Arthrobacter alpinus sp. nov., a psychrophilic bacterium isolated from alpine soil

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An aerobic, Gram-reaction-positive, non-motile, psychrophilic bacterium, designated strain S6-3^T, was isolated from alpine soil. Cells exhibited a rod–coccus growth cycle and produced a yellow pigment. Growth occurred at 1–25 °C. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain S6-3^T was related to members of the genus *Arthrobacter*, sharing highest sequence similarities with the type strains of *Arthrobacter psychrolactophilus* (97.9%) and *Arthrobacter stackebrandtii* (97.6%). Strain S6-3^T had MK-9(H₂) as the major menaquinone and anteiso-C_{15:0} as the major fatty acid. The cell-wall peptidoglycan was of type A3 α L-Lys–L-Thr–Ala₃. The predominant cell-wall sugars were galactose and rhamnose. The genomic DNA G+C content of strain S6-3^T was 61.9 mol%. On the basis of phenotypic characteristics, phylogenetic analysis and DNA–DNA relatedness data, strain S6-3^T is considered to represent a novel species of the genus *Arthrobacter*, for which the name *Arthrobacter alpinus* sp. nov. is proposed. The type strain is S6-3^T (=DSM 22274^T =CGMCC 1.8950^T).

The genus *Arthrobacter* was proposed by Conn & Dimmick (1947) to accommodate bacteria that show a rod–coccus growth cycle, aerobic metabolism and little or no acid production from glucose, have lysine in the peptidoglycan and a DNA G+C content of 59–66 mol% (Keddie *et al.*, 1986). Strains of the genus have been isolated from a variety of sources and belong to different thermal classes; several representatives are psychrophilic and are thus able to proliferate at low temperatures (Margesin *et al.*, 2008), such as *Arthrobacter psychrolactophilus* (Loveland-Curtze *et al.*, 1999), *A. stackebrandtii* (Tvřzová *et al.*, 2005), *A. flavus* and *A. roseus* (Reddy *et al.*, 2000, 2002), *A. psychrophenicus* (Margesin *et al.*, 2004b) and *A. chlorophenicus* (Westerberg *et al.*, 2000). We use the term psychrophile as a general term that describes a micro-organism that grows in a cold environment. The use of growth rates to define the optimum growth temperature as described by Morita (1975) has been shown to be ambiguous and inappropriate (Feller & Gerday, 2003; Cavicchioli, 2006). *A. psychrophenicus* and *A. chlorophenicus* have been reported to be useful for bioremediation in cold climates, as they are able to degrade

phenolic compounds at low temperatures (Backman & Jansson, 2004; Margesin *et al.*, 2004a).

Members of the genus *Arthrobacter* Conn and Dimmick 1947, the description of which was later emended by Koch *et al.* (1995), fall into at least two groups (Stackebrandt & Schumann, 2006) as revealed by chemotaxonomic studies (Minnikin *et al.*, 1978; Collins & Jones, 1981; Collins & Kroppenstedt, 1983; Stackebrandt *et al.*, 1983). The type species, *Arthrobacter globiformis* (Conn & Dimmick, 1947), represents the so-called ‘globiformis’ group, while the second, the ‘nicotianae’ group, is centred around the species *Arthrobacter nicotianae*. The globiformis group is characterized by the presence of peptidoglycan of the A3 α type (i.e. an interpeptide bridge made up of one to four residues of L-amino acids such as L-alanine, L-threonine or L-serine; Schleifer & Kandler, 1972) and of a dihydrogenated menaquinone with nine isoprenoid units [MK-9(H₂)]. The ‘nicotianae’ group comprises organisms that have peptidoglycan variation A4 α (i.e. an interpeptide bridge consisting of a dicarboxylic amino acid such as glutamic acid or aspartic acid; Schleifer & Kandler, 1972) and menaquinones that are completely unsaturated with eight to ten isoprenoid units.

Strain S6-3^T was isolated during the course of a study on microbial community composition and activity over an

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S6-3^T is GQ227413.

A table giving the cellular fatty acid compositions of strain S6-3^T and related type strains is available as supplementary material with the online version of this paper.

alpine altitude gradient (Margesin *et al.*, 2009). The strain was isolated from alpine soil collected in the Hohe Tauern/Grossglockner area (Fuschertörl), Austria, on the north slope at 2300 m above sea level. Soil was collected under sterile conditions in August 2006. Subsequently, 10 g sample was shaken with 90 ml sterile 1 % sodium pyrophosphate for 20 min at 150 r.p.m. Appropriate dilutions, prepared with sterile saline solution (0.9 % NaCl), were plated (0.1 ml) on R2A agar (Reasoner & Geldreich, 1985) and incubated at 20 °C. One of the pure cultures was yellow-pigmented and was designated strain S6-3^T.

A. stackebrandtii DSM 16005^T and *A. psychrolactophilus* DSM 15612^T were used as reference strains; they were routinely grown on trypticase soy-yeast extract medium (3 % trypticase soy broth, 0.3 % yeast extract, 1.5 % agar; pH 7.0–7.2) at 25 °C.

DNA was extracted and purified as described by Sambrook *et al.* (1989). The gene encoding the 16S rRNA was amplified by PCR with two universal primers (Zhang *et al.*, 2006). PCR products were cloned in pGEM-T vectors by using the pGEM-T easy vector system (Promega) according to the manufacturer's instructions. Sequencing reactions were carried out by using an ABI BigDye 3.1 sequencing kit (Applied Biosystems) and an automated DNA sequencer (model ABI 3730; Applied Biosystems). The nearly complete 16S rRNA gene sequence (1530 bp) of strain S6-3^T was submitted to GenBank and EMBL to search for similar sequences via the BLAST algorithm. Phylogenetic trees were constructed by using Kimura's two-parameter and

pairwise-deletion model analysis implemented in the program MEGA version 3.0 (Kumar *et al.*, 2004). The resulting tree topologies were evaluated by bootstrap analysis based on 1000 replicates. Phylogenetic analysis showed that strain S6-3^T was grouped with members of the genus *Arthrobacter*, and formed a distinct cluster with the type strains of *A. psychrolactophilus* (97.9 % 16S rRNA gene sequence similarity) and *A. stackebrandtii* (97.6 %) in the neighbour-joining tree (Fig. 1). Levels of 16S rRNA gene sequence similarity between strain S6-3^T and the type strains of all other recognized *Arthrobacter* species were also <98.0 %.

Strain S6-3^T was routinely cultured in low-strength R2A agar and was maintained as a suspension in skimmed milk (10 %, w/v) at –80 °C. Cell morphology was examined by phase-contrast microscopy (× 1000) of cells grown on R2A agar plates at 20 °C. Colony morphology was also observed on R2A agar plates. The API M system (bioMérieux) was used to evaluate cell motility. The Gram reaction was tested by Gram staining and was confirmed by the KOH lysis test. Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂; oxidase activity was determined by using 1 % (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. API strips (API 20 E, API 20 NE, API Coryne, API ZYM; bioMérieux) incubated at 15–20 °C were used according to the manufacturer's instructions to determine physiological and biochemical characteristics as well as enzyme activities of strain S6-3^T and reference strains *A. stackebrandtii* DSM 16005^T and *A. psychrolactophilus* DSM 15612^T. In addition, assimilation of lactose, Tween 40 and Tween 80 was tested in liquid culture by supplementing 10 ml of a pH-neutral

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic position of strain S6-3^T and the type strains of related *Arthrobacter* species. *Nocardioideis simplex* DSM 20130^T was used as an outgroup. Asterisks indicate nodes that were also found in the maximum-parsimony (Fitch, 1971) tree. Bootstrap values (percentages) are based on 1000 replicates and are shown for branches with more than 50 % support. GenBank accession numbers are given in parentheses. Bar, 0.01 expected changes per site.

phosphate-buffered mineral medium with a trace element solution (Margesin *et al.*, 2002) and the carbohydrates (final concentration 0.2 %, w/v) as the sole carbon source. Growth at 1, 5, 10, 15, 20, 25 and 30 °C was assessed on R2A agar plates and in R2A liquid medium. Growth at pH 5, 6, 7, 8 and 9 and 0, 1, 2, 3, 5, 7.5 and 10 % (w/v) NaCl was determined on R2A agar plates. Susceptibility to antibiotics was determined on R2A agar plates supplemented with various antibiotics; plates were incubated at 15 °C. Morphological, physiological and biochemical characteristics of strain S6-3^T are given in the species description or are shown in Table 1. The features that serve to differentiate strain S6-3^T from the phylogenetically most closely related members of the genus *Arthrobacter* are given in Table 1.

For analysis of the peptidoglycan structure and cell-wall sugars, cells of strain S6-3^T were cultured in shaken flasks of medium 92 (<http://www.dsmz.de>) at 20 °C for 3 days. The peptidoglycan structure was determined by using hydrolysates of purified cell walls according to Schleifer (1985). Amino acids and peptides were separated by two-dimensional ascending TLC on cellulose plates with the solvent systems of Schleifer & Kandler (1972). The molar ratio of the amino acids was determined by GC as

described by MacKenzie (1987). The N terminus of the interpeptide bridge was determined by dinitrophenylation according to Schleifer (1985). The peptidoglycan of strain S6-3^T contained the amino acids lysine, alanine, threonine and glutamic acid in an approximate molar ratio of 0.8:5.6:1.0:1.0. The total hydrolysate of the peptidoglycan (4 M HCl, 100 °C, 16 h) contained, in addition to these amino acids, the peptide L-Lys-L-Thr, which is relatively stable under these hydrolytic conditions. The partial hydrolysate (4 M HCl, 100 °C, 0.75 h) also contained the peptides Ala₃₋₄, L-Ala-D-Glu and D-Ala-L-Lys-L-Thr. Dinitrophenylation revealed that alanine represents the N terminus of the interpeptide bridge. From these data, it was concluded that the peptidoglycan of strain S6-3^T is of type A3α L-Lys-L-Thr-Ala₃ (A11.28 according to http://www.dsmz.de/microorganisms/main.php?content_id=35). Strain S6-3^T showed the same peptidoglycan structure as its phylogenetic neighbour *A. psychrolactophilus* but differed in this characteristic from *A. stackebrandtii*, which shows the peptidoglycan type A3α L-Lys-L-Ala₂ (Tvřzová *et al.*, 2005).

For analysis of sugars, cell walls were hydrolysed in 0.5 M H₂SO₄ at 100 °C for 2 h. H₂SO₄ was removed by shak-

Table 1. Phenotypic characteristics that differentiate strain S6-3^T from the type strains of phylogenetically related *Arthrobacter* species

Strains: 1, S6-3^T; 2, *A. psychrolactophilus* ATCC 700733^T (unless indicated, data from Loveland-Curtze *et al.*, 1999; Tvřzová *et al.*, 2005); 3, *A. stackebrandtii* CCM 2783^T (Tvřzová *et al.*, 2005); 4, *A. russicus* GTC 863^T (Li *et al.*, 2004). +, Positive; w, weakly positive; –, negative; NA, no data available.

Characteristic	1	2	3	4
Isolation source	Alpine soil	Soil from farm field	Poultry litter	Air in space laboratory
DNA G + C content (mol%)	61.9	60.6	NA	65.5
Cell-wall peptidoglycan type	A3α L-Lys-L-Thr-Ala ₃	L-Lys-L-Thr-L-Ala ₃	A3α L-Lys-L-Ala ₂	Lys-Ala ₂
Major fatty acids*	a-C _{15:0} , i-C _{16:0} , a-C _{17:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} †	a-C _{15:0} , i-C _{15:0} , i-C _{16:0} , a-C _{17:0} †	a-C _{17:0} , a-C _{15:0}
Growth temperature range (°C)	1–25‡	1–25†‡	1–30†‡	NA (T _{max} 30 °C)
Growth at 30 °C	–‡	w†‡	+†‡	+
Nitrate reduction	–	–†	+†	–
Hydrolysis of urea	+	–†	+†	–
Enzyme assays (API ZYM)				
α-Glucosidase	+	+†	w†	NA
β-Galactosidase	+	+†	w†	NA
β-Glucuronidase	+	w†	–†	NA
Assimilation of:				
L-Arabinose	+	–†	+†	+
Maltose	+	+†	+†	–
Lactose	+	+†	–†	–
Tween 40	w	–†	+†	+
Tween 80	–	–†	–†	+

*a, Anteiso-branched; i, iso-branched.

†Data from the present study for *A. psychrolactophilus* DSM 15612^T and *A. stackebrandtii* DSM 16005^T.

‡Data obtained on R2A agar plates.

ing with 20% *N,N*-dioctylmethylamine in chloroform according to Whiton *et al.* (1985) and the sugars in the hydrolysate were analysed by TLC on cellulose plates according to Stanek & Roberts (1974). The predominant cell-wall sugars of strain S6-3^T were galactose and rhamnose. An unidentified sugar component was also detected.

For analysis of fatty acid methyl esters, strain S6-3^T, *A. stackebrandtii* DSM 16005^T and *A. psychrolactophilus* DSM 15612^T were grown on trypticase soy-yeast extract agar plates at 25 °C for 3 days. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the Microbial ID system (MIDI, Inc.; Sasser, 1990). The predominant cellular fatty acids of strain S6-3^T were anteiso-C_{15:0} (61.2%), iso-C_{16:0} (17.9%), anteiso-C_{17:0} (10.1%) and iso-C_{15:0} (6.2%) (the complete fatty acid profile is given in Supplementary Table S1, available in IJSEM Online). This composition was consistent with those of members of the genus *Arthrobacter*, showing a predominance of anteiso-C_{15:0}.

Respiratory quinones were extracted and purified according to Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989). Strain S6-3^T contained MK-9(H₂) as the major menaquinone, with minor amounts of MK-8(H₂) and MK-10(H₂).

The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as the reference, and DNA–DNA hybridization experiments were performed by the liquid renaturation method (De Ley *et al.*, 1970) as modified by Huß *et al.* (1983); the two experiments were carried out by using a DU800 spectrophotometer (Beckman) with a thermal controller. The DNA G+C content of strain S6-3^T was 61.9 mol%. Levels of DNA–DNA relatedness between strain S6-3^T and *A. stackebrandtii* DSM 16005^T and *A. psychrolactophilus* DSM 15612^T were 10.7 ± 3.8% (mean ± SD from three replicate determinations) and 33.8 ± 5.2%, respectively.

Strain S6-3^T is a psychrophilic representative of the genus *Arthrobacter* and can be distinguished easily from its closest phylogenetic neighbours, *A. psychrolactophilus* and *A. stackebrandtii*, based on its inability to grow at 30 °C and from *A. stackebrandtii* based on its ability to utilize lactose and its different peptidoglycan type. Based on the phenotypic, phylogenetic and genomic evidence presented, strain S6-3^T is considered to represent a novel species of the genus *Arthrobacter*, for which we propose the name *Arthrobacter alpinus* sp. nov.

Description of *Arthrobacter alpinus* sp. nov.

Arthrobacter alpinus (al.pi'nus. L. masc. adj. *alpinus* alpine, referring to the isolation of the type strain from an alpine environment).

Cells are irregular rods showing a rod–coccus cycle, 0.8–1.0 × 1.3–1.9 µm after 3 days at 20 °C on R2A agar plates, often occurring in pairs as typical V-forms. Cells are Gram-

reaction-positive, aerobic and non-motile. Colonies on R2A agar and on nutrient agar are creamy yellow after 3 days and yellow after 6 days, round, convex, shiny and with an entire margin; colony diameter is 1–1.5 mm after 3 days at 20 °C on R2A agar. Growth occurs in liquid R2A medium and on agar plates at 1–25 °C, with fastest growth at 20–25 °C; growth is absent at 30 °C. On R2A agar plates, growth occurs at pH 6–9 and in the presence of 0–5% (w/v) NaCl. Produces catalase; cytochrome oxidase is not produced. Negative for nitrate reduction, indole production, H₂S production and citrate utilization. Hydrolyses aesculin, urea and starch, but not casein or Tween 80. Negative for activities of arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, lipase (C14), *N*-acetyl-β-glucosaminidase, trypsin, α-chymotrypsin, α-fucosidase and pyrrolidonyl arylamidase. Positive for activities of alkaline phosphatase, acidic phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase and α-mannosidase. Utilizes D-glucose, lactose, L-arabinose, maltose, D-mannose, D-mannitol and *N*-acetylglucosamine as sole carbon source, utilizes Tween 40 weakly and does not utilize citrate, capric acid, adipic acid or phenylacetic acid. Negative for fermentation of glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, glycogen, inositol, sorbitol, rhamnose, melibiose, amygdalin and L-arabinose. Sensitive to ampicillin, penicillin G, streptomycin, tetracycline and chloramphenicol (each at 30 µg ml⁻¹), but resistant to cyclosporin A (100 µg ml⁻¹). The predominant cellular fatty acids of the type strain are anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. Contains MK-9(H₂) as the major menaquinone, with minor amounts of MK-8(H₂) and MK-10(H₂). The cell-wall peptidoglycan is of type A3α L-Lys–L-Thr–Ala₃. The predominant cell-wall sugars are galactose and rhamnose. The DNA G+C content of the type strain is 61.9 mol%.

The type strain, S6-3^T (=DSM 22274^T =CGMCC 1.8950^T), was isolated from an alpine soil in Fuschertörl, Hohe Tauern, Austria.

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