Pseudomonas guineae sp. nov., a novel psychrotolerant bacterium from an Antarctic environment

Núria Bozal, M. Jesús Montes and Elena Mercadé

Laboratori de Microbiologia, Facultat de Farmacia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain

Two Gram-negative, cold-adapted, aerobic bacteria, designated strains M8^T and M6, were isolated from soil collected from the South Shetland Islands. The organisms were rod-shaped, catalase- and oxidase-positive and motile by means of polar flagella. These two psychrotolerant strains grew between −4 and 30 °C. 16S rRNA gene sequence analysis placed strains M8^T and M6 within the genus *Pseudomonas*. DNA–DNA hybridization experiments between the Antarctic isolate M8^T and type strains of phylogenetically related species, namely *Pseudomonas peli* and *Pseudomonas anguilliseptica*, revealed levels of relatedness of 33 and 37 %, respectively. Strain M6 showed 99 % DNA similarity to strain M8^T. Several phenotypic characteristics, together with data on cellular fatty acid composition, served to differentiate strains M8^T and M6 from related pseudomonads. On the basis of the polyphasic taxonomic evidence presented in this study, it can be concluded that strains M8^T and M6 belong to the same genospecies, representing a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas guineae* sp. nov. is proposed. The type strain is M8^T (=LMG 24016^T=CECT 7231^T).

Correspondence
Elena Mercadé
mmercade@ub.edu

In recent years, increasing attention has been devoted to cold-adapted micro-organisms and their enzymes (Antranikian *et al.*, 2005). Antarctica has become a great source of novel psychrophilic and psychrotolerant strains, some of which belong to the genus *Pseudomonas* (Kriss *et al.*, 1976; Shivaji *et al.*, 1989; Ma *et al.*, 2006; Maugeri *et al.*, 1996; Bruni *et al.*, 1999; Reddy *et al.*, 2004). The genus *Pseudomonas* once comprised more than 100 species, but, over the last decade, many of these have been reclassified into different genera (Kersters *et al.*, 1996; Anzai *et al.*, 2000). During a taxonomic investigation of cold-adapted bacteria from soil samples collected in the Antarctic area of the South Shetland Islands, two strains, M6 and M8^T, able to grow at −4 °C and capable of forming swarming colonies on trypticase soy agar (TSA) were isolated. In this study, the taxonomic status of these two strains was investigated by using a combination of phenotypic characterization, 16S rRNA gene sequencing, DNA G+C content determination, DNA–DNA hybridization and cellular fatty acid analysis. The data obtained show that strains M6 and M8^T belong to a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas guineae* sp. nov. is proposed, with M8^{T}as the type strain.

Strains M8^T and M6 were isolated from a soil sample collected from Deception Island (South Shetland Islands, Antarctica). Sample aliquots were removed with a platinum loop and diluted in a saline solution (pH 7) containing the following salts (g l^{-1}): NaCl, 0.56; KCl, 0.027; $CaCl_2$, 0.03; $NaHCO_3$, 0.01. TSA plates were inoculated with loopfuls of several sample dilutions by using the streak-plate method to obtain isolated colonies. Plates were incubated for 4 days at 15 °C. Isolates were maintained aerobically on TSA slopes at 4 °C and also at −80 °C on cryo-beads.

The morphology, cell size and shape of cells grown on TSA at 15 °C were determined by means of negative staining and transmission electron microscopy. Motility was determined by phase-contrast microscopy. Oxidase, catalase and urease activities, nitrate reduction and hydrolysis of casein, lecithin, gelatin, DNA, starch and Tween 80 were determined according to Cowan & Steel (1993). The presence of fluorescent pigments was tested under UV light after 8 days on King's B medium (King *et al.*, 1954). Acid production from carbohydrates, enzyme production and additional characteristics were determined by using API 50 CH, API ZYM and API 20NE strips (bioMérieux). Tolerance of NaCl was measured on nutrient agar

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains M8^T and M6 are AM491810 and AM491811, respectively.

An electron micrograph of a cell of strain M8^T, an extended phylogenetic tree for strains M8^T and M6 within the genus *Pseudomonas* and a table showing cellular fatty acid compositions are available with the online version of this paper.

containing 0.5–7.5 % (w/v) NaCl; plates were incubated at 15 °C for 30 days. The temperature range for growth was determined on TSA incubated for 14 days at temperatures from −4 to 37 °C. Anaerobic growth was determined on trypticase soy broth (TSB) plus 1.5 % agar-agar and on Marine agar (Difco) after incubation in an anaerobic chamber at 15 °C for 14 days.

The cells were Gram-negative, rod-shaped (0.4–0.5 μm wide and 1.5–2.0 μm long) and motile by means of polar flagella (see Supplementary Fig. S1, available in IJSEM Online). Colonies of the isolates grown on TSA at 15 °C for 72 h were non-pigmented, round with irregular edges, slightly convex, 1.5–2.0 mm in diameter and did not produce fluorescent pigment on King's B medium. After 1 week, colonies had swarmed over the plate, merging together and becoming more mucous. The isolates grew at temperatures ranging from −4 to 30 °C and tolerated NaCl concentrations up to 4 % (w/v) on TSA. The isolates were positive for the hydrolysis of lecithin and negative for the hydrolysis of casein, starch, Tween 80 and DNA. Other phenotypic characteristics of the Antarctic isolates and their closest phylogenetic relatives are shown in Table 1. These phenotypic studies showed that the isolates displayed characteristics consistent with those for the genus *Pseudomonas*.

Fatty acids were prepared from 40 mg wet cell material harvested from a TSB agar culture (30 g TSB l^{-1} and 15 g agar l^{-1}) incubated for 4 days at 15 °C. The whole-cell fatty acids were determined as described previously (Bozal *et al.*, 2002). The mean fatty acid compositions of strains M8^T and M6, together with those of type strains of the closest phylogenetic neighbours, are shown in Supplementary Table S1 (available in IJSEM Online). The most abundant fatty acids were $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH). The isolates had cellular fatty acid profiles similar to that of *Pseudomonas peli* LMG 23201^T, containing the same percentages of $C_{16:0}$ and $C_{18:1}\omega7c$, whereas the summed feature 3 content was lower for *P. peli*. *Pseudomonas anguilliseptica* LMG 21629^T contained a significantly higher proportion of $C_{16:0}$ and also had a smaller proportion of summed feature 3.

Total DNA for complete 16S rRNA gene sequence analysis was prepared according to the protocol of Niemann *et al.* (1997). Phylogenetic analyses were carried out by using the neighbour-joining method as described previously by Bozal *et al.* (2002), with the software package BioNumerics (Applied Maths). For DNA–DNA hybridizations and determination of the G+C content, total DNA was prepared according to a modification of the procedure of Wilson (1987). The G+C content was determined by using the HPLC technique, as described by Mesbah *et al.* (1989). The DNA–DNA hybridizations were performed at 47 °C according to a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the method described by Ezaki *et al.* (1989).

16S rRNA phylogenetic studies confirmed that the Antarctic isolates (strains M8^T and M6) were members of the genus *Pseudomonas*. The highest level of 16S rRNA gene sequence similarity (99.1 %) was found with *P. peli* LMG 23201^T; lower levels of similarity occurred with other *Pseudomonas* species with validly published names (Fig. 1; Supplementary Fig. S2, available in IJSEM Online, shows the complete phylogenetic tree). Strain M6 showed 100.0 % 16S rRNA gene sequence similarity to M8^T, indicating that these strains probably belong to the same species. To verify the taxonomic position of strain M8^T, DNA–DNA hybridizations were performed with *P. peli* LMG 23201^T and *P. anguilliseptica* LMG 21629^T. The low DNA–DNA reassociation values (33 % with *P. peli* LMG 23201^T and 37 % with *P. anguilliseptica* LMG 21629^T) and the 16S rRNA gene sequence data indeed showed that strain M8^T occupies a distinct position within the genus *Pseudomonas* (Wayne *et al.*, 1987). Strain M6 showed 99 % DNA similarity to M8^T and it can be concluded that they belong to the same genospecies. The DNA G+C contents of M8^T

Table 1. Phenotypic characteristics of strains M8^T and M6 and their closest phylogenetic neighbours

Strains: 1, M8^T; 2, M6; 3, *P. peli* LMG 23201^T [data from Vanparys *et al.* (2006)]; 4, *P. anguilliseptica* LMG 21629^T [data from Vanparys *et al.* (2006)]. All strains are positive for oxidase, catalase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase. All are negative for trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, indole production, acidification of glucose, arginine dihydrolase, urease, aesculin hydrolysis, gelatin hydrolysis and the assimilation of glycerol, L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, D-sucrose, D-trehalose, D-arabitol, gluconate, adipate, phenylacetate, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-lactose, D-melibiose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, L-arabitol and 2- and 5-ketogluconate. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4
Growth at 4 °C	+	+	−	+
Nitrate reduction	−	−	−	+
Enzyme activities				
Alkaline phosphatase	+	+	−	W
Esterase	+	+	−	−
Esterase lipase	+	+	+	−
Lipase	+	+	W	−
Valine arylamidase	+	+	−	−
Cystine arylamidase	+	+	−	−
Acid phosphatase	+	+	−	+
Assimilation of:				
D-Galactose	−	−	−	+
Caprate	+	+	−	+
Malate	+	+	+	+
Citrate	+	+	−	+

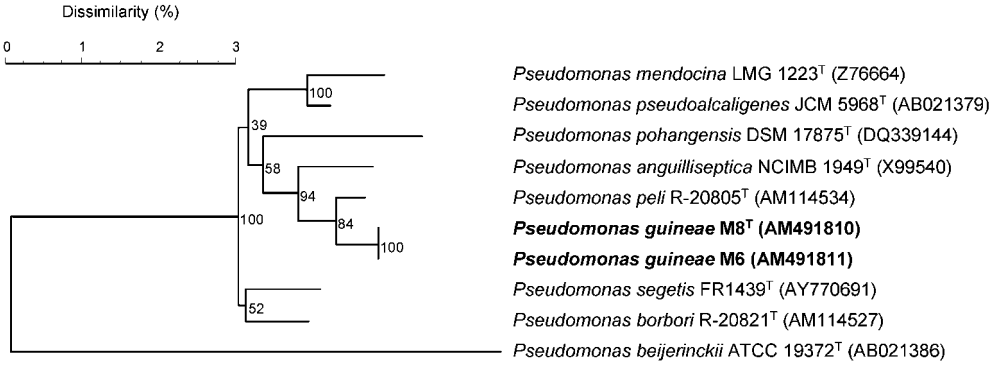

Fig. 1. Phylogenetic tree obtained by neighbour-joining analysis of 16S rRNA gene sequences, showing the position of the Antarctic isolates M8^T and M6 among neighbouring species of the genus *Pseudomonas*. Bootstrap values >70 % (based on 1000 replications) are shown at branch points.

and M6 (58.5 and 58.4 mol%, respectively) lie within the range described for members of the genus *Pseudomonas*.

The morphological, physiological, chemotaxonomic and phylogenetic data showed that strains M8^T and M6 belong to the genus *Pseudomonas*. The DNA–DNA hybridization analyses clearly distinguished strain M8^T from *P. peli* (Vanparys *et al.*, 2006) and *P. anguilliseptica* (Wakabayashi & Egusa, 1972). On the basis of the data from this polyphasic study, therefore, it is proposed that strains M8^T and M6 represent a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas guineae* sp. nov. is proposed.

Description of *Pseudomonas guineae* sp. nov.

Pseudomonas guineae (gui.ne′ae. N.L. gen. masc. n. *guineae* of Guinea, in honour of the late Professor Jesús Guinea, a prominent Spanish microbiologist, who isolated this strain).

Cells are rod-shaped (0.4–0.5 μm wide and 1.5–2.0 μm long), Gram-negative, non-spore-forming and do not produce fluorescent pigment on King's B medium. Cells are motile by means of polar flagella. After 72 h incubation at 15 °C on TSA, colonies are 1.5–2.0 mm in diameter, smooth and round with irregular edges. Growth occurs at temperatures between −4 and 30 °C, but not at 37 °C. NaCl is tolerated at concentrations up to 4 % (w/v). Growth is very poor under anaerobic conditions. Enzyme activities and details of the carbon sources utilized are given in Table 1. The DNA G+C content is 58.5 mol%.

The type strain, M8^T (=LMG 24016^T=CECT 7231^T), was isolated from a soil sample collected from Deception Island (South Shetland Islands, Antarctica).

Acknowledgements

This paper is dedicated, with sorrow, respect and gratitude, to the memory of Jesús Guinea Sánchez, who died on 29 September 2006. We would like to thank Josefina Castellví for providing Antarctic samples. We gratefully acknowledge the assistance of F. Garcia (Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Spain) with the fatty acid analysis. We thank the BCCM/LMG Identification Service (BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium) for performing hybridization analyses and 16S rRNA gene sequence analysis. We would also like to thank Dr H. G. Trüper for advice on Latin nomenclature. This research was supported by the Autonomous Government of Catalonia, Spain (grant 2005SGR00066).

References

Antranikian, G., Vorgias, C. E. & Bertoldo, C. (2005). Extreme environments as a source for microorganisms and novel biocatalysts. *Adv Biochem Eng Biotechnol* **96**, 219–262.

Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H. & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int J Syst Evol Microbiol* **50**, 1563–1589.

Bozal, N., Montes, M. J., Tudela, E., Jiménez, F. & Guinea, J. (2002). *Shewanella frigidimarina* and *Shewanella livingstonensis* sp. nov. isolated from Antarctic coastal areas. *Int J Syst Evol Microbiol* **52**, 195–205.

Bruni, V., Gugliandolo, C., Maugeri, T. & Allegra, A. (1999). Psychrotrophic bacteria from a coastal station in the Ross Sea (Terra Nova Bay, Antarctica). *New Microbiol* **22**, 357–363.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D. & Swings, J. (2002). Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. *Int J Syst Evol Microbiol* **52**, 1551–1588.

Cowan, S. T. & Steel, K. J. (1993). *Manual for the Identification of Medical Bacteria*, 3rd edn. Edited by G. I. Barrow & R. K. A. Feltham. Cambridge: Cambridge University Press.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol* **44**, 1148–1153.

Kersters, K., Ludwig, W., Vancanneyt, M., De Vos, P., Gillis, M. & Schleifer, K. H. (1996). Recent changes in the classification of the pseudomonads: an overview. *Syst Appl Microbiol* **19**, 465–477.

King, E. O., Ward, M. K. & Rainey, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44**, 301–307.

Kriss, A. E., Mitskevich, I. N., Rozanova, E. P. & Osnitskaia, L. K. (1976). Microbiological studies of the Wanda Lake (Antarctica). *Microbiology* (English translation of *Mikrobiologiya*) **45**, 1075–1081 (in Russian).

Ma, Y., Wang, L. & Shao, Z. (2006). *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. *Environ Microbiol* **8**, 455–465.

Maugeri, T. L., Gugliandolo, C. & Bruni, V. (1996). Heterotrophic bacteria in the Ross Sea (Terra Nova Bay, Antarctica). *New Microbiol* **19**, 67–76.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Niemann, S., Puehler, A., Tichi, H. V., Simon, R. & Selbitschka, W. (1997). Evaluation of the resolving power of the three different fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. *J Appl Microbiol* **82**, 477–484.

Reddy, G. S. N., Matsumoto, G. I., Schumann, P., Stackebrandt, E. & Shivaji, S. (2004). Psychrophilic pseudomonads from Antarctica: *Pseudomonas antarctica* sp. nov. and *Pseudomonas proteolytica* sp. nov. *Int J Syst Evol Microbiol* **54**, 713–719.

Shivaji, S., Rao, N. S., Saisree, L., Sheth, V., Reddy, G. S. N. & Bhargava, P. M. (1989). Isolation and identification of *Pseudomonas* spp. from Schirmacher Oasis, Antarctica. *Appl Environ Microbiol* **55**, 767–770.

Vanparys, B., Heylen, K., Lebbe, L. & De Vos, P. (2006). *Pseudomonas peli* sp. nov. and *Pseudomonas borbori* sp.nov., isolated from a nitrifying inoculum. *Int J Syst Evol Microbiol* **56**, 1875–1881.

Wakabayashi, H. & Egusa, S. (1972). Characteristics of a *Pseudomonas* sp. from an epizootic of pound-cultured eels (*Anguilla japonica*). *Bull Jpn Soc Sci Fish* **38**, 577–587.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Wilson, K. (1987). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Green Publishing & Wiley-Interscience.