Arthrobacter psychrochitiniphilus sp. nov., a psychrotrophic bacterium isolated from Antarctica

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A psychrotrophic *Arthrobacter* strain, GP3^T, was isolated from Adélie penguin guano from Antarctica and characterized. The 16S rRNA gene sequence of the novel strain showed the highest similarity (97.8 %) with that of *Arthrobacter psychrolactophilus* B7^T. The novel strain showed a morphological change from rod to coccus. The growth temperature range of strain GP3^T was 0–25 °C, with optimal growth at 20 °C. The strain grew over a salinity range of between 0 and 3 % (w/v) NaCl and the optimal pH for growth was pH 6–8. Strain GP3^T contained anteiso-C_{15:0} as the major fatty acid. The major menaquinone was MK-9(H₂). The cell-wall peptidoglycan type was of the A3 α variant. The DNA G + C content of strain GP3^T was 58.5 mol%. Strain GP3^T was able to hydrolyse chitin, Tween 80, starch, cellulose and lactose, but not gelatin, lecithin or urea. In addition to these physiological characteristics, DNA–DNA hybridization studies clearly differentiated strain GP3^T from *A. psychrolactophilus*. The combined results of phylogenetic, physiological and chemotaxonomic studies indicated that strain GP3^T represents a novel species of the genus *Arthrobacter*. The name *Arthrobacter psychrochitiniphilus* sp. nov. (type strain GP3^T=JCM 13874^T=CGMCC 1.6355^T) is proposed in recognition of the strain's strong chitin-utilizing ability.

Species of the genus *Arthrobacter* are Gram-positive actinomycetes with a high DNA G + C content (Keddie *et al.*, 1986; Jones & Keddie, 1992; Stackebrandt *et al.*, 1983). The majority of the species of the genus *Arthrobacter* have been isolated from soil, silt and environments such as sludge, sewage and mat samples (<http://www.bacterio-cict.fr/a/arthrobacter.html>). Most of the recognized species of the genus are mesophilic. However, psychrophilic members of the genus *Arthrobacter* have been increasingly isolated from Antarctica and other cold environments. Recently described cold-adapted species of the genus *Arthrobacter* include *Arthrobacter psychrolactophilus* (Loveland-Curtze *et al.*, 1999), *Arthrobacter flavus* (Reddy *et al.*, 2000), *Arthrobacter roseus* (Reddy *et al.*, 2002), *Arthrobacter psychrophenicus* (Margesin *et al.*, 2004) and *Arthrobacter ardleyensis* (Chen *et al.*, 2005). Cold active enzymes of industrial interest have been isolated and characterized from psychrophilic *Arthrobacter* species (Mavromatis *et al.*, 2003; Skalova *et al.*, 2005). Psychrophilic species of the genus *Arthrobacter* are potential sources of economically important cold active enzymes.

In our project involving the isolation of cold-adapted bacteria from various cold environments, several *Arthrobacter* strains were isolated from Antarctic and deep-sea samples (Chen *et al.*, 2005). In this study, strain GP3^T, isolated from penguin guano, is characterized.

Strain GP3^T was isolated from guano of Antarctic Adélie penguins. The sample was collected during the Nineteenth Chinese Antarctic Research Expedition in 2002 at Ardley Island. As penguin deposits are known to contain numerous chitinolytic bacterial strains (Xiao *et al.*, 2005), we used M9 plus chitin plates to isolate chitinolytic bacterial strains from fresh penguin guano samples. The samples were diluted at a ratio of approximately 1 : 5 (w/v) in distilled water and 100 μ l aliquots of the suspension were spread on basic M9 agar plates (1⁻¹ water; 12.8 g Na₂HPO₄ · 7H₂O, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl and 1.5 g agar) containing 1 % (w/v) colloidal chitin prepared as described previously (Xiao *et al.*, 2005). Plates were incubated for 7–28 days at 10 °C. Colonies were subcultured for purification. Strains were selected on the basis of differences in colony morphology. A yellowish, round-edged, smooth colony type with a large chitin-hydrolysing halo dominated in the isolation plates. A representative strain, designated GP3^T, was characterized. Strain GP3^T was routinely cultivated and maintained using Luria–Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract

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and 1 % NaCl, pH 7.4) in all further experiments, unless otherwise indicated.

DNA isolation and PCR amplification of the 16S rRNA gene fragment of the novel strain were performed as described previously (Chen *et al.*, 2005). A PCR product of around 1500 bp for the 16S rRNA gene was cloned into the pGEM-T vector by using a Rapid Ligation kit with 2 × Rapid Ligation buffer according to the manufacturer's instructions (Promega). The ligation mixture was used to transform competent cells of *Escherichia coli* XL1 Blue according to the manufacturer's recommendations (Promega). A positive clone was picked for sequencing (Sangon Inc.). The resulting sequence was used to search the NCBI database. Related sequences were aligned using CLUSTAL_X (version 1.83) software. A phylogenetic tree was constructed from a matrix of pairwise genetic distances by using the neighbour-joining and minimum-evolution methods as performed with the MEGA (molecular evolutionary genetics analysis; <http://www.megasoftware.net/index.html>) software, version 3.1 (Kumar *et al.*, 2004). A bootstrap analysis (1000 replicates) was used to provide confident estimates for phylogenetic tree topologies. The 16S rRNA gene sequence of strain GP3^T showed the highest similarity (97.8 %) with that of *A. psychrolactophilus* B7^T. The phylogenetic relationships between strain GP3^T and related reference strains from the genus *Arthrobacter* are shown in Fig. 1. Strain GP3^T and *A. psychrolactophilus* clearly clustered very closely in the phylogenetic tree. The type strain of *A. psychrolactophilus*, B7^T, was therefore included for later comparisons in this study (strain provided by Dr J. E. Brenchley).

Standard methods for morphological, biochemical, and physiological characterization were used as described by Dong & Cai (2001). Cultures of the strains in the lag, exponential and stationary phases of growth were observed under light microscopy. General utilization of carbon

sources was determined by using Biolog GP2 microplates (Biolog Inc.). All commercial kits were used according to the manufacturer's instructions. Tests for the utilization of lactose and chitin as sole carbon sources at a concentration of 0.5 % (w/v) were performed in 10 ml minimal medium containing 0.1 % (w/v) yeast extract, 0.01 % (w/v) FePO₄ and 3.4 % (w/v) NaCl. Colonies of strain GP3^T on LB plates were yellow and circular with entire margins. The strain showed a typical rod-coccus growth cycle (data not shown). Growth of cultures was monitored by measuring OD₆₀₀. The temperature range for growth of strain GP3^T was 0–25 °C. The optimal temperature for growth was 20 °C. The doubling times of cultures when grown at 0, 20 and 25 °C were 18.3, 4.5 and 6.0 h, respectively. The novel strain was able to grow over a salinity range of 0–3 % (w/v) NaCl. The optimum pH for growth was between pH 6 and pH 8. The antibiotic sensitivity of the novel strain was assessed by using antibacterial susceptibility discs (Oxoid). The main biochemical and physiological characteristics of the novel strain are given in the species description and in Table 1.

Cell walls were extracted by the TCA method and purified with trypsin according to Schleifer & Kandler (1972). The composition and molar ratio of the amino acids of the peptidoglycan was analysed with a Biochrom 20 system (Pharmacia). Isoprenoid quinones were extracted and purified according to Collins *et al.* (1977). The purified menaquinones were analysed by HPLC-MS (LCQ MAT; Finnigan) as previously described by Nishijima *et al.* (1997). The APCI ion source used was a mixture consisting of 2-propanol and acetonitrile (1.25:1, v/v). The major quinone of strain GP3^T was MK-9(H₂). Amino acid analysis showed that the cell-wall peptidoglycan contained lysine as the diagnostic diamino acid, as well as alanine, glutamic acid and threonine. The Ala-Glu-Lys-Thr molar ratio of the cell-wall peptidoglycan was about 3:1:1:1.

Fig. 1. Phylogenetic tree showing the relationships of strain GP3^T to reference strains of the genus *Arthrobacter* based on 16S rRNA gene sequences. GenBank accession numbers are given in parentheses. Bar, 5 nucleotide substitutions per 100 nucleotides.

Table 1. Major phenotypic characteristics that differentiate strain GP3^T and *A. psychrolactophilus* B7^T

Strains: 1, GP3^T; 2, *A. psychrolactophilus* B7^T. The optimal pH for growth for both strains was pH 6–8. Both strains were positive in tests for the production of lipase. +, Positive; –, negative.

Characteristic	1	2
Growth range (°C)	0–25	0–30
Nitrate reduction	+	–
NH ₃ production	+	–
Production of:		
Chitinase	+	–
Gelatinase	–	+
Utilization of:		
Raffinose	–	+*

*Data from Loveland-Curtze *et al.* (1999).

The peptidoglycan of strain GP3^T belonged to the A3α variant (Schleifer & Kandler, 1972).

Cells for cellular fatty acid analysis were harvested from 24 h cultures grown at 20 °C in LB medium (pH 7.0) with shaking. Fatty acids were extracted and analysed following the instructions of the Microbial Identification System operating manual (MIDI, Inc.). As found for other species of the genus *Arthrobacter*, strain GP3^T contained anteiso-C_{15:0} as the major fatty acid. Fatty acids anteiso-C_{17:0}, iso-C_{14:0}, iso-C_{15:0} and traces of straight-chain saturated fatty acids (C_{16:0} and C_{18:0}) were also present (Table 2).

The G+C content of the DNA was determined using a HPLC method. DNA was enzymically degraded into nucleosides as described by Tamaoka & Komagata (1984). The nucleoside mixture obtained was then separated by reverse-phase HPLC using a C₁₈ column (Kromasil ODS, 5 μm, 250 × 4.6 mm inner diameter) as described in a previous report (Tamaoka & Komagata, 1984). The solvent used was 0.05 mol NH₄H₂PO₄ l⁻¹, pH 4.0, containing 7 % acetonitrile. The DNA G+C mol% value was calculated based on the ratio of G/G+T mol%

Table 2. Fatty acid content

Strains: 1, GP3^T; 2, *A. psychrolactophilus* B7^T (data from Loveland-Curtze *et al.*, 1999). tr, <0.5 %.

Fatty acid (% of total)	1	2
anteisoC _{15:0}	75.6	~73
anteisoC _{17:0}	13.9	~13
isoC _{14:0}	1.0	tr
isoC _{15:0}	3.2	~1.4
isoC _{16:0}	tr	~8
C _{16:0}	tr	~2
C _{18:0}	tr	tr

according to Mesbah *et al.* (1989). The DNA G+C content of strain GP3^T was determined to be 58.5 mol%

Levels of DNA–DNA relatedness between strain GP3^T and *A. psychrolactophilus* B7^T were determined by DNA–DNA slot-blot hybridization as described by Xiao *et al.* (2007). The DNA–DNA relatedness value between strain GP3^T and *A. psychrolactophilus* B7^T was 33.8 %, well below the 70 % threshold value for the delineation of genomic species (Wayne *et al.*, 1987).

The results of the 16S rRNA gene sequence and peptidoglycan analyses placed strain GP3^T near *A. psychrolactophilus* B7^T. DNA–DNA hybridization, together with physiological and chemotaxonomic studies, clearly indicated that these two strains were different from each other. For example, strain GP3^T had a strong chitin-hydrolysing activity, but *A. psychrolactophilus* B7^T showed no chitinase activity (Table 1). It is concluded that strain GP3^T represents a novel species within the genus *Arthrobacter*. The name *Arthrobacter psychrochitiniphilus* sp. nov. is proposed.

Description of *Arthrobacter psychrochitiniphilus* sp. nov.

Arthrobacter psychrochitiniphilus (psy.chro.chi.ti.ni.phi'lus. Gr. adj. *psychros* cold; N.L. n. *chitinum* chitin; Gr. adj. *philos* loving; N.L. masc. adj. *psychrochitiniphilus* a cold, chitin-loving bacterium).

Individual cells show a distinct rod–coccus cycle. Cells are Gram-positive, aerobic, catalase-positive and oxidase-negative with motile rods. Spores or capsules are not seen. Colonies in LB medium at 20 °C are yellow, circular and convex. Growth occurs at 0–25 °C, the optimal growth temperature is around 20 °C. Grows well at between 0 % and 3 % NaCl. Optimal growth occurs at pH 6–8. Tween 80, starch, cellulose, lactose and chitin are hydrolysed. Gelatin, lecithin and urea are not hydrolysed. Nitrate is reduced and NH₃ production is positive. Production of indole and H₂S is negative. Sensitive to ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline. Growth occurs on lactose or chitin as the sole carbon source. The cellular fatty acid pattern is dominated by anteiso-C_{15:0}. The peptidoglycan type is A3α. The major menaquinone is MK-9(H₂). Biolog tests show that the following compounds are utilized for respiration: dextrin, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, cellobiose, D-fructose, D-galactose, D-glucose, α-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, melibiose, methyl β-D-galactoside, D-psicose, D-ribose, D-sorbitol, sucrose, D-tagatose, trehalose, turanose, xylitol, D-xylose, acetic acid, α-hydroxybutyric acid, α-ketovaleric acid, D-lactic acid methyl ester, L-lactic acid, 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-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, inosine, thymidine, uri-

dine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, D-glucose 6-phosphate and DL- α -glycerol phosphate.

The type strain, GP3^T (=JCM 13874^T=CGMCC 1.6355^T), was isolated from the guano of Adélie penguins, Antarctica. The DNA G+C content of the type strain is 58.5 mol%.

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