for decomposition of casein, starch and gelatin. Negative for indole, urease and hydrogen sulfide production, methyl red and Voges–Proskauer reactions, utilization of citrate, decomposition of aesculin and tyrosine and nitrate reduction. Acid is produced from L-rhamnose, D-fructose and trehalose. Acid is produced weakly from L-arabinose, but not from D-cellobiose, *myo*-inositol, D-mannitol, D-maltose, D-galactose, glycerol,

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1454 bases) showing the phylogenetic relationships between strain K22-21^T and other species of the genus *Agrococcus*. *Agromyces ramosus* DSM 43045^T was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are given at nodes. Bar, 0.5 % sequence variation.

salicin, sucrose or D-xylose. Utilization of various compounds as sole carbon sources is detailed in Table 1. The diagnostic cell wall diamino acid is diaminobutyric acid. The peptidoglycan contains Ala, Gly, Asp, Thr and diaminobutyric acid. The cell-wall sugars are glucose, rhamnose, ribose, mannose and galactose. The predominant lipids are diphosphatidylglycerol, phosphatidylglycerol, two unknown glycolipids and some non-characterized lipids. No mycolic acids are present. Contains major amounts of anteiso-branched C_{15:0} (48.44%) and C_{17:0} (27.61%) fatty acids and iso-branched C_{15:0} (9.94%) and C_{16:0} (5.80%) fatty acids. The major menaquinones are MK-10, MK-11 and MK-12. The acyl type is acetyl.

The type strain is K22-21^T (=MTCC 7154^T=DSM 17612^T), isolated from soil, 0.45 m below an ice glacier, 4200 m above sea level, in Lahaul Valley, Himachal Pradesh, India. The G+C content of strain K22-21^T is 74.1 mol%.

Acknowledgements

We would like to thank Ms Gabi Poetter and Jennifer Gregor, DSMZ, Germany, and Mr Malkit Singh, MTCC, for their excellent technical assistance. Financial assistance from DBT and CSIR, Government of India is duly acknowledged. This is IMTECH communication number 57/2005.

References

- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP 18) and a silver loaded ion exchanger as stationary phases. *J Liq Chromatogr* **5**, 2359–2367.
- Lanyi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Mayilraj, S., Prasad, G. S., Suresh, K., Saini, H. S., Shivaji, S. & Chakrabarti, T. (2005). *Planococcus stackebrandtii* sp. nov., isolated from a cold desert of the Himalayas, India. *Int J Syst Evol Microbiol* **55**, 91–94.
- Minnikin, D. E. & Goodfellow, M. (1976). Lipid composition in the classification and identification of *Nocardia* and related taxa. In *The Biology of the Nocardaceae*, pp. 160–219. Edited by M. Goodfellow, G. H. Brownell & J. A. Serrano. London: Academic Press.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, K. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Murray, R. G. E., Raymond, N., Doetsch, R. N. & Robinow, C. F. (1994). Determinative and cytological light microscopy. In *Methods for General and Molecular Bacteriology*, pp. 21–41. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Pandey, K. K., Mayilraj, S. & Chakrabarti, T. (2002). *Pseudomonas indica* sp. nov., a novel butane-utilizing species. *Int J Syst Evol Microbiol* **52**, 1559–1567.
- Park, Y. H., Suzuki, K., Yim, D. G. & 7 other authors (1993). Suprageneric classification of peptidoglycan group B actinomycetes by nucleotide sequencing of 5S ribosomal RNA. *Antonie van Leeuwenhoek* **64**, 307–313.
- Rainey, F., Weiss, N., Prauser, H. & Stackebrandt, E. (1994). Further evidence for phylogenetic coherence of actinomycetes with group B-peptidoglycan and evidence for the phylogenetic intermixing of the genera *Microbacterium* and *Aureobacterium* as determined by 16S rDNA analysis. *FEMS Microbiol Lett* **118**, 135–139.
- Saha, P., Krishnamurthi, S., Mayilraj, S., Prasad, G. S., Bora, T. C. & Chakrabarti, T. (2005). *Aquimonas voraii* gen. nov., sp. nov., a novel gammaproteobacterium isolated from a warm spring of Assam, India. *Int J Syst Evol Microbiol* **55**, 1491–1495.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Smith, N. R., Gordon, R. E. & Clark, F. E. (1952). *Aerobic spore-forming bacteria*. Agricultural Monograph no. 16. Washington, DC: US Department of Agriculture.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Suzuki, K., Sasaki, J., Uramoto, M., Nakase, T. & Komagata, K. (1997). *Cryobacterium psychrophilum* gen. nov., sp. nov., nom. rev., comb. nov., an obligately psychrophilic actinomycete to accommodate '*Curtobacterium psychrophilum*' Inoue and Komagata 1976. *Int J Syst Bacteriol* **47**, 474–478.
- Tourova, T. P. & Antonov, A. S. (1987). Identification of microorganisms by rapid DNA-DNA hybridization. *Methods Microbiol* **19**, 333–355.
- Uchida, K. & Aida, K. (1984). An improved method for the glycolate test for simple identification of the acyl type of bacterial cell walls. *J Gen Appl Microbiol* **30**, 131–134.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wieser, M., Schumann, P., Martin, K., Altenburger, P., Burghardt, J., Lubitz, W. & Busse, H.-J. (1999). *Agrococcus citreus* sp. nov., isolated from a medieval wall painting of the chapel of Castle Herberstein (Austria). *Int J Syst Bacteriol* **49**, 1165–1170.
- Zlamala, C., Schumann, P., Kämpfer, P., Rosselló-Mora, R., Lubitz, W. & Busse, H.-J. (2002). *Agrococcus baldri* sp. nov., isolated from the air in the 'Virgikapelle' in Vienna. *Int J Syst Evol Microbiol* **52**, 1211–1216.