Characterization of *Micrococcus antarcticus* sp. nov., a psychrophilic bacterium from Antarctica

Hongcan Liu, Yi Xu, Yanhe Ma and Peijin Zhou

Author for correspondence: Peijin Zhou. Tel: +86 10 625 53628. Fax: +86 10 625 60912.
e-mail: zhou@sun.im.ac.cn

Institute of Microbiology,
Chinese Academy of
Sciences, Beijing,
100080 China

A Gram-positive, cold-adapted, aerobic, spherical actinobacterium (strain T2^T) with a quite low cardinal growth temperature was isolated from Chinese Great-Wall station in Antarctica. Sequence comparisons of the 16S rDNA indicated the isolate to be a phylogenetic member of the genus *Micrococcus*, family *Micrococcaceae*, in which it represents a novel lineage. The phylogenetic distinctness of the isolate with respect to the type strains *Micrococcus luteus* and *Micrococcus lylae* was supported by DNA–DNA similarity values of less than 40%. Chemotaxonomic properties supported the placement of the isolate in the genus *Micrococcus*. The diagnostic diamino acid of the cell-wall peptidoglycan is lysine. The predominant menaquinones are MK-8 and MK-8(H₂). The G+C content of the DNA of the isolate is 66.4 mol%. Genotypic, morphological and physiological characteristics were used to describe a new species of *Micrococcus*, for which the name *Micrococcus antarcticus* is proposed. The type strain is T2^T (= AS 1.2372^T).

Keywords: cold-adapted bacteria, *Micrococcus antarcticus*, phylogeny

INTRODUCTION

Cold-adapted micro-organisms are more likely to be found in permanently cold environments such as polar regions, marine environments and other areas. They take on a process of nutrient cycling and primary biomass production (Bowman *et al.*, 1997). The ability to grow at low temperatures seems to have been acquired over evolutionary time by all the major groupings of micro-organisms, and the range of species within a particular cold habitat reflects those many different parameters (e.g. primary nutrient, pH, salinity) to which an organism must adapt if it is to compete successfully with others (Gounot, 1991). Morita proposed that cold-adapted micro-organisms were divided into two groups, i.e. obligately psychrophilic micro-organisms (psychrophiles) and facultatively psychrophilic micro-organisms (psychrotrophs). The psychrophiles, besides having the ability to grow at or below 0 °C, have an optimum growth temperature below 15 °C and a maximum growth temperature below 20 °C; the psychrotrophs have a minimum growth temperature at or above 0 °C and optimum and maximum growth temperatures above 20 °C (Russell, 1998). To date, the predominant

psychrophilic bacteria in Antarctica were found to belong to four phylogenetic groups, i.e. the α and γ subclasses of the *Proteobacteria*, the Gram-positive branch and the *Flexibacter–Bacteroides–Cytophaga* phylum (Nichols *et al.*, 1995). During an investigation into the bacterial diversity of Antarctic region, Gram-positive coccoid cells were frequently observed and isolated. In this study, the characterization of isolate T2^T was detailed and the results showed that strain T2^T represented a new species within the genus *Micrococcus*, for which the name *Micrococcus antarcticus* is proposed.

METHODS

Bacterial strains and cultural conditions. Strain T2^T was enriched by shaking in peptone/yeast extract/glucose (PYG) medium at 0–5 °C and then identified on PYG medium consisting of the following (g l⁻¹): peptone (5), yeast extract (10) and glucose (5) g, adjusted to pH 7.0 and then incubated at 10 °C. The growth temperature range was determined using a temperature-gradient incubator (TN-3F; Advantec). *Micrococcus luteus* (IFO 3333) and *Micrococcus lylae* (IFO 15355) were provided by the Institute for Fermentation, Osaka (IFO), Japan.

Morphological studies. Cell morphology was observed under a light microscope and under a scanning electron microscope by using cells in the exponential growth phase grown on PYG medium.

The GenBank/EMBL/DBJ accession number for the 16S rRNA sequence of strain T2^T is AJ005932.

Biochemical characterization. The biochemical and physiological characteristics of the isolate were investigated using the methods described by Barrow & Feltham (1993) and some physiological properties were tested using the BIOLOG GP-plate, System Release 3.50. Comparative investigations of biochemical and physiological characteristics and molecular features were carried out with *M. luteus* and *M. lylae*.

Chemotaxonomic characterization. Amino acid analysis of the cell wall was performed as follows. Cell wall was prepared from 500 mg dried cells by mechanical disruption with an ultrasonic oscillator and was purified as described by Schleifer & Kandler (1972). The amino acid composition of cell wall hydrolysates was determined by using an automatic amino acid analyser (model L-8500A; Hitachi) HPLC apparatus equipped with an ion-exchange column (model 2620MSC). For sugar analysis, cell wall was hydrolysed with 2 M HCl at 100 °C for 2 h, dried in a vacuum and then analysed by TLC on cellulose plates. Fatty acids were extracted from dry cells by methanolysis and were examined by using a gas chromatography apparatus (model GC-8A; Shimadzu).

Menaquinone profiles were examined using HPLC (Stackebrandt *et al.*, 1995) with apparatus (model 510; Waters) equipped with an IIS18 HG column (Wakoshi).

DNA base composition and DNA–DNA hybridization. Cells were grown in PYG at 10 °C on a rotary shaker. After incubation for 72 h, cells were harvested and washed with distilled water. DNA was isolated and purified by the method of Sambrook *et al.* (1989). The G+C content of the DNA was determined by the method of thermal denaturation (Sly *et al.*, 1986) with a UV-1206 UV-Vis spectrophotometer (Shimadzu). DNA–DNA hybridization was carried out as described by Tindall *et al.* (1984), with a minor modification. DNA fragments were labelled with [α - 32 P]dCTP according to the instructions provided with the Boehringer Mannheim nick translation kit.

16S rDNA sequence determination and phylogenetic analysis. The 16S rRNA gene was amplified by the PCR with primers 43F (5'-TCAGAACGAACGCTGGCGGC-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') using strain T2^T total DNA as the DNA template. Purified PCR products were cloned according to the manufacturer's instructions (Promega). The sequencing reactions were carried out using the ABI PRISM Dye primer cycle sequencing ready reaction kit (Applied Biosystems). Sequencing was performed on an ABI 373S DNA sequencer (Applied Biosystems) and the sequence was submitted to the EMBL nucleotide sequence database. The reference 16S rRNA gene sequence data used in this study were obtained from the GenBank nucleotide database.

The 16S rRNA gene sequence determined in this work was aligned with previously published sequences by using the multiple-sequence alignment program CLUSTAL W version 1.74 (Thompson *et al.*, 1994). Evolutionary distances were calculated with the Kimura two-parameter calculation model. Alignment positions with insertion or deletion were excluded for the calculations. The phylogenetic tree was constructed using the neighbour-joining method with the Kimura two-parameter calculation model in TREECON for Windows version 1.2 (Van de Peer & De Wachter, 1994). As references, other phylogenetic trees were also constructed with other techniques in PHYLIP version 3.572, such as the parsimony and maximum-likelihood methods (Felsenstein, 1993).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of the test strains have the following GenBank/EMBL/DBJ accession numbers (in parentheses): *Arthrobacter protophormiae* (X80745); *Arthrobacter nicotianae* (X80739); *Arthrobacter sulfureus* (X83409); *Arthrobacter ramosus* (X80742); *Arthrobacter globiformis* (M23411); *Arthrobacter atrocyaneus* (X80746); *Arthrobacter crystallopoietes* (X80738); *Arthrobacter citreus* (X80737); *Arthrobacter oxydans* (X83408); *Arthrobacter ilicis* (X83407); *Arthrobacter histidinolovorans* (X83406); *Kocuria varians* (X87754); *Kocuria kristinae* (X80749); *Kocuria erythromyxa* (Y11330); *Kocuria rosea* (X87756); *Micrococcus lylae* (X80750); *Micrococcus luteus* (M38242); *Rothia dentocariosa* (M59055); *Stomatococcus mucilaginosus* (X87758). The tree was rooted by using the 16S rDNA sequence of *Kytococcus sedentarius* (X87755) as an outgroup.

RESULTS AND DISCUSSION

Cultural characteristics

On PYG medium, the cells of strain T2^T were spherical (0.5–0.8 μ m), occurred in pairs and packets and were non-motile, Gram-positive and aerobic. Endospores were not detected. Colonies were yellow, mucoid and fluffy with entire margins. The catalase test was positive. The optimum temperature for growth was 16.8 °C (Fig. 1) and growth occurred at 0 °C. The physiological and biochemical characteristics are summarized in Table 1. Differentiation of the new species from other *Micrococcus* species is achieved by means of some physiological properties (Table 1); for example, it differs from *M. luteus* and *M. lylae* by hydrolysis of Tween and starch. In some respects (e.g. indole formation, Voges–Proskauer reaction, nitrate reduction), the isolate can be biochemically distinguished from the known species.

Chemotaxonomic and genotypic characteristics

Chemotaxonomic investigations revealed that the amino acids of peptidoglycan in the cell wall were glutamic acid, alanine, lysine and glycine in the molar ratio of 1:1.88:0.93:0.97. The amino sugar in the cell wall polysaccharide was mannosamine. The predominant menaquinones were MK-8 (63.7%) and MK-

Fig. 1. Growth curve of *Micrococcus antarcticus* T2^T at different temperatures.

Table 1. Comparisons of phenotypical and physiological properties of members of genus *Micrococcus*

+, Positive test result; –, negative test result. All three species shared test results for the following: morphology (coccoid); motility (–); Gram stain (+); catalase (+); oxidase (+); aesculin hydrolysis (–); acid production from glucose (–), fructose (–), maltose (–), sucrose (–) and glycerol (–); acetoin (–); arginine dihydrolase (–); lysozyme susceptibility (+); antibiotic susceptibility to penicillin (+), erythromycin (+), streptomycin (+), methicillin (+), novobiocin (+), tetracycline (+), chloramphenicol (+), neomycin (+) and polymyxin B (+); and peptidoglycan type (lysine).

Characteristic	<i>M. luteus</i>	<i>M. lylae</i>	<i>M. antarcticus</i> T2 ^T
Optimal growth temp. (°C)	37	37	16·8
Hydrolysis of Tweens:			
20	–	–	+
40	–	–	+
80	–	–	+
Starch hydrolysis	–	–	+
Urease	+	+	–
Indole formation	–	–	+
Nitrate reduction	–	–	+
Voges–Proskauer reaction	–	–	+
Methyl red test	+	–	+
Quinone system	MK-8, MK-8(H ₂)	MK-8(H ₂)	MK-8, MK-8(H ₂)
Cellular fatty acids*	S, A, I	A, I	anteiso-C _{15:0} , iso-C _{15:0}
Cell wall amino sugar	Mannosamine	Galactosamine	Mannosamine
G + C content (mol %)	70·0	69	66·4

* S, Straight-chain saturated; A, anteiso-methyl-branched; I, iso-methyl-branched.

8(H₂) (7·4%). Major amounts of anteiso-methyl-branched acid (anteiso-C_{15:0} 49·7%), smaller amounts of iso-methyl-branched acid (iso-C_{15:0} 19·9%) and 12-methyl tetradecenoic acid (anteiso-C_{15:1} 12·3%) were present. The G + C content of the DNA was 66·4 mol%. These data were compatible with the assignment of the strain to the genus *Micrococcus*, thereby confirming the identity of the isolate T2^T as a member of the genus *Micrococcus*.

Phylogenetic analysis

To determine the phylogenetic position of isolate T2^T, the 1497-base 16S rDNA sequence was determined and a phylogenetic tree based on *K*_{nuc} values was created by comparison of the new sequence with other known relevant sequences in the GenBank database. The tree (Fig. 2) clearly indicated that strain T2^T and the other two known species of genus *Micrococcus* should be grouped into the same lineage. The apparent closest relative to strain T2^T was *M. luteus*, with a sequence similarity of 94·4%; this branch received 100% bootstrap support. It is evident from both sequence divergence values and tree analysis that the isolate does not belong to any other described

species, and that the 16S rRNA sequence divergence (>3%) unequivocally demonstrates that isolate T2^T represents a new *Micrococcus* species (Collins *et al.*, 1999).

DNA–DNA hybridization

To verify the phylogenetically distinct position of strain T2^T as determined by 16S rDNA sequence analysis, the degree of DNA–DNA similarity was determined for strain T2^T and two *Micrococcus* species. The DNA–DNA similarity between strain T2^T and *M. luteus* and *M. lylae* was 34·4 and 28·8%, respectively. DNA similarities were clearly below 70% (which is considered to be the threshold value for the delineation of genospecies).

The genus *Micrococcus* was first introduced by Cohn (Kocur *et al.*, 1991) and since then the description has been revised several times. Stackebrandt *et al.* (1995) proposed an emended description of the genus *Micrococcus*. To date, *M. luteus* and *M. lylae* are the only species that remain in this genus. Phylogenetic and chemotaxonomic evidence indicated that strain T2^T should be placed in the genus *Micrococcus* but that it differed from the other species and represented a new

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence divergence, showing the relationships between isolate T2^T and other relatives. The tree was constructed using the neighbour-joining method and the Kimura two-parameter calculation model. The numbers represent the confidence levels from 100-replicate bootstrap sampling. Bar, 0.02 K_{nuc} .

species within the genus. The low DNA–DNA reassociation value confirmed the new species status of strain T2^T. Although the above characteristics were consistent with assignment to the genus *Micrococcus*, the isolate did not conform exactly to any currently validly published species of this genus. On the basis of the phylogenetic findings, in conjunction with the phenotypic distinctiveness of the isolate, we propose the assignment of strain T2^T to a new species, i.e. *Micrococcus antarcticus* sp. nov.

Our results also indicated members of the genus *Micrococcus* to be successful in colonizing low-temperature habitats. The psychrophilic isolate *M. antarcticus* AS 1.2372^T (strain T2^T), showed unique characteristics, particularly in its cellular fatty acid profile, which contained a significant amount of an anteiso-branched monounsaturated acid (12-methyl tetradecenoic acid) and was reasonably well adapted to low temperatures. Clearly, the combination of relatively rapid growth at low temperatures and broad metabolic versatility has given this isolate a strong competitive edge in polar environments.

Description of *Micrococcus antarcticus* sp. nov.

Micrococcus antarcticus (ant.arc'ti.cus. L. adj. *antarcticus* opposite the North Star, referring to the Antarctic habitat of the bacterium).

Cells are spherical, have a diameter of 0.5 μ m and are non-motile. Endospores are not formed. Gram-positive, aerobic. Colonies are yellow, mucoid and fluffy with entire margins. Optimal growth occurs at 15–17 °C. Catalase- and oxidase tests are positive. Positive for the following biochemical tests: indole formation, Voges–Proskauer, methyl red, and nitrate reduction. Starch, Tween 20, Tween 40 and Tween 80 are hydrolysed. The following carbon sources are utilized: arabinol, fructose, fucose, gluconic acid, mannitol, rhamnose, turanose, xylitol, hydroxybutyric acid, L-lactic acid, L-malic acid, D-malic acid, methyl pyruvate, mono-methyl succinate, succinamic acid, succinic acid, N-acetyl L-glutamic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, glycerol, adenosine, inosine, thymidine and uridine. The following biochemical tests are negative: urease, acetoin, arginine dihydrolase. Gelatin and aesculin are not hydrolysed. Acid production from carbohydrates is negative. The utilization of the following carbon sources is negative: dextrin, glycogen, inulin, mannan, amygdalin, L-arabinose, arbutin, cellobiose, D-galactose, D-galacturonic acid, gentiobiose, D-glucose, myo-inositol, D-lactose, lactulose, maltose, maltotriose, D-mannose, D-melezitose, D-melibiose, 3-methyl glucose, palatinose, psicose, raffinose, D-ribose, salicin, sorbitol, stachyose,

sucrose, D-tagatose, D-trehalose, D-xylose, acetic acid, α -ketoglutaric acid, α -ketovaleric acid, lactamide, propionic acid, pyruvic acid and 2,3-butanediol. Susceptible to lysozyme, penicillin, tetracycline, erythromycin, novobiocin, streptomycin, methicillin, chloramphenicol, polymyxin and neomycin. Cell wall peptidoglycan type is L-lysine (the diagnostic amino acid). The predominant menaquinones are MK-8 and MK-8(H₂). The amino sugar in the cell wall polysaccharide is mannosamine. The major cellular fatty acids are anteiso-C_{15:0} (49.7%) and iso-C_{15:0} (19.9%). The G+C content of the DNA is 66.4 mol% (according to the *T_m*). Closely related phylogenetically to *Micrococcus luteus* and *Micrococcus lylae* as determined by 16S rDNA analysis. The type culture is deposited in the China General Microbiological Culture Collection Centre under the accession number AS 1.2372^T.

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