NOTE

***Arthrobacter roseus* sp. nov., a psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample**G. S. N. Reddy,¹ J. S. S. Prakash,¹ G. I. Matsumoto,² E. Stackebrandt³ and S. Shivaji¹¹ Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India² Department of Environmental and Information Science, Otsuma Women's University, Tamashi, Tokyo 206, Japan³ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 38124 Braunschweig, Germany

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Strain CMS 90r^T, a red-pigmented bacterium, was isolated from a cyanobacterial mat sample from a pond located in McMurdo, Antarctica. Based on its chemotaxonomic and phylogenetic properties, strain CMS 90r^T was identified as a member of group I of *Arthrobacter*. It shared 16S rDNA similarity of 98% with *Arthrobacter oxydans* ATCC 14358^T and *Arthrobacter polychromogenes* ATCC 15216^T, while DNA–DNA similarities determined for these three organisms were less than 70%. It also differed from all 17 reported *Arthrobacter* species with A3 α -variant peptidoglycan in that it possessed a unique peptidoglycan (Lys–Gly–Ala₃) and contained galactose, glucose, ribose and rhamnose as cell-wall sugars. These data and the presence of diagnostic phenotypic traits support the description of CMS 90r^T as a novel species of *Arthrobacter*, for which the name *Arthrobacter roseus* sp. nov. is proposed. The type strain is strain CMS 90r^T (= MTCC 3712^T = DSM 14508^T).

Keywords: *Arthrobacter*, psychrophile, Antarctica, cyanobacterial mat

Prokaryotes are the predominant biomass in Antarctica (Wynn-Williams, 1996; Franzmann, 1996) and they influence the food chain and the biogeochemical cycles (Friedmann *et al.*, 1993; Matsumoto *et al.*, 1989). Among the prokaryotes, cyanobacteria belonging to the genera *Phormidium*, *Anabaena*, *Calothrix*, *Nostoc*, *Lyngbya* and *Synechococcus* are the most abundant and they form mats in the polar lakes (Parker & Wharton, 1985; Vincent *et al.*, 1993). These mats contain a large biomass of bacteria that contributes to the mineral and nutrient cycling in the lakes. However, these mat-associated, heterotrophic bacteria have rarely been identified. In an attempt to establish the microbial biodiversity of the cyanobacterial mat communities of the Antarctic lakes, a red-pigmented bacterium (CMS 90r^T), isolated from a cyanobacterial mat sample, was studied in detail with respect to its phenotypic characteristics and phylogenetic relationships.

Source and isolation

Strain CMS 90r^T was isolated from a cyanobacterial mat sample collected from pond L4 (77° 32' 18" S,

160° 45' E) located in the Wright valley, McMurdo, Antarctica (Matsumoto, 1993; Matsumoto *et al.*, 1993). The mat sample was found to contain an *Aphanothece* sp. and coccoid green algae in low densities (Matsumoto *et al.*, 1993). About 200 mg of the sample was suspended in 1 ml sterile saline (150 mM NaCl) and an aliquot of the suspension (100 μ l) was plated on Antarctic bacterial medium (ABM) plates containing 0.5% (w/v) peptone, 0.2% (w/v) yeast extract and 1.5% (w/v) agar (pH 6.9) and incubated at 5 °C (Shivaji *et al.*, 1988, 1989, 1992; Reddy *et al.*, 2000). The appearance of colonies was monitored on a regular basis and pure cultures of the bacteria were established. Following incubation for 15 days, colonies appeared that were predominantly white in colour, with the exception of CMS 90r^T, which was red-pigmented (indicated by the suffix 'r').

Phylogenetic analysis

Amplification and sequence analysis of the 16S rRNA gene were done as published previously (Reddy *et al.*, 2000; Shivaji *et al.*, 2000). The almost complete sequence of strain CMS 90r^T (1470 nucleotides) was aligned with those deposited in EMBL using CLUSTAL v (Higgins *et al.*, 1992). Following an initial crude taxonomic affiliation, a more-detailed analysis was

The EMBL accession number for the 16S rDNA sequence of strain CMS 90r^T is AJ278870.

Fig. 1. Phylogenetic relationship between *A. roseus* sp. nov. (CMS 90r^T) and 26 reported species of *Arthrobacter* based on 16S rDNA sequence analysis using DNAPARS. Bootstrap values above 50% are given at the nodes. The branch lengths indicated in the tree are not to scale.

done with members of *Kocuria*, *Arthrobacter* and related genera. Pairwise evolutionary distances were computed using the DNADIST program with Kimura's two-parameter model (Kimura, 1980). To obtain confidence values for the rDNA sequence-based genetic affiliations, the original sequence dataset was resampled 1000 times using SEQBOOT and subjected to bootstrap analysis. Phylogenetic trees were obtained using parsimony analysis (DNAPARS) and various distance-matrix-based clustering algorithms such as FITCH, KITCH and UPGMA as compiled in PHYLIP (Felsenstein, 1993). The different algorithms gave consistent results.

Strain CMS 90r^T fell into the radiation of *Arthrobacter* species (94.7–98% similarity), showing the highest values to the sequences of the type strains of *Arthrobacter oxydans* and *Arthrobacter polychromogenes*. The topology of the consensus phylogenetic tree (Fig. 1) displays the phylogenetic position of the two strains next to their closest phylogenetic neighbours. The statistical significance of these new lineages is high.

In order to determine the overall genomic relatedness between the two isolates and their phylogenetic neighbours, DNA–DNA hybridization was performed by

the membrane-filter method (Shivaji *et al.*, 1992; Reddy *et al.*, 2000). The results indicated that CMS 90r^T shared 69.8 and 65.46% DNA similarity, respectively, with the type strains of *A. oxydans* and *A. polychromogenes*.

Chemotaxonomic properties

Peptidoglycan was prepared according to Rosenthal & Dziarski (1994) and hydrolysed with 4 M HCl at 120 °C for 60 min and the composition of the main chain was determined according to the method of Schleifer & Kandler (1972).

Cellular fatty acid methyl esters were prepared from lyophilized bacterial cell pellets (Sato & Murata, 1988) and separated by GC on a DB-23 capillary column (30 m × 0.25 mm) purchased from J & W Scientific. The fatty acids were identified by comparison with fatty acid standards run under similar GC conditions and also by mass spectrometry (Shivaji *et al.*, 1992; Reddy *et al.*, 2000). The carrier gas used for GC was N₂ (1 ml min⁻¹). The initial run temperature was maintained at 175 °C for 8 min and then raised to 200 °C at a rate of 4 °C min⁻¹ and held at 200 °C for 5 min. The injector port and FID detector temperatures were respectively 200 and 240 °C.

Menaquinones were extracted as described by Collins *et al.* (1977) and were separated by TLC using petroleum ether and diethyl ether (85:15, v/v) (Dunphy *et al.*, 1971). The individual menaquinones were also identified by mass spectrometry (Reddy *et al.*, 2000). The method of Minnikin *et al.* (1975) was used for the extraction of polar lipids, using lyophilized cell pellets washed free of medium; polar lipids were resolved by thin-layer chromatography using chloroform/methanol/water (65:25:4, by vol.).

Cell-wall sugars were prepared and analysed according to the method described by Komagata & Suzuki (1987).

Pigments were extracted from lyophilized bacterial cell pellets with chloroform/methanol (1:2, v/v), centrifuged at 10000 *g* and the clear, pigmented supernatant was flushed under a stream of N₂. The dry pigment thus obtained was dissolved in a minimal volume of chloroform/methanol (2:1, v/v) and separated by TLC using chloroform/methanol/water (65:25:4) in the dark. The individual pigments were then scraped from the plates, dissolved in methanol and their absorption spectra were recorded in a Hitachi 2000 spectrophotometer (Chauhan & Shivaji, 1994; Shivaji *et al.*, 1992; Jagannadham *et al.*, 1991).

The G+C content of the DNA was determined as described previously (Shivaji *et al.*, 1992).

The phylogenetic affiliation of CMS 90r^T to *Arthrobacter* was supported by its chemotaxonomic properties (Table 1).

Table 1. Phenotypic characteristics that differentiate strain CMS 90r^T from other *Arthrobacter* species with A3 α -variant peptidoglycan and MK-9(H₂) as the major menaquinone

Taxa are identified as: 1, *A. globiformis*; 2, *A. crystallopoietes*; 3, *A. pascens*; 4, *A. ramosus*; 5, *A. aureus*; 6, *A. histidinovorans*; 7, *A. ilicis*; 8, *A. ureafaciens*; 9, *A. citreus*; 10, *A. nicotinovorans*; 11, *A. flavus*; 12, *A. agilis*; 13, *A. atrocyaneus*; 14, *A. oxydans*; 15, *A. psychrophilus*; 16, *A. psychrolactophilus*; 17, *A. chlorophenolicus*; 18, strain CMS 90r^T. Data from known species of *Arthrobacter* were from Keddle *et al.* (1986), Kodama *et al.* (1992), Reddy *et al.* (2000), Koch *et al.* (1995), Loveland-Curtze *et al.* (1999) and Westerberg *et al.* (2000). +, Positive; –, negative; (±), weak reaction; NA, data not available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Peptidoglycan type	Lys-Ala ₃	Lys-Ala	Lys-Ala ₂	Lys-Ala ₃	Lys-Thr-Ala ₂	Lys-Thr-Ala ₂	Lys-Thr-Ala ₂	Lys-Thr-Ala ₂	Lys-Thr-Ala ₂	Lys-Thr-Ala ₂	Lys-Thr-Ala ₂	Lys-Thr-Ala ₂	Lys-Ser-Ala ₂	Lys-Ser-Thr-Ala	Lys-Ser-Thr-Ala	Lys-Ala ₃	Lys-Ser-Thr-Ala	Lys-Gly-Ala ₃
Cell-wall sugars*	Gal, Glu	Gal, Glu	Gal, Glu	Gal, Rha, Man	Gal, Glu, (Man)	Gal, Glu	Gal, Rha, Man	Gal, Glu, (Man)	Gal	Gal, Glu	Gal, Glu	Glc	Gal, Glu, (Man)	Gal, Glu	Gal	NA	NA	Gal, Glu, Rib, Rha
Growth at:																		
5 °C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
30 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Colony colour	White/cream	White	White	White	Yellow	White	Yellow	Grey	Yellow	Grey	Yellow	Red	White	Grey/white	Blue/green	Yellow	Grey	Red
Motility	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Utilization of nicotine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hydrolysis of:																		
Starch	+	–	+	–	+	–	–	–	–	NA	–	+	+	+	–	NA	NA	–
Aesculin	–	–	–	–	–	–	–	–	–	–	–	–	(±)	+	+	NA	NA	–
Nitrate reduction	–	+	+	–	–	–	NA	–	NA	–	–	–	+	+	+	–	NA	+

*Gal, Galactose; Glc, glucosamine; Glu, glucose; Man, mannose; Rha, rhamnose; Rib, ribose. Compounds in parentheses are present at low levels.

Morphology, motility and biochemical characteristics

The shape and motility of cultures from the lag, exponential and stationary phases of growth were observed by phase-contrast microscopy (1000 ×). Motility was determined by the hanging-drop method and staining of the flagellum was by the silver-impregnation method (Blenden & Goldberg, 1965). All biochemical tests were performed by growing the culture at 20 °C. The activities of catalase, oxidase, phosphatase, gelatinase, urease, lipase, arginine dihydrolase and β -galactosidase were determined according to standard methods (Holding & Collee, 1971). The production of indole, the methyl red test, Voges-Proskauer test, levan formation, reduction of nitrate to nitrite, hydrolysis of starch and aesculin and formation of acid and gas were also monitored according to standard methods (Hugh & Leifson, 1953; Stanier *et al.*, 1966; Holding & Collee, 1971; Stolp & Gadkari, 1981). Furthermore, the ability of the cultures to utilize carbon compounds as sole carbon sources, sensitivity to 24 different antibiotics and determination of optimal temperature, pH and salt for growth were done on ABM plates.

The properties of strain CMS 90r^T are indicated in the species description; diagnostic characters are indicated in Table 1.

Conclusions

Strain CMS 90r^T, unlike *Arthrobacter agilis* (Keddle *et al.*, 1986; Schleifer, 1986; Koch *et al.*, 1994, 1995), is non-motile, oxidase-negative, β -galactosidase-negative, aesculin-hydrolysis-negative, nitrate-reduction-positive, phosphatase-positive and halotolerant (tolerates up to 5.8% NaCl) and has a peptidoglycan of the A3 α type. Strain CMS 90r^T, which is psychrophilic (grows between 5 and 30 °C), red-pigmented and has Lys-Gly-Ala₃ as the peptidoglycan type, is also distinctly different from the psychrophilic species *Arthrobacter psychrolactophilus* and *Arthrobacter flavus* and other psychrophilic strains that have been tentatively identified as species of *Arthrobacter* (Shivaji *et al.*, 1989; Loveland-Curtze *et al.*, 1999; Reddy *et al.*, 2000). Table 1 shows the characteristics by which strain CMS 90r^T differs from all known *Arthrobacter* species with A3 α -variant peptidoglycan and MK-9(H₂) as the major menaquinone. Two phenotypic features by which it differs from the 17 *Arthrobacter* species with A3 α -variant peptidoglycan are the Lys-Gly-Ala₃ type of peptidoglycan and galactose, glucose, ribose and rhamnose as the cell-wall sugars. In addition, unlike 90% or more of *Arthrobacter* strains (Keddle *et al.*, 1986), CMS 90r^T did not utilize glycine, threonine, lysine, tyrosine or histidine. Furthermore, strain CMS 90r^T differed from many *Arthrobacter* species in that it contained both C_{16:1} and C_{18:2} and did not contain iso-C_{17:0}, unlike *Arthrobacter globiformis*, *Arthrobacter atrocyaneus*, *Arthrobacter crystallopoietes*, *Arthrobacter nicotinovorans*, *Arthrobacter pascens*, *Arthrobacter*

ramosus, *Arthrobacter chlorophenolicus*, *Arthrobacter aureus*, *Arthrobacter citreus*, *Arthrobacter histidinovorans*, *A. psychrolactophilus*, *Arthrobacter ilicis*, *A. oxydans*, *A. polychromogenes*, *Arthrobacter ureafaciens*, *A. agilis* and *A. flavus* (Kodama *et al.*, 1992; Koch *et al.*, 1995; Loveland-Curtze *et al.*, 1999; Reddy *et al.*, 2000; Westerberg *et al.*, 2000).

Furthermore, 16S-rDNA-based phylogenetic analysis clearly established strain CMS 90r^T to be a member of the genus *Arthrobacter* (Fig. 1) and, more specifically, a member of group 1 of *Arthrobacter*, defined by peptidoglycan variant A3 α (Keddie *et al.*, 1986) and which includes the type species, *A. globiformis*. In this background, it was most interesting to note that the analysed novel strain CMS 90r^T always formed a cluster with *A. polychromogenes* and *A. oxydans* in both phenetic and parsimony-based phylogenetic analyses, with respective bootstrap values of 23 and 53% by the DNAPARS and UPGMA methods (Fig. 1). These results further support its genetic affiliation as group I *Arthrobacter* with a minimum evolutionary distance of 2.0% from both *A. polychromogenes* and *A. oxydans*.

The observed evolutionary distance between strain CMS 90r^T and its nearest relatives *A. oxydans* and *A. polychromogenes* (2%) is significantly more than that seen between many of the other previously described known species of *Arthrobacter*. For instance, the distances between *A. pascens* and *A. globiformis*, *A. histidinovorans* and *A. nicotinovorans*, *A. oxydans* and *A. polychromogenes* and *A. ramosus* and *A. pascens* were respectively 0.34, 0.27, 0.07 and 0%. These observations suggest that CMS 90r^T is sufficiently differentiated to be considered a novel species of the genus *Arthrobacter*. Furthermore, DNA–DNA relatedness between strain CMS 90r^T and *A. oxydans* and *A. polychromogenes* was respectively 69.8 and 65.46%. Despite the high 16S rRNA gene sequence similarity (> 97%) and DNA–DNA relatedness (65–70%), the phenotypic differences are so evident that strain CMS 90r^T must be assigned to a novel species (Table 1). Therefore, we conclude that strain CMS 90r^T represents a novel *Arthrobacter* species that has been named *Arthrobacter roseus* sp. nov., and the novel species can be differentiated from all known species of *Arthrobacter* on the basis of a number of phenotypic characteristics (Table 1).

Description of *Arthrobacter roseus* sp. nov.

Arthrobacter roseus (ro.se'us. L. adj. *roseus* red-coloured).

Rod–coccus growth cycle with irregular rods in the exponential phase and predominantly coccoid cells (~ 1.0 μ m in diameter) in the stationary phase. Gram-positive, non-spore-forming and non-motile; colonies on peptone/yeast extract medium are round, smooth, convex, 0.2–1 mm in diameter and red-pigmented. Pigment is insoluble in water but soluble in methanol

and exhibits four absorption maxima, at 437, 467, 494 and 524 nm. Aerobic. Growth occurs between 5 and 30 °C and pH 6 and 12 and cells tolerate up to 5.8% NaCl. Optimum growth is observed at 22 °C and pH 7. Catalase, phosphatase, gelatinase and nitrate-reduction tests are positive; oxidase, urease, lipase, β -galactosidase, arginine dihydrolase, indole production, methyl red test, Voges–Proskauer test and levan formation are negative. Aesculin and starch are not hydrolysed. Positive for utilization of mannose, galactose, maltose, fructose, glucose, arabinose, ribose, xylose, rhamnose, raffinose, trehalose, succinic acid, fumaric acid, citric acid, mannitol, sorbitol, adonitol, sucrose, inulin, pyruvate, acetate, alanine, leucine, isoleucine, valine, serine, arginine, aspartic acid, glutamic acid, asparagine, glutamine, proline and phenylalanine. The following substrates are not utilized as sole carbon sources: dulcitol, inositol, melibiose, lactose, nicotine, cellulose, glycine, threonine, cysteine, methionine, lysine, tyrosine, histidine and tryptophan. No acid or gas from glucose, arabinose, xylose, rhamnose, fructose, galactose, mannose, lactose, maltose or sucrose. Sensitive to penicillin, chlortetracycline, chloramphenicol, oxytetracycline, tetracycline, erythromycin, nitrofurantoin, bacitracin, nitrofurazone, leucomycin, rifampicin, nystatin, cotrimoxazole, trimethoprim, ampicillin, carbenicillin, gentamicin, amoxycillin, tobramycin and polymyxin B but resistant to furazolidone, colistin, furoxone and kanamycin. The peptidoglycan type is Lys–Gly–Ala₃ (variation A3 α) and the major menaquinone is MK-9(H₂). Cell-wall sugars are galactose, glucose, ribose and rhamnose. Cellular fatty acids are iso-C_{14:0}, C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, C_{15:0}, iso-C_{16:0}, C_{16:0}, C_{16:1}, anteiso-C_{17:0}, C_{18:0} and C_{18:2}. The predominant polar lipids are phosphatidylglycerol, cardiolipin and phosphatidylethanolamine. The DNA G+C content is 66–69 mol%. Isolated from a cyanobacterial mat sample from McMurdo Dry Valleys, Antarctica (77° 32' 18" S, 160° 45' E).

The type strain is strain CMS 90r^T (= MTCC 3712^T = DSM 14508^T).

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