Psychrophilic pseudomonads from Antarctica: *Pseudomonas antarctica* sp. nov., *Pseudomonas meridiana* sp. nov. and *Pseudomonas proteolytica* sp. nov.

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Thirty-one bacteria that belonged to the genus *Pseudomonas* were isolated from cyanobacterial mat samples that were collected from various water bodies in Antarctica. All 31 isolates were psychrophilic; they could be divided into three groups, based on their protein profiles. Representative strains of each of the three groups, namely CMS 35T, CMS 38T and CMS 64T, were studied in detail. Based on 16S rRNA gene sequence analysis, it was established that the strains were related closely to the *Pseudomonas fluorescens* group. Phenotypic and chemotaxonomic characteristics further confirmed their affiliation to this group. The three strains could also be differentiated from each other and the closely related species *Pseudomonas orientalis*, *Pseudomonas brenneri* and *Pseudomonas migulae*, based on phenotypic and chemotaxonomic characteristics and the level of DNA–DNA hybridization. Therefore, it is proposed that strains CMS 35T (=MTCC 4992T =DSM 15318T), CMS 38T (=MTCC 4993T =DSM 15319T) and CMS 64T (=MTCC 4994T =DSM 15321T) should be assigned to novel species of the genus *Pseudomonas* as *Pseudomonas antarctica* sp. nov., *Pseudomonas meridiana* sp. nov. and *Pseudomonas proteolytica* sp. nov., respectively.

The genus *Pseudomonas* was originally created by Migula (1894). Over the years, the genus has been redefined to differentiate it from other genera (Stanier et al., 1966; Palleroni et al., 1973; De Ley, 1992; Anzai et al., 2000). Moore et al. (1996) further delineated the genus *Pseudomonas* into two major intrageneric clusters, namely the *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* clusters. Subsequently, based on phylogenetic analysis of 56 species of *Pseudomonas sensu stricto*, using 1063 bp of the 16S rRNA gene sequence, the genus was categorized into two main clusters (Anzai et al., 2000). The first cluster had six groups within it and these were designated as the *Pseudomonas syringae* group (with 12 species), the *Pseudomonas chlororaphis* group (with five species), the *P. fluorescens* group (with 18 species), the *Pseudomonas putida* group (with six species), the *P. aeruginosa* group (with 11 species) and the *Pseudomonas stutzeri* group (with three species). The second cluster had only one group, the *Pseudomonas putida* group, which contained two species.

Until now, about 100 species of the genus *Pseudomonas* have been reported from various habitats, including Antarctica. Kriss et al. (1976) were the first to report the existence of *Pseudomonas* species in Antarctica. However, they were not identified at species level until 1989, when *Pseudomonas* spp. isolated from Antarctic soil and water samples were identified as psychrophilic strains of *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. syringae* (Shivaji et al., 1989a). More recently, Maugeri et al. (1996) and Bruni et al. (1999) isolated bacteria that belonged to the genus *Pseudomonas* from sea water and freshwater samples from Terra Nova Bay and Wanda Lake, Antarctica. However, these were also not characterized at species level. In the present study, attempts were made to identify bacteria that...
belong to the genus *Pseudomonas* that were isolated from cyanobacterial mat samples collected from the McMurdo region, Antarctica.

**Source of the organisms, media and growth conditions**

Thirty-one bacterial isolates were obtained from cyanobacterial mat that were samples collected from ponds L1 (strains CMS 62–72) and L3 (CMS 33–36) of Wright Valley, Adam’s glacier stream 1 (CMS 43–50), Adam’s glacier stream 2 (CMS 37, CMS 38T and CMS 40) and Canada glacier stream (CMS 41–42) of Miers Valley and Lake Canopus (CMS 54, CMS 57 and CMS 60) in Antarctica. Pure cultures of the heterotrophic bacteria were set up as described previously (Reddy *et al.*, 2000). Optimum temperature, pH and salt concentration for growth of cultures were determined by using plates of Antarctic bacterial medium (ABM) that contained 0.5% (w/v) peptone, 0.2% (w/v) yeast extract and 1.5% (w/v) agar (pH 6.9) (Reddy *et al.*, 2002, 2003).

**Morphology, motility and biochemical characteristics**

Bacterial cultures in the lag, exponential and stationary phases of growth were observed under a phase-contrast microscope (×1000) to ascertain their shape and motility. All biochemical tests were performed by growing cultures at 22°C in appropriate medium (Hugh & Leifson, 1953; Stanier *et al.*, 1966; Holding & Collee, 1971; Stolp & Gadkari, 1981). Furthermore, ability of the cultures to utilize a carbon compound as sole carbon source, sensitivity to different antibiotics and DNA G+C contents were determined as described previously (Shivaji *et al.*, 1989b). Total protein profiles of the cultures were determined by SDS-PAGE. For this purpose, cultures were grown in 3 ml ABM broth at 25°C and harvested at 6000 r.p.m. for 10 min at room temperature; the pellets were resuspended in 100 μl water and 100 μl SDS/sample buffer. The suspension was then boiled for 5 min and centrifuged at 10000 r.p.m. for 10 min; 50 μl supernatant was loaded onto 12% SDS/polyacrylamide gel (Laemmli, 1970). Bands were visualized by staining with Coomassie blue.

**DNA–DNA hybridization and identification of fatty acids**

DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987) as described previously (Shivaji *et al.*, 1992; Reddy *et al.*, 2000). Fatty acids were identified from bacterial cell pellets by comparison with fatty acid standards that were run under similar GC conditions and also by mass spectrometry (Sato & Murata, 1988; Reddy *et al.*, 2003).

**Riboprinting**

A pure colony of each of strains CMS 35T, CMS 38T and CMS 64T and *Pseudomonas brenneri* was picked up with a sterile toothpick and suspended in a 1.5 ml microfuge tube that contained 200 μl riboprinting buffer (DuPont Qualicon). The tube was then heated to 70°C for 10 min in a model 480 DNA thermocycler (Perkin Elmer) and the contents were transferred to a sample carrier (DuPont Qualicon). Lysis reagent A and reagent B (5 μl each) were added before inserting the sample carrier into the characterization unit of the Qualicon Riboprinter system, where the samples were processed automatically according to the EcoRI standard protocol.

**16S rRNA gene sequencing**

Amplification of the 16S rRNA gene, purification of the 1.5 kb amplic and sequencing of the amplicons were carried out by the method of Lane (1991), as described previously (Shivaji *et al.*, 2000).

**Phylogenetic analysis**

16S rRNA gene sequences of the three bacteria that represented the 31 isolates were aligned with reference sequences of all species in the *P. fluorescens* group (obtained from GenBank/EMBL) by using the multiple sequence alignment program CLUSTAL V (Higgins *et al.*, 1992). The aligned sequences were then checked manually for gaps. The DNADIST program was used to compute pairwise evolutionary distances for the aligned sequences by applying the Kimura two-parameter model (Kimura, 1980). Furthermore, the original sequence dataset was resampled 1000 times by using SEQBOOT and subjected to bootstrap analysis to obtain confidence values for 16S rRNA gene sequence-based genetic affiliations. The multiple distance matrices thus obtained were used to construct phylogenetic trees by using various distance matrix-based clustering algorithms, such as FITCH, KITSCH and UPGMA, as compiled in the Phylogeny Inference Package (PHYLIP; Felsenstein, 1993). Parsimony analysis was also performed for the aligned sequence dataset by using DNAPARS. In all cases, the input order of species added to the topology being constructed was randomized by using the jumble option with a random seed of 7 and ten replications. Majority-rule (50%) consensus trees were constructed for the topologies by using CONSENSE. All these analyses were done by using the PHYLIP package, version 3.5c (Felsenstein, 1993).

**Reference strains**


**Conclusions**

Thirty-one individual bacterial colonies were isolated from cyanobacterial mat samples that were collected from various}

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water bodies in Antarctica. These 31 isolates could be categorized into three groups, based on their protein profiles as analysed by SDS-PAGE (data not shown), namely group I (CMS 33–36 and CMS 44–50), group II (CMS 38<sup>T</sup>) and group III (CMS 37, CMS 40–41, CMS 43, CMS 54, CMS 57, CMS 60 and CMS 62–72). Members of the same group exhibited identical protein profiles, indicating that they are probably clonal in origin. Therefore, strains CMS 35<sup>T</sup>, CMS 38<sup>T</sup> and CMS 64<sup>T</sup> were chosen as representative isolates of groups I, II and III, respectively.

These three isolates, namely CMS 35<sup>T</sup>, CMS 38<sup>T</sup> and CMS 64<sup>T</sup>, are aerobic, Gram-negative, rod-shaped and motile, possess a polar flagellum and have C<sub>16</sub>:0, C<sub>16</sub>:1ω7c, C<sub>16</sub>:1ω9c and C<sub>18</sub>:1 as their major fatty acids, indicating their affiliation to the genus *Pseudomonas*. They could all grow at 4–30°C and did not accumulate polyhydroxybutyric acid. Riboprinting analysis indicated that strains CMS 35<sup>T</sup>, CMS 38<sup>T</sup> and CMS 64<sup>T</sup> are distinctly different from each other (Tables 1 and 2; Supplementary Table A, available in IJSEM Online; Fig. 1).

Phylogenetic analysis of the three isolates, based on 1438 bp of the 16S rRNA gene sequence, indicated a close relationship with species that belong to the *P. fluorescens* group (Anzai et al., 2000) (Fig. 2). Evolutionary distances, as calculated by using the Kimura two-parameter model, indicated that the three isolates are related very closely to each other, with >99% 16S rRNA gene sequence similarity, and also to other species of the *P. fluorescens* group (Anzai et al., 2000). At the DNA–DNA level, there was 40% relatedness between strains CMS 35<sup>T</sup> and CMS 38<sup>T</sup>, 40% between CMS 35<sup>T</sup> and CMS 64<sup>T</sup> and 43% between CMS 38<sup>T</sup> and CMS 64<sup>T</sup> (Supplementary Table B, available in IJSEM Online).

The topology of the tree indicates that strain CMS 64<sup>T</sup> is related phylogenetically to the clade that represents

Table 1. Phenotypic characteristics that differentiate strains CMS 35<sup>T</sup> (*P. antarctica*) and CMS 38<sup>T</sup> (*P. meridiana*) from each other and from closely related species of the genus *Pseudomonas*

<table>
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<tr>
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<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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<td>+</td>
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<td>−</td>
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<td>NA</td>
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<td>−</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>Lactose</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>D-Maltose</td>
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<td>−</td>
<td>−</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannitol</td>
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<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D-Melibiose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NA</td>
<td>NA</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>L-Rhamnose</td>
<td>−</td>
<td>−</td>
<td>NA</td>
<td>NA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Sucrose</td>
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<td>−</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>D-Xylose</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
Identification of strain CMS 35T as *Pseudomonas antarctica* sp. nov.

Strain CMS 35T can be differentiated from strains CMS 38T and CMS 64T with respect to its protein profile, riboprint, phenotypic characteristics and low (40 %) DNA–DNA relatedness (Tables 1 and 2; Supplementary Table B, available in IJSEM Online; Fig. 1). Strain CMS 35T can also be differentiated easily, based on phenotypic characteristics (Table 1). Thus, based on the above differences between strains CMS 38T, CMS 35T and *P. orientalis*, CMS 38T is proposed as the type strain of a novel species of the genus *Pseudomonas*, to which the name *Pseudomonas meridiana* sp. nov. is assigned.

Identification of strain CMS 38T as *Pseudomonas proteolytica* sp. nov.

Strain CMS 38T, which is different from CMS 35T (Table 1), shares 99.46 % 16S rRNA gene sequence similarity with *P. orientalis* (Fig. 2); however, it differs from *P. orientalis* (Dabboussi et al., 1999) in a number of phenotypic traits (Table 1) and its protein profile and exhibits 64 % relatedness at the DNA–DNA level (Supplementary Table B, available in IJSEM Online). In addition, strain CMS 38T exhibits 52, 58 and 49 % DNA–DNA relatedness (Supplementary Table B, available in IJSEM Online) respectively with *P. marginalis*, *P. rhodesiae* and *P. veronii*, which are part of the main cluster of the phylogenetic tree (Fig. 2). DNA–DNA hybridization was not performed between strain CMS 38T and *Pseudomonas extremorientalis* (Ivanova et al., 2002), *P. tolaasii* (Bradbury, 1987) or *Pseudomonas costantinii* (Munsch et al., 2002), but they can be differentiated easily, based on phenotypic characteristics (Table 1).

Table 2. Phenotypic differences between strain CMS 64T (*P. proteolytica*), *P. brenneri* and *P. migulae*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<tbody>
<tr>
<td><strong>Growth characteristics</strong></td>
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<tr>
<td>Temperature range for growth (°C)</td>
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<td>4–37</td>
<td>4–35</td>
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<tr>
<td>Optimum growth temperature (°C)</td>
<td>22</td>
<td>25</td>
<td>30</td>
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<tr>
<td><strong>Phenotypic characteristics</strong></td>
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<tr>
<td>Phosphatase</td>
<td>–</td>
<td>+</td>
<td>NA</td>
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<tr>
<td>Lipase</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Gelatinase</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Levan formation on sucrose</td>
<td>–</td>
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<tr>
<td><strong>Utilization of carbon compounds</strong></td>
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<tr>
<td>Adonitol</td>
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<tr>
<td>L-Arabinose</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>Erythritol</td>
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<tr>
<td>Fumaric acid</td>
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<td>+</td>
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<tr>
<td>meso-Inositol</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>L-Rhamnose</td>
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<td>Sorbitol</td>
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<td>D-Xylose</td>
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<td>L-Aspartic acid</td>
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<tr>
<td>L-Proline</td>
<td>–</td>
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<tr>
<td>L-Tryptophan</td>
<td>–</td>
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</table>

*P. brenneri* (Baïda et al., 2001) and *P. migulae* (Verhille et al., 1999) (Fig. 2), with a bootstrap value of 86 %. The other two Antarctic isolates, CMS 35T and CMS 38T (Fig. 2), appear to be related more closely to *P. orientalis* (Dabboussi et al., 1999).

Identification of strain CMS 38T as *Pseudomonas antarctica* sp. nov.

Strain CMS 38T can be differentiated from strains CMS 35T and CMS 64T with respect to its protein profile, riboprint, phenotypic characteristics and low (40 %) DNA–DNA relatedness (Tables 1 and 2; Supplementary Table B, available in IJSEM Online). In addition, strain CMS 38T exhibits 52, 58 and 49 % DNA–DNA relatedness (Supplementary Table B, available in IJSEM Online) respectively with *P. marginalis*, *P. rhodesiae* and *P. veronii*, which are part of the main cluster of the phylogenetic tree (Fig. 2). DNA–DNA hybridization was not performed between strain CMS 38T and *Pseudomonas extremorientalis* (Ivanova et al., 2002), *P. tolaasii* (Bradbury, 1987) or *Pseudomonas costantinii* (Munsch et al., 2002), but they can be differentiated easily, based on phenotypic characteristics (Table 1). Thus, based on the above differences between strains CMS 38T, CMS 35T and *P. orientalis*, CMS 38T is proposed as the type strain of a novel species of the genus *Pseudomonas*, to which the name *Pseudomonas meridiana* sp. nov. is assigned.

Identification of strain CMS 64T as *Pseudomonas proteolytica* sp. nov.

Strain CMS 64T can be differentiated from strains CMS 35T and CMS 38T by phenotypic characteristics (Tables 1 and 2). In addition, these isolates differ in their protein profiles, riboprints and DNA–DNA relatedness (Supplementary Table B, available in IJSEM Online; Fig. 1). At the 16S rRNA gene sequence level, strain CMS 64T is related closely to *P. brenneri* (99-73 %) and *P. migulae* (99-75 %) (Fig. 2). Characteristics that differentiate strain CMS 64T from *P. migrulae* (Verhille et al., 1999) and *P. brenneri* (Baida et al., 2001) are listed in Table 2. Furthermore, strain CMS...
64T exhibits differences in protein profile and shows only 55% relatedness with \( P. \) migulae and 68% with \( P. \) brenneri at the DNA–DNA level (Supplementary Table B, available in IJSEM Online). The riboprint of strain CMS 64T is also different from that of \( P. \) brenneri (Fig. 1). Thus, based on phenotypic, genotypic and phylogenetic characteristics, strain CMS 64T is proposed as the type strain of a novel species of the genus \( Pseudomonas \), to which the name \( Pseudomonas \) proteolytica sp. nov. is assigned.

**Description of \( Pseudomonas \) antarctica sp. nov.**

\( Pseudomonas \) antarctica (an.tarc’ti.ca. N.L. fem. adj. antarctica pertaining to Antarctica).

Forms circular, convex, smooth and translucent colonies with a diameter of 1–2 mm. Cells are Gram-negative, motile with a polar flagellum, rod-shaped and psychrophilic (grow at 4–30°C), can tolerate 3% NaCl (w/v) and grow at an optimum pH of 7. Positive for catalase, oxidase, urease and phosphatase and weakly positive for lipase; reduces ni trate to nitrite; negative for \( \beta \)-galactosidase, gelatinase, arginine dihydrolase, arginine decarboxylase, lysine decarboxylase, indole production, methyl red and Voges–Proskauer test. Does not hydrolyse aesculin, starch or cellulose. Acid is produced from D-fructose, D-galactose, D-glucose, D-mannose and D-mannitol, but not from L-arabinose, lactose, L-rhamnose, sucrose or D-xylene. Utilizes acetate, adonitol, citrate, \( \alpha \)-erythritol, D-fructose, D-galactose, D-glucose, glycerol, \( \alpha \)-inositol, lactic acid, D-mannose, D-mannitol, pyruvate, D-ribose, sorbitol, trehalose, L-alanine, L-arginine, L-glutamic acid, L-glutamine, L-lysine, L-serine, L-tyrosine and L-tryptophan as sole carbon sources, but not L-arabinose, D-cellulose, cellulose, dextran, dulcitol, fumaric acid, inulin, lactose, D-maltose, D-melibiose, melezitose, D-ribose, L-rhamnose, D-sorbose, sucrose, succinic acid, thiglycollate, D-xylene, L-aspatic acid, L-asparagine, L-cysteine, L-glycerol, L-histidine, L-leucine, L-isoleucine, L-methionine, L-proline, L-threonine or L-valine. Sensitive to the antibiotics ampicillin, amoxycillin, bacitracin, carbenicillin, chloramphenicol, chlorotetracycline, colistin, cotrimoxazole, erythromycin, kanamycin, gentamicin, lincomycin, nitrofurazone, nitrofurantoin, nystatin, oxytetracyclin, penicillin, polymyxin B, rifampicin, tetracycline and tobramycin, but resistant to furazolidone, furoxone and trimethoprim. DNA G+C content is 60-7 mol%.

The type strain is CMS 35T (\( =\)MTCC 4992T = DSM 15318T).

**Description of \( Pseudomonas \) meridiana sp. nov.**

\( Pseudomonas \) meridiana (me.ri.di.a’na. L. fem. adj. meridiana of or belonging to the south or south side, southern, southerly, meridional; pertaining to the South Pole).

Forms circular, convex, smooth and translucent colonies with a diameter of 1–2 mm. Cells are Gram-negative, motile with a polar flagellum, rod-shaped and psychrophilic (grow at 4–30°C), can tolerate 3% NaCl (w/v) and grow at an optimum pH of 7. Positive for catalase, oxidase and lipase and weakly positive for urease; reduces nitrate to nitrite; negative for phosphatase, \( \beta \)-galactosidase, gelatinase, arginine dihydrolase, arginine decarboxylase, lysine decarboxylase, indole production, methyl red and Voges–Proskauer test. Does not hydrolyse aesculin, starch or cellulose. Acid is produced from D-fructose, D-galactose, D-glucose, D-mannose and D-mannitol, but not from L-arabinose, lactose, L-rhamnose, sucrose or D-xylene. Utilizes acetate, adonitol, citrate, \( \alpha \)-erythritol, D-fructose, D-galactose, D-glucose, glycerol, \( \alpha \)-inositol, lactic acid, D-mannose, D-mannitol, pyruvate, D-ribose, sorbitol, trehalose, L-alanine, L-arginine, L-glutamic acid, L-glutamine, L-lysine, L-serine, L-tyrosine and L-tryptophan as sole carbon sources, but not L-arabinose, D-cellulose, cellulose, dextran, dulcitol, fumaric acid, inulin, lactose, D-maltose, D-melibiose, melezitose, D-ribose, L-rhamnose, D-sorbose, sucrose, succinic acid, thiglycollate, D-xylene, L-aspatic acid, L-asparagine, L-cysteine, L-glycerol, L-histidine, L-leucine, L-isoleucine, L-methionine, L-proline, L-threonine or L-valine. Sensitive to the antibiotics ampicillin, amoxycillin, bacitracin, carbenicillin, chloramphenicol, chlorotetracycline, colistin, cotrimoxazole, erythromycin, kanamycin, gentamicin, lincomycin, nitrofurazone, nitrofurantoin, nystatin, oxytetracyclin, penicillin, polymyxin B, rifampicin, tetracycline and tobramycin, but resistant to furazolidone, furoxone and trimethoprim. DNA G+C content is 60-7 mol%.

The type strain is CMS 35T (\( =\)MTCC 4992T = DSM 15318T).
or L-valine. Resistant to ampicillin, amoxyccilin, bacitracin, carbenicillin, chloramphenicol, colistin, cotrimoxazole, erythromycin, furazolidone, furoxone, gentamicin, lincomycin, nitrofurantoin, nystatin, penicillin, polymyxin B and trimethoprim, but sensitive to chlorotetacycline, kanamycin, nitrofurazone, oxytetracycline, rifampicin, tetracycline and tobramycin. DNA G + C content is 63-2 mol%.

The type strain is CMS 38T (=MTCC 4993T = DSM 15319T).

### Description of Pseudomonas proteolytica sp. nov.

*Pseudomonas proteolytica* (pro.te.o.ly’ti.ca. N.L. fem. adj. proteolytic*o*ny proteolytic).

Forms circular, convex, smooth and translucent colonies with a diameter of 1–2 mm. Cells are Gram-negative, motile with a polar flagellum, rod-shaped and psychrophilic with a diameter of 1–2 mm. Cells are Gram-negative, motile forming circular, convex, smooth and translucent colonies proteolytic).

The type strain is CMS 38T (=MTCC 4993T = DSM 15319T).

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### References


